Mechanical tension and spontaneous muscle twitching precede the formation of cross-striated muscle in vivo

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Abstract

Muscle forces are produced by repetitive stereotyped acto-myosin units called sarcomeres. Sarcomeres are chained into linear myofibrils spanning the entire muscle fiber. In mammalian body muscles, myofibrils are aligned laterally resulting in their typical cross-striated morphology. Despite this detailed textbook knowledge about the adult muscle structure, it is still unclear how cross-striated myofibrils are built in vivo. Here, we investigate the morphogenesis of Drosophila abdominal muscles and establish them as in vivo model for cross-striated muscle development. Using live imaging, we find that long immature myofibrils lacking a periodic acto-myosin pattern are built simultaneously in the entire muscle fiber and then align laterally to mature cross-striated myofibrils. Interestingly, laser micro-lesion experiments demonstrate that mechanical tension precedes the formation of the immature myofibrils. Moreover, these immature myofibrils do generate spontaneous Ca\(^{2+}\) dependent contractions in vivo, which when chemically blocked result in cross-striation defects. Together, these results suggest a myofibrillogenesis model, in which mechanical tension and spontaneous muscle twitchings synchronise the simultaneous self-organisation of different sarcomeric protein complexes to build highly regular cross-striated myofibrils spanning throughout large muscle fibers.
Introduction

The muscular system is the major force-producing tissue of animals. In particular the skeletal muscles enable precise body movements of invertebrates and vertebrates. For these accurate movements, each muscle must be properly connected to the skeleton. This is achieved by the attachment of both muscle fiber ends to tendons, which in turn connect to the skeleton. In large animals, often hundreds of fibers are packed into muscle fiber bundles that run parallel to the long axis of the muscle. Thus, muscle is a highly polar tissue, which harbours a defined contraction axis between both tendon attachments (Hill and Olson, 2012).

The sarcomere is the contractile unit of each muscle fiber (Clark et al., 2002; Gautel and Djinovic-Carugo, 2016). Each sarcomere is symmetrically organised between two Z-discs, which cross-link antiparallel polar actin filaments, also called thin filaments. The centrally located thick filaments are comprised of bipolar myosin filaments. These thick filaments are permanently connected to the neighbouring Z-discs by connecting filaments, largely formed by the gigantic protein titin (Gautel, 2011; Tskhovrebova and Trinick, 2003). This results in a stereotyped length of each sarcomere that is characteristic for the muscle type, ranging from about 3.0 to 3.4 µm in relaxed human skeletal muscle in vivo (Ehler and Gautel, 2008; Llewellyn et al., 2008). As individual muscle fibers can be several centimetres long, hundreds, often thousands of sarcomeres require to assemble into long chains called myofibrils during muscle development (Hill and Olson, 2012; Sanger et al., 2010).

Despite detailed textbook knowledge about mature sarcomere and myofibril architecture, our understanding of myofibril and sarcomere formation during muscle development is still limited. A proposed ruler hypothesis suggests that titin, which spans from Z-disc to M-line across half a sarcomere in mammalian muscle, sets sarcomere length (Fürst et al., 1988; Tskhovrebova and Trinick, 2003; Tskhovrebova et al., 2015; Whiting et al., 1989). However, it is unclear how such a ruler defines the characteristic sarcomere length of
the different muscle types (Gokhin and Fowler, 2013). The ruler hypothesis is also challenged in insect muscle, as individual insect titin homologs are too short to span across half a sarcomere. Nevertheless, insect sarcomere sizes are set as precisely as in vertebrates (Bullard et al., 2005; Tskhovrebova and Trinick, 2012). Likewise, it is debated how a large number of sarcomeres assemble into linear myofibrils. Different models propose that either short, irregular premyofibrils slowly mature into regular myofibrils by exchanging nonmuscle myosin II with muscle myosin II (Rhee et al., 1994; Sanger et al., 2010; Sparrow and Schöck, 2009) or alternatively, thin and thick filaments assemble more independently and subsequently interdigitate (Ehler et al., 1999; Holtzer et al., 1997; Rui et al., 2010). Data supporting these models were often acquired in vitro by analysing cardiomyocytes or myotubes adhering to a Petri dish. This contrasts the in vivo situation, in which both defined muscle fiber ends attach to tendons and thus set the polarity and contraction axis of the muscle fiber. Hence, it is important to study myofibrillogenesis using an in vivo model.

In vivo, vertebrate skeletal muscles have the typical cross-striated appearance (Hill and Olson, 2012), which is essential for the mechanism of muscle contraction (Huxley and Niedergerke, 1954; Huxley and Hanson, 1954). These cross-striations are formed by a regular lateral alignment of the individual myofibrils. During this alignment the Z-bands grow significantly in width (Sanger et al., 2010) and neighbouring Z-discs might be linked by intermediate filaments (Gautel and Djinovic-Carugo, 2016). It has been found that even mature Z-disc dynamically exchange a number of Z-disc components with the cytoplasmic pool (Wang et al., 2005). This may contribute to the Z-disc growth and potentially to their gradual lateral alignment, resulting in the cross-striations of the muscle. However, the exact molecular mechanism of cross-striation formation in vivo remains elusive.

Recently, we have investigated myofibrillogenesis in vivo using the Drosophila indirect flight muscle model (Weitkunat et al., 2014). We found that after myotubes have
attached to tendons, myofibrils assemble simultaneously throughout the entire myofiber. This results in continuous early myofibrils that span across the entire 200 µm long muscle fiber, suggesting a self-organisation mechanism of actin, and myosin filaments, together with titin complexes. Importantly, myofibril formation is preceded by a build-up of mechanical tension within the flight muscle-tendon system, and if tension build-up is blocked or tension is released, myofibrillogenesis is severely compromised. This led to an extended model of myofibrillogenesis, which proposed tension as an essential coordinator for myofibrillar self-organisation in the flight muscles (Lemke and Schnorrer, 2016; Weitkunat et al., 2014). Tension and myosin contractility are also components of theoretical models aiming at predicting the dynamics of sarcomere assembly (Friedrich et al., 2012; Yoshinaga et al., 2010). However, the in vivo presence of tension was thus far only detected in indirect flight muscles of Drosophila, which display a specialised fibrillar organisation of their myofibrils that enables fast contraction cycles, but lack the typical cross-striated pattern of vertebrate skeletal muscles (Josephson, 2006; Schönbauer et al., 2011; Weitkunat et al., 2014).

