A cis-Regulatory Mutation in Troponin-I of Drosophila Reveals the Importance of Proper Stoichiometry of Structural Proteins During Muscle Assembly

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Running title: *fliH* is a Troponin-I mutation

**Key words:** Drosophila, flight muscles, troponin, muscle hypercontraction, Protein stoichiometry

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ABSTRACT

Rapid and high wing beat frequencies achieved during insect flight are powered by the indirect flight muscles, the largest group of muscles present in the thorax. Any anomaly during the assembly and/or structural impairment of the indirect flight muscles gives rise to a flightless phenotype. Multiple mutagenesis screens in *Drosophila melanogaster* for defective flight behavior have led to the isolation and characterization of mutations which have been instrumental in the identification of many proteins and residues, important for muscle assembly, function and disease. In this paper, we present molecular-genetic characterization of a flightless mutation, *flightless-H* (*fliH*), originally designated as *heldup-a* (*hdp-a*). We show that *fliH* is a cis-regulatory mutation of the *wings up A* (*wupA*) gene, which codes for the Troponin-I protein, one of the troponin complex proteins, involved in regulation of muscle contraction. The mutation leads to reduced levels of Troponin-I transcript and protein. In addition to this, there is also coordinated reduction in transcript and protein levels of other structural protein isoforms which are part of the troponin complex. The altered transcript and protein stoichiometry ultimately culminates in unregulated acto-myosin interactions and a hypercontraction muscle phenotype. Our results shed new insights into the importance of maintaining the stoichiometry of structural proteins during muscle assembly for proper function with implications in the identification of mutations and disease phenotypes in other species including humans.
INTRODUCTION

The indirect flight muscles (IFMs) of *Drosophila* serve as a good genetic model system to study muscle development, assembly of structural proteins and regulation of muscle contraction (Vigoreaux 2006; Nongthomba et al. 2007). Like the vertebrate skeletal muscles, IFM contraction is regulated by the influx of neurally stimulated intracellular Ca\(^{2+}\). Stretch activation allows IFM to contract at a much higher frequency and is similar to the asynchronous muscle contraction in the human heart (Peckham *et al.* 1990; Josephson *et al.* 2000; Agianian *et al.* 2004; Moore *et al.* 2006). In addition, major structural proteins like myosin, actin, tropomyosin (Tm), troponin (Tn), α-actinin etc. that are involved in the assembly of sarcomeres, are conserved in vertebrates and invertebrates, and dispense similar functions. Mutations in these sarcomeric proteins disrupt muscle structure and function (summarized in Vigoreaux 2006).

Since, IFMs are the only fibrillar muscles present in fly’s body, many of the myofibrillar proteins have IFM-specific isoforms (Cripps 2006; Nongthomba *et al.* 2007) and are dispensable under laboratory condition, enabling the isolation of mutations without affecting other physiological activities. Majority of mutations affecting the IFMs were isolated during mutagenesis screens for flightless behavior (Deak 1977; Homyk and Sheppard 1977; Mogami and Hotta 1981; Deak *et al.* 1982; Cripps *et al.* 1994; summarized in Cripps 2006). However, molecular lesions for many of these mutations are yet to be identified; as a result plausible mechanisms that give rise to muscle phenotype of these mutations remain elusive.

Many of these flightless mutants are known to show a muscle phenotype that has been categorized as “hypercontraction”. Hypercontraction is a phenomenon which leads to muscle defects like, thinning and tearing, following uncontrolled acto-myosin interactions of otherwise normally assembled sarcomeric structures (Nongthomba *et al.* 2003). Mutations leading to
hypercontraction have been localized in structural genes such as the *upheld* gene (*up^101*) (Fyrberg *et al.* 1990; Nongthomba *et al.* 2003), *flightin* (*fln^0*) (Reedy *et al.* 2000), *Actin88F* (An and Mogami 1996; Nongthomba *et al.* 2003), *Myosin heavy chain* (*Mhc^6*, *Mhc^13* and *Mhc^19*) (Kronert *et al.* 1995), *wings up A* (*wupA^{hdp-2}* ) (Beall and Fyrberg 1991; Nongthomba *et al.* 2003), protein phosphatase genes *flapwing* (*flw^1*, *flw^6* and *flw^7*) (Raghavan *et al.* 2000; Pronovost *et al.* 2013) and *calcineurin B2* (*canB2^{EP(2)}^{0774}* ) (Gajewski *et al.* 2006). Mutations producing hypercontraction fall in a large repertoire of proteins suggesting that there could be multiple grounds for reaching the phenotype. Suppressor studies of the hypercontraction phenotype have led to the identification of mutations in structural genes like *Tm2* (Niami *et al.* 2001), *Mhc* (Kronert *et al.* 1999; Nongthomba *et al.* 2003) and integrin adhesive complex protein PINCH (Pronovost *et al.* 2013), providing very fruitful insights into the structure–function relationship of these proteins. Studies on these hypercontracting alleles have led to the conclusion that defects in Ca^{2+} regulation, structural defects, troponin-tropomyosin (Tn-Tm) regulation defects and mechanical stress can lead to IFM hypercontraction (Nongthomba *et al.* 2003; Cammarato *et al.* 2004; Pronovost *et al.* 2013). However, further work is required to identify genes/proteins and pathways involved in the pathogenesis of hypercontraction, to unravel new players involved in the regulation of muscle contraction and possible mechanisms leading to muscle dysfunction, as is the case with most human myopathies.

Detailed characterization of the flightless mutations, which were isolated decades ago, has led to the identification of many proteins and residues that are important for muscle assembly, function and diseases (reviewed in Cripps 2006; Vigoreaux 2006). Previously, we have shown detailed characterization of a mutation (*up^1*), which was isolated in 1958 (Fahmy and Fahmy 1958), which yielded new insights into troponin-T (TnT) isoform switching, muscle
assembly and function (Nongthomba et al. 2007). In the present study, we have used a similar approach to characterize flightless-H (fliH), a flightless mutation generated by Homyk and Sheppard in 1977 using a chemical mutagen ethyl methanesulfonate (EMS). fliH was considered to be an allele of the heldup-a (hdp-a) mutation at the Beadex (Bx) locus. We found that fliH is a regulatory mutation of troponin-I (TnI) rather than Bx, with a hypercontraction muscle phenotype. All the isoforms of TnI in Drosophila are encoded by a single wupA gene. Many mutations already exist for the wupA gene which showed developmental defects during sarcomere assembly leading to muscle degeneration (Beall and Fyrberg, 1991; Nongthomba et al. 2004). wupA^{hdp-2}, as mentioned above, is a hypercontracting allele of TnI and involves a point mutation changing alanine to valine at position 116 (Beall and Fyrberg, 1991). Our study reveals that fliH is a unique allele which confers a temperature sensitive muscle phenotype. This is the first mutation found in the regulatory region of any structural gene which leads to muscle hypercontraction. This study also emphasizes the importance of maintaining proper stoichiometry of structural proteins for proper functioning of the muscle.
MATERIALS AND METHODS

Fly strains and Crosses: All flies were maintained on corn flour-glucose-yeast-agar-medium. Canton-S served as the wild type control, unless otherwise mentioned. The fliH stock was obtained from Bloomington Stock Centre (BS No. # 6028) and flies were raised at 25° and 18° for various experiments. UH3-Gal4 line specific for adult IFM was generated in our lab (Singh et al. 2014) and UAS-L9 was procured from Prof. Alberto Ferrus, Cajal Institute, Madrid, Spain. wee-P26 (Mhc-GFP) (Clyne et al. 2003), Tm2-GFP (Morin et al. 2001) and sls-GFP (Burkart et al. 2007) constructs are fusion constructs wherein GFP coding sequence has been inserted at C-terminal end of the native protein. Act88F-GFP transgenic line leads to GFP expression in IFM under Act88F promoter (Barthmaier and Fyrberg 1995). Mhc null allele and Y97 (transgenic fly line carrying Headless myosin) have been described previously (Cripps et al. 1999). up101 (Fyrberg et al. 1990), wupa\(^{hdp-3}\) (Barbas et al. 1993; Nongthomba et al. 2003) and wupa\(^{hdp-2}\) (Beall and Fyrberg 1991) are mutations of troponin-T and troponin-I respectively. All chromosomes and gene symbols are as in Flybase (http://www.flybase.org). Pupae were aged according to the method previously described (Fernandes et al. 1991).

