

# Functional redundancy and nonredundancy between two Troponin C isoforms in *Drosophila* adult muscles

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**ABSTRACT** We investigated the functional overlap of two muscle Troponin C (TpnC) genes that are expressed in the adult fruit fly, *Drosophila melanogaster*: *TpnC4* is predominantly expressed in the indirect flight muscles (IFMs), whereas *TpnC41C* is the main isoform in the tergal depressor of the trochanter muscle (TDT; jump muscle). Using CRISPR/Cas9, we created a transgenic line with a homozygous deletion of *TpnC41C* and compared its phenotype to a line lacking functional *TpnC4*. We found that the removal of either of these genes leads to expression of the other isoform in both muscle types. The switching between isoforms occurs at the transcriptional level and involves minimal enhancers located upstream of the transcription start points of each gene. Functionally, the two TpnC isoforms were not equal. Although ectopic *TpnC4* in TDT muscles was able to maintain jumping ability, *TpnC41C* in IFMs could not effectively support flying. Simultaneous functional disruption of both TpnC genes resulted in jump-defective and flightless phenotypes of the survivors, as well as abnormal sarcomere organization. These results indicated that TpnC is required for myofibril assembly, and that there is functional specialization among TpnC isoforms in *Drosophila*.

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## INTRODUCTION

Numerous genes in higher eukaryotes exist as multigene families. In some instances, such as ribosomal DNA genes, the existence of multiple gene copies is believed to enable the production of an increased amount of gene product. By contrast, in many instances, the existence of gene duplicates has allowed for either subfunctionalization, by which individual gene family members acquire specialized functions, or neofunctionalization, by which a duplicate copy acquires a new or novel function (reviewed in Conant and Wolfe, 2008; Katju, 2012).

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Abbreviations used: BDSC, Bloomington *Drosophila* Stock Center;  $\beta$ Gal,  $\beta$ -galactosidase; CRISPR, clustered regularly interspaced short palindromic repeats; IFM, indirect flight muscle; KD, knockdown; KO, knockout; MHC, myosin heavy-chain; RNAi, RNA interference; shRNA, short hairpin RNA; TDT, tergal depressor of the trochanter muscle; TpnC, Troponin C; TpnT, Troponin T; UAS, upstream activating sequence; UTR, untranslated region; VDRC, Vienna *Drosophila* RNAi Center; WT, wild type.

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Although examples of neofunctionalization are relatively rare (see, e.g., Zhang et al., 2004), there are a number of examples of subfunctionalization. In mammals, several muscle structural genes are represented as duplicate copies, such as myosin heavy chain (MHC) genes. The mouse genome encodes 11 muscle-specific MHC genes that are expressed in different regions of the body and at different times of development; moreover, the encoded proteins have biochemical properties that differ from one another (reviewed in Weiss and Leinwand, 1996).

The existence of subfunctionalization raises questions of the degree to which the duplicate gene copies are functionally distinct and how the differences in patterns of gene expression for duplicate copies has arisen. For the myosin genes, as with other examples, there is evidence for functional nonredundancy in gene duplicates: loss of function of the murine *Myh1* gene is partially compensated for by up-regulation of *Myh2* expression. However, the mutant mice show a number of severe phenotypes, including muscle weakness and kyphosis, indicating that the two isoforms are functionally nonequivalent (Sartorius et al., 1998; Allen and Leinwand, 2001). Similarly, different muscle-specific actin genes in *Drosophila* were shown to be nonequivalent when tested for function in the adult indirect flight muscles (IFMs; Fyrberg et al., 1998). These studies, alongside many others, underline the adaptive potential of subfunctionalization.

Another muscle structural gene family in *Drosophila* encodes the Troponin C (TpnC) proteins. TpnC is the Ca<sup>2+</sup>-binding moiety of the troponin complex that regulates Ca<sup>2+</sup>-activated skeletal muscle contraction (reviewed in Lieber, 2010). There are five TpnC genes in the *Drosophila* genome, which show distinct patterns of gene expression. In particular, *TpnC41C* is expressed at high levels in some adult tubular muscles, including the tergal depressor of the trochanter (TDT; or jump) muscle, whereas *TpnC4* is expressed almost exclusively in the adjacent IFMs (Qiu *et al.*, 2003; Herranz *et al.*, 2004).

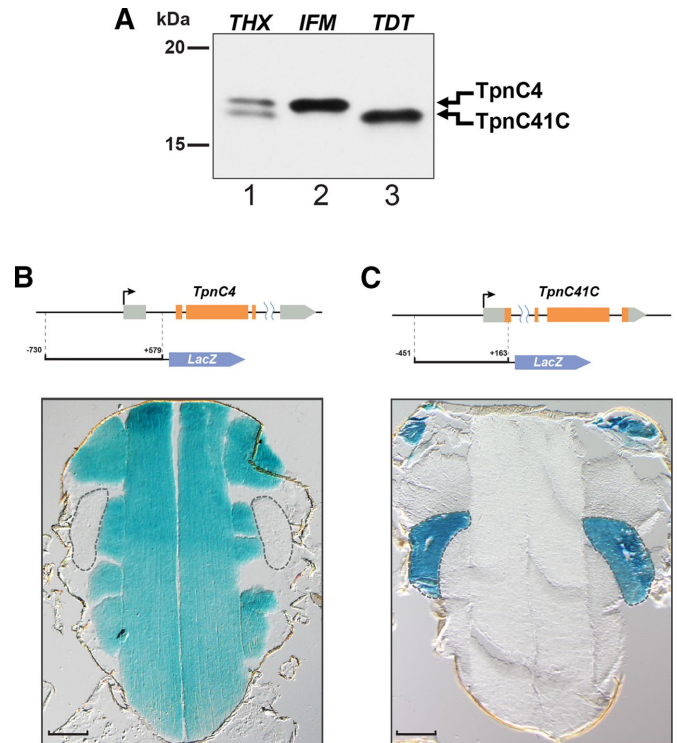
Two recent studies evaluated the roles of TpnC in *Drosophila* adult muscles. Eldred *et al.* (2014) studied the roles of the two adult TpnC isoforms in adult muscle function. They detected low levels of the TDT-enriched TpnC, TpnC41C, in the flight muscles; knockdown of *TpnC41C* expression, however, did not affect flight muscle function. By contrast, knockdown of *TpnC4* expression, encoding the predominant flight muscle isoform, resulted in up-regulation of TpnC41C in flight muscles and defects in muscle structure and function. In a similar study, Singh *et al.* (2014) demonstrated that flight muscle-specific knockdown of *TpnC4* expression resulted in a reduction in flight ability and myofibrillar defects. These findings suggested that, at least in the flight muscles, TpnC41C and TpnC4 were not functionally equivalent.

These two studies significantly enhanced our understanding of TpnC function and demonstrated that, at least in the context of the flight muscles, the two adult isoforms could not functionally replace one another. Nevertheless, several questions remain: are these results from RNA interference (RNAi) experiments supported by genetic loss-of-function experiments? Is there reciprocal functional nonredundancy between flight and jump muscle isoforms of TpnC in *Drosophila*? What is the phenotype of TpnC-null muscle? Finally, how is the up-regulation of expression of one protein in the absence of the other achieved? In this article, we demonstrate that *TpnC4* and *TpnC41C* are expressed almost exclusively in the IFM and TDT muscles, respectively, and that they show reciprocal cross-regulation. Using knockdown and knockout combinations, we confirm that TpnC41C cannot compensate for the loss of TpnC4 in the IFMs. We additionally demonstrate that, in the converse situation, loss of *TpnC41C* function in the TDT results in an expansion of *TpnC4* expression into the TDT, which enables normal jumping ability of adults. We also generate *TpnC41C TpnC4* double-null mutants, which can neither fly nor jump and fail to form normal myofibrils during development. Finally, we demonstrate that the expansion of gene expression in each case occurs through transcriptional up-regulation via identified enhancer sequences. These studies complement and significantly extend earlier findings by demonstrating the functional significance of TpnC isoform diversity and providing insight into the mechanisms by which loss of expression of one gene results in a change in the expression of a paralogous gene.

