REVIEW

The insect perspective on Z-disc structure and biology

Frieder Schöck1 and Nicanor González-Morales2,*

ABSTRACT
Myofibrils are the intracellular structures formed by actin and myosin filaments. They are paracrystalline contractile cables with unusually well-defined dimensions. The sliding of actin past myosin filaments powers contractions, and the entire system is held in place by a structure called the Z-disc, which anchors the actin filaments. Myosin filaments, in turn, are anchored to another structure called the M-line. Most of the complex architecture of myofibrils can be reduced to studying the Z-disc, and recently, important advances regarding the arrangement and function of Z-discs in insects have been published. On a very small scale, we have detailed protein structure information. At the medium scale, we have cryo-electron microscopy maps, super-resolution microscopy and protein–protein interaction networks, while at the functional scale, phenotypic data are available from precise genetic manipulations. All these data aim to answer how the Z-disc works and how it is assembled. Here, we summarize recent data from insects and explore how it fits into our view of the Z-disc, myofibrils and, ultimately, muscles.

KEY WORDS: Insects, Muscle, Sarcomere, Z-disc

Introduction
Together with a nervous system, muscles evolved in multicellular animals to provide the capacity to move freely. Muscles appeared in eumetazoans, which comprise bilateral animals (e.g. mammals and insects) and cnidarians (e.g. jellyfishes). Both bilateral animals and cnidarians have muscles (Burton, 2008; Seipel and Schmid, 2005). The muscles power movement by bridging two rigid skeleton structures and pulling them together (Sparrow and Schöck, 2009; Lemke and Schnorrer, 2017). Vertebrate muscles are linked to the bones, whereas insect muscles are attached to the exoskeleton (Gunage et al., 2017). A good example of muscle function in insects is the indirect flight muscle (IFM), which is attached to the thoracic exoskeleton and powers flight in several large insect groups, including Coleoptera, Hymenoptera and Diptera (Vigoreaux, 2001; Deora et al., 2017). Muscle contraction deforms the exoskeleton, indirectly causing the wings to move (Vigoreaux, 2001).

Muscle fibers are composed of long myofibrils, which are a tandem array of cylinder-shaped structures called sarcomeres. The sarcomere is the basic contractile unit in the muscles and is mainly composed of two antiparallel filament systems. The myosin thick filaments are composed of myosin heavy chains and light chains (Wang et al., 2018), and the actin thin filaments are composed of actin, tropomysosin and troponin (Szikora et al., 2022). Actin sliding on myosin filaments shortens the sarcomere. Because sarcomeres contract synchronously, the myofibrils shorten, powering muscle contraction (Fig. 1).

Microscopy of sarcomere longitudinal sections has revealed important defined regions or bands based on the presence of myosin and actin filaments. The A-band is the region where myosin filaments are present, the remaining region is called the I-band. In the middle of the A-band lies a region where actin filaments are excluded; it is called the H-zone. At the center of the H-zone, myosin filaments are anchored to a structure called the M-line. Most importantly, at the center of the I-band, actin filaments are anchored to an electron-dense disc, termed the Z-disc (Shafiq, 1963; Reedy and Beall, 1993a; Gunage et al., 2017). The Z-disc is the structure responsible for anchoring and recruiting actin filaments (Fig. 1).

In this Review, we summarize the recent contributions of insect biology to our understanding of the structure, function and assembly of Z-discs. We will focus on the components that are conserved in insects and vertebrates, as they are likely essential components. We will occasionally contrast the mechanistic details in insects with those from vertebrate species to provide a view of the flexibility of the components of the Z-disc.

A brief history of sarcomere descriptions
In 1949, the first high-resolution electron microscopy images of striated muscle appeared (Draper and Hodge, 1949). A few years later, in 1953, the filamentous nature of the sarcomere was established using phase-contrast and electron microscopy (Hanson and Huxley, 1953), and one year later the sliding of actin and myosin filaments was proposed (Huxley and Niedergerke, 1954; Huxley and Hanson, 1954). Soon after, researchers became interested in insects, reporting phase contrast and electron microscopy of a blowfly in 1955 (Hodge, 1955). Similar images were then obtained for other insect species – dragonflies (Smith, 1961), fruit flies (Shafiq, 1963) and cockroaches (Hagopian, 1966). The consensus from this work was that the general structure of sarcomeres is well conserved.