Behavioural test: Flight test was performed as described previously (Drummond et al. 1991). Walking and jumping test was performed as described previously in Naimi et al. (2001).

Polarized light microscopy: Fly hemithoraces were prepared for polarized microscopy as described in Nongthomba and Ramchandra (1999). Briefly, fly thoraces were frozen in liquid nitrogen, bisected longitudinally using a razor blade, dehydrated in alcohol series, cleared in methyl salicylate and mounted using DPX mounting medium. The hemithoraces were observed in Olympus SZX12 microscope and photographed using Olympus C-5060 camera under polarized light optics.
Confocal microscopy: Flies were bisected as mentioned above, fixed in 4% paraformaldehyde, washed four times with PBTx (0.3% Triton-X100 in Phosphate buffer saline) for 15 minutes each and stained with 1:200 diluted Phalloidin–Tetramethylrhodamine B isothiocyanate (Phalloidin–TRITC) (Sigma) (50 μg/ml stock) for 20 minutes. Anti-Mlp60A antibody raised in our lab was used as a marker for Z-disc staining. Finally, the sections were washed four times with PBTx and mounted in Vectashield mounting medium (Vector labs). Imaging was done using Carl Zeiss LSM 510 META confocal microscope.

Genomic DNA isolation and PCR: Genomic DNA isolation was done by following Berkeley Drosophila Genome Project (http://www.flybase.org) protocol. DNA was diluted 1:100 times in milli-Q water and quantified using a Bio-Rad spectrophotometer. Approximately 200 ng of DNA was used as template for amplifying the specified genomic region. Following primers were used: TnI-URE1039F5’-GGGATTCCCAATTATCTCTC-3’; TnI-URE1526R5’CCGCTTGGAATTCAATGC-3’; TnI-URE1818R5’-AACTGACATGGAACAGCACA-3’, TnI-URE57F5’-AACGCTCGGAACGAGAATGA-3’, TnI-URE2078R5’-CTGAACGGGCCGACGATCCA-3’, TnI-URE932R5’-TTCTTAGACCGTGCCACT-3’, TnI-IRE1453F5’-ACTATACGGATAGGCTAGCA-3’, TnI-IRE2021R5’-ATCGCACACGCCTACGATCT-3’, TnI-IRE7F5’-CGATCCGTATCTGTATCCGT-3’, TnI-IRE2495R5’-GGTTGCATGGCTGGTTGTTG-3’, TnI-IRE1111F5’-CCGAAGGTCATTGCATATTGTCAGAA-3’, TnI-IRE1158R5’-CTTAGCGAAGGTAAGGCCGTG-3’.

Gel purification, cloning and sequencing of PCR products: PCR products were purified using gel purification kit (Qiagen), ligated to pGEM-T Easy cloning vector (Promega) and transformed into E. coli DH5α cells. Plasmid preparations were done using Miniprep kit (Qiagen). DNA
sequencing was done at Macrogen Inc., Seoul, Korea; using T7 sequencing primers or primers used to generate the fragments. Both the DNA strands, for a minimum of three clones from each PCR, were sequenced; output was analyzed using Chromas Lite and ClustalW softwares.

**Semi-quantitative RT-PCR:** Newly eclosed flies were kept in 70% ethanol and frozen at -80°C freezer. IFMs were dissected from these flies, total RNA was isolated using Trizol reagent (Sigma). RNA amount was quantified by taking optical density (OD) at 260 nm and purity was assessed by calculating OD ratio at 260 nm and 280 nm wavelength. Total RNA (2ug) in a volume of 20 µl was used for cDNA preparation using First strand cDNA synthesis kit (Fermentas). cDNA (1ul) was used for carrying out the PCR. All the PCR reactions were performed at predetermined non–saturating cycle number for each gene. All the primers used have been described previously (Nongthomba et al. 2007). rp-49 (ribosomal protein encoding RNA) was used as internal control. The PCR products were resolved on 1.2% agarose gels and images were captured using JHBIO (JH BIO Innovations Pvt. Ltd.) gel documentation system and gel quantification was done using the SpotDenso tool of the AlphaEaseFC software (Alpha Innotech). The data was processed using MS Excel.

**Quantitative Real time RT-PCR:** Real time quantitation was performed using cDNA equivalent to 20 ng of total RNA isolated from fly IFM. All the PCR reactions were carried out using Dynamo™ SYBRgreen mix (Finnzymes, Finland) in ABI Prism 7900HT sequence detection system (Applied Biosystems, USA) and analyzed with SDS 2.1 software (Applied Biosystems, USA). rp-49 primers were used for normalization of RT-PCR data and fold change over control was calculated. Primers used in present study were tested before hand for the presence of single peak in the dissociation curve which suggests that there is a single amplicon. Following primers were used:
Ribosomal gene rp-49F 5'-AAGCTGTCGCACAAATGG-3', rp-49R 5'-ATCCGTAACCGATGTGG-3', TnI-Ex4F 5'-GGCTGATGATGAGGCTAAGA-3', TnI-Ex4R 5'-TACGCAGCAGCAACCTGAGT-3', TnI-Ex6F 5'-CCAGCGAAGGCGAATTG-3', TnI-Ex6R 5'-GATCGTTGATCTCCAGTCT-3'.

**Protein extraction and Western blot:** IFMs were removed from bisected flies preserved in 70% alcohol and homogenized in 1x Buffer (0.1 M NaCl, 10 mM Potassium phosphate pH 7.0, 2 mM EGTA, 2 mM MgCl$_2$, 1 mM DTT, 1 mM PMSF and 0.5% Triton-X). The IFM lysate was spun down to obtain protein pellet which was further washed with same 1x buffer without Triton-X and then boiled in SDS-sample buffer (0.0625 M Tris-Cl pH 6.8, 2% SDS, 10% glycerol, 5% 2-mercaptoethanol and 5 µg Bromophenol blue) for 4 minutes at 95°C. Samples were then resolved in 12% PAGE gel in mini electrophoresis unit (Amersham) at 100V. The protein was then transferred from gel to PVDF membrane (Immobilon-P, Millipore) in transfer buffer (20% Methanol, 25 mM Tris-base and 150 mM Glycine). The membrane was blocked with 8% milk solution in Tris buffer saline (TBS, pH 7.4) for 1h and then probed with primary antibody at prescribed dilution overnight at 4°C. The following anti-sera were used: anti-Drosophila Troponin-I (1:1000, raised in Rabbit, gift from Prof. Alberto Ferrus, Spain); anti-acetylated α-Tubulin (1:1000, raised in mouse (Sigma)). After washing three times with TBS, the membrane was incubated with HRP-conjugated secondary antibody (1:1000, Bangalore Genei, Bangalore) for 3 hours at room temperature. The membrane was then washed three times (15 minutes each) with TBST (TBS with 0.05% Tween 20) and 5 minutes with 0.5 M NaCl. Bands were detected by adding DAB substrate along with 0.5% H$_2$O$_2$ on the membrane or developed using enhanced chemiluminescence (ECL) method (Supersignal WestPico Chemiluminescent substrate, Pierce).
**Protein expression:** Mef-2 forward primer (5'-ATCTGTTCATATGGGCCGCA-3') and Mef-2 reverse primer (5'-CTGCTGCTCGAGATGGTGTT-3') were used to amplify the Mef-2 sequence from the pBluescript vector containing Mef-2 cDNA (provided by Prof. Richard Cripps, University of New Mexico, USA). The cDNA was cloned in pET15b expression vector (Novagen) under T7 promoter using NdeI and XhoI restriction enzymes. The protein was expressed in TNT® Quick Coupled Transcription/Translation systems (Promega) by adding 1µg of purified Mef-2-pET plasmid vector, 1 mM methionine and 40 µl of TNT® Quick Master Mix and incubating the cocktail at 30° for 30 minutes. The lysate from the reaction was used for further experiments.