Here, we set out to investigate myofibrillogenesis and tension formation in the Drosophila adult abdominal muscles, which are cross-striated, synchronously contracting muscles and thus resemble vertebrate skeletal muscles. Using in vivo imaging we detect simultaneous myofibril assembly in these muscles, and find that mechanical tension is not only present before but also during myofibril assembly. Remarkably, immature myofibrils, lacking an obvious periodic pattern, are already contractile when stimulated by Ca²⁺ influx, suggesting a sarcomere-like organisation of their components at this early stage. Importantly, we find that the conversion of immature myofibrils to cross-striated myofibrils coincides with a strong increase of spontaneous muscle twitchings, which are required to efficiently form cross-striations. Together, these results imply a general role of mechanical tension and Ca²⁺
dependent spontaneous twitchings to coordinate acto-myosin self-organisation to build regular cross-striated muscle fibers in vivo.

Results

Abdominal muscle morphogenesis – an overview

*Drosophila* abdominal muscles form by fusion of adult myoblasts to myotubes at about 24 h after puparium formation (APF) (Currie and Bate, 1991; Dutta et al., 2004; Krzemien et al., 2012; Weitkunat and Schnorrer, 2014). To analyse the development of the contractile apparatus in vivo we imaged abdominal dorsal muscle development using intact pupae. We labelled the actin cytoskeleton with Lifeact-Ruby (Hatan et al., 2011) and muscle myosin heavy chain (Mhc) using a GFP-trap within the endogenous Mhc gene (Clyne et al., 2003). At 30 h APF, the dorsal abdominal myotubes elongate along the anterior-posterior axis forming dynamic leading edges at both myotube tips. Filopodia at these tips point to the direction of elongation (Movie 1, Figure 1A). The filopodia at the posterior leading edge are less pronounced, suggesting that the posterior myotube tip is already in closer contact with its future epidermal tendon cells (Krzemien et al., 2012). Filopodia dynamics gradually reduces until 40 h APF, suggesting that myotube-tendon attachment is also initiated at the anterior myotube tip (Movie 1, Figure 1B). During this period Mhc-GFP is not yet detectable in the myotube and no obvious periodic actin pattern is found within the elongating myotubes (Figure 1A, B).

Shortly before 50 h APF, Mhc protein becomes detectable and localises in a periodic pattern throughout the myotube. Simultaneously with myosin, actin is also recruited into a similar period pattern (Movie 1, Figure 1C). Initially, both patterns are irregular; however, they refine until 60 h APF, to form two distinct periodic patterns along the entire contraction axis of the myofiber (Movie 1, Figure 1D). Taken together, these data suggest that actin is assembled into a periodic pattern when muscle myosin is expressed at significant levels
detectable by live imaging. Interestingly, this periodic assembly occurs largely simultaneously throughout the entire length of the myofiber, suggesting a self-organisation mechanism of actin and myosin filaments.

Abdominal muscle attachment

Studies in flight muscles suggested that muscle attachment is required for myofibrillogenesis (Weitkunat et al., 2014). In order to investigate myotube attachment of abdominal muscles before and during myofibrillogenesis in detail, we fixed pupae and stained them for the bona-fide attachment marker βPS-Integrin (Brown et al., 2000; Leptin et al., 1989) at different developmental stages. In accordance with the live imaging, β-Integrin first concentrates at the posterior tips of the myotubes at 36 h APF, with little integrin present at the anterior tips (Supplementary Figure 1A, A’). However, anterior myotube tips are in close proximity to the overlaying epidermis and are therefore likely to form dynamic contacts with the epidermis at 36 h APF (Supplementary Figure 1A’’). At 40 h APF, more β-integrin is present at the anterior myotube tips, suggesting that the myotube-epithelial tendon contacts are stabilised (Supplementary Figure 1B-B’’). At 46 h APF filopodia have largely disappeared from the myotube tips and more β-Integrin is localised at the tips, suggesting that the muscle-epithelial tendon contacts have further matured (Supplementary Figure 1C, C’). Interestingly, we detected epithelial cell extensions from 40 h onwards (Supplementary Figure 1B’’, C’’), which are similar to the tendon cell extensions produced during flight muscle morphogenesis when mechanical tension is built up (Weitkunat et al., 2014). At 52 h APF, even more integrin is localised at the muscle fiber tips, where it remains until 72 h APF. During this phase, the myofibers continue to grow in length, despite remaining stably attached to their epithelial tendons (Supplementary Figure 1D-F). Together, these data substantiate that
abdominal myotubes begin to stably attach to tendon precursors at 40 h APF and build periodic myofibrils after 46 h APF.

**Myofibrillogenesis of cross-striated muscle**

In order to investigate the dynamics of cross-striated myofibrillogenesis at high spatial resolution, we imaged intact pupae expressing Mhc-GFP from 48 h APF using multi-photon microscopy. This enabled us to follow individual muscle fibers *in vivo* over many hours of development. At 48 h APF Mhc-GFP is present at low levels, localising in a dotty pattern without obvious periodicity along the long axis of the muscle (Movie 2, Figure 2A). These Mhc-GFP dots become brighter and more organised by 50 h APF, building a defined periodic pattern along the entire muscle fiber by 52 h APF (Movie 2, Figure 2B, C). Moreover, the periodic Mhc-GFP aligns laterally to build the typical striated pattern that becomes more refined over time (Movie 2, Figure 2B - H). Importantly, the periodic Mhc-GFP pattern forms simultaneously along the future contraction axis of the muscle and also the cross-striations appear largely concurrently throughout the entire muscle fiber, again suggesting a self-organisation mechanism of the individual components to build the observed regular pattern.

