The nebulin repeat protein Lasp regulates I-band architecture and filament spacing in myofibrils

Isabelle Fernandes and Frieder Schöck

Introduction

Nemaline myopathy (NM) is one of the most common congenital skeletal muscle diseases. Mutations in the nebulin gene account for the majority of cases and, to date, 64 different mutations have been reported in nebulin (Pelin et al., 1999; Lehtokari et al., 2006; Wallgren-Pettersson et al., 2011). Therefore, understanding nebulin function in muscles will shed light on the underlying causes of NM.

Striated muscles consist of myofibrils, which are composed of a series of sarcomeres, the smallest contractile unit of muscles. Each sarcomere is bordered by Z-discs, to which the actin thin filaments are anchored. Thin filaments interdigitate with myosin thick filaments, which are anchored in the middle of the sarcomere at the M-line. Together with the elastic support protein titin, the sliding of thin and thick filaments generates the contractile force of muscles (Sparrow and Schöck, 2009). Nebulin is a giant actin-binding protein expressed in skeletal muscle, which is composed of 185 copies of a 35–amino acid sequence referred to as nebulin repeat (Pappas et al., 2010). Nebulin is now proposed to regulate thin filament length either through binding the barbed end capping protein CapZ (Pappas et al., 2008) or by stabilizing actin thin filaments through binding of multiple nebulin repeats along the I-band (Pappas et al., 2010; Gokhin and Fowler, 2013).

In vertebrates, the giant protein nebulin is the founding and largest member of a family of actin-binding proteins that also includes N-RAP (nebulin-related anchoring protein), nebulette, Lasp-1, and Lasp-2 (LIM and SH3 protein). These other members of the nebulin family contain significantly fewer nebulin repeats and are more widely expressed (Pappas et al., 2011). These repeats contain a conserved actin-binding SDxxYK motif (Labeit and Kolmerer, 1995). Immuno-EM has revealed that a single nebulin molecule spans the thin filaments with its C-terminal part anchored at the Z-disc and its N-terminal part directed toward the thin filament pointed end, with each nebulin repeat likely interacting with a single monomer of actin (Wright et al., 1993). Nebulin-deficient mice have shorter thin filaments resulting in shorter sarcomeres (Bang et al., 2006; Witt et al., 2006). Nebulin was originally believed to act as a molecular ruler, but this model is no longer favored because a smaller nebulin molecule with 59 nebulin repeats can specify thin filament length in skeletal myocytes as well as full-length nebulin does (Pappas et al., 2010). Nebulin is now proposed to regulate thin filament length either through binding the barbed end capping protein CapZ (Pappas et al., 2008) or by stabilizing actin thin filaments through binding of multiple nebulin repeats along the I-band (Pappas et al., 2010; Gokhin and Fowler, 2013).

Correspondence to Frieder Schöck: frieder.schoeck@mcgill.ca

Abbreviations used in this paper: BWM, body wall muscle; IFM, indirect flight muscle; NM, nemaline myopathy; TDT, tergal depressor of the trochanter.
In addition, Lasp modulates I-band architecture by interacting with titin family proteins and α-actinin and affects filament packing by interacting with both actin and myosin. These phenotypes are mediated by a dual localization of Lasp to the Z-disc edges and at the A-band. Physiologically, these defects result in substantially reduced crawling and flying ability. Importantly, a single amino acid change in the actin-binding motif of nebulin repeat 1 and 2 demonstrates different functions for each domain. Therefore, our work establishes Lasp as a model system for understanding the mechanism of nebulin repeat function.

Results

Thin filaments are shorter in Lasp mutants

One of the main functions of nebulin is the establishment of thin filament length (McElhinny et al., 2005; Bang et al., 2006; Witt et al., 2006; Ottenheijm et al., 2009). We therefore asked if the single nebulin family member in Drosophila, Lasp, also regulates thin filament length, even though Lasp has only two nebulin repeats. In humans, Lasp-2 is a shorter splice variant of nebulette with only three nebulin repeats that localizes in striated muscles to Z-discs (Zieseniss et al., 2008). N-RAP also localizes to Z-discs and together with α-actinin helps organize Z-disc structure (Lu et al., 2003). Finally, Lasp-1 has only two nebulin repeats and no described function in muscles: Lasp-1 knockout mice are viable with no overt phenotypic abnormalities (Chew et al., 2008).

