**Online Supplemental Methods**

*Fly lines, culture conditions and husbandry:*

*upheld*$^{101}$ (*up*$^{101}$) mutant flies, originally recovered from a mutagenesis screen of Canton-S *Drosophila*[1] 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*$^{101}$ IFM thin filament isolation, *up*$^{101}$ *f car*; *b el Mhc*$^{12}$ *cn* double mutants were generated by standard mating procedures. *Mhc*$^{12}$ ''IFM myosinless'' *Drosophila* served as starting material. The genetic alteration in the *Mhc*$^{12}$ strain prevents myosin heavy chain accumulation in the IFM. Thus thick filament assembly and *up*$^{101}$-induced IFM degeneration does not occur. The *Mhc*$^{12}$ strain has identifiable phenotypic markers, *b*, *el*, and *cn* flanking the myosin gene. These markers were used to follow the *Mhc*$^{12}$ lesion in subsequent crosses. Crosses of *b el Mhc*$^{12}$ *cn Drosophila* with *up*$^{101}$ flies possessing the *f* and *car* markers were carried out to obtain a stable double mutant *up*$^{101}$; *Mhc*$^{12}$ 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.[2-5]

*Confocal microscopy:*

Confocal microscopy was performed as detailed by Alayari et al. (2009).[6] Three-week old female wildtype Canton-S and *up*$^{101}$ *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).[7] 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.[8-11] 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.[10] 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.[10]

*Imaging of blebbistatin-induced changes in cardiac dimensions:*

Beating hearts from three-week old female Canton-S (n=15) and *up*$^{101}$ (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 *up*$^{101}$ (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 *up*$^{101}$ *f car; b el Mhc*$^{12}$ *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.

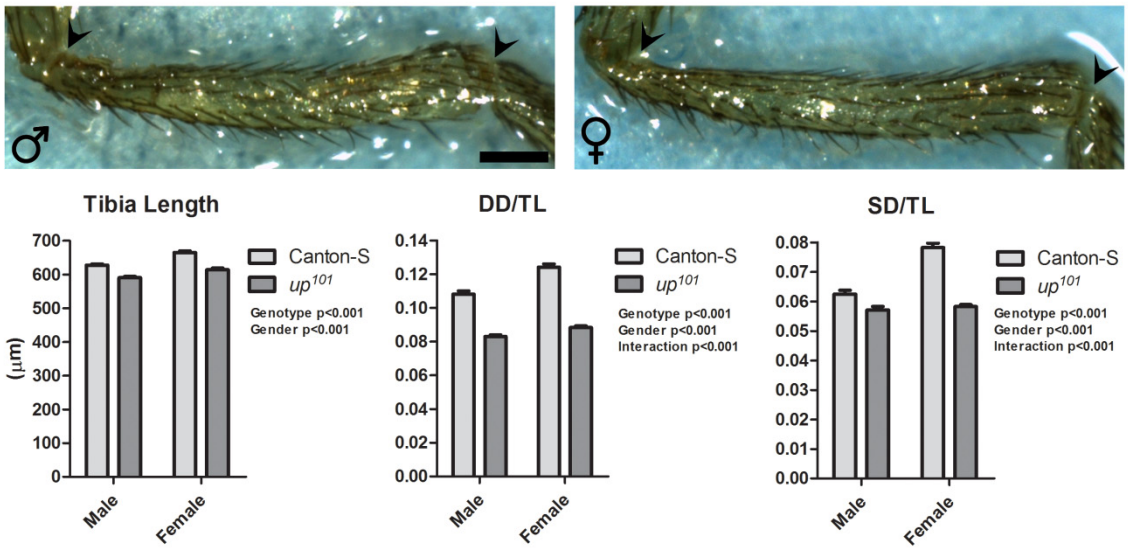
Micrographs were digitized using a Zeiss SCAI scanner at a pixel size corresponding to 0.7nm in the filaments, and well-preserved regions of the filaments were selected and straightened as previously.[13,14] Helical reconstruction, which resolves actin monomer structure and Tm strands, but not troponin, was performed on IFM thin filament segments which encompassed four-seven regulatory units from 18 *up*$^{101}$ filaments maintained in EGTA and from 14 *up*$^{101}$ filaments maintained in the presence of $Ca^{2+}$ according to standard methods.[13-15] Thus, structural information from roughly 80 *up*$^{101}$ 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). Sequence-specific forward and reverse primers targeting universally-transcribed regions of genes of interest were custom-designed (Integrated DNA Technologies; San Diego, CA). The calcium-handling protein-encoding genes assayed were: Ca-α1D, Rya-44F, Ca-P60A, CalX, Itp-r83A (see gene info and primer list in table below).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 *up*$^{101}$ 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.