**Electrophoretic mobility shift assays (EMSA):** Electrophoretic mobility shift assay was performed essentially as described in Sambrook et al. (1989). Probe DNA and unlabelled competitors were generated by annealing complementary oligonucleotides to generate dsDNA molecule with GG overhangs, thus creating recessed 3' ends. The following oligonucleotides were used for EMSA:

Dmef2(+) 5'-GGTGTCTATATTAGCCC-3', Dmef2(-) 5'-GGGGCTAAATATAGACA-3' (Cripps et al. 2004), CS1(+) (Control sequence1) 5'-'GCCATTTATCCTCATAAACATAACTAATATTTGATGAGATAATAGACA-3', CS1(-) 5'-GGGTATTTATCCTCATAAACATAACTAATATTTGATGAGATAATAGACA-3', CS2(+) 5'-GGAGTGACTGAATACAAATTACTGTTTTCA-3', CS2(-) 5'-GGTGAAAACAGTAATTTGTATTCAGTCAACT-3', FM1(+) (fliH mutant sequence1) 5'-GGCATTTATCCTCATAAACATAACTAATATTTGATGAGATAATAGACA-3', FM1(-) 5'-GGCATTTATCCTCATAAACATAACTAATATTTGATGAGATAATAGACA-3', FM2(+) 5'-GGTGAAAACAGTAATTTGTATTCAGTCAACT-3', FM2(-) 5'-GGAGTGACTGAATACAAATTACTGTTTTCA-3'.
GGTGAACAGTATTTGTAAATTCACTCAACT-3'. Annealed sequences were radioactively labeled with $^{32}$P-dCTP (BRIT, Hyderabad) using Klenow enzyme (Fermentas Life Sciences) and purified using G-25 columns. Mef-2 lysate was pre-incubated for 15 minutes on ice with 50 mg/ml of poly (dI-dC) and binding buffer (20 mM HEPES-KOH, 60 mM KCl, 200 µM EDTA, 10% (w/v) glycerol). Competitors were then added and incubated for 15 minutes on ice. Finally, labeled probes were added and reaction was incubated for 30 minutes. The entire reactions mixes were loaded onto an 8% non-denaturing polyacrylamide gel which was run at 100V at 4°C. The gel was then dried for 1 hr and exposed overnight. The image was captured using a Typhoon image scanner (Amersham Bioscience).

**Luciferase assay:** To determine the promoter activities of wild type and $fliH$ mutant, luciferase assay was performed. The 979 bp long DNA fragment from regulatory region of $wupA$ were PCR-amplified from wild type and $fliH$ mutant genomic DNA using the primer set TnI-URE840F 5'-GCGGCCAACATGCAAGATA-3' and TnI-URE1818R 5'-AACTGACATGGCAGAGCACA-3'. The resultant fragments were inserted into the pTZ57R/T vector (Fermentas) and sequenced. These fragments were further sub-cloned into the KpnI/XhoI sites of the pGL3-luciferase vector (Promega). The C2C12 myoblast cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Gibco) supplemented with 10% FBS and 1% penicillin/streptomycin (Gibco). The C2C12 cells (6x10$^4$ cells/well) were grown in a 24 wells plate (Nunc, Thermo Scientific, USA) for 24 hours till 70% confluence was reached. The cells were then transfected with pGL3 basic, pGL3 control, wild type 979 bp (Control-URE) and mutant 979 bp ($fliH$-URE) along with that of pRL-TK Renilla luciferase (Promega) using lipofectamine 2000 (Invitrogen, USA) as per manufacturer’s instruction. Luciferase reporter activity was estimated after 36 hours using the dual luciferase assay kit (Promega) and a TD-
20/20 luminometer (Turner Design, Sunnyvale, CA) following the manufacturer’s protocol and the Renilla luciferase signal was normalized to the firefly luciferase signal. HFF (Human foreskin fibroblasts) cells were used to reconfirm the promoter activation of wild type and the fliH mutant. The HFF cells (devoid of the transcription factor Mef2) were cultured in DMEM (Sigma Aldrich, USA) supplemented with 10% FBS and 100 units/ml penicillin and 100 µg/ml streptomycin (Invitrogen Life Sciences, USA). The HFF cells (6x10^4 cells/well) were grown in a 12-well plate (Nunc, Thermo Scientific, USA) for 24 hours until 70% confluence was reached. The cells were then transfected with the pGL3-Basic, wild type 979 bp (Control-URE) and mutant 979 bp (fliH-URE) plasmids along with pRL-TK (Promega) plasmid as transfection control. The cells were also transfected with pcDNA3.1(-) and pcDNA3.1(-)-Mef2 along with pGL3-Basic, Wild type 979 bp (Control-URE) and mutant 979 bp (fliH-URE) along that of the pRL-TK Renilla luciferase (Promega) using lipofectamine 2000 (Invitrogen, USA) as per manufacturer’s instructions. The luciferase reporter activity was measured after 24 hours using the dual luciferase assay kit (Promega) and a TD-20/20 luminometer (Turner Design, Sunnyvale, CA) following the manufacturer’s protocol. The Renilla luciferase signal was normalized to the firefly luciferase signal. DLR™ assay system kit (Promega Corp, USA) was used to perform dual luciferase assays. Briefly, HFF (human foreskin fibroblast) cells were grown in 12-well plates and transfected with 0.5 µg either of the pGL3-Basic, wild type 979 bp (Control-URE) and mutant 979 bp (fliH-URE) along with 0.5 µg of either the pCDNA3.1 empty vector or pCDNA3.1-Mef2 and50 ng of the pRL-TK (Renilla luciferase under the constitutively active Thymidine kinase promoter) construct for 6 hours in plain DMEM using lipofectamine 2000 (Invitrogen, USA) reagent. After 6 hours cells were washed with DPBS and fresh reconstituted media was added for a period of 24 hours. Cells were lysed in 200 µl of 1X passive lysis buffer.
The lysates were transferred into microfuge tubes and centrifuged at 10,000 rpm for 10 minutes at 4°C to pellet down the debris. The supernatant (10µl) was taken in a fresh microfuge tube to which 10 µl of the luciferase assay reagent II was added and the activity of the firefly luciferase was recorded using a luminometer (TD-20/20, Turner Designs). The activity of the renilla luciferase was also measured after adding the renilla substrate, ‘Stop and Glo’. The ratio of firefly and renilla (relative luciferase units, RLU) was calculated and plotted.

The cDNA was prepared from HFF cells alone and HFF cells transfected with pCDNA3.1 + pGCL3-Basic empty vector and pCDNA3.1-MEF2 + pGCL3-Basic empty vector. The following primers were used to see the expression of *Drosophila* Mef2 in HFF cells, RPL-35A-F 5'-GGGTACAGCATCACTCGGA, RPL-35A-R 5'-ACGCCCGAGATGAACAG, dMef2-F 5'-ACGAGTCCTCACCAACAAG and dMef2-R 5'-CGTGTAGCTGCTGTTTGAA.
RESULTS

The fliH mutation leads to IFM hypercontraction phenotype

The fliH mutant was isolated based on a recessive flightless phenotype in an ethyl methanesulfonate mutagenesis (EMS) screen for flightless behavior (Homyk and Sheppard 1977). fliH flies indeed showed complete flightlessness compared to their wild type Canton-S counterpart (Figure 1A). Mutations affecting flight muscles or neurons innervating these muscles are the major cause of decreased flight ability (reviewed in Lloyd and Taylor 2010). Two opposing groups of IFMs, the dorsal longitudinal muscles (DLMs) and dorso-ventral muscles (DVMs) assist the direct flight muscles linked to wing hinge, to produce higher wing beat frequencies required for flight by distorting the thorax (reviewed in Josephson 2006; Lehmann 2006). Upon examining the bisected thorax which houses these major muscles, we noticed that DLMs showed hypercontraction like muscle phenotype as reported earlier (Nongthomba et al. 2003), with muscles torn from the middle with few remnants attached at both ends (Figure 1C), compared to the six DLMs in wild type (Figure 1B).