In Drosophila melanogaster, Lasp encodes the single member of the nebulin family and exhibits homology to human Lasp-1. Lasp modulates the actin cytoskeleton in the germline and, similar to other nebulin family proteins, Lasp binds actin in vitro (Lee et al., 2008; Suyama et al., 2009). Analyzing Lasp will contribute to the understanding of all nebulin family members because Lasp-like proteins are found in at least one copy in all vertebrates and invertebrates, from sponges to humans, indicating that the evolution of nebulin repeat–containing proteins started with a Lasp-like protein (Nichols et al., 2006; Björklund et al., 2010).

Here we show that Lasp carries out the main function that nebulin fulfills in vertebrates, the setting of thin filament length. In addition, Lasp modulates I-band architecture by interacting with titin family proteins and α-actinin and affects filament packing by interacting with both actin and myosin. These phenotypes are mediated by a dual localization of Lasp to the Z-disc edges and at the A-band. Physiologically, these defects result in substantially reduced crawling and flying ability. Importantly, a single amino acid change in the actin-binding motif of nebulin repeat 1 and 2 demonstrates different functions for each domain. Therefore, our work establishes Lasp as a model system for understanding the mechanism of nebulin repeat function.
Function of the nebulin repeat protein Lasp in Drosophila • Fernandes and Schöck

74.2-kD isoforms are the most common ones in muscles we have analyzed (see Fig. S3). All isoforms have a LIM domain, nebulin repeats, and an SH3 domain and differ only in the length of the linker region between nebulin repeat 2 and the SH3 domain. Expressing the long isoform of Lasp, either carrying an N-terminal Flag and His tag (called L.Lasp) or a C-terminal GFP tag (L.LaspGFP) with Mef2-Gal4, a muscle-specific driver, in a Lasp1 mutant background rescues sarcomere and thin filament length (Fig. 1, D and E). Therefore, Lasp is specifying thin filament and sarcomere length, and our rescue assay is suitable to test the function of transgenes expressing different mutant Lasp proteins. We then tested the 56-kD isoform called S.Lasp, which we cannot detect in IFM. S.Lasp also rescues fully (Fig. 1, D and E), of Lasp1 null mutant adults stained for actin and Kettin, a titin family member used as a Z-disc marker, by confocal microscopy. No gross defects can be observed, but sarcomeres as well as thin filaments from Lasp1 mutants were 12% shorter than those of wild type (Fig. 1, A, B, D, and E). We also analyzed an independent Lasp null mutant allele, Lasp41 (Suyama et al., 2009), which shows the same reduction in sarcomere and thin filament length both homozygously and heterozygously (Fig. 1, D and E). These reductions are in the same range as those observed in nebulin-deficient mice (Bang et al., 2006; Witt et al., 2006).

FlyBase annotates four different Lasp isoforms with molecular masses of 32.2, 56, 71.9, and 74.2 kD. The 71.9- and 74.2-kD isoforms are the most common ones in muscles we have analyzed (see Fig. S3). All isoforms have a LIM domain, nebulin repeats, and an SH3 domain and differ only in the length of the linker region between nebulin repeat 2 and the SH3 domain. Expressing the long isoform of Lasp, either carrying an N-terminal Flag and His tag (called L.Lasp) or a C-terminal GFP tag (L.LaspGFP) with Mef2-Gal4, a muscle-specific driver, in a Lasp1 mutant background rescues sarcomere and thin filament length (Fig. 1, D and E). Therefore, Lasp is specifying thin filament and sarcomere length, and our rescue assay is suitable to test the function of transgenes expressing different mutant Lasp proteins. We then tested the 56-kD isoform called S.Lasp, which we cannot detect in IFM. S.Lasp also rescues fully (Fig. 1, D and E),

