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Review

Mechanical forces during muscle development

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ABSTRACT

Muscles are the major force producing tissue in the human body. While certain muscle types specialize in producing maximum forces, others are very enduring. An extreme example is the heart, which continuously beats for the entire life. Despite being specialized, all body muscles share similar contractile mini-machines called sarcomeres that are organized into regular higher order structures called myofibrils. The major sarcomeric components and their organizational principles are conserved throughout most of the animal kingdom. In this review, we discuss recent progress in the understanding of myofibril and sarcomere development largely obtained from *in vivo* models. We focus on the role of mechanical forces during muscle and myofibril development and propose a tension driven self-organization mechanism for myofibril formation. We discuss recent technological advances that allow quantification of forces across tissues or molecules *in vitro* and *in vivo*. Although their application towards muscle development is still in its infancy, these technologies are likely to provide fundamental new insights into the mechanobiology of muscle and myofibril development in the near future.

Contents

1. Introduction

Mature body muscles can produce very high forces. The 1992 Guinness Book of World Records reports an American with a masseter (jaw) muscle bite strength of 442 kg and at the 2016 Rio Olympics, a Georgian managed to lift 258 kg in a technique called 'clean and jerk' to win a gold medal. These maximum forces can only be produced for a few seconds until the muscles fatigue. However, body muscles can also produce forces over long time periods enabling body posture, walking or lifelong heart beating. Similarly enduring muscle forces support the flight of animals. During *Drosophila* flight, the indirect flight muscles contract at 200 Hz and sustain an estimated power of about 80 W/kg muscle mass over many hours of flight (Dudley, 2000; Götz, 1987; Lehmann and Dickinson, 1997).

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Although different body muscle types differ significantly in their physiology (Schiaffino and Reggiani, 2011; Schönbauer et al., 2011; Spletter and Schnorrer, 2014), the molecular basis for force production is shared amongst all of them. The contractile unit of all muscles is the sarcomere, which shortens using a sliding mechanism: bipolar myosin thick filaments pull themselves into cross-linked actin thin filaments and thus shorten the sarcomere (Huxley and Niedergerke, 1954; Huxley and Hanson, 1954). Sarcomeres are arrayed in series into linear myofibrils, which span the entire muscle. Thus, coordinated contraction of all sarcomeres along a myofibril shortens the entire muscle and produces a mechanical force. Interestingly, not only the mechanism of muscle contraction, but also most of its molecular components are evolutionarily conserved from worms and flies to humans, hence the sarcomere is an ancient molecular machine (Ehler and Gautel, 2008; Vigoreaux, 2006).

While the identity and in many cases the function of the sarcomeric proteins during muscle contraction is known in molecular detail (Hill and Olson, 2012), the mechanisms of sarcomere assembly during muscle development are much less well understood. Here, we review recent advances in understanding muscle development, with a particular focus on the role of mechanical forces in myofibril and sarcomere formation. We propose a tension-driven model of myofibrillogenesis and discuss recent technological advances to quantify mechanical forces *in vitro* or in developing muscles *in vivo*. These technologies should provide further mechanistic insight into how muscles are built during development to allow both the maximal strength and endurance observed in the amazing muscle performances during adult life.

2. The muscle 'dimension problem'

Mature skeletal muscles are connected at both ends via tendons to the skeleton (Fig. 1). This connection allows muscle contractions to move the skeleton of the animal, leading to locomotion. Large vertebrate muscles are generally composed of several hundred muscle fibers, which are the cellular units of the muscle. In humans, muscle fibers can be several centimeters long, and even in the small fruit fly *Drosophila*, the flight muscle fibers have a length of about 1 mm (Fig. 1). Every muscle fiber is filled with many myofibrils. Each myofibril linearly spans the entire length of the muscle fiber from one tendon attachment to the other. However, the sarcomeres, the repetitive units that build the

