A *Drosophila melanogaster* Model of Diastolic Dysfunction and Cardiomyopathy Based on Impaired Troponin-T Function

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**Rationale:** Regulation of striated muscle contraction is achieved by Ca\(^{2+}\)-dependent steric modulation of myosin cross-bridge cycling on actin by the thin filament troponin–tropomyosin complex. Alterations in the complex can induce contractile dysregulation and disease. For example, mutations between or near residues 112 to 136 of cardiac troponin-T, the crucial TnT1 (N-terminal domain of troponin-T)–tropomyosin-binding region, cause cardiomyopathy. The *Drosophila* upheld\(^{101}\) Glu/Lys amino acid substitution lies C-terminally adjacent to this phylogenetically conserved sequence.

**Objective:** Using a highly integrative approach, we sought to determine the molecular trigger of upheld\(^{101}\) myofibrillar degeneration, to evaluate contractile performance in the mutant cardiomyocytes, and to examine the effects of the mutation on the entire *Drosophila* heart to elucidate regulatory roles for conserved TnT1 regions and provide possible mechanistic insight into cardiac dysfunction.

**Methods and Results:** Live video imaging of *Drosophila* cardiac tubes revealed that the troponin-T mutation prolongs systole and restricts diastolic dimensions of the heart, because of increased numbers of actively cycling myosin cross-bridges. Elevated resting myocardial stiffness, consistent with upheld\(^{101}\) diastolic dysfunction, was confirmed by an atomic force microscopy–based nanoindentation approach. Direct visualization of mutant thin filaments via electron microscopy and 3-dimensional reconstruction resolved destabilized tropomyosin positioning and aberrantly exposed myosin-binding sites under low Ca\(^{2+}\) conditions.

**Conclusions:** As a result of troponin–tropomyosin dysinhibition, upheld\(^{101}\) hearts exhibited cardiac dysfunction and remodeling comparable to that observed during human restrictive cardiomyopathy. Thus, reversal of charged residues about the conserved tropomyosin-binding region of TnT1 may perturb critical intermolecular associations required for proper steric regulation, which likely elicits myopathy in our *Drosophila* model. ([Circ Res. 2014;114:e6-e17.])

**Key Words:** *Drosophila* ■ myosins ■ tropomyosin

**Muscle contraction** results from an orchestrated series of molecular events that culminate in transient interactions between myosin-containing thick and actin-containing thin filaments. The high propensity for cyclic ATP-dependent actomyosin associations indicates that without regulation, muscle would remain in a continuous state of contraction, and hence nonfunctional. Striated muscle regulation is primarily achieved by Ca\(^{2+}\)-dependent modulation of myosin cross-bridge binding on actin by the thin filament troponin–tropomyosin complex.\(^{1-3}\) The complex consists of an elongated coiled-coil tropomyosin dimer that extends along 7 monomers of the long-pitch actin helix and 3 troponin subunits: troponin-C (TnC, the Ca\(^{2+}\)-binding subunit), troponin-I (TnI, the inhibitory subunit), and troponin-T (TnT, the tropomyosin-binding subunit). These elements (7 actin monomers, 1 troponin complex, and 1 tropomyosin molecule) comprise regulatory units, which run continuously in series along thin filaments.

It is generally acknowledged that regulatory units adopt various structural states characterized by different tropomyosin positions that govern contraction.\(^{1-5}\) At rest, regulatory units are maintained predominantly in the blocked (B) state.\(^{5-7}\) Here, tropomyosin influenced by TnI (and TnT; see below) inhibits contraction by sterically occluding myosin-binding sites on actin. On activation, Ca\(^{2+}\) binding to TnC promotes...
a release of TnI-mediated inhibition and the movement of tropomyosin azimuthally from subdomains 1 and 2 and toward subdomains 3 and 4 of F-actin, resulting in the closed (C) state.5,7 Initial myosin binding to actin subsequently has allosteric effects on thin filaments such that there is further displacement of tropomyosin and spread of accessible myosin-binding sites along regulatory units to establish the open (M) state and promote the cooperative activation of contraction.3,5,8 Thus, as more cross-bridges are recruited, there is a proportional increase in active tension generation and transmission of contractile force. The positional states adopted by tropomyosin, however, seem to represent average locations of the coiled-coil because tropomyosin is thought to oscillate dynamically between B-, C-, and M-states at all Ca2+ levels, and it is the average azimuthal location of this equilibrium that actually is controlled by tropolin, Ca2+, and myosin.3,9,10

The TnT subunit of the troponin complex consists of 2 domains that can be separated by limited proteolysis.11,12 The C-terminal TnT2 domain binds TnI and TnC. Together, they form the globular head, core domain of the troponin complex, and act as the Ca2+ sensor of the thin filament. The N-terminal tropomin-T (TnT1) domain is highly helical, terminates in the head-to-tail tropomyosin–tropomyosin overlap region, enhances the cooperativity of myosin S1 binding to the thin filament, and, by binding to tropomyosin, increases its affinity for actin.13–17 Moreover, roles for TnT1 in cardiac myofilament regulation are supported by several findings, including a report describing that the N-terminal domain of TnT, in the absence of all other troponin components, inhibits myosin interaction with actin–tropomyosin filaments as a result of the TnT1 tail promoting the B-state.18 These results suggest that the tropolin tail may directly contribute to the inhibition of muscle contraction in the absence of Ca2+ and plays a critical role in modulating the average position of tropomyosin under different physiological, and potentially pathological, conditions.

TnT is the transducer that links the effects of Ca2+-binding interactions of the globular head of troponin to the physical location of tropomyosin along the thin filament. This fundamental property is likely compromised by disease-causing mutations. Alterations in the cardiac TnT (cTnT) gene account for 15% of documented familial hypertrophic cardiomyopathy–causing mutations with 70% of these localized to cTnT1 (Figure 1).19–23 cTnT1 mutations are also linked to dilated cardiomyopathy and restrictive cardiomyopathy (Figure 1).21,25–26 Many of these mutations have been investigated in vivo, in vitro, and in silico and have been shown to result in a wide range of complex molecular and physiological effects.20,21

The diverse consequences of cTnT1 mutations correlate with the clinical heterogeneity of the myopathic responses.20,21 A detailed understanding of such disorders can benefit from model systems that facilitate integrative interrogation of disease pathogenesis from the level of the whole organ down to the molecular machinery. Drosophila melanogaster is a powerful genetic model for investigating the structure and function of striated muscle hierarchically. The indirect flight muscle (IFM) and cardiac muscle of insects are, in fact, comprised of myofibrillar proteins that are highly homologous to those expressed in vertebrates.27,28 Direct structural evidence indicates that the
stERIC troponin–tropomyosin-based regulatory mechanism of contraction operates in insects.29 Furthermore, Drosophila myofibrillar mutants show disparate cardiomyopathic responses analogous to those observed in humans.30,31 Drosophila has a single TnT gene. Specific constitutively expressed TnT point mutations do not perturb assembly of IFM myofibrils but cause their deterioration within days after utilization.32,33 The ascribed as Glu88Lys, localizes to the TnT1 domain.33 The corresponding IFM degenerative syndrome was ascribed to abnormal acto-myosin interactions.31 Since TnT1 forms crucial tropomyosin associations, modulates the average tropomyosin position along regulatory units, and accumulates numerous cardiomyopathy-causing mutations, we tested the hypothesis that the TnT1 amino acid substitution induces cardiomyopathy in flies because of contractile dysinhibition and disrupted sterile regulation. Thus, we sought to resolve the initial molecular step that triggers TnT1 IFM myofibrillar degeneration, to evaluate cardiomyocyte contractile performance, and to examine the effects of the lesion on the entire Drosophila heart in order (1) to ascertain critical regulatory roles for conserved TnT1 regions and (2) to provide a possible mechanistic basis for cardiac dysfunction. We show that TnT1 charge reversal induces cardiomyopathy, prolongs periods of muscle activation, and causes excessive cross-bridge formation even at rest. We demonstrate that these changes are associated with altered diastolic dimensions and mechanical properties of the heart and its constituent myocytes, in vivo. Direct visualization of TnT1 thin filaments reveals that the mutation strongly promotes formation of the C-state in the absence of Ca2+, when a B-state is expected, which likely serves as the initial molecular factor for cardiac pathology. Similarly, human cardiomyopathy mutations dispersed throughout this conserved region likely disrupt critical TnT1–tropomyosin interactions needed to establish Ca2+-dependent thin filament regulatory states.