Figure 2. I-band architecture is altered in Lasp1 mutants. (A) EM of longitudinal sections of IFM myofibrils from 18-d-old wild-type (top) and Lasp1 mutant flies (bottom). Sarcomeres are shorter, but no gross structural defects are visible in Lasp1 mutants. Close-up view of Z-disc shows an enlargement of the I-band in Lasp1 mutants in 54% of sarcomeres (n = 49). I-band width is indicated by blue and pink arrows. White arrowheads mark a row of electron-dense material on both sides of the Z-disc, which is less dense in Lasp1 mutants. Schematic representation of the structure is shown in the side panel. Bars: (left) 500 nm; (right) 100 nm. (B) Structured illumination superresolution microscope images of wild-type IFM myofibrils stained with anti-α-actinin (magenta) and anti-Kettin (green). Kettin Klg16 signal now resolves as two lines on both sides of the Z-disc core protein α-actinin. Schematic representation of the staining is shown in the bottom panel. (C) Wild-type (top) and Lasp1 (bottom) IFM myofibrils stained with phalloidin (magenta) and anti-Kettin (green). Note that actin thin filament packing shows some defects in Lasp1 mutant myofibril. Bars: (B and C) 5 µm; (B, right) 1 µm. (D) Quantification of distance between Kettin Klg16 epitopes. ***, P < 0.0001. Data in B–D are from 5-d-old adults.
indicating that in IFM conserved domains are more important for muscle function than the linker region. Because of the similarity of the Lasp\textsuperscript{A} and Lasp\textsuperscript{W} phenotype as well as the rescue with different constructs, we continued our analysis with the Lasp\textsuperscript{A} allele and the L.Lasp transgene. We then tested the contractile properties of IFM with a flight assay. The flight ability of 3–5-d-old flies is partially impaired and deteriorates in older flies. Consistent with the rescue of thin filament length, flight ability is rescued by expressing L.Lasp (Fig. 1 C).

Next, we performed the same analysis in two very different muscles, the larval body wall muscle (BWM) and the tergal depressor of the trochanter (TDT), also called jump muscle. IFM is a stretch-activated muscle with fibrillar morphology, whereas TDT and BWM are muscles with tubular morphology (Schönbauer et al., 2011). Both BWM and TDT exhibit shorter sarcomere and thin filament length, as well as larval crawling and adult climbing defects, and these defects are completely rescued by reexpressing L.Lasp, except for thin filament length in BWM, which is partially rescued (Fig. S1). Thus Lasp is a general regulator of thin filament length in muscles.

### I-band is wider in Lasp\textsuperscript{A} mutants

We next analyzed Lasp\textsuperscript{A} mutant IFM by EM to determine if there are additional ultrastructural defects. Longitudinal sections confirm that sarcomeres are shorter, displaying a well-arranged sarcomeric organization regardless of their age (Fig. 2 A and Fig. S2, A and B). However, in 18-d-old Lasp\textsuperscript{A} mutants the I-band is wider (Fig. 2 A, compare areas marked by blue and pink arrows in close-up), and as a result the A–I junction no longer coincides with a row of electron-dense material on both sides of the Z-disc (Fig. 2 A, arrowheads). In 3-d-old flies, I-band appearance is almost wild type, suggesting that I-band stability progressively deteriorates (Fig. S2, A and B). An important role in I-band architecture is played by titin, a giant protein linking thick filaments to the Z-disc, where titin interacts with nebulin (Witt et al., 2006; Sparrow and Schöck, 2009). In Drosophila, the Sallimus isoform Kettin, a member of the titin family, is anchored at the Z-disc by its C terminus and extends toward the thick filaments (Bullard et al., 2006). Using immuno-EM, the Kettin Klg16 antibody has been shown to recognize an epitope located in the I-band, adjacent to the Z-disc edges (Fig. 2 B, cartoon; Burkart et al., 2007). We used structured illumination superresolution microscopy to compare Kettin localization in IFM of wild-type and Lasp\textsuperscript{A} mutant flies. In contrast to confocal microscopy we can now resolve two lines of Kettin, corresponding to its I-band epitope, on both sides of the Z-disc core protein α-actinin (Fig. 2 B; and compare with Fig. 1 A). Remarkably, Kettin epitopes are more widely separated in Lasp\textsuperscript{A} mutants compared with wild type (Fig. 2, C and D). Our data show that Kettin is improperly localized within the I-band, which may cause the observed widening of the I-band.

