Over-expression of miRNA-9 generates muscle hypercontraction through translational repression of the Troponin-T in Drosophila indirect flight muscles

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Abstract

miRNAs are small non-coding endogenous RNAs, typically 21-23 nucleotides long, that regulate gene expression, usually post-transcriptionally by binding to the 3’-UTR of target mRNA, thus blocking translation. The expression of several miRNAs is significantly altered during cardiac hypertrophy, myocardial ischemia, fibrosis, heart failure and other cardiac myopathies. Recent studies have implicated miR-9 in myocardial hypertrophy. However a detailed mechanism remains obscure. In this study, we have addressed the roles of miR-9 in muscle development and function using the genetically tractable model system, the indirect flight muscles (IFMs) of Drosophila melanogaster. Bioinformatics analysis identified 135 potential miR-9a targets, of which 27 genes were associated with Drosophila muscle development. Troponin-T (TnT) was identified as major structural gene target of miR-9a. We show that flies over-expressing miR-9a in the IFMs have abnormal wing position and are flightless. These flies also exhibit loss of muscle integrity and sarcomeric organization causing an abnormal muscle condition known as “hypercontraction”. Additionally, miR-9a over-expression resulted in the reduction of TnT protein levels while transcript levels were unaffected. Furthermore, muscle abnormalities associated with miR-9a over-expression were completely rescued by over-expression of TnT transgenes which lacked the miR-9a binding site. These findings indicate that miR-9a interacts with the 3’-UTR of the TnT mRNA and down-regulates the TnT protein levels by translational repression. The reduction in TnT levels leads to a cooperative down-regulation of other thin filament structural proteins. Our findings have implications for understanding the cellular pathophysiology of cardiomyopathies associated with miR-9 over-expression.
Key words: Drosophila, Troponin-T, Hypercontraction, miRNA, Indirect flight muscles

Running Title: miR-9 regulation of Troponin-T
**Introduction**

Muscle contraction is crucially dependent on the proper assembly, maintenance and function of myofibrils (Beall and Fyrberg 1991; Gordon *et al.* 2000). Myofibril assembly is a highly complex and coordinated process that requires the maintenance of appropriate stoichiometries of structural proteins and protein complexes, such as acto-myosin complex and the Troponin-Tropomyosin (Tn-Tm) complex (Laing and Nowak 2005; Firdaus *et al.* 2015). Defects genetically caused or otherwise, in muscle development, structure or function result in a number of disorders and diseases, collectively referred to as myopathies (Charge and Rudnicki 2003; Selcen 2011; Gautam *et al.* 2015). Congenital myopathies, including genetic heart diseases, comprise a wide variety of muscle disorders that are mostly due to mutations in the contractile proteins (Selcen 2011; Chawla 2011). For example, mutations of cardiac Troponin-T (cTnT), alpha-Tropomyosin and myosin cause hypertrophic cardiomyopathy (HCM) (Watkins *et al.* 1995; Karibe *et al.* 2001). Stoichiometric imbalances of structural proteins and altered isoform expression leading to myocardial damage are also seen in secondary cardiomyopathies, resulting from infection or other factors (Thierfelder *et al.* 1994, Watkins *et al.* 1996; Sisakian 2014). To achieve the appropriate stoichiometric balances, many levels of regulation are required. While transcriptional regulation of these components is extremely important, the significance of the roles of miRNAs in myopathies in general and in hypertrophy in particular are becoming increasingly recognized (Eisenberg *et al.* 2009; Parkes *et al.* 2015).

miRNAs are small non-coding endogenous RNAs typically 22-30 nucleotide long, that exert subtle control over gene expression, transcriptionally or post-
transcriptionally. This makes them an indispensable part of the regulatory network in almost all complex events in organisms ranging from plants to mammals, and even their viruses (Bartel and Chen 2004; Allen and Howell 2010; Carthew and Sontheimer 2010). In muscles, many miRNAs including both the muscle-specific “myomiRs” such as miR-1, miR-133 and miR-206 (McCarthy 2008), as well as those more widely expressed, such as miR-24, miR-29 and miR-181 (Erriquez et al. 2013), are involved in the regulation of both myoblast proliferation and differentiation (Chen et al. 2008). Importantly, the expression of several miRNAs are significantly altered during cardiac hypertrophy, myocardial ischemia, fibrosis, heart failure and other cardiac myopathies (Latronico and Condorelli 2011; Oliveira-Carvalho et al. 2012). Micro-RNA-9 (miR-9), a miRNA of recognized neural functions (Gladka et al. 2012; Krichevsky et al. 2003), reportedly plays a regulatory role in myocardial hypertrophy is antagonistic to myocardin, a positive mediator of cardiac hypertrophy (Wang et al. 2010). In addition, miR-9 was implicated in the regulation of PDGFR-β (platelet derived growth factor receptor-β), a regulator of cardiomyocyte angiogenesis (Zhang et al. 2011). Both studies reported down-regulation of miR-9 upon activation of the hypertrophic response. Recently, clinical studies revealed that miR-9 expression levels are significantly lower in hypertensive patients as compared to healthy controls, and appeared to be correlated with ventricular mass (Kontaraki et al. 2014). Thus, miR-9 appears to have more than one role in cardiac function. It is thus imperative to characterize its roles in the context of muscle development and function. There is high evolutionary conservation of the ultrastructure of striated muscles, their component proteins, and the mechanisms that regulate assembly of sarcomeres and myofibril
formation throughout the vertebrates; similarly there is substantial closeness to the muscles of invertebrates with complex locomotion (Taylor 2006). The *Drosophila* indirect flight muscles (IFMs) provide a good system with which to study muscle development, function and associated diseases (Sparrow *et al.* 2008). Particularly, with respect to the investigation of cardiomyopathic disorders, as the IFMs exhibit properties such as stretch activation and asynchronous contraction that are physiologically similar to those of cardiac muscles (Vigoreaux 2001). Specific mutations of the *Drosophila* contractile machinery (Kronert *et al.* 1995; Nongthomba *et al.* 2003), signaling cascades (Gajewski *et al.* 2006) and connective tissues (Pronovost *et al.* 2013), lead to muscle hypercontraction, associated with decreased structural integrity of the sarcomeres, which are similar to those seen in many myopathic conditions of higher organisms, including humans. Particularly, the Drosophila miR-9a is an exact copy of the human miR-9 (Yuva-Aydemir *et al.* 2011).