The connecting filaments, the filaments that span the entire half sarcomere and provide passive elasticity, now known to be strings of the titin protein, were reported in a series of papers from 1962 to 1965 (reviewed in Dos Remedios and Gilmour, 2017). In 1963, the first reports of connecting filaments in flies appeared (Auber and Couteaux, 1963), and later, several groups reported titin homologs as the main protein of the connecting filaments (Dos Remedios and Gilmour, 2017; Hu et al., 1990; Nave and Weber, 1990; Lakey et al., 1990; reviewed in Dos Remedios and Gilmour, 2017). Since then, given that most components of the sarcomere are highly conserved among animals (Table 1), insect models have become an essential part of muscle research.

The Z-disc structure
The Z-disc is a massive protein complex. The Z-disc from the IFM of honeybees is ∼120 nm thick and has a diameter of ∼2.5 μm (Rusu et al., 2017; Saide and Ullrick, 1973). The Z-disc diameter from the same muscle in Drosophila is slightly smaller, ∼90 nm thick, and has a diameter of ∼1.5 μm (Reedy and Beall, 1993a). An adult IFM sarcomere contains ∼2430 actin filaments and 825...
myosin filaments, with each myosin filament surrounded by six actin filaments (Fernandes and Schöck, 2014). Other muscles have the same overall architecture but with slight structural variations. For example, the Z-disc from larval body muscles is smaller, less dense and less regular compared to the IFM Z-disc (Wojtowicz et al., 2015; Jani and Schöck, 2007). Additionally, larval body Z-discs are not entirely connected and do not form a straight band (Wojtowicz et al., 2015; Jani and Schöck, 2007). Because of the high structural regularity of the IFM, muscle research is strongly focused on the IFM.

Actin filaments from opposing sarcomeres interleave and anchor at the Z-disc (Reedy and Beall, 1993a; Shaﬁq, 1963). The main protein responsible for anchoring actin filaments is α-actinin, a dimeric protein that is a member of the spectrin superfamily (Lakey et al., 1990; Murphy and Young, 2015). α-Actinin dimers organize the thin filaments by joining them together and providing equal spacing between them (Figs 1D and 2). Single-molecule localization microscopy of the Drosophila IFM has shown that there are two α-actinin-organizing pools flanking the Z-disc from a side view (Szikora et al., 2020). Because each myofibril has ∼2430 actin filaments (Fernandes and Schöck, 2014), each α-actinin pool consists of ∼1215 α-actinin dimers.

Viewed from the front, the Z-disc is a remarkably ordered lattice structure formed by repeated units (Rusu et al., 2017; Cheng and Deatherage, 1989; Saide and Ullrict, 1973). Vertebrate Z-discs have a semi-flexible tetragonal lattice that changes upon muscle contraction (Burgoyne et al., 2015; Perz-Edwards and Reedy, 2011; Luther, 2009). Insect Z-discs have a hexagonal lattice structure (Rusu et al., 2017; Cheng and Deatherage, 1989; Saide and Ullrict, 1973). A cryo-electron tomography model from isolated honeybee Z-discs has been solved to ∼6 nm, providing details about their structure (Fig. 1D,E). Each repeated unit of the lattice anchors six actin filaments, three on each side of the sarcomere (Rusu et al., 2017). Additionally, a large triangular channel per repeat unit extends through the entire Z-disc (Rusu et al., 2017). Overall, the Z-disc is extremely large, has a highly organized repetitive pattern, and is responsible for organizing and anchoring actin filaments.

The Z-disc protein core and the first steps of Z-disc assembly
Myofibril assembly occurs very fast in insects, taking at most several hours depending on the specific muscles. Regardless of the assembly speed, the Z-disc assembly steps are the same. First, the Z-disc grows from small actin-organizing centers called Z-bodies (Reedy and Beall, 1993a; Katzemich et al., 2013; Loison et al., 2018). These bodies condense from the cytoplasmic pool of proteins into discrete insoluble bodies (Katzemich et al., 2013; Orfano et al., 2015). The core Z-disc proteins Zasp and α-actinin are present as soon as the Z-bodies appear (Katzemich et al., 2013). Then, Z-bodies stably grow into mature Z-discs (Fig. 2).