Moreover, the phalloidin staining also shows defects in the packing of thin filaments because individual bundles of actin filaments can be distinguished in Lasp\textsuperscript{A} mutant IFM (Fig. 2 C). In young Lasp\textsuperscript{A} mutant IFM this defect is only visible by superresolution microscopy, but in 18-d-old flies confocal microscopy is sufficient to visualize the thin filament packing defect, indicating that thin filament stability or packing progressively deteriorates (Fig. S2 F). Thus Lasp is also essential to stabilize actin filaments.

### Lasp localizes to the Z-disc edge and the A-band

In vertebrates, nebulin extends from the Z-disc along most of the length of the thin filaments (Pappas et al., 2011). With only two nebulin repeats, Lasp cannot extend along thin filaments. We therefore asked where Lasp localizes within the sarcomere. We used full-length L.LaspGFP expressed with Mef2-Gal4 to assess Lasp localization in different muscles. Importantly, L.LaspGFP is fully functional (see Fig. 1, D and E). In BWM, L.LaspGFP colocalizes at the Z-disc with Kettin (Fig. 3 A, open arrowhead), but it also localizes at the A-band where thin and thick filaments overlap (Fig. 3 A, bracket). L.LaspGFP is excluded from the M-line in BWM. In TDT, L.LaspGFP shows a similar localization at the Z-disc and the A-band (Fig. 3 B). In IFM we can detect L.LaspGFP only at the Z-disc (Fig. 3 C, arrowhead). Lasp antibody stainings gave identical results, but with higher background (Fig. S3). We believe that Lasp is also present at the A-band region of IFM, but that L.LaspGFP cannot be properly incorporated, and the antibody cannot properly penetrate the densely packed A-band region of IFM. This is a common problem because even myosin cannot be detected at the thin–thick filament overlap zone of IFM (Fig. 3 D). In contrast, myosin can be detected at the A-band of BWM, demonstrating that the staining observed in IFM cannot be explained by the epitope recognized (Fig. 3 E). We then used superresolution microscopy to better define L.LaspGFP localization with respect to the Kettin Klg16 antibody. L.LaspGFP colocalizes with Kettin at the Z-disc periphery (Fig. 3 C). The close up of one Z-disc shows that L.LaspGFP is slightly closer to the Z-disc than the Kettin I-band epitope (Fig. 3 C, close-up). Thus, we propose that Lasp localizes at the Z-disc edges (Fig. 3 C, cartoon). Therefore, the localization of Lasp indicates that Lasp may function in two different structures, in the Z-disc and additionally at the A-band where thin and thick filaments overlap.

### Lasp nebulin repeats localize and function differentially

We then asked which domain of Lasp is responsible for regulation of thin filament length. Lasp contains three distinct protein domains, a LIM domain at the N terminus followed by two nebulin repeats and an SH3 domain at the C terminus. We asked Flag/His-tagged deletion transgenes for each domain, as well as the tyrosine to serine substitution in the actin-binding SDxxYK motif in nebulin repeat 1 (L.Lasp\textsuperscript{Y665}) and nebulin repeat 2 (L.Lasp\textsuperscript{Y121S}; Fig. 4 A). We generated these point mutations to assess the function of each nebulin repeat independently and also because several missense mutations in human patients with NM cause a substitution of this highly conserved tyrosine (Lehtokari et al., 2006).