In this study, we have investigated the regulatory role of miR-9a in *Drosophila* for IFM development and functioning. We show that *Drosophila* miR-9a plays a novel role in the regulation of TnT, a major structural protein, during myofibrillogenesis. This finding will lead to a better understanding of how human miR-9 may be involved in the pathogenesis of cardiac hypertrophy.

**Materials and Methods**

**Fly strains and crosses**
All flies were maintained on standard cornmeal-agar-yeast medium. Canton-S was used as control for most of the experiments unless specified. Crosses were performed at 25°C, unless otherwise indicated. UH3-Gal4 (X chromosome), expression from 36 hr APF onwards becomes IFM-specific (Singh et al. 2014). UAS- miR-9a (IIIrd chromosome - Bloomington #41138), UAS-miR-SP-9a (IIIrd chromosome kind gift from Dr. David Van Vactor, Harvard Medical School, USA) were used for over-expression and knocking down of miR-9a respectively, UAS-SlsRNAi (III chromosome), UAS-mbcRNAi (III chromosome), UAS-NeuralizedRNAi (III chromosome) were procured from Bloomington stock centre and UAS-TnTRNAi (III chromosome) was from, VDRC, Vienna (v27853). Green fluorescent protein (GFP) constructs: sls-GFP (III chromosome) has been described in Morin et al. (2001). All chromosomes and gene symbols are as mentioned in Flybase, http://flybase.org, unless specifically described.

**Generation of UAS-TnT lacking miR-9 binding site**

Two transgenic fly lines (UAS- TnT (10a) and UAS - TnT (10b)) were generated for the over-expression of either the adult isoform (10a) or the pupal isoform (TnT-10b). The TnT transcripts (both 10a and 10b) were amplified using cDNA extracted from wild type thoraces, using primers designed to target the 5’- and 3’-UTRs but to exclude the miR-9a binding site. The primers were also modified to incorporate EcoRI and KpnI restriction sites for sub cloning into the pUAST over-expression vector (TnT FP with EcoRI site: 5’-GAACCGCAGAATTCCGCTCCTAC-3’, TnT RP with KpnI site: 5’-GTGAAGGAAAGTGGTACCCGAG-3’). The transcripts were cloned into a TA vector and the clones were screened for the presence of 10a or 10b transcripts using reverse primers specific for the alternatively spliced exon (TnT FP + TnT10a RP: 5’-
TTGTGCGCTGAGTGAATC-3’ and TnT FP + TnT10b RP: 5’-CGGTGTATTGCTCTTCT-3’). The presence of the 10a or 10b transcripts in the respective clones was confirmed by sequencing. The sequenced clones and pUAST plasmid were digested with EcoRI and KpnI and the released inserts and the cut pUAST vector were ligated and the transgenic constructs were cloned and confirmed by sequencing. The construct, either pUAST-TnT-10a or pUAST-TnT-10b, along with λ-helper plasmid (encoding for a transposase) was injected into embryos of white eyed w^{1118} flies using the Olympus CK-X31- Narishige IM-9B microinjection system. The adult flies (G₀) that emerged were then crossed with white eyed flies to produce the F₁ generation. The transgenic flies were identified by their red eye phenotype.

**Behavioral test**

Flight ability was assayed using 2-3 day old individual flies as described previously (Drummond *et al.* 1991) and the flies were categorized as up (U); horizontal (H); down (D); or flightless (F). Each fly was flight tested three times.

**Polarized Microscopy**

2-3 days old flies were bisected and processed for polarized microscopy using a protocol described previously (Nongthomba and Ramachandra 1999). Images were captured using an Olympus SZX12 microscope fitted with an Olympus C-5060 camera.

**Haematoxylin and eosin (H & E) staining**

H & E staining of transverse sections of the adult thorax was done as previously described (Pantoja *et al.* 2013). Sections were mounted using DPX mounting medium
(Qualigens, Mumbai) and analyzed by light microscopy. Images were acquired using Leica DFC300FX camera and processed using inbuilt software.

**Confocal microscopy**

The bisected fly hemithoraces were processed for immunohistochemical analysis as described previously (Rai et al. 2014). Following blocking, samples were treated differently depending on the type of analysis required.