Methods

Fly Lines, Culture Conditions, and Husbandry

up101 mutant flies, originally recovered from a mutagenesis screen of Canton-S Drosophila,32 were obtained from the Bloomington Drosophila Stock Center at Indiana University. The Canton-S strain served as the wild-type control line.

For up101 IFM thin filament isolation, up101 f ccr, b el Mhc23 cn double mutants were generated by standard mating procedures. The Mhc23 mutation prevents myosin heavy chain accumulation in the IFM. Thus, thick filament assembly and up101-induced IFM degeneration do not occur.35

All up101 analyses were performed on the hearts and thin filaments of homozygous animals.

Confocal Microscopy

Confocal microscopy was performed as detailed by Alayari et al34 with an Olympus Fluoview FV10i Confocal Microscope system at ×10 magnification.

Cardiac Image Analysis of Semi-Intact Drosophila Preparations

Cardiac tubes of 3-week-old, male and female adult flies (n=40–45) were surgically exposed under oxygenated artificial hemolymph (AH) according to Vogler and Occor.15

Image analysis of heart contractions was performed using high-speed movies of semi-intact Drosophila preparations as previously described.11,16 Movies (30 seconds) were taken at ≈120 frames per second using a Hamamatsu Orca Flash 2.8 CMOS camera on a Leica DM5000B TL microscope with a ×10 (NA, 0.30) immersion lens. M-mode kymograms were generated using a MATLAB-based image analysis program.37 Measurements of cardiac dimensions and contractile dynamics were obtained as output from the MATLAB-based program. Two-way ANOVAs were used to test if the effects of sex or genotype (or if an interaction exists) for each cardiac parameter were significant. When measured values were not normally distributed, data were logarithmically transformed and significance confirmed via 2-way ANOVA. Large sample sizes mitigated concern regarding infrequent and mild evidence of variance heterogeneity. Significance was assessed at P<0.05.

Imaging of Blebbistatin-Induced Changes in Cardiac Dimensions

Beating hearts from 3-week-old female Canton-S (n=15) and up101 (n=20) flies were imaged as described previously using a ×20 (NA, 0.50) immersion objective lens. The hearts were recorded at various focal depths to resolve clear cardiac edges along the length of the tube. After filming, hearts were treated with 100 μmol/L blebbistatin (Cayman Chemical, Ann Arbor, MI) in AH for ≤30 minutes at room temperature. After complete blebbistatin-induced cessation of beating, cardiac tubes were fixed (8% formaldehyde in 1× PBS) for 20 minutes at 25°C and rinsed 3 times for 10 minutes in 1× PBS with continuous shaking. The hearts were filmed again posttreatment at various focal depths.

Movies of individual hearts, pre- and postblebbistatin treatment, were opened in HCImage Live software, Diastolic and blebbistatin-relaxed diameters were measured at identical longitudinal distances and focal depths, which permitted multiple clear-edge views along the tubes. Three distinct diameter measures were recorded across opposing cardiomyocytes of each heart tube and averaged for each fly. The effect of blebbistatin treatment on cardiac diameters was evaluated using a paired t test of the means of the matched groups before and after blebbistatin incubation. An unpaired t test was used to identify significant differences in the cardiac response to blebbistatin between genotypes. Significance was assessed at P<0.05.

Nanoindentation by Atomic Force Microscopy

Nanoindentation, to determine the transverse stiffness at the cellular seam of the conical chamber, was performed with an Asylum Research MFP-3D Bio Atomic Force Microscope mounted on a Nikon Ti-U fluorescence inverted microscope with a 120 pN/nm silicon nitride cantilever premounted with a 2-μm radius borosilicate sphere (Novascan Technologies, Ames, IA) as previously described.38 Before indentation, ≤3 semi-intact heart preparations were immobilized on glass coverslips and submerged in AH. Myogenic contractions were confirmed and then arrested with administration of 10 mmol/L EGTA in AH. Eight force curves per conical chamber were obtained from discrete locations at the ventral midline from 3-week-old female Canton-S (n=14) and up101 (n=15) flies. After indentation in EGTA, hearts were washed with fresh AH, and restoration of myogenic contraction was confirmed. Hearts were then incubated in 100 μmol/L blebbistatin in AH. Inhibition of contraction was verified ≤30 minutes of incubation. Indentation was repeated at the same cardiac locations. After final indentation, blebbistatin was photoactivated and resumption of myogenic contraction was visually confirmed to ensure that myocardium remained viable. Force indentation curves were analyzed with automated, custom-written software in MATLAB to calculate myocardial elastic modulus or stiffness (E, measured in Pa) as described previously.38 All force curves recorded from each heart tube were averaged for each fly. The effect of blebbistatin treatment on cardiac stiffness was evaluated using a paired t test of the means of the matched groups before and after incubation. Unpaired t tests were used to identify significant differences in stiffness between genotypes under each chemical condition and in the cardiac response to blebbistatin among genotypes. Significance was assessed at P<0.05.
Electron Microscopy and 3-Dimensional Reconstruction

IFM thin filaments were isolated from the thoraces of up\textsuperscript{101} f.car, b el Mhc\textsuperscript{17} cn Drosophila according to Cammarato et al.,\textsuperscript{29} with the following modifications: the chemically skinned thoraces were rinsed in 25 mL of rigor solution and homogenized in 0.5 mL of fresh buffer in a glass homogenizer. Homogenates were centrifuged at 16000 g for 60 minutes to sediment particulate material, including trace amounts of non-IFM thin filaments still bound in rigor to thick filaments derived from additional thoracic muscles. Then, 0.3 mL of the resultant supernatant, which contained IFM thin filaments, was diluted 2- to 10-fold with either rigor solution by itself or the buffer with 0.1 mmol/L CaCl\textsubscript{2} added in excess of the EGTA present, immediately before preparation for EM. Thin filaments were negatively stained and imaged as detailed previously.\textsuperscript{5,20,39}

Helical reconstruction, which resolves actin monomer structure and tropomyosin strands, but not troponin, was performed on IFM thin filament segments, which encompassed 4 to 7 regulatory units from 18 up\textsuperscript{101} filaments maintained in EGTA and 14 up\textsuperscript{101} filaments maintained in the presence of Ca\textsuperscript{2+}, according to standard methods.\textsuperscript{5,39,40} The statistical significance of densities in the reconstructions was computed from the standard deviations associated with contributing points as previously described.\textsuperscript{5,39,41,42}

Results

The Homologous Tropomyosin-Binding Region of TnT1 Is Associated With Multiple Disease Mutations