## RESULTS

### *Drosophila* tubular and fibrillar muscle fiber types express different TpnC isoforms

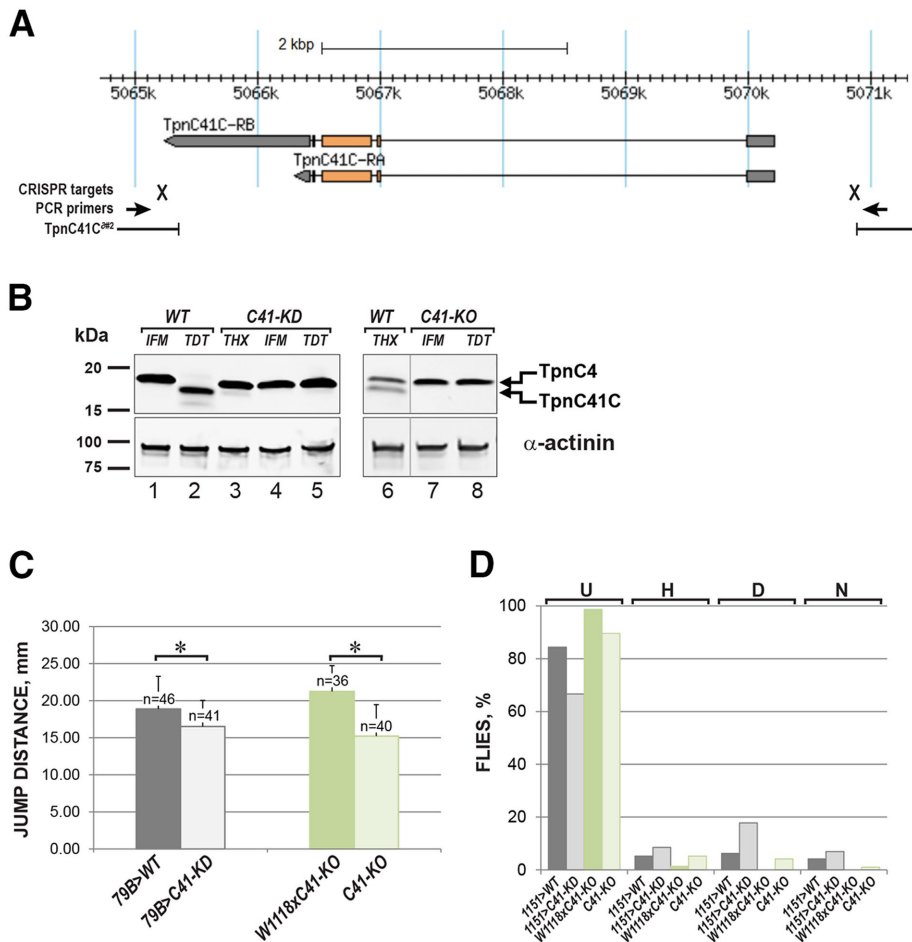
Previous studies demonstrated that TpnC4 is the predominant IFM isoform, whereas *TpnC41C* is most strongly expressed in the tubular muscles, including the jump muscle. More detailed studies also indicated that the flight muscles, although predominantly expressing *TpnC4*, also express *TpnC41C* to some degree (Qiu *et al.*, 2003; Herranz *et al.*, 2004; Eldred *et al.*, 2014). In our studies, we observed relatively little overlap in expression of the two genes: firstly, we assessed TpnC protein isoform composition in whole thoraces as well as dissected IFM and TDT muscles using Western blotting and probed with an antibody that recognizes both adult



**FIGURE 1:** *TpnC4* and *TpnC41C* are fiber-specific genes expressed in the muscles of the *Drosophila* thorax. (A) Western blot of protein samples prepared from *Drosophila* whole thoraces (THX, lane 1) and dissected IFM and TDT muscles (lanes 2 and 3, respectively) stained with TpnC antibody. Arrows indicate positions of TpnC4 and TpnC41C isoforms. Positions and molecular weights of protein standards are indicated on the left. (B, C) Top, diagrams of the *D. melanogaster TpnC4* and *TpnC41C* genes with their exon composition and the reporter constructs generated, in which the indicated genomic regions were fused to *lacZ*. Protein-coding regions are shown in orange; untranslated regions are shown in gray. Numbers indicate starting and terminal nucleotides relative to the transcription start site for each tested cloned promoter region. Bottom, representative sections of *Drosophila* thoraces expressing either *TpnC4-lacZ* (B) or *TpnC41C-lacZ* (C) and stained for  $\beta$ -Gal activity. IFMs stain blue in B, whereas TDTs stain blue in C. TDT muscles are outlined with dashed lines. Scale bar, 100  $\mu$ m.

isoforms of TpnC. We found that TpnC4 protein was the only isoform detected in the IFMs, whereas TpnC41C was a specific TpnC isoform of TDT muscles (Figure 1A). Although TpnC4 and TpnC41C were reported earlier as major and minor isoforms found in the IFM, respectively (Eldred *et al.*, 2014), we could not find any traces of TpnC41C expression on our Western blots when we loaded equal amounts of total protein of dissected muscles. Only when the flight muscle lane was overloaded to tenfold the amount of protein used in Figure 1A did we observe a faint band corresponding to TpnC41C.

Second, to complement this analysis, we tested the activities in the adult muscles of  $\beta$ -galactosidase ( $\beta$ -Gal) reporter constructs in which the regulatory sequences of either *TpnC4* or *TpnC41C* were fused to a *lacZ* reporter (Figure 1, B and C, diagrams). To locate the *TpnC4* regulatory sequences, we tested the enhancer activities of 5'- and 3'-adjacent sequences of the gene, as well as its introns, and the only construct that had reporter expression was -730/+579 *TpnC4-lacZ*. The *TpnC41C-lacZ* reporter was described in Chechenova *et al.* (2015).



**FIGURE 2:** Loss of TpnC41C is functionally compensated by expansion of *TpnC4* expression. (A) Design and analysis of a *TpnC41C* genomic deletion. Top, GBrowse image from flybase.org shows the *TpnC41C* locus and its annotated transcripts. Bottom, locations of CRISPR target sites (X marks), primers used to confirm the deletion (arrows), and deletion boundaries in the *TpnC41C*<sup>Δ#2</sup> allele (T-bars). (B) TpnC isoform composition in *TpnC41C*-knockdown and -knockout mutants. Proteins from whole thoraces and dissected IFM and TDT muscles were separated by SDS-PAGE, followed by Western blotting and staining with TpnC antibody (top) and  $\alpha$ -actinin antibody to serve as a loading control (bottom). Protein samples from *w*<sup>1118</sup> control flies (lines 1, 2, and 6) were analyzed alongside lysates obtained from either *Act79B>TpnC41C RNAi* flies (C41-KD, lines 3–5) or *TpnC41C*<sup>Δ#2</sup> homozygotes (C41-KO, lines 7 and 8). Vertical gray line between lanes 6 and 7 indicates that these samples were analyzed on the same blot but that intervening lanes were cropped out. (C) Jump test results. The offspring of *Act79B-Gal4* crossed to the RNAi control line 36303 (79B>WT, dark gray bar) were used as a control for the line with *TpnC41C* knockdown in jump muscles. *Act79B>UAS-TpnC41C RNAi* (79B>C41-KD, light gray bar) are the knockdown animals. Heterozygous flies from the cross of *w*<sup>1118</sup> x *TpnC41C*<sup>Δ#2</sup> (*w*<sup>1118</sup> x C41-KO, dark green bar) were used as controls for *TpnC41C*<sup>Δ#2</sup> homozygous mutants (C41-KO, light green bar). Error bars show SD for average jumping distance in millimeters. *TpnC41C*-knockout and -knockdown flies showed reduced jumping ability compared with controls ( $*p < 0.01$ ). (D) Flight test results. The offspring of *1151-Gal4* crossed with the RNAi control line 36303 (1151>WT, dark gray bars) were used as a control for flies with the *TpnC41C* knockdown in both flight and jump muscles ( $n = 96$ ). *1151>UAS-TpnC41C RNAi* (1151>C41-KD, light grey bars) represent knockdown animals ( $n = 129$ ). Heterozygous offspring from the cross of *w*<sup>1118</sup> with *TpnC41C*<sup>Δ#2</sup> (*w*<sup>1118</sup> x C41-KO, dark green bars) was used as a control for the *TpnC41C*<sup>Δ#2</sup> homozygous mutants (C41-KO, light green bars;  $n = 72$  and  $96$ , respectively). Letters on the top show direction of flight: D, downward; H, horizontally; N, no flight at all; U, upward. *TpnC41C*-knockdown and -knockout flies showed only slight reductions in flight ability compared with their controls.