**Scanning electron microscopy (SEM)**

SEM analysis of IFMs was used to visualize sarcomeric structure. Three day old flies dehydrated using an alcohol series (50%, 70%, 80%, 90%, 95% and 100%). Samples were incubated for 10 min in each dilution with the final dehydration step in 100% alcohol repeated twice. After twice incubating each sample in hexamethyldisilazane (HMDS) for 45 min they were dried in a desiccator for 24 hr. The head, wings, abdomen and legs were then removed, transferred to a glass slide and bisected sagittally. Bisected thoraces were mounted onto an aluminium stub with carbon tape and surface coated by gold sputtering (20 nm thick film) using a Baltec sputter to avoid charging. Zeiss, Ultra 55, Field Emission Scanning Electron Microscope with Secondary Electron Detector was used for imaging with an accelerating voltage of 2 - 5 keV and 8 mm working distance.

**RNA and PCR**

RNA was isolated from the IFMs of 2 - 3 day old flies. IFMs were removed from the bisected thoraces at 4°C and immersed in Trizol (Sigma). Next, RNA was extracted with the help of Trizol (Sigma) as per the manufacturer’s protocol. cDNA was made using 1
-2 µg of extracted RNA and the cDNA synthesis kit (Fermentas). The following primers were used, RP49 (FP: 5’-TTCTACCAGCTTCAAGATGAC-3’, RP: -5’-GTGTATTCCGACCACGTACAC-3’). upheld (FP: 5’-CTCGGGTGTCTCGGCTCAC-3’ RP: 5’-CTCGAAGCAGGATCTGGA-3’), Opal-like (FP: 5’-AACGTTGGAGCCAGTTTCG-3’; RP: 5’-TGATCTCCGTCTGCAGCGTC-3’)

Quantitative PCR was carried out using DyNAmoTM HS SYBR green mix (Thermo Scientific F - 410L). Fluorescence intensities were recorded and analyzed in an ABI Prism 7900HT sequence detection system (Applied Biosystems SDS 2.1, USA). The relative changes in gene expression were estimated after normalization to the expression of a housekeeping gene, RP49. For semi-quantitative PCR, reactions were set up using the 2x PCR Mastermix (Fermentas) and PCR amplification was carried out using Mastercycler Nexus (Eppendorf).

**Northern Blotting**

Detection of miRNA was carried out using the Northern blotting technique protocol of Varallyay et al. (2008). RNA was extracted as described earlier, and quantified using a Nano Drop 1000 spectrophotometer (Thermo scientific). Equal concentrations of samples were loaded on the gel. RNA bands were visualized under a UV transilluminator (JH BIO Innovations Pvt. Ltd) and transferred onto a Nitrocellulose membrane (Millipore) by semi dry transfer. Locked nucleic acid LNA Probe for miR-9a (5’-TCATACAGCTAGATAACCAAAGA-3’) and control probe for U6snoRNA (5’-GTCATCCTTGCAGCAGGGGCATGC-3’) was labeled using 1 µl T4 polynucleotide kinase (PNK), 1 µl [γ - 32P] ATP, 2 µl PNK buffer and the final volume was made up to 20 µl. Following incubation at 37°C for 60 min, the probe was purified using Sephadex G-50 column. The membrane was washed
twice with 0.5X TBE (TRIS Borate - EDTA) and allowed to cross-link under UV light for 60 sec and then incubated at 40°C for hybridization with LNA probe in 1X Perfect Hyb Plus buffer (Sigma) for 2 – 3 hr. The probed membrane was then washed thrice with 2X SSC, 0.1% SDS buffer at 40°C was and exposed to photographic film. The exposed film was scanned using the Phosphor image scanner (GE Typhoon 9500).

**Western blotting**

Dissected adult IFMs were homogenized in sample buffer (312.5 mMTris-HCl pH 6.8, 10% SDS, 0.5% Bromophenol Blue, 50% glycerol, 25% β-mercaptoethanol) and denatured for 3 minutes at 95°C. Samples were run on a 12.5% resolving gel and western blotting was carried out as described previously (Nongthomba et al. 2007). The primary antibodies used to detect specific proteins were: Drosophila anti- Troponin-T raised in rat 1:1000 (Gift from John Sparrow, UK), Drosophila anti- Troponin-I raised in rabbit 1:1000 (Gift from Alberto Ferrus, Spain), Drosophila anti- Flightin raised in rabbit 1:1000 (Gift from Jim O. Vigoreaux, Vermont, USA), Drosophila anti- Actin raised in rabbit 1:1000 (Gift from John Sparrow, UK), Drosophila anti- α- Tubulin raised in mouse 1:1000 (DHSB).

**Bioinformatics Analysis**

miR-9a target prediction was done using five different target prediction software suites. These were Miranda (http://www.microrna.org/, Enright et al. 2003), Pic - Tar (http://pictar.bio.nyu.edu, Krek et al. 2005), the method used by Stark et al. (2003, http://www.russell.embl.de/miRNAs/), EMBL target prediction and Target scan fly (http://www.targetscan.org/, Lewis et al. 2003). All five different algorithms to predict
microRNA targets, and hence, their results should be non-overlapping. We shortlisted targets recognized by three or more target prediction software as significant matches. These shortlisted genes were cross-referenced to a *Drosophila* IFM microarray dataset ([https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE70252](https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE70252)) to check for their expression levels in adult IFMs. The miR-9a targets thus obtained were then functionally annotated using DAVID (http://david.abcc.ncifcrf.gov/, Huang *et al.* 2008) and PantherDB software (http://www.pantherdb.org/about.jsp, Mi *et al.* 2005).

