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Abstract

Muscles generate forces for animal locomotion. The contractile apparatus of muscles is the sarcomere, a highly regular array of large actin and myosin filaments linked by gigantic titin springs. During muscle development many sarcomeres assemble in series into long periodic myofibrils that mechanically connect the attached skeleton elements. Thus, ATP-driven myosin forces can power movement of the skeleton. Here we review muscle and myofibril morphogenesis, with a particular focus on their mechanobiology. We describe recent progress on the molecular structure of sarcomeres and their mechanical connections to the skeleton. We discuss current models predicting how tension coordinates the assembly of key sarcomeric components to periodic myofibrils that then further mature during development. This requires transcriptional feedback mechanisms that may help to coordinate myofibril assembly and maturation states with the transcriptional program. To fuel the varying energy demands of muscles we also discuss the close mechanical interactions of myofibrils with mitochondria and nuclei to optimally support powerful or enduring muscle fibers.

Highlights

– Update on the pseudo-crystalline structure of the sarcomere

– Molecularly detailing the development of force resistant muscle-tendon attachments

– Model for tension-driven myofibril self-assembly and myofibril maturation

– Mechanical coordination of muscle morphogenesis by transcriptional feedbacks

– Muscle-type specific mitochondrial and myofibril architectures

Contents

1. The musculoskeletal system

An average human individual synthetises per day about half its body weight in form of ATP to power basic body functions. Each ATP/ADP molecule is turned over about 1000 times per day [1,2]. An exercising athlete, such as a Tour de France cyclist, can consume up to 32 Mega Joule during a five-hour stage, more than five times the energy used during a regular day [3]. This enormous amount of energy is largely needed to fuel the myosin motor proteins of the muscles that produce the mechanical forces to power cyclists up the climbs. Even more impressive, flying animals power themselves into the air, with mosquitos beating their wings up to 1000 times per second giving rise to their well appreciated tone [4].

For the efficient conversion of chemical energy into body movements it is critical that first, the energy is produced close to the consuming motors; second, the force generation of the many individual motor proteins is efficiently coordinated; and third, the force is converted into effective movements of the skeleton. If any of these conditions were not met, the muscles would run out of energy or largely produce heat instead of movement. Hence, the musculoskeletal system is a highly optimised biochemical apparatus that needs to form during animal development to match specific needs. In this review, we aim to cover the morphogenesis of muscles with a particular focus on the mechanobiology of its contractile machine, the sarcomere, the force transduction to the skeleton and lastly muscles' intracellular compartmentalisation optimised to fuel the molecular motors.

Skeletal muscles in vertebrates or body muscles in insects are large multinucleated cells, called muscle fibers, which can be tens of centimetres long in large vertebrates and are about 1 mm long in small fruit flies [5,6]. Both muscle fibers ends are connected via tendon cells to the skeleton, a bony endoskeleton in vertebrates or a chitin-based exoskeleton in insects [5-7]. The precise insertion sites of the tendons at the skeletal elements together with hinges placed between different skeletal elements ensure that shortening of the muscle fibers causes effective

movement of legs or wings (Figure 1A). Hence, the morphogenesis of skeleton, tendons and muscles must be precisely coordinated during animal development [8-10].

1.1 The sarcomere – a pseudo-crystalline force producing machine

At the 'heart' of every muscle fiber is the muscles' contractile machine, the sarcomere. We know since pioneering work in the 1950ies that sarcomeres contract by a sliding mechanism [11,12]: thick bipolar myosin filaments, which are anchored at the sarcomeric M-band, pull on precisely organised parallel actin filaments, which are cross-linked at the sarcomeric Z-discs bordering the sarcomere (Figure 1B). Hence, by pulling on the actin filaments the myosin motors slide into actin filaments, shorting the sarcomere and thus shortening the muscle fiber [13].

How is sarcomere length controlled? Actin and myosin filaments are stably connected by the largest human protein, titin, whose N-terminus is bound to α-actinin at the Z-disc, whereas its C-terminus is located in the middle of the sarcomere embedded into the M-band (Figure 1B) [14-17]. Thus, titin is thought to serve as the ruler of the sarcomere determining its resting length, usually between 2 and 3 μm in vertebrate muscle, depending on species and muscle type [18-20].

Mature sarcomeres display a pseudo-crystalline order of their components. In particular, insect flight muscles are so regular that structural changes of contracting sarcomeres can be observed in the flying animal (during tethered flight) using X-ray diffraction [21,22]. This provided molecular evidence for the long-proposed stretch activation mechanism of insect flight [23-25]: stretch-induced myosin and possibly troponin deformations are needed for efficient binding of myosin motors to actin and thus to trigger fast oscillations of the antagonistic insect flight muscle pairs. This showed that sarcomeres are excellently suited for structural studies. Understanding the mature structure is the first step to understand how a sarcomere is built

during muscle development. Hence, we briefly summarise our current structural knowledge of the sarcomeric parts.

Z-discs: despite its high regularity, resolving the native molecular structure of a sarcomere in muscles is still an ambitious goal, especially for protein dense and complex regions such as the Z-disc. However, significant progress has been made using X-ray crystallography of purified sarcomeric proteins that are present in the Z-disc, in particular its core member α-actinin. α-actinin contains an N-terminal actin binding domain, four central spectrin repeats and a C-terminal calmodulin-like domain. The latter binds to the N-terminal Z-repeats of titin and thus anchors titin directly at the Z-disc [26,27]. The high-resolution structure of the purified 200 kDa α-actinin dimer provides a first basis for how this interaction may be regulated [28 29]: the structure revealed that PIP2 binding to α-actinin's actin binding domain can release the calmodulin-like domain and hence make it available for titin binding [28], as has been suggested by biochemical experiments [30,31]. Interestingly, mutations in α-actinin that alter the titin interaction dynamics show defects in sarcomere formation in cell culture models [28]. Furthermore, the structural model of the anti-parallel α-actinin dimer places the two actin filaments bound to it 23 nm apart, which is in almost perfect agreement with the 24 nm spacing of tetragonal Z-disc lattice found in native mammalian muscle by electron microscopy [32]. The structure further suggested that the bound actin filaments have rotational freedom with respect to the stiff α-actinin rod, in analogy to a 'pivot and rod' structure, resulting in some structural flexibility of Z-disc [29].

The flexibility of the Z-disc can also be affected by its thickness, which varies depending on mammalian muscle type from 30 nm in fast fibers up to 100 nm in slow and cardiac fibers [33] and about 80 nm in insect flight muscles [34] strongly suggesting that multiple columns of crosslinked α-actinin exist at the Z-disc (Figure 1B). Differences might be regulated by alternatively spliced titin isoforms containing variable number of Z-repeats [29,35]

and thus more α-actinin binding sites or by presence of alternatively spliced Zasp (Z-disc Alternatively Spliced Protein) isoforms, part of the Alp/Enigma conserved family of scaffolding proteins, which can also interact with α-actinin via its PDZ domain and were recently proposed to regulate the diameter of the Z-disc in insect muscles by an oligomerisation mechanism [36]. The native sarcomeric Z-disc also houses a proposed titin cross-linker telethonin [37], the actin capper CapZ [38] and the actin cross-linker filamin [34,39,40]. However, their precise function for the molecular arrangements within the Z-disc need to await a molecular model of the Z-disc.

Thin and thick filaments: to investigate the architecture of thin and thick filaments as well as their regulated interaction structural studies using purified *in vitro* assembled subcomplexes have been very insightful. Cryo-electron microscopy has revealed the architecture of a F-actin–tropomyosin complex with and without a myosin head demonstrating how a tropomyosin shift enables myosin binding and how the myosin motor head interacts with the actin filament at the molecular level [41-43]. This provided a molecular model for how myosin forces are transduced to actin filaments in a regulated way in the sarcomere [43]. Thus, *in vitro* assembled structures of sarcomeric components can teach us about the likely *in vivo* arrangement of these components and will be key structural models to resolve the native sarcomere structure *in situ* [44].

