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A Restrictive Cardiomyopathy Mutation in an Invariant Proline at the Myosin Head/Rod Junction Enhances Head Flexibility and Function, Yielding Muscle Defects in *Drosophila*

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Abstract

An “invariant proline” separates the myosin S1 head from its S2 tail and is proposed to be critical for orienting S1 during its interaction with actin, a process that leads to muscle contraction. Mutation of the invariant proline to leucine (P838L) caused dominant restrictive cardiomyopathy in a pediatric patient (Karam et al., Congenit. Heart Dis. 3:138–43, 2008). Here, we use *Drosophila melanogaster* to model this mutation and dissect its effects on the biochemical and biophysical properties of myosin, as well as on the structure and physiology of skeletal and cardiac muscles. P838L mutant myosin isolated from indirect flight muscles of transgenic *Drosophila* showed elevated ATPase and actin sliding velocity *in vitro*. Furthermore, the mutant heads exhibited increased rotational flexibility, and there was an increase in the average angle between the two heads. Indirect flight muscle myofibril assembly was minimally affected in mutant homozygotes, and isolated fibers displayed normal mechanical properties. However, myofibrils degraded during aging, correlating with reduced flight abilities. In contrast, hearts from homozygotes and heterozygotes showed normal morphology, myofibrillar arrays, and contractile parameters. When P838L was placed in *trans* to *Mhc*^S, an allele known to cause cardiac restriction

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in flies, it did not yield the constricted phenotype. Overall, our studies suggest that increased rotational flexibility of myosin S1 enhances myosin ATPase and actin sliding. Moreover, instability of P838L myofibrils leads to decreased function during aging of *Drosophila* skeletal muscle, but not cardiac muscle, despite the strong evolutionary conservation of the P838 residue.

Keywords

myofibril; motility; electron microscopy; contraction; *Drosophila melanogaster*

Introduction

Restrictive cardiomyopathy (RCM) is a rare form of heart disease with a poor prognosis. Cardiac transplantation offers the only effective therapy for severe pediatric cases. RCM is characterized by the stiffening of the myocardium, which can be caused by infiltration (sarcoidosis, amyloidosis), fibrosis, or desmin abnormalities. This leads to impaired ventricular relaxation without severe effects on myofibrillar organization or gross level cardiac morphology (for reviews, see Refs. [1, 2]). Mutations in contractile proteins such as α -actin, troponin I, and troponin T have been identified as causative of RCM [3–6]. Cardiac β myosin heavy chain gene mutations were also recently linked to RCM in newborns [7,8] and children [9]. Furthermore, some myosin mutations that cause hypertrophic cardiomyopathy (HCM) with minimal left ventricular hypertrophy also produce a restrictive phenotype, suggesting a continuum between the HCM and RCM classifications, with RCM often considered an extreme form of HCM [10,11].

The pediatric RCM case reported by Karam and coworkers arose from a *de novo* proline to leucine mutation at residue 838 of β myosin heavy chain [7]. This “invariant proline” is conserved in myosin II molecules; it separates the S1 head/neck of myosin (containing binding sites for actin, ATP, and myosin light chains) from the S2 fragment that forms the N-terminal portion of the coiled-coil rod dimer capable of assembling into thick filaments. The invariant proline is proposed to aid in the formation of a fork-like structure at the S1/S2 junction of the dimeric heavy chain molecule [12]. This is presumably critical for presenting the S1 motor domain to the actin molecule during the contractile cycle [12] and may be important in regulating myosin head–head interactions during muscle relaxation [13].

Change in contractile power output has been associated with several cardiomyopathies (for reviews, see Refs. [14, 15]), with the general observation that decreased power (force $\times$ velocity) makes cardiac muscle prone to dilated cardiomyopathy, whereas increased power yields HCM [16–18]. Limited data suggest that RCM shares a common mechanism with HCM, in that, RCM mutations in troponin I and troponin T result in increased calcium sensitivity of contraction, as is seen for HCM mutations (for review, see Ref. [19]). Since increased myosin function can cause HCM, particular over-functional alleles might cause RCM. In this regard, we reported previously that the *Mhc*⁵ mutation, which results in higher basal MgATPase activity and increased sliding velocity of actin filaments *in vitro*, results in constriction of the *Drosophila* cardiac tube and decreased fractional shortening [20]. The concordance between the increased biochemical/biophysical parameters and the cardiac

restriction suggests that modeling human cardiomyopathies in the fly may yield mechanistic insights into disease causality and progression.

The *Drosophila* model system is powerful for studying both cardiovascular diseases [21] and skeletal muscle myopathies [22]. Flies have a simple tubular heart [23,24], and many genes coding for heart differentiation and function in *Drosophila* are evolutionarily conserved [25]. For skeletal muscle, a major focus of assessment in *Drosophila* is the thoracic indirect flight muscles (IFM), which are responsible for powering flight (for reviews, see Refs.[26, 27]). IFM sarcomeric structure is well characterized [28] and similar to sarcomeres of vertebrates. A further advantage of the *Drosophila* system is that the effect of severe mutations on the heart or IFM can be studied readily because these muscles can be compromised without causing immediate death.

Drosophila is particularly advantageous for examining the effects of myosin heavy chain (MHC) mutations in that only one muscle *Mhc* gene exists, with MHC isoforms generated through alternative RNA splicing [29–32]. For mutant analysis, the single gene can be mutagenized *in vitro*, and *Mhc* transgenes can be expressed in lines with *Mhc* mutations that obviate endogenous myosin expression. This prevents non-mutated isoforms from being expressed in muscles of interest, as may occur in vertebrates with complex *Mhc* multigene families (for reviews, see Refs. [33–35]).

For this study, we produced the analog of the human *P838L* RCM mutation in *Drosophila* in an attempt to understand the effects of the mutation on myosin function, myofibril assembly/stability, and flight muscle and cardiac function. We were particularly interested in determining whether P838L myosin would have increased biochemical and biophysical parameters akin to those found in many human myosin HCM mutations and whether the *Drosophila* heart would recapitulate the narrowed phenotype observed for the overactive myosin produced by the *Mhc*⁵ allele [20]. Our integrative analysis included expression in IFM to study the isolated mutant protein in regard to ATPase, *in vitro* motility, rotational flexibility, and head orientation. We used electron microscopy, flight tests, and mechanical assays to define the effects of the mutation on myofibril assembly, stability, and function. We performed heart structure and physiology measurements in both the myosin null and *Mhc*⁵ backgrounds to determine the effects of the mutation on cardiac muscle. Our results demonstrate that mutating the invariant proline increases rotational flexibility of the myosin head, the average angle of separation between the dimeric heads, and the myosin basal Mg-ATPase and induced actin filament *in vitro* motility. The mutant protein causes defects in myofibril stability and function in the IFM, but its expression does not recapitulate the cardiac defects seen in the *Mhc*⁵ mutant. It is remarkable that mutating the invariant proline yielded essentially no cardiac dysfunction in *Drosophila* compared to the severe effect observed in the human patient [7].