**Data Availability**

We have provided the details of all the web-links of data resources that we made use in this study. Other data which support our findings have been included as supplementary figures and are described in the result section.

**Results**

**Knockdown of miR-9a in the IFMs during myofibrillogenesis does not affect muscle structure and function.**

To investigate the role of miR-9a during myofibrillogenesis, we performed IFM-specific knockdown of miR-9a from 36 hrs after puparium formation (APF). Sarcomeres of the IFMs are established by an organized assembly of their structural proteins between 37 to 46 hrs APF (Reedy and Beall 1993; Nongthomba *et al.* 2004). miR-9a is expressed in developing IFMs and the muscle attachment sites (Yatsenko and Shcherbata 2014), expression is highly reduced in adult IFMs (Figure S1.A). The knockdown of miR-9a during myofibril assembly had no detrimental effect on flight (Figure S1.B). Flies with knockdown of miR-9a (UH3>miR-SP-9a) exhibited close to
normal flight ability with 74.2% of flies capable of upward flight (U) and 25.8% exhibited horizontal flight (H) (n=31) which is similar to wild type, where 80.6% of flies were up-flighted and 19.4% were horizontally flighted (n=31) (Figure S1.B). Further, both wild type (Figure S1.C) and miR-9a knocked down flies (Figure S1.D) had six normal DLM fibers in each hemi-thorax with normal sarcomeric structures (Figure S1.C’-D’’). 

**Over-expression of miR-9a in the IFMs during myofibrillogenesis causes hypercontraction.**

To determine whether the inherently low expression of miR-9a during myofibrillogenesis (Figure S1.A) is important for the critical roles of its targets during this stage of muscle development, we over-expressed miR-9a in the IFMs throughout myofibrillogenesis. While miR-9a expression was barely detectable in wild type IFMs, *UH3-Gal4* (Singh *et al.* 2014) mediated miR-9a over-expression clearly increased miR-9a levels (Figure S1.A) and adversely affected both wing posture and flight performance (Figure 1A-A’ and B-B’ and E).

The hemi-thoraces of wild type flies showed presence of six dorsal longitudinal muscles (DLMs) (asterisks) with conventional sarcomeric structure, with well demarcated Z-discs (arrows) (Figure 1C-C’’’). Whereas flies over-expressing miR-9a exhibited broken muscle fibers that appeared to be hypercontracted and pulled towards attachment sites (highlighted in Figure 1D-D’’’). Control wild type adult hemi-thoraces showed the typical six well organized DLM fascicles (Figure 1C) and at higher magnification individuals showed well-arranged fibers (Figure 1C’). However, over-expression of
miR-9a resulted in abnormal muscle and loss of myofibril integrity in the IFMs, with defects in sarcomeric organization and no organized Z-discs (Figure 1D-D’’’). Unlike those of the wild type flies (Figure S2.A-A’), flies over-expressing miR-9a had severe muscle disorganization (Figure S2.B), with whole fascicles missing (black arrowheads, Figure S2.B’). Flies of both sexes, over-expressing miR-9a, also exhibited a complete loss of flight ability (100% flightless (n= 35)) unlike their control counterparts (+; +; UAS miR-9a/+) (90 % up-flighted and 10 % horizontally flighted (n=31) (Figure 1E).

The loss of muscle integrity and sarcomeric structure caused by miR-9a over-expression (Figure 1D-D’’’) is very similar to the hypercontraction phenotype reported earlier (Nongthomba et al. 2003). This phenotype is usually associated with mutations in genes encoding sarcomeric structural proteins. It is well established in Drosophila IFMs that mutation in some of the structural proteins like Troponin-T, Actin and Troponin-I, can lead to the coordinated reduction in other thin filament proteins. These result in loss of sarcomeric structure and muscle hypercontraction, as a consequence of mis-regulated acto-myosin interaction (Nongthomba et al. 2003; 2004; 2007; Firdaus et al. 2015). The hypercontraction phenotype can be suppressed in flies with the MhcP401S mutation (Figure 1G-G’) (Nongthomba et al. 2003). This mutation is in the actin binding head region of the myosin heavy chain which prevents the interaction of the thin and thick filaments, thus reducing muscle contraction. Flies carrying MhcP401S mutation in the miR-9a over-expression background showed suppression of the muscle structural defects associated with the miR-9a over-expression (Figure 1F-H). The DLMs and sarcomeric structure in adult flies (Figure 1G-G’) were comparable to those of wild type controls (Figure 1F-F’). Compared to flies with over-expression of miR-9a alone
(Figure 1H-H’), the hypercontraction suppressed flies possessed six DLMs (asterisks) (Figure 1G) with close to normal sarcomeric structures (Figure 1G’, white arrow shows a Z-disc) confirming that the miR-9a muscle phenotype results from unregulated acto-myosin interactions.

To confirm that the muscle defects observed resulted directly from miR-9a over-expression in the IFMs during myofibrillogenesis, we studied the effect of suppressing miR-9a expression in the over-expression background. Knockdown of miR-9a in the over-expression background rescued the flight ability to levels similar to wild type flies (Figure S1.B). The adult flies had a normal arrangement and pattern of DLMs (Figure S2.D-D’ and E-E’). Thus, the hypercontraction was rescued and the IFMs showed ordered sarcomeres (Figure S2.D-D’ and E-E’) in contrast to those with the phenotype from over-expression of miR-9a alone (Figure 1.D-D’).