Taking advantage of the stability of sarcomere subcomplexes, *Drosophila* or *Lethocerus* flight muscle thick filaments have been isolated and analysed with cryo-electron tomography. Although not yet at atomic resolution, these studies visualised the long α-helices of the myosin rods in their native environment and revealed how their parallel arranged tails are packed together: interestingly, the individual myosin tails run in an angle from the main direction of the thick filament, forming a layered or 'ribbon-like' super-structure slowly

wrapping around the thick filament axis [45,46], similar to what has been proposed in the 1970ies [47].

M-band: thick filament associated proteins, such as myosin binding protein C in vertebrates and myofilin or paramyosin in insect muscles, have not yet been fully resolved structurally. Similarly, the native structure of the key M-band components myomesin and obscurin are unknown. Myomesin forms C-terminal dimers with a suggested role to bridge between neighbouring thick filaments (Figure 1B) [48,49]. Interestingly, these myomesin dimers can be elastically stretched to more than double their length at forces around 30 pN that might be physiologically relevant during muscle contractions [50]. This is consistent with a proposed strain absorber function of myomesin at the M-band [51]. Myomesin is not present in insect muscles, however the large M-band protein obscurin (Unc-89) is conserved and required for M-band alignment in *Drosophila* flight muscles and *C. elegans* body muscles [52-55]. In mammalian muscle, obscurin binds to the C-terminus of titin as well as to myomesin and hence also localises to the M-band [56-58]. However, its role in sarcomerogenesis is unclear [59].

Towards a first native sarcomere structure: one recent approach towards resolving the native sarcomere nanostructure with all its components included was to perform cryo-focused ion beam-milling electron tomography on intact myofibrils isolated from mouse muscles. This revealed that α-actinin dimers indeed cross-link anti-parallel actin filaments at native Z-discs [60]. It also provided a structural explanation of how actin filaments, helped by stabilising myosin cross-bridges, transition from their square-like symmetry at Z-discs to their hexagonal pattern in the A-band [60]. Future cryo-electron tomography studies should reveal further details of the native sarcomere nanostructure.

1.2 Titin – a ruling and force sensing giant spring protein

Titin is a key sarcomeric protein that in vertebrate muscle spans across half the sarcomere, with its N-terminus anchored at the Z-disc and its C-terminus embedded into the M-band. Its function in muscle architecture as well as muscle mechanics has been excellently reviewed recently [61,62], thus, we focus here on some recent highlights providing overwhelming evidence that titin does rule sarcomere length and that titin can be a force sensing spring.

In mammalian skeletal or cardiac muscle, the myosin filament is 1.6 μm long (30 nm diameter) [63], consisting of about 300 muscle myosin II hexamers [64-66]. The A-band part of titin consists of immunoglobulin (Ig) and fibronectin type 3 (Fn3) domain super-repeats that are constitutively present in the classical long titin isoforms in all muscle types. Hence, the length of these titin super-repeats was long suggested as a blueprint, or ruler, for the observed stereotypic length of the thick filaments. Indeed, genetic reduction of the number of A-band Ig-Fn3 titin super-repeats caused the expected reduction of thick filament length and as consequence, these muscle fibers produce lower forces [67]. This provided direct evidence for titin's role regulating thick filament length and thus A-band length of mammalian sarcomeres (Figure 1C).

If titin determines A-band length, which is constant during the sarcomere contraction cycle, how about the I-band, whose length changes during contraction? The I-band part of titin consists of a long series of Ig domains followed by an unstructured region rich in amino acids proline, glutamic acid, valine and lysine (PEVK), which are known to function as an elastic spring, extending and relaxing during sarcomere contraction cycles (Figure 1D) [61,62,68].

The length of the I-band part of titin is muscle-type specific and regulated largely by alternative splicing, with the general correlation that long Ig series and flexible PEVK containing titin isoforms result in long I-bands present in mammalian skeletal and insect body muscles, whereas short titin I-band versions, containing few PEVK and less Ig domains, result in short I-bands present in cardiac muscle or insect flight muscle [61,69-71]. As a result, cardiac or

flight muscles are much stiffer compared to skeletal muscle. This has important functional consequences, particularly prominent during mammalian heart beat or insect flight, both of which are regulated with a large stretch-activation component [72,73]. It is hypothesized that this stretch-activation (or length-dependent activation) requires the shorter and stiffer titin isoform to communicate stretch effectively to fully activate actin and myosin filaments for the next contraction cycle [22,61,69,74].

Nevertheless, these findings provided only indirect evidence that titin rules I-band length in muscles. Direct evidence was recently obtained by deleting a large part of titin's PEVK spring using mouse genetics. Interestingly, the resulting mice are viable, grow to normal size and have muscles with normal lengths, however, their sarcomeric I-bands are shorter, resulting in shorter sarcomeres (Figure 1C). As a consequence, the muscle fiber assembles more sarcomeres, likely by a tension-driven assembly mechanism (see section 3), to mechanically connect over the same length, forming a muscle fiber that is stiffer compared to wild type [75]. Surprisingly, these mice are performing equally well, at least in a simple running wheel assay [75]. This provided direct evidence that titin's flexible PEVK spring determines the length of the I-band and largely contributes to the elasticity and thus passive stiffness of the muscle fiber.

The elastic properties of the PEVK spring have been investigated using single molecule atomic force microscopy or optical tweezers on native titin or recombinantly expressed protein domains. This established that titin's PEVK sequence acts as a perfectly elastic spring at low pico-newton forces [76-78], which are likely present on endogenous titin during sarcomere contraction-relaxation cycles *in vivo* (Figure 1D) [17,79]. However, initial pulling experiments suggested that much larger forces are needed to unfold titin's Ig domains [77,80,81], questioning whether or not Ig domain unfolding may also happen during normal muscle contraction cycles. Interestingly, recent experiments pulling with magnetic tweezers on recombinantly expressed

titin fragments showed that at least some titin Ig domains can already unfold and refold at forces around 10 pN [82-84], which are likely present across individual titin molecules *in vivo*. Refolding of Ig and PEVK domains can produce work and hence can contribute to force produced by a contracting sarcomere, in addition to the active myosin force (Figure 1D), similarly to how a stretched spring stores elastic energy released when recoiling back to normal its normal length [82-84]. It has been estimated that the refolding of titin's domains under load can produce a work of about 40zJ per titin molecule [84], which is comparable to the work produced by one myosin motor cycle [85]. On the other hand, during muscle contraction cycles an antagonistic muscle is usually stretched (not shown in Figure 1A), hence work is needed to unfold PEVK and potentially Ig domains in the antagonistic muscle. Thus, the net contribution of titin refolding to the active contractile force *in vivo* remains unclear.

The range of forces present across a titin molecule in a native sarcomere has not yet been determined. However, the recent introduction of a TEV protease cleavage site at the end of the I-band part of mouse titin provided very strong evidence that titin does transduce forces *in vivo*: cleaving titin in the I-band in permeabilised muscles *ex vivo* resulted in quick dislocation of the myosin filaments and a splitting of the sarcomeric A-band, when muscle contractions were induced [86]. Passive tension in these titin cleaved muscles is reduced by more than 50% [84,86]. This demonstrated the key role of titin as an elastic buffer for the forces exerted by myosin during muscle contraction by stably linking the Z-disc to the sarcomeric M-band. Hence, titin is in the perfect position to sense the molecular forces present in a native sarcomere.