All hypercontraction muscle phenotypes can be rescued by removing myosin or reducing acto-myosin interaction through headless myosin construct (Y97) or myosin mutations (Nongthomba et al. 2003). Mhc^{2B} (Mhc^P401S) is a mutation in the actin binding loop (Nongthomba et al. 2003) of myosin which inhibits its interaction with actin and this mutation completely rescued the fliH (Figure 1D) muscle phenotype. Headless myosin construct (Y97) completely rescued fliH phenotype (Figure 1E). Both these experiments confirmed that the fliH muscle phenotype is due to ‘hypercontraction’, which may result from increased or unregulated acto-myosin interaction or mechanical stress.
Early IFM myofibrillogenesis proceeds normally in *fliH* and hypercontraction of muscles is seen at eclosion

During IFM development, well demarcated sarcomere structures are seen by 42 hours after puparium formation (APF) and increase in the length and breadth of the sarcomere takes place throughout the pupal development stages (Reedy and Beall 1993; Nongthomba *et al.* 2004). Development and assembly of sarcomeric structure in *fliH* was tracked using confocal microscopy utilizing *wee-P26*, a “protein trap construct” expressing GFP tagged myosin heavy chain (Mhc-GFP) fusion protein, which enables tracking of thick filaments in sarcomere (Clyne *et al.* 2003). The muscles from this genetic background were counterstained with Phalloidin-TRITC to visualize F-actin. Assembly of sarcomeres in *fliH* IFM during early developmental stages i.e. 55 hours APF (Figure 2, B-B”) and 75 hours APF (Figure 2, D-D”), were normal with properly organized thick and thin filaments that are comparable to controls (Figure 2, A-A” and C-C”). However, sarcomere organization in just eclosed adult mutant flies showed completely disrupted structures (Figure 2, F-F” and Supporting information, Figure S1, B-B”), suggesting that hypercontraction of the muscles takes place in late pupal stage, like the other hypercontracting alleles (Nongthomba *et al.* 2003). The normal F-actin banding pattern was lost in mutant IFMs (Figure 2F’, compare with Figure 2E’). Mutant IFMs also showed disarrayed myofibrils moving in and out of the confocal plane (Figure S1B”) as opposed to their parallel arrangement in wild type (Figure S1A”).

The mutant muscle phenotypes in adult IFM were also studied using Tropomyosin-2-GFP (TM2-GFP, a ‘protein trap’ expressing GFP-tagged Tropomyosin-2), a thin filament protein (Figure S1, C-D); and sallimus-GFP (SLS-GFP, a ‘protein trap’ expressing GFP-tagged sallimus), a Z-disc protein (Figure S1, E-G). Thin filament proteins are seen in small clumps in
the *fliH* hypercontracted muscles (Figure S1D”). The regular banding patterns of Z-discs and size and spacing of the thin filaments were lost in freshly eclosed *fliH* IFMs (Figure S1, F-F”) when compared to control (Figure S1E”). More pronounced dissolution of Z-discs and myofilaments are observed in 2-day old flies (Figure S1, G-G”).

**fliH shows temperature dependent IFM and other locomotor behaviour defects**

Most of the mutations isolated during Homyk and Shephard’s (1977) screen showed temperature dependent flight behaviour. Since, *fliH* was also isolated from the screen; we wanted to know if *fliH* is a temperature sensitive mutant. Indeed the muscle hypercontraction phenotype of *fliH* was a temperature dependent phenotype (Figure S3). As expected, polarized light microscopy showed that DLM morphology is completely rescued in the mutant flies raised at lower temperature (data not shown). Confocal micrograph of the DLM fibres of these mutants showed proper assembly of the thick filaments (Figure S2, A-A”), thin filaments (Figure S2, B-B”) and Z-discs (Figure S2, C-C”) reflecting normal sarcomere development. Since, flight behavior directly reflects the muscle organization, it can be concluded that hypercontraction of the IFMs in *fliH* is temperature dependent.

Many muscle mutants show phenotype restricted to IFMs thus affecting only flight. Such mutations in question have genetic lesion in the protein isoforms which are IFM specific (Nongthomba *et al.* 2004; Nongthomba *et al.* 2007). We wanted to check if the phenotype associated with *fliH* is restricted to IFMs or not. Survival test was performed to assay for any associated lethality during development. We found that the *fliH* mutation leads to partial lethality at various stages of development at 25° (Figure 3A) compared to much reduced lethality at 18° (Figure 3B). Jumping (Figure 3C) and walking (Figure 3D) abilities were also found to be significantly compromised in *fliH* as compared to Canton-S, suggesting that tergal depressor of
trochanter (TDT) and leg muscles are also affected. Like the flight ability, we also found reduced jumping and walking abilities in wild type and fliH flies when raised at 18°. However, unlike flight, walking and jumping abilities were not rescued in fliH raised at 18°, instead it showed more pronounced phenotype (Figure 3, C and D), suggesting that TDT and leg muscles may have abnormalities which are independent of temperature. Jumping and walking abilities when monitored for several days post eclosion did not show any deterioration, ruling out any enhanced age dependent progressive TDT damage or leg muscle defects (data not shown), which have been shown for other hypercontracting alleles (Nongthomba et al. 2003). Overall, we found that the fliH mutation also affects other muscles but the most pronounced phenotype is seen in the IFMs.

**The fliH mutation is present in wupA regulatory region**

fliH is considered as heldup-a (hdp-a) mutation for Beadex (Bx) locus with cytogenetic map position at 17A1-18A2 (Flybase report). However, we did not find any communication which has reported the cytogenetic mapping or locus associated with fliH or hdp102 (which is synonym for fliH reported in Homyk and Emerson 1988). Hypomorphs of Bx do show held up wing phenotype (Lifschytz and Green 1979; Shoresh et al. 1998), but IFMs do not show hypercontraction phenotype (our unpublished data). The fliH mutation was reported to be proximal to forked (f), close to wup$_{hdp^2}$ and wup$_{hdp^3}$ (Homyk and Shephard 1977). Using forked (f) and carnation (car), recessive markers, we found the recombination map location of fliH to be at 1-57.69± 0.87 (16F chromosomal locus) (Figure 4A). Unlike the Flybase report, fliH is not uncovered by Df(1)N19 which deletes the chromosomal segment 17A1-18A2, which includes the Bx gene (Figure 4A). Recombination mapping suggested that fliH could be localized near the chromosome loci 16F. We have used multiple translocation and duplication lines to fine map the
mutation. We could not rescue the mutant phenotype with Y chromosome translocation \(T(1;Y)V7\) (translocated chromosome regions of \(16F5-16F7; h18-h25\) (Ferrus et al. 1990)) (Figure 4B). However, \(fliH\) was rescued by lines \(T(1;Y)B18\) (16F-17A; \(h1-h17\)) (Stewart and Merriam, 1973) and \(Dp(1;Y)W39\) (duplicated region spanning 16F1-18A7 (Prados et al. 1999) (Figure 4A). Rescued flies showed six DLM fascicles like the wild type counterpart in polarized light imaging of hemithoraces (Figure 4, B-D). These flies also showed slight rescue of the flight ability (Figure 4, E and F). Confocal micrographs of the rescued flies showed marked regularity in Z-discs spacing as well as regular banding pattern of thin filaments (Figure 4, G-H). Thus, genetic analyses using duplication and deletion mapping place \(fliH\) mutation to the cytogenetic map location 16F1-17A. The only structural gene reported in this region which gives muscle hypercontraction similar to \(fliH\) is \(wup\) gene, which codes for Troponin I (TnI), an inhibitory component of the troponin complex.

The \(wup\) gene function is impeded in the case of mutations such as in alleles, \(wup^{hdp-2}\) (Beall and Fyrberg, 1991) and \(wup^{hdp-3}\) (Barbas et al. 1993; Nongthomba et al. 2004). \(wup^{hdp-2}\) is a recessive mutation involving single amino acid change A116V in constitutive exon 5 in the region which interacts with TnC. This mutation is recessive in nature and homozygous flies show muscle hypercontraction (Figure 4I). However, \(wup^{hdp-3}\) is a splice mutation in the intron preceding the alternative exon 6b1, which is IFM and TDT specific. It leads to TnI null condition in IFM and TDT. \(wup^{hdp-3}\) heterozygotes are flightless and show hypercontraction muscle defects in 30% of the flies (Nongthomba et al. 2004). \(fliH\) fails to complement \(wup^{hdp-3}\) and all the individuals show muscle tearing (data not shown). Similarly, \(fliH/wup^{hdp-2}\) heterozygotes show muscle hypercontraction suggesting that the interaction between the two mutations could be intragenic in nature (Figure 4J). \(wup^{hdp-2}\) mutant is known to show muscle tearing in trans-
heterozygous condition with another hypercontracting mutation, $up^{101}$ in the *upheld* gene (Figure 4K). The *upheld* gene codes for troponin T (TnT) and $up^{101}$ is a recessive single point mutation (Fyrberg *et al*. 1990). Such interaction studies have revealed that mutation in one component of muscle can either enhance or relieve the effect of mutation at any other locus that takes part in normal muscle assembly and function. These studies have been very fruitful in deciphering gene functions and the pathways involved (Mogami and Hotta 1981; Homyk and Emerson 1988).