We first assessed the localization of the mutant Lasp proteins encoded by these transgenes with an anti-Flag antibody staining in both IFM and BWM (Fig. 4). All mutant Lasp proteins localize to the Z-disc, although L.LaspΔSH3 is weaker at
the Z-disc than the other Lasp proteins (Fig. 4, B and C), and L.LaspΔLIM is more spread out along the thin filaments in IFM (Fig. 4 B). L.LaspΔNEB12, L.LaspY121S, and L.LaspΔSH3 do not localize to the A-band in BWM (Fig. 4 C). This indicates that the SH3 and LIM domains contribute to Z-disc localization, and the SH3 domain and nebulin repeats, in particular nebulin repeat 2, are required for A-band localization of Lasp. Intriguingly, we notice accumulations of Lasp and Kettin in L.LaspY121S IFM myofibrils and bent Z-discs (Fig. 4 B, asterisk). EM confirms that these accumulations are Z-disc material (Fig. 5 A), resulting in partially and abnormally enlarged Z-discs.

We next quantified how far the mutant Lasp proteins can rescue Lasp' IFM defects. We measured sarcomere length, thin filament length, and myofibril diameter as a readout of myofibril area (Fig. 5, C–E). We also tested adult flying ability as a physiological readout (Fig. 5 B). L.LaspΔLIM rescues thin filament length, myofibril diameter, and adult flying ability (Fig. 5, B–E). L.LaspY121S, L.LaspΔNEB12, and L.LaspΔSH3 cannot rescue any of the Lasp' mutant muscle phenotypes. Sarcomere length, thin filament length, myofibril diameter, and adult flying ability are all unchanged compared with Lasp' mutants (Fig. 5, B–E). Surprisingly, L.LaspY66S rescues all phenotypes except myofibril diameter (Fig. 5, B–E). We observed very similar rescue results in BWM for all mutant Lasp proteins (Fig. S4), demonstrating that Lasp domains have the same function in different muscles. Therefore, these results show that the nebulin repeats and the SH3 domain are essential for Lasp localization and thin filament length determination, whereas the LIM domain of Lasp is dispensable for the parameters assayed here. Moreover, the dramatically different phenotypes of L.LaspY86S and L.LaspY121S demonstrate that nebulin repeats carry out distinct functions.

Figure 3. Lasp localizes to the Z-disc edge and the A-band. Wild-type muscles stained with phalloidin (magenta) to visualize thin filaments and with anti-Kettin (blue) to mark Z-discs (open arrowheads). LLaspGFP (green) is used to observe the localization of the Lasp long isoform. (A) In BWM, LLaspGFP localizes to both Z-disc (open arrowheads) and A-band, where thin and thick filaments overlap (bracket), but not the M-line (closed arrowheads) and I-band. (B) In DTC, LLaspGFP localizes at the Z-disc and the A-band (bracket). (C) In IFM, LLaspGFP colocalizes with Kettin at the Z-disc (open arrowheads). At superresolution level, LLaspGFP partially colocalizes with Kettin antibody, with Lasp being closer to the Z-disc, which we interpret as localization at the Z-disc edge. Mel2-Gal4 driver is used to drive UAS-LLaspGFP in a wild-type background. (D) Wild-type IFM myofibril stained with phalloidin (magenta) to visualize thin filaments, anti-myosin (blue) to visualize thick filaments, and anti-obscurin (green) to visualize the M-line. Myosin staining is only detected at the M-line and surrounding the Z-disc, not where thin and thick filaments overlap. (E) Wild-type BWM stained as in D. The zone where thin and thick filaments overlap is stained by the anti-myosin antibody. Bars: (main) 5 µm; (close-up) 1 µm. Data shown are from larvae or 3-d-old adults. SIR, structured illumination superresolution microscopy.