Next, we explored the relationship of actin and myosin filaments – the two major myofibril components – during myofibril assembly at high resolution using fixed images. We used Mhc antibodies and phalloidin to visualise Mhc and Actin, respectively. While the Mhc-GFP trap line only labels particular Mhc isoforms (Clyne et al., 2003; Orfanos and Sparrow, 2013), the antibody should label most Mhc isoforms, allowing a better visualisation of the thick filaments. Phalloidin stainings showed that actin filaments are present at 40 h APF. These actin filaments display an obvious polar orientation along the long myotube axis; however, they are still rather short and discontinuous. Importantly, the low levels of Mhc that are detectable by antibodies at 40 h APF reveal a dotty Mhc pattern throughout the myotube,
without an obvious enrichment on actin filaments (Figure 3A). This pattern changes until 46 h APF, when Mhc levels have increased and Mhc dots are recruited onto the actin filaments, which themselves appear longer and more continuous (Figure 3B). Although Mhc is still present in small dots without periodic pattern, we termed these actin-myosin structures present at 46 h APF immature myofibrils.

Consistent with the live imaging, Mhc expression increases further until 50 h APF when Mhc assembles into a periodic pattern that alternates with the actin pattern (Figure 3C). As observed in the Mhc-GFP movies, the Mhc filament pattern is not yet laterally aligned at this stage. However, this changes rapidly and cross-striated myofibrils with a prominent lateral alignment of actin and myosin filaments are detectable at 52 h APF (Figure 3D). Consistent with our live imaging data, these striations further refine during the next hours of development, resulting in distinct but overlapping actin and myosin filaments, which are laterally aligned (Figure 3E, F). Taken together, these data show a gradual maturation of the myofibrils throughout the muscle fiber and suggest that actin and myosin filaments self-organise to form cross-striated myofibrils.

**Mechanical tension precedes myofibrillogenesis**

In the non cross-striated *Drosophila* flight muscles we have demonstrated that mechanical tension precedes the formation of myofibrils. However, we had not been able to determine tension during the myofibril assembly or myofibril maturation itself (Weitkunat et al., 2014). It also remained unclear if tension build-up generally precedes myofibril formation, also in cross-striated muscle types. To investigate tension formation before and during myofibrillogenesis of cross-striated muscles, we performed laser lesion experiments using a pulsed UV-laser (Mayer et al., 2010) and cut within abdominal myotubes at 36 h and 40 h APF. When performing a large lesion, to cut the myotube entirely, both myotube halves
recoil significantly within the first second after the cut (Movies 3, 4 and Figure 4). Additionally, the myotube ends move outwards after the cut, supporting that the myotube has indeed made mechanical contacts with the overlaying epithelium during these stages and has built up mechanical tension across the muscle (Figure 4A’, B’, C, D). A similar recoil is also detected after a smaller micro-lesion, which only partially severs the myotube (Movies 5, 6 and Supplementary Figure 2). These data demonstrate that mechanical tension is indeed present within the myotubes from 36 to 40 h APF, which is the stage before immature myofibrils are assembling. This suggests that mechanical tension generally precedes myofibril assembly in developing muscle, including cross-striated muscle types.

**Imature myofibrils are contractile**

In order to investigate if tension is also present at 46 h, when immature myofibrils have assembled, we performed the same micro-lesion experiments as above, leading to a surprising result – the injured myofiber starts to contract after the laser lesion (Movie 7 and Supplementary Figure 3). To explore this interesting result in more detail, we only induced a nano-lesion in the muscle, which does not result in a visible rupture. Such a nano-lesion has no effect on overall muscle morphology at 40 h APF (Movie 8, Figure 5A, C). Strikingly however, the nano-lesions induce muscle fiber contractions at 46 h APF, resulting in both fiber ends moving closer together, instead of further apart (Movie 8, Figure 5B, D). As an influx in Ca\(^{2+}\) ions is the trigger of sarcomere contractions in mature muscles, we tested if nano-lesions result in a cytoplasmic Ca\(^{2+}\) peak in the developing muscles. By applying the Ca\(^{2+}\) indicator GCaMP6 (Chen et al., 2013), we indeed detected a strong Ca\(^{2+}\) increase within the muscles following the nano-lesions, both at 40 h and 46 h APF (Movie 9 and Figure 5E, F). Supposedly, Ca\(^{2+}\) is released from laser-fragmented intracellular stores into the cytoplasm, where it triggers muscle fiber contraction at 46 h but not at 40 h APF. These data demonstrate
that the immature myofibrils that have started to incorporate Mhc, but not the actin filaments present at 40 h APF, are capable of contracting upon release of Ca\(^{2+}\). Moreover, the entire muscle fiber must be mechanically coupled at 46 h APF as the fiber contraction is present at both muscle ends (Figure 5B’, B’’). These results are consistent with a self-organisation of actin and myosin filaments into myofibrils across the entire muscle fiber.

**Myofibril contractility increases before striations appear**

Since laser induced nano-lesions may induce other changes than solely increasing Ca\(^{2+}\) ions, we aimed to increase cytoplasmic Ca\(^{2+}\) concentrations directly using optogenetics. We expressed the light-gated cation channel Channelrhodopsin (Boyden et al., 2005) in muscles and activated it with 488 nm light, the same wavelength used to image muscle morphology. Interestingly, upon channel activation at 46 h APF, we indeed observed small muscle contractions in about 60% of the stimulated muscle fibers (Movie 10, Figure 6A, D). Both, the intensity of the induced contractions, as well as the incidence increased with development, resulting in strong contractions along the entire muscle fiber in all stimulated muscles at 50 h or 52 h APF (Movie 10, Figure 6B - D). These data show that a depolarisation induced Ca\(^{2+}\) peak efficiently induces myofiber contractions from 50 h APF onwards. Interestingly, this matches the developmental time period when immature myofibrils (50 h APF) transition to cross-striated myofibrils (52 h APF).