2. Muscle attachments

2.1 Force-resistant muscle attachments: how to link a sarcomere to the skeleton

As discussed above, effective body movements require not only effective force production in the muscle fibers but also an efficient force transduction to the skeleton (Figure 1A). As bone

or chitinous skeleton is generally hard, whereas muscle tissue is soft, this generates high demands for the material interfaces connecting the two tissues [87]. Nature solved this challenge by placing tendon cells between muscles and skeleton, hence allowing for a more graded transition between these different cell types with such different stiffness. In mammals, tendons cells are surrounded by a graded extracellular matrix (ECM) containing triple helical collagen I, as well as collagen II, together with increasing amounts of mineralised calcium phosphate (apatite) closer to the bone surface [5,88]. In *Drosophila*, which only possess a single tendon cell layer between the cuticle and the flight muscles (Figure 2A), the ECM facing the apical surface of the tendons is formed by the gigantic, fiber forming protein Dumpy [89-91], whereas its basal side connecting the tendons with the muscle fibers is an integrin-based adhesion with tiggrin, laminins and possibly collagen IV as ECM components [92]. Thus, a material with a graded stiffness is used to stably anchor soft muscles to the stiff skeleton.

The terminal sarcomere of each myofibril faces a similar challenge as it needs to stably attach to the ECM, which links muscle cells to tendons. One strategy to increase the anchoring stability is to increase the surface contact. Hence *Drosophila* flight muscles and also the intercalated discs of cardiomyocytes linking neighbouring sarcomeres, contain extensively folded membrane indentations that largely increase the interaction surface (Figure 2A) [93,94]. Recently, super-resolution microscopy of flight muscle-tendon attachments revealed an interesting layered organisation between the ECM and the terminal Z-disc of a myofibril [94]: proximal to the membrane is the integrin signalling layer with an extended integrin adaptor talin. Talin, in its extended conformation, recruits vinculin in a force dependent manner [95,96]. Additionally, focal adhesion kinase (Fak) is present in the signalling layer (Figure 2A). This is followed by an actin-rich layer with Arp2/3, filamin, Zasp and α-actinin nucleating and cross-linking actin into a dense but not well-ordered actin network that links to the terminal Z-disc of the myofibril (Figure 2A) [94]. This complex organisation can enable various feedback

mechanisms to optimally adjust adhesion in response to the high force produced by muscle fibers.

2.2 Quantifying molecular forces at muscle attachments

Integrin adhesions are very well characterised force transducing macro-molecular assemblies [97]. One key component linking integrin tails to the actin cytoskeleton and hence mediating the force transduction between integrins and the cytoskeleton is the integrin adaptor talin [98,99]. Talin binds with its head to the beta-integrin tail and with its rod to the actin cytoskeleton, which upon the application of force opens to expose vinculin binding sites that in turn enable more actin filaments to pull on talin and induce focal adhesion growth [100,101]. Hence, talin is at a key location to sense the range of forces present at muscle attachment sites.

Recent developments of genetically encodable FRET-based molecular force sensors made it possible to quantify molecular forces across proteins in cell culture or *in vivo* [102]. These sensors contain a small mechanosensitive peptide between the FRET pair with a calibrated force range between 2 to 11 pN [102]. The application of high forces unfolds the elastic peptide, resulting in low FRET values (Figure 2B).

Such molecular tension sensors have been inserted into the endogenous talin gene in *Drosophila* to quantify forces across talin at flight muscle attachments [103]. Surprisingly, it was found that in mature adult flight muscles at rest, only a small amount of the talin molecules is under force. However, if talin amounts are reduced to about half, the flight muscle-tendon junction mechanically ruptures. This suggests that recruiting a large pool of talin molecules to mature attachments is an efficient way to share peak forces likely present during active flight muscle contractions [103]. We can wonder how recruitment of such a large pool of talin molecules, and all other sarcomeric components, is coordinated temporally and spatially during

muscle development in a regulated way? We are addressing this question in the following section.

3. Muscle and sarcomere morphogenesis

Muscle development with a focus on muscle patterning or myoblast fusion has been excellently reviewed recently [104-109]. Here we focus on recent advances in mechanical aspects of muscle morphogenesis and sarcomere development in particular. We use the well-studied *Drosophila* indirect flight muscles as one model system to elaborate common concepts of muscle and sarcomere morphogenesis as well as to highlight specific differences to mammalian skeletal muscle development.

3.1 Muscle-tendon attachment

Each of the *Drosophila* indirect flight muscles is built during pupal stages by fusion of several hundred myoblasts to large syncytial myotubes [110,111]. These myotubes elongate at both ends towards their future attachment sites to which they establish a force-resistant integrin dependent attachment at 24 h after puparium formation (24 h APF, Figure 3A) [112,113]. During myotube elongation, myoblast fusion is largely restricted to the lateral membranes and absent from the dynamic leading edges, which are extending towards the tendons [113,114]. A similar myotube elongation process towards developing tendon attachments initiates myotube development in other *Drosophila* muscle types and also in a wide range of vertebrate muscles [8,115-118]. Hence, elongation and attachment are common principles of early muscle morphogenesis that define the long axis of each muscle fiber. This axis also determines the future muscle contraction axis, which will not be changed anymore during later steps of muscle morphogenesis.

During myotube-tendon attachment, mechanical tension is built-up within the myotendinous system: initially, tissue tension is low and only small amounts of integrin, talin and other integrin associated proteins are present at myotube attachment sites. When tissue tension increases, large amounts of integrin and talin are recruited and attachments mature over the next 6 h (until 30 h APF) [103,113]. This tension increase causes the compaction of the flight muscle myotube and its transition into an immature myofiber at 30 h APF (Figure 3A), coinciding with the self-assembling of immature myofibrils that connect both attachments (see section 3.2) [103,113,119]. At this stage, attachments have matured enough to be able to resist the first active muscle twitchings [120]. This indicates that attachment maturation and myofibril assembly must be coordinated during development.

How is attachment maturation triggered molecularly and how is it coordinated with tissue tension increase? A first step to understand this coordination was undertaken by quantifying molecular forces across talin at developing muscle attachments (see Figure 2B). Interestingly, molecular tension across each talin molecule is larger at the early stages when few talin molecules are present and gradually reduces when more molecules are recruited [103]. This suggests that force dependent recruitment of talin is one important mechanism of attachment maturation. Integrin associated proteins are also recruited in a force dependent manner in *Drosophila* embryonic muscles. In particular the more peripheral members vinculin and paxillin strongly depend on muscle myosin activity, and thus muscle twitching, for effective recruitment and maturation of embryonic muscle attachments [121]. This fits with the interpretation that force-induced stretching of the talin rod will enable vinculin recruitment [95]. This is also well established in mammalian cells [98,101,102] and in beating cardiomyocytes, in the latter the oscillating forces across talin have even been directly quantified *in vitro* [122]. Together, this suggests that the tension dependent recruitment of large amounts of integrin complex proteins provides a buffer for peak forces produced by muscle contractions. This mechanical

feedback mechanism coordinates muscle-tendon attachment morphogenesis with formation of contractile sarcomeres and prevents a fatal rupture of attachments.

3.2 Myofibril and sarcomere assembly

Developing myofibers are very long cells connecting two skeletal elements. However, each contractile sarcomere is only a few micrometres long (see Figure 1). How do myofibers manage to assemble the correct number of sarcomeres into long myofibrils to bridge the long distance, maintaining but not exceeding the correct tension level in each of its sarcomeric components? One possible mechanism to solve this 'dimension problem' is suggested by the tension-driven myofibril self-organisation hypothesis [68,113]. This hypothesis, postulated in *Drosophila*, is likely to also apply to mammalian muscles [123-126]. It suggests that mechanical tension is coordinating the assembly of many smaller molecular complexes into higher order periodic structures throughout the entire large muscle fiber. These long continuous and periodic myofibrils mechanically bridge both ends of the entire muscle (Figure 3B). Live imaging has shown that a periodic pattern in myofibrils indeed appears simultaneously over long distances in *Drosophila* muscles as well as in cultured cardiomyocytes, suggesting a self-organisation mechanism [113,117,123,124]. How could such a phenomenon work molecularly?