**Over-expression of miR-9a results in down-regulation of Troponin-T (TnT)**

Hypercontraction results from the sarcomeres being unable to withstand the forces produced within them due to changes in the structural proteins of the sarcomere and/or their regulation. Therefore, we investigated if any such proteins are target of miR-9a. Bioinformatics analysis identified 135 potential miR-9a targets which were then functionally annotated. Five different prediction programs were used (see Materials and Methods) and we chose only those predicted targets that were detected by three or more programs. Functional annotation revealed that these genes are involved in a variety of cellular and developmental functions such as transcriptional regulation, protein degradation, apoptosis, endocytosis, neuronal specification, imaginal disc development,
etc., but 29 genes are involved in muscle development (Figure 2A). All the putative miR-9a targets associated with muscle development prove to be involved in muscle function. Of these miR-9a targets in muscles, 4 genes are reported to be involved in larval muscle development, 19 in the development of pupal and adult muscles (Figure 2A) (Schnorrer et al. 2010) and 6 genes are associated with muscle development (Madan et al. 2017; https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE70252). These putative targets are expressed in the IFMs as demonstrated using the Drosophila IFM micro-array (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE70252) (Figure 2B).

Among the putative targets of miR-9a genes involved in muscle development and function is the upheld (up) gene, which codes for all the Troponin-T (TnT) isoforms in Drosophila melanogaster. upheld (up) showed a very high level of expression in adult IFMs (blue bar in Figure 2B, expression validated by real-time PCR in Figure 2C). The 3’-UTR of up gene has the miR-9 binding site sequence (Figure 2D). Given that up mutations have been implicated in muscle hypercontraction (Nongthomba et al. 2003; 2007), we investigated whether miR-9a can cause the down regulation of TnT. Possibly through binding to its target sequence at the 3’-UTR of TnT mRNA and suppressing the translational process as represented in Figure 2D. Indeed there was significant reduction in TnT protein levels (P<0.0017) in the DLMs of flies over-expressing miR-9a, compared to wild type (Figure 2E). There was also a concomitant decrease in the levels of other structural proteins (which are not targets of miR-9a) that are part of the thin filament (Actin), and including the troponin complex (TnI) (Figure S3.A). On the other hand, Flightin, a thick filament component, was not reduced in flies over-expressing
miR-9a compared to wild type flies (Figure S3.A). These results were expected since reduction of one thin filament protein is known to lead to a coordinated reduction of other thin filament proteins but does not affect thick filament components (Nongthomba et al. 2004, 2007).

**Repression of TnT by miR-9a is responsible for the hypercontraction phenotype**

Since several miR-9a putative targets are important during muscle development, we asked whether the hypercontraction phenotype resulted directly from the down-regulation of TnT by miR-9a or was due to the repression of other targets. Firstly, we observed that the knockdown of TnT in IFMs during myofibrillogenesis generated a phenotype very similar to the over-expression of miR-9a. Knockdown of TnT resulted in the disruption of muscle structures (Figure S3.E-E’), which was comparable to the muscle defects seen in over-expression of miR-9a (Figure S3.F-F’). We further tested if the knockdown of some other predicted miR-9a targets can lead to similar defects in muscle structure and function. The down-regulation of neuralized, an E3 ubiquitin ligase, failed to show any muscle defects (Figure S3. C-C’). Flies with a knockdown of Sallimus (Sls), showed six DLMs (Figure S3.D). However, reduction in Sls, which is a structural component of the Z – disc, did result in some tearing of the sarcomeres (rectangle, Figure S3.D’), but not nearly comparable to the damage following miR-9a over-expression.

To further confirm that that the muscle hypercontraction resulting from over-expressing miR-9a is a direct result of knockdown of TnT alone, we carried out a rescue of the muscle phenotype by over-expressing TnT devoid of the miR-9a binding site in flies
with elevated levels of miR-9a in their IFMs. TnT transgenic lines were driven using *UH3-Gal4* and shifted to 29ºC at 50- 52 hr APF. Over-expression of transgenic TnT, either the TnT 10a or 10b isoform, devoid of the miR-9a binding sequence, restored TnT protein levels in these flies compared to flies over-expressing miR-9a alone (Figure 3A). Importantly, the restoration of TnT levels in the background of miR-9a over-expression, completely rescued the hypercontraction phenotype. This was evidenced by the presence of six DLMs (Figure 3B’) and the complete absence of any hypercontracted DLMs in the hemithoraces of these flies (Figure 3C). Further, the flight ability of these flies was also partially rescued; where control flies (UH3/++;++;+) Gal4 flies): 100% flies up flighted (n=31). TnT 10a: 19.6% flies horizontally flighted, 28.2% down-flighted, 52.2% flightless (n=46) and TnT 10b: 4.8% flies were up-flighted, 37.1% horizontally flighted, 22.6% down-flighted and 35.5% were flightless (n=62) as compared to the flies with over-expression of miR-9a alone which were all flightless (n=60) (Figure 3D). Both of these transgenic lines (TnT 10a and TnT 10b) showed almost complete restoration of muscle integrity and sarcomeric structure (Figure 4B-B’ and D-D’) comparable to that of wild type controls (Figure 4A-A’), in stark contrast to the muscles in the flies over-expressing miR-9a (Figure 4C-C’).