Step 1 – α-actinin crosslinking
The core of the Z-disc comprises actin crosslinked by α-actinin, and this crosslinking is the first step of Z-disc formation. Most of our knowledge of crosslinking between α-actinin and actin comes from in vitro studies (Murphy and Young, 2015). α-Actinin is a dimer; each monomer contains two actin-binding calponin homology (CH) domains, four spectrin domains and two EF-hand domains (Almeida Ribeiro et al., 2014). In Drosophila, muscles without α-actinin or actin cannot form Z-discs (Rui et al., 2010; González-Morales et al., 2019b; Karlik et al., 1984). Vertebrate α-actinin exists as muscular and non-muscular forms that are encoded by separate genes (reviewed in Murphy and Young, 2015). Both forms...
are actin crosslinkers and their main difference is their ability to bind Ca\(^{2+}\) at their EF-hand domains. The muscle \(\alpha\)-actinin is insensitive to Ca\(^{2+}\), while actin-binding by the non-muscle form is inhibited by Ca\(^{2+}\) (Burridge and Feramisco, 1981; Waites et al., 1992). In contrast, Drosophila \(\alpha\)-actinin has only one \(\alpha\)-actinin gene (Actn) (Fyrberg et al., 1990). It encodes at least two isoforms, one restricted to the ovaries and one enriched in muscles (Wahlström et al., 2004). Structurally, the two forms differ in just five amino acids in the \(\alpha\)-actinin crosslinking activity to antiparallel filaments. The IFM has two crosslinking sites per actin filament pair (Szikora et al., 2020), whereas other muscles have more than two (Luther, 2009). It is unclear which factor restricts \(\alpha\)-actinin to the Z-disc.

A good candidate are Zasp proteins, scaffolding proteins that organize Z-disc assembly; these have been shown to bind actin filaments and \(\alpha\)-actinin directly, and localize in between two actin monomers in the Z-disc (Szikora et al., 2020; Liao et al., 2016, 2010).

Step 2 – setting up a scaffolding center, the Zasp proteins

Drosophila has three Zasp-encoding genes – Zasp52, Zasp66 and Zasp67. Zasp52 and Zasp66 are present in all muscle types, whereas Zasp67 is specific to the IFM (González-Morales et al., 2019a). Zasp proteins have a conserved function in developing and maintaining the Z-disc by acting as a scaffold for recruiting proteins. Structurally, the Zasp proteins typically contain an N-terminal PDZ domain, a Zasp-motif (ZM) domain and one to four C-terminal LIM domains, as well as large unstructured flexible linkers in between (Zheng et al., 2010).

The complex arrangement between actin, \(\alpha\)-actinin and Zasp proteins provides the structural repetitive unit that ultimately forms the Z-disc. In this context, a combination of structural studies using vertebrate proteins and functional studies using Drosophila have provided important insights into how the ordering of these proteins occurs. \(\alpha\)-Actinin binds actin through its CH domains (Borrego-Hoppman et al., 1996), whereas other muscles have more than two (Luther, 2009). It is unclear which factor restricts \(\alpha\)-actinin to the Z-disc.

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protein binds to α-actinin through an extended PDZ domain, which contains a conserved PWGFRL motif in between two β-sheets on the outside of the PDZ core (Liao et al., 2016; Au et al., 2004). Independently of α-actinin, Zasp can also join the Z-bodies through direct binding to actin filaments (Liao et al., 2020). LIM domains, like those in Zasp, are known protein–protein interaction domains (Kadrmas and Beckerle, 2004), and the flexible unstructured linkers between the LIM and the PDZ domains of Zasp might provide the spacing for simultaneous binding of multiple targets. Importantly, Zasp LIM domains are not involved in binding to actin or α-actinin and are located at the center of the Z-disc (González-Morales and Schöck, 2020; Szikora et al., 2020; Liao et al., 2020, 2016), making them an ideal scaffold for subsequent protein recruitment.

**Step 3 – Z-disc growth**

Once stable Z-bodies are formed and aligned, they grow to their final size recruiting additional proteins that form a mature Z-disc. The Z-disc grows in diameter by the coordinated oligomerization of Zasp proteins mediated by direct binding of their ZM and LIM domains (González-Morales et al., 2019b). In the IFM, the Z-bodies are 0.45 µm in diameter and grow to 1.2 µm (Orfano et al., 2015).

The oligomerization process is tightly controlled, and the Z-disc diameter is equal for all sarcomeres in a muscle (Reedy and Beall, 1993a; Spletter et al., 2018). There are two types of Zasp isoforms, ‘growing’ isoforms with LIM domains and ‘blocking’ isoforms without LIM domains. Later during myofibril development, the blocking Zasp isoforms are expressed and bind to full-length Zasp52, thereby halting any further recruitment of Z-disc proteins and blocking Z-disc growth (González-Morales et al., 2019b). Indeed, depletion of blocking isoforms results in the Z-disc growing larger than normal, whereas the depletion of growing isoforms makes it smaller than normal (González-Morales et al., 2019b).

Although Zasp oligomerization has been described in *Drosophila*, mice with mutations in their two Zasp homologs, Zasp and Enigma (also known as PDLIM3 and PDLIM7), also have smaller sarcomeres than normal, suggesting that a similar oligomerization mechanism occurs in vertebrates (Cheng et al., 2010; Zhou et al., 2001).