In early stage myotubes, actomyosin is organised in a polar network along the long muscle axis possessing a rather low molecular order without distinct myofibrils [127,128]. As most major sarcomeric components such as titin, muscle myosin or troponin are absent at this stage [120], the main force-producer is non-muscle myosin (Figure 3B). Non-muscle myosin molecularly orders actin filaments into parallel arrays and is needed for efficient assembly of immature periodic myofibrils in flight muscles and mammalian cardiomyocytes [124,125,128]. However, non-muscle myosin assembles into rather short, up to 300 nm long, bipolar filaments [129], too short to span the entire A-band of a mature sarcomere. On the other hand, muscle

myosin assembles several hundred hexamers (composed of 2 heavy chains, 2 essential and 2 regulatory light chains) into 1.6 μm long filaments in mammalian muscle or into more than 3.0 μm long bipolar filaments in insect muscles [65]. Hence with the onset of muscle myosin expression, non-muscle myosin filaments are either replaced with muscle myosin ones, as suggested by the pre-myofibril model [130,131], or the simple increase in muscle myosin levels results in the dominant incorporation of muscle myosin into periodic immature myofibrils that span the entire muscle fiber length (Figure 3B). These myofibrils are chains of immature sarcomeres, containing periodic cross-linked actin filaments of increasing molecular order as development progresses [128], as well as titin, α-actinin, obscurin, Zasp and likely other components [54,113,132,133]. Thus, not only actin and myosin but additional, possibly preassembled, protein complexes are organising into these continuous immature myofibrils [134-136]. They may use mechanical tension as a compass to orient themselves properly to assemble the correct number of immature sarcomeres within each myofibril. The better the assembly, the higher the active force that myosin can produce by pulling on ordered actin filaments (Figure 3A, B) [68].

3.3 Myofibril and sarcomere maturation

Immature sarcomeres are contractile, but they are not yet fully adapted to the specific needs of the respective muscle type, be it a slow or fast mammalian fiber or an insect flight muscle fiber. Sarcomere maturation is particularly well illustrated in *Drosophila* flight muscles, which simultaneously assemble all their 2000 myofibrils per muscle fiber that mature synchronously without addition of new myofibrils until the adult stage (Figure 3A, B) [120]. During these maturation steps sarcomere length grows from about 2 μm to 3.4 μm and myofibril diameter progressively increases to reach about 1.5 μm [120,133,137,138]. How is such a defined sarcomere growth achieved?

Although not understood in molecular detail, it is likely that the relative amounts and the specific sarcomeric protein isoforms present at any time point determine the precise sarcomere architecture during sarcomere maturation. Expression of muscle myosin heavy chain isoforms changes via a switch in alternative splicing in flight muscles [137,139] or via a transcriptional switch during mammalian muscle development from embryonic to fetal and finally to adult isoforms (Figure 3B) [140]. Actin filament length growth is controlled by the expression of capping proteins like Tmod and Sals [141-143], as well as actin nucleators, including the formin family member Fhos or the short nebulin homolog Lasp during flight muscle sarcomere maturation [144,145]. Similar proteins are needed to control actin filament length in mammalian muscle development, such as the formin homolog FHOD3 [146], and in particular the gigantic protein nebulin whose proposed function as actin filament ruler has been reviewed in detail [62,147-149].

A particularly impressive way of how myofibril diameter can be regulated was recently proposed for flight muscles: the three distinct Zasp genes (Zasp52, Zasp66 and Zasp67) encode either short Zasp isoforms only containing a PDZ-domain and the Zasp-motif (ZM) domain or long isoforms with additional 1 to 4 LIM domains [132,138]. Interestingly, these Zasp proteins are core components of the Z-discs and can form aggregates by an interaction of their ZM domain with one of the LIM domains, whereas their PDZ domain interacts with α-actinin [132,150]. Consequently, Zasp isoforms with one or more LIM domains promote ZASP complex growth and hence Z-disc growth, whereas short isoforms, lacking any LIM domain, block further growth. Strikingly, expression of 'growing' isoforms is high during myofibril maturation and diameter growth, whereas 'blocking' isoforms are upregulated only very late to terminate growth. Consistently, 'growing' isoforms are found located more centrally in the Z-discs, compared to blocking isoforms [138]. These findings underscore the importance to coordinate

transcriptional regulation with sarcomere morphogenesis to form functional sarcomeres (see sections 3.4 and 3.5).

3.4 Muscle fiber growth

In mammalian embryonic and adult muscles, muscle fiber growth is largely driven by continuous myoblast fusion to existing fibers [151,152] resulting in a fiber volume per nucleus correlation termed the myonuclear domain size [153]. Careful volume measurements in *Drosophila* larval muscles also revealed a striking correlation of muscle fiber volume with nuclear volume [154]. This correlation is maintained during the rapid insulin-induced growth of the *Drosophila* larval muscles: instead of additional myoblasts fusing, the larval muscle nuclei undergo several rounds of DNA endo-replication to produce very large nuclei, with up to 64 copies of each chromosome [154,155]. Interestingly, the number of endo-replication cycles appears to be regulated mechanically via the LINC complex, which links the lamin meshwork below the nuclear membrane to the sarcomere and microtubule cytoskeleton [156]. This provides a possible mechanical feedback mechanism to control nuclear endo-replication cycles during larval muscle fiber growth.

Further support for a significant mechanical input to the muscle nuclei is given by their specific positions. In *Drosophila* larval muscles, nuclei are initially transported by microtubules to clusters located close to the fiber ends [157,158]. When sarcomeres have assembled and the fibers grow, the nuclei are redistributed and equally spaced along the fiber length, suggesting that a force-balance mechanism equally positions them per muscle volume [154,159]. Furthermore, the larval nuclei are pushed to the surface of the larval muscles by the growing myofibrils and microtubules [160]. Very similarly, nuclei in mature mammalian muscle fibers are also equally spaced along the fiber axis and are pushed to the fiber surface by the maturing and contracting myofibrils [161,162]. In developing flight muscles, most nuclei are located centrally at

24 h APF before myofibrils are forming (Figure 3A). Upon myofibril assembly, they relocate and fill the space between the growing myofibril bundles [163]. As a consequence, all nuclei adopt an elongated shape along the myofibril axis. Together, this strongly suggests that myofibrils impact nuclear shape and mechanics during muscle fiber growth.

Drosophila flight muscles grow tremendously after myoblast fusion has ceased, increasing their volume more than ten-fold in three days (Figure 3A) [163]. Since their final number of myofibrils is already determined at 30 h APF, flight muscle length growth coincides with the addition of new sarcomeres to each existing myofibril, while their increase in diameter causes muscle diameter growth (Figure 3A, see section 3.3). Additionally, the mitochondrial content increases enormously (see section 4) [164]. It is unknown if flight muscle nuclei undergo endoreplication, however a classical tissue growth pathway was recently shown to be essential during postmitotic flight muscle growth. Loss of the transcriptional regulator Yorkie (YAP, TAZ in mammals) or gain of the Hippo or Warts kinases, which are negatively regulating Yorkie, result in a block of flight muscle fiber growth [163]. While these fibers finish myoblast fusion normally, they assemble fewer myofibrils, which then fail to mature, resulting in a dramatic muscle growth defect [163]. The Hippo pathway has a well described key mechanosensory function at the epithelial cell cortex regulating stretch-dependent epithelial cell growth [165-168]. However, how can the Hippo pathway sense stretch in muscle fibers to induce muscle growth?

Interestingly, the classical upstream Hippo regulators Expanded, Merlin or Kibra that are involved in sensing stretch at the epithelial cell cortex do not seem to be required in flight muscles. Instead, the STRIPAK phosphatase complex controls Hippo activity. Slmap, one member of the STRIPAK complex binds to Dlg5, both of which are present in internal membranes forming the future T-tubules in proximity to the assembling myofibrils [163]. Thus, it was speculated that in analogy to sensing stretch at the epithelial cell membrane, mechanical

stretch might be sensed at these internal muscle membranes to control Hippo kinase activity before and after myofibril assembly. Consequently, expression of key sarcomeric components is reduced upon loss of Yorkie, suggesting that mechanics and possibly myofibril assembly status may feedback on the transcriptional state of the developing muscle fiber to induce its further growth [163].

