

RESEARCH ARTICLE

Roles of the Troponin isoforms during indirect flight muscle development in *Drosophila*

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Abstract

Troponin proteins in cooperative interaction with tropomyosin are responsible for controlling the contraction of the striated muscles in response to changes in the intracellular calcium concentration. Contractility of the muscle is determined by the constituent protein isoforms, and the isoforms can switch from one to another depending on physiological demands and pathological conditions. In *Drosophila*, a majority of the myofibrillar proteins in the indirect flight muscles (IFMs) undergo post-transcriptional and post-translational isoform changes during pupal to adult metamorphosis to meet the high energy and mechanical demands of flight. Using a newly generated Gal4 strain (*UH3-Gal4*) which is expressed exclusively in the IFMs, during later stages of development, we have looked at the developmental and functional importance of each of the troponin subunits (Troponin-I, Troponin-T and Troponin-C) and their isoforms. We show that all the troponin subunits are required for normal myofibril assembly and flight, except for the Troponin-C isoform 1 (TnC1). Moreover, rescue experiments conducted with Troponin-I embryonic isoform in the IFMs, where flies were rendered flightless, show developmental and functional differences of TnI isoforms and importance of maintaining the right isoform.

Introduction

All the muscles in *Drosophila* are striated and their contraction is regulated by the Troponin-Tropomyosin (Tn-Tm) complex like their vertebrate counterparts. However, muscle contraction in the indirect flight muscles (IFMs) is activated by mechanical stretch/applied strain in addition to the Ca^{2+} activation, to produce and sustain the high wing beat frequency during the flight (Peckham *et al.* 1990; Agianian *et al.* 2004; Moore 2006; Bullard and Pastore 2011). Most of the structural proteins of the IFMs are homologous to their vertebrate counterparts, performing similar function during muscle contraction (Vigoreaux 2006). A majority of these proteins undergo isoform switch during later stages of development to meet the physiological demands of the adult flight (Marden 2006; Orfanos and Sparrow 2013). The IFMs are dispensable for survival under laboratory condition, providing an effective genetic system to study the developmental and functional importance of different isoforms (Nongthombaet *et al.* 2004; Vigoreaux 2006). Isoform replacement studies in the IFMs suggest that most of these isoforms complement each other and do not hamper the myofibril assembly *per se* but have different mechanical properties as reflected from compromised flight (Miller *et al.* 1993; Wells *et al.*

1996; Fyrberget *et al.* 1998; Swank *et al.* 2002). Though the factors/signals, which lead to the isoform switch, are not clearly understood, these isoforms are spatio-temporally regulated by cis-regulatory factors or alternative transcript splicing (Marin *et al.* 2004; Mas *et al.* 2004; Marden 2006).

In *Drosophila*, both Troponin-I (TnI) and Troponin-T (TnT) proteins are encoded by a single gene in each case and all their respective isoforms are produced by differential alternative splicing. TnI has been shown to produce 10 different isoforms, of which the exon 6b1 containing isoforms are solely expressed in the IFMs and tergal depressor of trochanter (TDT), with or without exon 3 (Barbas *et al.* 1993). The isoform that includes exon 3 is the major constituent of the adult IFMs (Nongthombaet *et al.* 2004). Whereas, the TnT gene has 11 exons, of which exons 3, 4 and 5 containing isoforms are excluded from the IFMs and TDT (Benoist *et al.* 1998). Exon 10 is alternatively spliced to produce exon 10a and 10b isoforms, both of which are expressed in IFMs and TDT (Herranz *et al.* 2005b; Nongthombaet *et al.* 2007). Unlike TnI and TnT, TnC isoforms are produced by five independent genes, of which TnC1 and TnC4 isoforms are expressed in the IFMs in the ratio of 1:5 (Qiu *et al.* 2003; Herranz *et al.* 2005a).

The defective splice site mutation (*heldup*³ – *hdp*³) in TnI exon 6b1 results in the absence of TnI and subsequently, the IFMs are never formed due to unregulated actomyosin interactions during early myofibril assembly (Nongthombaet *et al.* 2004). Similarly, a mutation in the TnT exon 10a splice site (*upheld*¹ – *up*¹) leads to abnormal myofibrils (Nongthombaet *et al.* 2007). There is no reported mutation for either TnC1 or TnC4, though biochemical studies suggest that TnC1 is required for isometric contraction and TnC4 for stretch activation (Linari *et al.* 2004; Krzic *et al.* 2010; Bullard and Pastore 2011). Most of the isoform switches happen during later stages of IFM development, around 65-75 hours after puparium formation (APF) (Nongthombaet *et al.* 2004; Orfanos and Sparrow 2013). Developmental and functional consequences of reduction in the expression of specific troponin isoforms during the isoform-switching stage have not been addressed before. Defects in the isoform switch have been implicated in many pathological conditions in higher vertebrates including humans, particularly for the TnT (Wei and Jin 2011). Using a newly isolated enhancer trap Gal4 strain (*UH3-Gal4*), which is expressed exclusively in the IFMs during the isoform switching stage, we have knocked down troponin isoforms to study their myofibrillar assembly and functional roles. We show that all the troponin proteins are required for normal myofibril assembly and flight, except for the Troponin-C isoform 1 (TnC1), which showed normal myofibrils with reduced flight. We also show that expression of

embryonic TnI isoform in the place of adult isoform in the IFMs allows the assembly of myofibrils but is not functionally equivalent to the adult isoform.