How is the switch in the Zasp isoform expression regulated? Differential isoform usage is typically controlled by splicing factors or by distinct transcription start sites and is particularly abundant in muscles (Spletter and Schnorrer, 2014; Venables et al., 2012; Nikonova et al., 2020). Bruno (also known as Bruno 1), a conserved RNA-binding protein is a major regulator of alternative splicing in flies and has been shown to control the splicing of many proteins during muscle development, including that of Zasp proteins (Oas et al., 2014; Spletter et al., 2015). Bruno is under transcriptional control of Salm, the major transcription factor that specifies muscle identities (Schönbauer et al., 2011; Bryantsev et al., 2012). Bruno, as well as possibly other yet unidentified splicing factors, regulates a global change in isoform usage that is required during muscle development, including that of switching the growing Zasp isoform to the blocking one (Spletter et al., 2018).

During the Z-disc growth phase, protein recruitment to the Z-disc is likely achieved in several ways. Proteins might join by binding to α-actinin, the LIM domains of Zasp, the actin filaments or other additional proteins. The *Drosophila* interaction database (http://www.droidb.org/) lists 67 distinct actinin-binding proteins, including Z-disc proteins, such as Lap, MSP300, MLP84B and Rols (Yu et al., 2008; Kreiskoth et al., 2006; Fernandes and Rols, 2008).
Schöck, 2014). Overexpression of a Zasp isoform containing four LIM domains causes huge protein aggregates, suggesting that this is an important recruitment mechanism (González-Morales et al., 2019b).

Roles of the Z-disc

Anchoring actin filaments to resist contractile forces

The Z-disc provides strong anchoring support for actin filaments to resist the pulling forces of myosin filaments. Actin attachment to the Z-disc starts with α-actinin crosslinking, but α-actinin crosslinks are not sufficient to hold actin filaments in place during muscle contraction. To resist the contraction forces while maintaining a rigid structure, the Z-disc incorporates several spring-like proteins (Lemke and Schnorrer, 2017; Bullard et al., 2006; Kulke et al., 2001), with titin and filamin as two notable examples (Fig. 3).

Vertebrate titin is a large modular protein that spans half of the sarcomere from the Z-disc to the H-zone; it establishes a direct connection between the Z-disc and myosin filaments and has a role in setting the length of the sarcomere (Tskhovrebova and Trinick, 2017; Tonino et al., 2017; Linke, 2018). In the insect IFM, the function of titin is provided by at least two proteins – Sallimus (Sls), which connects the Z-disc to the beginning of the A-band, and Bent (also called Projectin), which overlaps with Sls at the interface between the I-band and the A-band and extends into the A-band along the myosin filaments until ~350 nm from the Z-disc, covering ~20% of the A-band (Schueder et al., 2022 preprint; Ayme-Southgate et al., 2004; Bullard et al., 2006; Ayme-Southgate et al., 2005). In other muscles, Bent extends further into the A-band where it almost reaches the M-line (Loreau et al., 2022 preprint). Thus, vertebrate titin reaches the M-line and covers the entire A-band, whereas in insects, neither Sls nor Bent reaches the M-line. In the IFM, targeted removal of Sls by calpain digestion severely decreases sarcomere stiffness (Kulke et al., 2001). Like vertebrate titin, Sls is composed of a variable number of immunoglobulin (Ig) and fibronectin (FN) domains, from 59 to 82 repeated domains, and one C-terminal SH3 domain (Bullard et al., 2002; Granates et al., 2017). The repeated domains constitute a force-sensing spring that folds and unfolds during the contraction cycles (Bullard et al., 2006). In addition, vertebrate titin is also responsible for setting up the number of α-actinin layers in muscles (Gautel et al., 1996). Titin splice variants differ in their number of ‘Z-repeats’. Each of these repeats binds a single α-actinin dimer directly, thereby setting the number of actinin layers. Multiple layers are required since individual interactions are weak (Grison et al., 2017). Z-repeats (Pfam: PF09042) are unique to chordates and not present in insects (El-Gebali et al., 2019), suggesting a different binding site exists between Sls and α-actinin.