These data argue that TnT is the major target of the miR-9a responsible for the muscle hypercontraction phenotype. We also confirmed that the rescue of the hypercontraction phenotype by expressing TnT isoforms in IFMs is indeed because of restoration Troponin-T (TnT) and not due to Gal4 dilution. When *UH3-Gal4* was used to drive both UAS-miR-9a and UAS-GFP, the progeny still exhibited hypercontracted muscles in hemithoraces with loss of sarcomeric structure (Figure S4.B-B’), similar to the
phenotype that results from driving UAS-miR-9a alone using UH3-Gal4 (Figure 1D-D’).

Discussion

The present study throws light on a new role played by miR-9a during muscle development and function. Previously, Drosophila miR-9a has only been shown to be involved in neuronal differentiation, wing margin patterning and myotendinous junction formation (Biryukova et al. 2009; Bejarano et al. 2010; Yatsenko and Shcherbata 2014). We have now shown that miR-9a is involved in the translational regulation of Troponin-T (TnT) levels during sarcomeric assembly.

**Troponin-T is a major target of miR-9a during myofibrillogenesis in the IFMs**

In general, microRNAs and their targets have been observed to exhibit mutually exclusive expression (Stark et al. 2005). While, miR-9a is strongly expressed in all developmental stages, its expression is reduced in adult flies, including the adult IFMs where its expression is much reduced compared to earlier developmental stages (Sempere et al. 2003). We have confirmed that miR-9a is barely detectable in adult IFMs (Figure S1.A). These data suggest that the expression of some miR-9a targets of could be required for IFM development and function.

We report here that over-expression of miR-9a causes a hypercontraction phenotype in the IFMs. We identified TnT, a structural component of the thin filament of the sarcomere, as a major target for miR-9a in muscles and have shown that miR-9a over-
expression leads to repression of TnT and that this is sufficient to explain the IFM hypercontraction phenotype. In *Drosophila*, Troponin-T (TnT) is encoded by the *upheld* gene, and is a key component in coordinating the Tn-Tm complex (Fyrberg *et al.* 1994; Nongthomba *et al.* 2007) and serves as an anchor for the other components of the complex which comprise Troponin-I, Troponin-C and Tropomyosin (Gordon *et al.* 2000). Whereas the *up*¹ mutation is characterized by the absence of the adult IFM-specific TnT isoform, TnT-10a, resulting in hypercontraction (Nongthomba *et al.* 2007), the *up*¹⁰¹ mutation in *upheld* leads to increase calcium sensitivity and the irregular acto-myosin interactions which also causes hypercontraction which produces damaged muscles (Beall and Fyrberg 1991; Nongthomba *et al.* 2003; 2007). However, previously there has been no report that epigenetic regulation of TnT can also contribute to the maintenance of stoichiometric balance. To the best of our knowledge, this is the first report on the miRNA-mediated post-translational regulation of Troponin-T.

Clearly down-regulation of TnT by miR-9a phenocopies the mutation in *upheld* via the same mechanism of stoichiometric imbalance driven mis-regulation of acto-myosin interaction. Importantly, this demonstrates that not only defects in transcriptional control, but also the derailing of other regulatory processes, such as miRNA-mediated control, can result in the same defects. Thus, our finding that miR-9a can alter levels of thin filament components via translational control of TnT demonstrates that miRNAs are not just “regulators of regulators” but can act as direct regulators in coordinating a complex process such as myofibrillar assembly. We show here, that a major structural protein, TnT, can be in fact regulated by miRNA.
miR-9 is required for maintaining protein stoichiometry and may have implications in etiology of myopathies

Studies on IFM mutants indicate that structural integrity of IFMs is highly dependent on interactions between thin and thick filaments as well as the ratio of individual myofibrillar contractile components. Any change in gene dosage and corresponding protein stoichiometry in the thin filaments translates into defects in normal myofibrillar assembly leading to hypercontraction (Tansey et al. 1991; Nongthomba et al. 2003, 2007). This explains the myofibrillar defects that results from over-expression of Troponin-T (Marco-Ferreres et al. 2005), Mhc (Cripps et al. 1994), and in most of the heterozygotes carrying mutations in genes encoding structural proteins (Prado et al. 1995; Gajewski and Saul 2010).

The phenomena of both hypercontraction and hypertrophy, although observably different, are both responses to the muscle contraction and the imbalance of structural proteins. In hypercontraction, the muscles show properly arranged sarcomeres during early development, but the subsequent uncontrolled acto-myosin interactions lead to stress and muscle tearing (Nongthomba et al. 2003). Hypertrophy, on the other hand, is characterized by increase in muscle volume. Myocardial hypertrophy for instance is associated with cardiac remodeling where there is an increase of muscle wall thickness, but not through any increase in myocyte number (hyperplasia). However, cardiac hypertrophy is also a physiological response to stress induced by ischemia, mitochondrial defects, mutations in sarcomeric components, etc. Importantly, mutations in the same gene orthologs which cause hypercontraction in Drosophila are the ones mutated in cardiac hypertrophy patients as well. For example, mutations in the
Troponin-T gene are one of the predominant causes of hypertrophy (Seidman and Seidman 2001; Di Pasquale et al. 2012). Most of these TnT mutations exhibit increased calcium sensitivity and activation of muscle contractility (Harada and Potter 2004; Parvatiyar and Pinto 2015; Gilda et al. 2016) a similar pathogenesis to the hypercontraction produced by TnT mutants in Drosophila (Nongthomba et al. 2003, 2007). Viswanathan et al. have shown that the up101 mutation generates a muscle abnormality similar to human cardiomyopathy through sensitive calcium regulation in the Drosophila heart (Viswanathan et al. 2014).