Materials and Methods

Fly strains

The fly strains used in the study were procured from Bloomington Drosophila Stock Center, Indiana University, USA; Vienna Drosophila RNAi Centre, Austria; and Fly Facility, National Centre for Biological Sciences, Bangalore. Flybase IDs along with the specific strain numbers are given within the brackets. The fly strains used in this study are - *P{GawB}c747* (FBti0007258 - 6494), *UAS-GFP* (FBti0003040 - 1521), *UAS-RedStinger* (FBtp0018199 - 8547), *UAS-dcr2* (FBti0100276 - 24651), and *tub-Gal80^{ts}* (FBtp0017264 - 7019). RNAi lines used are: *UAS-TnI IR* (FBst0460508), *UAS-TnI V10 (VALIUM 10)* (FBti0130301 - 31893), *UAS-TnT IR* (FBst0457162), *UAS-TnT V20* (FBst0032949 - 32949), *UAS-TnCI V10* (FBst0027053) and *UAS-TnC4 IR* (FBst0469555). $\Delta_{2-3}Ki$, *hdp³* and *UAS-TnI-L9* were a kind gift from Prof. John Sparrow, University of York, UK. *Canton-S* was used as a wild type strain unless specified. All stocks were maintained in cornmeal-yeast-sugar-agar medium at 22°C, and crosses were set up at 25°C. All temperature sensitive crosses were set at 18°C in the presence of *tub-Gal80^{ts}* and later moved to 29°C at specific hours.

Enhancer trap Screen

Screen for enhancer trap lines was followed according to O’Kane and Gehring(1987). *P{GawB}c747* was crossed with flies carrying transposase source $\Delta_{2-3} Ki$. Male flies of filial 1 (F₁) generation or “jump starters” were crossed with reporter *UAS-GFP* and their progenies were screened under fluorescent stereo microscope (Olympus SZX12 fluorescence stereozoom microscope). F₂ - individual fly showing GFP expression in adult thorax were crossed to different balancer lines to establish stable lines. Progenies from each cross were selfcrossed, and the chromosome with *P{GawB}* insertion was identified based on the eye marker in the next generation. Each of these isolated strains was later crossed with reporter *UAS-GFP* to confirm their expression.

Insertion localization by inverse PCR

The inverse PCR protocol given in the Berkeley Drosophila Genome Project (<http://www.fruitfly.org/about/methods/inverse.pcr.html>) was followed except for changes in the primers. Following primers were designed to amplify the 5' region of Gal4 encoding sequences using the Gal4 Enhancer Trap Database - *PGaw2* (5' – CAGATAGATTGGCTTCAGTGGAGAC – 3') and *PGaw3* (5' – CGCATGCTTGTTTCGATAGAAGAC – 3'). The genomic DNA was digested with enzyme *Sau3A1* and ligated to form circularized DNA. Inverse PCR amplification was performed with above mentioned primers. The resulting amplified PCR product was cloned into the sequencing vector *pTZ57R/T* (Fermentas) and transformed into bacterial cells following standard protocol. Positive clones were screened and plasmids were sequenced using universal *M13F/R* primers (Macrogen, Korea). The resulting nucleotide sequences were BLAST analyzed against the *Drosophila melanogaster* genome using the Flybase database.

Real time PCR

Using Tri Reagent® (Sigma), total RNA from 5-days old adult IFMs was isolated from control and gene specific knocked down flies. Complementary DNA was prepared using the RevertAid First Strand cDNA synthesis kit following manufacturer's protocol (Thermo Scientific). The mRNA expression level of target genes was PCR quantified using the DyNAmo SYBR Green kit (Thermo Scientific) on Eppendorf Master cycler® eprealplex S. The oligo primers used for the thermal amplifications are as follows: – *rp49*: forward 5' - AGATCGTGAAGAAGCGCACCAAG - 3', reverse 5' - CACCAGGAACTTCTTGAATCCGG - 3', *TnI*, forward 5' - TCGCGGCAAGTTCGTCAAGC – 3', reverse 5' - GGACACTAGTGGACGTGTGG - 3', *TnT*, forward 5' - AGCTCTTCGAGGGTTTGA – 3', reverse 5' - TTGTGCGCTGAGTGAATC - 3', *TnCI*, forward 5' - CGCGTCAATACCAAGTTTATTTCTCGTC – 3', reverse 5' - CTTTTGATATTGTTTTAGTCGTCGCCAC - 3', *TnC4*, forward 5' - CCTAAACCTTAGCGGTGTAATTTG – 3', reverse 5' - CTTATCTGCTTTTGGCCCGATATTTG - 3'. All quantifications were from two independent biological samples. To calculate the fold changes of the expression level of mRNA, Ct values were normalized to *rp49* expression as endogenous control. P-values were calculated by one way-ANOVA using GraphPad Prism 5 software.

Other *TnI* primers used for thermal cycler amplifications are as follows: forward 5' – AACACAAATCAAATGGCTG – 3' designed at the 5'UTR, reverse 5' – CACATCAAATCTCTGATCAAG – 3' specific to exon 6a1, forward – 5' – GTGAAGGCCAGAAATGGGAT – 3' specific to exon 6b1 and reverse 5' - GGACACTAGTGGACGTGTGG - 3' designed at the 3'UTR.