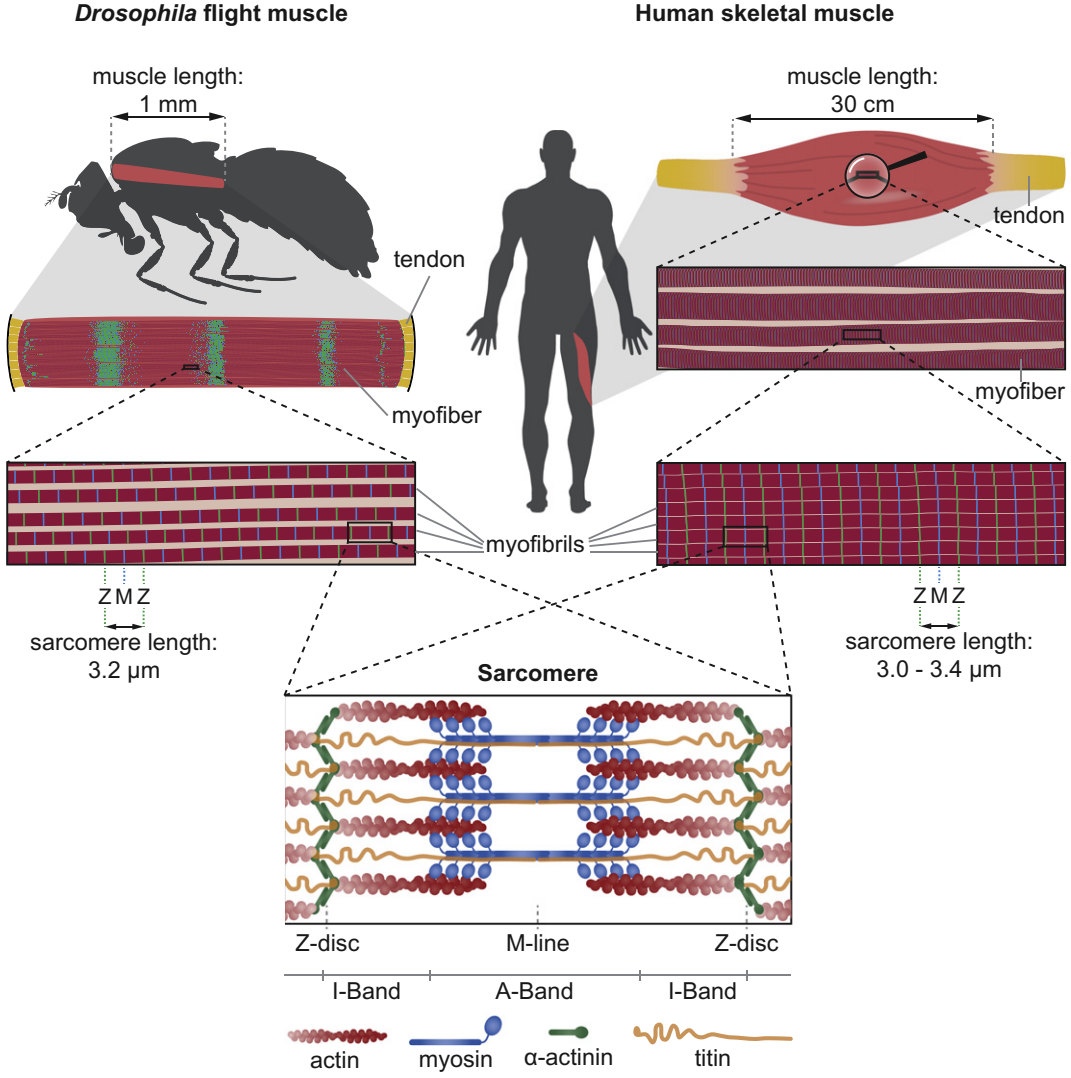

Fig. 1. 'The muscle dimension problem': structure and dimensions of muscles in fly *versus* human. Top: Schematic representation of *Drosophila* and human muscles in a series of magnifications. Each muscle fiber contains hundreds of myofibrils that span the entire length of the myofiber. Sarcomeres are several orders of magnitude shorter but must be assembled perfectly into a myofibril to connect both muscle-tendon attachments at the fiber ends. In fly and human, sarcomeres are similar in length (3.2 µm in flight muscles and 3.0 to 3.4 µm in relaxed human muscles) and many sarcomeric proteins are well conserved. **Bottom:** Schematic of the sarcomere. Polar actin filaments (also called thin filaments) are anchored at the Z-disc (Z, green) by α-actinin. Thick filaments comprised of myosin bundles are centred at the M-line (M, blue) and interact with actin with their myosin heads. Titin, a connecting filament, is anchored at the Z-disc and spans through the I-band all the way to the M-line.

myofibrils, are several orders of magnitude shorter than the muscle fiber. Depending on the muscle type, the sarcomere length in relaxed human skeletal muscle *in vivo* varies from 3.0 to 3.4 μm (Ehler and Gautel, 2008; Llewellyn et al., 2008; Regev et al., 2011), which is similar to the 3.2 μm sarcomere length of *Drosophila* indirect flight muscle (Vigoreaux, 2006). This creates a 'dimension problem': about 300 sarcomeres must be linearly arrayed to form one myofibril spanning the entire flight muscle fiber in *Drosophila*, whereas large vertebrate muscles can require 100,000 sarcomeres to build a sufficiently long myofibril (Fig. 1).

It is important to note that most relaxed muscles are under passive tension, even if they do not produce active contractile forces. The source of this passive tension are the myofibrils themselves (Magid and Law, 1985), with a major contribution from the gigantic elastic molecule titin, which – in vertebrates – extends across half a sarcomere from the Z-disc to the M-line (Fig. 1) and thus stably connects thin with thick filaments (Fürst et al., 1988; Gautel, 2011a; Gautel and Goulding, 1996; Linke et al., 1996; Maruyama et al., 1976; Tskhovrebova and Trinick, 2003). This property of titin coined the term 'connecting filaments' for titin filaments. In fact, the historical name for titin was connectin, which was originally isolated from rabbit muscle depleted of actin and myosin (Maruyama, 2002; Maruyama et al., 1977). The passive tension ensures that each myofibril spans the muscle fiber linearly, similar to a slackline spanned between two trees, with a sarcomere length that is optimal for the next active contraction. The challenging consequence is that a myofibril rupture is deleterious and cannot be repaired easily, as the passive tension prevents the myofibril from simply reattaching to the muscle end. Similarly, the entire myofiber faces a challenge during development, as the fiber cannot simply measure its length and then decide to assemble 10, 100, 1000 or 10,000 sarcomeres per myofibril. The requirement for passive tension also rules out the developmental strategy to just assemble one sarcomere after the next onto loose myofibril ends, as such a myofibril would immediately collapse. Thus, nature chose a smarter solution to solve the dimension problem.

3. Muscle and myofibril development – a balance of forces

Muscle development is a multi-step process beginning with the fate specification of myoblasts, which fuse to form a myotube. Each myotube elongates and stably attaches its ends to tendon cells. Only after attachment does the myotube assemble its myofibrils and thus transition to a muscle fiber. At each of these steps, mechanical forces and the actomyosin cytoskeleton play important roles. We refer the reader to excellent recent reviews detailing both myoblast patterning (Dobi et al., 2015) and myoblast fusion (Kim et al., 2015; Rochlin et al., 2010). Here, we focus on the later steps of myogenesis, starting with the elongating myotube.

During myotube elongation in *Drosophila*, both myotube tips extend towards their future tendon cells (Fig. 2A) (Schnorrer and Dickson, 2004; Schweitzer et al., 2010). During this phase, the myotube tips resemble leading edges of migratory cells with numerous dynamic filopodia extending in the direction of myotube elongation (Schnorrer and Dickson, 2004; Schnorrer et al., 2007; Weitkunat et al., 2014). The actin cytoskeleton is polarized along the long axis of the myotube and supposedly produces a force that drives myotube elongation towards the future tendon attachments. It is likely that similar principles enable myotube elongation across somites in vertebrates, in order to reach their future tendons at the somite borders (Gros et al., 2008; Schweitzer et al., 2010). However, tendon development in vertebrates is more complex and requires an interplay between myogenic and osteogenic cells (Brent and Schweitzer, 2003; Brent and Tabin, 2004; Schweitzer et al., 2001).

After myotube elongation, myotubes need to attach to tendons. Recent studies of myotube attachment using the large *Drosophila* flight muscles showed that the tendon cells are more active than initially thought. They also form dynamic filopodia on their basal side, which interdigitate with the myotube filopodia to initiate myotube-tendon attachment (Fig. 2A and B). During the next few hours of development, filopodia formation at the myotube tips stops and the initially dynamic attachment matures. Interestingly, during this phase of attachment maturation, the myotube compacts in length while the tendon cells form long cellular extensions to maintain the stable connection with both myotube ends (Fig. 2B and C). These cellular extensions, which originate from the basal side of the tendon epithelium, appear as straight lines, indicating strong tissue tension produced by the compacting myotube. Indeed, *in vivo* laser cutting experiments demonstrated that after attachment initiation, mechanical tension within the muscle-tendon system is gradually built up as the attachment matures and the myotube compacts (Weitkunat et al., 2014). Importantly, the tension axis is aligned with the long muscle axis, which will be the future muscle contraction axis. Therefore, tension can act as a guiding compass to ensure that the myofibrils form along this axis and not in any other direction.