Results

Production and verification of *P838L* transgenic lines

We constructed a mutant *P838L Mhc* transgene and obtained 11 transgenic *P838L* lines by *P* element-mediated germline transformation. We crossed those that did not map to the second

chromosome (the location of the endogenous *Mhc* gene) into the IFM MHC-null *Mhc*¹⁰ background [36] and assessed levels of myosin accumulation by polyacrylamide gel electrophoresis. Two lines (*P838L-6F* and *P838L-1 M*) yielded near wild-type levels of MHC relative to actin (Table 1). We constructed an additional line with a strongly expressing transgene insert on the second chromosome (*P838L-3 M*) by genetic recombination with an *Mhc*¹⁰-containing chromosome (see Materials and Methods). Reverse transcription-polymerase chain reaction (RT-PCR) of RNA isolated from dissected IFM verified the mutant transcript, replaced the wild-type transcript in each line, and demonstrated that the alternative splicing pattern was not altered by the mutation (see Materials and Methods).

The P838L mutation enhances basal ATPase rates and *in vitro* motility

We assessed the steady-state ATPase parameters of P838L myosin isolated from dissected IFM (Fig. 1; mean $\pm$ SD). Both Ca-ATPase ($10.80 \pm 0.76 \text{ s}^{-1}$) and basal Mg-ATPase ($0.35 \pm 0.09 \text{ s}^{-1}$) rates were significantly enhanced ($p = 0.013$ for both parameters) compared to wild-type transgenic myosin values ($9.78 \pm 0.81 \text{ s}^{-1}$ and $0.24 \pm 0.02 \text{ s}^{-1}$, respectively) (Fig. 1A and B). In contrast, P838L myosin values for Mg-ATPase V_{max} of actin activation ($1.95 \pm 0.38 \text{ s}^{-1}$), K_m of actin concentration relative to ATPase activity ($0.32 \pm 0.16 \mu\text{M}$) and catalytic efficiency, defined as V_{max}/K_m ($5.86 \pm 2.52 \text{ s}^{-1}/\mu\text{M}$), did not differ significantly ($p = 0.354$, $p = 0.540$, $p = 0.221$, respectively) from wild-type transgenic ($1.78 \pm 0.32 \text{ s}^{-1}$, $0.29 \pm 0.09 \mu\text{M}$ and 6.82 ± 1.99 , respectively) (Fig. 1C–E).

To determine the ability of P838L myosin to drive actin filament sliding, we performed *in vitro* motility assays using isolated myosin in conjunction with fluorescently labeled actin filaments (Fig. 1F; mean $\pm$ SD). Analysis of captured video images indicated a statistically significant ($p = 0.011$) increase in motility for P838L myosin ($6.89 \pm 0.25 \mu\text{m/s}$) compared to wild-type transgenic myosin ($6.56 \pm 0.18 \mu\text{m/s}$). Overall, the P838L ATPase and *in vitro* motility results are interesting in that they mimic the increased basal Mg-ATPase and *in vitro* motility we observed for *Mhc*⁵ myosin, a mutant protein that yields a constricted adult heart [20,37].

Changes in myosin torsional stiffness and S1 head orientation

To test if the P838L mutation at the junction between the S1 head domain and the S2 rod affects myosin head mobility, we used video microscopy to measure the rotational motion of fluorescent actin filaments bound to either P838L or wild-type transgenic myosin (Fig. 2A). At times, the rotational motion of the myosin-bound actin filaments did not appear completely random where filaments were observed to rotate in a single direction for several seconds. As described previously [64], both single-headed and double-headed myosin molecules yielded similar unwinding behavior, indicating that the flexibility observed here with intact mutant and wild-type myosin molecules likely reflects the properties of residues located where the two heavy chains join to form a coiled-coil dimer [64]. We found that the thermally induced variance in actin angle for filaments attached to P838L mutant myosin heads was larger, indicated by a significantly lower torsional stiffness compared to wild-type myosin [Fig. 2B (mean $\pm$ SD): $0.907 \pm 0.631 \text{ pN nm/rad}$ ($n = 78$) *versus* $1.891 \pm 1.880 \text{ pN nm/rad}$ ($n = 86$), respectively; $p < 0.001$]. This indicates that the replacement of the invariant proline by leucine increased molecule flexibility at the S1/S2 junction.

To determine if the orientation of the myosin heads relative to the S1/S2 junction is altered by the P838L mutation, we imaged heavy metal coated myosin molecules ($n = 111$) via transmission electron microscopy and measured the inter S1 angle (θ) between the myosin heads relative to the myosin rod (Fig. 2C). Figure 2D (mean $\pm$ SD) shows that there is a significant ($p < 0.001$) increase in the average angle between the myosin heads in P838L ($88.9 \pm 22.0^\circ$) relative to wild-type control myosin ($74.8 \pm 22.1^\circ$).

The P838L mutation impairs the flight ability of transgenic *Drosophila*

To determine the effects of the P838L mutation on indirect flight muscle function, we measured flight ability at two days, one week, and two weeks of age (Table 1). All three mutant lines showed impairment compared to the control at all the ages. Flight ability deteriorated with age for all mutant lines and the control line, particularly after one week. Although the mutant lines showed impaired flight, the degree of impairment was not consistent, with lines showing reduced levels of flight by 20% (*P838L-1 M*), 44% (*P838L-6F*), and 81% (*P838L-3 M*) at two weeks of age.

The P838L mutation affects indirect flight muscle ultrastructure during aging

We used transmission electron microscopy to assess the ultrastructure of IFM in homozygous *P838L* young and aged adults. Two-day-old adult transgenics expressing wild-type or P838L myosin displays myofibrils with normal sarcomeric structures (Fig. 3A–D), although occasional minor abnormalities in Z-disk regularity were seen in particular mutants. Myofilaments are organized in the standard double hexagonal arrangement (Fig. 3A'–D'). Average sarcomere lengths among the lines did not show a consistent difference compared to wild-type transgenic IFMs (*P838L-6F*: $2.59 \pm 0.122 \mu\text{m}$; *P838L-1 M*: $3.07 \pm 0.26 \mu\text{m}$; *P838L-3 M*: $3.15 \pm 0.31 \mu\text{m}$; *pwMhc2* control: $2.99 \pm 0.26 \mu\text{m}$), with *P838L-6F* being significantly shorter ($p < 0.001$), *P838L-1 M* showing no significant difference, and *P838L-3 M* being significantly longer ($p < 0.01$) than control (mean $\pm$ SD, $n = 60$ per line). In longitudinal sections from three-week-old control flies (Fig. 3E), wild-type structure is maintained. However, *P838L* mutants consistently show areas with Z-band irregularities, as well as abnormalities in myofibrillar shape and orientation (Fig. 3F–H). A cross-section of control IFM shows essentially intact myofibrils (Fig. 3E'), whereas some degradation of IFM myofilament packing is present in all mutant lines (Fig. 3F'–H'). In summary, P838L myosin assembles into fundamentally normal myofibrils, but progressive moderate deterioration occurs during aging.