Imaging

Samples for polarized light imaging were prepared from 3-5 days old adult thoraces following the protocol described in Nongthomba and Ramachandra (1999). To take the fluorescent images, wing discs were dissected from third instar larva and mounted in 20% glycerol. Aged pupae were removed from pupal case at specific hours after puparium formation (APF), and adult flies were briefly anaesthetized and fluorescence images were captured by digital camera (Leica DFC 300 FX) attached to the Olympus SZX12 stereomicroscope. Confocal microscopy was done following a protocol described in Rai and Nongthomba (2013). Briefly, using a sharp razor, flies were bisected after snap freezing in liquid nitrogen and fixed in 4% paraformaldehyde in PBS. Tissue samples were washed thoroughly with 0.3% PBTx and then stained with Phalloidin TRITC in 1:250 dilutions (P1951-TRITC, Sigma). After washing thoroughly, tissues were mounted in Vecta shield media (Vector Laboratories, USA), and images were taken using a Zeiss confocal microscope (LSM 510). Images were later assembled using the Adobe Photoshop CS3.

Behavioural assays

The flight test was performed following the method described previously by Drummond *et al.* (1991), and their ability to fly up, horizontal, down or flightless was plotted as a percentage. The walking ability test was performed with slight modification from Nongthomba *et al.* (2003). The time taken to walk (negative geotaxis) a distance of 15cm towards the light source was measured in a transparent 15ml falcon tube. For each fly, the test was repeated thrice, and the average time taken was plotted on the graph. The test for the jumping ability was done following a protocol described in Nongthomba *et al.* (2007).

Results

Screen for Gal4 strains that express in adult thoracic muscles

With an aim to generate the enhancer trap Gal4 strains that spatio-temporally express in the subsets of fly thoracic muscles, we crossed the *P{GawB}c747* Gal4 line, in which the P-element is located at the 41F region on the second chromosome, with a stably inserted P-element transposase source on the third chromosome. F₁ male flies carrying both the *P{GawB}* and transposase source, also known as jump starters, were crossed with the reporter strain carrying the *UAS-GFP* construct. Progenies (~30,000) from more than 200 such crosses were screened under a fluorescent microscope. All the flies that showed mild to strong expression in the thorax region were selected for further analysis. Selected individual lines were crossed to different chromosomal balancers to create stable lines. The expression pattern of each stabilized strain was confirmed after crossing with the reporter *UAS-GFP* strain again. A total of 30 strains were isolated from the screen as summarized in Table 1.

Isolation and characterization of UH3-Gal4

One of the enhancer trap lines, *UH3-Gal4*, isolated during the screen showed expression in the adult IFMs. A detailed expression profiling showed that it was expressed in certain pockets of the whorl region of the wing imaginal disc in third instar larvae (figure 1a). No remarkable GFP expression was seen in the notum region where the myoblasts are harbored. A ubiquitous expression was observed during early developmental stages of pupae (figure 1b-c). Over time, the expression was found restricted to the IFMs (figure 1d-e) and by the time the adult fly emerges, the expression was found only in the IFMs as visualized through reporter proteins (figure 1g). It showed expression both in the dorsal longitudinal muscles (DLMs) and dorso-ventral muscles (DVMs) (figure 1h). Visible expression could not be detected during embryonic stages. However, over-expression of the toxic protein - ricin (using *UAS-ricin* transgene) caused embryonic lethality (data not shown), indicating that the *UH3-Gal4* line could have an early embryonic expression also. The insertion of 11.2 kb long P-element construct itself did not affect the flight (figure 2h), walking or jumping (data not shown) and showed normal sarcomeric structures (figure 1i), suggesting that *UH3-Gal4* could serve as an elegant tool for studying the IFM myofibrillogenesis.

Analysis of the flanking sequences recovered from inverse PCR indicated that *UH3-Gal4* is inserted at cytological region 9B5 on the X chromosome at nucleotide position 10151572 of the scaffold GB:AE014298. This corresponds to an intron common to five transcripts of *Hyperkinetic (Hk)* gene, which has annotated function of voltage-gated potassium channel and

oxidoreductase activity (Flybase). The insertion site also falls within the intron of a non-coding RNA, *CR43959*. However, the orientation of the *P{GawB}* points towards minus strands which encodes *Hk* (figure 1j). The flanking genomic DNA has been submitted to the GenBank with accession number KF682142.

Knock down of troponin isoforms during development of the IFM

As described above, the *UH3-Gal4* expression is restricted to the IFMs in later stages of development, which coincides with isoform switching for most of the structural proteins. Muscle structural proteins - TnI, TnT, TnC1 and TnC4 were knocked down using *UH3-Gal4* and RNAi construct for each gene at an optimum temperature of 29°C, after growing the flies at 18°C till 50 hours after puparium formation (APF). Quantification of mRNA levels for the targeted genes by real time PCR showed significant reduction (figure 2a-d). The knock down specific to later stages of IFM development was evident from the fact that confocal images of the developing IFMs taken before the temperature shift showed normal muscles (figure 2e-g). However, the adult flies that eclosed after the temperature shift experiment (as mentioned above), showed flightless phenotype (figure 2i) compared to controls (figure 2h). Enhanced reduction in flight ability was achieved by addition of a copy of *UAS-dcr2* (Dietzlet *et al.* 2007) (figure 2i). The case of TnC1 was an exception, where only a small reduction in flight ability was observed. As expected, the walking and jumping behaviors were not significantly affected (figure 2j-k), also supporting the fact that the knock downs were IFM specific.