Vertebrate Troponin-T (TNNT) has a vital role in anchoring of Tn-Tm to actin and also essential for Ca^{2+} mediated activation and inhibition of acto-myosin activity during muscle contraction (Potter et al. 1995; Schiaffino and Reggiani 1996; Perry 1998; Domingo et al. 1998; Oliveira et al. 2000). Mutations in TNNT in C. elegans result in defects in embryonic body wall muscle contractions and sarcomere organization (Myers et al. 1996). Mutations in the cardiac isoform of Troponin-T (TNNT2) are associated with familial hypertrophic cardiomyopathy, dilated cardiomyopathy or arthrogryposis (Thierfelder et al. 1994; Kamisago et al. 2000; Sehnert et al. 2002; Sung et al. 2003). TNNT2 was also found to be up-regulated in cardiac hypertrophic or myocardial infarctions conditions (Salic and Windt 2012). However, there are still unanswered questions pertaining to the mechanisms by which cardiac TNNT2 up-regulation is brought about during hypertrophy. Hence, the upstream players that regulate the level of cardiac TNNT2 during muscle development and function are very important.

Drosophila miR-9a is identical to human miR-9 and the human Troponin-T (TNNT) is has significant homology of the Drosophila TnT (Lagos-Quintana et al. 2001). Since
miR-9a is capable of regulating TnT levels in *Drosophila*, it is possible that the human miR-9 may also play a role in regulating TNNT levels. Interestingly, sequence analysis of the human skeletal and cardiac isoforms of TNNT reveals that only the cardiac TNNT possesses the miR-9 binding site (Figure S.4C) while the skeletal isoforms lack it. Incidentally, bioinformatic analysis failed to find a miR-9 target site in the mRNA sequence of mouse TNNT. It is important to note that the initial report of miR-9’s role in muscle hypertrophy were from studies on mice (Wang *et al.* 2010) so miR-9a could be playing varied roles in different organisms. Our study suggests that miR-9a might be involved in specifically regulating the levels of cardiac Troponin T in humans. It would be interesting to know if the increase in cardiac TNNT that occurs in response to hypertrophic stimulus is mediated by miR-9. Many mutations of the human cardiac Troponin-T give rise to the hypertrophic condition (Di Pasquale *et al.* 2012). However, cardiac hypertrophy is a genetically and clinically heterogeneous disorder and its etiology in many instances has not been determined (Gilda *et al.* 2016). The present study provides a plausible candidate in the form of miR-9 to explore in the etiology of idiopathic cardiomyopathies. It would be interesting to determine what represses miR-9 during myofibril assembly. Its continuous expression would be deleterious to myofibril assembly through its repression of the expression of very important structural proteins such as TnT. Taurine-upregulated gene-1 (TUG1) negatively regulates miR-9 in a human cancer cell line (Zhao and Ren 2016). Whether TUG1 or similar protein(s) are involved in muscle hypertrophy/hypercontraction and myofibril assembly requires further investigation.

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Literature Cited


78. Thierfelder L., Watkins H., MacRae C., Lamas R., McKenna W., Vosberg H. P., Seidman J. G., Seidman C. E., 1994 Alpha-tropomyosin and cardiac troponin T


**Figure 1**

Figure 1: IFM-specific over-expression of miR-9a causes muscle hypercontraction. (A) Wild-type adult flies with normal wing posture. (A’) Regular wing position along the body axis in a wild type fly. (B) Adult flies over-expressing miR-9a have upheld wings. (B’) Upright wing phenotype in a fly over-expressing mir-9a. (C) Polarized light micrograph of dissected wild-type adult hemi-thorax showing six DLMs (asterisks). (C’-C’’) Adult myofibrils stained with Phalloidin-TRITC (F-actin) and Sls-GFP (arrows indicate Z-disc). (C’’) SEM micrograph of wild-type myofibril (arrow indicates Z-disc). (D) Polarized light image of hemi-thorax of adult male with over-expression of miR-9a showing broken muscles (arrow) and clumped muscle (outlined in black and orange). (D’-D’’) Loss of sarcomeric structural integrity (box) in flies over-expressing miR-9a. (D’’) SEM micrograph showing absence of proper sarcomeres (box) after miR-9a over-expression (Scale bar 2 µm). (E) Flight assay of the miR-9a over-expression flies. (F) Images of wild-type hemi-thorax showing six DLMs (asterisks) under polarized light. (F’) Phalloidin-TRITC stained myofibril showing normal sarcomeric structure (arrow) in wild type flies. (G) Adult flies carrying a myosin MhcP401S mutation in the over-expression of miR-9a background show six DLMs (asterisks) in the hemi-thorax and (G’) normal sarcomeres (arrows) in the myofibrils. (H) Polarized light image of hemi-thorax from flies with over-expression of miR-9a. (H’) Abrogated sarcomeric structure (box) in the myofibrils of flies over-expressing miR-9a (Scale bar 2 µm).
Figure 2