During attachment initiation, when attachments are dynamic and tension is low, primarily short actin filaments are present within the myotube (Fig. 2B). These dynamic actin filaments are preferentially oriented along the tension axis, but are not yet periodic or striated. When tissue tension is built up and the myotube is maximally compacted, the first periodic muscle myosin pattern arises within the myotube as it transitions to a myofiber. This periodic muscle myosin pattern is located on a continuous actin fibril that represents an immature myofibril (Fig. 2C). Importantly, live imaging showed that both the formation of these immature myofibrils as well as the periodic muscle myosin pattern occurs simultaneously across the entire muscle fiber. As a consequence, each myofibril spans the entire myofiber from one attached end to the other and is indistinguishable from its neighboring fibrils in terms of maturity (Weitkunat et al., 2014). This strongly implies that a self-organization mechanism transitions the actomyosin cytoskeleton from an unordered to an ordered state, somewhat similar to a phase transition from freely diffusing proteins in a liquid to a liquid crystal or gel-like state. In this state, the proteins are ordered and tend to keep the same neighboring proteins – at least for a while, similar to what has been observed in the mitotic spindle (Hyman et al., 2014; Shimamoto et al., 2011). Interestingly, if the tension build-up in the myotube is disturbed genetically or acutely by laser cutting, myofibrillogenesis is severely compromised, suggesting that tension coordinates the myofibril self-organization process (Weitkunat et al., 2014). Using such a mechanism guarantees that the developing myotube is properly attached to tendons, as only then tension can be built up. Additionally, it ensures that the immature myofibrils are already anchored at the myotube-tendon junctions and only there, thus setting the correct muscle contraction axis required for coordinated body movements.

Similar long periodic myofibrillar arrays were found during development of the zebrafish somites (Sanger et al., 2009) or recently, during differentiation of human cells into myofibers attached to culture dishes *in vitro* (Chal et al., 2015). In the mammalian heart, myofibrils are also attached during development. In this case, the myofibrils of the neighboring rather small cardiomyocytes are mechanically coupled by specialized adhesion structures across membranes to produce coordinated forces (Perriard et al., 2003). If this coupling is blocked *in vitro*, myofibrillogenesis is severely compromised (Marino et al., 1987). Together, these observations strongly suggest that a similar tension driven self-organization mechanism may also coordinate myofibrillogenesis in mammalian muscle.

Once built, the immature myofibrils grow in width as well as in length, with the sarcomeres refining to a regular pseudo-crystalline array and adopting their final size in the mature adult muscle fiber (Fig. 2D) (Spletter et al., 2015). During this maturation phase, the myofibrils further adjust to the needs of the muscle fiber type, *e.g.* being a more enduring or high force producing fiber-type, by incorporation of particular sarcomeric proteins or protein isoforms (Schiaffino and Reggiani, 2011; Spletter et al., 2015; Spletter and Schnorrer, 2014).

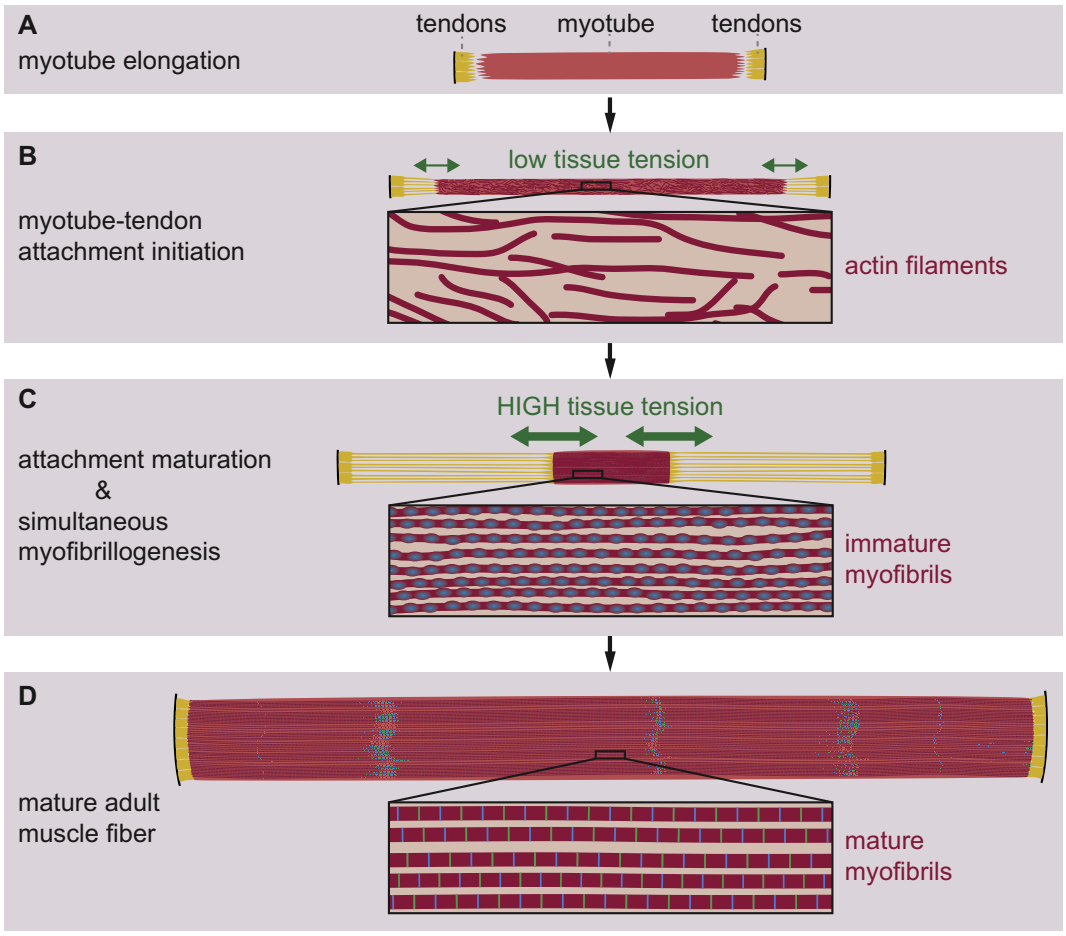

Fig. 2. Development of *Drosophila* indirect flight muscles. (A) The myotube (red) elongates towards its tendon targets (yellow). **(B)** The myotube initiates attachment to the tendon cells. Actin filaments in the myotube are short but preferentially oriented along the long muscle axis; tissue tension is low (green arrows). **(C)** During attachment maturation, the myotube compacts and high tissue tension is generated (green arrows). At the same time, immature myofibrils are formed simultaneously throughout the myotube. **(D)** The muscle fiber grows in length and width and the myofibrils mature to their final size.