Since the flight was defective, we analyzed the IFM morphology through polarized light imaging. TnI and TnT knock down flies showed remarkable abnormalities of the IFM fascicles (figure 3b-c) as compared to the wild type (figure 3a). *TnC4* knock down flies however showed no visible phenotype at the IFM fascicle level (figure 3d). TnC1 flies had completely normal fascicles (not shown), exhibiting only a slight reduction in their flight. When the myofibrillar structures of these flightless flies were observed under confocal microscope, the sarcomeres were found to be disorganized (figure 3j-l). It was also quite evident that co-expression of *dcr2* and *UAS-RNAi* enhanced the severity of these sarcomeric defects (figure 3j'-l') compare to controls (figure 2e-i). Knock down of *TnC1* gave normal myofibrils like the wild type (data not shown). When *UH3-Gal4* was brought together with *Gal80^{ts}* - an antagonist of Gal4, at 18°C, the defective phenotypes seen while growing at 29°C temperature could be completely evaded (figure 3m-p). These flies also exhibited normal flight similar to controls (data not shown). These

results demonstrate the suitability of this Gal4, in combination with Gal80^{ts}, to be used for targeted knocked down of troponin isoforms or other muscle genes in the later stages of IFM development.

Embryonic isoform of TnI rescues myofibril structure of null allele

*hdp*³, a null allele of the TnI gene in the IFMs, is caused by a mutation in the splice site preceding exon 6b1, which is specific to IFMs and TDT (Barbaset *al.* 1993). In the absence of TnI, an inhibitory component within the troponin-complex proteins, the unregulated actin-myosin interactions cause hypercontraction of the IFMs and TDT during early myofibrillogenesis (Nongthombaet *al.* 2004). Using the *UH3-Gal4* line, we attempted to rescue the *hdp*³ allele with a non-flight muscle isoform of TnI, TnI-L9 (embryonic isoform) (Sahota *et al.* 2004), to study the importance of isoform switching. Since, the driver *UH3-Gal4* and the mutant allele *hdp*³ are located on the X chromosome, we first recombined both alleles into a single chromosome and confirmed the muscle phenotype (figure 4b), which retained the *hdp*³ phenotype. It was then crossed with the embryonic isoform transgene, *UAS-TnI-L9*. The analysis of the polarized light images from succeeding progenies showed that the TnI-L9 isoform could rescue the *hdp*³ muscle structural defects (figure 4c-d), though not completely. The expression level of the TnI embryonic isoform was confirmed by PCR method using primers designed for exon 6a1 sequences, which are specific to embryonic isoforms. The expression level of exon 6a1 isoform in rescued flies was albeit less compared to the 6b1 expression levels in the controls (figure 4i-j). Rescued flies were completely flightless (data not shown). As revealed by confocal images, the sizes of myofibrils were comparatively smaller in the rescued flies (figure 4g-h) as compared to the controls (figure 4e). The major thin filament protein actin is found accumulated abnormally in IFMs of *UH3-Gal4 hdp*³ heterozygous (figure 4f) and *hdp*³ hemizygous flies (figure 4g, arrows). In comparison, the rescued heterozygous flies (*UH3-Gal4 hdp*^{3/+}; *UAS-TnI-L9/+*) showed a better myofibrillar structure where the sarcomeres looked near normal (figure 4h). However, the inability of these rescued flies to fly even in the heterozygous condition highlights the importance of the presence of the right isoform.

Discussion

Two of the troponin subunits -TnT and TnI, undergo isoform switching during later stages of IFM development (Nongthombaet *al.* 2004; 2007). For TnT, the 10b isoform, which is

the major isoform during pupal development, is completely replaced by the 10a isoform in the adult IFMs. In the *up¹* mutant, the TnT 10a isoform variant, which is expressed only in the IFMs and TDT, is completely absent in the adult IFMs (Nongthombaet *al.* 2007). Since, both the isoforms co-exist in equal proportion in the TDT, loss of the 10a isoform is compensated up to some extent by the 10b isoform. Knocking down TnT during the isoform switching stage gives rise to abnormal myofibrils, more or less similar to what was observed in the *up¹* mutant, suggesting that TnT is important for assembly and maintenance of the myofibrils. In the absence of the proper troponin complex (due to lack of TnT), the thick and thin filaments will interact in an unregulated manner to produce disassembly of the myofibrils and sarcomeres (figure 3k, k'). The 10a isoform has more number of residues which can be phosphorylated, which may be necessary for increased power production during flight but this needs experimental validation.

The TnI isoform of the adult IFM has an extended N-terminal sequence of 60 residues, encoded by exon 3 (Barbaset *al.* 1993; Nongthombaet *al.* 2004). The exon 3 isoform is always found with the exon 6b1 isoform. Therefore, the mutation in the exon 6b1 splice site results in the absence of both the isoforms (containing exons 6b1 and 3) and the IFMs degenerate just after differentiation, which may result from unregulated acto-myosin interactions (Barbaset *al.* 1993; Nongthombaet *al.* 2004). As expected, knocking down of TnI also gives abnormal myofibrils and sarcomeres (figure 3j, j'), but slightly less severe than TnT knock down. The less severe phenotype may be attributed to the fact that in the absence of TnT, whole of the troponin complex will be lost as TnT anchors the troponin complex to the tropomyosin (Farah and Reinach 1995; Gordon *et al.* 2000), allowing the thick and thin filament to interact in an unregulated manner. However, in the case of TnI, there could be still a few functional troponin complexes formed as TnI will be intact. As a result, uncontrolled interactions between the thick and thin filaments will be less severe. The muscle phenotype achieved by knocking down of TnI is also less severe than *hdp³* or *hdp³/+*. This supports our hypothesis that the inhibition of the acto-myosin interaction during early assembly of the thick and thin filament proteins is very important for sarcomere formation, which is the case in the *hdp³* mutation (Nongthombaet *al.* 2004). As reported previously for other structural protein isoforms (Miller *et al.* 1993; Wells *et al.* 1996; Fyrberget *al.* 1998; Swank *et al.* 2002), replacement of the IFM isoform by the TnI embryonic isoform (TnI-L9) led to normal assembly of myofibrils, except for mild structural defects in few sarcomeres (figure 4f-g). The embryonic isoform was equally potent in replacing reduced amount of TnI in *hdp³/+* and no myofibrillar or sarcomeric defect was observed (figure

4h). This also suggests non-equivalent functional properties of the isoforms. The differences may be ascribed to exon 6b1 and/or exon 3. The exon 3 codes for extended N-terminal region at the protein level with more phosphorylation sites which could be important for more power production. The exon 6b1 region is the most variable region of TnI and is known to interact with the C-lobe of the TnC (De Nicola *et al.* 2007).