Filamin is a much shorter protein; it contains two CH actin-binding domains and 22 to 24 Ig domains. Filamin is a conserved homodimer linker by the final C-terminal Ig domain (Razinina et al., 2012). Ig domains 14 to 19 are the mechanosensing region of filamin (Huelsmann et al., 2016). They typically exist in a closed conformation that is maintained by direct interaction between Ig domains; masking protein-binding sites at the mecano-sensing region. However, upon a pulling force of 2–5 pN, the mechanosensing region unfolds into an open conformation, unmasking those binding sites (Ehrlicher et al., 2011; Huelsmann et al., 2016). The C-terminal region of filamin localizes to the center of the Z-disc where it binds Sls. The N-terminal actin-binding region of filamin localizes to the edge of the Z-disc where it binds actin filaments from opposing sarcomeres, serving as an inter-sarcomere bridge (González-Morales et al., 2017; Szikora et al., 2020). Accordingly, Drosophila filamin mutants have unstable Z-discs that break easily (González-Morales et al., 2017). Filamin function at the Z-disc is likely conserved. In humans, mutations in filamin-c constitute a common type of myopathy that affects the heart and the skeletal muscles (Verdonschot et al., 2020). Accumulation of filamin-c at damaged Z-discs has been observed in mouse muscles after prolonged exercise, in cultured muscle cells after electrical stimulation and after laser-induced general damage (Leber et al., 2016; Orfano et al., 2016).

Coordinating sarcomere maintenance

Elastic proteins at the Z-disc that resist contractile forces inevitably become damaged, so that the Z-disc also incorporates a multicomponent chaperone system that selectively degrades them. This system is called the chaperone-assisted selective autophagy (CAS) pathway and is composed of Starvin (BAG-3 in
vertebrates), HSC70 (also known as HSPA8) and HSPB8, as well as CHIP (also known as STUB1), a ubiquitin ligase (Arndt et al., 2010). The degradation of filamin by the co-chaperone Starvin is particularly well studied in insects. The cochaperone Starvin initiates the formation of the chaperone complex at the Z-disc, which consists of the chaperones Hsc70 and HspB8 and the ubiquitin ligase CHIP (Arndt et al., 2010). Filamin is phosphorylated by the NUAK kinase (Brooks et al., 2020), possibly upon damage or mechanical unfolding, and this phosphorylation serves as a signal for the Starvin chaperone complex to mediate the selective autophagy of filamin (Arndt et al., 2010). It is unclear how the damaged unfolded state of filamin that is recognized by NUAK is different from the open state constantly occurring during contractions.

The CASA pathway is not the only sarcomere maintenance system. The CryAB chaperone localizes to the Z-discs, binds filamin and maintains myofibril integrity in insects (Wojtowicz et al., 2015). The mechanistic details of CryAB function are not entirely understood, but one hypothesis is that it stabilizes filamin at the Z-disc. Besides the CASA pathway and CryAB, other chaperones might be involved; indeed, the *Drosophila* genome encodes more than 100 different predicted chaperone proteins and some might be Z-disc components (Gramates et al., 2017). We do not know the exact number of proteins with continuous turnover at the Z-disc, but there are some clues. By knocking down genes in differentiated muscles and not during development, a recent study showed that there is a continuous turnover of some myofibril proteins, such as filamin, Slr, Bent, actin and myosin, in addition to that of endocytosis-related proteins and cell adhesion proteins (Perkins and Tanentzapf, 2014). Future studies will undoubtedly shed light on the extent of myofibril repair systems.

Serving as an enzymatic hub

Insects adapt their IFM metabolism to sustain long flights. The tephritid fruit fly can fly for more than 1 h (Chen et al., 2015), and *Drosophila* tephritid fruit fly can fly for more than 1 h (Chen et al., 2015), and *Insects adapt their IFM metabolism to sustain long flights. The tephritid fruit fly can fly for more than 1 h (Chen et al., 2015)*. The mechanistic details of CryAB function are not entirely understood, but one hypothesis is that it stabilizes filamin at the Z-disc. Besides the CASA pathway and CryAB, other chaperones might be involved; indeed, the *Drosophila* genome encodes more than 100 different predicted chaperone proteins and some might be Z-disc components (Gramates et al., 2017). We do not know the exact number of proteins with continuous turnover at the Z-disc, but there are some clues. By knocking down genes in differentiated muscles and not during development, a recent study showed that there is a continuous turnover of some myofibril proteins, such as filamin, Slr, Bent, actin and myosin, in addition to that of endocytosis-related proteins and cell adhesion proteins (Perkins and Tanentzapf, 2014). Future studies will undoubtedly shed light on the extent of myofibril repair systems.