The strongest association between tropomyosin and troponin involves a C-terminal recognition site of striated muscle tropomyosin and TnT1.\textsuperscript{33,44} Likewise, evolutionarily conserved sequences within TnT1 presumably mediate interaction with tropomyosin.\textsuperscript{33,44} In particular, multiple sequence alignments identified a highly conserved and highly charged domain corresponding to residues 112 to 136 of human cTnT1 that likely comprise a critical tropomyosin-binding element (Figure 1).\textsuperscript{44} The importance of this TnT1 domain is further underscored by the high number of disease-inducing mutations that reside within and closely flank this region (Figure 1). The Drosophila up\textsuperscript{101} TnT glutamic acid to lysine amino acid substitution is located just downstream of this element and likely perturbs TnT1–tropomyosin interactions.

up\textsuperscript{101} Mutant TnT Alters Cardiac Morphology

Adult Drosophila melanogaster possess a 1-mm-long pulsatile heart tube, which is located in the abdomen along the insect’s body axis and is composed of a single layer of contractile cardiomyocytes (Figure 2A).\textsuperscript{49,50} The cardiomyocytes form bilateral rows that join together through specialized cell junctions to create a simple linear tube that extends down the dorsal body wall.\textsuperscript{47,48}

Drosophila cardiac morphology is sensitive to mutation. Alexa594-phalloidin staining of fixed Canton-S Drosophila heart tubes revealed typical spiraling myofibrillar arrangements within the cardiomyocytes (Figures 2B and 2C). The fibers were tightly packed with myofilamentous F-actin. Compared with Canton-S hearts, up\textsuperscript{101} TnT mutant hearts exhibited an apparent loss of myofilaments as noted by reduced F-actin staining relative to that in the retractors of tergite muscles. The mutant heart tube also appeared substantially narrower than that of the wild-type control. The overall morphological effects of the up\textsuperscript{101} mutation on the heart, however, seem less severe than the autodestructive effects of the lesion on IFM.\textsuperscript{33}

up\textsuperscript{101} Mutant TnT Induces Restrictive Physiology and Diastolic Dysfunction

Beating Drosophila heart tubes were imaged to further investigate the pathophysiologial effects of the up\textsuperscript{101} TnT1 mutation on live cardiac muscle. M-mode traces, which illustrate the positions of the heart wall edges over time, reveal cardiac dimensions and cardiac contraction dynamics (Figure 3A). up\textsuperscript{101} hearts were characterized by reduced diameters and sustained periods of systole relative to control hearts.

We quantified the effects of altered TnT1 on cardiac dimensions and contractile performance and compared the data to those obtained from Canton-S flies (Figure 3B; Online Table I). Mutant TnT1 expression resulted in significantly reduced diastolic and systolic diameters for both male and female up\textsuperscript{101} relative to control flies. Consistent with 6% (Canton-S) and 4% (up\textsuperscript{101}) sex-related differences in body size (Online Figure I), female Drosophila had significantly larger cardiac diameters relative to male flies. A significant interaction effect was determined between genotype and sex for diastolic and systolic diameter measurements. This suggests the up\textsuperscript{101} mutation differentially perturbs male versus female heart dimensions. The effect of the TnT1 lesion on diastolic diameters was greater than that on systolic diameters, which resulted in a significant decrease in mutant fractional shortening relative to control. The mutation influenced male versus female cardiac shortening differentially (interaction P value <0.0001). Overall up\textsuperscript{101} hearts appeared restricted and unable to relax during diastole adequately.

We additionally evaluated inherent myogenic contractile dynamics using motion analysis software (Figure 3B; Online Table I).\textsuperscript{37} The heart period, which is the combined length of time required for a single diastolic and subsequent systolic
event of the cardiac cycle, was significantly shorter for \textit{up\textsuperscript{101}} mutant flies relative to control. Therefore, the mutants display a faster myogenic heart rate. Moreover, a significant interaction effect was determined between genotype and sex, which suggests that the TnT1 lesion differentially perturbs the heart rate of male flies relative to female flies. The \textit{up\textsuperscript{101}} systolic interval was significantly prolonged relative to that of Canton-S. The degree of prolonged contractile events of the mutant hearts was determined to be sex-dependent (interaction \(P=0.003\)). Dividing the systolic interval by the heart period demonstrates the extent of time during the cardiac cycle the hearts are contracting. Regardless of sex, the \textit{up\textsuperscript{101}} TnT1 mutation promoted a significant increase in systolic interval/heart period ratio.

Thus, as observed in humans exhibiting restrictive cardiac physiology and diastolic dysfunction, the \textit{up\textsuperscript{101}} TnT1 mutation seemingly perturbs the ability of the myocardium to re-establish resting diastolic diameters and it prolongs systolic contractile events.

\textbf{\textit{up\textsuperscript{101}} Mutant TnT Reduces Cardiac Tube Diameters During Diastole by Increasing Actively Cycling Cross-Bridges}

Blebbistatin is a small-molecule inhibitor of several striated muscle myosins and impedes acto-myosin interaction in cardiac preparations from multiple species.\textsuperscript{49,50} Blebbistatin treatment of wild-type \textit{Drosophila} cardiac tubes completely prevented heart wall motion within 15 to 30 minutes (Figure 4A). Interestingly, the diameter across the heart wall in the presence of blebbistatin appeared slightly larger than the maximum diameter across the wall during peak diastole. This implies that in \textit{Drosophila} cardiac muscle, not all regulatory units are sterically preventing acto-myosin interactions at diastolic Ca\textsuperscript{2+} levels. To quantify the extent of basal, diastolic...
shortening, wild-type Canton-S fly hearts were filmed before and after incubation with blebbistatin. The average diameter after drug treatment was ≈3% greater than that determined during diastole (Figure 4B). This difference was highly significant. These data suggest that a small population of residual cross-bridges is actively cycling, generating force, reducing myocyte length, and establishing basal mechanical tone in wild-type myocardium during diastole. Interestingly, a paired analysis of \textit{up101} cardiac diameters during diastole and after blebbistatin treatment revealed a highly significant increase of ≈13% (Figure 4B). Comparing the change in diameters for each genotype revealed that the response to blebbistatin was significantly greater for \textit{up101} hearts than that for control hearts (Figure 4B). These observations are consistent with diastolic dysfunction and restrictive physiology due to excessively dysinhibited cross-bridge cycling, enhanced mechanical tone, and incomplete relaxation during diastole for \textit{up101} TnT mutant hearts relative to control hearts.

**Figure 4.** Blebbistatin has differential effects on diameters of control vs \textit{up101} TnT1 mutant hearts. Beating hearts were treated with 100 μmol/L blebbistatin (Bleb) to identify a potential contribution of disproportionate, strong acto-myosin interactions to \textit{up101}-mediated diastolic dysfunction. A, M-modes generated from an identical region of the same Canton-S (cs) control heart during its cardiac cycle and after blebbistatin-induced relaxation (top). Note the complete, blebbistatin-generated cessation of cardiomyocyte movement and a slight increase in diameter across the heart tube relative to that during diastole. Individual frames from movies of cs and \textit{up101} hearts during peak diastolic and postblebbistatin treatment time points (bottom). The cell-lengthening response to blebbistatin seems greater for mutant hearts relative to control. B, The diameter across discrete locations of cs hearts increased from 87.9±2.2 μm (mean±SEM) during diastole to 90.5±2.1 μm postblebbistatin incubation, whereas that for \textit{up101} hearts increased from 51.7±1.6 to 58.3±1.7 μm. A paired \(t\) test revealed that these responses were highly significant (**P<0.0001). The average change in diameter for \textit{up101} hearts (6.60±0.29 μm) was significantly greater (unpaired \(t\) test; ****P<0.0001) than that determined for cs hearts (2.50±0.43 μm). This is consistent with a greater number of dysinhibited strong acto-myosin associations during diastole, which promote enhanced myocyte shortening for \textit{up101} hearts relative to control. Scale bar, 50 μm.