Together, this generates muscle fibers that perfectly match the biomechanical demands of the animal.

4. Integrins – central force sensors at the muscle-tendon junction

How can cells – and myotubes in particular – sense mechanical tension molecularly and what molecular consequences does tension induce? The integrin family of receptors is likely to act as important force sensors. Integrins are essential for muscle-tendon attachment as mutations lead to round or detached muscles in worms and flies (Leptin et al., 1989; Williams and Waterston, 1994) and severe sarcomerogenesis defects in mice (Schwander et al., 2003). Integrins are α-, β-heterodimers, containing large extracellular domains, which interact with the extracellular matrix (ECM), and short cytoplasmic tails that bind to intracellular adaptor proteins such as talin and kindlin, which in turn link to the actin cytoskeleton (reviewed in Moser et al., 2009). Integrins are present both in the tendon cell membrane as well as the myotube tip membrane. They mediate muscle-tendon attachment by generating a force resistant muscle-ECM-tendon junction (reviewed in Brown, 2000).

Although forces across integrin molecules have not yet been measured *in vivo* at muscle-tendon junctions, the combination of several insights – partially gained in other systems – strongly suggest that integrin molecules at muscle-tendon junctions do bear forces. First, using cells plated on glass functionalized with a FRET-based molecular tension sensor attached to an artificial integrin ligand, individual integrin complexes were estimated to experience pulling forces in the low piconewton (pN) range (Morimatsu et al., 2013). These values fit well with forces measured within focal adhesions of plated cells using a genetically encoded molecular tension sensor in the integrin adaptor protein talin (Austen et al., 2015). Second, *in vivo* evidence from talin mutant muscles showed a detachment of the actin cytoskeleton from the muscle-tendon junction, strongly suggesting that the assembling actin cytoskeleton is producing mechanical forces transmitted onto integrin tails by talin (Brown et al., 2002). Third, recent *in vivo* FRET analysis in *Drosophila* embryonic muscles confirmed that integrin tails bind to the talin head domain, while actin is binding along the rod domain, pulling talin into an extended conformation (Klapholz et al., 2015). Together, this is solid evidence that both integrins and talin are experiencing mechanical forces during muscle development *in vivo*.

What molecular consequences do these forces have for integrins and talin at the muscle-tendon junction? It appears that forces result in a stabilization and clustering of integrins at the muscle-tendon junction membrane (Fig. 3), similarly as proposed for focal adhesions (Kanchanawong et al., 2010; Legate et al., 2009). As a consequence, integrin levels increase during muscle-tendon attachment maturation and thus can counteract the higher forces. This model is supported by several *in vivo* observations. First, when flight muscles initiate attachment, mechanical tension is low and both integrin and talin levels at the junction are low. A few hours later, when tension is higher, attachments have matured and both integrin and talin are present in elevated amounts at the junction (Weitkunat et al., 2014). Second, *in vivo* fluorescence recovery after photobleaching (FRAP) experiments at the muscle-tendon junction demonstrated that higher contractile forces

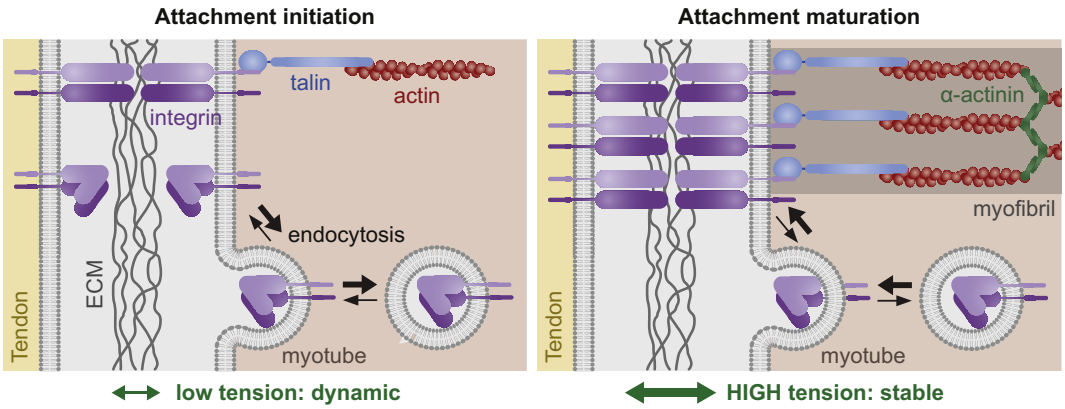

Fig. 3. Force-dependent integrin clustering and turnover at the muscle-tendon junction. During attachment initiation, tissue tension is low. The amount of integrin at the muscle-tendon junction bound to the extracellular matrix (ECM) is also low, whereas the mobile fraction of integrin is high. During attachment maturation and myofibrillogenesis, the tissue tension increases, resulting in reduced endocytosis. Therefore, integrin clusters are stabilized at the tips of myofibrils.

produced by the muscle correlate with lower integrin adhesion complex turnover by clathrin-mediated endocytosis (Pines et al., 2012). This suggests that forces can directly stabilize the clustered integrin complexes at the muscle-tendon junction (Fig. 3). Third, it was recently discovered in an elegant series of experiments that the Ilk-Pinch-Parvin (IPP) complex, a crucial regulator of integrin adhesion and integrin signalling (Legate et al., 2005), directly regulates integrin turnover in response to mechanical forces (Vakaloglou et al., 2016). If the IPP complex is mutated, integrins at the junction turn over faster than normal, resulting in an abnormally fuzzy muscle-tendon junction upon force. Interestingly, this morphological defect is rescued if muscle contraction is blocked, suggesting that the IPP complex reinforces integrin adhesion in response to muscle forces. Altogether, these and many other observations place integrins as central force sensors at the muscle-tendon junction that must integrate and adapt to dramatically dynamic forces during muscle development when the myofibrils assemble as well as during muscle function when the myofibrils contract.