**Serving as an enzymatic hub**

Insects adapt their IFM metabolism to sustain long flights. The tephritid fruit fly can fly for more than 1 h (Chen et al., 2015), and *Drosophila* can fly up to 12 km (Leitch et al., 2021). Specialized mitochondria, variations of classic metabolic pathways and the localization of metabolic enzymes to the Z-disc are some of the adaptations required for insect flight (Teulier et al., 2016; Wojtas et al., 1997; Rai et al., 2014; Wang et al., 2016). As most of the cytoplasm in muscle cells is occupied by bulky myofibrils, cytosolic enzymes concentrate in discrete regions that function as catalytic hubs. For example, some enzymes localize to the Z-disc, which provides a condensed environment that promotes the assembly of metabolic complexes that require multiple subunits and thus favors enzymatic reactions (González-Morales et al., 2021 preprint; Sullivan et al., 2003). Concentrating these reactions in one place keeps metabolites away from undesired locations (Menard et al., 2014; Sweetlove and Fernie, 2018). For instance, Zap proteins recruit the tricarboxylic acid cycle enzyme oxoglutarate dehydrogenase (OGDH), which together with other tricarboxylic acid cycle enzymes coordinates amino acid catabolism to sustain myofibril growth (González-Morales et al., 2021 preprint). Vertebrate myofibril proteins are translated at the Z-disc (Denes et al., 2021). A pool of amino acids at the Z-disc might be required for local translation.

Another well-documented example of enzymatic usage of the Z-disc backbone are glycolytic enzymes. At least five out of ten glycolytic enzymes localize to the Z-disc (Wojtas et al., 1997; Sullivan et al., 2003). Glyceraldehyde-3-phosphate dehydrogenase, which links carbohydrate and lipid metabolism, also localizes to the Z-disc, where it coordinates the recruitment of glycolytic enzymes (Sullivan et al., 2003). Flies that only have the mitochondrial glyceraldehyde-3-phosphate dehydrogenase are flightless, demonstrating a functional requirement for its Z-disc localization (Sullivan et al., 2003; Wojtas et al., 1997).

The Z-disc localization of metabolic pathway components is conserved. Vertebrates have pools of glycogen particles associated with the Z-disc, and their metabolism is different from that of the other glycogen pools (Ortenblad et al., 2011; Nielsen et al., 2022), suggesting compartmentalization of glycolytic metabolism. Furthermore, the phosphoglycerate mutase enzyme localizes to the Z-disc in mammalian skeletal muscles (Kowalski et al., 2009), and some glycolytic enzymes directly bind actin filaments (Menard et al., 2014). In addition, a recent proximity labeling method revealed many metabolic enzymes that are associated with myofibrils in mice (Rudolph et al., 2020).

Organizing actin filaments

Actin filaments polymerize in vitro in a two-step process. First, a complex comprising three actin monomers nucleates, which then grows by recruiting additional actin monomers into a forming filament (Szikora et al., 2022). In contrast to what has been observed in vitro, in muscles, actin filament elongation mainly occurs at the minus end and filament elongation proceeds through the incorporation of short actin polymers into the growing filament, as well as by the addition of individual actin monomers (reviewed in Szikora et al., 2022). Filament length is controlled by capping proteins both at the minus ends located at the M-line and the plus ends located at the Z-disc (Shwartz et al., 2016; Bai et al., 2007; Littlefield et al., 2001).

At the M-line, the recruitment of actin polymers is regulated by the minus-end-capping protein tropomodulin (Fig. 4A). In *Drosophila*, tropomodulin binds to the minus ends and blocks actin elongation (Mardahl-Dumesnil and Fowler, 2001). In contrast, the WH2-domain actin regulator Sals and the DAAM formin family homolog dDAAM promote filament elongation by inhibiting tropomodulin and by recruiting actin polymers into the growing filament (Molnar et al., 2014; Bai et al., 2007). The balance between these proteins sets the final actin filament length.

At the Z-disc, several parallel mechanisms fine-tune actin filament organization. α-Actinin determines filament spacing and recruitment of actin filaments into the Z-disc. In insects and vertebrates, CapZ, a heterodimeric actin-binding protein, prevents the spontaneous turnover of actin monomers at the plus ends and localizes to the Z-disc (Cooper and Sept, 2008; Hopmann and Miller, 2003; Szikora et al., 2020). Fhos, a homolog of the FHOD sub-family of formins, mediates the incorporation of actin polymers into early sarcomere units and coordinates the recruitment of actin filaments at the Z-disc, thus coordinating Z-disc growth with actin filament incorporation (Shwartz et al., 2016). Lasp, the only member of the nebulin family in insects (Lee et al., 2008; Suyama et al., 2009), is an actin-binding scaffolding protein that stabilizes muscle actin filaments and sets proper filament spacing (Fernandes and Schönck, 2014).