I-band width is set by Lasp α-actinin interaction

To identify mechanisms of Lasp function at the I-band, we searched for Lasp-interacting partners by affinity purification and mass
Figure 4. Localization of mutant Lasp proteins in Lasp\textsuperscript{1} mutant background in IFM and BWM. (A) Schematic representation of all Lasp proteins. Orange box, Flag/His tag; blue box, LIM domain; pink boxes, nebulin repeats; green box, SH3 domain. Localization of mutant Lasp proteins is shown in a Lasp\textsuperscript{1} mutant background in IFM myofibrils (B) or larval BWM (C). Z-discs are visualized by anti-Kettin antibody staining (blue). Lasp proteins with anti-Flag antibody staining (green), and thin filaments with phalloidin (magenta) in the merged image. (B) In IFM, all Lasp proteins localize to the Z-disc (open arrowheads). Deletion of the LIM domain (L.Lasp\textsuperscript{ΔLIM}) slightly impairs localization, which appears more diffuse across the thin filaments. (C) In BWM, all Lasp proteins localize to the Z-disc (open arrowheads). The LIM domain deletion (L.Lasp\textsuperscript{ΔLIM}) and the point mutation in the first nebulin repeat (L.Lasp\textsuperscript{Y121S}) also localize to the A-band (bracket). The nebulin domain deletion (L.Lasp\textsuperscript{ΔNEB12}), the SH3 domain deletion (L.Lasp\textsuperscript{ΔSH3}), and the point mutation in the second nebulin repeat (L.Lasp\textsuperscript{Y211S}) disrupt A-band localization. Moreover, the deletion of the SH3 domain leads to a weaker Z-disc localization of Lasp. Closed arrowheads indicate the M-line. Bars, 5 µm. Data shown are from larvae or 3-d-old adults.

Figure 5. Nebulin repeats have different functions. (A) EM longitudinal section of LLasp\textsuperscript{Y211S} IFM myofilibr showing abnormally widened Z-disc. Bars: (left) 500 nm; (right) 100 nm. (B) Rescue of flight ability of 3–5- or 15–18-d-old Lasp\textsuperscript{1} flies with all Lasp proteins compared with wild-type and Lasp\textsuperscript{1}. L.Lasp and L.Lasp\textsuperscript{ΔLIM} fully rescue. L.Lasp\textsuperscript{ΔNEB12}, L.Lasp\textsuperscript{ΔSH3}, and L.Lasp\textsuperscript{Y211S} do not rescue. L.Lasp\textsuperscript{Y86S} partially rescues at 3–5 d and barely rescues at 15–18 d. The wild-type bar in 3–5-d-old flies is not visible because 0% of flies are flight impaired. Error bars indicate SD. P-values are compared with Lasp\textsuperscript{1}.

(C–E) Sarcomere measurements from IFM of 3-d-old adults depicted as dot plot graphs showing all data points. (C) Sarcomere length. (D) Thin filament length. (E) Myofibril diameter. L.Lasp fully rescues all parameters assayed. L.Lasp\textsuperscript{ΔLIM} rescues fully except for sarcomere length. L.Lasp\textsuperscript{ΔNEB12}, L.Lasp\textsuperscript{ΔSH3}, and L.Lasp\textsuperscript{Y211S} do not rescue any of the phenotypes. L.Lasp\textsuperscript{Y86S} rescues sarcomere and thin filament length, but not myofibril diameter. "***", P < 0.0001; "**, P < 0.001; *, P < 0.01; no p-value indicates no significant difference to Lasp\textsuperscript{1}. n refers to sarcomeres measured or flies tested for each genotype.
Function of the nebulin repeat protein Lasp in Drosophila • Fernandes and Schöck

Moreover, pull-downs with mutant Lasp proteins show that α-actinin interacts with the LIM domain and the nebulin repeats, possibly interacting more strongly with nebulin repeat 1 than nebulin repeat 2 (Fig. 6 B). The direct interaction of Lasp with actin was already demonstrated in vitro (Suyama et al., 2009). Our interaction data extend these results by showing that Lasp interacts with actin through the nebulin repeats and, surprisingly, interacts more efficiently with nebulin repeat 2 than nebulin repeat 1 (Fig. 6 B).