$up^{101}$ and *wup* $^hdp-2$ mutants show genetic interaction possibly due to the fact that they are mutations in the TnT and TnI proteins respectively, which are part of the same complex that regulates muscle contraction. Since, *fliH* fail to complement *wup* $^hdp-2$ allele and *wup* $^hdp-2$ in turn interacts with $up^{101}$, the *fliH* interaction with $up^{101}$ was studied. *fliH*/$up^{101}$ trans-heterozygotes showed gradation of phenotypes from flightlessness to individuals with normal flight (Figure 4M). In addition, none of the flies showed any muscle defect (Figure 4L). This suggests that the nature of mutation involved in *fliH* is completely different from *wup* $^hdp-2$, if they are intragenic.

Targeted over-expression of TnI using UAS TnI L9 (embryonic isoform of TnI, Prado *et al*. 1999) under the control of UH3-GAL4 which expresses in developing IFM (Singh *et al*. 2014) brings about rescue of the *fliH* muscle phenotype (Figure 4, N-O). Six DLM fascicles were seen (Figure 4N) with normal banding pattern sarcomeres (Figure 4O). Rescued flies also showed functional recovery of the muscles as indicated by their flight ability (Figure 4P). Overall, genetic mapping, interaction and rescue experiments showed that *fliH* is a *wupA* allele. Keeping the flybase genetic nomenclature of the *wupA* gene, *fliH* is renamed as *wupA* $^{fliH}$. However, for clarity we will still address the mutation with the original name *fliH* in this paper.

The *wupA* gene consists of 13 exons and alternative splicing gives rise to isoforms containing exon 6b1 in the IFMs. There are two major isoforms in IFMs, one with exon 3 and
another lacking it (Nongthomba et al. 2004). Sequencing of the TnI coding region as well as UTR’s of mRNA did not reveal any mutation (data not shown). Quantitative expression of TnI is controlled by two regulatory sequences, upstream regulatory element (URE) present upstream to the 5’UTR and intronic regulatory element (IRE) lying within first intron of the wupA gene. Both these elements work synergistically to control the correct expression of TnI in most of the Drosophila muscles (Marin et al. 2004). Both the regulatory regions were amplified and sequenced using genomic DNA isolated from the fliH and Canton-S. Sequencing results revealed no mutation in IRE but few changes in URE region. A schematic showing mutations as well as changes in the chromatogram are compiled in Figure 4Q. Changes include TTA insertion at one site (Site A) and changes in another site (Site B) that include insertion of a T at one location and deletion of T from a nearby sequence. However, for clarity these mutations are addressed as site A and site B mutation. MatInspector (Genomatix Software Gmbh, Munich, Germany), a program to find transcription factor binding sites, predicted Myocyte enhancer factor-2 (Mef-2) binding sites present near site A and other falling at site B. The predicted Mef2 sites are not similar to previously published consensus sequence (YTAWWWWWTAR, Cripps et al. 2004). However, Mef2 has also been known to bind to different TA rich sequences (Andre’s et al. 1995).

**Mutation abrogates Mef-2 binding to URE leading to reduced TnI transcript and protein.**

Gene regulation is an elaborate process involving multiple players at various levels. The Mef-2 transcription factor is known to bring about the transcription of the muscle genes and muscle differentiation (Lin et al. 1996; Black and Olson 1998; Kelly et al. 2002; Sandmann et al. 2006; Elgar et al. 2008; Tanaka et al. 2008). Mef-2 is also involved in spatio-temporal expression of downstream targets by altering its own activity levels (Elgar et al. 2008). Previous studies done on wupA gene regulation in *Drosophila* by Marin et al. (2004) have shown
significance of Mef-2 (dMef-2) transcription factor binding in TnI IRE for quantitative wupA expression pattern in different muscle sets. They also suggested multiple Mef-2 binding sites in URE region which could work synergistically with IRE to bring about optimal expression levels of TnI in the IFMs. To assay for the Mef-2 binding affinity, CS1 and CS2 oligos (for the DNA sequence of wupA gene regulatory region containing site A and site B Mef-2 binding sites predicted near fliH mutation, Figure 4Q; check materials and methods) were used as control. Their corresponding mutant fragments (carrying the in-vivo mutation present in fliH) are labeled as FM1 and FM2, respectively. dMef2 oligos which have already been shown to bind dMef2 protein were used as positive controls (Cripps et.al. 2004). dMef2, CS1 and CS2 oligos bind to the dMef-2 lysate and the binding gets competed-out by their respective cold oligos (Figure 5A). Mutant FM1 oligos showed no binding whereas FM2 showed lesser complex formation as compared to CS2 when equal amount of radioactivity was taken (Figure 5B). CS1 and CS2 oligos could compete out when its own specific cold was used. Excess of wild type CS2 cold was able to compete-out the FM2 complex formation. To verify the quantitative difference in dMef2 binding between the control and mutant sequences, we performed cross-competition EMSAs. Our results shown in Figure 5C, indicate that, the cold dMef2 probe gradually competes-out the labeled dMef2 at 50- and 100-fold excess, thus confirming the specificity of the protein binding to the control dMef2 sequence. The cold CS1 and CS2 probe was able to compete the labeled dMef2 at 50- and 100-fold excess (Figure 5D) but the cold FM1 and FM2 can only compete at 200-fold excess but remain same at a 100-fold (Figure 5E). More amount of mutant cold is required to compete out the dMef2-protein complex. These results verify that CS1 and CS2 binds to dMef2 protein in a specific and stronger fashion than the mutant FM1 and FM2 sequence. The cross competition experiments show that the binding is not an all or none phenomenon.
To confirm the above results further, the 979 bp stretch of genomic DNA harboring fliH mutation (fliH-URE) was checked for promoter activity in C2C12 myoblast cell culture by subcloning it in luciferase vector pGL3 Basic. C2C12 is a mouse myoblast cell line which expresses the Mef-2 orthologue of Drosophila dMef2 (Tomczak et al. 2003). fliH-URE showed 50% reduction in luciferase activity compare to the control (Control-URE) (significant at p<0.0001 (Figure 5F). To verify whether Drosophila Mef2 protein alone can activate the promoter we used HFF cells (Human foreskin fibroblasts), where it is known that the vertebrate MEF2 protein is very minimally expressed (Neelam et al. 2005), thus serving as a good model to study promoter activity in a vertebrate Mef2 null background. There was no significant difference in the luciferase activity when Control-URE and fliH-URE constructs were transfected in HFF cells (data not shown). When these same constructs were co-transfected in the Drosophila Mef2 over expression background a significant level of luciferase activity was seen in the Control-URE suggesting that the activation of promoter is due to Drosophila Mef2 protein binding to the promoter region (Figure 5G). The confirmation of the over expression of dMef2 was seen in the transcript level of the plasmid transfected cells as compared to the naive HFF cells (Figure 5H). Thus, electrophoretic mobility shift assay, supported by promoter activity assay, suggests that the fliH mutation abrogates the proper binding of dMef-2 transcription factor to TnI URE, which may cause decreased expression of TnI and also confirms that Drosophila Mef2 binding activates the promoter of TnI.

To assess the relative levels of TnI transcript accumulation in mutant versus the wild type control, quantitative RT-PCR was done using mRNA isolated from 2 days old adult fly IFMs (Figure 6A). Transcripts were detected by primers which amplified exon 6b1 (IFM and TDT specific exon) and exon 4 (Constitutive exon). Transcript level was found to be significantly
reduced in \textit{fliH} mutants raised at 25° as compared to \textit{Canton-S} wild type. There were no differences in the TnI expression pattern between the constitutive exon and IFM-TDT specific primers, supporting that the URE mutations could be the reason. The level of TnI transcript in \textit{fliH} mutant raised at 18° was higher reflecting the normal muscle phenotype. Flies heterozygous for \textit{wupA}^{hdp-3} mutation were used as the internal control and results showed that transcript accumulation was much reduced in \textit{wupA}^{hdp-3}/+ as compared to \textit{fliH} at 25°. Consistent to the RNA levels, western blot from the mutant also showed reduced accumulation of TnI protein in adult the IFMs raised at 25° as compared to wild type \textit{Canton-S} flies (Figure 6B). Protein accumulation was checked in 70-80h pupae IFMs which have not undergone hypercontraction. Protein accumulation in the mutant pupae grown at 25° and 18° is comparable (Figure 6C), suggesting that accumulation of TnI is similar in both the temperatures before the initiation of hypercontraction. However, the \textit{fliH} genotype where there is no hypercontraction as a result of removal of thick filaments, still showed slight decrease in expression of TnI when raised at 25° (Figure 6D) as compared to 18° flies, suggesting that temperature has a bearing on the expression pattern of TnI as a result of the URE mutations.