5. Titin – a force sensor with a tuneable spring at the heart of the sarcomere

Not only muscle-tendon junctions experience forces. Tension is likely present homogeneously along developing and mature myofibrils. As a consequence, every assembling and mature sarcomere is under force, even in the relaxed muscle state. As mentioned above, this tension is called passive tension and titin is one of its major sources. Titin is a gigantic protein larger than 3 MDa in size. Its modular architecture largely consists of immunoglobulin (Ig) and fibronectin type III (Fn3) domains, and in its fully extended state, titin is more than 1.5 μm long (Labeit and Kolmerer, 1995). This allows titin to span half a sarcomere, with its N-terminus embedded at the Z-disc and its C-terminus at the M-line (Fürst et al., 1988). It has been suggested to act as a molecular ruler or blue print for sarcomeric architecture (reviewed in Tskhovrebova and Trinick, 2003). Interestingly, the titin I-band region (between Z-disc and beginning of the thick filament, see Fig. 4) consists of different spring elements: the Ig-domain series and the so-called PEVK region. The latter largely consists of the amino acids Pro (P), Glu (E), Val (V) and Lys (K), which under low force form a coiled confirmation, stabilized by salt-bridges and hydrophobic interactions (Linke et al., 2002). If tension is applied on titin, these spring elements gradually extend; the Ig-domain series straightens (without unfolding any of the Ig-domains) and the PEVK domain gradually stretches, resulting in the elongation of the I-band region of the sarcomere under increasing strain (Gautel and Goulding, 1996; Linke et al., 1999) (Fig. 4). This mechanically induced elongation occurs at a low pN range and is fully reversible during the contraction cycle of a muscle (Linke et al., 2002). In a resting mature or developing muscle, titin is always present in a partially extended conformation and thus produces passive tension within the muscle-tendon system.

Importantly, the spring constant of titin is under tight regulation during muscle development and depends on the muscle type. Heart

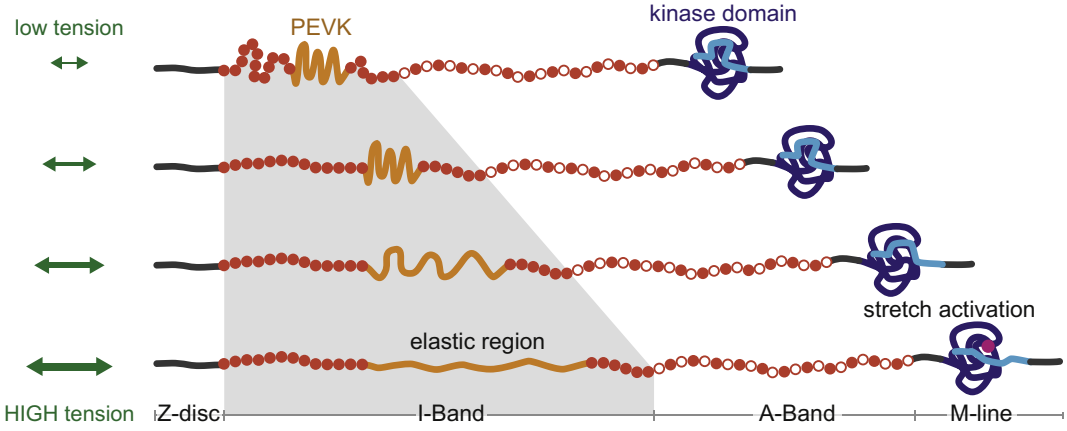

Fig. 4. Titin – a force sensor in the sarcomere. Schematic representation of titin and its response to increasing tension in the sarcomere (green arrows). Titin mainly consists of Immunoglobulin (Ig) and Fibronectin type III (Fn3) domains (red and white dots, respectively). The PEVK region (orange) is an elastic element that stretches with increasing tension, allowing the I-band region of titin to extend. The kinase domain of titin (blue) is thought to be activated upon stretch by the removal of a regulatory tail (light blue) allowing ATP binding (pink) at the active site. Therefore, titin can act as a stretch sensor in the sarcomere.

muscle is stiffer than skeletal muscle and the primary source of this difference is that skeletal muscle titin contains a significantly longer PEVK sequence as compared to heart muscle, making skeletal muscle titin more compliant (Gautel et al., 1996; Guo et al., 2010; Lange et al., 2006). As humans have only one titin gene, this isoform regulation occurs by alternative splicing (Bang et al., 2001). Misregulation of titin splicing, for example by a mutation in the RNA-binding protein RBM20, results in severe hereditary cardiomyopathies (Guo et al., 2012; Spletter and Schnorrer, 2014). Titin stiffness can also be fine-tuned by phosphorylation of the PEVK domain, which may play an important role during human heart failure (Kötter et al., 2013). Another important function of titin and its elasticity is that it can position the myosin filament centrally by pulling more strongly to one side, in case the filament is displaced (Agarkova et al., 2003). This balance of forces ensures that the myosin motor activity cannot cause a collapse of the sarcomere to one side and thus maintains symmetrical sliding between thin and thick filaments. Together, these data highlight the importance of titin's mechanical properties and their precise modulation for normal muscle development and function.

Beyond its role as a tuneable spring, titin was also proposed as an active force sensor that may regulate contractility or signalling in response to strain (reviewed in Gautel, 2011a, 2011b). This hypothesis is supported by the discovery of a C-terminally located autoinhibited kinase domain in titin (Mayans et al., 1998). The kinase domain could be activated *in vitro* by pulling on a titin fragment consisting of the kinase domain flanked by its neighboring Ig- and Fn-domains using an atomic force microscope (AFM). These pulling forces 'open' the kinase domain by pulling a regulatory tail away from the active site, enabling ATP binding to the kinase domain (Fig. 4) and thus supposedly kinase activity (Puchner et al., 2008). The forces required for ATP binding and kinase activation are within the physiological range and well below forces needed to unfold Ig-domains. These data suggest that the titin kinase may act as a biological force sensor *in vivo*. However, it is unclear if kinase activity is required for the function of the titin kinase domain. Recent data support the hypothesis of titin being a pseudokinase that may potentially act as a mechanically regulated scaffold to control protein turn-over (Bogomolovas et al., 2014; Lange et al., 2005). However unexpected for a pseudokinase, the catalytic core of titin is largely conserved (Gautel, 2011b) and also the insect titin kinases are active, at least *in vitro* (Fährmann et al., 2002). Thus, it remains an open question whether vertebrate titin is an active kinase or not and future research is required to fully understand how the gigantic sarcomeric force sensor titin communicates changes in tension with its partner proteins.