Given that Kettin localization is altered in Lasp1 mutants (Fig. 2 D), we first asked how Kettin localization correlates with the protein interactions we observed. We therefore measured the distance between Kettin epitopes in rescue assays with all mutant Lasp proteins (Fig. 6 C). L.Lasp rescues Kettin epitope spacing (Fig. 6 C). In the absence of the LIM domain, Kettin epitopes as well as Lasp itself are spaced further apart (Fig. 6, C and D), which correlates with the requirement of the LIM domain for interaction with α-actinin (Fig. 6 B). Rescue is more efficient with wild-type L.Lasp than with any of the mutant Lasp proteins (Fig. 6 C). Moreover, pull-downs with mutant Lasp proteins show that α-actinin interacts with the LIM domain and the nebulin repeats, possibly interacting more strongly with nebulin repeat 1 than nebulin repeat 2 (Fig. 6 B). The direct interaction of Lasp with actin was already demonstrated in vitro (Suyama et al., 2009). Our interaction data extend these results by showing that Lasp interacts with actin through the nebulin repeats and, surprisingly, interacts more efficiently with nebulin repeat 2 than nebulin repeat 1 (Fig. 6 B).

We expressed a GFP-tagged L.Lasp transgene in muscles and affinity-purified L.LaspGFP protein from a total protein extract of dissected thoraces. As a control, we purified GFP alone from the same tissue. We identified five groups of proteins by mass spectrometry, which associate with L.Lasp protein: three different titin family members (Sallimus, Projectin, and Stretchin), α-actinin, troponins, actin, and myosin (Fig. 6 A). In vertebrates the interaction of nebulin with titin is already well described. In vitro studies indicate that these two giant proteins interact at the Z-disc edge (Witt et al., 2006). The mass spectrometry data combined with the I-band phenotype suggest the same interaction occurs in Drosophila. We therefore first focused on the interaction of Lasp with α-actinin to uncover a possible mechanism for the I-band defect. To confirm the mass spectrometry data, we performed an independent pull-down assay, with transgenes expressing Flag/His double-tagged Lasp proteins, first over Flag beads, then over nickel beads, followed by immunoblotting. Full-length L.Lasp interacts specifically with α-actinin (Fig. 6 B). Moreover, pull-downs with mutant Lasp proteins show that α-actinin interacts with the LIM domain and the nebulin repeats, possibly interacting more strongly with nebulin repeat 1 than nebulin repeat 2 (Fig. 6 B). The direct interaction of Lasp with actin was already demonstrated in vitro (Suyama et al., 2009). Our interaction data extend these results by showing that Lasp interacts with actin through the nebulin repeats and, surprisingly, interacts more efficiently with nebulin repeat 2 than nebulin repeat 1 (Fig. 6 B).
intermediate for the other transgenes, but two observations are noteworthy: L.LaspY121S, which interacts more strongly with α-actinin, rescues the Kettin epitope distance significantly better than L.LaspY86S (Fig. 6, C and D); and L.LaspΔSH3 protein collapses into a narrow stripe at the center of the Z-disc (Fig. 6 D). These data indicate that the Lasp α-actinin interaction modulates Kettin localization within the I-band.

Finally, the phalloidin staining shows that L.LaspΔNEB12, L.LaspY121S, and L.LaspΔSH3 incompletely rescue actin thin filament defects, with L.LaspΔNEB12 being least able to rescue (Fig. 6 D).