3.5 Transcriptional dynamics recapitulates morphogenesis phases

As highlighted above, muscle fiber development can be divided into multiple phases such as early muscle fate choice, myoblast fusion, myotube elongation and attachment, myofibril assembly and maturation, as well as muscle fiber growth. Flight muscles are a suitable model to study how transcriptional dynamics may impact the different phases, as their development is largely homogenous. A systematic transcriptomic time-course covering flight muscle development from myoblast stage in third instar larvae to mature adult flight muscles revealed that the different morphogenesis phases are indeed preceded by important transcriptional transitions [120]. As expected, genes implicated in patterning and cell division dominate the early phase when myoblasts are dividing and their future muscle fate is determined (Figure 3C). Then cell adhesion and muscle attachment genes become upregulated, fitting with the next morphogenesis step, the attachment of the myotubes to tendons. At this stage, the expression of sarcomeric genes is still very low, showing that only after successful myotube attachment the expression of the key sarcomeric genes is initiated enabling myofibril assembly (Figure 3C) [120]. As discussed above, one signalling pathway that may react to mechanical input is the Hippo pathway needed for effective sarcomere gene expression [163]. However, as Hippo signalling appears to be particularly important only in flight muscles it remains to be seen how other muscles regulate this important transition.

A subset of sarcomeric genes or gene isoforms, including the titin-like protein Stretchin-MLCK, are only expressed at late stages during sarcomere maturation, once the general sarcomere scaffold is built [120]. How could this late boost of sarcomere expression be controlled? General muscle-specific regulators such as Mef2 are needed throughout flight muscle development [169] and also the flight muscle identity transcription factor Spalt is needed both at the beginning to set up muscle fate and for the efficient boost at late stages [120]. Interestingly, E2F, known for its key role during the cell cycle in many cell types [170], is dispensable for myoblast proliferation or fusion in *Drosophila* muscles and only needed at late stages for myofibril assembly and, in particular, for myofibril maturation and fiber growth [171]. Together with the results from the Hippo pathway, this demonstrates that key transcriptional regulators controlling cell cycle and growth in dividing tissue can be repurposed allowing postmitotic muscle cell growth.

It is important to note that flight muscle morphogenesis is not only controlled at the transcriptional level, but alternative splicing is also critical in many different muscle types [70,172]. In flight muscles, the splicing regulator Bruno (arrest), a CELF family member, is transcriptionally induced by Spalt at the stage of myofibril assembly. However, Bruno is largely needed to instruct flight muscle specific splicing during sarcomere maturation, for example enabling splicing of the correct Stretchin-MLCK isoform. Consequently, *bruno* mutant flight muscles show severe myofibril maturation defects [139,173]. This further illustrates that transcriptional dynamics must be well synchronised with the morphogenesis phases of the muscle fibers.

4. Morphogenesis coordination within a myofiber

As illustrated above, maturing muscle fibers are very densely packed cells with a defined tension axis. While it is well established that the mechanical properties of the extracellular

environment can directly impact cell fate, including muscle fate choice [174,175], it is less clear how tension and mechanical interactions between various intracellular organelles and molecular machines are sensed and coordinated [176]. For muscle fibers, it particularly matters to correctly partition their myofibrils, their mitochondria and their nuclei in a fiber-type specific way to efficiently support insect flight or long-distance running (Figure 4).

The increasing crowdedness of differentiating muscle fibers strongly restricts diffusion of mRNAs [177]. Thus, in mature muscle fibers most mRNAs undergo microtubule-based transport when exiting the nucleus before they are translated [177]. Interestingly, mature cardiomyocytes and skeletal muscle fibers show a strong enrichment of ribosomes close to their sarcomeric Z-discs, at which not only mRNAs coding for sarcomeric proteins, but most mRNAs appear to be translated. [177-179]. If proteins are mostly synthetised at these 'translation hubs' [177], how do they efficiently reach their destinations in the nuclei, mitochondria or muscle attachments, as protein diffusion in dense myofibers is restricted [180]? This is a particular challenge for the most abundant organelle inside muscle fibers, the mitochondria, which import most of their proteins from the cytoplasm [181]. The answer might be resolved by live imaging of individual protein dynamics in muscle in the future.

Three-dimensional electron microscopy techniques [182] are now allowing to reconstruct the detailed 3D morphology of muscle fibers revealing precise mitochondria shapes and their relation to the myofibrils. Interestingly, in mammalian muscles mitochondria form elongated networks whose locations and shapes depend on the muscle fiber type. In glycolytic fibers, elongated mitochondria are largely oriented perpendicular to the contraction axis, whereas in oxidative slow fibers mitochondria form a grid like network and in cardiomyocytes the network is exclusively oriented in the direction of the myofibrils [183]. This leads to an increased proximity between mitochondria and myosin filaments in myofibrils of oxidative and cardiac

myofibers compared to glycolytic ones and hence increase the efficiency of ATP delivery for their function [183].

Oxidative fibers and in particular cardiomyocytes contain up to 35% of mitochondria per total muscle volume [183,184], making it likely that mitochondria are not only providing the myofibers with energy but also influence them mechanically. Indeed, in mammalian skeletal muscles these mitochondrial networks form thin extensions or 'nanotunnels' close to the aligned myofibrils at their sarcomeric I-bands, in proximity to the sarcoplasmic reticulum and the T-tubule network [183,185,186]. Some of these tunnels are connecting two distant larger mitochondria generating an extensive network (Figure 4, right panel) [186]. Interestingly, also in *Drosophila* muscles the mitochondrial shapes and networks strongly depend on muscle fiber-types: cross-striated leg muscles contain complex shaped mitochondrial networks with similar mitochondrial nanochannels along their sarcomeric I-bands as in mammalian muscle fibers [164]. In contrast, flight muscle mitochondria are elongated along each of the individual myofibrils adopting ellipsoid-like shapes that ensheath myofibrils and isolate them from their neighbouring myofibrils (Figure 4, left panel) [164]. This creates a large mitochondria-myofibril interface that will enable an optimised ATP/ADP exchange to feed the high energy demands during flight. Mitochondria-myofibril proximity is emphasized by the finding that myofibrils are pushing the mitochondria into the elongated shapes since releasing the pressure or disassembling the myofibrils results in a rounding of the mitochondria [164]. How can such close ties arise in a coordinated way during muscle development?

Recent studies of developing *Drosophila* flight muscles showed that in myotubes, before myofibrils assembly, mitochondria are thin elongated tubes and assemble to local networks largely excluded from the actin filament bundles [164]. Concomitantly with the assembly of the immature myofibrils (see Figure 3B), mitochondria intercalate between them and insulate each myofibril from its neighbours [164,187]. When myofibrils start to mature and

grow in diameter, mitochondria also dramatically increase in volume and grow until they are pushed from the myofibrils into ellipse shapes (Figure 4, left panel) [164]. At the same time, expression of mitochondrial genes is strongly boosted (Figure 3C) [120,188] and mitochondria dramatically increase their cristae density [128,187], which are invaginations of the internal mitochondria membranes that house the electron transport chain components [189]. The higher the cristae surface, the higher the respiration capacity of the mitochondria, a feature particularly associated with oxidative fibers [190]. Similarly dynamic mitochondria remodelling has been observed in mammalian cardiomyocytes shortly after birth when myofibrils grow in size [191]. This demonstrates that mitochondria morphogenesis is intimately coordinated with myofibril morphogenesis.