(A) Lists of genes which are putative targets of miR-9a and their functions in muscles. The upheld gene that encodes Troponin T is highlighted in red (within blue box) (B) Expression profile of the putative miR-9a targets in the IFMs obtained from the microarray data from IFM of wild type flies. The upheld gene showed highest expression in IFM (highlighted by the blue bar). (C) Relative expression of the target genes validated by real-time PCR. (D) Schematic representation of miR-9a binding site at 3'-UTR of upheld (TnT) and the mechanism of translation repression of Troponin T. (E) Quantification of the relative expression of TnT after miR-9a over-expression, using α-Tubulin as loading control.
Figure 3: Transgenic lines with over-expression of TnT (10a or 10b isoform) restore Troponin-T levels and rescue the muscle hypercontraction phenotype resulting from over-expression of miR-9a. (A) Western blots and the quantification of the relative expression of Troponin-T (loading control α-Tubulin). (B) Polarized light micrograph showing normal six DLMs following the over-expression of TnT-10a or (B’) TnT-10b isoforms in the background of miR-9a over-expression. (B’’) Polarized image showing hypercontracted muscles after miR-9a over-expression. (C) Quantification of the percentage of flies over-expressing miR-9a that present with hypercontraction phenotype after restoration of TnT levels. (D) Flight data for the flies over-expressing TnT 10a or 10b isoforms in the background of over-expression of miR-9a.
Figure 4: Rescue of the loss of muscle integrity. (A) Polarized light micrograph of wild type hemi-thorax with six DLMs. (A’) Confocal microscopy image of wild type muscles stained for F-actin along with Sls-GFP. The sarcomeres (box) and Z- discs (white arrows) are highlighted. (B-B’) 6 DLMs (asterisks) and close to normal sarcomeric structure (box) with Z- discs (white arrows) in muscles of flies expressing TnT-10a in mi-9a over-expression background. (C-C’) Hypercontracted muscles (circled) and lack of sarcomeres (box) in muscles of flies over-expressing miR-9a. (D-D’) Six DLMs (asterisks) and restored muscle structure (box) with Z- discs (white arrows) in flies over-expressing TnT-10b in miR-9a over-expression background (Square Box). (Scale bar 2 µm).
Supplementary figures

Figure S1: miR-9a depletion did not affect normal muscle structure and function. (A) Northern blot analysis of miR-9a expression in IFM of various genotypes. (B) Flight data following knockdown of miR-9a and knockdown of miR-9a in the over-expression background. (C) Polarized light image of hemi-thorax of adult wild type flies showing six DLMs (asterisks). (C’) Confocal images of wild type myofibrils stained with Ph-TRITC (F-actin) showing normal sarcomeres and Z- discs (arrows) (C’’) SEM micrograph of wild-type myofibril. Z- discs are highlighted (red arrows). (D) Adult hemi-thorax of a fly with miR-9a depletion shows 6 DLMs (asterisks). (D’) Ph-TRITC stained muscles of flies with knockdown of miR-9a show normal sarcomeric structure (arrow). (D’’) SEM micrographs of myofibrils from IFM of flies with miR-9a depletion (C’-D’, scale bar 2 μm).
Figure S2: (A) H & E staining of transverse sections (TS) of wild-type hemi-thorax. (A’) Single DLM from the TS of wild type hemi-thorax. (B) H & E staining of hemi-thorax of a fly with miR-9a over expression. (B’) TS of a single DLM in flies over-expressing miR-9a showing disintegration of muscle structure (Black arrows). (C) Polarized light images of hemi-thorax of adult wild type flies show six DLMs (asterisks). (C’) Confocal images of wild-type myofibrils with normal sarcomeric integrity (arrow). (D) Adult hemi-thorax of a male fly with miR-9a depletion in the miR-9a over-expression background shows 6 DLMs (asterisks). (D’) Ph-TRITC stained myofibrils of male flies with knockdown of miR-9a in the over-expression background show normal sarcomere structure (arrow). (E) Hemi-thorax of a female fly with knockdown of miR-9a in the over-expression background showing 6 normal DLMs (asterisks). (E’) Myofibrils from female flies with miR-9a depletion in the miR-9a over-expression background with normal sarcomeric structure (arrow). Scale bar 2 μm.
Figure S3: miR-9a knock down under miR-9a over expression rescues muscle structure and function. (A) Western blots and the quantification of the relative expression of thin filament proteins (Actin and TnI) and thick filament protein (Flightin). (B) Adult wild-type hemi-thorax of a fly showing 6 DLMs (asterisks). (B’) Confocal images of wild type myofibrils stained with Ph-TRITC showing normal sarcomeric structure (white arrows). (C) Flies with knock down of neuralized show 6 DLMs (asterisks) in the hemi-thorax and (C’) show intact sarcomere structures (white arrows) in myofibrils. (E) Adult flies with knockdown of Salimus (Sls) show six DLMs and (E’) slightly irregular sarcomeres respectively (rectangle). (F) Flies with knock down of Troponin-T (upheld) show hypercontracted muscles and (F’) complete absence of sarcomeres (box). (G) Hemi-thorax of a fly with miR-9a over-expression shows hypercontracted muscles and (G’) absence of sarcomeres (box). Scale bar 2 μm.
Figure S4: (A) Presence of normal 6 DLMs in the wild type hemi-thorax where UAS-GFP is driven by UH3-Gal4. (A’) Well-arranged sarcomeric structure (white arrows) in the myofibrils of wild type IFMs. (B) Hemi-thoraxes of flies with UH3-Gal4 driven over-expression of miR-9a along with UAS-GFP showed hypercontracted muscles and (B’) complete loss of sarcomere structures (box). Scale bar 2 μm. (C) Only the human cardiac muscle-specific Troponin T isoform (TnnT 2) has a binding site for human miR-9.