Regulation of the Evolutionarily Conserved Muscle Myofibrillar Matrix by Cell Type Dependent and Independent Mechanisms

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Abstract

Skeletal muscles play a central role in human movement through forces transmitted by contraction of the sarcomere. We recently showed that mammalian sarcomeres are connected through frequent branches forming a singular, mesh-like myofibrillar matrix. However, the extent to which myofibrillar connectivity is evolutionarily conserved as well as mechanisms which regulate the specific architecture of sarcomere branching remain unclear. Here, we demonstrate the presence of a myofibrillar matrix in the tubular, but not indirect flight (IF) muscles within *Drosophila melanogaster*. Moreover, we find that loss of transcription factor *H15* increases sarcomere branching frequency in the tubular jump muscles, and we show that sarcomere branching can be turned on in IF muscles by *salm*-mediated conversion to tubular muscles. Finally, we demonstrate that *neurochondrin* knockdown results in myofibrillar connectivity in IF muscles without conversion to tubular muscles. These data indicate an evolutionarily conserved myofibrillar matrix regulated by both cell-type dependent and independent mechanisms.

Keywords

Sarcomere branching, myofibril splitting, *Drosophila*, fiber type specification, neurochondrin

Introduction

Sarcomeres are the basic force production unit of the striated muscle cell and make up the bulk of the muscle volume^{1, 2}. Not surprisingly, improper sarcomere assembly, maintenance, and contractile function have been linked to a wide variety of pathologies including dystrophy, aging, and heart failure^{3, 4, 5, 6, 7}. We recently demonstrated that mammalian sarcomeres are not arranged end-to-end into many individual, parallel myofibrils as had long been thought^{8, 9, 10}, but instead, sarcomeres frequently branch to create a mesh-like myofibrillar matrix thereby providing a direct pathway for active force transmission across both the length and width of the muscle cell¹¹. Sarcomere branching frequency has been shown to vary according to cell type^{11, 12}, though the mechanisms regulating the degree of connectivity have yet to be determined. Additionally, evidence of sarcomere branching can be seen in muscle images from fish¹³, chickens^{14, 15}, and frogs¹⁶ in addition to mice^{12, 17} and humans^{11, 18, 19} suggesting that myofibrillar networks may be conserved across vertebrate species. Conversely, insect fibrillar muscles, such as the commonly studied *Drosophila* indirect flight (IF) muscle, are known to be made up of the textbook individual myofibrils running the entire length of the muscle cell^{20, 21, 22}, implying that a myofibrillar matrix may not occur in invertebrate muscles. However, the majority of *Drosophila* muscle cells are tubular, rather than fibrillar in nature²³, and the connectivity of sarcomeres within tubular muscles remains unclear.

Here, we use high-resolution, 3D electron microscopy to show that the myofibrillar matrix is conserved in the invertebrate tubular muscles of *Drosophila melanogaster*. The frequency of sarcomere branching in tubular muscles is cell-type dependent with greater myofibrillar connectivity in the relatively more oxidative leg and direct flight (DF) muscles than in the glycolytic jump (tergal depressor of the trochanter, TDT) muscles. Further, we demonstrate that the muscle-specific loss of cell type specification factor *H15* leads to conversion of the jump muscles to a leg muscle-like phenotype with increased sarcomere branching and smaller myofibrils while maintaining the tubular nature of the contractile apparatus. The complete lack of sarcomere branching in the fibrillar IF muscles allowed for the demonstration of two types of regulatory on/off switches for myofibrillar connectivity. First, loss of IF muscle specification factor *salm*²⁴ leads to conversion of the IF muscles to a tubular

phenotype, resulting in sarcomere branching linking the contractile apparatus across the width of the cell. Second, we identify *neurochondrin* (*NCDN*) as a cell-type independent regulator of sarcomere branching in the IF muscles. Loss of *NCDN* leads to the formation of thin myofibrillar segments that link the larger fibrillar segments of the IF contractile apparatus together, resulting in the formation of a mesh-like myofibrillar matrix without conversion to a tubular phenotype. Thus, we find that connectivity of the evolutionarily conserved myofibrillar matrix can be regulated by both cell-type dependent (*salm*, *H15*) and cell-type independent (*NCDN*) mechanisms.

Results

Myofibrillar matrix is conserved in Drosophila tubular muscles

To investigate myofibrillar connectivity in the invertebrate striated muscles of *Drosophila melanogaster*, we used focused ion beam scanning electron microscopy (FIB-SEM) to visualize muscle cell ultrastructure with 10 nm pixels along all three dimensions (3D)^{11, 25}. The 26 IF muscles (12 dorsal longitudinal + 14 dorso-ventral muscles) are the largest muscle group in *Drosophila* and are known as fibrillar muscles due to the individual fiber-like nature of their myofibrils which can be observed under a light microscope^{20,21, 23}. Not surprisingly, our FIB-SEM analyses of the fibrillar dorsal longitudinal muscles (n = 3 cells, 114 myofibrils, and 982 sarcomeres) corroborates the individual nature of the circular myofibrils running the length of the IF muscle cell without any connecting sarcomere branches (**Figure 1a,e,f**). On the other hand, *Drosophila* tubular muscles have centralized nuclei surrounded by cross-striated myofibrils in tube-like arrays that make up the majority of the adult muscle cells²³. To evaluate tubular muscles with varying developmental backgrounds and functional demands^{26, 27, 28, 29}, we chose to assess three different tubular muscle types: DF, TDT, and leg muscles. While the tightly packed nature of the tubular muscle contractile apparatus has prohibited analyses of myofibrillar connectivity using standard light microscopy approaches (>200 nm resolution), we are able to clearly delineate the sarcomere boundaries in tubular muscles due to the high resolution of FIB-SEM as well as the relatively higher content of the sarcotubular system (sarcoplasmic reticulum + t-tubules, SRT) which runs in between each sarcomere

(**Supplementary Figure 1**). Tracking the myofibrillar structures along the length of the tubular muscles revealed that sarcomere branching does indeed occur in invertebrate muscles, as myofibrillar networks are found in the *Drosophila* DF, TDT, and leg muscles (**Figure 1b-d**). Moreover, the amount of sarcomere branching varied among the three tubular muscle types. The percentage of myofibrils within the field of view of our datasets (up to 50x30x27 μm) with at least one branching sarcomere (**Figure 1e**), as well as the percentage of branching sarcomeres (**Figure 1f**) was highest in the leg muscles followed by the DF and TDT muscles (94 $\pm$ 3.5% (mean $\pm$ SE) of myofibrils, 65.9 $\pm$ 3.4% of sarcomeres, n = 3 cells, 150 myofibrils, 690 sarcomeres in leg muscles (**Supplementary Video 1**); 77 $\pm$ 4.4% of myofibrils, 32.4 $\pm$ 5.0% of sarcomeres, n = 4 cells, 200 myofibrils, 1461 sarcomeres in DF muscles; and 50 $\pm$ 3.1% of myofibrils, 10.8 $\pm$ 3.1% of sarcomeres, n = 3 cells, 150 myofibrils, 1713 sarcomeres in jump muscles (**Supplementary Video 2**)). Thus, the myofibrillar matrix is evolutionarily conserved in invertebrate muscles, and like mammalian muscle¹¹, the connectivity of the myofibrillar matrix is cell-type dependent.

Fig. 1 Myofibrillar networks in *Drosophila* skeletal muscles. **a** 3D rendering of isolated myofibrils in fibrillar indirect flight (IF) muscles. **b-d** 3D rendering of unified myofibrillar matrix structures in tubular muscles. **b**: jump (tergal depressor of the trochanter, TDT) muscles; **c** – direct flight (DF) muscles; **d** – leg muscles. Individual colors represent different myofibrillar segments separated by sarcomere branches. **e** Percentage of myofibrils with at least one branching sarcomere in wild type IF, TDT, DF, and leg muscles. **f** Percentage of sarcomeres with at least one branch in wild type IF, TDT, DF, and leg muscles. **g** Total percentage of cellular volume occupied by contractile, mitochondrial, and sarcotubular structures in wild type IF, TDT, and leg muscles. *N* values for **e**, **f**, **g**: IF—3 muscle

cells, 3 datasets, 114 myofibrils, 982 sarcomeres; TDT—3 muscle cells, 2 datasets, 160 myofibrils, 1863 sarcomeres; DF—4 muscle cells, 1 dataset, 200 myofibrils, 1461 sarcomeres; and leg—3 muscle cells, 2 datasets, 150 myofibrils, 690 sarcomeres. Larger shape symbols represent data from a single cell and smaller shape symbols represent data from a single myofibril. Bars represent muscle cell overall mean $\pm$ SE. Asterisk (*): Significantly different from fibrillar indirect flight (one-way ANOVA, $P < 0.05$). Pound sign (#): Significantly different from tubular TDT. Plus sign (+): Significantly different from tubular DF. Scale bars – 2 μ m.

Also similar to mammalian muscle¹¹, sarcomere branching in *Drosophila* muscle can occur through different physical mechanisms. The simplest type of sarcomere branch is termed a single branching event where one sarcomere segment transitions into two segments (**Supplementary Figure 2b,d, Supplementary Video 3**). More complex branching types can be grouped as multi-branching events and occur when a single sarcomere segment transitions to three or more resultant segments (**Supplementary Figure 2c,e, Supplementary Videos 4**). Thus, the total number of sarcomere branches can be greater than the number of sarcomeres, such as in the leg muscle where $38.7 \pm 2.9\%$ of sarcomeres undergo a multi-branching event (**Supplementary Figure 2e**) and $27.2 \pm 1.2\%$ of sarcomeres undergo a single branching event (**Supplementary Figure 2d**) resulting in 13.0 ± 1.2 sarcomere branches per 10 sarcomere lengths (**Supplementary Figure 2f**). Further, to determine whether sarcomere branching was related to subcellular location, we assessed the positions of all sarcomere branches in the highly branching leg muscle and the low branching TDT muscle relative to the cell boundary (**Supplementary Figure 3**). In both the leg and TDT muscles, sarcomere branches were more likely to occur near the cell periphery than in the interior (**Supplementary Figure 3c,d**) similar to previous findings in fish muscle¹³.

To evaluate how myofibrillar connectivity may be related to muscle function in *Drosophila*, we assessed the overall cellular content of the contractile apparatus, mitochondria, and the SRT system as estimates of the capacity for force production²⁵, oxidative phosphorylation³⁰, and calcium cycling³⁰, respectively. TDT muscles have the highest contractile content ($85.0 \pm 0.5\%$ of cell volume, $n=3$ cells) and the lowest mitochondrial content ($2.6 \pm 0.1\%$, $n=3$), whereas the IF muscles have the lowest contractile content ($45.1 \pm 3.6\%$, $n=3$) and highest mitochondrial content ($37.6 \pm 3.9\%$, $n=3$) (**Figure 1g**). However, the less frequent sarcomere branching in the IF and TDT muscles compared to the DF and leg muscles (**Figure 1f**) suggests that the degree of sarcomere branching is not directly related to myofibrillar or mitochondrial

content, and thus the capacities for force production or oxidative energy conversion, respectively. Conversely, the non-branching IF muscles have much lower SRT content ($1.0 \pm 0.2\%$, $n=3$) than each of the tubular muscles (TDT: $12.4 \pm 0.4\%$, $n=3$; DF: $10.0 \pm 0.9\%$, $n=4$; leg: $11.9 \pm 0.5\%$, $n=3$) (**Figure 1g**) implying that myofibrillar connectivity may be related to SRT content and perhaps calcium cycling. Indeed, IF muscles are asynchronous in nature³¹ meaning that a single cycle of calcium can result in tens of muscle contractions, whereas the tubular muscles, like their mammalian counterparts, are synchronous and require calcium to cycle for each contraction. However, no differences in SRT content are observed across the three tubular muscles here despite the more than six-fold variation in sarcomere branching percentage (**Figure 1f**). Thus, these data suggest that while SRT content and perhaps calcium cycling may be related to the need for initiation of myofibrillar networks, there is no apparent relationship between SRT content and the degree of connectivity within myofibrillar networks.

H15 regulates sarcomere branching frequency in TDT muscles through cell type conversion

We recently identified T-box transcription factor *H15* as capable of specifying between the TDT and leg muscle phenotypes based on conversion of the mitochondrial network configuration in muscle-specific *H15* knockdown (KD) tubular muscles (*Mef2-Gal4; UAS-H15 RNAi*) as observed with confocal microscopy³². Based on the large difference in the magnitude of sarcomere branching between the TDT and leg muscles shown here (**Figure 1**), we hypothesized that *H15* KD would also regulate myofibrillar network connectivity through conversion of the TDT muscles to a leg muscle phenotype. *H15* KD in the TDT muscles resulted in a reduction in cross-sectional area (CSA) and circularity per myofibril ($0.73 \pm 0.15 \mu\text{m}^2$, 0.48 ± 0.05) compared to the wild type TDT muscles ($0.97 \pm 0.02 \mu\text{m}^2$, 0.53 ± 0.01) and closer to the level of the leg muscles ($0.56 \pm 0.15 \mu\text{m}^2$, 0.46 ± 0.02) (**Figure 2b**). Moreover, loss of *H15* led to the formation of holes in the TDT muscle z-disks (**Supplementary Videos 5-6**). Importantly, *H15* KD in the TDT raised the percentage of myofibrils with at least one sarcomere branch ($95.0 \pm 5.0\%$, $n=2$ cells) to levels similar to those observed in the wild type leg ($94.0 \pm 3.5\%$, $n=3$) (**Figure 2a,c, Supplementary Video 7**). The percentage of sarcomeres with a branch in the *H15* KD TDT muscles ($78.2 \pm 2.3\%$) increased 7.2-fold over the wild type TDT muscles ($10.8 \pm 3.1\%$) and

were also no different from the wild type leg ($65.9 \pm 3.4\%$) (**Figure 2c**). Additionally, the frequency of sarcomere branches was also similar between the *H15* KD TDT (14.0 ± 0.4 branches per 10 sarcomere lengths) and leg muscles (13.0 ± 1.2 branches per 10 sarcomere lengths) and higher than in the wild type TDT muscles (1.8 ± 0.5 branches per 10 sarcomere lengths) (**Figure 2d**). Overall, these data suggest that *H15* regulates the connectivity of myofibrillar networks in the tubular TDT muscle by converting these muscles towards a leg muscle phenotype.

Fig. 2 Loss of *H15* increases TDT muscle sarcomere branching to leg muscle levels. **a** 3D rendering of myofibrillar matrix in TDT muscles with RNAi knockdown of *H15*. Bottom two images show clippings through the long axis of the muscle directly above. **b** Assessment of myofibril size and shape for TDT, *H15* KD TDT, and leg muscles. **c** Percentage of myofibrils with at least one branching sarcomere (left) and percentage of sarcomeres with a branch (right). **d** Frequency of sarcomere branching in TDT, *H15* KD TDT, and leg muscles. *N* values: TDT—3 muscle cells, 2 datasets, 160 myofibrils, 1863 sarcomeres; *H15* KD TDT—2 muscle cells, 1 dataset, 70 myofibrils, 403 sarcomeres; and leg—3 muscle cells, 2 datasets, 150 myofibrils, 690 sarcomeres. Larger shape symbols represent data from a

single cell and smaller shape symbols represent data from a single myofibril. Bars represent muscle cell overall mean $\pm$ SE. Pound sign (#): Significantly different from tubular TDT. Plus sign (+): Significantly different from H15 KD TDT (one-way ANOVA, $P < 0.05$). Scale bars $-2 \mu\text{m}$.

Loss of salm initiates IF muscle myofibrillar networks by a fibrillar to tubular conversion

Due to the absence of sarcomere branching in the IF muscles and the presence of myofibrillar networks in each of the three tubular muscles assessed above (**Figure 1**), we hypothesized that factors which control the fibrillar fate of IF muscles such as *extradenticle* (*exd*)³³, *homothorax* (*hth*)³³, *vestigial* (*vg*)²⁴, *salm*²⁴, and *H15*³² would also serve effectively as a switch for myofibrillar connectivity in IF muscles. To test this hypothesis, we evaluated sarcomere branching in the IF muscles of muscle-specific *salm* KD (*Mef2-Gal4; UAS-salm RNAi*) flies which are known to convert to a tubular muscle phenotype^{24, 34}. 3D rendering of the *salm* KD contractile apparatus confirmed that knocking down *salm* in IF muscles changes its organization to resemble the cross-striated nature of tubular muscles and initiated myofibrillar connectivity (**Figure 3a, Supplementary Video 8**). Consistently, loss of *salm* leads to a reduction in CSA ($0.86 \pm 0.11 \mu\text{m}^2$) and circularity (0.66 ± 0.06) per myofibril compared to the wild type IF muscles ($2.15 \pm 0.03 \mu\text{m}^2$, 0.88 ± 0.00) (**Figure 3b**). Moreover, similar to the loss of *H15* in the TDT muscle, *salm* KD results in the formation of holes in the z-disks of the IF muscle (**Supplementary Video 9**). The high degree of myofibrillar connectivity across the length and width of the *salm* KD muscle is apparent in the 3D rendering by the frequent changes in color representing the location of sarcomere branches (**Figure 3a**). Quantitative assessment of myofibrils within the field of view of our datasets revealed the extent sarcomeres connect with one another, and its frequency was comparable to that of tubular muscles we evaluated. Assessing 3 cells, 180 myofibrils, and 932 sarcomeres, $85.3 \pm 10.1\%$ of myofibrils had at least one sarcomere that branched to connect with adjacent sarcomeres and $45.2 \pm 17.5\%$ of sarcomeres were branched (**Figure 3c**), yielding a total of 6.9 ± 3.8 sarcomere branches per 10 sarcomere lengths when accounting for both single and multi-branching events (**Figure 3d**). Thus, the role of *salm* as a master regulator of IF muscle cell type^{24, 34} also involves the inhibition of myofibrillar network formation.

Fig. 3 Loss of *salm* initiates sarcomere branching through tubular conversion of indirect flight muscles. **a** 3D rendering of myofibrillar matrix in IF muscles with RNAi knockdown of *salm*. Bottom two images show clippings through the long axis of the muscle directly above. **b** Assessment of myofibril size and shape for IF and *Salm* KD IF muscles. **c** Percentage of myofibrils with at least one branching sarcomere (left) and percentage of sarcomeres with a branch (right). **d** Frequency of sarcomere branching in IF and *Salm* KD IF muscles. *N* values: IF—3 muscle cells, 3 datasets, 114 myofibrils, 982 sarcomeres; *Salm* KD IF—3 muscle cells, 3 datasets, 180 myofibrils, 932 sarcomeres. Larger shape symbols represent data from a single cell and smaller shape symbols represent data

from a single myofibril. Bars represent muscle cell overall mean $\pm$ SE. Pound sign (#): Significantly different from fibrillar IF (independent t-test, $P < 0.05$). Scale bars $-1 \mu\text{m}$.

Loss of NCDN induces sarcomere branching while maintaining fibrillar IF muscles

We identified *neurochondrin* (*NCDN*) as a gene potentially involved in sarcomere branching by filtering our recent proteomic database of *Drosophila* IF, TDT, leg, and *salm* KD IF muscles³² for proteins which are upregulated in muscles proportional to the sarcomere branching frequencies described above (leg>TDT>IF and *salm* KD>IF). *NCDN* is a cytoplasmic, leucine-rich protein conserved in humans and primarily expressed in the brain with a role in neurite outgrowth through modulation of metabotropic glutamate receptor 5 signaling, Ca^{2+} /calmodulin-dependent protein kinase II phosphorylation, and actin dynamics^{35, 36, 37, 38}. Little is known about the role of *NCDN* in striated muscle though its expression is upregulated in a *Drosophila* model of hypercontraction-induced myopathy³⁹. To investigate the role of *NCDN* in myofibrillar network connectivity, we generated muscle-specific *NCDN* KD flies (*Mef2-Gal4; UAS-NCDN RNAi*) which are viable but unable to fly, indicating a critical role for *NCDN* in muscle function. *NCDN* KD was confirmed by the more than ten-fold decrease in *NCDN* mRNA expression in the KD muscles (**Figure 4j**), and *NCDN* KD does not alter the expression of fibrillar muscle specification factors *Salm* and *H15* compared to wild type IF muscles nor alter the configuration of mitochondrial networks running in parallel to the myofibrils (**Supplemental Figure 4**). We initially screened the *NCDN* KD IF muscle contractile apparatus under the light microscope by visualizing sarcomeric actin stained with phalloidin (**Figure 4a-d**). While the *NCDN* KD muscles appear to largely maintain their individual fibrillar nature (**Figure 4b,d**), sarcomere branching can be seen in the longitudinal images, and many thin actin projections are observed in the cross-sectional images (white arrows, **Figure 4b,d**).

Fig. 4 Loss of *NCDN* turns on myofibrillar connectivity in fibrillar indirect flight muscles. a-d Light microscopy images of longitudinal sections (a,b) and transverse sections (c,d) of wild type and *NCDN* KD IF muscles. White arrows highlight branching structures. e-i 3D renderings of *NCDN* knockdown IF muscles showing myofibrillar connectivity at different angles. j *NCDN* mRNA expression in wild type IF and *NCDN* KD IF muscles. Each point represents an independent biological replicate. k Assessment of myofibril size for IF and *NCDN* KD IF muscles (left) and myofibril shape for tubular muscles, IF, and *NCDN* KD IF muscles (right). Pound sign (#): Significantly different from fibrillar IF. Plus sign (+): Significantly different from *NCDN* KD IF (one-way ANOVA, $P < 0.05$). l Percentage of

myofibrils with at least one branching sarcomere (left) and percentage of sarcomeres that branch (right) in wild type IF and *NCDN* KD IF muscles. *N* values: IF—3 muscle cells, 3 datasets, 114 myofibrils, 982 sarcomeres and *NCDN* KD IF —3 muscle cells, 2 datasets, 100 myofibrils, 878 sarcomeres. Larger shape symbols represent data from a single cell and smaller shape symbols represent data from a single myofibril. Bars represent muscle cell overall mean $\pm$ SE. Pound sign (#): Significantly different from fibrillar IF (independent t-test, $P < 0.05$). Scale bars – 2 μm .

As a result, we proceeded to the higher resolution FIB-SEM analyses to better visualize and quantify myofibrillar connectivity in the *NCDN* KD IF muscles. The majority of the contractile volume in *NCDN* KD IF muscle is comprised of large, roughly circular sarcomeres in series similar to the wild type IF muscle. However, thin fiber-like myofibrillar segments containing as few as four myosin filaments project out from the larger segments and unite them together (**Figure 4e-i, Supplementary Videos 10-11**). As a result, the average CSA per myofibril is smaller in the *NCDN* KD IF muscles ($1.14 \pm 0.32 \mu\text{m}^2$) compared to wild type IF muscles ($2.15 \pm 0.03 \mu\text{m}^2$) though some myofibrils are much larger ($6+ \mu\text{m}^2$) in the *NCDN* KD muscle (**Figure 4k**) and the volume-weighted average myofibril CSA is also larger for *NCDN* KD ($2.86 \mu\text{m}^2$) than in the wild type ($2.16 \mu\text{m}^2$). The circularity of the *NCDN* KD IF muscle myofibrils (0.74 ± 0.02) is higher than in any of the tubular muscles assessed here but not as high as the nearly perfect circular profiles of the wild type IF muscles (0.88 ± 0.00) (**Figure 4k**). Additionally, unlike the *H15* KD TDT and *salm* KD IF muscles, the formation of holes in the z-disks in the *NCDN* KD IF muscles is rare (**Supplementary Video 12**). Instead, many z-disks retain a circular profile similar to the wild type IF muscle, while others appear stretched or pulled along the longitudinal axis of the muscle often forming thin fiber-like strands several microns in length and connecting to adjacent z-disks (**Supplementary Video 13**). To quantify the connectivity of myofibrillar networks in the *NCDN* KD IF muscles, we assessed the structures of 100 myofibrils and 878 sarcomeres across three different cells. Every single myofibril had at least one sarcomere branch and $53.8 \pm 7.9\%$ of sarcomeres branched in the *NCDN* KD IF muscles (**Figure 4l**). Overall, these data demonstrate that *NCDN* acts as a regulator of myofibrillar network connectivity without altering the fibrillar fate of IF muscles.

Discussion

Here, we show that the highly connected myofibrillar networks linked together across the width of the muscle cell by branching sarcomeres recently described in mammalian muscles¹¹ are conserved in the tubular muscles of the invertebrate *Drosophila melanogaster*. In both mammalian skeletal muscles¹¹ and *Drosophila* tubular muscles, a large variability in the degree of connectivity exists across muscle types (**Figure 1**), where the relatively more oxidative and lower force producing muscles (i.e. mammalian slow-twitch and fly DF and leg muscles) have sarcomeres which branch 3-6 times more frequently than in the high force producing, glycolytic muscles (i.e. mammalian fast-twitch and fly TDT muscles). These data combined with the similarities in cellular volume occupied by the contractile apparatus, mitochondria, and SRT between the *Drosophila* leg (**Figure 1g**) and mammalian slow-twitch oxidative muscles^{1, 25} and between the TDT (**Figure 1g**) and mammalian fast-twitch glycolytic muscles^{6, 16} suggest that *Drosophila* tubular muscles provide an ideal model system to investigate the mechanisms of myofibrillar connectivity. Conversely, the near perfect circular cylindrical shape and complete lack of sarcomere branching in the fibrillar IF muscle myofibrils (**Figure 1a,e**), in addition to the very low SRT content (**Figure 1g**) are unlike any known mammalian muscle, including the often compared cardiac muscle cell^{22, 24, 34, 40, 41} in which myofibrillar networks are indeed formed¹¹. However, we demonstrate that myofibrillar connectivity can be initiated in the IF muscles through either cell-type dependent (e.g. *salm* KD) or cell-type independent mechanisms (e.g. *NCDN* KD), indicating that the *Drosophila* IF muscles offer a unique model for dissecting mechanisms which inhibit sarcomere branching.

The conserved nature of myofibrillar network structures across invertebrate and mammalian muscles may also be extended to the cell-type dependent regulators of network connectivity. Muscle-specific loss of transcription factors *exd*, *hth*, *vg*, *salm*, or *H15* each leads to conversion of the IF muscle contractile apparatus from fibrillar to tubular with *exd*, *hth*, and *vg* operating upstream of *salm*^{24, 33, 34} and *H15* downstream of *salm*³². Based on the lack of myofibrillar networks in the fibrillar IF muscles^{20,21, 23} (**Figure 1**), the presence of myofibrillar networks in each of the three tubular muscles assessed here (**Figure 1**), and the initiation of myofibrillar connectivity in the *salm* KD IF muscles (**Figure 3**), we propose that each of these

regulators of muscle cell type also acts as an inhibitor of myofibrillar network connectivity in IF muscles. Additionally, while the muscle-specific role of *salm* orthologs (*Sall*1-4) in vertebrates has yet to be reported, orthologs for *exd* (*Pbx*1-4), *hth* (*Meis*1-4), *vg* (*Vgll*1-4), and *H15* (*Tbx*15) are each known to be involved in the specification of muscle fiber type^{42, 43, 44, 45}. Thus, both the cell type dependence of myofibrillar connectivity (**Figure 1**) and the cell type dependent mechanisms regulating myofibrillar connectivity (**Figures 2,3**) appear to be widely conserved across species.

Transcription factors that regulate muscle cell type often act on the overall design of the cell and influence both the contractile and metabolic machineries^{24, 33, 34, 46, 47}. Conversely, cell-type independent regulators of the myofibrillar matrix are likely to operate more directly on the contractile apparatus. Indeed, muscle-specific loss of *NCDN* had no effect of the IF muscle expression of cell type regulators *Salm* and *H15* nor on the configuration of mitochondrial networks running parallel in between the myofibrils (**Supplementary Figure 4**). Additionally, tracheoles were still present within the *NCDN* KD muscle interior (**Supplemental Video 10**) as only occurs in fibrillar muscles in a *salm* dependent manner^{48, 49}. However, while the majority of the myofibrillar volume in *NCDN* KD IF muscles retained the large, roughly circular profile characteristic of fibrillar muscles (**Figure 4**), many thin myofibrillar branches were formed which provided connections between the larger myofibrils. Actin (**Figure 4b,d**), myosin filaments (**Supplementary Video 10**), and z-disk structures (**Supplementary Video 12**) can each be observed in the thin myofibrillar segments suggesting that the major sarcomeric components are retained by these branches. While there have been few volume electron microscopy studies of *Drosophila* muscle³⁴ with which to compare our data, the light microscopic images of *NCDN* KD muscles (**Figure 4b,d**) are reminiscent of previous reports describing IF muscle sarcomere fraying^{22, 50}. In fact, loss of *diaphanous* (*Dia*), an actin polymerization factor from the formin family, has been shown to induce fraying of *Drosophila* IF muscle myofibrils²². Interestingly, mammalian orthologs of *Dia* (*Dia*1) and *NCDN* (*NCDN*) are known to be interacting partners where *NCDN* can inhibit *Dia*1-induced neurite outgrowth, albeit not through regulation of actin polymerization activity³⁸. Determining whether *NCDN* interacts with *Dia* or other proteins involved in sarcomere fraying⁵⁰ to mediate myofibrillar connectivity in *Drosophila* muscle will

be critical to the identification of mechanisms acting directly on the sarcomere to induce branching in a cell type independent manner.

The forces generated by contraction of the muscle sarcomere are critical for the coordinated movement of animals from insects to humans. Force transmission down the longitudinal axis of the muscle fiber through sarcomeres arranged in series has long been considered the primary means by which sarcomeric forces arrive at the muscle tendon and ultimately act upon the skeleton to create movement^{8, 9, 10, 16, 51, 52}. Transmission of force laterally from the sarcomere to the sarcolemma and extracellular matrix which is then propagated to the tendon is now also appreciated, at least in vertebrate muscles, although the specific structures which permit lateral force transmission have remained elusive^{53, 54, 55, 56}. The presence of a singular, mesh-like myofibrillar matrix linked together through branching sarcomeres shown previously in mammals¹¹ and amphibians¹⁶ and now here in invertebrates provides a direct structural pathway for lateral force transmission across the width of the muscle cell. However, additional structural components such as the cytoskeleton^{53, 54, 55, 56} and the costamere^{57, 58, 59} are still needed for transmission of forces from sarcomeres located at the periphery of the cell to the sarcolemma and extracellular matrix. Thus, elucidating the structural and functional interactions among the active (myofibrillar matrix) and passive (cytoskeleton/costamere) force transmitting elements of the muscle cell will be of particular importance, especially under pathological conditions such as in muscles lacking the costameric protein dystrophin^{60, 61}. The large dynamic range of myofibrillar network connectivity shown across muscle types here combined with the widely available genetic tools suggests that *Drosophila* offers a promising model system for investigating the integrated nature of muscular force transmission^{62, 63}.

Methods

Drosophila strains and genetics. Genetic crosses were performed on yeast corn medium at 22°C. *W¹¹¹⁸* were used as controls for the respective genetic backgrounds. *Mef2-Gal4* (III) (BS# 27390) was used to drive muscle specific gene knockdown of respective genes. *UAS-mito-GFP* (II chromosome BS# 8442) was used as a UAS control. RNAi lines for knockdown of *salm* (*UAS-salm RNAi*, V101052), *H15* (*UAS-H15 RNAi*, V28415, V106875), and *Neurochondrin* (*NCDN*) (*UAS-NCDN RNAi*, V109002) were purchased from the Vienna Drosophila Resource Center. All other stocks were obtained from the Bloomington (BS#) Drosophila Stock Center. All chromosomes and gene symbols are as mentioned in Flybase (<http://flybase.org>).

Muscle preparation. Muscles from 2-3 days old flies were dissected on standard fixative solution (2.5% glutaraldehyde, 1% formaldehyde, and 0.1 M sodium cacodylate buffer, pH 7.2) and processed for FIB-SEM imaging as described previously²⁵. Muscles were then transferred to the fresh fixative Eppendorf tube and fixed overnight at 4 °C. Samples were washed in 0.1 M cacodylate buffer for 10 min, three times. Later, fixed tissues were immersed in a solution of 1.5% K₃[Fe(CN)₆] in 0.1 M cacodylate buffer with 2% OsO₄ on ice for 1 hr. Samples were washed three times with distilled water for 10 min each and were then incubated in filtered thiocarbonylhydrazide (TCH) solution for 20 min at room temperature. This was followed by three washes with water for 10 min each, then incubation for 30 min in 2% OsO₄ on ice. Incubated samples were washed with distilled water for 10 min, three times each. Tissues were transferred to 1% uranyl acetate at 4 °C overnight. Furthermore, samples were washed with distilled water three times for 10 min, then incubated with Walton's lead aspartate solution at 60 °C for 30 min. Samples were again washed with warm distilled water and then dehydrated with increasing order of alcohol percentage (20%, 50%, 70%, 90%, 95%, 100%, 100%) at room temperature. Later, samples were transferred to the freshly made 50% epoxy resin in alcohol and incubated for 4-5 hr at room temperature and then incubated in 75% exoxy overnight at room temperature. Samples were transferred to freshly prepared 100% Epon812 resin and incubated for 1 hr, then transferred to fresh 100% Epon812 resin for another 1 hr incubation, and finally transferred to 100 % Epon812 for a 4 hr incubation. Tissues were then transferred to

the stub with as little resin as possible and incubated for polymerization on the stub for 48 hr at 60 °C.

FIB-SEM imaging. FIB-SEM images were acquired using a ZEISS Crossbeam 540 with ZEISS Atlas 5 software (Carl Zeiss Microscopy GmbH, Jena, Germany) and collected using an in-column energy selective backscatter detector with filtering grid to reject unwanted secondary electrons and backscatter electrons. Images were acquired at 1.5 keV, 2 nA probe current at the working distance of 5.0 mm. FIB milling was performed at 30 keV, 700 pA current, and 10 nm thickness. Image stacks within a volume were aligned using Atlas 5 software (Fibics Incorporated, Ontario, Canada) and exported as TIFF files for analysis. Voxel size was set at 10 x 10 x 10 nm.

Image segmentation. Raw FIB-SEM image volumes were rotated in 3D so that the XY orientation represented the cross section of the muscle. The image stack volume was imported into the TrakEM2 plugin in ImageJ. A myofibril was chosen in the first slice and cross-sections of sarcomeres were traced along the longitudinal axis of the muscle. Tracings were then stitched together using the interpolation feature in TrakEM2. When sarcomeres branched or merged during tracing via the presence or absence of the sarcoplasmic reticulum/t-tubules, cross-sections were traced immediately before and after the connection point to ensure accurate interpolation. For example, when arriving at a sarcomere that splits into two, the previously traced segment prior to the branch point was continued along one part of the branch using the same label and a new label was created to trace the other branched segment. In the case of a merging event, the method applied was in reverse from that of a splitting event. When two sarcomeres merged at a connection point, only one of the two labels used to keep track of the two previously traced segments were chosen to trace downstream segments. Therefore, each myofibrillar segment of the matrix was identified as a different label. Accuracy of interpolated tracings was assessed by overlaying the exported tracings from TrakEM2 with the raw FIB-SEM file. A multi-color representation of the myofibrillar matrix was obtained following a series of steps. First, a myofibrillar mask with myofibrillar pixels assigned a value of 1.0 and background pixels assigned as null (NaN or ignored in all mathematical analyses) values was created by

dividing the myofibrillar image by itself using the Image Calculator tool while choosing 32-bit (float) result. The myofibrillar mask image was then multiplied by the raw 32-bit FIB-SEM data. Finally, the resulting grayscale image stack was then merged with the myofibrillar labels file, with each segment shown as a different color.

For analysis of the overall contractile, mitochondrial, and SRT volumes, FIB-SEM volumes were segmented in semi-automated fashion using Ilastik⁶⁴ machine learning software as described previously²⁵. Briefly, FIB-SEM image volumes were binned to 20 nm in ImageJ, saved as 8-bit HDF5 files, and imported into Ilastik. Pixel classification training using all available features was performed for the contractile apparatus, mitochondria, SRT, and all other pixels and exported as 8-bit probability files. The resultant HDF5 files were imported back into ImageJ and binarized using a 50% threshold. The binary structures were filtered using the Remove Outliers plugin in ImageJ using a 3-10 pixel radius and 1.5-2 standard deviations and the number of resultant pixels were counted for each structure and compared to the total number of cellular pixels.

Quantitative analysis of myofibrillar networks. Raw FIB-SEM image volumes were rotated in 3D so that the muscle cell's cross-section was visible. Every sarcomere in parallel in the first image within the dataset was numbered and subsequently tracked along the longitudinal axis for sarcomere connectivity. Only sarcomeres which stayed within the field of view of our datasets were used for analysis. We define branching as interconnections between nearby sarcomeric structures. Analyses were done per sarcomere and as such, the number of times that a sarcomere split, merge, or had no event(s) were counted. Tracking proceeded down the length of the muscle with the portion of the myofibrillar structure which best maintained the same relative location within the muscle fiber (i.e., distance from the cell membrane) as the original numbered sarcomere from the first image.

Myofibril cross-sectional area (CSA) and circularity were measured from the traced structures by converting them to binary images and running the Analyze Particles plugin in ImageJ for each slice throughout the volume. The volume-weighted average myofibril CSA was calculated for the wild type and *NCDN* KD IF muscles by multiplying the CSA of each myofibril by

its percentage of the total CSA for all myofibrils and then summing the resultant value for all myofibrils.

Image rendering. 3D animations of the myofibrillar networks were created in Imaris 9.7 (Bitplane) using the horizontal and vertical rotation features and/or the clipping tool. Pictures of 3D renderings were created using the Volume Viewer plugin in ImageJ.

Real-time Quantitative PCR

Total RNA was extracted from 1-2 day old adult thorax muscles (n=20 per trial) from wildtype (*UAS-mito-GFP; Mef2 Gal4*) and *neurochondrin* (*NCDN*) KD (*UAS-NCDN RNAi; Mef2 Gal4*) flies using the TRIzol™ Plus RNA Purification Kit (A33254) according to the manufacturer's instructions. DNA digestion was carried out using TURBO DNA-free™ Kit (AM1907). First-strand synthesis was performed on 500 ng of total RNA using SuperScript™ VILO™ cDNA Synthesis Kit (Thermo Fisher Scientific, 11754050) according to the manufacturer's instructions. Concentrated cDNA from all samples was used to generate standard curves. Amplification was detected using the QuantStudio Real-Time PCR System (Thermo Fisher Scientific). The measures were normalized to *Rpl32* reference gene. All reactions were performed in triplicates. Probes used: *Neurochondrin* (Assay ID Dm02137509_s1) and *Rpl32* (Assay ID Dm02151827_g1) (Thermo Fisher Scientific).

Immunofluorescent analysis. IF muscles were dissected from adult thoraces in 4% formaldehyde and were processed as described previously⁶⁵. Briefly, thoraces with IF muscles were fixed in 4% formaldehyde for 2h at room temperature on a rotor. Samples were washed three times with PBS + 0.03% Triton X-100 (PBSTx) for 15 min and blocked for 2 h at room temperature or overnight at 4°C using 2% BSA (Bovine Serum Albumin, Sigma). Samples were incubated with respective primary antibody (Ab) at 4°C overnight and later washed three times for 10 min with PBSTx and incubated for 2.5 h in respective secondary Ab at 25°C or overnight at 4°C. Samples were incubated for 40 min with Phalloidin TRITC (2.5 µg/ml) (P1951, Sigma, USA) and mounted using Prolong Glass Antifade Mountant with NucBlue stain and incubated

for 20 min. Images were acquired with a ZEISS 780 confocal microscope and processed using ImageJ and ZEN software (version 3.2.0.115) respectively. Antibodies used for the staining: Rabbit anti-salm-1 (1:500, gift from Dr. Tiffany Cook⁶⁶), Rabbit anti-nmr1 (H15) (1:200, gift from Dr. James B. Skeath⁶⁷), and Alexa-Fluor-488-labeled anti-rabbit IgG (1:500, Cat# A32731, Thermo Fisher Scientific).

Statistical analysis. Statistical analyses were conducted using Excel 2016 (Microsoft, Redmond, WA) and Prism 9.0.0 (GraphPad, San Diego, CA). When appropriate, data points were overlaid with their corresponding summary plots to create a SuperPlot. All comparisons of means between wildtype muscles of the indirect flight, jump, direct flight, and leg muscles were performed by one way ANOVA. Brown-Forsythe tests were performed to determine the equality of group variances. When the variances were determined to be different, a Dunn's multiple comparisons test was performed. When variances were determined to not be different, a Tukey's HSD *post hoc* test was performed. Differences between the gene knockdowns and corresponding wildtype muscles were evaluated on the means from each dataset using a two-sided independent t-test for data that passed a Shapiro-Wilks normality test. A Wilcoxon signed rank test was performed in lieu of the Shapiro-Wilks normality test if insignificant. Statistical significance was set at a p-value of 0.05.

Data availability. All raw data used in this work are available upon reasonable request.

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Author Contributions. PTA, PK, CKEB, and BG designed, PK and YS prepared samples, YS and CKEB imaged samples, and PTA, TBW, and BG analyzed images for all electron microscopy studies. PK and BG designed, and PK performed and analyzed all light microscopy experiments. PTA and BG wrote the manuscript draft and PTA, PK, TBW, YS, CKEB, and BG edited and approved the manuscript.

References

1. Hoppeler H, Hudlicka O, Uhlmann E. Relationship between mitochondria and oxygen consumption in isolated cat muscles. *J Physiol* **385**, 661-675 (1987).
2. Hoppeler H, Lüthi P, Claassen H, Weibel ER, Howald H. The ultrastructure of the normal human skeletal muscle. *Pflügers Archiv* **344**, 217-232 (1973).
3. Murgia M, *et al.* Single muscle fiber proteomics reveals fiber-type-specific features of human muscle aging. *Cell reports* **19**, 2396-2409 (2017).
4. Lim GB. Drugs targeting the sarcomere in heart failure and hypertrophic cardiomyopathy. *Nature Reviews Cardiology* **18**, 71-71 (2021).
5. Mou YA, *et al.* Altered myofilament structure and function in dogs with Duchenne muscular dystrophy cardiomyopathy. *Journal of molecular and cellular cardiology* **114**, 345-353 (2018).
6. Vignier N, *et al.* The non-muscle ADF/cofilin-1 controls sarcomeric actin filament integrity and force production in striated muscle laminopathies. *Cell reports* **36**, 109601 (2021).
7. Balakrishnan M, *et al.* Cofilin loss in *Drosophila* muscles contributes to muscle weakness through defective sarcomerogenesis during muscle growth. *Cell reports* **32**, 107893 (2020).
8. Lieber RL. *Skeletal muscle structure, function, and plasticity*. Lippincott Williams & Wilkins (2002).
9. Patel TJ, Lieber RL. Force transmission in skeletal muscle: from actomyosin to external tendons. *Exerc Sport Sci Rev* **25**, 321-363 (1997).

- 574
- 575 10. Squire JM. Muscle contraction: Sliding filament history, sarcomere dynamics and the
- 576 two Huxleys. *Glob Cardiol Sci Pract* **2016**, e201611 (2016).
- 577
- 578 11. Willingham TB, Kim Y, Lindberg E, Bleck CKE, Glancy B. The unified myofibrillar matrix for
- 579 force generation in muscle. *Nature communications* **11**, 3722 (2020).
- 580
- 581 12. Goldspink G. Changes in striated muscle fibres during contraction and growth with
- 582 particular reference to myofibril splitting. *J Cell Sci* **9**, 123-137 (1971).
- 583
- 584 13. Patterson S, Goldspink G. Mechanism of myofibril growth and proliferation in fish
- 585 muscle. *J Cell Sci* **22**, 607-616 (1976).
- 586
- 587 14. Shear CR. Effects of disuse on growing and adult chick skeletal muscle. *J Cell Sci* **48**, 35-
- 588 54 (1981).
- 589
- 590 15. Ashmore CR, Summers PJ. Stretch-induced growth in chicken wing muscles: myofibrillar
- 591 proliferation. *Am J Physiol* **241**, C93-97 (1981).
- 592
- 593 16. Street SF. Lateral transmission of tension in frog myofibers: a myofibrillar network and
- 594 transverse cytoskeletal connections are possible transmitters. *J Cell Physiol* **114**, 346-364
- 595 (1983).
- 596
- 597 17. Goldspink G. The proliferation of myofibrils during muscle fibre growth. *J Cell Sci* **6**, 593-
- 598 603 (1970).
- 599
- 600 18. Roth SM, *et al.* Ultrastructural muscle damage in young vs. older men after high-volume,
- 601 heavy-resistance strength training. *Journal of Applied Physiology* **86**, 1833-1840 (1999).
- 602
- 603 19. Vincent AE, *et al.* Quantitative 3D Mapping of the Human Skeletal Muscle Mitochondrial
- 604 Network. *Cell reports* **26**, 996-1009 e1004 (2019).
- 605
- 606 20. Schonbauer C, *et al.* Spalt mediates an evolutionarily conserved switch to fibrillar muscle
- 607 fate in insects. *Nature* **479**, 406-409 (2011).
- 608
- 609 21. Katti P, Thimmaya D, Madan A, Nongthomba U. Overexpression of miRNA-9 Generates
- 610 Muscle Hypercontraction Through Translational Repression of Troponin-T in *Drosophila*
- 611 *melanogaster* Indirect Flight Muscles. *G3 (Bethesda)* **7**, 3521-3531 (2017).

22. Deng S, *et al.* The actin polymerization factor Diaphanous and the actin severing protein Flightless I collaborate to regulate sarcomere size. *Dev Biol* **469**, 12-25 (2021).
23. Bernstein SI, O'Donnell PT, Cripps RM. Molecular genetic analysis of muscle development, structure, and function in *Drosophila*. *International review of cytology* **143**, 63-152 (1993).
24. Schönbauer C, *et al.* Spalt mediates an evolutionarily conserved switch to fibrillar muscle fate in insects. *Nature* **479**, 406-409 (2011).
25. Bleck CKE, Kim Y, Willingham TB, Glancy B. Subcellular connectomic analyses of energy networks in striated muscle. *Nature communications* **9**, 5111 (2018).
26. Fyrberg EA, Mahaffey JW, Bond BJ, Davidson N. Transcripts of the six *Drosophila* actin genes accumulate in a stage-and tissue-specific manner. *Cell* **33**, 115-123 (1983).
27. Elliott CJ, Sparrow JC. In vivo measurement of muscle output in intact *Drosophila*. *Methods* **56**, 78-86 (2012).
28. Swank DM. Mechanical analysis of *Drosophila* indirect flight and jump muscles. *Methods* **56**, 69-77 (2012).
29. Peckham M, Molloy J, Sparrow J, White D. Physiological properties of the dorsal longitudinal flight muscle and the tergal depressor of the trochanter muscle of *Drosophila melanogaster*. *Journal of Muscle Research & Cell Motility* **11**, 203-215 (1990).
30. Schwerzmann K, Hoppeler H, Kayar SR, Weibel ER. Oxidative capacity of muscle and mitochondria: correlation of physiological, biochemical, and morphometric characteristics. *Proc Natl Acad Sci U S A* **86**, 1583-1587 (1989).
31. Wang Q, Zhao C, Swank DM. Calcium and stretch activation modulate power generation in *Drosophila* flight muscle. *Biophysical journal* **101**, 2207-2213 (2011).
32. Katti P, Ajayi PT, Aponte AM, Bleck CK, Glancy B. An Evolutionarily Conserved Regulatory Pathway of Muscle Mitochondrial Network Organization. *BioRxiv*, (2021).

33. Bryantsev AL, *et al.* Extradenticle and homothorax control adult muscle fiber identity in *Drosophila*. *Developmental cell* **23**, 664-673 (2012).
34. Avellaneda J, *et al.* Myofibril and mitochondria morphogenesis are coordinated by a mechanical feedback mechanism in muscle. *Nature communications* **12**, 1-18 (2021).
35. Dateki M, *et al.* Neurochondrin negatively regulates CaMKII phosphorylation, and nervous system-specific gene disruption results in epileptic seizure. *Journal of Biological Chemistry* **280**, 20503-20508 (2005).
36. Istvanffy R, Weisenhorn DV, Floss T, Wurst W. Expression of neurochondrin in the developing and adult mouse brain. *Development genes and evolution* **214**, 206-209 (2004).
37. Fatima A, Schuster J, Akram T, Sobol M, Hoeber J, Dahl N. Generation of a human Neurochondrin deficient iPSC line KICri002-A-3 using CRISPR/Cas9. *Stem cell research* **44**, 101758 (2020).
38. Schwaibold EM, Brandt DT. Identification of Neurochondrin as a new interaction partner of the FH3 domain of the Diaphanous-related formin Dia1. *Biochemical and biophysical research communications* **373**, 366-372 (2008).
39. Montana ES, Littleton JT. Expression profiling of a hypercontraction-induced myopathy in *Drosophila* suggests a compensatory cytoskeletal remodeling response. *Journal of Biological Chemistry* **281**, 8100-8109 (2006).
40. Spletter ML, *et al.* A transcriptomics resource reveals a transcriptional transition during ordered sarcomere morphogenesis in flight muscle. *eLife* **7**, e34058 (2018).
41. Roy S, VijayRaghavan K. Developmental biology: taking flight. *Curr Biol* **22**, R63-R65 (2012).
42. Maves L, *et al.* Pbx homeodomain proteins direct Myod activity to promote fast-muscle differentiation. *Development* **134**, 3371-3382 (2007).
43. Honda M, *et al.* Vestigial-like 2 contributes to normal muscle fiber type distribution in mice. *Scientific reports* **7**, 7168 (2017).

44. Lee KY, *et al.* Tbx15 controls skeletal muscle fibre-type determination and muscle metabolism. *Nature communications* **6**, 8054 (2015).
45. Heidt AB, Rojas A, Harris IS, Black BL. Determinants of myogenic specificity within MyoD are required for noncanonical E box binding. *Mol Cell Biol* **27**, 5910-5920 (2007).
46. Handschin C, *et al.* Skeletal muscle fiber-type switching, exercise intolerance, and myopathy in PGC-1 α muscle-specific knock-out animals. *Journal of Biological Chemistry* **282**, 30014-30021 (2007).
47. Shintaku J, *et al.* MyoD regulates skeletal muscle oxidative metabolism cooperatively with alternative NF- κ B. *Cell reports* **17**, 514-526 (2016).
48. Sauerwald J, Backer W, Matzat T, Schnorrer F, Luschign S. Matrix metalloproteinase 1 modulates invasive behavior of tracheal branches during entry into Drosophila flight muscles. *eLife* **8**, e48857 (2019).
49. Peterson SJ, Krasnow MA. Subcellular trafficking of FGF controls tracheal invasion of Drosophila flight muscle. *Cell* **160**, 313-323 (2015).
50. Schnorrer F, *et al.* Systematic genetic analysis of muscle morphogenesis and function in Drosophila. *Nature* **464**, 287-291 (2010).
51. Huxley AF, Niedergerke R. Structural changes in muscle during contraction; interference microscopy of living muscle fibres. *Nature* **173**, 971-973 (1954).
52. Huxley H, Hanson J. Changes in the cross-striations of muscle during contraction and stretch and their structural interpretation. *Nature* **173**, 973-976 (1954).
53. Pardo JV, Siliciano JD, Craig SW. A vinculin-containing cortical lattice in skeletal muscle: transverse lattice elements ("costameres") mark sites of attachment between myofibrils and sarcolemma. *Proc Natl Acad Sci U S A* **80**, 1008-1012 (1983).
54. Dalakas MC, Park KY, Semino-Mora C, Lee HS, Sivakumar K, Goldfarb LG. Desmin myopathy, a skeletal myopathy with cardiomyopathy caused by mutations in the desmin gene. *N Engl J Med* **342**, 770-780 (2000).

55. Bermudez-Jimenez FJ, *et al.* Novel Desmin Mutation p.Glu401Asp Impairs Filament Formation, Disrupts Cell Membrane Integrity, and Causes Severe Arrhythmogenic Left Ventricular Cardiomyopathy/Dysplasia. *Circulation* **137**, 1595-1610 (2018).
56. O'Neill A, Williams MW, Resneck WG, Milner DJ, Capetanaki Y, Bloch RJ. Sarcolemmal organization in skeletal muscle lacking desmin: evidence for cytokeratins associated with the membrane skeleton at costameres. *Molecular biology of the cell* **13**, 2347-2359 (2002).
57. Danowski BA, Imanaka-Yoshida K, Sanger JM, Sanger JW. Costameres are sites of force transmission to the substratum in adult rat cardiomyocytes. *The Journal of cell biology* **118**, 1411-1420 (1992).
58. Bloch RJ, Gonzalez-Serratos H. Lateral force transmission across costameres in skeletal muscle. *Exercise and sport sciences reviews* **31**, 73-78 (2003).
59. Rybakova IN, Patel JR, Ervasti JM. The dystrophin complex forms a mechanically strong link between the sarcolemma and costameric actin. *The Journal of cell biology* **150**, 1209-1214 (2000).
60. van der Plas MC, Pilgram GS, de Jong AW, Bansraj MR, Fradkin LG, Noordermeer JN. Drosophila Dystrophin is required for integrity of the musculature. *Mechanisms of development* **124**, 617-630 (2007).
61. Taghli-Lamalle O, *et al.* Dystrophin deficiency in Drosophila reduces lifespan and causes a dilated cardiomyopathy phenotype. *Aging cell* **7**, 237-249 (2008).
62. Henkin JA, Maughan DW, Vigoreaux JO. Mutations that affect flightin expression in Drosophila alter the viscoelastic properties of flight muscle fibers. *American Journal of Physiology-Cell Physiology* **286**, C65-C72 (2004).
63. Lemke SB, Weidemann T, Cost A-L, Grashoff C, Schnorrer F. A small proportion of Talin molecules transmit forces at developing muscle attachments in vivo. *PLoS biology* **17**, e3000057 (2019).
64. Sommer C, Straehle C, Kothe U, Hamprecht FA. Ilastik: Interactive Learning and Segmentation Toolkit. *I S Biomed Imaging*, 230-233 (2011).

65. Rai M, Katti P, Nongthomba U. Drosophila Erect wing (Ewg) controls mitochondrial fusion during muscle growth and maintenance by regulation of the Opa1-like gene. *J Cell Sci* **127**, 191-203 (2014).
66. Xie B, Charlton-Perkins M, McDonald E, Gebelein B, Cook T. Senseless functions as a molecular switch for color photoreceptor differentiation in Drosophila. *Development* **134**, 4243-4253 (2007).
67. Leal SM, Qian L, Lacin H, Bodmer R, Skeath JB. Neuromancer1 and Neuromancer2 regulate cell fate specification in the developing embryonic CNS of Drosophila melanogaster. *Dev Biol* **325**, 138-150 (2009).

Supplementary Figure 1: The variety in size, shape, and content of the contractile apparatus and surrounding organelles across *Drosophila* muscles. Representative scanning electron microscopy images of *Drosophila* muscle cross-sections. Red S in each image signifies a single sarcomere. Scale bar – 2 μm.

Supplementary Figure 2: Types of events within a sarcomere. a) 3D rendering of longitudinal and cross sections of isolated myofibrils in fibrillar indirect flight muscles. b) 3D rendering of single branching event in tubular muscles of TDT, DF, and leg muscles. c) 3D renderings of multi-branching events (more than one event) in tubular muscles. Examples of one sarcomere branching into three segments (Trichotomy) and into four segments (Tetrachotomy) are shown. For a-c, individual colors represent different myofibrillar segments. d) Percentage of sarcomeres with just one branching event in wild type muscles. e) percentage of sarcomeres with just one branching event in wild type muscles. f) Frequency of sarcomere branching in wild type muscles. N values for d, e, f: IF—3 muscle cells, 3 datasets, 114 myofibrils, 982 sarcomeres; TDT—3 muscle cells, 2 datasets, 160 myofibrils, 1863 sarcomeres; DF—4 muscle cells, 1 dataset, 200 myofibrils, 1461 sarcomeres; and leg—3 muscle cells, 2 datasets, 150 myofibrils, 690 sarcomeres. Larger shape symbols represent data from a single cell and smaller shape symbols represent data from a single myofibril. Bars represent muscle cell overall mean $\pm$ SE. Asterisk (*): Significantly different from fibrillar indirect flight (one-way ANOVA, $P < 0.05$). Pound sign (#): Significantly different from tubular TDT. Plus sign (+): Significantly different from tubular DF. Scale bars—2 μ m.

Supplementary Figure 3: Sarcomere branching occurs more frequently near the muscle cell boundary. a) 3D rendering of the leg muscle sarcolemma (red) and the location of the branch points within the muscle (colored based on distance to sarcolemma). b) 3D rendering of the jump muscle sarcolemma (orange) and the location of the branch points within the muscle (colored based on distance to sarcolemma). c) Histogram of the location of sarcomere branches in the leg muscle relative to the sarcolemma using 2 μm bins. d) Histogram of the location of sarcomere branches in the jump muscle relative to the sarcolemma using 2 μm bins.

Supplementary Figure 4: Neurochondrin knockdown does not alter indirect flight muscle fibrillar fate. a-h) Locations of mitochondria (a,e), Salm protein expression (b,f) and nuclei (c,g) in wild type (a-d) and *neurochondrin* (*NCDN*) knockdown (KD) (e-h) indirect flight muscles as imaged under a confocal microscope. Representative of four muscle cells. i-p) Locations of mitochondria (i,m), H15 protein expression (j,n) and nuclei (k,o) in wild type (i-l) and *neurochondrin* (*NCDN*) knockdown (KD) (m-p) indirect flight muscles as imaged under a confocal microscope. Representative of four muscle cells. q-r) Quantification of Salm (q) and H15 (r) immunofluorescence. Circles represent individual muscle cells, bars equal mean $\pm$ SE.