Mechanical properties of young IFM fibers are not altered by the P838L mutation

We performed mechanical assays on 2-3 day old *Drosophila* IFM fibers expressing P838L myosin to probe for altered muscle fiber mechanical properties prior to the initiation of muscle structural abnormalities. We chose the *P838L-1 M* homozygous line to analyze, since it yielded the best flight index (Table 1) and therefore best demonstrated the *in vivo* functional ability of the transgene. We report mean values $\pm$ SEM. We measured IFM power generation by driving the fibers through small amplitude sinusoidal length changes [38] because these muscles, like heart muscles, generate power through cyclical contractions. The average values for power generated by the *P838L-1 M* mutant fibers over the range of frequencies where IFM fibers produce positive power did not show a statistically significant

difference compared to control average values (Fig. 4; $p = 0.223$, mixed ANOVA). Furthermore, the frequency at which maximum power was generated did not differ (Fig. 4; Table 2), suggesting that the overall rate of flux through the strongly bound steps of the cross-bridge cycle was not altered by the mutation. Increasing the amplitude of muscle length oscillations during the sinusoidal power measurements yielded the same results, that is, the frequency of maximum power and the amount of power produced did not differ from the control values. The fibers showed no change in their active stiffness properties as both elastic (E_e) and viscous ($-E_v$) stiffness components were not changed (Table 2). There were also no changes in isometric tension or rigor stiffness (Table 2). The rigor stiffness results suggest that the stiffness of the cross-bridge was unchanged in the direction of force propagation, that is, in parallel to the thick and thin filaments. We also found that the *P838L* mutation did not change wing beat frequency at 15 °C, the same temperature at which the muscle mechanical assays were performed. Control fly wing beat frequency and SEM were 164 ± 2.6 Hz ($n = 12$), compared to 163 ± 1.6 Hz, ($n = 15$) for *P838L-1 M* flies. Wing beat frequency is usually very sensitive to changes in muscle mechanical properties such as stiffness [39,40]. Thus, our wing beat frequency results support the absence of a statistically significant change in muscle mechanical function. While the lack of change in wing beat frequency in young flies appears at odds with the observed decrease in flight performance for the mutants (Table 1), the decrease may arise from reduced wing stroke amplitude, as this is also an important contributing factor to aerodynamic power [41,42].

Heart ultrastructure is intact in *P838L* mutants

To determine the effects of the *P838L* mutation on cardiac ultrastructure and function, we crossed the *P838L-6F* transgene into the *Mhc^l* background. This was readily done since it is not on the same chromosome as *Mhc^l*. Furthermore, the *P838L-6F* transgene might more readily yield cardiac defects since it was less able to rescue flight ability than *P838-1 M* (Table 1). The *Mhc^l* allele is null for myosin in all tissues and must be maintained over a balancer chromosome [43]. Two copies of the *P838L-6F* transgene rescued lethality of the *Mhc^l* homozygotes. Hence, we were able to examine the effects of the mutation on the heart (dorsal vessel) in the homozygous condition and in heterozygotes. The dorsal vessel is located in the abdomen of the adult and is covered ventrally by a layer of skeletal muscle. In transverse sections taken between the second and third abdominal segments, the cardiomyocyte layer is distinct from the ventral layer (Fig. 5A). Myofibrils are arranged in a circumferential array around the heart tube, with most myofibrils oriented perpendicular to the anterior–posterior axis. A similar pattern is seen in *P838L* homozygous mutant hearts (Fig. 5B). High magnification transverse sections show that intact myofilamentous arrays exist in homozygous controls and *P838L/P838L* 4-day-old hearts (Fig. 5C–F). In longitudinal orientation, discontinuous Z-disks (arrows) separate similarly arranged sarcomeric structures in control (Fig. 5C) and homozygous mutant samples (Fig. 5D), as is the case in wild-type hearts [24]. Sarcomeres from *pwMhc2/+* and *P838L-6F/+* heterozygotes also show normal morphologies (Fig. 5G and H). Overall, the *P838L* mutation did not cause obvious myofibrillar defects in the cardiomyocytes, suggesting that skeletal muscles are more susceptible to myofibrillar degradation induced by this myosin mutation.

Cardiac physiology is unaltered by the *P838L* mutation

We examined whether cardiac restriction and functional defects are associated with *P838L* myosin expression in 3-week-old female heterozygous or homozygous *P838L-6F* flies by using high-speed video microscopy and motion analysis software[‡] [44]. Parameters determined included heart period, diastolic diameter (DD), and systolic diameter (SD). These measurements were used to calculate the fractional shortening [(DD-SD)/DD]. For every parameter determined, no statistically significant difference was observed between mutant and the corresponding control hearts, for both homozygotes and heterozygotes (Fig. 6; mean $\pm$ SEM). Thus, it appears that the *P838L* mutation does not cause *Drosophila* heart restriction and that it does not affect heartbeat periodicity.

Given our previous observation that the strongly disruptive *Mhc*⁵ mutation yields a restricted cardiac phenotype in the homozygous state [20], we assessed whether placing one copy of *Mhc*⁵ in combination with one copy of the “weak” *P838L* allele would also yield a restrictive phenotype. As a control, we used *Mhc*⁵ in combination with the wild-type *Mhc*⁺ allele, a genotype that does not affect diastolic or systolic diameters compared to wild-type controls (Fig. 6). Replacing the wild-type copy of *Mhc* with the *P838L* transgene did not yield the restricted phenotype that is normally seen in *Mhc*⁵ homozygotes [20]. Interestingly, *P{P838L}; Mhc*⁵/*Mhc*^L heterozygotes showed an increased systolic diameter and decreased fractional shortening compared to the *P{Mhc*⁺*}; Mhc*⁵/*Mhc*^L configuration. While the significance of these observations is not obvious, it is clear that the “restriction-sensitized” background provided by one copy of *Mhc*⁵ did not permit heart constriction in combination with *P838L*.