One possible mechanism how to coordinate morphogenesis of both organelles has recently been suggested from studies of developing flight muscles. Changing the mitochondrial fusion and fission dynamics during development in the direction of increased fusion and hence larger mitochondrial clusters prevents the intercalation of the mitochondria between the developing myofibrils [164]. Interestingly, this intercalation block causes the alignment of the otherwise individualised flight muscle myofibrils changing them to a cross-striated morphology normally found in leg muscles. Strikingly, the transcriptional status of these flight muscles also changes and sarcomeric protein isoforms that are normally flight muscle specific are reduced, whereas leg muscle specific proteins are boosted. This suggested a mechanical feedback mechanism from the mitochondria and myofibrils back to the nuclei adapting their transcriptional program [164]. As the nuclei are in close proximity to and pushed by the myofibrils to their fiber-type specific positions (Figure 4 and see section 3), it is conceivable that the nuclei themselves, possibly using the LINC complex in the nuclear membrane, sense the mechanical changes and adapt their transcriptional program accordingly [156,192]. Appropriate mitochondrial fusion and fission dynamics are also needed during mammalian muscle and heart development

[193-195] leading us to speculate that similar mechanical feedback mechanisms may coordinate mitochondria with myofibril development in mammalian muscle.

5. Concluding remarks

Cellular mechanics controls the function and the development of most eukaryotic cell types and tissues, with a prime role in muscle and heart. The development of the muscle contractile machinery directly impacts the mechanical properties of muscle cells and hence feedbacks to coordinate its development with its surrounding tissues as well as with the other muscle cell organelles. We are just beginning to understand how such feedback mechanisms work molecularly to coordinate the formation of functional muscle cells. In the future, muscle and heart biology will continue to profit from novel high-resolution microscopy techniques, in particular electron tomography, to investigate the native muscle nanostructure and similarly from the *in vivo* application of live imaging tools that will allow to quantify individual protein dynamics and turnover rates as well as mechanical forces in developing muscle cells. Combining these technologies with genetic models and automated analysis tools will hopefully create the required synergism to reveal the nanostructure of the muscle and understand how biomechanical coordination between its components can assemble such beautiful contractile machines.

Competing interests

The authors declare that no competing interests exist.

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Box 1.

Sarcomere - defined as the region flanked by two Z-discs with a M-band at its center. It is composed of bipolar myosin filaments intercalated with polar actin filaments. Sliding of actin filaments by pulling myosin motors results in sarcomere shortening.

Myofibril - a concatenation of sarcomeres that spans the length of a muscle fiber. Myofibrils form bundles which laterally align in cross-striated mammalian skeletal muscle or insect leg muscle. In fibrillar insect flight muscle, each myofibril is isolated from its neighbours by surrounding mitochondria.

I-band - or 'isotropic' band region, is the zone of the sarcomere without any myosin filaments. It spans across one Z-disc into to the adjacent sarcomere. When the muscle fiber contracts the I-band shortens.

A-band - or 'anisotropic' band region, is the region encompassing myosin bipolar filaments. Myosin filaments are cross-linked at the central M-band by M-band proteins, such as myomesin. A-band length is constant during sarcomere contraction.

M-band - the central part of the sarcomere composed of proteins that laterally cross-link the myosin filaments, likely providing rigidity and stability.

Z-disc - the connection between two sarcomeres. It is composed of several actin cross-linking proteins that stably anchor actin filaments at their plus ends. It also anchors the N-terminus of the gigantic titin protein.

Titin - the gigantic spring protein of the sarcomere. It connects actin to myosin filaments, by spanning in mammals from the Z-disc (titin N-terminus) to the M-band (titin C-terminus). It rules the length of the sarcomere, thus is shortens during sarcomere contraction.

Figure legends.

Fig. 1. (A) The musculoskeletal system: a biomechanical apparatus. The muscle fibers attach via tendons to two bones. Muscle contraction generates force to move the two skeletal elements closer together and thus powers body movement. **(B) The sarcomere: a force producing machine.** Scheme of the basic architecture of a vertebrate sarcomere with myosin filaments (blue) and actin filaments (red), which are stably linked by titin spanning from the Z-disc (dark green) to the M-band (light blue). Note that the myosin containing region is also called the A-band, whereas the myosin free actin region of the sarcomere is called the I-band. The scheme was modified from [68]. **(C) Titin: a molecular ruler.** Titin spans half the length of a vertebrate sarcomere providing a direct template for sarcomere length: PEVK spring domain deletions result in shorter sarcomeres with shorter I-bands, whereas deletions of titin A-band parts lead to shorter myosin filaments and thus shorter A-bands. **(D) Titin: a molecular spring and possible force sensor.** The elasticity of titin is generated by its Ig domain series and PEVK rich region, which extend and possibly unfold in relaxed muscle to store elastic energy. Upon contraction the domains compact or refold again elastically.

Fig. 2. (A) Muscle-tendon attachment: a force resistant connection at molecular resolution. Top: transmission electronic micrograph of a *Drosophila* flight muscle tendon cell connecting one flight muscle myofibril to the chitin exoskeleton (cuticle). Note the zig-zag pattern of the interface between tendon and muscle. Scale bar is 500 nm. **Bottom:** molecular scheme of a terminal Z-disc connecting to a tendon cell. A terminal integrin signalling mechanically connects via an actin-rich layer to the terminal Z-disc of a flight muscle myofibril. Scheme was inspired by [94,103]. **(B) Tension sensors: measuring molecular forces at muscle attachments.** A talin FRET-based tenson sensor allows to quantify molecular forces across talin. The sensor is composed of a mechanosensitive peptide inserted between FRET

donor (D) and acceptor (A). Low tension results in a closed sensor and high FRET, while high force stretches the peptide and reduces FRET. Scheme was inspired by [103] and modified from [102].

Fig. 3. (A) *Drosophila* flight muscle morphogenesis. Myotubes have attached to tendons at 24 h APF and contain a cortical actomyosin network under lower tension. At 30 h APF tension has increased, myotubes have converted to myofibers and have assembled all their immature myofibrils into myofibril bundles. High tension produced long tendon extensions. After 30 h APF each myofibril and hence the entire myofiber grow in length and diameter resulting in a large muscle volume increase. Tendon extensions shrink. **(B) Myofibrillogenesis: sarcomere assembly and sarcomere maturation.** Schemes of actomyosin filament network without periodic patterns which assembles to long immature myofibrils containing periodic muscle myosin and titin. Each myofibril matures by isoform exchange and recruitment of more sarcomeric components to a mature sarcomere. **(C) Transcriptional dynamics recapitulates morphogenesis phases.** Complex transcriptional regulation patterns generate the necessary proteins at precise stages and sufficient quantities to the various muscle morphogenesis phases. Scheme was inspired by [120].

Fig. 4. Muscle fibers: coordinating morphogenesis in a crowded space. Two examples of different mitochondria-myofibril architectures. Left: Adult *Drosophila* fibrillar flight muscles contain round individual myofibrils (red) isolated from each other by large ellipsoid shaped mitochondria (green). Nuclei (blue) are located between the myofibril bundles. Note that myofibrils are squeezing mitochondria into their elongated shapes. **Right:** Mammalian skeletal muscle contain bundles of vertically aligned cross-striated myofibrils with mitochondria concentrated in groups squeezed in between. These mitochondria form long thin

extensions along the sarcomeric I-bands that build networks. In both muscle types the crowded cellular environment generates mechanical pressure that positions the nuclei at the periphery of myofibril bundles adopting an elongated shape.