In the thoraces of transgenic flies, the cloned *TpnC4* enhancer selectively directed reporter expression in the fibrillar IFMs, whereas reporter expression was excluded from tubular TDT muscles

but the appearance of a band corresponding to TpnC4 (Figure 2B, lane 8). Together these data indicate that loss of TpnC41C from the jump muscles caused an isoform switch in the TDT.

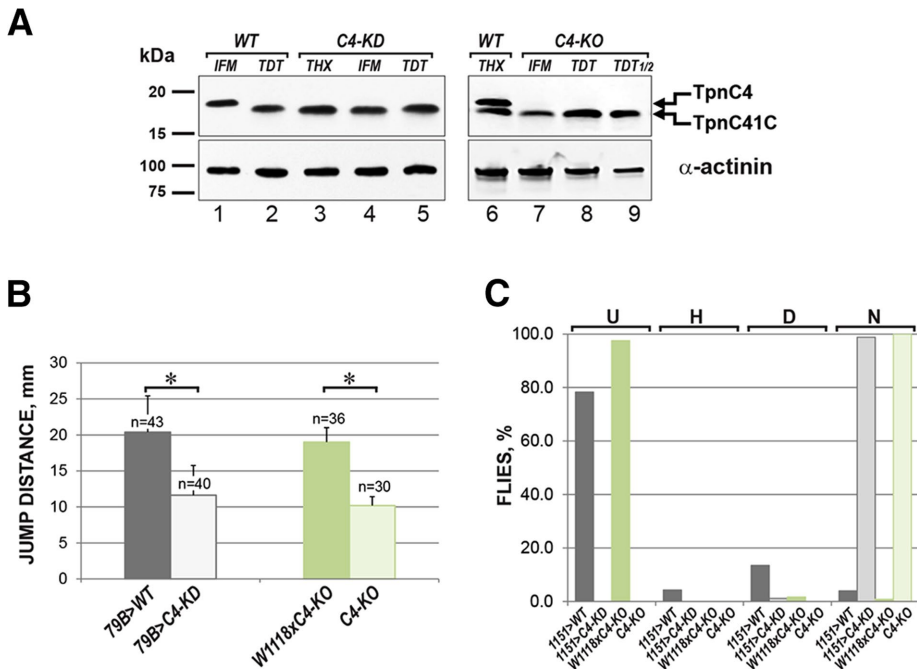
(Figure 1B). By contrast, the *TpnC41C* enhancer was active in TDT muscles but not in the IFMs (Figure 1C). These findings underline the published observations that different thoracic muscle fibers use different isoforms of TpnC protein, which probably reflect the differences in functional demands for different types of highly specialized adult muscles. Our data differ to a small extent from those of Eldred *et al.* (2014), in that we show that the contribution of *TpnC41C* in the flight muscles is very minor, which might simply reflect line-specific differences in gene expression.

### The TpnC4 isoform functionally substitutes for TpnC41C

To analyze the functional importance of *TpnC41C* in TDT muscles, we sought to inactivate its expression. We achieved gene inactivation by two methods: expressing a short hairpin RNA (shRNA) targeting *TpnC41C* and generating a *TpnC41C*-null allele.

The *TpnC41C*-null allele was created using the clustered regularly interspaced short palindromic repeats (CRISPR)/Cas9 genome-editing approach. We generated two plasmids containing sequences to express single guide RNAs targeting 5' and 3' regions of the gene (Figure 2A). The plasmids were injected into embryos expressing Cas9 in their germline, and potential mutant lines were identified as homozygotes in the second generation. Western blotting identified one line, *TpnC41C*<sup>Δ#2</sup>, that did not accumulate TpnC41C in the thorax, and we determined that this line had arisen through the designed deletion of the genomic sequence between the two target sites based on PCR and sequencing genomic DNA from this line (Figure 2A, bottom).

We next compared the effects of RNAi-mediated knockdown (KD) of *TpnC41C* with the *TpnC41C*-null allele. For the KD, we observed a loss of TpnC41C protein from whole thoraces (Figure 2B, lane 3), yet when we analyzed the IFM and TDT muscles separately, we observed that the jump muscles accumulated a TpnC with the same electrophoretic mobility as the flight muscle TpnC4 isoform (Figure 2B, lanes 4 and 5). This result suggested that loss of TpnC41C from the jump muscles caused an expansion of *TpnC4* expression into the TDT. To confirm that this result was not an artifact of the RNAi system being used, we studied TpnC accumulation in the muscles of homozygous *TpnC41C*<sup>Δ#2</sup>-null mutants. Again, we observed a loss of the TpnC41C-specific band in TDT samples



**Fig. 3**

**FIGURE 3:** Loss of TpnC4 is not functionally compensated by TpnC41C, despite *TpnC41C* expression expanding into the IFMs. (A) TpnC isoform composition in *TpnC4*-knockdown and -knockout mutants. Proteins from whole thoraces (THX) and dissected IFM and TDT muscles were separated by SDS-PAGE, followed by Western blotting and staining for TpnC (top) and  $\alpha$ -actinin as a loading control (bottom). Protein samples from *w<sup>1118</sup>* control flies (WT, lanes 1, 2, and 6) were analyzed alongside lysates obtained from either *1151>TpnC4 RNAi* flies (C4-KD, lanes 3–5) or *TpnC4<sup>F2-3</sup>* mutant homozygotes (C4-KO, lanes 7–9). Lane 9 has TDT lysate loaded at 50% of the total protein amount used for other lanes. (B) Jump test results. The offspring of the *Act79B-Gal4* driver line crossed to the RNAi control line 60100 (1151>WT, dark gray bar) were used as a control to compare to *TpnC4* knockdown in jump muscles. *Act79B>UAS-TpnC4 RNAi* (79B>C4-KD, light gray bar) are the knockdown sample. Heterozygous adults from the cross *w<sup>1118</sup> x TpnC4<sup>F2-3</sup>* (*w<sup>1118</sup>xC4-KO*, dark green bar) were used to compare with *TpnC4<sup>F2-3</sup>* homozygotes (C4-KO, light green bar). Error bars show SD for average jumping distance in millimeters. Note that the jumping ability is reduced in both knockdown and knockout lines compared with controls, \**p* < 0.01. (C) Flight test results. The offspring of *1151-Gal4* crossed with the RNAi control line 60100 (1151>WT, dark gray bars) were used as a control for the line with *TpnC4* knockdown in both flight and jump muscles, *1151>UAS-TpnC4 RNAi* (1151>C4-KD, light gray bar; *n* = 304 and 174, respectively). Heterozygotes from the cross of *w<sup>1118</sup> x TpnC4<sup>F2-3</sup>* (*w<sup>1118</sup>xC4-KO*, dark green bar) were used to compare with *TpnC4<sup>F2-3</sup>* homozygotes (C4-KO, light green bar; *n* = 243 and 89, respectively). Letters on the top show direction of flight: D, downward; H, horizontally; N, no flight at all; U, upward. Note that knockdown and knockout flies of *TpnC4* are completely flightless.

To define the functional consequences of this isoform switch, we jump tested the *TpnC41C* KD and null homozygotes because the TDT is the sole muscle responsible for jumping in the adult (Elliott et al., 2007). Of interest, although the KD and mutant animals showed a small but significant reduction in jumping distance compared with controls, they maintained jumping ability (Figure 2C), indicating that there is a functional overlap between TpnC4 and TpnC41C in the context of jump muscle function.

As mentioned earlier, some publications reported a low expression of TpnC41C in IFMs (Eldred et al., 2014). To determine whether such minor TpnC41C presence had an effect on flight ability, we performed flight testing on flies with *TpnC41C* deficiency. We found that 90% of homozygotes for the *TpnC41C* deletion were able to fly (Figure 2D, C41-KO), which was comparable to a heterozygous line lacking one copy of the *TpnC41C* gene (99% able to fly; Figure 2D, w1118xC41KD). To down-regulate *TpnC41C* expression in flight

muscles, we crossed the *1151-Gal4* driver to a *UAS-TpnC41C RNAi* line. This resulted in a complete loss of TpnC41C from TDT muscles (unpublished data) and therefore presumably also the loss of any small amount of TpnC41C present in the flight muscles. Among the offspring of this cross, 18% were flight impaired and flew downward, although the majority of the flies were able to fly normally (67%; Figure 2D). These results confirmed the observations of Eldred et al. (2014) that the TpnC41C isoform does not play a noticeable role in the function of the IFMs. Overall our studies demonstrate that, in the absence of TpnC41C in the jump muscles, there is an expansion of *TpnC4* expression into the jump muscles that rescues jump muscle function.