**Figure 5.** Excessive cross-bridge cycling increases the transverse stiffness of \textit{up101} cardiomyocytes. An atomic force microscopy-based nanoindentation technique was used to measure basal myocardial stress and to assess residual active cross-bridges. A, Illustration portraying the nanoindentation scheme of \textit{Drosophila} myocardium. Indentations were made at the ventral midline of the heart tubes under 10 mmol/L EGTA-relaxed and 100 μmol/L blebbistatin-relaxed (Bleb) conditions. Longitudinal stress, derived from active and unimpeded force-generating acto-myosin associations is transmitted to the midventral seam and can be detected as elevated transverse stiffness via nanoindentation. B, Stiffness at identical midventral locations of Canton-S (cs) conical chambers decreased from 1.97±0.25 kPa (mean±SEM) in EGTA to 1.49±0.16 kPa postblebbistatin incubation, whereas that for \textit{up101} hearts decreased from 3.28±0.32 to 1.68±0.19 kPa. A paired \(t\) test revealed that these responses were significant for both cs (**P<0.01) and \textit{up101} (****P<0.0001) cardiomyocytes. Furthermore, the transverse cardiomyocyte stiffness for \textit{up101} hearts in EGTA was significantly greater (unpaired \(t\) test; \#P<0.01) than that determined for cs control hearts. The average blebbistatin-induced change in stiffness for \textit{up101} hearts (1.61±0.3 kPa) was significantly greater (unpaired \(t\) test; **P<0.01) than that for cs hearts (0.46±0.12 kPa). This is consistent with a higher number of basally dysinhibited acto-myosin interactions, which promote increased myocyte transverse stiffness under low Ca\(^{2+}\) conditions in \textit{up101} hearts relative to control.
Absence of Ca\(^{2+}\) Stiffness Under Low Ca\(^{2+}\) hence unlikely to sterically prevent contraction. We suspect IFM thin filaments, distal to known myosin-binding sites and tropomyosin is most associated with the inner aspect of actin (Ai) in the absence of Ca\(^{2+}\) within the average structure remaining in the B-state. Under both chemical conditions, however, mutant IFM thin filaments were primarily in the C-state with the outer domains of actin exposed. The average phase residuals (±SD), a measurement of the agreement among thin filaments generating reconstructions in each data set (EGTA and Ca\(^{2+}\)), were 61.6°±5.8° and 62.0°±7.8°, respectively. The average up–down phase residuals, a measure of the filament polarity, were 19.4°±5.1° and 20.4°±8.6°, respectively, values comparable to those previously reported. The average numbers of actin subunits per turn of the genetic helix of the IFM thin filaments were 2.155±0.007 and 2.156±0.008, respectively, consistent with a 28/13 helix. B, Helical projections for F-actin and up\(^{101}\) IFM thin filaments. Helical projections are formed by projecting component densities of the reconstructions down the long-pitch actin helices onto a plane perpendicular to the filament axis. Thus, the projections reveal the axially averaged positions of F-actin and tropomyosin, which appear symmetrical on either side of the filament. The up\(^{101}\) TnT1 mutation causes tropomyosin (Tm) to associate primarily with the inner domains of actin (Ai) in the absence of Ca\(^{2+}\). The helical projections revealed no difference in average tropomyosin position in the absence or presence of Ca\(^{2+}\), and thus steric regulation is disrupted as a result of the mutation. Densities in all maps were shown to be significant over background noise at greater than the 99.95% confidence levels.

**Figure 6. 3-Dimensional reconstructions of up\(^{101}\) thin filaments reveal perturbed steric regulation.** A, Surface views of reconstructed up\(^{101}\) indirect flight muscle (IFM) thin filaments showing the position of tropomyosin strands on actin. The 4 subdomains of a single actin monomer are labeled on the F-actin control reconstruction (subdomains-1 and -2, outer domain; subdomains-3 and -4, inner domain of F-actin). Note the presence of helically wound, continuous strands of tropomyosin density (double-headed black arrows) running longitudinally along the up\(^{101}\) thin filaments that are not seen on the F-actin control. Under both chemical conditions, tropomyosin clearly associated with the inner domains (subdomain-3) of successive actin monomers (triple gray arrows) on up\(^{101}\) thin filaments. In the absence of Ca\(^{2+}\), however, the surface view reveals a small amount of extra density extending from tropomyosin (black arrowheads) to the extreme inner edge of the outer domains (subdomain-1). This additional density is due to tropomyosin on 17% of mutant thin filaments within the average structure remaining in the B-state. Under both chemical conditions, however, mutant IFM thin filaments were primarily in the C-state with the outer domains of actin exposed. The average number of actin subunits per turn of the genetic helix of the IFM thin filaments were 2.155±0.007 and 2.156±0.008, respectively, consistent with a 28/13 helix. B, Helical projections for F-actin and up\(^{101}\) IFM thin filaments. Helical projections are formed by projecting component densities of the reconstructions down the long-pitch actin helices onto a plane perpendicular to the filament axis. Thus, the projections reveal the axially averaged positions of F-actin and tropomyosin, which appear symmetrical on either side of the filament. The up\(^{101}\) TnT1 mutation causes tropomyosin (Tm) to associate primarily with the inner domains of actin (Ai) in the absence of Ca\(^{2+}\). The helical projections revealed no difference in average tropomyosin position in the absence or presence of Ca\(^{2+}\), and thus steric regulation is disrupted as a result of the mutation. Densities in all maps were shown to be significant over background noise at greater than the 99.95% confidence levels.

**up\(^{101}\) Mutant TnT Promotes the C-State Even in the Absence of Ca\(^{2+}\)**

To determine the primary consequence of the up\(^{101}\) glutamic acid to lysine substitution and to provide a mechanistic basis for myopathy, we generated 3-dimensional reconstructions of purified thin filaments expressing the TnT mutation. Thin filaments were isolated from IFM of up\(^{101}\) Drosophila and either maintained in EGTA or treated with Ca\(^{2+}\) before negative staining. Electron micrographs showed that the thin filaments were not obviously different from control filaments and possessed the characteristic double helical array of actin subunits and additionally displayed periodic tropomyosin bulges and elongated tropomyosin strands (not shown). Three-dimensional reconstructions of mutant filaments were determined by helical analysis. Surface views showed that Ca\(^{2+}\)-dependent tropomyosin strand movement, typical of control filaments, was not a general feature of the mutants (Figure 6A). The majority of IFM thin filaments from up\(^{101}\) flies revealed tropomyosin strands that contacted subdomains 3 and 4 of successive actin monomers in both the absence and presence of Ca\(^{2+}\). However, 17% of individual Ca\(^{2+}\)-free mutant thin filaments differed and exhibited tropomyosin strands that contacted actin monomers along the inner edge of subdomains 1 and 2 of F-actin. When the 2 sets of Ca\(^{2+}\)-free data were combined, the tropomyosin density in helical projection can be seen to contact only the inner domains of actin (Figure 6B). Therefore, tropomyosin is most associated with the inner aspect of up\(^{101}\) IFM thin filaments, distal to known myosin-binding sites and hence unlikely to sterically prevent contraction. We suspect this is responsible for the destructive hypercontraction of IFM and for the excessive residual cross-bridge cycling in relaxed hearts.
Discussion