**Spontaneous contractions precede striations**

Next, we asked the question, if contractions occur spontaneously in the muscles during this critical developmental period between 40 and 52 h APF. To address this question, we imaged developing muscles expressing Lifeact-Ruby and GCaMP6 at high time resolution to monitor muscle morphology and cytoplasmic Ca\(^{2+}\) levels at the same time. We find that at 40 h APF
muscles do not contract spontaneously (Figure 6H, I). At 46 h APF, 30% of muscles do show small spontaneous contractions within a 20 min observation period. These contractions are always associated with a transient strong increase in cytoplasmic \( \text{Ca}^{2+} \) levels (Movie 11, Figure 6E, H, I). Importantly, at 50 h APF most (81%) and at 52 h APF all imaged muscles strongly contract at least once within the 20 min observation period (Movie 11, Figure 6F - I). The average contraction frequency increases during development from 0.8 contractions within 20 min at 46 h APF to 8.6 contractions within 20 min at 52 h APF (Figure 6I) This demonstrates that spontaneous muscle twitchings occur frequently during the developmental period preceding the appearance of cross-striated myofibrils. It also shows that immature myofibrils at 50 h APF are already highly contractile. Together, these data strongly support the hypothesis that the periodic actomyosin arrays in the assembling myofibrils are mechanically coupled throughout the entire muscle fiber and are responsive to stimulatory \( \text{Ca}^{2+} \) signals.

**Spontaneous contractions contribute to cross-striation formation**

In order to functionally investigate the role of the spontaneous contractions for cross-striation formation we aimed to block the contractions from 46 h APF onwards and investigate the consequences for Mhc-GFP localisation in the muscles. We tried to optogenetically block the contractions using Halorhodopsin (Fenno et al., 2011), but failed to do so reliably and continuously over several hours of muscle development (data not shown). As an alternative approach, we used Thapsigargin, a chemical inhibitor of SERCA, the main \( \text{Ca}^{2+} \) pump located in the membrane of the sarcoplasmatic reticulum (Treiman et al., 1998). To assess the potency of Thapsigargin, we injected it into the abdomen of pupae between 52 h and 53 h APF and imaged these at 55 h APF, a stage after which spontaneous contractions have been initiated (Figure 6H, I). Indeed, we find that Thapsigargin is a potent blocker of these spontaneous contractions (Movie 12).
To test the impact of the contractions on cross-striation formation, we injected Thapsigargin into pupae at 46 h APF, when the contractions normally begin to occur, incubated them for 10 h and imaged Mhc-GFP distribution at 56 h APF at high resolution using a multi-photon microscope. We find that 87 % of the control injected pupae show normal cross-striations at 56 h APF (Figure 7A-C, G), whereas 73 % of the Thapsigargin injected pupae fail to build cross-striations in their abdominal muscles close to the injections site (Figure 7D-G). These data demonstrate that Ca\(^{2+}\) induced contractions after 46 h APF are required to assemble regular cross-striations in *Drosophila* abdominal muscles.

**Discussion**

Myofibrils displaying a periodic sarcomere pattern are built during muscle development. Muscle fibers can be very long, more than 20 cm for a number of human muscles, while sarcomeres are below 4 µm in most animals (Burkholder and Lieber, 2001). Therefore, the precise periodic assembly of hundreds or often thousands of sarcomeres into long linear myofibrils is a challenging task. Our results demonstrate that muscles approach this task by first attaching both muscle fiber ends to tendons cells. When attachment is initiated the actin cytoskeleton is polarised along the long axis of the muscle but has no periodic order yet. When muscle attachments have matured, a periodic actomyosin pattern assembles largely simultaneously across the entire muscle fiber length, suggesting sarcomeric self-organisation to build long continuous myofibrils. The concurrence of attachment maturation and myofibril self-organisation is not only observed in body wall muscles resembling vertebrate skeletal muscles, but also in the specialised flight muscles (Weitkunat et al., 2014), strongly suggesting that myofibril self-organisation is a general mechanism to assemble myofibrils within muscle fibers *in vivo*. The beauty of such a mechanism is that it always results in periodic myofibrils spanning across the entire muscle fiber, independently of total fiber...
length. A similar periodic acto-myosin self-organisation has been predicted by theoretical models (Friedrich et al., 2012; Yoshinaga et al., 2010) and was also found in nonmuscle cells, such as the stress fibers of cultured cells (Pellegrin and Mellor, 2007) or the peri-junctional actomyosin belts present in certain epithelial cell sheets \textit{in vivo} (Ebrahim et al., 2013). Hence, simultaneous self-organisation appears to be a general mechanism to create periodic acto-myosin structures, with developing muscles being a particularly prominent example.

The synchrony of pattern formation suggests that the individual components are cooperating during the assembly process. We have shown that mechanical tension is required to build the highly regular myofibrils of the specialised flight muscles (Weitkunat et al., 2014). Here, we expanded these studies to the cross-striated body muscles of the adult fly and show that tension is not only present before but also during simultaneous myofibril assembly. Importantly, we found that immature myofibrils, which have started to incorporate muscle myosin but do not display a periodic pattern yet, are already twitching in response to increased \(\text{Ca}^{2+}\) levels. This demonstrates that the individual components within immature myofibrils are already mechanically coupled along the fiber axis. The active contractions also suggest that myosin motors create forces, which contribute to the tension build-up during myofibril assembly. This is supported by myosin inhibitor studies \textit{in vitro} (Kagawa et al., 2006) and by the expression of motor-deficient myosin variants \textit{in vivo} (Weitkunat et al., 2014), both resulting in severe myofibrillogenesis defects. The \(\text{Ca}^{2+}\) induced twitching of immature myofibrils also implies that the \(\text{Ca}^{2+}\) dependent troponin, tropomyosin machinery, which regulates mature muscle contractions (Ohtsuki and Morimoto, 2008), is co-assembling together with the periodic actomyosin pattern and is controlling active myofibril twitching already during development.

We have incorporated these data into an updated myofibrillogenesis model, which proposes two roles for mechanical tension, a local and a global one. Locally, tension can act
as a molecular compass to orient individual myofibrillar components, like bipolar actin and myosin mini-filaments, along the long axis of the muscle. Thereby, it creates linear myofibrils with periodically arranged sarcomeres. Globally, tension can coordinate the self-organisation process across the entire muscle fiber. This global coordination synchronises the assembly process and results in balanced forces throughout the system. This synchrony appears analogous to phase transitions from unordered to more ordered states, when tension is large enough, or molecularly speaking, when enough myosin has been recruited onto the myofibrils to pull cross-linked bipolar actin filaments into a periodic order (Figure 8).