### The TpnC41C isoform does not functionally substitute for TpnC4

The finding that the *TpnC4* gene was activated in TpnC41C-deficient TDTs encouraged us to examine the possibility of a reciprocal effect. We asked whether the tubular muscle TpnC isoform can substitute for its fibrillar-muscle paralogue in flight muscles of TpnC4-deficient flies. To achieve this, we analyzed a *TpnC4*-knockdown mutant created by crossing the *1151-Gal4* driver with the *UAS-TpnC4 RNAi* line. We also analyzed the *TpnC4*-null mutant, termed *TpnC4<sup>F2-3</sup>*, described in Adame et al. (2016). Thoracic muscles of both lines were dissected and assessed for TpnC expression by Western blotting.

In our Western blots, we observed that knockdown of *TpnC4* in the flight muscles resulted in a loss of the corresponding protein in whole-thorax samples (Figure 3A, lane 3). Moreover, we observed that flight muscles of these knockdown animals accumulated a TpnC isoform with mobility matching that of TpnC41C (Figure 3A, lane 4). A similar observation was made in the *TpnC4*-null animals, in which loss of TpnC4

in the IFMs resulted in accumulation of TpnC41C in those muscles (Figure 3A, lanes 7–9). Of interest, in the *TpnC4*-knockout mutants, the accumulation of TpnC41C in the IFMs appeared to be reproducibly reduced relative to the accumulation of TpnC4 in control animals. This difference was apparent when compared with a lane in which half as much of the TDT sample was loaded (Figure 3A, lane 9). On the other hand, the accumulation of TpnC41C in a *TpnC4* knockdown was not noticeably less than in controls (Figure 3A, lane 4; Eldred et al., 2014). As described in the Discussion, it is possible that these reduced levels of TpnC in the *TpnC4* mutants could contribute to the behavioral defects detailed later. Nevertheless, our data overall demonstrate that there is mutual compensation in expression between *TpnC41C* and *TpnC4*, although the precise levels of expression in control and compensating animals may differ.

We next analyzed the jump and flight capabilities of *TpnC4* KD and mutant animals. In this instance, we compared the jumping

abilities of control animals, KD animals generated by combining the TDT-specific driver line *Act79B-Gal4* with *UAS-TpnC4 RNAi*, and *TpnC4* nulls. We observed a significant reduction in the jumping distance of *TpnC4*-deficient animals (Figure 3B). This result was unexpected because we do not detect significant levels of *TpnC4* expression in the TDT muscles, and this will be addressed in the Discussion. Of greater importance, when we subjected the *TpnC4* mutants to a flight test, we observed that both knockdown and knockout mutants were completely flightless (Figure 3C). These results established that whereas *TpnC41C* expression could expand into the flight muscles of *TpnC4*-deficient flies, *TpnC41C* could not substitute for the lack of *TpnC4*.

### TpnC4 and TpnC41C isoforms are essential for thoracic muscle function

The foregoing experiments showed that two *TpnC* genes, expressed in large thoracic muscles, can genetically substitute for the expression of each other, and that one isoform, *TpnC4*, can take over both flight and jump functions. Although *TpnC41C* does not substitute for *TpnC4* in flight muscle function, its expression in *TpnC4*-deficient flight muscle could be important for supporting muscle structural integrity. Therefore we investigated whether simultaneous removal of *TpnC4* and *TpnC41C* would be detrimental to adult muscle morphology and viability. Again, we used two independent techniques to down-regulate troponin C genes: RNAi knockdown and genetic knockout.

To create a double knockdown, we generated lines containing IFM and TDT-specific drivers (*Act88F-Gal4* and *Act79B-Gal4*, respectively; Bryantsev *et al.*, 2012a), as well as UAS-driven RNAi constructs targeting *TpnC4* and *TpnC41C* transcripts. These lines were crossed to generate *Act88F+Act79B>TpnC4 RNAi + TpnC41C RNAi* adults.

To generate a double mutant, we initially considered combining the *TpnC41C<sup>Δ#2</sup>* and *TpnC4<sup>F2-3</sup>* alleles by recombination. However, because the genes are located within 440 kb of each other in a centromere-proximal part of the second chromosome right arm, we felt it would be more practical to simply generate a *TpnC4* mutation on the chromosome that already carries the *TpnC41C<sup>Δ#2</sup>* allele. We used the same targeting plasmids as described in Adame *et al.* (2016) to mutate *TpnC4* and obtained two independent mutant lines. Line *TpnC<sup>Δ#25</sup>* contained a 2–base pair deletion plus a 9–base pair insertion in exon 3 at the location of the 5′ CRISPR target. The insertion carried a stop codon resulting in translation termination after the sixth amino acid of the polypeptide (Figure 4A). Line *TpnC<sup>Δ#6</sup>* contained two single-nucleotide insertions in exon 3, in close proximity to the 5′ CRISPR target. One of the insertions produced a premature stop codon after amino acid 14 of the polypeptide chain (unpublished data). Overall both mutant coding sequences contained in-frame stop codons that were predicted to terminate translation early in the coding sequence of *TpnC4*. Through these approaches, we were able to assess the effects upon IFM and TDT function of removing all *TpnC* from those muscles.

For the double-knockdown approach, analysis of protein lysates from IFM and TDT muscles showed complete elimination of *TpnC4* and a marked decrease of *TpnC41C* (Figure 4B, lanes 4 and 5). Although these double-knockdown flies survived until adulthood, they were flightless and unable to jump (Figure 4, C and D).

For the double-mutant approach, we observed that homozygotes of the two independent *TpnC41C TpnC4* double-knockout lines were able to reach late pupal stages, but only a few adults could successfully eclose. The surviving adult flies showed very low

mobility, could not jump, and were flightless (Figure 4, C and D). These results highlight the biological importance of the *TpnC* isoform switching observed because when only one isoform was disabled, the animals showed strong viability and jumping ability. Thus the *TpnC* genes complemented each other for viability and jumping ability, but there was no complementation for flight muscle function.

### Myofibril structure of TpnC double mutants

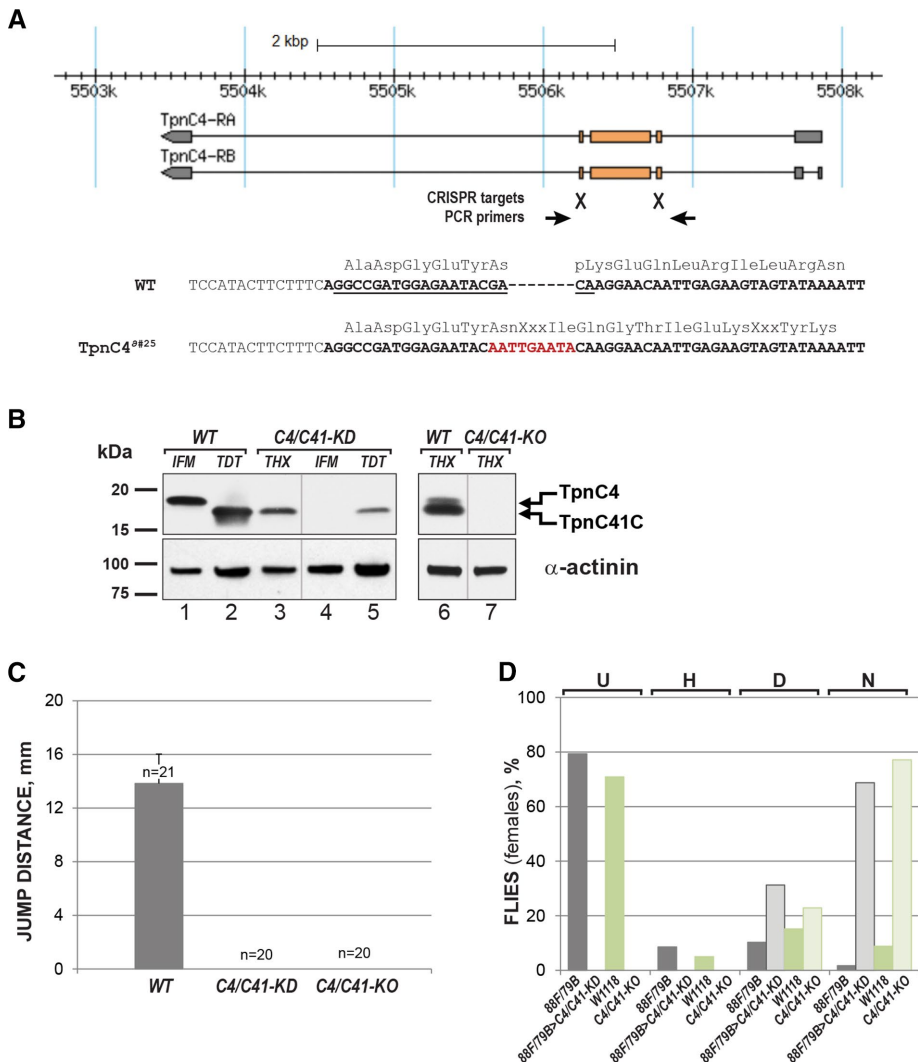
It was reported that reduced expression of *TpnC4* in flight muscle resulted in abnormalities in sarcomere structure and loss of Z- and M-line integrity (Eldred *et al.*, 2014). This phenotype represented the effects of switching *TpnC* isoforms between the IFM and TDT. In contrast, depletion of *TpnC41C* did not show any changes in the organization of the fibrillar muscle (Eldred *et al.*, 2014; Singh *et al.*, 2014). To identify the effects of a complete lack of adult *TpnCs* in the IFM and TDT, we analyzed myofibril structure in each muscle fiber type in double *TpnC4/TpnC41C* knockout mutants. To specifically visualize the formation of the Z-line and M-line of the sarcomere, we stained muscle cryosections of pharate adults with antibodies recognizing the muscle structural proteins Sallimus (Sls; also known as kettin) to label the Z-line and Obscurin to label the M-line. The overall organization of myofibrils was evaluated by staining for F-actin using a fluorescent conjugate of phalloidin.