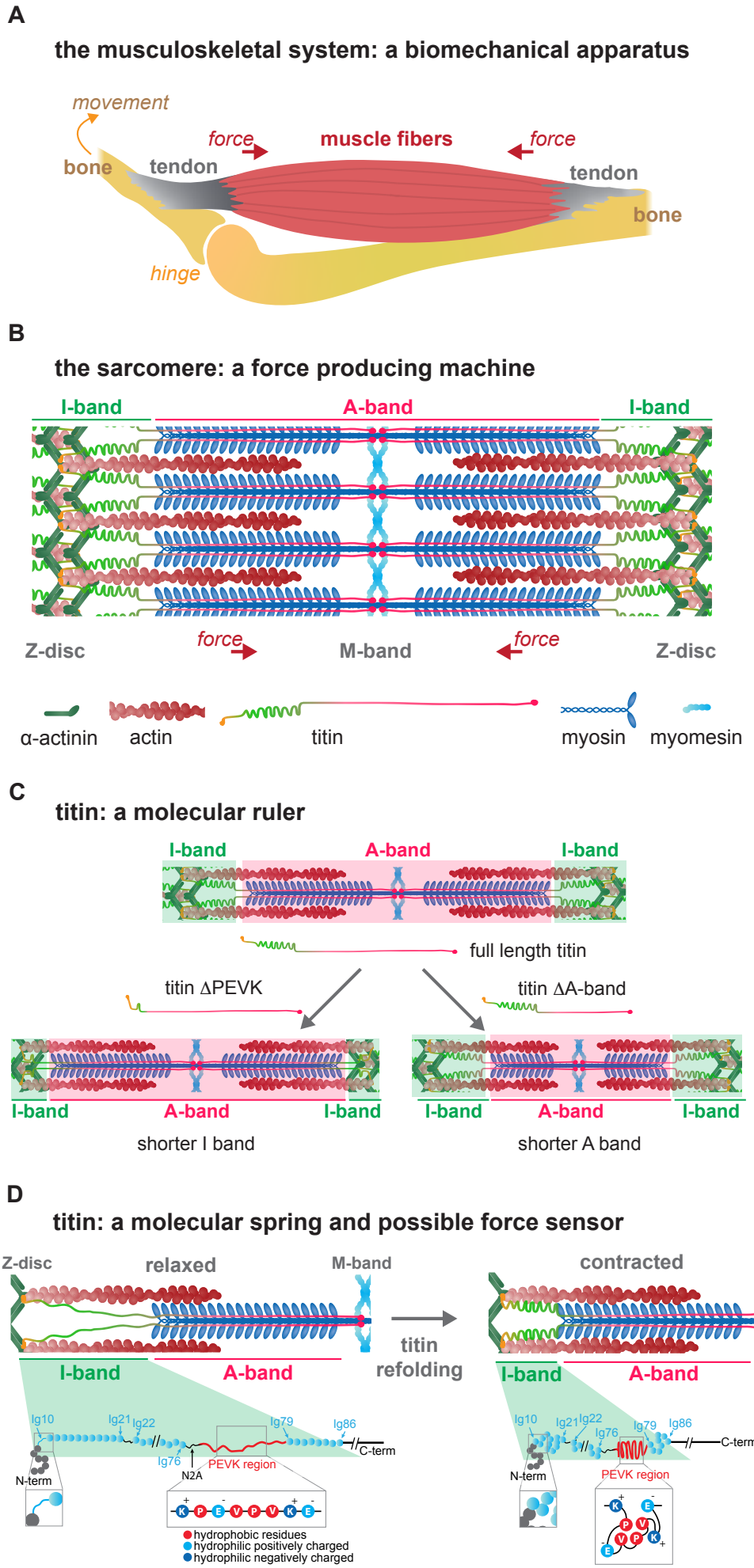
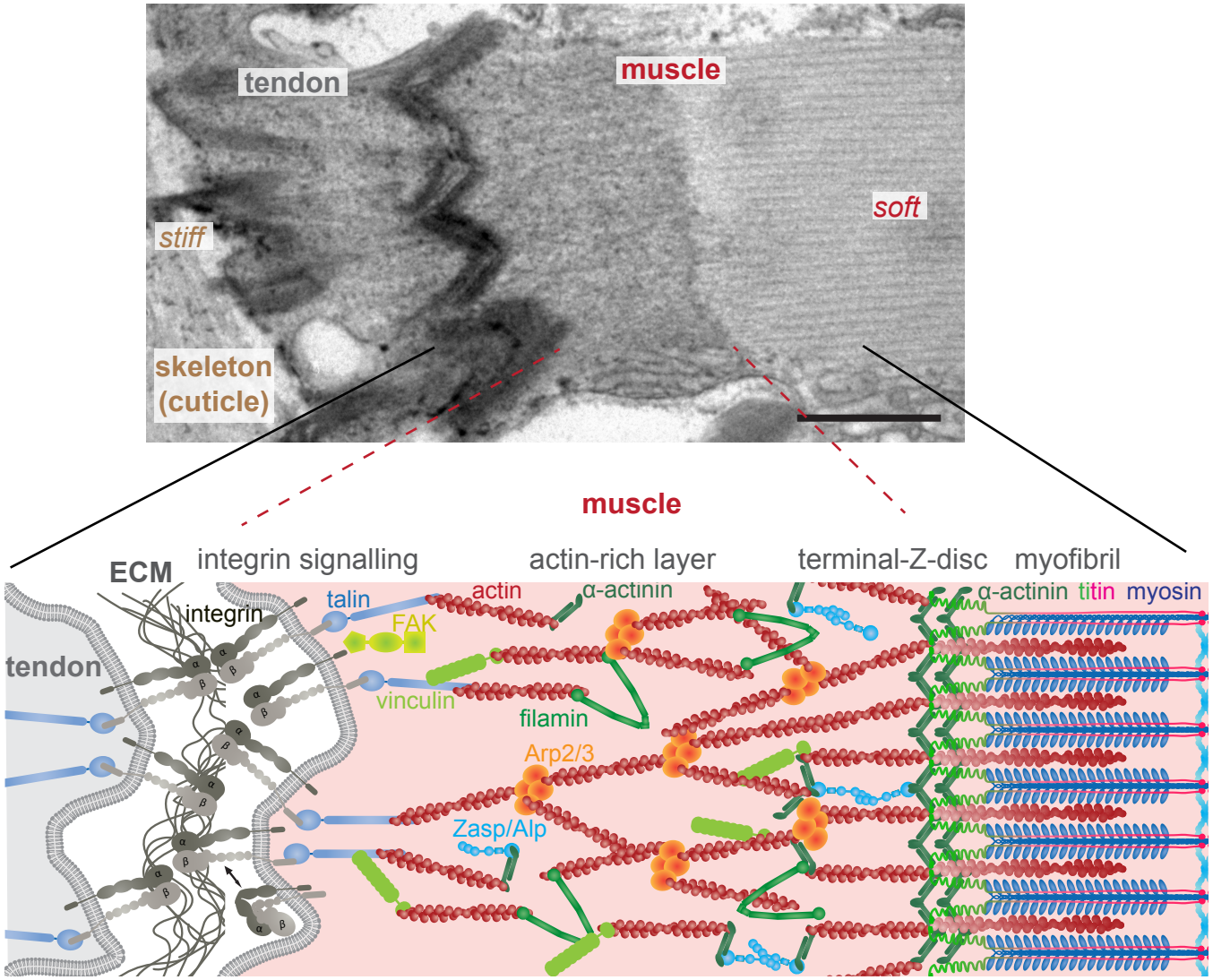


Figure 1

A **muscle-tendon attachment:**
a force resistant connection at molecular resolution