Two isoforms of TnC: - TnC1 and TnC4; are expressed in the IFMs in the ratio of 1:5 (Quiet *et al.* 2003; Herranz *et al.* 2005a). TnC1 has two Ca^{2+} binding sites and TnC4 has one (Agianian *et al.* 2004). Based on Ca^{2+} affinity studies, it has been predicted that TnC1 is required for isometric contraction and TnC4 for stretch activation (Linari *et al.* 2004; Krzic *et al.* 2010; Bullard and Pastore 2011; Martin *et al.* 2011). However, our *in vivo* data suggest that TnC4 is required for the proper assembly of the myofibrils and function, and TnC1 cannot compensate its loss. Whereas, the knock down of the TnC1 does not affect the assembly of myofibrils or sarcomere except for the mild reduction in flight ability (figure 2i), suggesting that the major isoform TnC4 can compensate the loss of TnC1. The reduction in flight could be attributed to the loss of TnC1 which had higher Ca^{2+} affinity. Such a compensation mechanism has been reported for TnT isoforms in TDT muscle of the *up¹* mutant (Nongthombaet *et al.* 2007). Our *in vivo* knock down data of TnC1 also suggest that TnC4 is capable of regulating muscle contraction. In other words, its single Ca^{2+} binding domain, which has high affinity for Mg^{2+} (Potter and Gergely 1975), can also binds to Ca^{2+} to regulate muscle contraction.

Mutations of the troponin complex proteins are prevalent in many species, from *Caenorhabditiselegans* (Myers *et al.* 1996; McArdle *et al.* 1998), zebrafish (Sehnert *et al.* 2002; Ohtet *et al.* 2009) to humans (Perry 1998; Johnston *et al.* 2000; Roberts and Sigwart 2001; Morimoto *et al.* 2002; Towbin and Bowles 2002; Marston and Redwood 2003; Wei and Jin 2011). Our previous studies in *Drosophila* (Nongthombaet *et al.* 2003; 2004; 2007) have contributed to mechanisms that could be responsible for the development of cellular structures like zebra-bodies which are widely seen in many human myopathic conditions. In the present study, a newly isolated enhancer trap Gal4 strain that expresses in the IFMs is reported and using it, we have been able to illustrate the importance of all the troponin proteins and their importance during isoform switching stage. Considering the importance of muscles in preventing ageing and other physiological activities that have been discovered in recent times, we believe that the isolated strain can serve as a potential tool for studies where the IFM system is used as a model. We have also shown the functional differences of isoforms and compensatory mechanisms.

Further experimental studies, both *in vivo* (rescue experiments with different isoforms), and *in vitro* (chemo-mechanical studies of muscles) will be required to fully understand the physiological and evolutionary importance of isoform switching in muscles.

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Table 1. Summary of enhancer trap Gal4 strains isolated from the screen

| | Chromosome X | Chromosome 2 | Chromosome 3 | Homozygous/ partially lethal |
|----------------|--------------|--------------|--------------|---------------------------------|
| No. of strains | 1 | 18 | 7 | 4 |