In wild-type IFMs, the myofibrils were cylindrical, and bright phalloidin staining represented the Z-lines, which accumulate Sls protein (Figure 5, A–A′). In the double-mutant animals, the overall sarcomere architecture was retained but the Z-lines were frequently indistinct, and the myofibrils did not retain a consistent diameter along their length (Figure 5, B–B′). In addition, there was background Sls staining in the mutant, suggesting that some of the Sls might have been relocalized from myofibrils to the sarcoplasm. Similarly, in the TDTs, wild-type fibers show striking organization into parallel arrays of myofibrils, with Z-lines of adjacent myofibrils kept in close register (Figure 5, C–C′). In the double-mutant animals, however, there was significant disorganization of the TDT myofibrils, with reduced and patchy Z-line-specific signal, plus diffuse Sls accumulation (Figure 5, D–D′).

The M-line in wild-type IFMs corresponds to the absence of strong phalloidin staining and was visualized as a distinct signal using anti-Obscurin immunostaining (Figure 5, E–E′). In the double *TpnC* mutant homozygotes, the M-line staining in the IFMs was still detected but was somewhat disorganized compared with the result in control animals (Figure 5, F–F′). Similarly in the TDTs, anti-Obscurin immunostaining labeled the organized register of M-lines in control animals (Figure 5, G–G′), and whereas mutant animals showed evidence of M-line assembly, there was still some disorganization to the muscles (Figure 5, H–H′). These findings indicate that the presence of *TpnC* in flight and jump muscles is required for normal myofibril assembly.

### The TpnC isoform switching is regulated at the transcriptional level

Since our cloned *TpnC4-lacZ* and *TpnC41C-lacZ* reporters contained the necessary regulatory sequences to direct correct fiber-specific expression in nonmutant animals (Figure 1), we asked whether the isoform switching occurred at the transcriptional level and whether it was controlled via the regulatory sequences that we had identified. We achieved this by combining the *TpnC-lacZ* reporter for one gene with a knockdown for the other gene and vice versa (described in more detail in *Materials and Methods*). Upon down-regulation of *TpnC41C*, the expression of the *TpnC4-lacZ*



**FIGURE 4:** *TpnC41C* and *TpnC4* double mutants cannot jump and are flightless. (A) Design and analysis of a *TpnC4* mutation that was created in the *TpnC41C*<sup>Δ#25</sup> background. Top, GBrowse image from flybase.org showing the *TpnC4* locus and its annotated transcripts. Bottom, positions of CRISPR targets (X-marks) and primers used for analysis of mutations (arrows), showing the sequence analysis of *TpnC4* in WT and *TpnC4*<sup>Δ#25</sup> that was generated in the *TpnC41C*<sup>Δ#25</sup> genetic background. This new *TpnC4* allele has a 9-base pair insertion (red) that includes an in-frame stop codon. The allele also has a 2-base pair deletion. Underlined sequence in WT corresponds to the site targeted by CRISPR/Cas9 mutagenesis. (B) TpnC isoform composition in TpnC double-knockdown and double-knockout mutants. Proteins from whole thoraces (THX) and dissected IFM and TDT muscles were separated by SDS-PAGE, followed by Western blotting and immunostaining with TpnC antibody (top) and α-actinin antibody as a loading control (bottom). Protein samples from *w*<sup>1118</sup> control flies (lines 1, 2, and 6) were analyzed alongside lysates obtained from females carrying *Act79B-Gal4* and *Act88F-Gal4* drivers together with *UAS-TpnC41C RNAi* and *UAS-TpnC4 RNAi* (*C4/C41-KD*, lines 3–5) or alongside sample from *TpnC41C*<sup>Δ#25</sup> *TpnC4*<sup>Δ#25</sup> double-mutant homozygotes (*C4/C41-KO*, line 7). Vertical gray lines between lanes 3 and 4 and lanes 6 and 7 indicate that the samples were analyzed on the same blot, but intervening lanes were removed. (C) Jump test results. The *TpnC4/TpnC41C* double-knockdown line (*C4/C41-KD*) and the *TpnC41C*<sup>Δ#25</sup> *TpnC4*<sup>Δ#25</sup> double-knockout line (*C4/C41-KO*) showed no jumping ability compared with *w*<sup>1118</sup> controls (WT, dark grey bar). Error bars show SD for average jumping distance in millimeters. (D) Flight test results. Heterozygous females carrying both *Act79B-Gal4* and *Act88F-Gal4* drivers (88F/79B, dark grey bars) were used as a control for the line with double *TpnC4/TpnC41C* knockdown (88F/79B>*C4/C41-KD*, light grey bars; *n* = 58 and 34, respectively); *w*<sup>1118</sup> flies (dark green bars) were used as a control for the *TpnC41C*<sup>Δ#25</sup> *TpnC4*<sup>Δ#25</sup> homozygotes (*C4/C41-KO*, light green bars; *n* = 79 and 29, respectively); letters on the top show direction of flight: D, downward; H, horizontally; N, no flight at all; U, upward. Note that the knockdown and knockout mutants were unable to jump or fly.

reporter was activated in the TDTs, where normally this reporter was not expressed (Figure 6, A and B). This result indicated that the accumulation of *TpnC4* in the TDTs of *TpnC41C* KD animals resulted from a change in the pattern of transcription for *TpnC4*. Similarly, when *TpnC4* was down-regulated in the IFMs, expression of the *TpnC41C-lacZ* reporter was detected in these muscles, although potentially at reduced levels compared with that in the jump muscles (Figure 6, C and D). Collectively these results indicated that the enhancers of both troponin genes contain regulatory sequences required for their expression in both IFMs and TDTs. However, normal expression of one TpnC isoform apparently inhibits transcription of the paralogous gene.