B **tension sensors:**
measuring molecular forces at muscle attachments

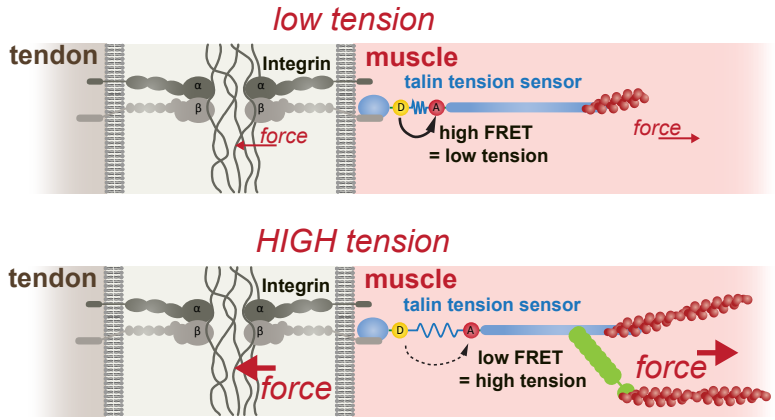


Figure 2

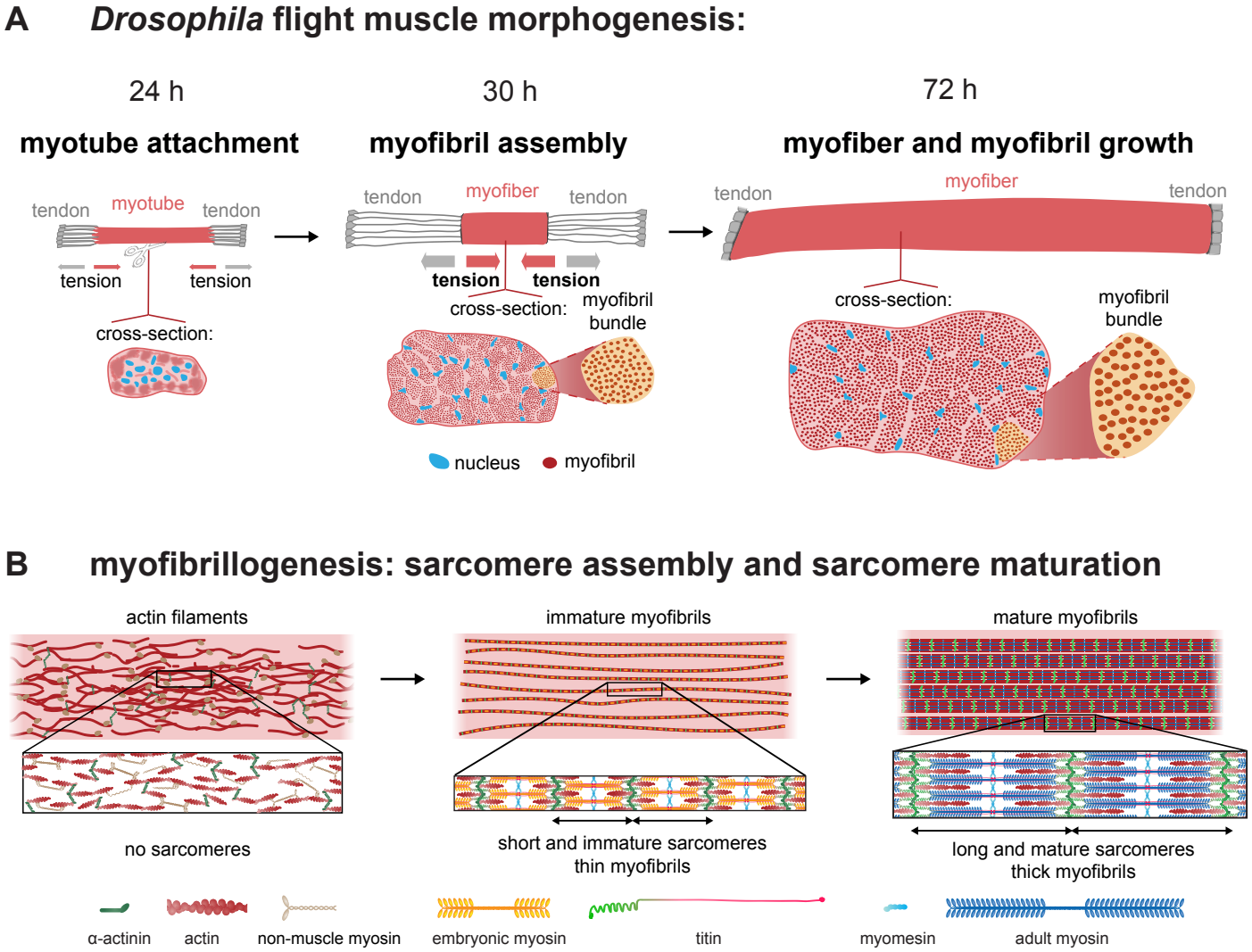
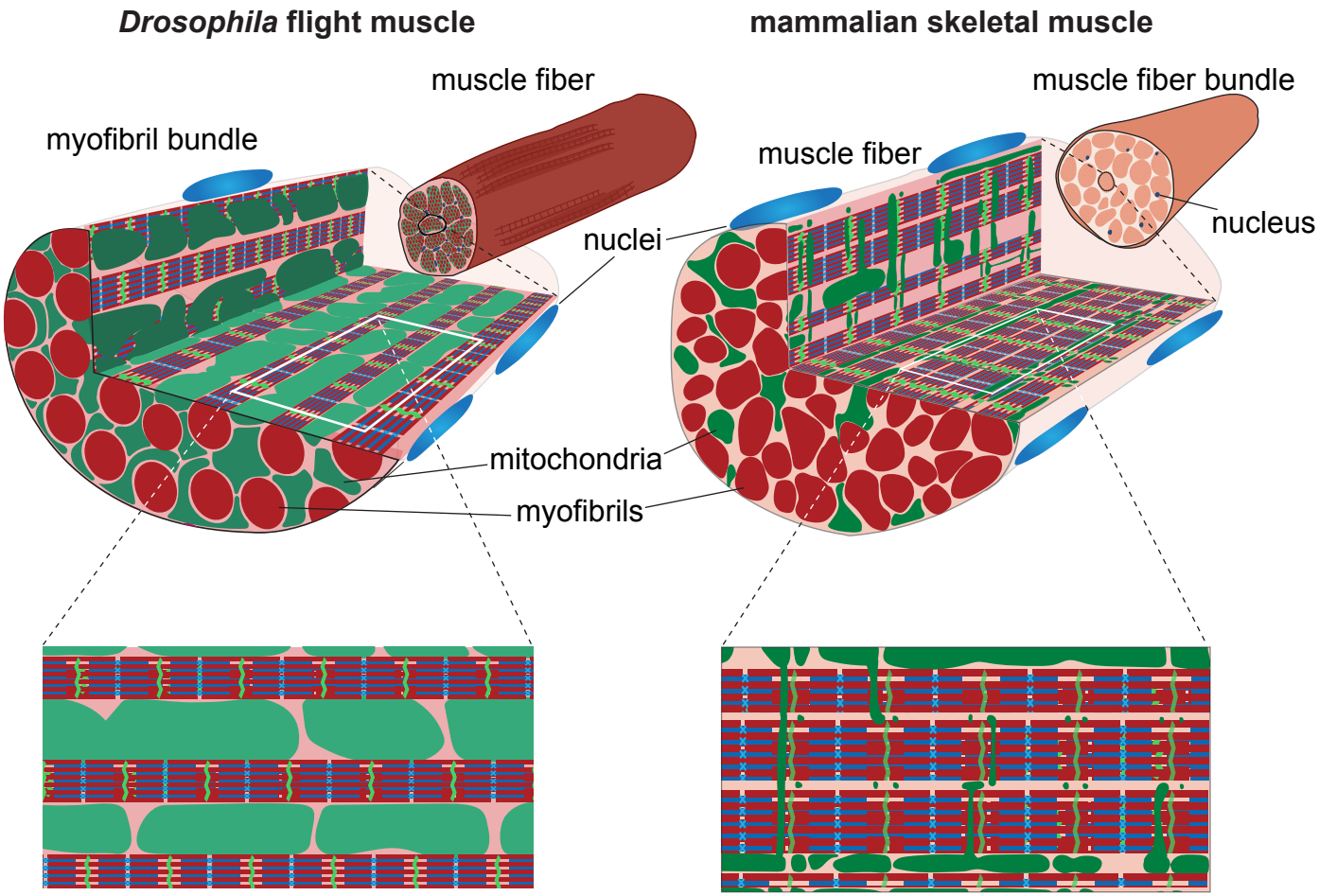


Figure 3

muscle fibers: coordinating morphogenesis in a crowded space



myofibrils: individual 'fibrillar' type
mitochondria: squeezed by myofibrils, isolated & compact

myofibrils: cross-striated type
mitochondria: highly networked, elongated & branched

Figure 4

Credit Author Statement

Nuno M Luis and Frank Schnorrer conceptualised the content, prepared the figures and drafted and revised the main text for this review together.