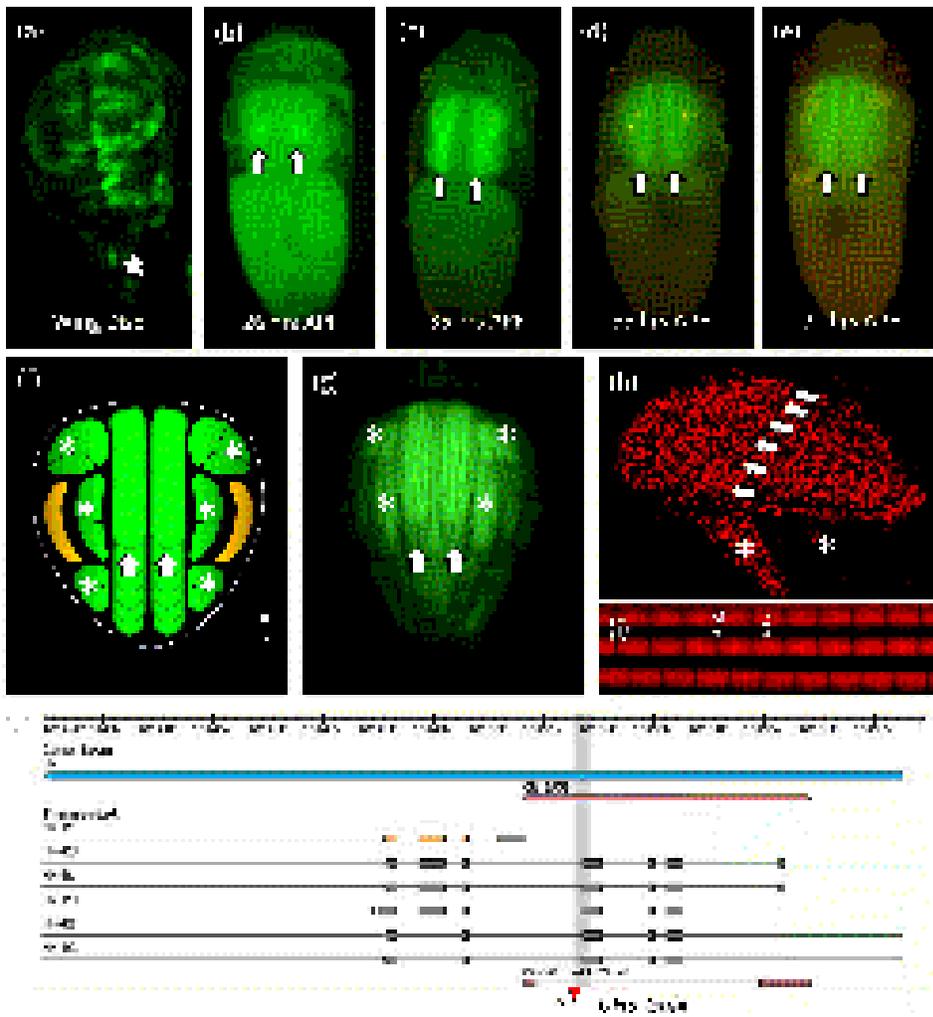


Figure 1. Expression pattern and localization of *UH3-Gal4*. Fluorescent images of the reporter GFP expressed under *UH3-Gal4*. (a) Wing imaginal disc from a third instar larva showing a pronounced expression in whorl region (whorl region is encircled by dotted lines and star indicates the notum region). (b-e) Pupae showing GFP expression at different stages of IFM development (APF: after puparium formation). (f) A schematic representation of dorsal coronal-plane view of thorax. A pair of six vertically tiered dorso-longitudinal muscle (DLM) fascicles aligned along the antero-posterior median axis (arrows) is bracketed by three separate dorso-ventral muscle (DVM) fascicles bundled up in 3: 2: 2 muscle fibres (asterisks). The tergal depressor of trochanter or the jump muscle is represented by two orange colour crescent shaped structures, 'A' on scale map depicts anterior. (g) Adult thorax showing strong GFP in the IFMs.

Confocal images of (h) hemithorax showing *UH3-Gal4* driven nuclear localized RedStinger in DLMs and DVMs and (i) phalloidin TRITC labeled myofibrils of *UH3-Gal4*. DLMs and DVMs are represented by arrows and asterisks respectively. M and Z indicate M-line and Z-disc respectively. Scale = 5 μ m. (j) The BLAST analysis of flanking genomic DNA sequences recovered from an inverse PCR (grey region) indicates that *UH3-Gal4* (represented by red triangle below the grey region) is inserted in an intron common to five annotated transcripts of *Hyperkinetic* with an orientation towards the minus strand (indicated by arrow). The insertion site also falls within the intron of a non-coding RNA gene - *CR43959*, encoded by the opposite strand. (Snapshot from Flybase).

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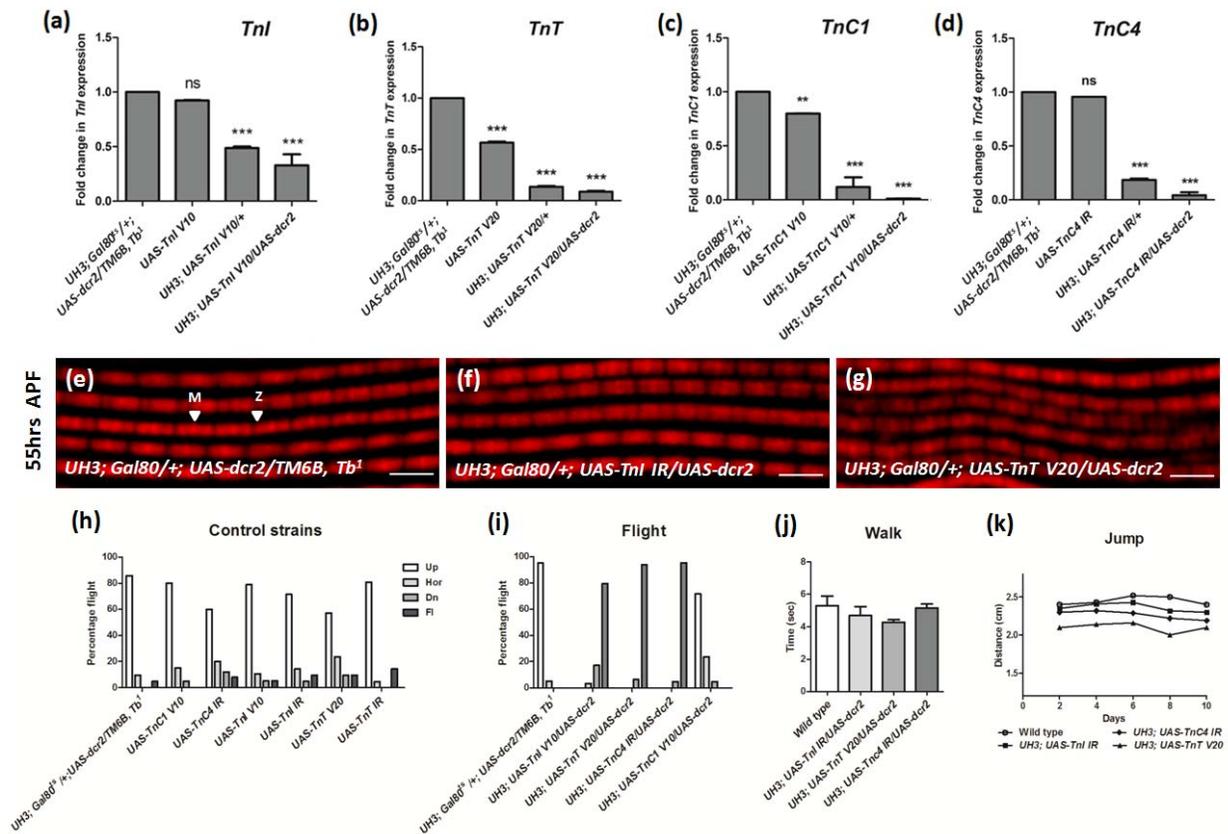