TnT makes extensive associations with multiple thin filament components, including the TnI–TnC binary complex, tropomyosin, and actin, and therefore is central to thin filament–mediated regulation of striated muscle contraction. TnT mutations, which predominately localize to the N-terminal TnT1 domain, are frequently associated with a host of myopathic responses (Figure 1). Several investigative efforts have shown that cardiomyopathic cTnT lesions in vitro induce a range of complex effects, including altered myofilament in vitro sliding velocity, disturbed Ca$^{2+}$ sensitivity of force generation, decreased cTnT–tropomyosin affinity, impaired ability to stabilize tropomyosin overlap, perturbed efficacy of promoting tropomyosin binding to actin, disrupted folding stability and secondary structure of cTnT1, and mutation-specific changes in peptide flexibility, all of which likely contribute to disease pathogenesis.14,51–55

Drosophila muscles are also sensitive to TnT alterations. Previously, constitutive TnT1 mutations were shown to drastically disrupt both the structure and function of IFMs within days of adulthood.33 We speculate that because the up101 mutation resides in the N-terminal region of TnT and it induces myosin-dependent IFM degeneration, the likely causes cardiomyopathy in flies due to contractile dysinhibition and perturbed steric regulation.

TnT-binding tropomyosins from vertebrates and invertebrates share a well-conserved amino acid segment thought to be a critical TnT1 recognition site.43 Similarly, multiple sequence alignments illustrate a high degree of evolutionary conservation among stretches of TnT1 postulated to be critical for tropomyosin associations (Figure 1).44 Residues 112 to 136 of human cTnT were found to be 70% homologous across analyzed sequences, which suggests an essential tropomyosin-binding role for this element throughout the animal kingdom. This region is highly charged, and intramolecular electrostatic associations likely dictate proper function. Importantly, engineered troponin constructs bind tightly to the thin filament only if they contain the entire 112 to 136 TnT1 domain.15,17,44,56 The Drosophila up101 mutation lies just downstream of this region. Introduction of basic charges could disturb conserved interactions immediately at the tropomyosin–TnT1 interface that are required for proper steric regulation. Similarly, cTnT cardiomyopathy mutations located in and adjacent to this region (Figure 1) are also expected to influence tropomyosin–TnT1 associations. Interestingly, our data illustrate that the up101 charge reversal mutation results in a cardiac phenotype reminiscent of human restrictive cardiomyopathy.

The up101 amino acid substitution may also promote molecular pathogenesis by potentially altering overall TnT performance, including mutation-driven propagated effects that could influence TnT function at a distance. These effects may involve (1) changes in helical stability of TnT1 and subsequently the flexibility of this region, which could compromise effective interactions with tropomyosin36; (2) alterations in local helical electrostatic compaction and consequently distal helical expansion that drives unwinding and flexibility changes at remote distances along TnT55.57; and (3) disruptions that possibly propagate through a complex structural pathway to perturb the affinity of cardiac TnC for Ca$^{2+}$.58 The latter could potentially contribute to changes in myofilament Ca$^{2+}$ sensitivity, as frequently observed in other models of TnT-based cardiomyopathies.21,54,58,59

Regardless of sex, the up101 Drosophila mutation markedly reduced diastolic volumes and extended systole in mutant relative to control hearts (Figure 3B). These changes are consistent with diastolic dysfunction, a hallmark of hypertrophic cardiomyopathy and restrictive cardiomyopathy. Diastolic dysfunction is characterized by impaired relaxation, decreased distensibility, and increased myocardial stiffness, which can result from excessive acto-myosin interactions.50,61 Thus, up101 Drosophila serve as a unique model to investigate the root of these pathological alterations in myocyte properties.

The diameter changes across the Drosophila heart, in response to the myosin-specific inhibitor blebbistatin, suggest that diastole in flies is accompanied by a small but significant population of residual, force-generating cross-bridges that actively shorten the cardiomyocytes and impact diastolic tone (Figure 4). Detection of cycling myosin cross-bridges at submaximal, diastolic levels of Ca$^{2+}$, which affect cardiomyocyte mechanical properties, is not unprecedented.62,63 Although beyond the scope of the current study, these basal acto-myosin associations could bolster the prominent stretch activation response common to insect muscle and may have important implications in myofilament length dependent activation of cardiomyocytes. Interestingly, specific TnT-induced restrictive cardiomyopathy and tropomyosin-causing hypertrophic cardiomyopathy mutations are associated with excessive cross-bicycle bridging during diastole.64,65 These thin filament mutations were shown to reduce basal sarcomere length and elevate diastolic tension of cardiomyocytes. Likewise, the reduced diastolic diameter of up101 hearts is due, in part, to excessive, less inhibited acto-myosin interactions that promote enhanced cell shortening relative to control hearts. Application of the myosin inhibitor, however, did not restore mutant heart diameter to that of blebbistatin-treated control flies (Figure 4B). Thus, additional remodeling events must transpire during the 3-week preanalysis period that influence up101 myocyte dimensions. Changes in cellular dimensions are consistent with pathological responses and tissue disorganization that accompany cardiac disease. For example, pathological stimuli associated with several cardiomyopathies induce changes in myocyte geometry and shape that help determine contractile function and whole heart morphology.66

The myosin-dependent changes in up101 cardiac dimensions are accompanied by differences in resting myocardial stiffness. We used a novel atomic force microscopy–based technique to resolve Drosophila myocardial tension disparities, with single cell resolution (Figure 5A).34,47 The geometric nature of the cardiac tube allows indentation and transverse stiffness determination from discrete cellular loci in the intact organ with no mechanical artifacts due to myocyte isolation, seeding, or plating. By determining the transverse stiffness at cell junctions, we can directly assess the degree of active longitudinal tension generated and transmitted to the
connections between the ends of coupled conical chamber myocytes. Thus, we can quantify the relative extent of contractile dysinhibition by comparing the transverse stiffness at the midventricular septum from different genotypes before and after incubation with blebbistatin. Control cardiomyocytes showed roughly a 25% drop in transverse stiffness at the midline on incubation with the myosin inhibitor, consistent with a small number of residual force-generating acto-myosin associations under low Ca$^{2+}$, diastole-like conditions (Figure 5B). The transverse stiffness of the up$^{101}$ cardiomyocyte junction was over 60% greater than that of control and showed $\approx$50% decrease after blebbistatin treatment. The large discrepancies in resting transverse stiffness and in response to the myosin-specific inhibitor indicate that the TnT1 mutation promotes a greater number of actively cycling, force-generating cross-bridges in up$^{101}$ myocytes relative to Canton-S myocytes, and these contribute to the diastolic dysfunction observed under relaxing conditions.

TnT is pivotal in modulating the average position of tropomyosin between B-, C- and M-states along thin filaments. Disease-causing mutations in troponin subunits may lead to changes in the distribution of these states and, therefore, disrupt regulation of contractile force. To assess a mutation-specific redistribution of regulatory states directly, we purified and imaged up$^{101}$ IFM thin filaments. In the complete absence of Ca$^{2+}$, surface views of 3-dimensional reconstructions of up$^{101}$ thin filaments revealed tropomyosin stands, on average, making contact with the inner domains of successive actin monomers along the long-pitch helices of the filament (Figure 6A). However, a small amount of density could be seen extending from the extreme inner edge of the outer domains of actin to the tropomyosin strands on these filaments (Figure 6A, black arrowheads). This extra density is the result of a small population of up$^{101}$ thin filaments, which in the absence of Ca$^{2+}$ exhibited tropomyosin in the B-state, as seen with wild-type filaments lacking Ca$^{2+}$,28 Nonetheless, the vast majority of up$^{101}$ thin filaments in the absence of Ca$^{2+}$ were shown to be in the C-state. Thus, TnT products of the up$^{101}$ allele may alter the equilibrium of tropomyosin position at rest such that, at any given time, the majority of thin filament regulatory units are in the C-state and not the B-state. This is consistent with the mutation disrupting the inherent, and possibly vital, B-state–promoting property of the TnT1 tail,18 a property that seems dependent on a TnT1 subdomain that includes the up$^{101}$ locus. When the up$^{101}$ mutation is expressed in vivo and in the presence of thick filaments, excessive cross-bridge cycling would occur in a dysregulated fashion. Over time, this could lead to destruction of IFM and drive restrictive remodeling in the heart.