Such a tension supported myofibrillogenesis model likely also applies to mammals. In the mammalian heart, myofibrils are anchored at specialised adherens junctions that mechanically couple myofibrils across cell membranes of neighbouring cardiomyocytes (Perriard et al., 2003). If cardiomyocytes are grown individually in suspension and are therefore not mechanically coupled, effective myofibrillogenesis is blocked (Marino et al., 1987). Similarly, skeletal muscles that are defective in integrin function and thus cannot effectively generate tension, fail to assemble normal myofibrils during embryonic development of mice (Schwander et al., 2003). However, direct in vivo evidence for an instructive role of mechanical tension during myofibrillogenesis awaits live in vivo imaging of myofibril formation in developing mammalian muscle.

Mature mammalian heart or skeletal muscles as well as Drosophila body wall muscles are cross-striated. Formation of cross-striations requires the lateral alignment of neighbouring myofibrils into register, an essential process that is not well investigated in developing muscles in vivo. Both our live imaging and our immunohistochemistry data demonstrate that the transition from immature, non-aligned myofibrils to cross-striated myofibrils occurs simultaneously across the entire myofiber. This again strongly argues for a globally coupled system. Interestingly, the incidence of the spontaneous muscle fiber
contractions coincides with myofibril alignment. Myofiber contractions are detectable at 46 h APF \textit{in vivo} and their frequency strongly increases until 50 h APF, shortly before regular actomyosin cross-striations are detected. Indeed, when the contractions are blocked by blocking Ca$^{2+}$ cycling with the SERCA inhibitor Thapsigargin, the formation of cross-striations is severely impaired. Although it is difficult to rule out an indirect effect of potential ER-stress induced by the SERCA block, these data strongly suggest that Ca$^{2+}$-dependent actomyosin twitches refine the actomyosin periodicity and result in efficient lateral alignment of neighbouring myofibrils, an essential maturation step to build cross-striated muscle (Figure 8).

A role for Ca$^{2+}$ dependent twitches has also been suggested for mammalian myofibrillogenesis in \textit{in vitro} experiments. Blocking membrane depolarisation and spontaneous twitching in cultured rat myoblasts resulted in severe sarcomerogenesis defects (De Deyne, 2000). Conversely, electrically induced Ca$^{2+}$ peaks could effectively promote sarcomere assembly in C2C12 cell derived myotubes \textit{in vitro} (Fujita et al., 2007). Further, it has been shown that neuronal innervation and thus spontaneous muscle twitching results in increased cross-striations in cultured Xenopus myotubes (Kidokoró and Saito, 1988). Similar to the twitchings we found in developing \textit{Drosophila} muscles \textit{in vivo}, the contractions present or induced in cell culture also resemble contractions of mature muscle, as they require ryanodine receptor (RyR) dependent Ca$^{2+}$ cycling (Ferrari et al., 1998). Interestingly, either blocking the RyR \textit{in vitro} (Harris et al., 2005) or knocking it out \textit{in vivo} results in severe myofibrillogenesis defects, with RyR mutant mice having only small muscles that lack cross-striations (Barone et al., 1998; Takeshima et al., 1994). Together, these observations strongly suggest that Ca$^{2+}$ dependent myofibril twitching is important for myofibril cross-striation formation during mammalian muscle morphogenesis. As mammalian muscle fibers are often at least one magnitude larger than \textit{Drosophila} muscle fibers, tension dependent self-
organisation is likely even more critical to form regular cross-striated mammalian muscle. As muscle growth and muscle regeneration continues through human life time, defects in tension supported myofibril self-organisation may result in severe myofibril disarrays and fatal myopathies (Clarke, 2008; Tajsharghi and Oldfors, 2012; Udd, 2008).

Materials and Methods

Fly strains

All fly work, unless otherwise stated, was performed at 27 °C to enhance GAL-4 activity. Muscle-specific expression was achieved using Mef2-GAL4 (Ranganayakulu et al., 1996). Abdominal muscles were labelled with Mef2-GAL4, UAS-GFP-Gma (Dutta et al., 2002), UAS-Lifeact-Ruby (Hatan et al., 2011), UAS-Cherry-Gma (Millard and Martin, 2008) or Mhc-GFP (MhcWee-P26) (Clyne et al., 2003). Ca^{2+} was imaged using UAS-GCaMP6f (BL#42747, gift of Alex Mauss) (Akerboom et al., 2012) and muscles were depolarised with UAS-Channelrhodopsin2-H134R-mCherry (UAS-ChR2-H134R, gift of Alex Mauss) (Pulver et al., 2009).

Fixed analysis of developing abdominal muscles

Staged wild-type pupae (white^{118}) were dissected as described (Weitkunat and Schnorrer, 2014). To relax the myotubes, the dissections were performed in cold relaxing solution followed by fixation in relaxing solution with 4% paraformaldehyde (PFA). After washing in PBS containing 0.3 % TritonX (PBST) dissected pupae were blocked for 30 min with normal goat serum (1:30), stained with primary antibodies over night at 4 °C and washed 3 times in PBST. Secondary antibodies (1:500), rhodamine phalloidin (1:500), or phalloidin-Alexa488 (1:500) (all from Molecular Probes) were added for 2 h at room temperature, followed by 3 washing steps in PBST, before samples were embedded in Vectashield. Primary antibodies:
mouse anti-β-PS-Integrin 1:500 (CF.6G11, DSHB), mouse anti-Mhc 1:100 (J. Saide, Boston University). Images were acquired with a Zeiss LSM 780 and processed with Fiji (Schindelin et al., 2012) and Photoshop.

**Time-lapse movies**

GFP expressing pupae were staged and a small opening was cut into the pupal case on the dorso-lateral side of the abdomen using sharp forceps and scissors. Pupae were transferred into a custom-made slide with a slit fitting the pupa and turned 20 - 30° resulting in abdominal myotubes facing up. The opening was covered with a thin layer of 86 % glycerol and a cover slip to prevent evaporation. Z-stacks were acquired every 5 to 20 min with a multi-photon set up (LaVision) using a long distance 20x objective (NA = 1.0, Zeiss) or spinning disc confocal microscope (Zeiss, Visirion) using a 40x long distance objective (NA = 1.0, Zeiss). The microscope stage was heated to approximately 27°C.