6. Force production – the usual suspects?

For many decades, it has been established that the motor activities of muscle myosin heavy chains produce the active forces responsible for sarcomere shortening during mature muscle contractions (reviewed in Cooke, 2004; Szent-Györgyi, 2004). Like all myosin II motors, muscle myosin is a hexamer consisting of two heavy chains, two essential light chains and two regulatory light chains (Howard, 2001). About 300 of these hexamers are assembled into surprisingly stereotyped 1.65 μm long bipolar myosin filaments present in mature sarcomeres of vertebrate muscles (Gokhin and Fowler, 2013; Tskhovrebova and Trinick, 2003). The pseudo-crystalline regularity of mature sarcomeres ensures that myosin motors only contact actin filaments of correct polarity to readily move towards their plus ends, which are anchored and cross-linked at the Z-discs (Fig. 1). Thus, the thick filaments can very efficiently produce forces along the axis of muscle contraction.

During muscle development and myofibril formation in particular, it is less clear how mechanical forces are being generated. At the early developmental stages, when myotubes elongate and myotube-tendon attachment is initiated, muscle myosin is not yet expressed at detectable levels (Spletter et al., 2015; M. Spletter & F.S. unpublished data). However, early myotubes do express nonmuscle myosin, which is responsible for regulated force production in numerous cell types in various developmental contexts (reviewed in Howard, 2001). Evidence from cultured Xenopus myotubes suggests a role for nonmuscle myosin light chain kinase during myofibrillogenesis, both for correct myosin and titin assembly (Ferrari et al., 1998; Harris et al., 2005). Yet, mutations in nonmuscle myosin-IIA in mice only result in mild muscle phenotypes (Tullio et al., 1997), likely because other nonmuscle myosin isoforms compensate (Berg et al., 2001). In *Drosophila*, mutations in the single nonmuscle myosin heavy chain (*zipper*) present result in severe myofibrillogenesis defects in embryonic muscles (Bloor and Kiehart, 2001), supporting a direct role for nonmuscle myosin in myofibrillogenesis. Thus, it is possible that nonmuscle myosin is responsible for the observed tension before myofibrils start to assemble (Fig. 2C) (Weitkunat et al., 2014).

7. Myofibrillogenesis model – tension driven self-organization

Myotubes face the challenge of organizing myofibrils throughout their entire volume. This is different in epithelial cells forming a monolayer, which is one of the best models to study the requirement of actin-myosin contractility for tissue self-organization. In these cells, actomyosin is largely restricted to belt-like structures at the apical cell cortex (reviewed in Heisenberg and Bellaiche, 2013; Lecuit et al., 2011). In order to fill the entire myotube with myofibrils, it is likely that the actomyosin network is mechanically coupled across large areas, possibly across the entire myotube from one integrin attachment site to the other (Fig. 5). The subsequent mechanical tension across the myotube appears largely isotropic, as it does not result in obvious actomyosin flows (Weitkunat et al., 2014), contrasting observations from the cell cortex of polarizing cells (Mayer et al., 2010) or during epithelial spreading and remodelling (Behrndt et al., 2012; Rauzi et al., 2010).

Historically, two distinct myofibrillogenesis models have been established. The premyofibril model, which is largely based on *ex vivo* culture data, suggests that short bipolar nonmuscle myosin filaments assemble with short α-actinin cross-linked actin filaments to form premyofibrils (Rhee et al., 1994; White et al., 2014). This is consistent with the observation of actin filaments *in vivo* before periodically patterned immature myofibrils are detected (Fig. 5A). The premyofibril model further suggests that nonmuscle myosin is gradually exchanged for muscle myosin in the mature myofibrils. Although observed in a dotted pattern on pre-myofibrils *in vitro* (White et al., 2014), it is unclear if nonmuscle myosin is present in a detectable periodic pattern *in vivo* during early steps of myofibrillogenesis (Bloor and Kiehart, 2001). By contrast to muscle myosin, nonmuscle myosin only forms bipolar mini-filaments with approximately 10 heads on each side (reviewed in Vicente-Manzanares et al., 2009) and thus a periodic spacing is unlikely to be detected with current microscopy techniques. The observation that muscle myosin is detectable *in vivo* as periodic dots on immature myofibrils (Weitkunat et al., 2014) suggests that the myosin exchange must occur at an early developmental stage (Fig. 5B). These myosin dots are also consistent with the second myofibrillogenesis model, which proposes that muscle myosin containing thick filaments (precursors) and titin containing α-actinin cross-linked actin filaments, termed I-Z-I bodies, are first built independently and only assemble to myofibrils in a second step (Ehler et al., 1999; Holtzer et al., 1997). Data supporting a coordinated assembly of prebuilt protein complexes during sarcomerogenesis have also been gained in *Drosophila* (Rui et al., 2010). In our opinion, both models are rather complementary and represent two aspects of the complex myofibrillogenesis process, both depending on mechanical tension caused by bipolar myosin filaments pulling on bipolar cross-linked actin filaments to self-organize immature periodically patterned myofibrils.

We integrate the old models with recent *in vivo* data to suggest the following revised tension-driven model for *in vivo* myofibril self-organization (see also Sparrow and Schöck, 2009). In a first phase (Fig. 5A), myotubes need to establish a stable integrin-mediated attachment to