Finally, not all actins are alike. *Drosophila* has six actin genes, and the small sequence variations in them result in different functions and localization patterns within the sarcomere (Fyryberg et al., 1983; Röper et al., 2005). Actin88F is the main actin present in IFM sarcomeres, but overexpression studies suggest that it is excluded from the Z-disc; instead, Actin5C is present only at the Z-disc (Röper et al., 2005; Shwartz et al., 2016). Interestingly, Fhos mediates the elongation of filaments containing Actin88F but not Actin5C monomers (Shwartz et al., 2016). However, the exact
contribution of the different actin isoforms to sarcomere architecture is still largely unresolved and requires further studies. Overall, regulating actin filament growth and organizing filaments is an incredibly complex feat that requires the simultaneous action of several processes to guarantee a stereotypical pattern.

The evolution of the Z-disc

Much of what we know about Z-discs comes from bilateral animals. The overall shape of sarcomeres from vertebrates and insects is mostly identical; however, there are some differences in the myofibril components. Some important vertebrate muscle proteins are not present in insects at all, and conversely, insects have their own set of specific proteins (Box 1). Therefore, muscles can build almost identical structures even when some components are different. Looking beyond Bilateria holds more surprises – jellyfishes and ctenophores are non-bilateral animals, and their sarcomeres appear almost identical to their bilateral counterparts by electron microscopy (Tanaka et al., 2018; Blanquet and Riordan, 1981; Mackie et al., 1988). But intriguingly, their muscles lack titin entirely and have only one Zasp family member without the oligomerization ZM domain (Steinmetz et al., 2012; González-Morales et al., 2019b; Koch et al., 2012). Therefore, either a disc-like structure that is molecularly distinct from a bilateral Z-disc holds actin filaments in place, or a supportive disc is not entirely required for sarcomere assembly.

Some evidence suggests the existence of sarcomeres without Z-discs, even in bilateral animals. For instance, mathematical modeling of the contractile properties of actin and myosin suggests that some degree of striation spontaneously appears when accounting for filament sliding and the coalescence of actin filaments (Friedrich et al., 2012). In Drosophila, depletion of α-actinin prevents Z-disc formation, but some degree of myosin filament spacing and striation is maintained (Rui et al., 2010). In C. elegans, α-actinin is also dispensable for muscle striation, whereas a homolog of titin is required (Ono et al., 2019; Moulder et al., 2010). Furthermore, the actin cytoskeleton in non-muscle cells exhibits structures that resemble miniature sarcomeres but without Z-discs (Coravos and Martin, 2016; Hu et al., 2017). Thus, Z-discs of bilateral animals might have evolved from striated muscles without canonical Z-disc proteins.

The myofibril attachment site – a modified Z-disc

Translation of myofibril contraction into muscle contraction requires the fibrils to be physically linked to the exoskeleton through the tendons (Fig. 4B). The modified terminal Z-disc (MTZ) anchors the extracellular matrix (ECM) to the myofibril (Reedy and Beall, 1993b). The MTZ shares components and functions with focal adhesions (FAs) and Z-discs. Like an FA, it relies on integrins to establish force-resistant attachments. Structurally, the MTZ is composed of four different layers: (1) the integrin signaling layer, (2) the force-transduction layer, (3) the actin-regulatory layer and (4) the first Z-disc (Green et al., 2018). A proper attachment also plays a developmental role by aligning and straightening myofibrils during pupal IFM assembly (Lemke et al., 2019; Lemke and Schnorrer, 2017). Finally, stochastic muscle contractions occur during the last steps of sarcomere maturation (Katzemich et al., 2013; Spletter et al., 2018; Weitkunat et al., 2017), and these appear to be important for proper muscle development given that genetic or physical disruption of the tension between tendons and muscles during development severely affects sarcomere maturation and the coordinated sarcomere assembly into myofibrils (Lemke et al., 2019; Weitkunat et al., 2014).

The M-line

The M-line (Fig. 4C), located at the center of the sarcomere, anchors and stabilizes myosin filaments (Manring et al., 2017; Lange et al., 2020). The Z-disc and M-line are connected by their interactions with actin and myosin filaments and, in vertebrates, through titin.
C. elegans have an obscurin homolog. Obscurin was originally identified in myomesin myosin and myomesin (Fukuzawa et al., 2008; Pernigo et al., 2017). Subsequently, the large multidomain protein obscurin further (Schoenauer et al., 2008; Pinoutsis et al., 2012; Lange et al., 2005). 2021 preprint; Orfanos et al., 2015). 2012). The largest obscurin isoform is 475 kDa; it has fewer Ig domains than the vertebrate counterpart and lacks the calmodulin-binding motif (Katzemich et al., 2012). Drosophila obscurin forms a complex with Mask, a protein containing multiple ankyrin repeats, and with the serine/threonine kinase Ball to maintain the M-line structure (Katzemich et al., 2015).