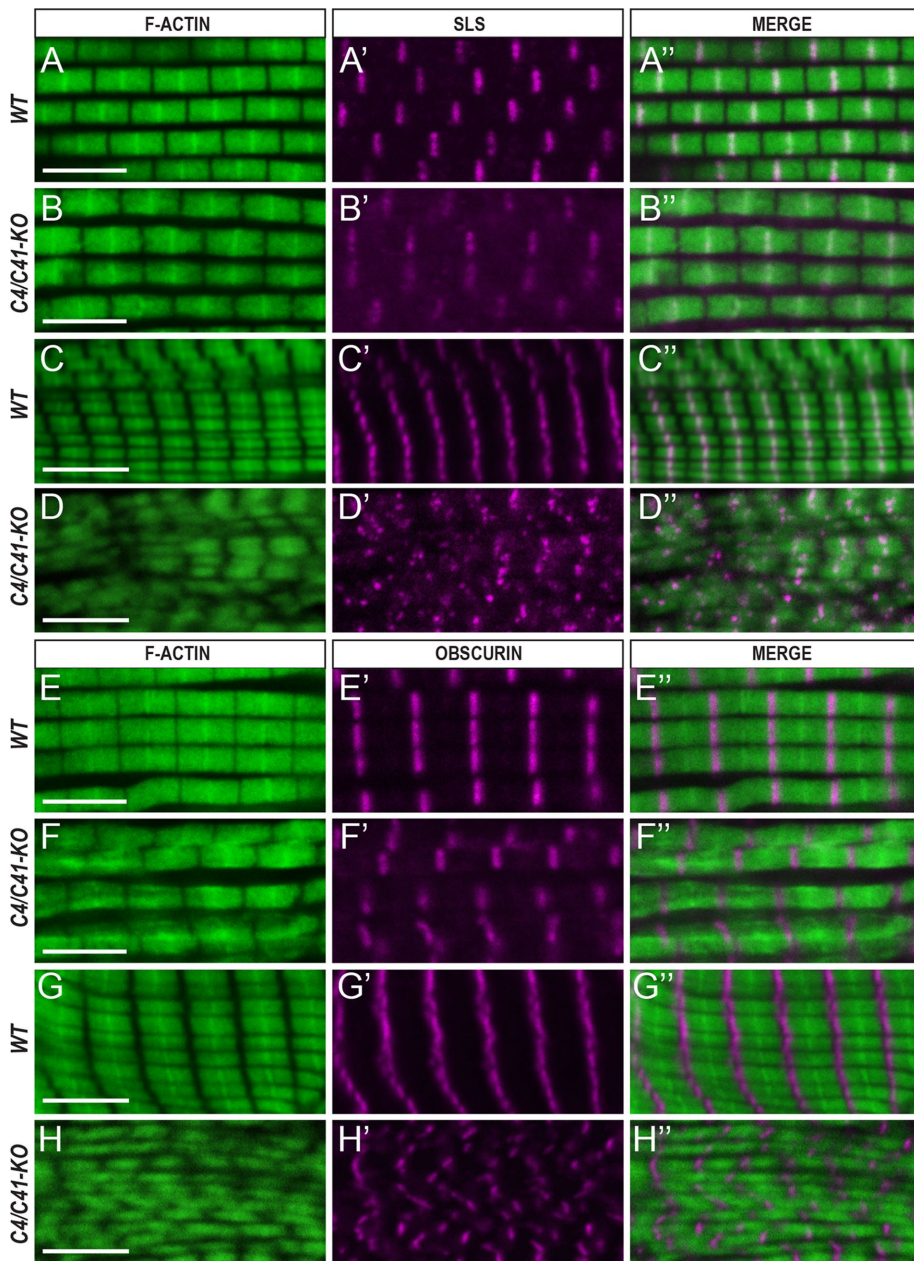
## DISCUSSION

We showed that there is partial functional redundancy between TpnC isoforms expressed in the adult *Drosophila* thorax. When either *TpnC4* or *TpnC41C* level was reduced, flies were viable, and expression of the complementary gene expanded to compensate. At the functional level, whereas the flight muscle isoform, *TpnC4*, could rescue jump muscle function in *TpnC41C* mutants, the reverse was not the case. These studies are important for understanding how genetic redundancy might occur and they also define the degree to which these different TpnC isoforms are interchangeable.

### Functional compensation between TpnC isoforms

Both our work and that of Eldred et al. (2014) demonstrate that, when *TpnC41C* expands its expression into the flight muscles of *TpnC4*-deficient flies, the flies are flightless. The inability of *TpnC41C* to rescue loss of *TpnC4* flight ability might have a number of reasons. In one scenario, there might be corresponding changes in the levels or isoforms of other myofibrillar proteins that are not consistent with normal flight muscle function. Although we cannot test this possibility exhaustively, we analyzed by Western blotting Troponin T (TpnT) accumulation in control and mutant flight muscles and did not observe a change in TpnT isoform abundance (unpublished data). Note that flight and jump muscles accumulate different isoforms of TpnT that differ in apparent relative mass (Benoist et al., 1998; Domingo et al., 1998).

In a second scenario, it could be argued that even though *TpnC41C* accumulates in the *TpnC4*-knockout flight muscles, there



**FIGURE 5:** TpnC is required for myofibril assembly. Confocal images of cryosectioned IFM and TDT muscles from WT and *TpnC41C<sup>Δ#2</sup> TpnC41C<sup>Δ#25</sup>* double-knockout mutants stained for F-actin, Sallimus (Sls), and Obscurin. (A–A'', B–B'', C–C'', D–D'') Staining with phalloidin (green) and anti-Sls antibody (magenta). (A–A'', B–B'') IFM. (C–C'', D–D'') TDT myofibrils. (E–E'', F–F'', G–G'', H–H'') Immunostaining with phalloidin (green) and anti-Obscurin antibody (magenta). (E–E'', F–F'') IFM. (G–G'', H–H'') Images of TDT. Scale bar, 5 μm. All images represent longitudinal sections of the muscles. Note that myofibril organization is disrupted in the mutants compared with controls.

are insufficient levels of accumulation to rescue muscle function. Some support for this possibility comes from the observation that TpnC41C levels in the IFMs of TpnC4 knockouts appeared reduced. On the other hand, TpnC41C levels in the IFMs of RNAi-induced TpnC4 knockdowns did not appear different from controls (Figure 3A, lane 4), and the same can be observed in the results of Eldred *et al.* (2014).

A third possibility, which we feel to be most likely, is that functional differences in the TpnC proteins affect their ability to rescue muscle function. Herranz *et al.* (2005) used a bioinformatic approach

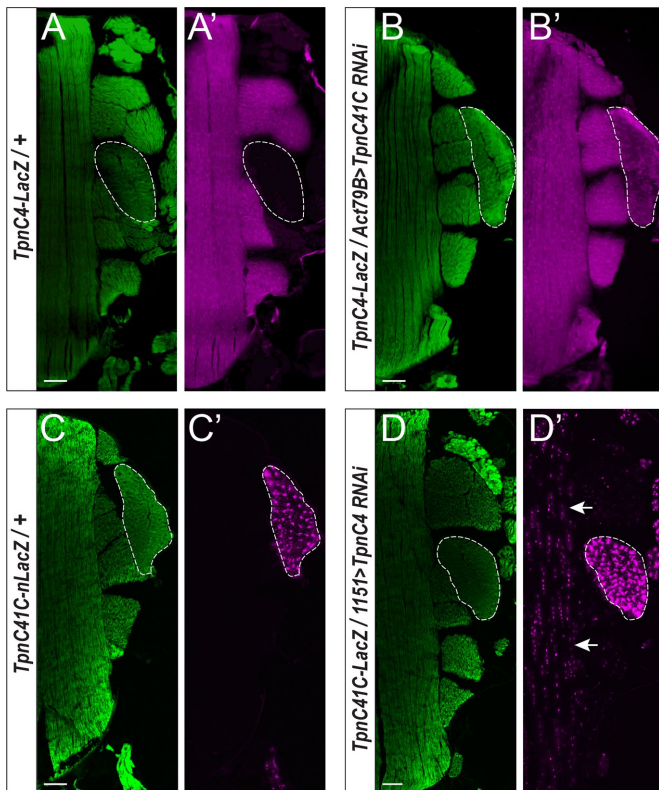
to predict functional differences between TpnC4 and TpnC41C isoforms and suggested that they would differ in their Ca<sup>2+</sup>-binding ability. Moreover, Eldred *et al.* (2014) detected significant differences in isometric tension and power production in the flight muscles expressing TpnC41C that would seem unlikely to result simply from a reduction in TpnC levels. Accordingly, we propose that whereas TpnC4 can substitute for loss of TpnC41C in the TDTs, the reciprocal is not true.

It is also interesting to note that knockdown and knockout *TpnC4* mutants jumped less well than wild type, even though TpnC4 was not detected in the TDTs. One explanation for this result is that there are low levels of TpnC4 in the jump muscles that are required for maximal muscle function, although these levels must be very low since we do not detect TpnC4 in the jump muscles of control animals on Western blots. Alternatively, the differences in jumping ability might simply reflect differences in genetic background because different control lines can have significantly different jumping abilities (see, e.g., Cripps *et al.*, 1994).