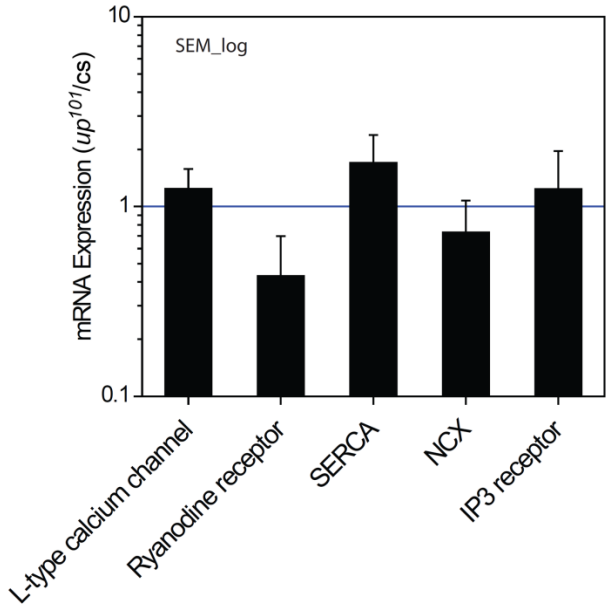
**qPCR Primers:**

| | Forward Primer (5'-3') | Reverse Primer (5'-3') |
|---|---|---|
| L-type Calcium Channel (Ca-α1D; CG4894; NCBI ID# 34950) | CAACAGCAACAGAGAGAGAGAG | GAACTCGGAGTCGCAGTATTT |
| Ryanodine Receptor (Rya-44F; CG10844; NCBI ID# 49090) | AAGTGGACTGGTGGCTTTATC | GTTTCTCCTCGTGCTCCATATC |
| SERCA (Ca-P60A; CG3725; NCBI ID# 49297) | GCCACTGAACGAAGAGGATAA | GACGGCGATCTTGAAGTAGTAG |
| NCX (CalX; CG5685; NCBI ID# 42481) | CTCAAAGTCCAGGAGACAGAAG | CCCACAAACAGGTAGATCAGTAG |
| IP3 Receptor (Itp-r83A; CG1063; NCBI ID# 40664) | CTTCTTCCTCCTCACCGTATTC | CACCACAGCTCGTCCATATT |
| Housekeeper (Rpl32; CG7939; NCBI ID# 43573) | CCAAGGGTATCGACAACAGAG | GTGTATTCCGACCACGTTACA |
| Standard (Fibronectin 1; NCBI ID# 2335) | AGGCTTGAACCAACCTACGGA | GCCTAAGCACTGGCACAACAG |




**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 *up*$^{101}$ 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 *up*$^{101}$. 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.

| | **Canton-S Male** ***n*=41** (Mean ± SEM) | ***up*$^{101}$ Male** ***n*=41** (Mean ± SEM) | **Canton-S Female** ***n*=45** (Mean ± SEM) | ***up*$^{101}$ Female** ***n*=44** (Mean ± SEM) |
|---|---|---|---|---|
| **Heart period (sec)** | 0.58 ± 0.037 | 0.53 ± 0.016 | 0.70 ± 0.047 | 0.49 ± .0200 |
| **Systolic interval (sec)** | 0.20 ± 0.004 | 0.27 ± 0.006 | 0.23 ± 0.004 | 0.26 ± 0.008 |
| **SI/HP** | 0.38 ± 0.016 | 0.53 ± 0.011 | 0.38 ± 0.018 | 0.56 ± 0.018 |
| **Diastolic diameter (μm)** | 67.88 ± 1.208 | 49.01 ± 0.588 | 82.62 ± 1.300 | 54.29 ± 0.592 |
| **Systolic diameter (μm)** | 39.24 ± 0.808 | 33.71 ± 0.700 | 52.08 ± 0.995 | 35.77 ± 0.426 |
| **Fractional shortening** | 0.42 ± 0.007 | 0.31 ± 0.009 | 0.37 ± 0.006 | 0.34 ± 0.005 |

**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.

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