Analysis of myocytes from several TnT-based cardiomyopathy models has revealed significant alterations in cellular Ca$^{2+}$ handling.69,70 Moreover, a Drosophila TnT mutant, which is characterized by an enlarged cardiac chamber and cardiac contractile dysfunction, displays abnormalities in the cytosolic Ca$^{2+}$ transient as well as changes in transcription of proteins associated with Ca$^{2+}$ handling.71 Therefore, the up$^{101}$ TnT mutant may similarly exhibit downstream alterations in Ca$^{2+}$ kinetics and homeostasis that potentially contribute to the observed cardiac phenotype. Quantitative polymerase chain reaction analysis was performed to assay possible changes in the Ca$^{2+}$-handling biosignature of up$^{101}$ relative to Canton-S hearts (Online Figure II).71 No significant differences were identified between the lines in transcript levels of L-type Ca$^{2+}$ channels, ryanodine receptors, SERCA, Na/Ca exchangers, or in inositol-3-phosphate receptors. Although this assay does not preclude possible posttranscriptional or posttranslational modifications that could influence the encoded proteins, nor does it completely rule out all potential adaptive Ca$^{2+}$ responses, the results suggest that the cardiac phenotype we observed is primarily due to the direct, Ca$^{2+}$-independent, C-state–promoting effect the up$^{101}$ TnT mutation exerts on thin filaments.

Our integrative data are consistent with a mechanism of diastolic dysfunction and restrictive cardiac pathology based on a fundamental inability of the homozygous up$^{101}$ TnT1 mutant thin filaments to properly block myosin cross-bridge cycling at rest (Figure 7). Here, a disproportionately large number of up$^{101}$ regulatory units adopt the C-state under low Ca$^{2+}$ conditions. This permits an exceedingly high number of strong stereospecific acto-myosin associations and excessive formation of the M-state that would promote decreased diastolic heart chamber volumes and elevated diastolic myocardial stiffness. Moreover, inordinate myosin binding increases the affinity of troponin for Ca$^{2+}$,1,72 Thus, fewer mutant regulatory units required to undergo Ca$^{2+}$-dependent unblocking combined with enhanced Ca$^{2+}$ sensitivity primes...
the system for systole. As a result, for a given Ca2+ transient, systole would commence earlier and terminate later, which is consistent with the highly prolonged systolic intervals observed in up101 hearts relative to control.

We anticipate similar, potentially less severe responses in up101 heterozygotes. Because of cooperativity of contractile activation and the continuous nature of regulatory units, the effects of the mutation could potentially be transmitted to neighboring, nonmutant regulatory units and thus influence the regulatory status of regions up- and downstream of the lesion. These propagated effects along the thin filament may also be sufficient to promote myosin cross-bridge cycling, decreased diastolic chamber volumes, and elevated diastolic myocardial stiffness, but potentially to a lesser extent than that found in homozygotes because of the presence of some normally functioning, wild-type regulatory units. Thus, as with other models of cardiomyopathy, we expect a relationship between the number of mutant up101 alleles and phenotype severity.74–77

TnT1 is essential for proper transduction of Ca2+ signals and modulation of tropomyosin position along regulatory units in striated muscle. Here, we examined the effects of the constitutively expressed up101 TnT1 mutation at multiple levels in Drosophila. We provide the first direct structural evidence of how a mutation, adjacent to the conserved tropomyosin-binding element of TnT1, perturbs steric regulation, promotes contractile dysinhibition and diastolic dysfunction, and drives cardiac remodeling. Our results emphasize the potential significance of tropomyosin–TnT1 electrostatic associations for proper steric regulation throughout the animal kingdom. Numerous indices of up101 cardiac function suggest a shift in equilibrium status of thin filament regulatory units to a mutation-induced preponderance of the C-state even at rest. We propose that human cardiomyopathy mutations located in and close to the homologous tropomyosin-binding element of TnT1 may also alter the fundamental B-state–promoting role of TnT118 and thereby activate pathological remodeling cascades. Overall, our study indicates that Drosophila is a valuable tool for investigating the most proximal, direct effects of thin filament lesions and that flies are an effective and unique model to resolve, at multiple levels, how such mutations perturb the normal distribution of force-generating regulatory states and elicit cardiac dysfunction.

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Disclosures

None.

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What Is Known?
- The N-terminal troponin-T tail promotes a conformational state of the thin filament that inhibits muscle contraction.
- Mutations in and around the highly conserved tropomyosin-binding region of the N-terminal cardiac troponin-T tail result in diverse human cardiomyopathies.
- The Drosophila melanogaster glutamic acid to lysine up101 troponin-T mutation resides just downstream of the homologous tropomyosin-binding element and results in skeletal muscle degeneration.

What New Information Does This Article Contribute?
- up101 Drosophila exhibit a cardiac phenotype that is characterized by restrictive physiology and diastolic dysfunction.
- Under relaxing conditions, up101 cardiomyocytes are shorter and stiffer compared with control cardiomyocytes because of elevated numbers of basally cycling myosin cross-bridges.
- The up101 troponin-T mutation disrupts the thin filament conformational state that suppresses myosin activity under low Ca2+ conditions.

Troponin-T mutations result in heterogeneous and diverse myopathies, and in vivo models of such disorders facilitate understanding of the underlying pathophysiology. This study describes an integrative approach to characterize the effects of the up101 mutation on the Drosophila heart. We show how a troponin-T lesion, adjacent to the evolutionarily conserved and highly charged N-terminal tropomyosin-binding element, perturbs muscle regulation, promotes contractile dysinhibition and diastolic dysfunction, and drives cardiac remodeling. Our results highlight the potential significance of troponin-T–tropomyosin electrostatic associations for proper contractile regulation. Human cardiomyopathy mutations that localize in, and close to, the well-conserved tropomyosin-binding domain of troponin-T may also alter thin filament regulatory conformational states and thereby contribute to activation of pathological remodeling cascades.
A *Drosophila melanogaster* Model of Diastolic Dysfunction and Cardiomyopathy Based on Impaired Troponin-T Function
Meera Cozhimuttam Viswanathan, Gaurav Kaushik, Adam J. Engler, William Lehman and Anthony Cammarato

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Correction

In the Circulation Research article by Viswanathan et al (A Drosophila melanogaster model of diastolic dysfunction and cardiomyopathy based on impaired troponin-T function. Circ Res. 2014;114:e6–e17. DOI: 10.1161/CIRCRESAHA.114.302028), the following changes were missed in the final publication:

1. Page e8, 1st column, line 23, sentence changed to “We show that up101 charge…”
2. Page e8, 1st column, line 26, sentence changed to “We demonstrate that these changes are…”
3. Page e9, 2nd column, 4th paragraph, line 15, sentence changed to “Inhibition of contraction was verified <30 minutes…”
4. Page e10, Figure 2 legend, lines 14–15, sentence changed to “Depending on sex and on genotype, on average the remainder of wild-type heart tubes is…”
5. Page e10, 1st column, 4th line from bottom, sentence changed to “…F-actin staining relative to that in the retractors…”
6. Page e13, 1st column, 1st heading changed to: “up101 Mutant TnT Enhances Myocardial Stiffness Under Low Ca2+”
7. Page e13, 1st column, 2nd heading changed to: “up101 Mutant TnT Promotes the C-State Even in the Absence of Ca2+”
8. Page e13, 1st column, 1st sentence under 2nd heading, sentence changed to “To determine the primary consequence of the up101 glutamic acid to lysine substitution and to provide…”
9. Page e14, 2nd column, 3rd paragraph, 7th line from bottom, sentence changed to “…3-week pre-analysis period that influence up101 myocyte…”

The errors have been corrected in the online version of the article, which is available at http://circres.ahajournals.org/content/114/2/e6.full.