#### Requirement of TpnC for myofibril assembly

IFM myofibrillar defects were previously observed in animals that had undergone knockdown of *TpnC4* (Eldred *et al.*, 2014; Singh *et al.*, 2014). Since knockdown or knockout of *TpnC4* results in up-regulation of *TpnC41C* expression in the IFMs, the observed defects might arise through one of two mechanisms: either TpnC41C lacks some molecular function that is required for normal IFM myofibril structure, or TpnC41C has an antimorphic effect upon flight muscle structure. Moreover, it was not clear from the previous studies whether muscle structure was evaluated in animals that had already used the muscles (i.e., after emerging from the pupal case). The latter condition might be important because mechanical strain can provide an additional test on myofiber integrity (Firdaus *et al.*, 2015). Thus the contribution of TpnC to myofibril assembly, rather than myofibril maintenance, remained unclear.

Here we demonstrate that myofibrils from double-null pharate adults showed severe defects in muscle structure and function. Are these muscles completely devoid of TpnC protein, which, if true, would indicate a requirement for TpnC in myofibril assembly? This question is germane because expression of *TpnC4* and *TpnC41C* compensates for the absence of each other in the flight and jump muscles. Therefore it might be that in their absence, other TpnC proteins are produced in the double-null IFMs and TDTs, although no other TpnC species were observed in our Western blots. Qiu *et al.* (2003) demonstrate that the MAC352 antibody that we use



**FIGURE 6:** The TpnC isoform switch is regulated at the transcriptional level. (A–D, A'–D') Frozen sections of control and experimental flies were stained with phalloidin (green) and with antibody to  $\beta$ -Gal (magenta) and imaged using confocal microscopy. (A, A') Control line carrying a genomic insertion of *TpnC4-lacZ* shows reporter expression in the IFMs and not the TDT (TDT is outlined with a dashed line). (B, B') *TpnC41C*-knockdown flies carrying the *TpnC4-lacZ* reporter show *lacZ* expression expanding into the TDT. (C, C') Control line carrying genomic insertion of *TpnC41C-nLacZ* shows reporter expression in the TDT. (D, D') *TpnC4*-knockdown flies carrying the *TpnC41C-nlacZ* reporter show *lacZ* expression expanding into the IFM (arrows). Note that in C' and D', the  $\beta$ -Gal reporter carries a nuclear localization signal and accumulates in muscle nuclei, creating a punctate pattern. Scale bars, 50  $\mu$ m.

cross-reacts with TpnC4 and TpnC41C (as we also show here), as well as with a third TpnC, named TpnC47D. In addition, we observed that MAC352 stains preparations of *Drosophila* embryos (R.M.C., unpublished observation), in which the predominant isoform is TpnC73F (Herranz et al., 2004). Cross-reactivity with the remaining TpnC isoform, TpnC25D, has not been tested. Thus, if another TpnC gene were expressed in the double-null muscles, because our Western blots were negative for TpnC protein, the only possibility is that *TpnC25D* is expressed there.

Given that MAC352 also cross-reacts with a number of *Lethocerus* TpnC species (Qiu et al., 2003), the most reasonable conclusion is that MAC352 cross-reacts with all *Drosophila* TpnCs. Therefore the IFM and TDT of the double-null mutants that we describe here are devoid of TpnC, making this study one of the first to evaluate the requirement of TpnC to muscle assembly. Our study complements an earlier finding that point mutations in the *Caenorhabditis elegans* TpnC gene *pat-10* cause defects in muscle organization (Terami et al., 1999).

We also note with interest that whereas the TpnC double-knock-out animals are largely flightless (70–80% in the N category upon

flight testing; Figure 4D), the *TpnC4*-knockout animals are completely flightless (>95% in the N category; Figure 3C). This indicates that *TpnC41C*-expressing flight muscles are even less functional than double-null flight muscles, also supporting the idea that *TpnC41C* might be having an antimorphic effect in the flight muscles. Whether this antimorphic effect is on myofibril assembly or function (or both) remains to be determined.

### Mechanisms by which duplicate genes diverge in their expression

One common feature of subfunctionalization is the divergence in expression patterns for duplicate genes. As *TpnC41C* and *TpnC4* are the most recent TpnC gene duplicates in the *Drosophila* lineage (Herranz et al., 2005), it seems likely that a primordial gene, before duplication, was expressed in both IFM and TDT muscles or their equivalents. After duplication, each gene acquired different patterns of expression and different functions, such that they are now not functionally identical. Bioinformatic studies by Herranz et al. (2005) determined that the *Apis mellifera* genome contains only a single TpnC gene, which is classified as most similar to *Drosophila* *TpnC41C* and *TpnC4*. It would be interesting to determine whether this gene is expressed in both flight and jump muscles of the honeybee, and, if so, how the sequence of the encoded protein contributes to the unique properties of the different muscles.

Our data demonstrate that a likely mechanism for divergence in expression is through the repression of gene expression in the tissue in which the gene is not normally expressed. There are two lines of evidence for this. First, we show that each gene is capable of expanding its expression into the noncognate muscle in the absence of expression of the other gene. This result indicates that there are still transcriptional mechanisms that can promote broader expression of each gene; however, these mechanisms are suppressed under normal conditions.

Second, we recently characterized the enhancer that controls TDT-specific expression of *TpnC41C*. In that study, we identified a repressor element that, when mutated, resulted in an expansion of *TpnC41C-lacZ* expression from the TDT into the flight muscles (Chechenova et al., 2015). The identity of the factor that mediates the repression has not been identified. It is tempting to speculate that transcription factors that promote flight muscle fate, such as Spalt-major, Extradenticle, and Homothorax (Schönbauer et al., 2011; Bryantsev et al., 2012b), might do so at least in part by suppressing jump muscle-specific genes. However, consensus binding sites for those factors are not found in the short repressor element that we defined (Chechenova et al., 2015).

More broadly, a working model for how the divergence in expression of TpnC genes has been achieved, at least for *TpnC41C*, could be through the acquisition of this repressor element. Our model also predicts that another repressor element must be present in the *TpnC4* enhancer that we identify in this study, although, given that the 1.3-kb regulatory region for *TpnC4* that we identified has not been further delimited, we do not have insight into the factors that might regulate the expression of this gene.

How is loss of expression of one TpnC gene detected in the cell and transduced into an alteration in gene regulation? The mechanism of this phenomenon, termed paralogous compensation, is yet to be determined and is a relatively understudied process (reviewed in Diss et al., 2014). Defining how this process works at the mechanistic level for TpnC genes studied here might provide significant insight into gene regulatory mechanisms and the processes that account for divergence in expression of duplicated genes. Moreover,



investigating this process could generate broader insight into how muscle protein homeostasis is achieved.

## MATERIALS AND METHODS

### *Drosophila* methods and fly stocks

All fly stocks and crosses were cultured at 25°C, and crosses with Gal4 driver lines were carried out at 29°C. Targeted gene expression was accomplished using the UAS/Gal4 system (Brand and Perrimon, 1993). Tissue-specific Gal4 driver lines used were *Act79B-Gal4* for expression in jump muscles (Bryantsev et al., 2012a), *1151-Gal4* for adult myoblasts (Roy and Vijayraghavan, 1997), and *Act88F-Gal4* for flight muscle-specific expression (Bryantsev et al., 2012a). In experiments with down-regulation of single *TpnC41C* or *TpnC4* genes, UAS-RNAi lines 27053 (Bloomington *Drosophila* Stock Center [BDSC]) and 102031 (Vienna *Drosophila* RNAi Center [VDRC]) were used, respectively. Line 36303 (BDSC), which carries an attP landing site but no inserted UAS-RNAi element, was used in crosses with *Act79B-Gal4* or *1151-Gal4* driver lines to produce control offspring for UAS-*TpnC41C* RNAi in the jump and flight tests. Line 60100 (VDRC), which similarly carries only attP landing sites, was crossed with *Act79B-Gal4* or *1151-Gal4* driver lines, and the progeny was used as control for UAS-*TpnC41C* RNAi in the jump and flight tests. The laboratory strain *w<sup>1118</sup>* was used as control for all protein samples. In crossing schemes, balancer lines used were *w*; *CyO/In(2LR) Gla, Gla Bc* (line 5439, BDSC), *w*; *CyO/Bl*; *TM2/TM6* (3704, BDSC), and *Mef2<sup>44-5</sup>/CyO, Cy Kr>GFP* (Lovato et al., 2009).