Discussion

Here, we developed a novel *Drosophila melanogaster* model to investigate the biochemical, structural, and physiological effects of a *P838L* myosin mutation proposed to cause human restrictive cardiomyopathy. This mutation in the human cardiac β -myosin heavy chain gene (*MYH7*) was reported in a postmortem patient with early-onset RCM [7]. Our study involved substituting the analogous mutation into the *Drosophila* gene encoding muscle myosin heavy chain and determining the effects upon myosin biochemical, structural, and biophysical properties; indirect flight muscle structure and function; and cardiac structure and physiology. We observed that the mutation increased basal myosin ATPase, *in vitro* actin motility, torsional flexibility, and degree of separation of the dimeric myosin heads. We found that the mutant myosin assembled normally into myofibrils in both IFM and cardiac muscle, and that IFM, but not cardiac muscle, showed structural and functional degeneration.

RCM cases caused by myosin mutations might be expected to enhance myosin function and result in increased force, since muscle fibers containing RCM-inducing thin filament proteins show increased Ca-sensitivity, which results in increased force production (for review, see Ref.[19]). This is similar to the phenomenon seen with HCM mutations affecting thin filament proteins (where there is increased Ca-sensitivity) or HCM mutations affecting

[‡]<http://sohasoftware.com/>

myosin (where there is increased enzymatic activity) (for review, see Ref. [45]). The increases observed for *Drosophila Mhc*⁵ basal ATPase and *in vitro* motility rates and the generation of a constricted heart tube also suggested a correlation between increased myosin function and RCM [20]. As the increased biochemical parameters for P838L myosin were much milder than those exhibited by *Mhc*⁵ myosin, the lack of a constricted phenotype with myofibrillar defects in the *P838L* cardiac tube is not completely unexpected. Furthermore, the *P838L* patient biopsy showed only mild cardiomyocyte disarray with no hypertrophy [7]. P838L myosin had only minor effects at the ultrastructural level in *Drosophila*, in concordance with the normal ventricular mass typically associated with RCM.

The *P838L* mutation affects the “invariant proline” at the S1/S2 junction of myosin. This residue is at the N-terminal end of a heptad repeat region responsible for dimerization of heavy chains into the alpha-helical coiled-coil rod [46,47]. This proline has been proposed to aid in the formation of a forked structure emanating from the dimeric coiled-coil rod, allowing the globular heads to diverge [12]. Proline–proline interaction in the two alpha helices may be necessary for both the proper orientation of the heads and for head–head interactions at their bases, which must occur in the absence of steric hindrance for effective cross-bridge cycling [12,48]. Replacement of the compact prolines with leucines in dimeric myosin might cause the residues to clash. Alternatively, since P838 is at the *d* position of the heptad repeat of the S2, its replacement with a hydrophobic leucine might stabilize the S2 into a coiled-coil configuration. Furthermore, the invariant proline may play a role in assuring correct head flexibility and orientation for interactions between the head and rod that contribute to inactivation of myosin IIs in a switched-off state [13, 49]. It is also notable that the invariant proline lies close to hydrophobic WXW sequences known to interact with the hydrophobic core of the N-terminal lobe of the regulatory light chain (RLC) [46,49]. As discussed below, disruption of any of these structural configurations by the *P838L* mutation could account for the various mutant phenotypes in our *Drosophila* model and in the RCM patient.

The increased flexibility at the S1/S2 junction that we observed in P838L myosins (Fig. 2B) may reduce head constraints. There is also increased head separation compared to wild-type myosins (Fig. 2D). This could lead to an increased motor step size or changes in rates of attachment/detachment to actin and the observed increase in *in vitro* motility (Fig. 1F). Notably, however, our IFM fiber mechanical studies found no change in $f_{\max}$, or other muscle kinetic measurements (Table 2), suggesting that when the mutant myosin is constrained within the myofibrillar lattice, there are no mechanically significant changes in rate constants of the cross-bridge cycle that involve binding to actin (we note, however, that other changes in muscle mechanical function, such as activation and relaxation rates and maximum shortening velocity, were not measured, since this is not readily feasible with IFM). In support of the lack of mechanical changes, we observed no alterations in actin-activated ATPase activity ($V_{\max}$) or actin binding as measured via ATPase (K_m) that are attributable to the *P838L* mutation (Fig. 1C and D). Another possibility to consider is that the mutation could destabilize the interacting head motif (the switched-off state of myosin in the absence of actin [13,49]), resulting in an increased number of heads available to participate in basal ATP hydrolysis.

Observed changes in basal ATPase activity that are induced by the *P838L* mutation may arise from alterations in RLC configuration. Biochemical [50] and modeling [12] studies suggest that the RLCs of myosin II dimers interact. The *P838L* mutation may reconfigure RLC–RLC or RLC–MHC interactions due to its proximity to the RLC binding site. This could indirectly feed back to the motor domain such that the basal Mg-ATPase and Ca-ATPase activities of P838L myosin are significantly increased compared to wild type (Fig. 1A and B). Another possibility is that the state of RLC phosphorylation might be indirectly affected by the mutation, which could affect myosin function [51,52]. However, we did not observe changes in levels of phosphorylated forms of the RLC (data not shown).

Despite the changes in P838L myosin that are concordant with increased myosin function predicted in RCM-causing protein, the relatively mild phenotypes engendered by the mutation in *Drosophila* are unexpected given the conservation of this residue in all myosin II proteins. It is possible that the *Drosophila* myosin protein backbone is less sensitive to the P838L perturbation than the human backbone. Such a phenomenon was observed for the R403Q HCM mutation placed in two different mouse cardiac myosins, in that opposite effects on ATPase activity were documented [53]. Interestingly, and in agreement with our *in vitro* observations, a study that involved deletion of the invariant proline in smooth muscle myosin II showed only minor effects upon myosin basal ATPase, actin-activated ATPase activity, or actin-sliding velocity, although deletion of the two immediately C-terminal leucines or substitution of the first leucine with alanine affected actin-activated ATPase activity dramatically [54]. Interestingly the changed properties were seen only for dimeric myosin, not S1, suggesting that head cooperativity is affected by the mutation [54].

The changes in torsional flexibility at the S1/S2 junction, ATPase parameters, and actin sliding velocity induced by the *P838L* mutation might be expected to affect muscle structure and function in *Drosophila*. While we did not observe ultrastructural or functional defects in the *Drosophila* heart or structural abnormalities in IFMs from young flies, older flies showed progressive mild deterioration of IFM structure and further decrease in flight ability. There was some variability in IFM deterioration among the mutant lines, which might be explained by differences in mutant myosin expression levels (as seen for *P838L-3 M*, Table 1) and by the possibility that the genomic insertion site could disrupt expression of another gene that impacts IFM structure or function. Overall, it is clear that in the context of the highly organized IFMs and their fast MHC isoform, the *P838L* mutation is capable of producing a *Drosophila* phenotype *in vivo*. The relatively mild *Drosophila* phenotype starkly contrasts to the severe *in vivo* effects of the human *P838L* mutation [7]. It is important to note, however, that the identification of the human mutation involved sequencing of select candidate genes. Hence, it is possible that a mutation in another genetic locus, alone or in conjunction with P838L myosin, is responsible for the severe phenotype observed in the human patient.

Materials and Methods

Production of the *P838L* construct

The *P838L* construct was created by changing the proline codon (CCC) to a leucine codon (CTC) at the *Drosophila* equivalent of the 838th amino acid coding position of the human beta-cardiac myosin heavy chain gene (codon 836 located in exon 12 of *Drosophila Mhc*).

To accomplish this, a previously constructed vector containing the *Mhc* 3' region inserted in the pKS vector was digested with Eag I to excise the insert from the pKS vector. The insert was then sub-cloned into the Eag I site of pKSmod [pKSmod was constructed from the pBluescript KS vector (Stratagene, La Jolla, CA) for sub-cloning purposes by eliminating the Apa I restriction site]. The resulting sub-clone was digested by Apa I and Nde I to liberate the 0.2-kb Nde I-Nde I and the 0.7-kb Apa I-Nde I sub-fragments. The 0.7-kb sub-fragment, which contains exon 12, was sub-cloned into the Apa I-Nde I site of the pGEM vector for site-directed mutagenesis. Mutagenesis was carried out using forward (5'-GCAGAAGGTCAAGCTCCTCCTCA-3') and reverse (5'-CTGACGTTGAGGAGGAGCTTGAC-3') primers in conjunction with the Agilent Technologies Quickchange® II kit (La Jolla, CA). The presence of the mutation was verified by DNA sequencing (Microchemical Core Facility, SDSU). The mutagenized 0.7-kb sub-fragment and the 0.2-kb Nde I-Nde I sub-fragment were ligated back into the 3' region of *Mhc* in the pKS mod vector. Next, the entire *P838L* 3'*Mhc* fragment was excised by Eag I digestion and ligated at the Eag I site of the 5' region of *Mhc* in the pCaSpeR vector. pCaSpeR is a *P*element vector [55] with the miniwhite gene, *w*⁺, as a selectable eye color marker. *P838L* DNA was purified using a QIAfilter Plasmid Maxi kit (Qiagen Inc., Valencia, CA) following the manufacturer's instructions.

***Drosophila* transformation and genetics**

BestGene, Inc. (Chino Hills, CA) performed *P*element-mediated transformation to produce the transgenic fly lines [56]. G₀ generation *y w*¹¹¹⁸ embryos carrying the white eye mutation were injected with the *P838L* plasmid at 0.4 µg/µl and 2–3 helper plasmid at 0.08 µg/µl. The resulting G₀ larvae, upon reaching adulthood, were individually crossed with *y w*¹¹¹⁸ flies. The G₁ transformants were mapped to chromosome insertion sites by standard genetic crosses using multiple balancer chromosomes. Fly lines with insertions on the X and third chromosome were crossed into the *Mhc*¹⁰ background that is null for the IFM myosin isoform [36]. The viable homozygous *P838L* lines in the *Mhc*¹⁰ background and *PwMhc2* wild-type control lines [57] were used for further experiments.

To move an insert on the second chromosome into the *Mhc*¹⁰ genetic background, we chose a fly line with a homozygous viable transgene for genetic recombination. Male homozygotes were crossed with female *Mhc*¹⁰/*Mhc*¹⁰; *MKRS/TM2* flies. *Mhc*¹⁰/*Mhc*⁺ *P838L*; *TM2*/+ female progeny were selected, with the goal of having the transgene move to the *Mhc*¹⁰ chromosome by genetic recombination during meiosis. These females were crossed with *Mhc*¹⁰/*Mhc*¹⁰; *MKRS/TM2* males. Fifty individual male offspring, each possibly carrying a recombinant second chromosome, were crossed with female *Mhc*¹⁰/*Mhc*¹⁰; *MKRS/TM2* flies. Orange-eyed progeny with the *TM2* marker from each individual cross were self-crossed to yield transgene homozygotes (displaying darker orange eyes). Each homozygous line was assessed for flight ability. Flightless lines were eliminated as non-recombinants with two transgenes and two wild-type *Mhc* genes, since *Drosophila* with four copies of *Mhc* are flightless [58]. Flies with two copies of the transgene in the presence of the *Mhc*¹⁰ allele do show flight ability (see Results), and a recombinant line was thereby selected. Expression of the transgene and absence of wild-type *Mhc* transcripts was verified by RT-PCR (see below).

RNA isolation and reverse transcription-polymerase chain reaction (RT-PCR)

Flight muscle was isolated following the methods previously described [59]. Total RNA was isolated using the RNeasy Plus Mini Kit (Qiagen, Valencia, CA). The manufacturer's protocol was followed except for the following modifications. Upper thoraces from 50 very young adult flies were dissected out and immediately put in 350 µl of Buffer RLT premixed with 3.5 µl of β-mercaptoethanol. Fly tissue was disrupted with a homogenizer followed by disruption through a 20-gauge 1-inch needle attached to a 1 ml syringe. In the final step, RNA was eluted by addition of 30 µl of RNase-free water.

First strand cDNA was synthesized using the First Strand cDNA Synthesis Kit (Protoscript™) following the manufacturer's instructions. Double-stranded cDNA was synthesized using PuRe Taq™ Ready-To-Go™ PCR beads (Illustra™, GE Healthcare, Pittsburgh, PA) following the manufacturer's protocol. Then, 2.5 µl of the first strand cDNA, 1.5 µl each of forward and reverse primers were used for the synthesis of double-stranded cDNA. Forward (+) and reverse (−) primer pairs used were as follows: Exon 2 (+) 5'-TGGATCCCCGACGAGAAGGA-3' and Exon 8 (−) 5'-GTTCGTCACCCAGGGCCGTA-3'; Exon 5 (+) 5'-GGCTGGTGCTGATATTGAGA-3' and Exon 8 (−) 5'-GTTCGTCACCCAGGGCCGTA-3'; Exon 8 (+) 5'-TCTGGATACCCAGCAGAAGCGT-3' and Exon 13 (−) 5'-GCGGTCTTCTCAGCCAAAAGC-3'; Exon 10 (+) 5'-ATGCACAGCTGACCTGTAACGG-3' and Exon 13 (−) 5'-GCGGTCTTCTCAGCCAAAAGC-3'; Exon 13 (+) 5'-GGATCTGGAATTGAACGTCAG-3' and Exon 16 (−) 5'-GGGTGACAGACGCTGCTTGGT-3'; Exon 18 (+) 5'-GGCCGCAAGAGCGCGCTGC-3' and Exon 19 (−) 5'-GGTCGAATCTTGGTGGGAAGGCC-3'.

PCR products were electrophoresed on a 0.8% agarose gel and the significant bands were isolated and purified using Ultra Clean™ Gel Spin DNA Purification Kit (Mo Bio Laboratories, Carlsbad, CA) following the manufacturer's instructions. The purified DNA was sequenced (Microchemical Core Facility, SDSU) using the appropriate primers: Exon 2(+) 5'-TGGATCCCCGACGAGAAGGA-3', Exon 5 (+) 5'-GGCTGGTGCTGATATTGAGA-3', Exon13 (−) 5'-GCGGTCTTCTCAGCCAAAAGC-3', Exon 16 (−) 5'-GGGTGACAGACGCTGCTTGGT-3', Exon 18 (+) 5'-GGCCGCAAGAGCGCGCTGC-3'.

Quantification of transgenic protein expression level

SDS polyacrylamide gel electrophoresis [60] was used to quantify the MHC expression level in *yw* and each of the transgenic fly lines. Upper thoraces from at least six 1–2-day-old female flies were individually homogenized in 60 µl of sample buffer. Samples were boiled for 3 min and then centrifuged. Twelve µl samples of supernatants were electrophoresed on a 10% acrylamide separating, 4% stacking 12-well precast Tris–glycine minigel (Bio-Rad, Richmond, CA). Wild-type standards (*yw* upper thorax extracts) at various concentrations were loaded in 5–6 wells. Gels were stained with Coomassie blue, destained, scanned on an Epson Expression 636 flatbed scanner, and analyzed using the gel plotting macros of the

NIH image plotter package[§]. For each lane, the myosin to actin ratio was determined and expressed relative to the average *yw* myosin to actin ratio.

***In vitro* motility assay and ATPase assay**

Myosin was purified from the dorso-longitudinal indirect flight muscles of 100–200 young adults. As the expression of the *P838L* mutation was verified in all three transgenic lines, all were used for myosin preparations (*n* = 9 each, for mutant and for control). Ca-, Mg-, and actin-activated Mg-ATPase assays were carried out as previously described, with V_{max} and K_m values determined by fitting Mg-ATPase activities at increasing actin concentrations to the Michaelis–Menten equation using SigmaPlot [61]. Ca-ATPase levels are non-physiological but assess maximal ATPase activities in the absence of actin. The *in vitro* motility assay involves video capture of stabilized fluorescent actin filaments moving on myosin-coated cover slips in the presence of ATP. This assay was performed as previously described, with at least 40 smoothly moving filaments analyzed per sample [59,62,63]. *In vitro* motility and ATPase assays were performed immediately after the myosin purification. Mutant and wild-type results were compared for statistical significance by Student's *t*-tests (significance = $p < 0.05$).

Myosin torsional stiffness measurements

The rotational motion of surface immobilized actomyosin was determined similarly to the method described previously [64,65]. For these experiments, flow cell loading and preparation were analogous to that of *in vitro* motility assays, except that flow cells were loaded with one volume of myosin at concentrations ranging from 100 ng/ml to 1 µg/ml. The lower myosin concentrations assured that the neighboring actin filaments would not interfere with each other and that a single actin filament would only bind to a single myosin molecule. Flow cells were washed with one volume (50 µl) of 10 mg/ml BSA to remove unbound myosin and block any uncovered areas on the nitrocellulose-coated coverslip surface. Next, flow cells were washed by flowing through two volumes of low salt buffer followed by one volume of TRITC-phalloidin actin (10 nM in low salt buffer) that had been vortexed vigorously to produce relatively small filaments (1–4 µm). Actin was incubated for 5 min to ensure myosin binding. Movies of actin filament rotation from a single myosin-mediated surface attachment point were captured at 30 frames per second for a total of 30 s.

The torsional stiffness, the thermally generated rotation of the actin while attached to myosin, was derived via the variance in actin angles. For each frame, the angle of the swiveling actin filament was quantified based on the secant line formed between the filament ends using a custom-written Matlab program. The principle of energy equipartition was used to calculate the torsional stiffness of the myosin head as $\kappa = k_B^* T / \langle \theta^2 \rangle$ for each myosin head and averaged overall samples, as described previously [64–66]. Here, k_B denotes the Boltzmann's constant, T is the absolute temperature, and $\langle \theta^2 \rangle$ is the measured variance in the angles of the swiveling actin filament. Torsional stiffness was determined for ~80 molecules per group. Data were logarithmically transformed to establish normal distribution,

[§]<http://rsb.info.nih.gov/nihi-image/>

and a statistical difference in torsional stiffness between transgenic control and *P838L-6F* myosin was determined using Student's *t*-test (significance = $p < 0.05$).

Myosin molecule imaging and determination of head orientations

Control and P838L myosin molecules were purified, rotary shadowed, and imaged at room temperature with a FEI Tecnai 12 transmission electron microscope operating at 120 kV at a magnification of 21,000-fold, as previously described [67]. Images were acquired with a TVIPS (Tietz) TemCam-F214 digital camera.

The analysis of the inter S1 angle (θ) between myosin heads was performed manually using FIJI image analysis software [68]. Measurements were performed with the user blinded as to control or *P838L-6F* samples. For each myosin molecule, the user selected a single point for each myosin head and one for the junction of the two head domains with the tail. The angle was calculated from the inner product of the two lines made by the points connecting the junction of the two head domains with the tail and each myosin head (Fig. 2C). Molecules where either myosin head was not visible were excluded from analysis. Angles from 111 myosin molecules from each group were measured. Data were logarithmically transformed to establish normal distribution and a statistical difference in θ between control and P838L myosin was determined using Student's *t*-test (significance = $p < 0.05$).

Flight testing

Flight testing was performed at room temperature [69]. Approximately 100 flies from each line were collected and aged in plastic vials containing food for 2, 7, and 14 days at 25 °C and then kept at room temperature for two h prior to testing. Individual flies were released in the center of a Plexiglas box (43 cm H $\times$ 27.5 cm W $\times$ 43 cm L) with a bright light source at the top. Each was scored for its ability to fly upward (U), horizontally (H), downward (D), or for no flight (N). Flight index was calculated by assigning a value of 6 for U, 4 for H, 2 for D, and 0 for N [51]. Flight Index was calculated as $6*U/T + 4*H/T + 2*D/T + 0*N/T$, with T being the total number of flies tested for each line. Wing beat frequency was determined, as described by Hyatt and Maughan [70], with statistical comparison made via Student's *t*-test (significance = $p < 0.05$).

Skinned IFM fiber preparation and mechanical analysis

Fiber preparation and mechanical evaluation were performed, as described previously [38]. Briefly, a bundle of dorsal longitudinal IFM fibers was isolated from a 2–3-day-old fly and chemically demembranated in dissection solution. A single fiber was separated from the bundle, split lengthwise, and secured on either end with aluminum foil t-clips. The length, width, and height of the fiber were measured in relaxing solution. The cross-sectional area was calculated using the formula for the area of an ellipse. Using the t-clips, the fiber was mounted on a mechanics rig, in 15 °C relaxation solution, between a length motor and force transducer. The fiber was activated in 12-mM ATP by increasing calcium to pCa 5.0 and it was lengthened until maximum power production was achieved. All mechanical measurements were performed at this length. Power, the frequency at which maximum power generation occurred (f_{max}), elastic modulus and viscous modulus were measured by imposing small amplitude sinusoidal length changes, 0.125%, on the fiber over a range of

frequencies, 0.5 to 650 Hz. To measure active isometric tension, we released the muscle fiber until it went slack. This tension level was defined as 0 (tension baseline) and the muscle was re-lengthened. Isometric tension was recorded ~15 s after the fiber was returned to its original length. The fiber was put into rigor by decreasing ATP to 0 mM, and rigor stiffness was measured as the elastic modulus value at 500 Hz. Statistical significance for all parameters was assessed by Student's *t*-test (significance = $p < 0.05$).

Electron microscopic analyses of IFM and cardiac muscles

For electron microscopy, late stage pupae were clipped anteriorly and posteriorly, relative to IFMs prior to fixation. Upper thoraces were dissected to prepare adult IFM samples. Dorsal vessels were isolated in an oxygenated artificial hemolymph solution, and normal rhythmic cardiac beating was confirmed to avoid hearts with structural perturbations [71]. All samples were relaxed in 10 mM EGTA and treated with primary fixative [3% formaldehyde, 3% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4)] overnight, secondary fixative [1% OsO₄, 10 mM MgCl₂, 100 mM phosphate buffer (pH 7.4)] for 2 h, and block stain (2% uranyl acetate) overnight. Samples were dehydrated with an acetone series, oriented, and embedded in Epon-filled BEEM capsules, followed by Epon polymerization at 60 °C under vacuum. Thin sections (20–50 nm) were obtained and stained with 2% uranyl acetate for 10 min and Sato's lead stain [72] for 2 min. Images were obtained at 120 kV on a FEI Tecnai 12 Transmission Electron Microscope. IFM sarcomere lengths (the distances between Z-disks) were measured in 2-day-old transgenic flies using ImageJ software. Twenty sarcomeres were measured from non-overlapping images for each of three flies per genetic line, yielding a total of 60 sarcomeres from each line. A one-way ANOVA with Dunnett corrections for multiple comparisons was employed for statistical analysis (significance = $p < 0.05$).

Cardiac functional analysis

Cardiac functional analysis was performed on *P838L-6F Drosophila* crossed into the *Mhc^L*-endogenous myosin null background (*P{P838L}/P{P838L}; Mhc^L/Mhc^L*). *PwMhc2 Drosophila* [57] crossed into the *Mhc^L* [43] null background (*P{Mhc⁺}/P{Mhc⁺}; Mhc^L/Mhc^L*) served as controls. In addition, hearts of *P838L-6F Drosophila* were examined in the heterozygous state, by crossing a single copy into the *Mhc^L* heterozygous background (*P{P838L}; Mhc^L/+* compared to *P{Mhc⁺}; Mhc^L/+*). Cardiac analysis was also performed on *Drosophila* carrying a *P{Mhc⁺}* or *P{P838L}* allele in *trans* with the *Mhc^S* allele (*P{P838L}; Mhc^S/Mhc^L* vs. *P{Mhc⁺}; Mhc^S/Mhc^L*).

Using FlyNap (Carolina Biological, Burlington, NC), 20–35 flies per group were anesthetized, and hearts were microdissected in oxygenated artificial hemolymph, as described previously [71]. Myogenic contractions of cardiac tissue in abdominal segments 2–3 were recorded using a high-speed digital camera (120–140 frames/s, Hamamatsu EM-CCD 9300) and image capture software (Simple PCI, Hamamatsu Corp., Bridgewater, NJ) on a Leica DM LFSA microscope. M-modes of heart wall movements over time were obtained from videos using Semi-automatic Optical Heart Beat Analysis software[¶] [44]. A

[¶]<http://sohasoftware.com/>

specialized computer algorithm [44] was used to determine heart period, chamber dimensions during mid-diastole and mid-systole and fractional shortening. Significant differences in heart period and chamber dimensions were assessed via one-way ANOVA followed by a Bonferroni post hoc test to compare each *P838L*-expressing line with its appropriate control.

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Abbreviations used

RCM	restrictive cardiomyopathy
HCM	hypertrophic cardiomyopathy
IFM	indirect flight muscles
MHC	myosin heavy chain
RT-PCR	reverse transcription-polymerase chain reaction
RLC	regulatory light chain

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Fig. 1.

ATPase and *in vitro* motility values for wild-type transgenic and P838L myosins. Ca-ATPase (A) and basal Mg-ATPase (B) levels were significantly enhanced (*) for P838L myosin relative to control ($p < 0.05$). In contrast, V_{max} (C), K_m (D), and catalytic efficiency (E) showed no statistically significant differences (ns). *In vitro* motility of actin filaments (F) is significantly increased (*) for the P838L mutant myosin compared to wild-type transgenic myosin ($p < 0.05$). Statistical comparisons made by Student's *t*-test; error bars represent SD.

Fig. 2.

Torsional stiffness and S1 head orientation of wild-type transgenic and P838L myosins. (A) Video microscopy was employed to determine the torsional stiffness of wild-type transgenic and P838L myosins by evaluating the rotational motion of fluorescently labeled F-actin bound to surface immobilized myosin. (B) The reduced torsional stiffness (*** $p < 0.001$) for P838L myosin indicates that the mutation yields increased flexibility of the S1 head relative to the S2 region. (C) Orientation of the two myosin heads relative to the S1/S2 junction (outlined in blue) was determined by measuring the angle (θ) from the inner product of two lines made by points connecting the junction of the two head domains with the tail and each S1 head of rotary shadowed molecules. (D) There is a significant increase (*** $p < 0.001$) in the inter S1 angle (θ) in P838L myosin compared to control. Statistical comparisons made by Student's t -test; error bars represent SD.