\textbf{Coordinated down-regulation of structural gene transcripts working together in a complex}

Mogami and Hotta (1981) showed for the first time that mutations in a single myofibrillar protein gene affect accumulation of other structural proteins. This has been specially studied elaborately in the case of the null alleles, \textit{Act88F} and \textit{Mhc} alleles, which lead to their corresponding protein null in the IFMs (Beall \textit{et al.} 1989). Similar results have been reported in case of TnI null allele \textit{wupA}^{hdp-3} (Nongthomba \textit{et al.} 2004) and TnT major isoform TnT10a null in IFM (Nongthomba \textit{et al.} 2007). However, hypercontracting alleles present in the coding region of TnI (\textit{wupA}^{hdp-2}) or TnT (\textit{up}^{101}) do not show such down regulation (Nongthomba \textit{et al.} 2007).
2003). Since, fliH is a hypercontracting allele that does not fall in above categories of mutations, transcript levels of other structural proteins was checked through semi-quantitative RT-PCR.

Coordinated down-regulation of RNA levels of the genes, which function together in muscle to generate regulated force, was observed in the case of fliH mutation (Figure 7). The Tm2 gene which encodes for tropomyosin showed down-regulation of transcript in fliH raised at 25° (Figure 7A). However, no significant difference was seen in Act88F RNA levels (Figure 7B). Semi-quantitaive RT-PCR also showed reduced levels of troponin-C (TnC4) transcript in fliH (Figure 7C). TnC4 is the major isoform of TnC expressed in IFMs (Qui et al. 2003). Like TnC4, TnT10a is the major TnT isoform specific for IFM and is expressed at much higher levels than other ubiquitous TnT10b isoforms (Nongthomba et al. 2007). TnT10a transcript was found to be significantly reduced in fliH (Figure 7D). No reduction in the transcript levels for minor isoforms of TnC (TnC1) and TnT (TnT10b) was observed (Figure 7, E and F).
DISCUSSION

Maintaining the right stoichiometry of the structural proteins is important for the proper assembly of myofibrillar structure and function. This is evident from the myofibrillar defects shown by overexpression of TnT (Marco-Ferreres et al. 2005), Mhc (Cripps et al. 1994) and heterozygotes for most of the structural proteins (Prado et al. 1999). Beall and co-workers (1989) showed in an elegant experiment that concomitant reduction of both actin and myosin restores normal myofibrillar structure and flight ability than the haploinsufficiency of the either protein alone (Beall et al. 1989). Many factors are responsible for regulating the right levels of expression of the structural proteins in different muscles, including the transcription factors Mef-2 and Chorion factor-2 (CF-2) (Elgar et al. 2008; Garcia-Zaragoza et al. 2008; Tanaka et al. 2008). Mef-2 has been shown to dictate the differential expression of its downstream target genes through its spatio-temporal activities (Elgar et al. 2008), whereas, CF-2 has been shown to be involved in regulation of the Actin and Myosin filaments stoichiometry (Gajewski and Schulz 2010). For TnI and TnT, correct levels of expression in different muscles are brought about by two enhancer elements, namely the upstream regulatory element (URE) and the intronic regulatory element (IRE) that work synergistically (Marin et al. 2004; Mas et al. 2004). Both the elements harbor multiple Mef-2 binding sites, suggesting that integration of Mef-2 in these regions could be important for bringing the correct levels of expression of associated genes. In fliH, two Mef-2 binding sites in the URE region are compromised; leading to 50% reduction in promoter activity and overall reduction in TnI transcripts and protein levels (Figure 6 A-D). Over-expression of larval isoform of TnI (L9) rescued not only the muscle phenotype but also the flight abilities, suggesting that correct stoichiometry of TnI is restored. It is likely that reduction in the TnI levels impedes proper troponin complex formation thus leading to
uncontrolled acto-myosin interaction, leading to IFM hypercontraction (Figure 8). Even though function of other muscles is also affected, more pronounced phenotype is seen in the IFMs. Other hypercontracting alleles like \( wupA^{hdp-2} \) and \( up^{101} \), with mutations in constitutive exons, also show more pronounced phenotype in the IFMs (Nongthomba et al. 2003), which may result from the fact that IFMs are the only muscles which require stretch activation and have to sustain the mechanical stress and strain produced through cuticular movement. Such heterogeneity in the myopathic phenotypes in different muscles in a single individual is also observed in other species including humans (reviewed in Emery 2002). Reduction of TnI level is also seen in the case of flies heterozygous for the \( wupA^{hdp-3} \) mutation that is null for TnI in the IFMs. \( wupA^{hdp-3}/+ \) flies also show muscle hypercontraction (Nongthomba et al. 2003) suggesting that the mechanistic aspects of muscle dysfunction and tearing are similar between \( wupA^{hdp-3}/+ \) and \( fliH \). Unlike \( wupA^{hdp-3} \) (Nongthomba et al. 2004), \( fliH \) does not show any defect in sarcomeric assembly until 75 hours APF suggesting that TnI protein levels during pupal development is sufficient to drive proper assembly. This implies that a certain threshold of TnI is required for the proper assembly; however, a proportion of TnI-containing troponin complexes may not be adequate for proper regulation of muscle contraction. Thus, mutants will not be able to sustain prolonged unregulated force leading to muscle damage once activated at around 72-75 hours APF.

Another interesting observation was the difference in the penetrance of hypercontraction phenotype in \( fliH \) and \( wupA^{hdp-3}/+ \). As opposed to 100% in \( fliH \) only 30% of flies showed hypercontraction phenotype in \( wupA^{hdp-3}/+ \) (Nongthomba et al. 2003). Molecular data shows that \( fliH \) has elevated TnI levels than \( wupA^{hdp-3}/+ \), which will explain normal myofibril assembly during pupal development stages whereas, \( wupA^{hdp-3}/+ \) has been shown to have defective assembly during early development (Nongthomba et al. 2004), which will not allow proper
generation of force to produce hypercontraction. Based on heterogeneity in development, some of the flies that generate enough uncontrolled acto-myosin force may produce the hypercontraction phenotype in $wupA^{hdp-3/+}$. Other factors like the difference in the rate of accumulation of the proteins cannot be ruled out. The rate of actin accumulation with respect to myosin is slower in $Act88F^{KM88/+}$ flies, as a result, the flies are rendered flightless even though actin amounts are comparable to wild type. This can be rescued, up to some extent, by balancing the stoichiometry with myosin ($Mhc^{10}/+$; Myosin null) heterozygotes (John Sparrow and UN; unpublished data). It is likely that in $wupA^{hdp-3/+}$, the rate of accumulation of TnI could be slower with respect to other thin filament proteins with which it forms a complex. One can speculate based on the difference in TnI levels that there could be a difference in the sarcomeric assembly and the troponin complex formed between the two genotypes. We propose that this difference leads to more compromised sarcomeric assembly in $wupA^{hdp-3/+}$ thus producing less acto-myosin interaction owing to sarcomeric defects and manifestation of muscle hypercontraction in fewer flies.

Hypercontracting alleles such as $wupA^{hdp-2}$ and $up^{101}$ harbor point mutations in their coding sequence that leads to defects in regulation of muscle contraction, owing to which IFMs degenerate once they start functioning (Nongthomba et al. 2003; Cammarato et al. 2004). Both these mutations are recessive in nature but when brought in trans-heterozygous condition show genetic interaction leading to muscle hypercontraction in all the flies. It is likely that more than half the troponin complexes formed in this genetic background will carry one or the other mutation (Figure 8). These complexes once activated by $Ca^{2+}$ will move away from steric blocking sites on actin leading to uncontrolled acto-myosin interactions. Since the troponin complex plays a central role in $Ca^{2+}$ regulation of muscle contraction, absence of it will lead to
muscle hypercontraction. *fliH* and *wupA*\(^{hdp-3/+}\) show reduced accumulation of TnI thus impeding formation of functional troponin complex which leads to unregulated acto-myosin interaction and hence IFM hypercontraction. Similarly, one can explain the intragenic interaction between *fliH* and *wupA*\(^{hdp-2}\) based on formation of the functional troponin complex. In this case too, troponin complex formed will either lack TnI or will carry a mutated version leading to hypercontraction. *fliH* and *up\(^{101}\)* transheterozygotes show reduced flight ability with no obvious muscle tearing or hypercontraction. In this genetic background, *up\(^{101}\)* contributes a normal copy of TnI locus and one copy of regulatory mutations is enough to express sufficient protein (*fliH/+* flies are normal); very few troponin complexes that carry mutant TnT will be nonfunctional (like *up\(^{101}/+\)*), so flies show partial complementation.