(Circ Res. 2014;114:e28.)
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Online Supplemental Methods

Fly lines, culture conditions and husbandry:

_**upheld**<sup>101</sup> (up<sup>101</sup>) mutant flies, originally recovered from a mutagenesis screen of Canton-S _Drosophila_<sup>1</sup> were obtained from the Bloomington _Drosophila_ Stock Center at Indiana University. The Canton-S strain served as the wildtype control line. All _Drosophila_ were maintained on a standard yeast-sucrose-agar medium at 25°C. Newly eclosed flies were collected and sexually segregated, over an eight hour span. Flies were transferred to fresh vials every two-three days over a three-week period.

For _up<sup>101</sup> IFM thin filament isolation, _up<sup>101</sup> f car; b el Mhc<sup>12</sup> cn_ double mutants were generated by standard mating procedures. _Mhc<sup>12</sup>''IFM myosinless'' _Drosophila_ served as starting material. The genetic alteration in the _Mhc<sup>12</sup>_ strain prevents myosin heavy chain accumulation in the IFM. Thus thick filament assembly and _up<sup>101</sup>_ induced IFM degeneration does not occur. The _Mhc<sup>12</sup>_ strain has identifiable phenotypic markers, _b, el_, and _cn_ flanking the myosin gene. These markers were used to follow the _Mhc<sup>12</sup>_ lesion in subsequent crosses. Crosses of _b el Mhc<sup>12</sup> cn_ _Drosophila_ with _up<sup>101</sup>_ flies possessing the _f_ and _car_ markers were carried out to obtain a stable double mutant _up<sup>101</sup>_; _Mhc<sup>12</sup>_ stock. This stock was used exclusively for native IFM thin filament purification. The filaments are replete with all regulatory components and are thus well-suited for structural and biochemical analyses.<sup>2-5</sup>

Confocal microscopy:

Confocal microscopy was performed as detailed by Alayari et al. (2009).<sup>6</sup> Three-week old female wildtype Canton-S and _up<sup>101</sup>_ _Drosophila_ hearts were surgically exposed and arrested using 10mmol/L EGTA in artificial hemolymph. Relaxed hearts were fixed (4% formaldehyde in 1X PBS) and washed thrice with 1X PBST (PBS with 0.1% Triton X-100). Fixed hearts were then stained with Alexa594 TRITC-phalloidin (1:1000 in PBST), rinsed thrice in 1X PBST, mounted and imaged with an Olympus FluoView FV10i Confocal Microscope system at 10x magnification.

Preparation of semi-intact _Drosophila_ and cardiac image analysis of beating hearts:

Cardiac tubes of three-week old male and female adult flies (n=40-45) were surgically exposed under oxygenated artificial hemolymph according to Vogler and Ocorr (2009).<sup>7</sup> Briefly, flies were anesthetized and the heads, ventral thoraces, and ventral abdominal cuticles were removed, revealing beating heart tubes. All internal organs and abdominal fat were carefully discarded leaving the heart and associated cardiac tissues.

Image analysis of heart contractions was performed using high speed movies of semi-intact _Drosophila_ preparations as previously.<sup>8-11</sup> 30 second movies were taken at ~120 frames per second using a Hamamatsu Orca Flash 2.8 CMOS camera on a Leica DM5000B TL microscope with a 10x immersion lens. M-mode kymograms were generated using a MATLAB-based image analysis program.<sup>10</sup> This provides a trace that documents the movement of the heart tube edges on the y-axis over time on the x-axis.

Measurements of cardiac diameters at peak diastolic and systolic time points were made at two locations along the third abdominal segment of each heart tube, directly from individual movie frames, and averaged together. These individual mean values for all flies of a particular genotype or gender were then used to establish average cardiac diameters. Fractional shortening as well as heart periods and systolic intervals were obtained as output from the MATLAB-based program.<sup>10</sup>
Imaging of blebbistatin-induced changes in cardiac dimensions:

Beating hearts from three-week old female Canton-S (n=15) and up101 (n=20) flies were imaged as described above using a 20x (0.50 NA) immersion objective lens. The hearts were recorded at various focal depths to resolve clear cardiac edges along the length of the tube. After filming, hearts were treated with 100µmol/L blebbistatin (Cayman chemical, Ann Arbor, MI) in artificial hemolymph for ~30 minutes at room temperature. Following complete blebbistatin-induced cessation of beating, cardiac tubes were fixed (8% formaldehyde in 1x PBS) for 20 minutes at 25°C and rinsed three times for 10 minutes in 1xPBS with continuous shaking. The hearts were filmed again post-treatment at various focal depths.

Movies of individual hearts, pre- and post-blebbistatin treatment were opened in HCImage Live software. Diastolic and “blebbistatin-relaxed” diameters were measured at identical longitudinal distances and focal depths, which permitted multiple clear edge views, along the tubes. Three distinct diameter measures were recorded across opposing cardiomyocytes of each heart tube and averaged for each fly. The effect of blebbistatin treatment on cardiac diameters was evaluated using a paired t-test of the means of the matched groups before and after blebbistatin incubation. An unpaired t-test was used to identify significant differences in the cardiac response to blebbistatin between genotypes. Significance was assessed at p<0.05.

Nanoindentation by atomic force microscopy (AFM):

Nanoindentation, to determine the transverse stiffness at the cellular seam of the conical chamber, was performed with an Asylum Research MFP-3D Bio Atomic Force Microscope mounted on a Nikon Ti-U fluorescence inverted microscope with a 120pN/nm silicon nitride cantilever pre-mounted with a two µm-radius borosilicate sphere (Novascan Technologies, Ames, IA) as previously described.12 Prior to indentation, up to three surgically exposed, beating fly hearts were immobilized on glass coverslips and submerged in freshly oxygenated artificial hemolymph. Myogenic contraction was confirmed and then arrested with administration of 10mmol/L EGTA in hemolymph. Eight force curves per conical chamber were obtained from discrete locations at the ventral midline from three-week old female Canton-S (n=14) and up101 (n=15) flies. Following indentation in EGTA, hearts were washed with fresh hemolymph and restoration of myogenic contraction was confirmed. Hearts were then incubated in 100µmol/L blebbistatin in hemolymph. Inhibition of contraction was confirmed within 30 minutes of incubation. Indentation was repeated at the same cardiac locations. Following final indentation, blebbistatin was photo-inactivated and resumption of myogenic contraction was visually confirmed to ensure myocardium remained viable. Indentation was performed with an approach and retraction velocity of 1µm/s and <5nN of total load at maximum indentation. No hysteresis or adhesion was observed. Force-indentation curves were analyzed with automated, custom-written software in MATLAB to calculate myocardial elastic modulus or “stiffness” (E, measured in Pascal; Pa) as described previously.12 All force curves recorded from each heart tube were averaged for each fly. The effect of blebbistatin treatment on cardiac stiffness was evaluated using a paired t-test of the means of the matched groups before and after incubation. Unpaired t-tests were used to identify significant differences 1) in stiffness between genotypes under each chemical condition and 2) in the cardiac response to blebbistatin among genotypes. Significance was assessed at p<0.05.
**Electron microscopy and three-dimensional reconstruction:**