A distant Drosophila protein called myomesin and myosin-binding protein (MnM) has a protein domain structure similar to myomesin and myosin-binding proteins H and C; it is highly expressed in the heart and muscles and might perform some of the functional roles of myomesin (Auxerre-Plantie et al., 2020; Gramates et al., 2017). However, given that muscles are only minimally affected in the absence of MnM, it is unlikely that MnM fulfills all myosin functions in insects. Instead, it seems that, in Drosophila, the obscurin homolog fulfills the role of assembling and tethering myosin filaments at the M-line by physically interacting with myosin thick filaments (Katzemich et al., 2012). It is recruited into periodic sarcomere-like structures before other M-line components, including the myosin heavy chain (Katzemich et al., 2012). Consistent with this, in muscles that lack obscurin, the M-line is misplaced (Katzemich et al., 2012) suggesting that obscurin is the physical link between myosin filaments that maintains them aligned.

**Conclusions**

In this Review, we summarized recent advances in our understanding of the structure and the function of the Z-disc, mainly in insects, and contrasted them with their vertebrate counterparts. The Z-disc is a critical structure that controls many aspects of myofibril ultrastructure, size, metabolic state and contractility.

Muscle research is not new; indeed, the sarcomere ultrastructure from transmission electron microscopy has been around for more than 50 years. Although the majority of the abundant structural Z-disc proteins are known, the current challenge is to decipher their intricate interactions that assemble and maintain the Z-disc structure. Traditional loss-of-function analysis can only go so far in a highly interconnected complex, as if a piece is missing, the entire complex collapses. Here, technological advances in genomic engineering are highly valuable in separating discrete protein functions and interrogating the precise molecular mechanisms (Bier et al., 2018; González-Morales et al., 2021 preprint). Similarly, novel imaging methods, such as cryo-electron microscopy and super-resolution microscopy, will allow the observation of structural details that were previously inaccessible. Cryo-electron microscopy and cryo-electron tomography have already provided the structure of Z-discs from honeybees (Rusu et al., 2017), the myosin tails of native thick filaments from Lethocerus (Rahmani et al., 2021) and the entire mouse psos muscle sarcomere (Wang et al., 2021)! Moreover, super-resolution microscopy has provided amazing details on protein localization within the Z-disc (Fernandes and Schöck, 2014; Szikora et al., 2020; González-Morales et al., 2021 preprint; Schueder et al., 2022 preprint). Now is the perfect time to study insect muscles.

**Competing interests**
The authors declare no competing or financial interests.

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**Box 1. Sarcomere proteins found specifically in either vertebrates or insects**

**Vertebrate-specific sarcomere proteins**

**Telethonin:** crosslinks the N-terminal end of two titin molecules at the core of the Z-disc (Gautel and Djinovic-Carugo, 2016).

**FATZ:** also called myozin; a structural protein that connects Z-disc proteins, it binds to filamin, telethonin, and α-actinin (Faulkner et al., 2000; Gontier et al., 2005; Sponga et al., 2021).

**Desmin:** an intermediate filament protein that surrounds the Z-disc and provides support (Paulin and Li, 2004).

**Myotillin:** a Z-disc protein that interacts and stabilizes F-actin, coordinates Z-disc assembly and moves to the M-line upon exercise-induced damage (Carlsson et al., 2007; Kostant et al., 2021).

**MyBP-C:** accessory myosin-binding protein that coordinates the interactions between actin and myosin filaments (Heling et al., 2020).

**Xin:** binds and stabilizes actin filaments found almost exclusively at intercalated discs and myotendinous junctions (Pacholsky et al., 2004).

**Myopodin:** bundles actin filaments and is speculated to be involved in the very early steps of Z-disc assembly (Linnemann et al., 2013).

**Chap:** an essential actin-binding Z-disc protein that can translocate to the nucleus (Beqqali et al., 2010).

**Myomesin:** an M-line elastic protein that forms bridges between myosin filaments and serves to resist the deformation caused by sarcomere contractile forces (Tskhovrebova and Trinick, 2012).

**Insect-sarcomere proteins**

**Flightin:** a protein specific to the IFM that controls the assembly of myosin filaments and is required for correct contraction frequency (Vigoreaux et al., 1993; Chakravorty et al., 2017).

**Myofilin:** a protein of unknown function that binds and localizes to the core of myosin filaments (Qiu et al., 2005).

**Paramyosin:** a structural coiled-coiled dimer composed of a myosin-tail domain that occupies the interior of myosin filaments (Bullard et al., 1977) The small isoform of the paramyosin gene is called Miniparamyosin (Maroto et al., 1995).

**Sals:** a WH2 domain-containing protein that binds actin pointed ends and promotes filament elongation (Bai et al., 2007).