Fig. 3.

Transmission electron microscopy of IFM from transgenic control and *P838L* lines during aging. IFMs from control *pwMhc2* homozygotes in longitudinal (A) and transverse (A') orientations at 2 d following eclosion show highly organized sarcomeres with repeating Z- and M-lines, as well as myofibrils with a regular myofilament lattice. Each of the mutant transgenic lines, *P838L-6F* (B, B'), *P838L-1 M* (C, C'), and *P838L-3 M* (D, D'), displays normal myofibril morphology at 2 days. At 3 weeks post-eclosion, *pwMhc2* IFM retains normal patterns of sarcomere and myofilament organization (E, E'), while each of the mutants (F, F': *P838L-6F*; G, G': *P838L-1 M*; H, H': *P838L-3 M*) shows abnormal structural features such as sarcomere gaps (F, arrowhead), misshapen myofibrils (F', asterisk), and Z-line non-linearity (asterisks, G,H). M, mitochondrion. All of these flies lack endogenous IFM MHC, due to being homozygous for the *Mhc*¹⁰ allele. Bar for A and adjacent sections,

2 μm . Bar for A' and adjacent sections, 0.5 μm . Bar for E and adjacent sections, 2 μm . Bar for E' and adjacent sections, 0.5 μm .

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Fig. 4.

Mechanical power output *versus* frequency for IFM fibers from transgenic control and *P838L* lines. Chemically demembranated dorsal longitudinal IFM fibers from a 2–3-day-old flies were assessed for power generation by imposing small amplitude sinusoidal length changes on the fiber over 0.5 to 650 Hz with calcium at pCa 5.0. No statistically significant differences in power output was observed between fibers from *pwMhc2* homozygous control and homozygous *P838L-1 M* ($p = 0.223$, mixed ANOVA); error bars represent SEM. See Table 2 for details.

Fig. 5.

Transmission electron microscopic images of 4-day-old hearts from transgenic control and *P838L* homozygotes and heterozygotes. Transverse sections were prepared from cardiac tissue located between the second and third abdominal segments. Similar to *pwMhc2* homozygous control (A), *P838L-6F* homozygous mutant hearts (B) contain a normal cardiomyocyte layer thickness with non-degraded myofibrils. CM, cardiomyocyte layer; VL, ventral layer. Both *pwMhc2* control homozygote hearts (C, E) and *P838L-6F* mutant homozygote hearts (D, F) contain non-degraded myofibrils with discontinuous Z-lines (C, D arrows) and normal myofilamentous arrays (E, F). All homozygote transgenics are in the *Mhc^l* null background. Sarcomeres from *pwMhc2*⁺ (G) or *P838L-6F*⁺ (H) heterozygotes

(in the *Mhc^I/+* background) show normal morphology with discontinuous Z-lines (arrows). All bars represent 500 nm.

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Fig. 6.

Cardiac structure and physiology of 3-week-old transgenic control and *P838L* homozygotes and heterozygotes. High-speed video image analysis was used to determine various parameters of cardiac structure and function. No statistically significant differences for heart period, systolic interval, diastolic diameter, systolic diameter, and fractional shortening were observed between $P\{Mhc^{+}\}/P\{Mhc^{+}\}; Mhc^1/Mhc^1$ and $P\{P838L\}/P\{P838L\}; Mhc^1/Mhc^1$ or between $P\{Mhc^{+}\}; Mhc^1/+$ and $P\{P838L\}; Mhc^1/+$ *Drosophila*. The effects of placing the *P838L-6F* transgene in combination with *Mhc*⁵, an allele that causes cardiac constriction in the homozygous state [20] were also examined. $P\{P838L\}; Mhc^5/Mhc^1$ shows a statistically significant ($*p < 0.05$) increase in systolic diameter and a decrease in fractional shortening compared to $P\{Mhc^{+}\}; Mhc^5/Mhc^1$. Statistical significance was assessed by one-way ANOVA followed by a Bonferroni post hoc test; error bars represent SEM.

Table 1

Chromosomal locations of *P838L* transgenes, MHC expression levels, and flight indices

Transgenic Line	Chromosome	% MHC $\pm$ SEM (n)	Flight Index $\pm$ SEM (n)		
			2 days	1 week	2 weeks
<i>pw/Mhc2</i> (control)	X	100 (5) *	4.18 $\pm$ 0.17 (100)	3.72 $\pm$ 0.22 (97)	3.06 $\pm$ 0.22 (100)
<i>P838L-6F</i>	X	99.2 $\pm$ 2.2 (6)	2.32 $\pm$ 0.20 (100)	2.38 $\pm$ 0.19 (105)	1.71 $\pm$ 0.17 (106)
<i>P838L-1 M</i>	3	102.5 $\pm$ 2.3 (6)	3.45 $\pm$ 0.23 (99)	3.39 $\pm$ 0.23 (101)	2.44 $\pm$ 0.19 (101)
<i>P838L-3 M</i>	2	91.5 $\pm$ 2.4 (6)	1.30 $\pm$ 0.14 (106)	1.31 $\pm$ 0.14 (108)	0.58 $\pm$ 0.10 (100)

* *yw* strain with wild-type endogenous *Mhc* genes.

Table 2

IFM mechanical parameters of *P838L* versus wild-type transgenic (*pwMhc2*). The stiffness parameters, viscous (–Ev) and elastic (Ee) moduli were measured at the frequency at which maximum power generation occurred (f_{max}). Rigor stiffness is the elastic modulus value at 500 Hz when ATP = 0 mM. All other values were measured at pCa 5.0 and 12-mM ATP. Mean $\pm$ SEM, n = 8 for *pwMhc2*, n = 6 for *P838L-I M*. None of the mutant values was statistically different from *pwMhc2*, as assessed by Student's *t*-test (significance = $p < 0.05$)

	Max Power W/m ³	f_{max} s ⁻¹	–Ev kN/m ²	Ee kN/m ²	Isometric Tension kN/m ²	Rigor stiffness kN/m ²
<i>pwMhc2</i> (control)	165 $\pm$ 15	210 $\pm$ 7	178 $\pm$ 13	304 $\pm$ 28	3.15 $\pm$ 0.26	702 $\pm$ 84
<i>P838L-I M</i>	141 $\pm$ 21	213 $\pm$ 5	144 $\pm$ 20	283 $\pm$ 35	3.18 $\pm$ 0.39	598 $\pm$ 93