IFM thin filaments were isolated from the thoraces of *up101 f car; b el Mhc12 cn Drosophila* according to Cammarato et al., (2004)\(^2\) with modifications. 5µl of IFM thin filament suspension in either EGTA or Ca\(^{2+}\)-containing rigor buffer was applied to carbon coated EM grids (at ~25°C), negatively stained and rinsed with 1% (w/v) uranyl acetate, and dried at 80% relative humidity to aid in spreading the stain.\(^{13,14}\) EM images were recorded at 80kV on a Philips CM120 EM at 60,000X magnification under low dose conditions (~12e-/Å) at a defocus of 0.5µm.

Micrographs were digitized using a Zeiss SCAI scanner at a pixel size corresponding to 0.7nm in the filaments, and well-preserved regions of the filaments were selected and straightened as previously.\(^{13,14}\) Helical reconstruction, which resolves actin monomer structure and Tm strands, but not troponin, was performed on IFM thin filament segments which encompassed four-seven regulatory units from 18 *up101* filaments maintained in EGTA and from 14 *up101* filaments maintained in the presence of Ca\(^{2+}\) according to standard methods.\(^{13-15}\) Thus, structural information from roughly 80 *up101* regulatory units was included in each of the final reconstructions. The statistical significance of densities in reconstructions was computed from the standard deviations associated with contributing points as previously described.\(^{13,14,16,17}\) Note subtle differences in appearance of overall Tm density and in actin substructure can be introduced as a result of staining inconsistencies as well as from the inclusion of distinct B- and C-state subpopulations of filaments in an average three-dimensional reconstruction.

**Quantitative polymerase chain reaction (qPCR):**

Total RNA was isolated from dissected hearts of 3 week old flies using the Quick-RNA microprep kit (Zymo Research Corp., Irvine, CA). Contaminating DNA was removed with RNase free DNase I (Qiagen Inc, Valencia, CA). Reverse transcription polymerase chain reactions were performed using Qiagen QuantiTect Reverse Transcription Kits (Qiagen Inc, Valencia, CA) and 10ng of RNA per reaction. Quantitative (Real Time) polymerase chain reactions were carried out on a Bio-Rad CFX96 Touch Real-Time PCR Detection System (Bio-Rad Laboratories Inc.; Hercules, CA). The following components were used per reaction: 1µL (1-2µg/µL starting concentration) cDNA, 12.5µL 2X Power SYBR Green PCR Master Mix (Life Technologies; Carlsbad, CA), 2.5µL (100µmol/L starting concentration) Forward Primer, 2.5µL (100µmol/L starting concentration) Reverse Primer, and 6.5µL DEPC water for a final reaction volume of 25µL. Reactions with 1µL DNAase/RNAase-free water in lieu of 1µL cDNA in 25µL reaction volume served as negative controls for each gene. The following reaction conditions were used: 10 min at 95°C followed by 40 cycles of a) 95°C for 15 sec and b) 60°C for 60 sec. Starting-quantity of each reaction was calculated by comparing Ct to a standard curve generated from known concentrations of human Fibronectin 1 cDNA and then subtracting negative-control quantity. Each replicate was then normalized to Rpl32 quantity. Finally, we report fold-change as the final average *up101* quantity for a given gene normalized to final average Canton-S quantity. Six independent experiments were performed per gene in duplicate using pools of 12 different hearts per reaction.
qPCR Primers:

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<td></td>
</tr>
<tr>
<td>Standard</td>
<td>AGGCTTGAACCACTACGGA</td>
<td>GCCTAAGCAGCTGGCACAACAG</td>
</tr>
<tr>
<td>(Fibronectin 1;</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NCBI ID# 2335)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Online Figure I: The contribution of gender and genotype to Drosophila body and heart size. **Top** The legs of Drosophila consist of 9 segments separated by flexible joints. Average tibia contour length was used as an index of body size. We carefully measured tibia length from the flanking joints (arrow heads) of the right middle leg of over 35 Canton-S and up101 male and female flies. Scale bar = 100µm. **Bottom** Average tibia length for female flies was significantly greater than that determined for males while average Canton-S tibia length was significantly greater than that for up101. Thus, both gender and genotype significantly influence Drosophila body size. The genotype and gender effects on tibia length were determined by two-way
ANOVA. However, normalizing diastolic (DD) and systolic (SD) cardiac diameters to average tibia length (TL) prior to two-way ANOVA illustrates differences in body size have little influence on the effects of gender and genotype on cardiac diameters.

**Online Figure II: The $up^{101}$ TnT mutation does not influence Ca$^{2+}$-handling gene expression.** Quantitative polymerase chain reaction measurements of transcript expression levels in hearts of $up^{101}$ relative to Canton-S flies for L-type Ca$^{2+}$ channels, ryanodine receptors, sarco/endoplasmic reticulum Ca$^{2+}$-ATPase, Na/Ca exchangers and inositol-3-phosphate receptors. Each replicate was normalized to Rpl32 quantity. Six independent experiments were performed per gene in duplicate using pools of 12 different hearts per reaction. Independent unpaired t-tests of normalized values for each gene revealed no significant differences in the expression of Ca$^{2+}$-handling genes between Canton-S control and $up^{101}$ mutant hearts.

<table>
<thead>
<tr>
<th></th>
<th>Canton-S Male</th>
<th>$up^{101}$ Male</th>
<th>Canton-S Female</th>
<th>$up^{101}$ Female</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heart period (sec)</td>
<td>0.58 ± 0.037</td>
<td>0.53 ± 0.016</td>
<td>0.70 ± 0.047</td>
<td>0.49 ± 0.020</td>
</tr>
<tr>
<td>Systolic interval (sec)</td>
<td>0.20 ± 0.004</td>
<td>0.27 ± 0.006</td>
<td>0.23 ± 0.004</td>
<td>0.26 ± 0.008</td>
</tr>
<tr>
<td>SI/HP</td>
<td>0.38 ± 0.016</td>
<td>0.53 ± 0.011</td>
<td>0.38 ± 0.018</td>
<td>0.56 ± 0.018</td>
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<tr>
<td>Diastolic diameter (µm)</td>
<td>67.88 ± 1.208</td>
<td>49.01 ± 0.588</td>
<td>82.62 ± 1.300</td>
<td>54.29 ± 0.592</td>
</tr>
<tr>
<td>Systolic diameter (µm)</td>
<td>39.24 ± 0.808</td>
<td>33.71 ± 0.700</td>
<td>52.08 ± 0.995</td>
<td>35.77 ± 0.426</td>
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<tr>
<td>Fractional shortening</td>
<td>0.42 ± 0.007</td>
<td>0.31 ± 0.009</td>
<td>0.37 ± 0.006</td>
<td>0.34 ± 0.005</td>
</tr>
</tbody>
</table>

**Online Table I: The effects of the $up^{101}$ TnT mutation on the Drosophila heart.** Two-way ANOVA results for Online Table I can be found in Fig 3B.
Supplemental References: