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Transcriptional regulation and alternative splicing cooperate in muscle fiber-type specification in flies and mammals

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Abstract

Muscles coordinate body movements throughout the animal kingdom. Each skeletal muscle is built of large, multi-nucleated cells, called myofibers, which are classified into several functionally distinct types. The typical fiber-type composition of each muscle arises during development, and in mammals is extensively adjusted in response to postnatal exercise. Understanding how functionally distinct muscle fiber-types arise is important for unraveling the molecular basis of diseases from cardiomyopathies to muscular dystrophies. In this review, we focus on recent advances in *Drosophila* and mammals in understanding how muscle fiber-type specification is controlled by the regulation of transcription and alternative splicing. We illustrate the cooperation of general myogenic transcription factors with muscle fiber-type specific transcriptional regulators as a basic principle for fiber-type specification, which is conserved from flies to mammals. We also examine how regulated alternative splicing of sarcomeric proteins in both flies and mammals can directly instruct the physiological and biophysical differences between fiber-types. Thus, research in *Drosophila* can provide important mechanistic insight into muscle fiber specification, which is relevant to homologous processes in mammals and to the pathology of muscle diseases.

Keywords

Drosophila; Muscle; Sarcomere; Transcription; Alternative splicing

Introduction

Animals from jellyfish to humans use contractile muscle cells to perform coordinated movements. Higher animals possess distinct muscle classes that are specialised for certain tasks: the vertebrate heart pumps blood life-long without rest, smooth muscles ensheathing the gut propel food without voluntary control, and body muscles move in a precise, consciously controlled manner to enable body movements, body posturing and facial expressions. To optimally fulfill these different tasks, each muscle class requires distinct contractile, metabolic and electrophysiological properties.

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The molecular basis for these functional distinctions is generated during development and results in a dramatically different morphology for each of the three muscle classes. Smooth muscles are mononucleated and can be activated by a variety of neuronal, hormonal, autocrine/paracrine signals or changes in load and length. Their contractile elements lack a regularly striated structure [30,75]. Cardiomyocytes are also mononucleated. They are activated through electrical coupling after neuronal firing and show regular striations along their myofibrils [30]. Skeletal muscle is built of many large, syncytial muscle fibers. Each muscle fiber contains many, often hundreds, of nuclei and has a defined neuromuscular junction that triggers contractions. Each fiber houses many highly ordered myofibrils that are laterally aligned to form stereotypical cross-striations [30].

In this review, we discuss recent progress on mechanisms of differential transcription and alternative splicing that instruct functional differences between muscle types. We focus on mammalian skeletal muscle and *Drosophila* body muscle as the best understood model systems. Mammalian skeletal muscle fibers are historically classified as slow (type 1, red muscle) or fast (type 2, white muscle) fibers. Fast fibers are further subdivided into type 2A, 2B and 2X. They generally can produce higher forces than slow fibers, are glycolytic and fatigue rather quickly. In contrast, slow fibers produce lower forces, primarily use oxidative metabolism and are more fatigue-resistant (reviewed in [63]). Each individual human skeletal muscle consists of many, often several hundred, muscle fibers with a characteristic fiber-type composition. For example, the extensor digitorum longus (EDL) muscle in the foot is mainly composed of fast fibers, whereas the soleus muscle in the lower leg contains mainly slow fibers. However, the individual fiber composition of each muscle will adapt to exercise regime, such that the soleus muscle of a sprint athlete will incorporate more fast fibers as compared to that of a marathon runner, which will be “slower” [12].

Patterning of mammalian muscle fiber-types

The different functional properties of skeletal muscle fiber types in mice arise during fetal muscle development and are further modified during postnatal life. The general myogenic transcription factors MyoD, Myf5, Mrf4 and Myogenin are required for the correct development of most, if not all, skeletal muscles early in embryogenesis (reviewed in [5,7]). Subdivision into distinct muscle fiber types arises during late fetal development in mice through initiation of the fetal myogenic program. It was recently shown that the expression of nuclear factor one X (Nfix) switches the embryonic to the fetal program by repressing embryonic and activating fetal myogenic genes such as muscle creatine kinase (MCK) or β -enolase [49]. This enables the next steps of fiber-type specification by the differential expression of additional transcription factors. The best studied factors are Six1 and Six4, which promote the fast fiber fate, together with their cofactor Eya1 ([25,53]). Their action is supported by the transcriptional repressor Sox6, which represses slow genes in fast fibers [28,31]. Together, this complex interplay between general and specific transcription factors establishes the typical fiber-type distribution at the end of murine fetal muscle development.

Postnatally, muscle fiber-type distribution is significantly reorganized, coinciding with substantial muscle growth after birth. Neuronal innervation, together with calcium-calcineurin signaling, is a key player at this stage. Increased calcineurin signaling promotes

the slow fiber fate [67], potentially through the downstream cooperation of Mef2d with the transcriptional coactivator PGC-1 α , which induces the expression of slow fiber genes, such as myoglobin, or genes required for mitochondrial oxidative metabolism [42]. Varying levels of neuronal activity, and thus calcineurin signaling, also promote the differential recruitment of NFAT family members to the promoters of activity-dependent genes. An NFATc2/3/4 complex specifies transcription of fast fiber genes, while the nuclear import of NFATc1 driven by slow nerve activity redirects the complex to activate transcription of slow genes [10]. As in embryogenesis, general muscle transcription factors cooperate with fiber type-specific transcription factors to achieve differential expression of fiber type-specific genes during adult muscle differentiation.

Fiber-type specific effectors

How do muscle fibers achieve their specific contractile properties? The best-studied examples of differentially expressed sarcomeric components in mammalian body muscle are the myosin heavy chain (MyHC) isoforms. Different fiber types express different MyHC isoforms from the various muscle myosin II genes in mammals. During the embryonic to fetal myogenic switch, embryonic MyHC is gradually replaced by neonatal MyHC. After birth, neonatal MyHC is lost and type 2A fast fibers express MyHC-2A, while slow fibers express MyHCbeta/ slow (reviewed in [63]). MyHC expression is at least partially regulated by NFAT family members downstream of neuronal activity, as MyHC-slow is cooperatively controlled by all four NFAT family members, while MyHC-2A is controlled by NFATc2/3/4 [10].

While further details of upstream regulation are unclear, the expression of MyHC isoforms with different molecular properties, for example variable cross-bridge lengths with actin during contraction, underlies part of the functional differences between fiber types (reviewed in [11,24]). Additionally, specific MyHC isoforms are combined with fiber type-specific isoforms of the Troponin–Tropomyosin complex to adjust the calcium sensitivity of different fiber types. As a consequence, slow fibers already start contracting at low cytoplasmic calcium concentrations; whereas, fast fibers require higher calcium levels to initiate contraction and show a steeper tension increase upon further calcium influx due to a larger cooperativity in their calcium response [4,63].

However, the physiological differences between fast and slow fibers depend on more than just differences in myosin, troponin or tropomyosin isoforms. A recent proteomics analysis in mice compared the slow soleus muscle with the fast EDL muscle and identified 551 proteins that vary significantly between the two muscles. Most notably, these differentially expressed proteins were enriched for proteins involved in contraction, ion homeostasis, glycolysis and oxidation, emphasizing their roles in the different physiologies of these muscles [17]. Despite this significant progress, our mechanistic understanding of how a combination of transcription factors and signaling molecules assembles functionally distinct muscle types and regulates such a broad repertoire of cellular genes remains limited. This is in part due to the high complexity and plasticity of the mammalian system: every muscle consists of many muscle fibers of different fiber types and is patterned by a complex

interplay between autonomous and non-autonomous inputs on its transcriptional machinery at various stages during embryonic and adult development.

***Drosophila* muscle fiber-types**

Complementary to vertebrate studies, research in *Drosophila* has added valuable insights into how different body muscle types are generated during development. Adult *Drosophila* possess at least two functionally distinct body muscle fiber types. Tubular muscle fibers in the head, legs, thorax and abdomen are responsible for most movements of the fly. Tubular muscles are very similar to vertebrate skeletal muscles, as they contain laterally aligned myofibrils that lead to a typical cross-striated pattern (Fig. 1a and e). Nerve stimulation results in calcium release, which triggers actomyosin contraction and thus leads to a synchronous stimulation-contraction pattern.

In stark contrast, *Drosophila* indirect flight muscles (IFMs) are asynchronous muscles that require stretch-activation in addition to calcium stimulation to induce their contractions [9,34,59,72]. This stretch-activation mechanism allows very fast (200 Hz) IFM oscillations, coupled with high force production (up to 80 W/kg muscle), enabling flight [40]. Calcium does not cycle during contractions, but instead remains at an elevated level during the entire flight period due to continuous low frequency nerve stimulation. IFMs have high mitochondrial content for longlasting oxidative metabolism, little sarcoplasmic reticulum, and a fibrillar stretch-sensitive organization of their myofibrils (Fig. 1c) [33].

The muscle fiber-type selector gene *salm*

How the striking morphological and physiological differences between tubular and fibrillar muscle arise during development can be mechanistically investigated with modern *Drosophila* genetics. The *Drosophila* tool kit includes systematic, tissue-specific loss of function studies with genome-wide RNAi approaches using recently established transgenic genome-wide RNAi libraries [16]. The binary *GAL4-UAS* system allows tissue-specific gene knockdown at a given developmental time period, e.g. during pupal stages, thereby preventing pleiotropic phenotypes. A systematic muscle-specific RNAi screen identified more than 2000 genes with a putative role in muscle, about 300 of which are required for normal flight behavior and thus likely function in IFMs [64].

Detailed morphological analysis of the IFMs upon knock-down of these 300 genes identified the conserved zinc-finger transcription factor *spalt major* (*salm*) as a muscle-type specific selector gene for the fibrillar muscle fate. In *salm* knock-down animals, the fibrillar flight muscles are morphologically transformed to tubular, leg-like muscles (Fig. 1b and d) [65]. *Salm* is expressed specifically in fibrillar muscle during development and, if misexpressed, is sufficient to switch tubular muscle to the fibrillar fate (Fig. 1g), making it a bone-fide muscle-type selector gene. Importantly, this function of Spalt is not restricted to *Drosophila*, but is conserved over at least 280 million years of evolution in most flying insects [65]. Interestingly, spalt-like (SALL) proteins are conserved to mammals and some SALL family members are expressed in the mammalian heart [54]. The heart is a very stiff muscle, whose contraction is also stretch-modulated, a phenomenon described as the Frank–Starling mechanism, which links cardiac ejection to cardiac filling with stronger ejection upon larger

filling [69]. Mutations in *SALL1* lead to Townes-Brocks syndrome [71], a multi-organ syndrome that includes heart abnormalities, suggesting an evolutionarily conserved role of SALL family members in stretch-activated muscle.

How does *salm* execute this switch from tubular to fibrillar muscle development and to what extent does *salm* control the characteristic properties of stretch-activated IFMs? *salm* is the founding member of the conserved family of Spalt zinc-finger transcription factors, which play important roles during the development of many organ systems, such as the eyes, wings and trachea in insects [15,20,51]. In mammals, Spalt-like (SALL) genes are similarly required for the development of various organs including the heart and for specification of the mouse inner cell mass-derived lineages [14,18,37]. SALL proteins are localized to the nucleus in mammals and *Drosophila* and have been reported to act as both transcriptional repressors [36,62] and activators [77], depending on the system studied. Despite their crucial roles in organ development, specific DNA binding motifs for most of the Spalt family members, in particular for *Drosophila* Salm, remain elusive. In IFMs, Salm either directly or indirectly activates the expression of fibrillar muscle specific genes such as *flightin (fln)* or *Troponin C isoform 4 (TpnC4)* and at the same time represses tubular muscle-specific genes like *Troponin C41 (TpnC41)* or *Muscle protein 20 (Mp20)* [65]. Importantly, it has been shown that all of these proteins directly regulate the contractile properties of the IFMs: TpnC4 is critical for stretch-activation of the myofibrils [1], whereas Fln is required for proper assembly of the thick filaments in IFMs contributing to their high stiffness, an essential mechanical property for stretch-activation [61]. In addition, Salm up-regulates a wide range of mitochondrial genes specifically in IFMs, which mainly rely on oxidative metabolism, thereby adapting them to the high energy demand of flight [65]. Thus, Salm not only switches myofiber fate by changing the transcriptional profile of core sarcomeric genes, it also determines most of the physiological differences between tubular and fibrillar muscle fibers.

It is poorly understood how Salm achieves IFM-specific target gene expression, especially when considering that *salm* function is also essential to activate distinct sets of targets in eye, wing or trachea cells. One possible mechanism is that Salm cooperates with or modifies the activity of the essential muscle-specific transcription factor Mef2 at particular Mef2-dependent enhancers in IFMs. Since vertebrate Mef2 family members are well-known to cooperate with bHLH factors of the MyoD family [50], as well as with HDACs and HATs [47,48], to achieve different regulatory outputs in distinct muscle types, it is plausible that Salm similarly cooperates with or modifies the activity of *Drosophila* Mef2 at particular Mef2-dependent enhancers in IFMs. Such cooperative interactions of Mef2 to instruct spatio-temporally restricted expression of target genes have also been demonstrated with the fusion competent myoblast-specific transcription factor *Lame duck (Lmd)* [13], the cardiogenic transcription factor *Tinman* and the general mesodermal factor *Twist* [78]. Mef2 is also required for IFM differentiation, being essential for proper formation and maturation of contractile filaments [70]. However, whether cooperative interaction of Salm with Mef2 at enhancers of fibrillar muscle genes occurs awaits further investigation.

Salm may additionally act downstream or cooperatively with the homeodomain proteins *Homothorax (Hth)* and *Extradenticle (Exd)*. Despite broad expression in adult tubular leg

and abdominal muscle as well as fibrillar IFMs, adult muscle-specific loss of *hth* or *exd* results in a specific transformation of IFMs to tubular muscles, and mis-expression of Hth or Exd in jump muscle, another large tubular muscle in the thorax, induces its fibrillar transformation [6]. Notably, while IFMs express high levels of Salm, wild-type jump muscle expresses low levels of Salm; thus, it is attractive to hypothesize that the fibrillar fate results from transcriptional cooperativity of Salm with Hth and Exd, which in wild type only occurs during development of the fibrillar IFMs. These examples suggest that the cooperation of general myogenic transcription factors with muscle-type specific transcriptional regulators constitutes a basic principle for muscle fiber-type specification conserved from *Drosophila* to vertebrates.

Alternative splicing in insect muscle

Interestingly, the fibrillar IFM fate is not only determined by a transcriptional switch, but also by a change in the splicing pattern between fibrillar and tubular muscle. Again, this switch in splicing is controlled by *salm* [65]. One clear example for this mechanism is the regulation of *Myofilin* (*Mf*). *Mf* is a muscle-specific thick-filament associated protein conserved in insects that is putatively involved in the assembly of thick filaments [60]. Muscle-specific RNAi-mediated knock-down of *Mf* is pupal lethal, supporting an important role in fiber assembly [64]. In *Drosophila*, the *Mf* locus generates multiple gene isoforms, including a short isoform that is specifically expressed in IFMs and longer isoforms that are expressed in tubular muscles [60]. mRNA sequencing of IFMs, *salm* knock-down IFMs as well as tubular leg and jump muscles reveals that the major difference between long and short *Mf* isoforms is a regulated splicing event joining exon 5 with 6 in fibrillar muscles or exon 5 with 7 in tubular muscles (Fig. 2a and M.L.S, D. Gerlach, A. Stark, F.S. unpublished data). The exon 5–6 junction is preferentially used in fibrillar IFMs, whereas this splice event rarely occurs in tubular muscles or *salm* knock-down IFMs (Fig. 2b). The exon 5–7 junction is preferentially used in tubular muscles (Fig. 2b), leading to larger *Mf* proteins (Fig. 2c). Although two specific *Mf* splicing events in larval muscles are regulated by *muscleblind* (*mbl*) [32], the splicing factor regulating the switch from exon 5–6 to 5–7 splicing in fibrillar *versus* tubular muscle remains to be identified. In addition to *Mf*, several other structural genes are reported to be alternatively spliced between tubular and fibrillar muscle, including *Drosophila* Troponin-T (*upheld*, *up*), Tropomyosin 1 (*Tm1*), Myosin alkali light chain (*Mlc1*), Myosin heavy chain (*Mhc*) (for review see [74]) and Projectin (*bent*, *bt*), a *Drosophila* titin homolog [2]. In *salm* knock-down animals, splicing of all these genes is likewise switched to the tubular instead of the fibrillar pattern [65], consistent with the hypothesis of a functional transition in the alternative splicing landscape to that normally found in tubular instead of fibrillar muscle.

How do differences in alternative splicing affect muscle type morphology and physiology? In general, inclusion or exclusion of particular coding sequences due to alternative splicing likely leads to the production of proteins with different regulatory or biophysical properties. While the functional significance of long *versus* short *Mf* isoforms remains to be determined, aberrant splicing of *Drosophila* Troponin T (TnT, *up*) in IFMs results in functional impairment (flightlessness) caused by defects in the myofibrillar apparatus, with diffuse Z-lines and the formation of so-called “zebra bodies”, a major hallmark in a variety

of human myopathies [68]. These morphological and functional defects are presumably due to changes in TnT activity by differential phosphorylation of C-terminally located alternative exons, which affects TnT function and sarcomere stability [55].

Another target of extensive splicing regulation is the large *Drosophila* titin homolog, *projectin* (*bt*). Projectin is one of the largest *Drosophila* proteins composed of 47 annotated exons, including multiple exons encoding a conserved proline-glutamic acid-valine-lysine (PEVK)-rich domain. Interestingly, the PEVK domain from vertebrate titin was shown to be elastic *in vitro* and *in vivo*, likely due to entropic changes upon stretch, suggesting a spring-like function during sarcomere contraction cycles [21,39,43,73]. This implies that a difference in PEVK domain length between fiber types may modulate the stiffness of vertebrate muscle [44]. Indeed, vertebrate heart contains shorter titin isoforms with shorter PEVK-rich domains resulting in an increased passive stiffness of the adult heart compared to skeletal muscle ([45,46]; reviewed in [26]). Mice with a deletion of the short PEVK domain of the heart-specific isoform display a hypertrophic cardiomyopathy most likely caused by the changed elastic properties of titin [23]. The *Drosophila* titin-like protein projectin also displays elasticity and is proposed to function as a molecular spring [8]. Although titin's fiber-type specific splice events might not be directly conserved between insects and vertebrates, IFMs do possess a high passive resting stiffness that is required to facilitate stretch-activation with minimal sarcomeric displacement [57]. IFMs normally contain the shortest observed PEVK domain of only a few amino acids, and presumably a shift to one of the longer 363 or 464 amino acid domains found in tubular muscle would decrease passive resting stiffness and likely disrupt the stretch-activation mechanism powering flight [2,52].

A final example of alternative splicing directly affecting sarcomeric function is *Drosophila Myosin heavy chain* (*Mhc*), the motor protein that produces contractile force in muscle, which in contrast to vertebrates is encoded by the single *Mhc* gene in *Drosophila*. Alternative splicing generates the wide diversity of Mhc protein isoforms expressed in *Drosophila* muscle fibers. IFMs express a different complement of alternatively spliced exons than larval *Mhc* isoforms, resulting in Mhc proteins with distinct physiological properties [56,76]. In particular, the relay domains encoded by exon 9 variants result in variations in MgATPase activity and actin sliding velocity and affect myofibril assembly and stability, while variants in the converter domain encoded by exon 11 affect CaATPase, MgATPase, and actin sliding velocity [38]. Thus, alterations in Mhc splicing in IFMs would affect myofibril assembly and stability and also change the fine-tuning of the myosin ATPase and actin filament motility. Taken together, these three examples strongly suggest that muscle fiber-type selector genes switch not only the transcriptional but also the splicing status of the muscle to ultimately control muscle fiber-type specific morphological and physiological properties.

Alternative splicing in vertebrates and its impact on muscle disease

Regulation of splicing in muscle is not limited to insects and is also well documented in vertebrate muscle. One elegant example is the transcription factor family Mef2 itself. In vertebrates, Mef2 family members modulate the differentiation of many tissues including muscle, and Mef2D produces a unique muscle-specific splice isoform, Mef2D α 2. While the

broadly expressed Mef2D α 1 isoform inhibits expression of late muscle differentiation genes due to phosphorylation by PKA followed by association with corepressors, the muscle-specific isoform Mef2D α 2 escapes PKA phosphorylation due to exon switching and thus can recruit the transactivator Ash2L to activate transcription of late muscle targets [66]. Sarcomeric effectors are also regulated by alternative splicing in vertebrates. Vertebrates have three Troponin T (TnT) genes encoding cardiac, slow and fast TnT isoforms. The fast TnT gene undergoes extensive muscle fiber-type specific splicing to produce different isoforms that affect its Ca²⁺ sensitivity and thus regulate actomyosin interactions [3]. These examples strongly suggest that isoform switching is a basic principle in both vertebrate and *Drosophila* muscle to fine-tune muscle fiber-type specific functional properties.

Proper control of the splicing machinery is relevant for muscle function in mice and humans, as mis-regulation of splicing can lead to major muscle and heart diseases. While strong evidence supports the involvement of splicing factors such as Muscleblind-like [19] and Rbfox [58] in muscular dystrophies, the relationship between RNA-binding motif protein 20 (RBM20) and titin in heart disease is perhaps the most provocative link to-date between alternative splicing and muscle disease. As discussed above, titin is the elastic component of the myofibril, maintaining the precise structural arrangement of thick and thin filaments and generating passive muscle stiffness. Alternative splicing of titin's PEVK region modifies the “spring” property, with shorter, stiffer versions typically found in the adult vertebrate heart [22,45,46,26]. Mutations affecting titin splicing that reduce passive stiffness are associated with dilated cardiomyopathy (DCM) and account for nearly one third of all cases of familial DCM [29]. Titin splicing is at least partially regulated by RNA-binding motif protein 20 (RBM20), which promotes exon skipping by repressing particular splicing events, normally resulting in the shorter titin isoforms expressed in the heart [41]. Mutation of the RBM20 gene results in mis-splicing, notably resulting in the expression of longer titin isoforms (Fig. 2d), which results in symptoms of dilated cardiomyopathy including ventricular enlargement, arrhythmia, extensive fibrosis and increased rate of sudden death in both rat and human [27]. Moreover, RBM20 regulates conserved splicing events in at least 30 additional genes, including the enigma protein ZASP/Cypher, that are involved in sarcomere organization and ion transport in the sarcoplasmic reticulum, several of which are also associated with DCM [27]. This example illustrates that correct alternative splicing is critical to muscle-type physiology and function.

Future directions

Muscle fiber-type specification is a complex process controlled by coordinated regulation of both transcription and alternative splicing. Research in model organisms such as flies provides an important complement to vertebrate studies in understanding the basic principles contributing to the development of different muscle-types. In the case of the evolutionarily conserved fibrillar selector gene *salm* from *Drosophila*, mechanisms of transcriptional regulation of not only sarcomeric components but also genes dictating the physiologic status of the muscle, in addition to pervasive regulation of alternative splicing, are likely relevant to the underpinnings of cardiomyopathy in humans. While recent studies have started to provide insight into how functional and morphological differences are generated during development, many important questions await detailed clarification. One important

challenge is to generate a complete network of both transcriptional and splicing regulators expressed in different muscle types, as factor cooperativity and feed-forward or feed-back mechanisms are important in fine-tuning muscle fiber-type specific physiological properties. The differences in physiological properties between fiber-types are functionally dictated by the biophysical properties of alternatively spliced forms of signaling and structural proteins. Thus, characterizing first which alternate protein isoforms are present in each fiber-type and second how inclusion or exclusion of particular protein domains affects muscle physiology will be essential to understand muscle fiber-type specific function. The ultimate goal is to molecularly define how different fiber fates are specified during normal development and how these fiber-types instruct the construction of physiologically different molecular machines. These insights may be applied for treating disease symptoms such as muscle wasting and cachexia, or to increase muscle fatigue resistance for occupational reasons.

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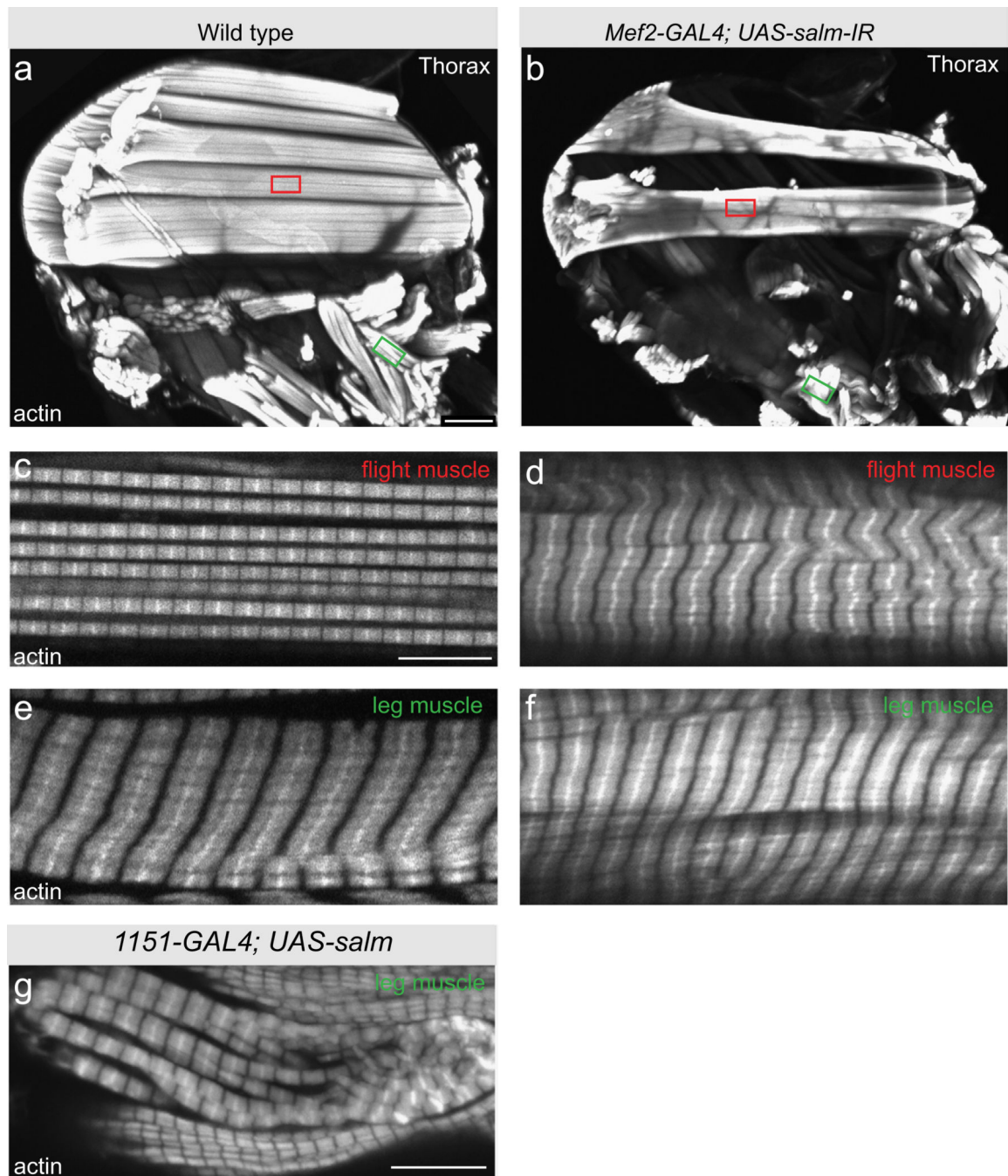


Fig. 1. *salm* specifies fibrillar versus tubular muscle morphology in insects. *Drosophila* wild type (a) and muscle-specific *salm* knock-down hemi-thoraxes (*Mef2-GAL4, UAS-salm-IR*) (b) stained with phalloidin. Colored boxes indicate regions magnified in c–f. Fibrillar IFMs in wild type (c) and tubular *salm* knock-down IFMs (d). Tubular leg muscles in wild type (e) and *salm* knock-down (f). Gain of fibrillar fate in leg muscles mis-expressing *salm* (*1151-GAL4 UAS-salm*) (g). Scale bars 100 μm in a, b, 10 μm in c–g.

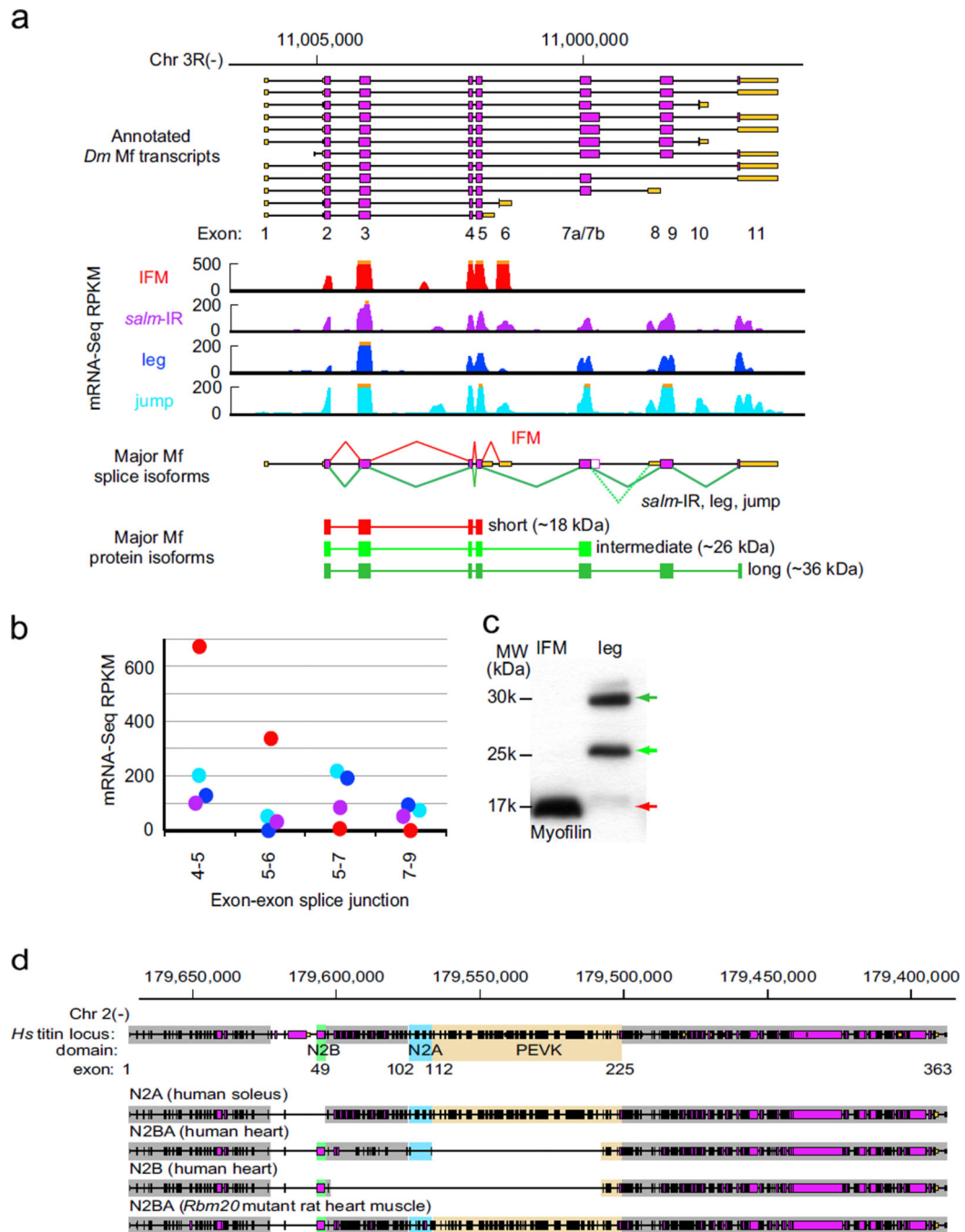


Fig. 2. Muscle-type specific alternative splicing of *Drosophila Myofilin* and vertebrate titin. (a) Illustration of a subset of predicted *Mf* transcripts, with coding exons in magenta and UTRs in gold. mRNA-Seq RPKM values for wild-type IFMs (red), *salm* knock-down IFMs (*salm*-IR in purple), whole legs (blue) and jump muscles (teal) samples reveal differential expression of individual *Mf* exons. Orange lines indicate RPKM values greater than the scale shown. Major *Mf* splice and protein isoforms are illustrated, based on use of exon-exon splice junctions. (b) Preferential use of the exon 5–6 junction in fibrillar IFMs results in a

short Mf isoform, while preferential use of the exon 5–7 junction in tubular muscles and *salm* knock-down IFMs (*salm*-IR) results in longer Mf isoforms. (c) This difference is observed *in vivo* at the protein level, as shown by western blot for Myofilin in IFMs and legs. As predicted, IFMs express a short Mf isoform of about 18 kDa (red arrow), while an intermediate isoform of about 26 kDa (light green arrow) and a long isoform of about 36 kDa (dark green arrow) are expressed in legs. (d) Illustration of the human titin locus, with exons shown in magenta and UTRs in gold, modified from [26,41]. Domain regions are labeled and boxed regions denote variable patterns of skipped exons. Titin isoforms in human heart contain the N2B region and shorter PEVK domains (tan box), while skeletal muscle titin contains the N2A region (blue box) and longer PEVK domains. Mutation of splicing regulator Rbm20 results in the inclusion of additional PEVK exons and longer titin isoforms. Diagrams are oriented 5' to 3'