Figure 2. Knock down of muscle structural proteins and behavioural assays. (a-d) Quantification of the mRNA expression levels of all the troponin genes in 1-2 days old adult IFMs by real time PCR. Each of the troponin genes was knocked down during later stages of IFM development using *UH3-Gal4* driver in combination with *Gal80^{ts}*. One-way ANOVA with Dunnett's multiple comparison post test revealed a significant decrease of transcript levels (** $p < 0.01$, *** $p < 0.001$ and ns – not significant). (e-g) Confocal images showing normally developed adult IFMs when the muscle structural genes (*TnI* and *TnT*) were knocked down in the presence of *Gal80^{ts}* at 18°C. Knock down of all the Troponin isoforms gives (i) flightless phenotype, except for TnC1 (Up-up flighted, Hor-horizontal flighted, Dn-down flighted, Fl-flightless) as compared to (h) control parental lines. (j) Walking and (k) jumping abilities though are not significantly affected. Genotypes are given at the lower panel of each image. Red is Phalloidin TRITC. Scale = 5µm.

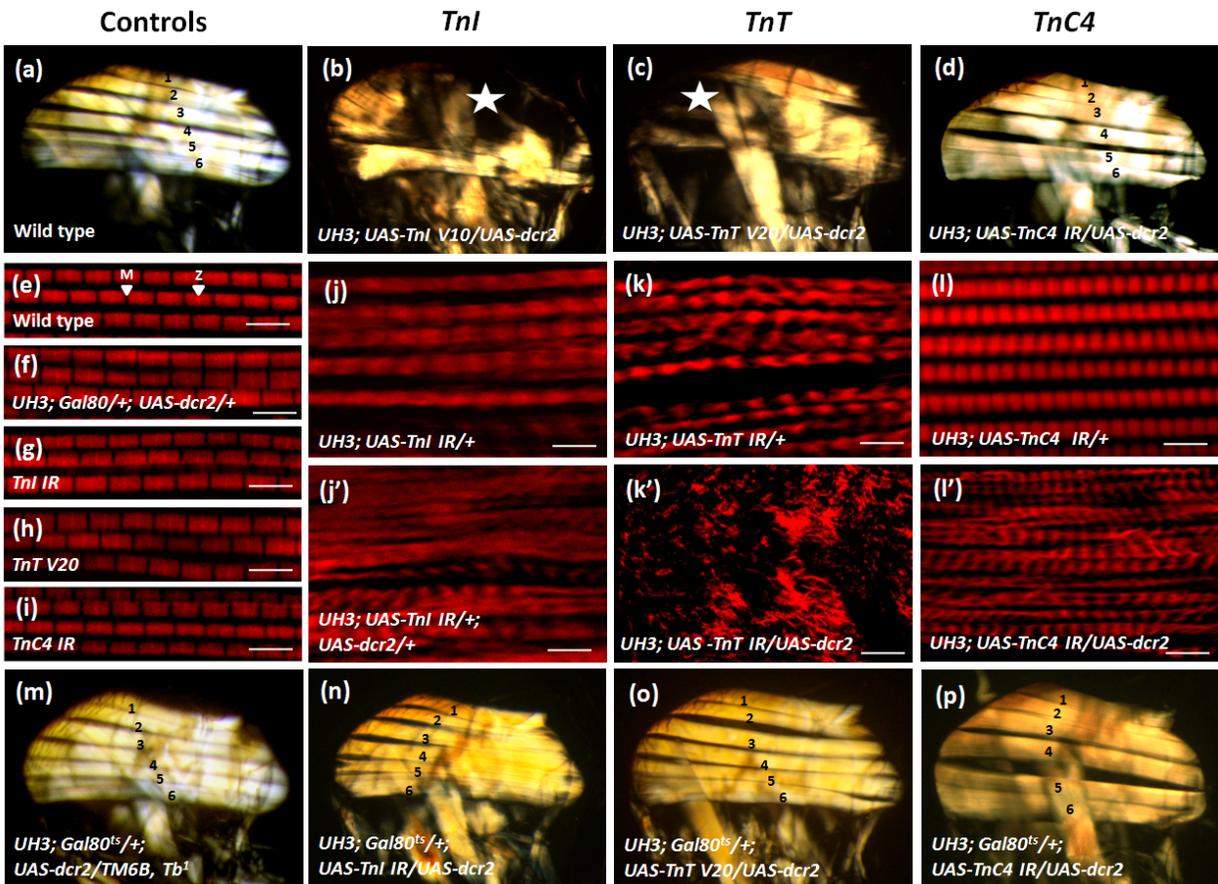


Figure 3. Myofibrillar morphology associated with the knock down of each of the troponin gene (TnI, TnT and TnC4) using *UH3-Gal4*. (b-d) Polarized images show remarkable abnormalities in TnI and TnT knock down as compared to (a) wild type (1-6 represents six DLM fascicles). The myofibrillar structure of DLMs shows disorganized sarcomeric structures (j-l) which become severe when the knock down was enhanced by adding a copy of *UAS-dcr2* (j'-l'). (e-i) images show the normal myofibrils of the wild type and control parent strains. M and Z indicate M-line and Z-disc respectively. (m-p) Co-expression of *Gal80^{ts}* at 18°C can prevent defective knock down effects. Red is Phalloidin TRITC. Scale = 5µm.

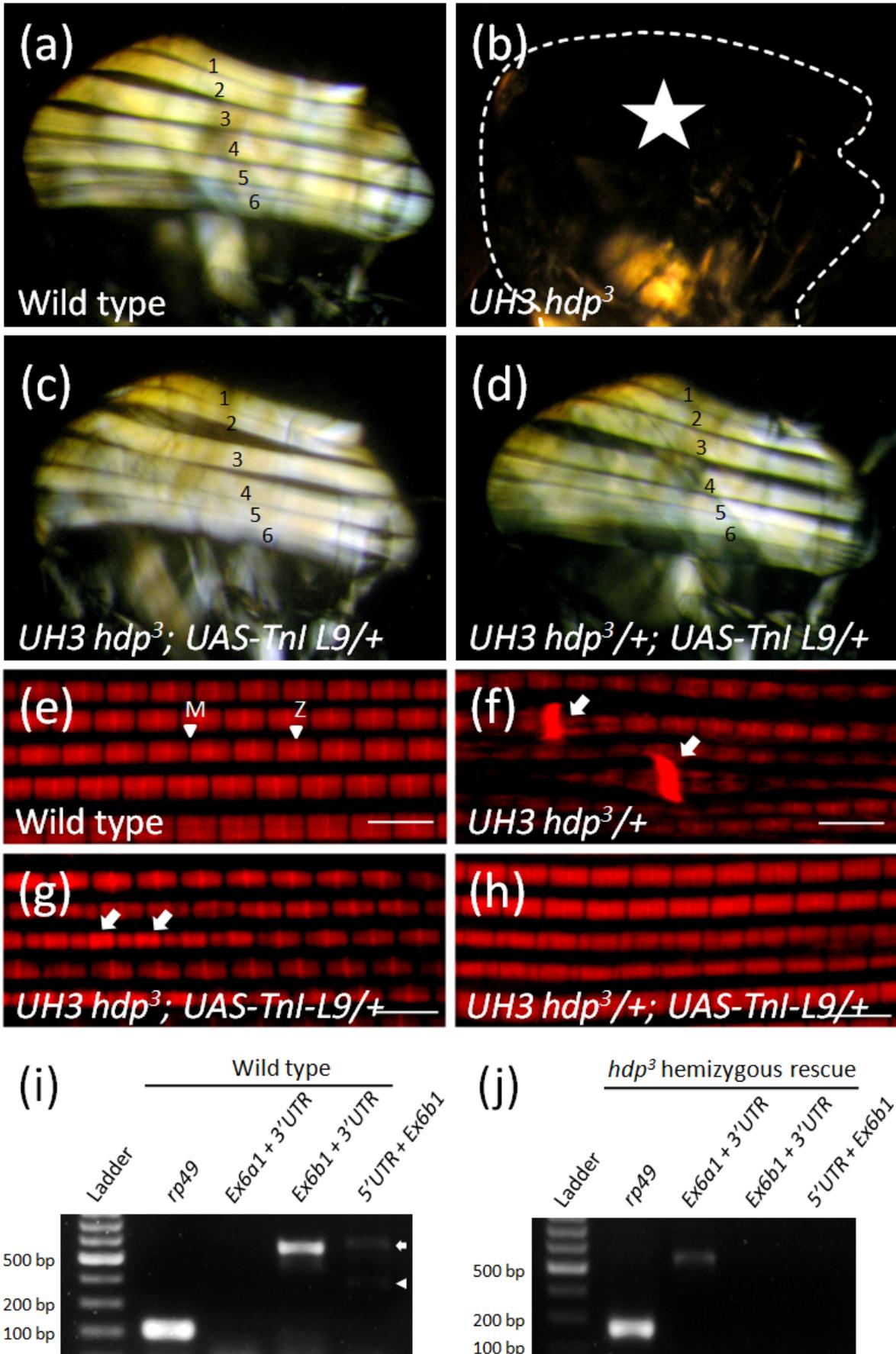


Figure 4. Rescue of the TnI null adult IFM phenotype with an embryonic isoform of TnI. Polarized images of (a) wild type thorax showing six normal DLMs and (b) *UH3-Gal4* recombined with *hdp³* without any IFMs and TDT (indicated by star). Expression of non-flight muscle TnI embryonic isoform (TnI-L9) under *UH3-Gal4* can rescue the *hdp³* phenotype as seen in (c) hemizygous and (d) heterozygous conditions. (g) and (h) show confocal images of myofibrils rescued with TnI-L9 in *hdp³* hemizygous and heterozygous conditions as compared to control (e) wild type and (f) recombined *UH3-Gal4 hdp³* heterozygous counterpart. Arrows indicate abnormal accumulation of actin. M and Z indicate M-line and Z-disc respectively. (i-j) Agarose gel showing the amplified DNA products of TnI containing exon 6a1 and exon 6b1 in rescued and wild type IFMs respectively. The last lane in i) shows the two isoforms of TnI which are with (arrow) or without (arrowhead) exon 3 in wild type IFMs. Genotypes of each image are given at the lower panel. Red is Phalloidin TRITC. Scale = 5 μ m.

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