Coordinated down regulation of the expression of thin filament genes in response to mutation in a single thin filament protein has been very well documented in the case of null alleles of myosin (*Mhc\(^7\)*), actin (*Act88F\(^{KM88}\)*), TnI (*wupA*\(^{hdp-3}\)) and TnT (*up\(^1\)*). Such phenomenon has not been reported for known hypercontracting alleles, like *wupA*\(^{hdp-2}\) and *up\(^{101}\)*. Our result emphasize that there is coordinated down-regulation of major isoforms of TnC and TnT (*TnC4* and *TnT10a* respectively) as compared to minor isoforms, *TnC1* and *TnT10b* which do not show any change in RNA levels in the IFMs. This could be explained based on the formation of functional troponin complex and interdependence of the expression level of each member of the complex by an unknown mechanism. Thus, in the absence of a single player of the complex, the message for the other major interacting partners is also down-regulated. Studies involving coordinated down-regulation of proteins which functions together in the same complex have also been reported in other model organisms like Zebrafish (Sehnert *et al.* 2002).
Genetic variations lead to changes at cellular and molecular level affecting performance of the flight muscles. Apart from the intrinsic variables, extrinsic factor such as temperature also plays an important role in the flight ability. Certain species of moth cannot fly until they have prewarmed their flight muscles (Esch 1988). Flight performance of the Canton-S flies, used as control in the present study, showed reduced flight at 18° (Figure 3). They were better flighted at elevated temperature suggesting physiological difference at the two extreme temperatures. *fliH* mutation also shows temperature dependent effect in its viability, walking and jumping. However more profound phenotype is seen in the IFMs which are raised at 25° which correlates with reduced level of TnI transcript and protein as compared to those at 18° and wild type. Another hypercontracting alleles like, *flapwing*, are known to show increased viability and reduced hypercontraction when flies are cultured in reduced temperature (Pronovost *et al.* 2013). Initiation of hypercontraction correlates with the movement of the thorax of the pupae within the pupal case, which is more pronounced in 25° raised flies than those raised at 18°. Moreover, one needs to keep the 18° raised flies for at least 2-3 hours at 25° to see flight suggesting for a limited movement and lethargic nature of pupae and muscles in lower temperature. It is likely that the reduced activity of the muscles and less mechanical stress will allow the muscles to assemble completely, so that it will show suppression of the hypercontraction after eclosion. Our work has shown that binding of Mef-2, a trancription factor, which brings about correct expression of the structural genes, is affected in *fliH* mutant. However, defective binding of Mef-2 or any other transcription factors or changes in local structure of the DNA that brings about the temperature sensitive effect, need to be worked out. Little is known about the mechanisms that confer different temperature dependent phenotypes. Works done on lower organisms like bacteria (Tamai *et al.* 1998) and yeast (Nouraini *et al.* 1996) and in plants (Gilmour *et al.* 1998;
Zhu et al. 2007) have shown that mutations in the cis-regulatory region show temperature dependent phenotype. Work done on the W3133 operon in bacteria showed that an insertional mutation in the promoter region could confer temperature sensitive phenotype and affect transcription efficiency by stabilizing the DNA stem loop structure (Tamai et al. 1998). Such results have not been reported in higher organism, may be due to higher order organization of the genome. However, temperature sensitive mutations in protein coding sequence have been isolated in eukaryotes wherein temperature difference might affect the folding, stability and function of the proteins (Reese and Katzenellenbogen 1991; Jantti et al. 2001; Mondal et al. 2007; Hoeberichts et al. 2008).

In this study, we have shown that mutation in the regulatory region of TnI can lead to IFM hypercontraction. Mutations in the coding region of myofibrillar protein causing myopathies in humans are very well documented. However, there is no record of a mutation or single nucleotide polymorphism (SNP) in the non coding region and regulatory region being associated with disease. This may be one reason for the large number of myopathic cases where the causative nature of mutation remains unknown. We propose from our study that regulatory mutations as well as mutations leading to stoichiometric changes (splice site mutants or nonsense mutations resulting in protein null) that may cause myopathic conditions can easily be identified by simple quantitation of transcripts and proteins by applying the whole genome approach. In humans, many mutations in sarcomeric proteins have been identified that lead to myopathic conditions. Cellular fibre disarray seen in hypercontracted IFM is also observed in the case of human hypertrophic cardiomyopathies (Seidman and Seidman 2001) and dystrophic muscles (Amato et al. 1998), suggesting that there may be parallel genetic pathways for hypercontraction induced cellular phenotypes. Conservation of expression of many remodeling proteins has been
already shown for hypercontracting $Mhc$ alleles (Montana and Littleton 2006). Mutations have also been uncovered in human TnI that lead to various cardiomyopathies and skeletal myopathies (Kimura et al. 1997; Murphy et al. 2004; Gomes et al. 2005). The molecular mechanism by which these mutations lead to pathogenesis of myopathies remains unclear. One needs to study the pathogenesis of these mutations in a model organism and follow the effects of other factors like environmental stress as well as different genetic backgrounds. Overall, our results shed new insights into the importance of maintenance of structural protein stoichiometry during muscle assembly for proper function with implications in identification of mutations and disease phenotypes in other species including humans.
ACKNOWLEDGEMENTS

We would like to thank Mrs. Sneha Raghuram and Mrs. Meenakshi Sen at IISc Confocal Facility for their technical assistance. We would like to acknowledge anonymous reviewers, Prof. John Sparrow, University of York, UK, Prof. S. Mahadevan and our lab members for their critical comments and suggestions. We extend our gratitude to Prof. Richard Cripps, University of New Mexico, USA for the Mef-2 construct and Prof. Alberto Ferrus, Cajal Institute, Madrid, Spain for flies and antibodies. We thank Prof. Sathees C. Raghavan and Ms. M. Nishana, Dept of Biochemistry, Indian Institute of Science, India, for help with EMSA experiments. We thank Dr. Sunita Chopra for her help in the co-transfection luciferase assays. The Bloomington Drosophila Stock Centre and the NCBS stock centre, Bangalore, India for providing flies. We acknowledge Indian Institute of Science (IISc); Department of Science and Technology (DST) and Department of Biotechnology (DBT), Govt. of India, for financial assistance.
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FIGURE LEGENDS

Fig. 1. IFM abnormalities in 3- to 5-days old fliH flies. (A) Flight data of Canton-S and fliH flies grown at 25°, where all the mutant flies show flightless phenotype (n=50). Flight ability was measured using Sparrow Box, with gradation on their ability to fly U- upward, H- horizontal, D- downward and F- non-flighted. (B) Polarized light micrograph of wild-type DLMs. (C) fliH hemithorax showing hypercontraction muscle phenotype (arrows). Arrowhead points to normal jump muscles in the mutant. (D) Rescue of fliH phenotype. Mhc\(^{2B}\) harbors mutation in actin binding loop and completely rescues fliH hypercontraction phenotype. (E) Significant rescue of the muscle phenotype is also achieved with headless myosin construct (Y97) and a copy of myosin null (to reduce functional myosin). All the 6 DLMs are marked with asterisks. Anterior is to the left and dorsal on the top.

Fig. 2. Developmental profile of the fliH DLMs. The sarcomeric structure of muscles was observed using confocal microscope. Inter-digitating thick filaments was followed with wee-P26 (green, see materials and methods for details) and thin filaments (red) with Phalloidin-TRITC. (A-D”) Myofibril develops normally in fliH pupa till 75 hours APF. (E-E””) Wild-type sarcomeres show uniform distribution of thick filaments (E, arrowhead). (F-F”) Myofibrillar disorganization is seen in the IFMs of freshly eclosed fliH flies. (F) Thick filaments are diffused with increased spacing between them (arrows). (F””) Regular arrangement of thin filaments is also disturbed (compare with E”). (Scale bar, 5µm).