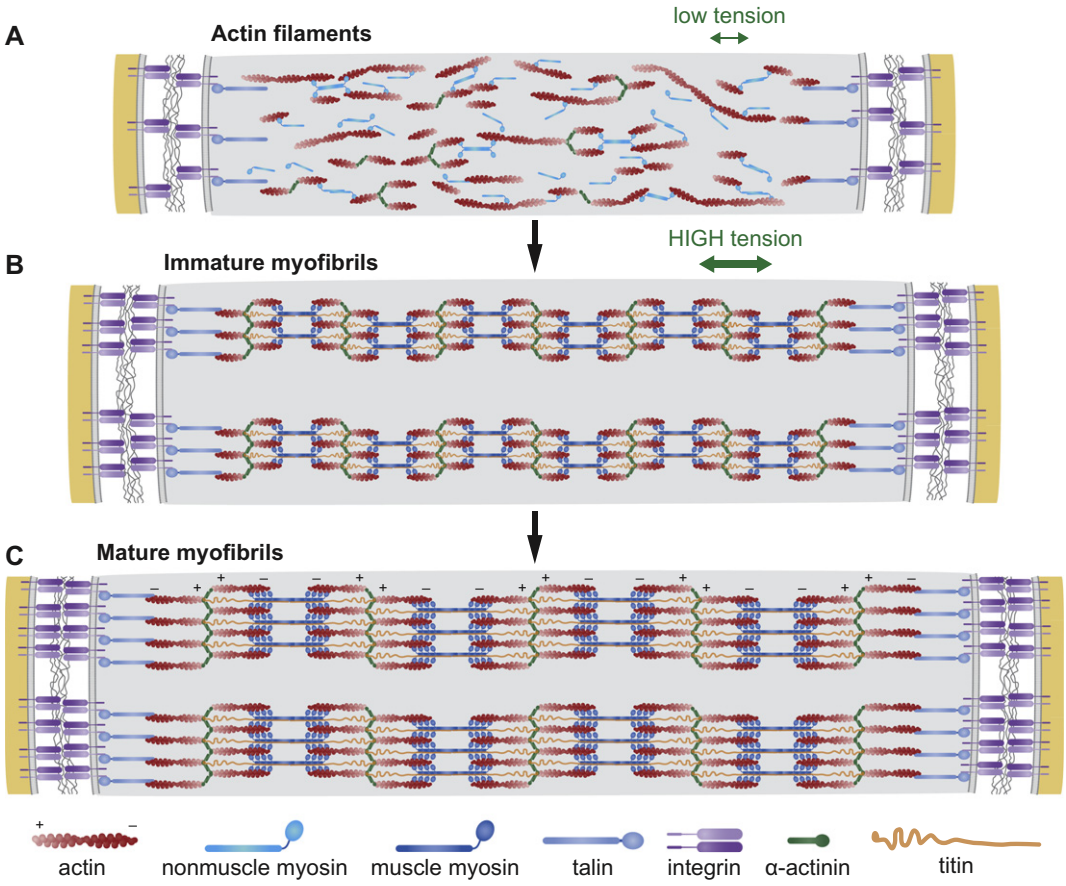

Fig. 5. Tension-dependent model of myofibril development. (A) Early in development when tissue tension is low (green arrows), bipolar non-muscle myosin (light blue) and actin filaments (red) are loosely and dynamically interacting. The actin filaments are oriented with respect to the muscle axis but not yet striated. **(B)** As development proceeds, tension increases and immature myofibrils form across the entire myofiber. Muscle myosin is expressed and forms larger bipolar filaments (dark blue) stably binding to bipolar actin filaments, cross-linked by α-actinin (red and green). Titin (orange) stably connects both. This creates a striated appearance of the immature myofibrils, each one anchored to the muscle-tendon junction by integrin and talin at both ends, allowing tension to be transmitted across the entire myofibril. **(C)** In a final step, myofibrils and sarcomeres grow both in length and thickness by incorporating additional molecules until they reach the mature size.

tendons at both myotube ends. During this phase, mechanical tension is initially low but steadily increases, likely through the pulling of nonmuscle myosin filaments on short cross-linked actin filaments. Here, tension fulfills two important roles: it locally orients the actin and myosin filaments towards each other along the muscle axis and it globally coordinates the assembly process across the entire myotube. As a consequence, the actin filament network orients along the muscle axis but is not yet periodic. In a second phase (Fig. 5B), muscle myosin and titin are expressed in high quantities and tension induces integrin clustering at the muscle ends. Together, this drives the simultaneous self-organization of periodic immature myofibrils, each of which spans the entire myofiber, thus mechanically linking both integrin attachments. These immature myofibrils contain muscle myosin and titin in a periodic pattern and require both myosin motor activity and the titin scaffold or its passive tension activity for their formation (Rui et al., 2010; Schnorrer et al., 2010; Weitkunat et al., 2014). In a third phase (Fig. 5C), this self-organization process continues and the myofibrils mature. Large amounts of actin, muscle myosin, α-actinin, titin and many other components are incorporated and form the final pseudo-crystalline sarcomere morphology.

8. How experimentalists sense tension

As discussed throughout this review, mechanical tension at the tissue and single molecule level is essential for muscle morphogenesis. The same is true for many other developmental processes: most cells continuously probe the stiffness of their environment to instruct developmental decisions, *e.g.* myotubes only build myofibrils efficiently on a substrate that recapitulates the *in vivo* stiffness and is neither too soft nor too stiff (Engler, 2004). The experimentalist can take advantage of this continuous exploratory behaviour and measure the forces generated by cells *in vitro* by applying a technique called traction force microscopy (Fig. 6A). To achieve this, cells are plated on a micropillar substrate of defined stiffness. By determining the deflection angle of the micropillars, cellular pulling forces can be measured in a quantitative way and thus a traction map of the cell is acquired (reviewed in Ribeiro et al., 2016). A similar technique was successfully applied to measure substrate tension during myotube differentiation and myofibrillogenesis using the C2C12 myoblast cell line. Consistent with the *in vivo* observations, mechanical tension is present along the long axis of forming myotubes and tension significantly increases during myotube to myofiber differentiation (Li et al., 2008). Thus, traction force microscopy can measure global cellular forces *in vitro*.

A complementary method that allows determination of the biomechanical properties of individual proteins or protein domains at the single molecule level *in vitro* is single molecule atomic force microscopy (AFM) (reviewed in Hughes and Dougan, 2016). A protein of interest is purified and attached to a rigid substrate on one end and to the tip of a calibrated AFM cantilever on the opposite end. The deflection angle of a laser beam is used to quantify the forces applied as the cantilever moves away from the substrate and thus pulls and eventually unfolds the protein or peptide of interest (Fig. 6B). This technique was