**Tension measurements**

Muscle severing and imaging was performed on a custom made nano-dissection device based on (Colombelli et al., 2009), including a spinning-disc unit (CSU-X1, Yokogawa) with an Andor NEO sCMOS camera and a 63x 1.20 water or a 63x 1.40 oil objective (Leica Microsystems). Laser output: 355 nm, 350 psec pulse duration, 72 kW peak power, 25 mW average power. Imaging was done with the spinning disc unit and a COBOLT MLD™ 488 nm laser. Movies were taken at frame rates between 2 fps and 12.5 fps. Images and movies were processed with Fiji. Tension release in severed muscles was inferred from the response of cut edges, structures along the muscle and attachment sites to severing. For Ca$^{2+}$ imaging during laser-cutting muscles were labelled using *Mef2-GAL4, UAS-Cherry-Gma* or *UAS-CD8-Cherry* and Ca$^{2+}$ was imaged with *UAS-GCaMP6f*. Pupae at the respective time points
were positioned with a 561 nm laser (COBOLT Jive 50TM), optically stimulated by the 355nm laser (1 pulse) and imaged with the 488 nm laser on the nano-dissection device.

**Quantification of spontaneous contractions**

Muscles were labelled using *Mef-GAL4, UAS-Lifeact-Ruby* and Ca\(^{2+}\) was imaged with *UAS-GCaMP6f*. Pupae of the respective age were prepared for life-imaging and imaged for 20 min using 600 msec intervals on a spinning disc microscope. Contractions were counted manually. Intensity of the GCaMP6f signal was quantified using Fiji. Contractions per minute were calculated using Excel and graphs were designed using Adobe illustrator and Prism (GraphPad).

**Induction of contractions using channelrhodopsin.**

*UAS-Channelrhodopsin2-H134R-mCherry* was expressed using *Mef2-GAL4* and muscles were labelled with *UAS-GFP-Gma*. Yeast paste containing 1 mM all-trans-retinal (Sigma) was mixed into the fly food containing the larvae one day before the pupae were staged for imaging. Pupae were then kept in dark until imaging. CHR2 was activated using 488 nm; this wavelength was simultaneously used for GFP excitation and 40 time points were imaged using 50 msec intervals on a spinning disc microscope. This was repeated 8 times on the same pupa with 60 sec breaks in-between repetitions. The 2nd repeat was used for analysis.

**Pupal injections**

Similar to the time lapse movies, a small opening was cut into the pupal case of 46 h APF *Mhc-GFP* pupae. A small amount of either DMSO or 2.5 - 5mM Thapsigargin (Sigma) dissolved in DMSO was injected using a self-made glass needle and a FemtoJet injection system (Eppendorf). Injected pupae were transferred into custom-made imaging slides, put
back into the incubator and imaged with a multi-photon microscope at high resolution at 56 h APF. Injections were usually performed into the left half of abdominal segment A2 and all visible dorsal longitudinal muscles in abdominal segments A2 and A3 were used to quantify the cross-striations.

For the initial tests of drug efficiency, Mef-GAL4, UAS-Lifeact-Ruby and Mhc-GFP expressing pupae were similarly injected at 52 h - 53 h APF and imaged to assess the spontaneous contractions at 55 h APF using 300 msec intervals on a spinning disc microscope.

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Author contributions

M.W. performed the experiments for and largely generated Figures 1, 3 and 6 with input from F.S. M.L performed the experiments for and largely generated Figures 4 and 5 with input from A.B. F.S. generated the data and made Figures 2, 7 and 8. F.S and A.B. conceived and supervised the project. F.S. wrote the manuscript with input from M.W. and A.B.
Competing interests

The authors declare that no competing interests exist.
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Figures.

Figure 1. Simultaneous sarcomerogenesis in *Drosophila* abdominal body muscles

(A - D) 30 h (A), 40 h (B), 50 h (C) and 60 h (D) APF time points from a spinning disc confocal movie of developing dorsal abdominal muscles expressing Lifeact-Ruby in red and Mhc-GFP in green (arrowheads, Movie 1). (A’ - D’) Relative Mhc-GFP intensities from representative longitudinal lines drawn within an abdominal dorsal muscle at the respective time points; Mhc-GFP expression appears between 40 h and 50 h APF simultaneously across the muscle fiber (B’ - C’). (A’’ - D’’) Schemata of developing dorsal abdominal muscles, Mhc-GFP is indicated in white. Scale bar 25 µm.
Figure 2. Formation of cross-striated abdominal body muscles - live

(A - H) 48 h (A), 50 h (B), 52 h (C), 54 h (D), 56 h (E), 58 h (F), 60 h (F) and 62 h APF (H) time points from a multi-photon movie of two developing dorsal abdominal muscles expressing Mhc-GFP (Movie 2). (A’ - H’). Contrast-adjusted Mhc-GFP intensities within one abdominal dorsal muscle (yellow lines in A - H) at the respective time points. Note the simultaneous appearance of Mhc-GFP periodicity from 50 - 52 h onwards and its lateral alignment. Scale bar 10 µm.
Figure 3. Myofibril assembly of abdominal body muscles

(A - F) Dissected wild-type abdomen at 40 h (A), 46 h (B), 50 h (C), 52 h (D), 56 h (E) and 72 h APF (F). Actin (green) and Mhc (red) were labelled by phalloidin and anti-Mhc antibodies, respectively. Actin filaments are visible at 40 h (A, white arrowheads). Mhc is recruited to immature myofibrils (B, C white arrowheads) in dots at 46 h and in lines at 50 h.
APF. Both Actin and Mhc organise into striated patterns that align laterally and refine from 52 h - 72 h APF (D - F, red and green arrowheads). Sale bar 5 µm.
Figure 4. Abdominal body muscles develop under mechanical tension

(A - B’’) Time points from spinning disc confocal movies of myotubes labelled by *Mef2-GAL4, UAS-GFP-Gma* at 36 h and 40 h APF before (A, B) and after complete myotube severing using laser cutting (A’ - B’’, Movies 3, 4). Newly created myotube ends (orange arrowheads) move away from the cutting site (marked by + in A, B). Anterior and posterior myotube ends move outwards; compare pre-cut (green arrowheads in A, B) with post-cut (blue arrowheads in A’, B’). (A’’, B’’’) Kymographs of movies 3 and 4 displaying intensities at the red lines indicated in A and B. (C, D) Schemata of the laser cuts; myotube movement after laser severing is indicated by arrows. Scale bar 10 µm.
Figure 5. Laser-induced myotube contractions during development.