The stable lines *Act79B>UAS-TpnC4 RNAi*, *1151>UAS-TpnC4 RNAi*, and *Act88F>UAS-TpnC41C RNAi* were created by crossing the Gal4 and UAS-RNAi lines listed here with balancer-containing lines listed earlier. In experiments with simultaneous down-regulation of *TpnC41C* and *TpnC4*, we analyzed female progeny from *Act88F>UAS-TpnC41C RNAi* crossed with *Act79B>UAS-TpnC4 RNAi*. As a control for these flies in flight testing, we used female progeny of the cross between *Act88F-Gal4* and *Act79B-Gal4*. The *TpnC4<sup>F2-3</sup>*-knockout line was described in Adame et al. (2016). The line CAS-0002, expressing the Cas9 nuclease used for genome editing (Kondo and Ueda, 2013), was obtained from the National Institute of Genetics, Japan.

*Drosophila* transgenic lines expressing cytoplasmic *lacZ* or nuclear *nlacZ* controlled by *TpnC41C* promoter regions were created earlier (Chechenova et al., 2015). Transgenic lines expressing cytoplasmic *lacZ* under control of *TpnC4* regulatory sequences were created by P-element-mediated transformation (Rubin and Spradling, 1982) of *w<sup>1118</sup>* with the *TpnC4(5+6)-CHAB* construct (described later). In experiments to assess *TpnC4-lacZ* expression in *TpnC41C* KDs, we first crossed female *Act79B-Gal4* with male *TpnC4-lacZ*. The resulting male offspring were crossed with UAS-*TpnC41C* RNAi adults to obtain *TpnC4-lacZ/Act79B>TpnC41C RNAi* females, which were used in the experiment. The male progeny of the latter cross were used as controls (*TpnC4-lacZ/+*). In experiments to assess *TpnC41C-lacZ* expression in *TpnC4* KDs, we crossed lines *TpnC41C-lacZ* and *1151>UAS-TpnC4 RNAi* adults, and female progeny were used in the experiment (*TpnC41C-lacZ/1151>TpnC4 RNAi*). Male offspring of this cross were used as controls (*TpnC41C-lacZ/+*).

In the flight and jump tests, 1- to 2-d-old males and females were used. For fly lines carrying two Gal4 drivers (*Act88F-Gal4* and *Act79B-Gal4*), only female progeny were tested. We analyzed 30–300 animals per experimental set in flight tests and 20–50 animals in jump tests. Flight testing was performed according to Drummond et al. (1991). The jump test was performed as described in Zumstein et al. (2004) with some modifications. Briefly, flies were anesthetized with CO<sub>2</sub>, and their wings were clipped off; flies were then returned

to vials and left at room temperature for at least 20 h to recover. Jump tests were performed on a piece of white paper; flies were stimulated to jump by touch with a paintbrush from the rear, and the points of takeoff and landing were marked with a pencil. The distances of three successful jumps were measured and recorded for each fly, and the average distances and SDs calculated from at least 20 male and female flies per line are plotted on the graphs. Statistical analyses were performed using two-tailed Student's *t* tests.

### CRISPR/Cas9 genome editing

To generate a line with a deletion of the *TpnC41C* gene, we created two plasmids expressing different single guide RNAs (sgRNAs) by cloning annealed oligonucleotides into the *BbsI* site of pBFv-U6.2 (Kondo and Ueda 2013). Plasmid pBFv-U6.2-TC41(-652) contained the annealed oligonucleotides 5'-CTTCGCTATTCAGCTAGT-GAGAGC-3' and 5'-AAACGCTCTCACTAGCTGAATAGC-3' to target a site in the 5' upstream region of *TpnC41C*. Plasmid pBFv-U6.2-TC41(+69) contained the annealed oligonucleotides 5'-CTTCG-TATTTTCGACTGCTTAGAT-3' and 5'-AAACATCTAAGCAGTCGAA-AATAC-3' to target a site in the 3'UTR of *TpnC41C*. These two plasmids were mixed in a 1:1 ratio and injected into embryos of CAS-0002 line following the protocols of Rubin and Spradling (1982). To purify incorporated mutations, we performed consecutive crosses of the progeny with balancer lines. Candidate lines containing homozygous *TpnC41C* deletions were analyzed by Western blotting and probed for TpnC protein. Among 14 lines tested, only the line *TpnC41C<sup>Δ#2</sup>* showed loss of the corresponding protein. Genomic DNA from this line was amplified using the primers 5'-GTGC-GTATCAGCCTATTG-3' and 5'-GTCTCCTCTAGCTCTTGG-3'. Sequencing of the obtained PCR product confirmed deletion of the corresponding genomic region.

To generate a line with double *TpnC4/TpnC41* deletion, we first created a *TpnC41C<sup>Δ#2</sup>* line that also carried a third chromosome expressing Cas9 under control of *vasa* regulatory sequences from BDSC line 51324. Embryos from this line were injected with two pBFv-U6.2-based plasmids expressing sgRNA for deletion of the *TpnC4* third exon (described in Adame et al., 2016). Injected progeny were analyzed by consecutive crossing with balancer lines and assessed for deletion of *TpnC4* using Western blotting. Of all lines tested, we selected two (*TpnC4<sup>Δ#6</sup>* and *TpnC4<sup>Δ#25</sup>*) that were defective in both flight and jumping ability. Sequencing of the affected genomic regions revealed that in both lines we obtained frameshift mutations resulting in premature termination of *TpnC4* protein synthesis (Figure 4).

### DNA methods

Standard protocols for PCR amplification and either conventional cloning or Gateway recombination were used for plasmid construction. *Drosophila* genomic DNA was isolated according to Huang et al. (2009) with minor modifications. The *TpnC4-lacZ* reporter construct was created as follows: a 1.3-kb genomic region containing ~200 base pairs of the most immediate upstream region of the *TpnC4* gene and the entire first intron up to the first coding exon was amplified using the primers C4(5 + 6)-F 5'GGGGACAAGTTTG-TACAAAAAAGCAGGCTACAAACAATGAGATCACACA and C4(5 + 6)-R 5'-GGGGACCACTTTGTACAAGAAAGCTGGGTTAAGGTAATA TCTTCTTCACTG. The underlined sequences within each primer indicate attB sequences compatible with Gateway integration technology (Hartley et al., 2000). The amplified fragment was recombined into pDONR-CHAB, which is a modified pCHAB vector (Thummel and Pirrotta, 1992) that had its multiple cloning site replaced with the Gateway recombination cassette, flanked with attP1 and attP2 sequences, which were subcloned from pDONR221

(Invitrogen). For recombination, we used BP Cloning Mix II (Invitrogen) and followed the manufacturer's protocol. The resulting construct, pC4(5+6)-CHAB, was used for producing transgenic flies. Plasmid pBFv-U6.2, used for genome editing, was obtained from the National Institute of Genetics, Japan.

### Protein methods and Western blotting

Dissected IFM and TDT muscles were homogenized in Laemmli SDS sample buffer and heated at 100°C for 5 min, and lysates were centrifuged at 14,000 × g for 2 min. Protein concentration in clarified extracts was measured using the RC DC Protein Assay Kit (Bio-Rad). Protein extracts were loaded on 4–20% gradient TGX gels (Bio-Rad) in a volume corresponding to 9–10 µg of total protein per well. Separated proteins were transferred to nitrocellulose membrane and probed with MAC352 antibody to TpnC (concentration 1:1,000) or MAC276 antibody (concentration 1:100,000) to α-actinin (Abcam). An anti-rat horseradish peroxidase-immunoglobulin G conjugate (Invitrogen) was used as the secondary antibody (concentration 1:1,000), and chemiluminescence signal was detected using the Amersham Biosciences ECL Western blot analysis system (GE Healthcare).

### Cryosectioning, immunofluorescence, and histochemistry

Cryosectioning, histochemistry, and immunostaining of frozen sections were performed as described in Morriss *et al.* (2012). Rabbit anti-Obscurin antibody (used at a concentration of 1:200) was generously provided by Belinda Bullard (University of York, York, United Kingdom). Mouse anti-β-Gal antibody, used at a concentration of 1:1,000, was obtained from Promega. MAC155 antibody to Sallimus (kettin), used at a concentration of 1:400, was obtained from Abcam. Alexa Fluor-conjugated phalloidin and secondary antibodies were obtained from Molecular Probes and used at dilution 1:500 and 1:2000, respectively. Confocal images were produced using a Zeiss LSM780 confocal microscope.

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