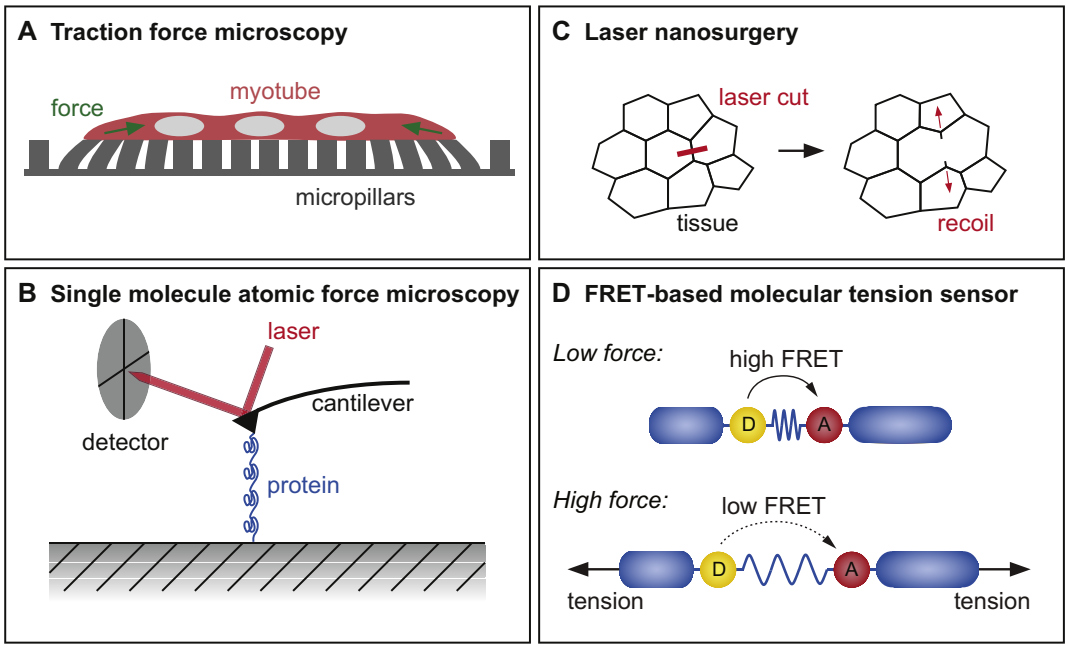

Fig. 6. How experimentalists sense tension. (A) Traction force microscopy: Cells are plated on a surface of stereotyped micropillars. Forces generated by the cells are calculated by measuring the deflection of the micropillars. **(B) Single molecule atomic force microscopy:** A single protein is attached to a surface and a cantilever. Bending of the cantilever can be detected as the cantilever is moved away from the surface and stretches the protein. This allows the quantification of forces needed to stretch and unfold protein domains. **(C) Laser nanosurgery:** Tissue tension is measured by severing of for example cell-cell edges with a pulsed UV-laser and quantifying the tissue recoil velocity. **(D) FRET-based molecular tension sensor:** A tension sensor module, consisting of a donor 'D' and an acceptor fluorescent protein 'A' connected by a flexible linker peptide, is inserted into a protein of choice. The higher the forces across a protein, the lower the FRET efficiency.

applied extensively to study the properties of individual titin fragments. It was shown that 150 to 300 pN forces are required to unfold individual titin Ig-domains *in vitro* (Rief et al., 1997). This appears higher than what an individual titin molecule may experience *in vivo*; however, the same study also showed that Ig-domain unfolding is reversible and thus may be a way to balance very high forces *in vivo* (Rief et al., 1997). Similar single molecule measurements established titin's PEVK domain as a flexible spring that elastically opens and closes at a much lower force range and thus likely contributes to the mechanical properties of the sarcomere *in vivo* (Li et al., 2001; Linke et al., 2002).

Measuring tension *in vitro* always has the disadvantage of being artificial. Thus, we need techniques that can also be applied *in vivo* within the intact developing organism. One technique widely used within recent years is *in vivo* laser nanosurgery or laser cutting (Fig. 6C). A pulsed UV laser (355 nm) or infrared laser is used to locally generate a low energy plasma and precisely sever protein connections, for example of the actin or microtubule cytoskeleton, without affecting the rest of the cell (Colombelli et al., 2009; Colombelli et al., 2005; Vogel et al., 2009). Laser cutting was also applied widely *in vivo* to sever cell-cell junctions or cortical actin networks. The velocity of the resulting recoil can be used to quantify relative tissue tension comparing different cells, mutants or developmental stages (Behrndt et al., 2012; Mayer et al., 2010; Weitkunat et al., 2014). Thus, laser nanosurgery is a powerful method to assess and quantify tissue tension *in vivo*.

A more challenging task is to determine forces across individual molecules in intact cells or even within an intact developing organism. One method that has gained significant interest in recent years uses genetically encoded Förster resonance energy transfer (FRET)-based molecular tension sensors. These sensors consist of a FRET donor-acceptor pair that is connected by a short flexible spring-like peptide that stretches or unfolds under force (Fig. 6D). Low forces applied to this peptide produce high FRET, whereas increasing forces reduce FRET. Various linker peptides were recently developed and calibrated using single molecule force spectroscopy to quantify forces in the range of 1 to 11 pN (reviewed in Freikamp et al., 2016). As these sensors are genetically encoded and can be inserted into proteins of choice, piconewton forces across specific proteins can be quantified in cells and in principle also within organisms. Such tension sensors have been successfully applied to quantify forces across talin and the talin binding protein vinculin in focal adhesions of cells plated in a dish. These studies nicely showed that assembling focal adhesions experience larger forces than disassembling ones (Grashoff et al., 2010) and that talin molecules are subjected to forces ranging from 7 to 10 pN (Austen et al., 2015). Thus far, molecular tension sensors have not been used to investigate forces during muscle development *in vivo*, however, we believe developing muscles will be a fantastic system to test them and quantify forces across proteins that build the contractile sarcomere.

9. Conclusions and perspectives

Mechanical forces are important for many, if not most, morphogenetic processes that build a higher organism. In particular during muscle formation, the correct timing, location and magnitude of mechanical forces subjected onto cells and molecules are critical. We are only beginning to adapt techniques that were developed *in vitro* to measure forces *in vivo*. In many cases, we do not know the magnitude of forces that tissues or molecules encounter during animal development. It also remains a challenge to determine how many molecules within a given pool, for example at the muscle-tendon junction, do experience forces. Novel techniques such as FRET-based tension sensors may open new avenues to measure tension across proteins *in vivo*. It will be similarly challenging to manipulate forces *in vivo* in a controlled manner in order to assess their impact. Tackling and hopefully overcoming some of these challenges may eventually unravel the mechanisms muscles use to sense and interpret tension to form the highly ordered contractile apparatus that fits the biomechanical needs of each muscle type.

Competing interests

The authors declare that no competing interests exist.

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