(A - B’’) Time points from spinning disc confocal movies of myotubes labelled by *Mef2-GAL4, UAS-GFP-Gma* at 40 h and 46 h before (A, B) and after laser induced nano-lesion (A’ - B’’, Movie 8). At 46 h APF anterior and posterior myotube ends move inwards after nano-lesion indicating myotube contraction; compare pre-cut (green arrowheads, B) with post-cut (blue arrowheads B’). (A’’, B’’) Kymographs of movie 7 displaying intensities at red lines.
indicated in A and B. (C, D). Schemata of the laser cuts; myotube movement after nano-lesion is indicated with arrows. (E - F’’’) Ca^{2+} imaging of myotubes labelled with Mef2-GAL4; UASGCaMP6 at 40 h and 46 h APF before (E, F) and after laser induced nano-lesion (E-F’’, Movie 9). Both, after nano-lesions at 40 h (E’, E’’) and 46 h APF (F’ and F’’’) a Ca^{2+} signal is visible in myotubes. Scale bars 10 μm.
Figure 6. Optogenetically-induced and spontaneous myotube contractions during development.

(A - C’) Time points from spinning disc confocal movies of myotubes labelled by Mef2-GAL4, UAS-GFP-Gma and additionally expressing UAS-Channelrhodopsin at 46 h (A), 50 h (B) and 52 h APF (C, Movie 10). Ca$^{2+}$ influx and contractions are induced while imaging with 488 nm laser light. Bulges are marked by yellow arrows (A’, B’, C’) and myotube end movements with green vs. blue arrowheads. Note that myotube contractions increase at 50 h APF. (D) Quantification of myotube contractions; number of contracting myotubes increases from 46 h to 50 h APF. (E - G’’) Time points from spinning disc confocal movies of myotubes labelled with Mef2-Gal4, UAS-Lifeact-Ruby and UAS-GCaMP6 imaged for a 20 min interval at 46 h (E - E’), 50 h (F - F’’) and 52 h APF (G - G’’). Ca$^{2+}$ influx is visualised in green and muscles in red (Movie 11). Bulges are marked by yellow arrows (E’, F’, G’) and myotube end movements with green vs. blue arrowheads. Note that myotube contractions increase at 50 h APF. (H) Number of myotubes that contract within 20 min intervals at 40 h, 46 h, 50 h and 52 h APF. (I) Contraction frequency during development. Each point represents number of contractions of one myotube within a 20 min interval. The mean contraction frequency (blue line) increases with time. Scale bars 50 µm.
Figure 7. Contractions contribute to cross-striation formation.

(A - F) Mhc-GFP pupae, either injected with DMSO (A - C) or with Thapsigargin (D - F) at 46 h APF and imaged at 56 h APF. Three representative examples of the phenotypic spectrum, ranging from normal to irregular and absent cross-striations, are shown for control and Thapsigargin injection. (G) Quantification of the cross-striation phenotypes of the injected pupae according to the phenotypic range shown in A - F. Scale bar 10 μm.
Figure 8. Tension driven model of myofibrillogenesis.

Locally, tension orients actin and myosin filaments along the axis of the muscle to assemble linear myofibrils. Globally, it coordinates the synchronous formation of periodic acto-myosin filaments across the entire muscle fiber. Spontaneous muscle twitchings contribute to the self-organisation of perfectly ordered striated myofibrils. Further details are elaborated in the discussion part of the text.
| Time  | Actin, β-Integrin | β-Integrin | Actin, β-Integrin zoomed | Actin, high gain, zoomed |
|-------|-------------------|------------|-------------------------|--------------------------|
| 36 h  | ![Image](A)        | ![Image](B) | ![Image](A')           | ![Image](A'')            |
| 40 h  | ![Image](B)        | ![Image](C) | ![Image](B')           | ![Image](B'')            |
| 46 h  | ![Image](C)        | ![Image](D) | ![Image](C')           | ![Image](C'')            |
| 52 h  | ![Image](D)        | ![Image](E) | ![Image](D')           | ![Image](D'')            |
| 56 h  | ![Image](E)        | ![Image](F) | ![Image](E')           | ![Image](E'')            |
| 72 h  | ![Image](F)        |            | ![Image](F')           |                          |
Supplementary Figure 1. Attachment of Drosophila adult body muscles

(A - F) Dissected wild-type abdomen at 36 h (A), 40 h (B), 46 h (C), 52 h (D), 56 h (E) and 72 h APF (F). Actin (green) and β-Integrin (red) were labelled with phalloidin and anti-β-PS-Integrin antibodies, respectively. (A’- F’) Magnifications of anterior myotube tips at respective time points; β-Integrin is accumulating at smoothening myotube tips over time (arrowheads). (A’’- C’’) Anterior tips at high magnification and high Actin gain; Myotubes are attached to the epidermis at 40 h and 46 h APF. Scale bars 25 μm (A - F), 5 μm (A’- F’, A’’ - C’’).
Supplementary Figure 2. Abdominal body muscles develop under tension

(A - B'') Time points from spinning disc confocal movies of myotubes labelled by Mef2-GAL4, UAS-GFP-Gma at 36 h and 40 h APF before (A, B) and after partial myotube severing using laser cutting (A’ - B’’, Movies 5, 6). Wounded ends (orange arrowheads) move away from the cutting site (yellow lines in A, B). (A’’, B’’)