Fig. 3. Behavioral experiments. (A) Survival curve of fliH raised at 25° shows partial lethality dispersed over all the developmental stages, though they do not have any associated lethality at permissive temperature (18°) (B). (C) Jumping ability is severely compromised in mutants at 25° and 18°. (D) Mutant flies take longer time to walk a distance of 10 cm as compared to wild type
counterpart at 18° and 25°. Error bar denotes SEM. Statistical analysis was performed using unpaired two-tailed t-test.

**Fig. 4. Genetic mapping and rescue of fliH.** (A) X-chromosome map showing cytological location 15F-19A (adapted from Flybase). Green lines denote X-chromosome segments duplicated on Y chromosome for various transposition lines used in the study. Meiotic recombination mapping with forked (f) and carnation (car) places fliH mutation at 1-57.69± 0.87 recombination map unit. (B) fliH was not rescued with T(1;Y)v7. Complete rescue of the DLM fibre morphology with T(1;Y)B18 and T(1;Y)W39 transposition line (C and D respectively). (E and F) Partial rescue of the flight ability of fliH mutants with above mentioned transposition lines. (G and H) Confocal micrograph showing normal sarcomeric organization of DLMs in fly rescued with duplicated segments on Y chromosome. (I-M) fliH interaction with other hypercontracting alleles. (I) wupA<sup>hdp-2</sup> is a hypercontracting allele of TnI that shows muscle tearing. (H) fliH genetically interacts with wupA<sup>hdp-2</sup> at 25° and all the flies show muscle hypercontraction. (K) wupA<sup>hdp-2</sup> genetically interacts with TnT hypercontracting allele up<sup>101</sup> in trans leading to muscle rupturing. (L) fliH and up<sup>101</sup> transheterozygotes do not show muscle tearing. Arrows point to muscle tearing due to hypercontraction. (M) Flight data reveals that all the flies are flightless in case of fliH/wupA<sup>hdp-2</sup> and wupA<sup>hdp-2</sup>/up<sup>101</sup> transheterozygotes, whereas fliH/up<sup>101</sup> females show gradation in flight ability. Flies heterozygous for fliH mutation serve as control for the flight test. (N-P) fliH mutants rescued by targeted overexpression of TnI transgenic line in IFM. (N) Polarized light image showing the rescue of DLM fibres with overexpression of a copy of TnI (w, UH3, fliH/Y; UAS-TnI-L9). (O) Myofibre showing complete rescue of the sarcomere organization as visualized by confocal imaging of w, UH3, fliH/Y; UAS-TnI-L9 flies. (P) Rescued fliH flies with TnI transgene show normal flight. (Q). Chromatogram
showing mutations in fliH as analyzed by DNA sequencing. Mutations lie in upstream regulatory region of the wupa gene coding for TnI. Box indicates transcription factor Mef-2 binding site as predicted by MatInspector. Phallodin-TRITC for F-actin (red) and anti-Mlp60A antibody localizing on Z-discs (green). Flight data and muscle analysis: n=25, and all the flies were grown at 25°.

**Fig. 5. Mef-2 binding is affected in fliH, leading to reduced TnI transcript and protein.** (A) The dMef2, CS1 and CS2 oligos bind to the Mef2 protein lysate to form complex, and the binding get compete-out by their respective cold oligos. (B) Mutation in fliH abrogates binding of dMef-2 to FM1 oligo whereas level of binding is much reduced in FM2 as compared to CS2. The binding of FM2 is competed out by CS2 cold. Cross- competition EMSAs. (C) Published dMef2 probe (Cripps et al. 2004) was used as a control for the competition assay. Cross competition using cold dMef2 oligos (25-, 50- and 100-fold excess) shows sequential abrogation of the complex. (D) Cross-competition with cold CS1 and CS2 (25-, 50- and 100-fold excess) shows better competition then the (E) Cold FM1 and FM2 (25-, 50-, 100 and 200-fold excess) (Compare the lanes with 100-fold excess). But at 200-fold excess even FM1 cold competes out the complex but not FM2 These results were estimated quantitatively as the results do not show all or none kind of competition.(F) 979 bp upstream region of TnI gene was used for promoter activity studies by subcloning the region into luciferase vector pGL3 basic. The assay was carried out using C2C12 myoblast cell line. The fliH-URE shows 50% reduction compared to the control (Control-URE) in luciferase activity and was significant at p<0.0001. (G) HFF cells were co-transfected with pcDNA3.1 and pcDNA3.1–Mef2 condition with Mutant and Control promoters independently. There is a significant increase in the luciferase activity in the Control-URE where the Mef2 protein was overexpressed compared to that of fliH-URE at p<0.0001. (IH
RT-PCR shows that dMEF2 transcripts are present only in the HFF cells where the MEf2 protein was overexpressed. HFF cells have vertebrate MEf2 protein present in very basal levels (Neelam et al. 2005)

**Fig.6. Reduction in TnI transcripts and protein levels.** (A) Quantitative RT-PCR from 1-2 days old adult IFM shows significant reduction in the level of TnI transcript in fliH background at 25° with slight elevation in transcript levels at 18°. Error bar denotes SD. Statistical analysis using one way ANOVA reveals that means of fold expression of TnI transcript of different genotypes when analyzed in groups of two show significant difference in mRNA accumulation in fliH (p<0.001). (B) TnI protein accumulation in mutant adult IFMs. Western blot analysis using Drosophila TnI antibody reveals that final accumulation of protein in 1-2 days old adult IFMs is much reduced in fliH raised at 25° (p<0.05). However, protein levels are comparable to wild type in fliH flies grown at 18°. wupA^hidp-3/+ flies also show significantly reduced TnI accumulation (p<0.05). Anti-Tubulin antibody was used as control. Error bar denotes SEM. (C) TnI accumulation in 70 to 80 hours old pupae. The protein accumulation is not significantly different in all the genotypes. Error bar denotes SEM. (D) TnI protein accumulation in fliH flies in Mhc^7 background. Mhc mutations do not affect the thin filaments protein accumulation (Nongthomba et al. 2004). Mhc^7 was used to suppress the hypercontraction at 25° grown fliH flies to get enough muscle materials for protein and RNA assays.

**Fig. 7. Coordinated downregulation of transcripts of other thin filament proteins in fliH.**

Semi-quantitative RT-PCRs of (A) Tm2, (C) TnC4 and (D) TnT10a show reduced levels in fliH flies raised at 25°. Significant rescue in RNA levels is observed at 18° raised fliH flies. (E) TnC1, (F) TnT10b and (B) Act88F RNA levels remain unchanged both at 25°C and 18°C. Semi-
quantitative RT-PCR plot showing relative RNA levels in arbitrary units. Error bar represent mean from standard deviation for minimum of three RT-PCR runs.

**Fig. 8. Schematics for genetic interactions between hypercontracting alleles.** (A) Thin filament protein organization in the wild type flies. (B) Troponin complex is functionally abnormal in \( wupA^{hdp-2} \) (Troponin I mutant) flies leading to aberrant regulation of muscle contraction and relaxation and hence resulting to tearing of muscles. (C) Recessive mutations \( up^{101} \) (Troponin T mutant) and \( wupA^{hdp-2} \) interact in trans rendering most of the troponin complex abnormal as they must be harbouring either of the mutation. (D) \( fliH \) shows downregulation of TnI hence troponin complex formation is impeded. (E) \( fliH \) shows –cis interaction with \( wupA^{hdp-2} \). (F) \( fliH \) being recessive regulatory mutation does not interact with the TnT mutation \( up^{101} \) to bring about significant IFM tearing.
Figure-1

Figure-2
Figure 3

A. Survival of Canton-S and fliH strains at 25°C.

B. Survival of Canton-S and fliH strains at 18°C.

C. Distance jumped by Canton-S (25°C) and fliH (25°C) at 25°C and Canton-S (18°C) and fliH (18°C) at 18°C.

D. Time taken to walk 10 cm by Canton-S (25°C) and fliH (25°C) at 25°C and Canton-S (18°C) and fliH (18°C) at 18°C.
Figure-4
Figure-5
Figure-6
Figure 7
Figure-8

Wild type

\(\text{wupA}^{\text{hp-2}}\)

\(\text{wupA}^{\text{hp-2}}, +/+, \text{up}^{302}\)

\(\text{fliH}\)

\(\text{fliH}/\text{wupA}^{\text{hp-2}}\)

\(\text{fliH}, +/+, \text{up}^{301}\)

- Actin monomer
- Troponin C
- Troponin I
- Mutant Troponin I
- Troponin T
- Mutant Troponin T