Kymographs of movies 5 and 6 displaying intensities at the red lines indicated in A and B. (C, D) Schemata of the laser cuts, myotube movement after laser severing is indicated with arrows. Scale bar 10 µm.
Supplementary Figure 3. Laser-induced myotube contractions during development

(A - A’’) Time points from spinning disc confocal movie of myotubes labelled by Mef2-GAL4, UAS-GFP-Gma at 46 h before (A) and after partial myotube severing using laser-cutting (A’, A’’, Movie 7). Wounded ends (orange arrowheads) move away from the cutting site (yellow line in A). Induced bulges are marked by white arrowheads. (A’’) Kymograph of Movie 7 displaying intensities at the red line indicated in A. (B) Scheme of the laser cut; myotube movement after laser severing is indicated with arrows. Scale bar 10 µm.
Movie 1. Simultaneous sarcomerogenesis in *Drosophila* abdominal body muscles

Z-projection of spinning disc confocal movie of developing dorsal abdominal muscles expressing Lifeact-Ruby (red) and Mhc-GFP (green) shown as merge on the right and Lifeact-Ruby in grey on the left. Note the simultaneous establishment of the periodic Mhc-GFP pattern. Large red structures are remaining and degrading larval muscles. Movie plays with 5 frames per second. Time is indicated in hh:mm APF.
Movie 2. Formation of striated abdominal body muscles

Z-projection of a multi-photon movie showing developing dorsal abdominal muscles expressing Mhc-GFP. Note the simultaneous establishment of the periodic Mhc-GFP pattern that aligns at about 52h APF across the entire muscle. Round moving cells are hemocytes digesting larval Mhc-GFP. Movie plays with 5 frames per second. Time is indicated in hh:mm APF.
Movie 3. Myotubes at 36 h APF are under mechanical tension

Single plane spinning disc confocal movie of two myotubes labelled by Mef2-GAL4, UAS-GFP-Gma at 36 h. The lower one is cut with a UV laser. Note the recoil of the wounded ends and also the movement of the distal myotube ends. Time is indicated in seconds and starts at the cut. Movie plays with 5 frames per second.
**Movie 4. Myotubes at 40 h APF are under mechanical tension**

Single plane spinning disc confocal movie of a myotube labelled by *Mef2-GAL4, UAS-GFP-Gma* at 40 h, which is cut with a UV laser. Note the recoil of the wounded ends and also the movement of the left distal myotube end (the right end is not visible in this single plane). Time is indicated in seconds and starts at the cut. Movie plays with 5 frames per second.
Movie 5. Myotubes at 36 h APF are under mechanical tension

Single plane spinning disc confocal movie of two myotubes labelled by *Mef2-GAL4, UAS-GFP-Gma* at 36 h; the lower one is partially severed by a UV laser at the right side (see Supplementary Figure 2). Note the recoil of the wound indicating tension. Time is indicated in seconds and starts at the cut. Movie plays with 5 frames per second.
Movie 6. Myotubes at 40 h APF are under mechanical tension

Single plane spinning disc confocal movie of three myotubes labelled by *Mef2-GAL4, UAS-GFP-Gma* at 40 h. The middle one is partially severed by a UV laser at the right side (see Supplementary Figure 2). Time is indicated in seconds and starts at the cut. Movie plays with 5 frames per second.
Movie 7. Myotubes at 46 h APF are contractile upon laser lesion

Single plane spinning disc confocal movie of two myotubes labelled by *Mef2-GAL4, UAS-GFP-Gma* at 40 h. The lower one is partially severed by a UV laser in the middle (see Supplementary Figure 3). Note the induced contraction after the cut. Time is indicated in seconds and starts at the cut. Movie plays with 5 frames per second.
**Movie 8. Myotubes at 46 h but not 40 h APF are contractile upon laser lesion**

Single plane spinning disc confocal movies of myotubes labelled by *Mef2-GAL4, UAS-GFP-Gma* at 40 h (upper movie) and 46 h APF (lower movie). The muscles in the center of the movies were severed by a UV nano-lesion (see Figure 6 A, B). Note the induced contraction after the cut at 46 h but not at 40 h APF. Time is indicated in seconds and starts at the cut. Movies plays with 5 frames per second.
Movie 9. Laser severing induces $\text{Ca}^{2+}$ release

Single plane spinning disc confocal movies of myotubes labelled with Mef2-GAL4, UAS-GCaMP6 at 40 h (upper movie) and 46 h APF (lower movie). Both muscles were severed by a UV nano-lesion (see Figure 6 E, F). Note the induced $\text{Ca}^{2+}$ release at both time points, with induced contraction only at 46 h APF. Time is indicated in seconds and starts at the cut. Movies plays with 10 frames per second.
Movie 10. Optogenetically induced muscle contractions

Single plane spinning disc confocal movies of myotubes labelled with *Mef2-GAL4*, *UAS-GFP-Gma* and *UAS-Channelrhodopsin* at 46 h (upper movie), 50 h (middle) and 52 h APF (lower movie). \( \text{Ca}^{2+} \) influx is induced while imaging with 488 nm laser light and induces a small contraction at 46 h APF and strong contractions at 50 h and 52 h APF. Time is indicated in seconds. Movies plays with 10 frames per second.
Movie 11. Spontaneous muscle contractions

Single plane spinning disc confocal movies of myotubes labelled with *Mef2-Gal4*, *UAS-Lifeact-Ruby* and *UAS-GCaMP6* at 46 h (upper movie), 50 h (middle) and 52 h APF (lower movie). Spontaneous Ca$^{2+}$ influx is found at all stages, and induces a small contraction at 46 h APF and strong contractions at 50 h and 52 h APF. Time is indicated in seconds. Movies plays with 5 frames per second.
**Movie 12. Thapsigargin blocks muscle contractions**

Single plane spinning disc confocal movies of myotubes labelled with *Mef2-Gal4, UAS-Lifeact-Ruby* and Mhc-GFP (not shown), either injected with DMSO (left movie) or with Thapsigargin (right movie) at 52 h - 53 h APF and imaged at 55 h APF. Time is indicated in seconds. Movies plays with 10 frames per second.