To further clarify the importance of the interaction with α-actinin, we performed a FRAP assay in third instar larvae with L.LaspGFP (Fig. 6, E–G; and Fig. S5 C). We compared Lasp dynamics in wild-type larvae with α-actinin null mutant larvae (Actm14). Actm14 larvae entirely lack α-actinin (Fig. S5, A and B). By confocal microscopy, L.LaspGFP still localizes at the Z-disc in Actm14 larvae, indicating that α-actinin cannot be the only protein recruiting Lasp to the Z-disc (Fig. S5 A). As indicated by the steeper slope, the recovery rate of L.LaspGFP is faster in Actm14 mutants than in wild type (Fig. 6 E). We recently demonstrated that Zasp52, a core protein of the Z-disc, interacts directly with α-actinin and is required for Z-disc assembly (Katzemich et al., 2013). The recovery rate of Zasp52GFP is slower than of LaspGFP, but identical in wild-type and Actm14 larvae, demonstrating that Zasp52 dynamics are not affected by the absence of α-actinin (Fig. 6 F). Moreover, Lasp is much more mobile compared with Zasp52 (Fig. 6 G). These data indicate that Lasp is a peripheral Z-disc protein, in part recruited by α-actinin.

### Discussion

By analyzing *Drosophila* Lasp, we can show that a nebulin family member with only two nebulin repeats is sufficient to regulate thin filament length. In addition, Lasp localization and biochemical interactions indicate that Lasp uses different protein domains to mediate filament spacing at the A-band versus I-band architecture and maintenance at the Z-disc edge.

Lasp or its orthologues is one of the few muscle proteins conserved in all Eumetazoans and therefore in all organisms with striated muscles (Steinmetz et al., 2012). This indicates that nebulin repeats play an ancient and conserved role in modulating muscle function. This notion is supported by the Lasp mutant phenotype, which exhibits several similarities with vertebrate nebulin mutants, in particular the partial widening of Z-discs (Fig. 5 A) and shorter sarcomere and thin filament length leading to impaired muscle function (Figs. 1 and S1).

As Lasp does not coalign with thin filaments across the I-band (Fig. 3), neither the nebulin ruler nor the two-segment model (Gokhin and Fowler, 2013) can explain thin filament length regulation in *Drosophila*. However, our data show that Lasp stabilizes actin filaments in agreement with what has been reported for nebulin (Pappas et al., 2010). In contrast to superresolution or confocal analysis (Figs. 2 C and S2 F), ultrastructural analysis of Lasp mutant myofibrils revealed no apparent thin filament defect in the longitudinal sections regardless of their age (Fig. 2 A and Fig. S2, A and B). This is likely explained by different dissection procedures. For EM imaging, half thoraces
Function of the nebulin repeat protein Lasp in Drosophila • Fernandes and Schöck

In older mice, and myofibrils are misaligned (Bang et al., 2006; Witt et al., 2006; Tonino et al., 2010). Similar defects are observed in biopsies from human patients (Ottenheijm et al., 2009). Thus, the functional roles of nebulin extend beyond thin filament length regulation. Similarly, we observe the occasional breakdown of Z-disc width regulation in L.LaspY121S mutants, which show abnormally widened Z-discs, usually at the periphery of myofibrils (Fig. 5 A). In vertebrates, titin and nebulin interact at the Z-disc edge, and this interaction is believed to specify Z-disc width (Witt et al., 2006). We propose that at the Z-disc edge Lasp links the Z-disc core protein $\alpha$-actinin with thin filaments and anchors the Sallimus isoform Kettin. Sallimus forms an elastic link between the Z-discs and the ends of thick filaments similar to titin in vertebrates (Bullard et al., 2006).

Our affinity purification and mass spectrometry analysis uncovered all three proteins: $\alpha$-actinin, actin, and Sallimus (Fig. 6 A).

Structural studies on nebulin-deficient mice showed that in the absence of nebulin, Z-discs occasionally widen, especially in older mice, and myofibrils are misaligned (Bang et al., 2006; Witt et al., 2006; Tonino et al., 2010). Similar defects are observed in biopsies from human patients (Ottenheijm et al., 2009). Thus, the functional roles of nebulin extend beyond thin filament length regulation. Similarly, we observe the occasional breakdown of Z-disc width regulation in L.LaspY121S mutants, which show abnormally widened Z-discs, usually at the periphery of myofibrils (Fig. 5 A). In vertebrates, titin and nebulin interact at the Z-disc edge, and this interaction is believed to specify Z-disc width (Witt et al., 2006). We propose that at the Z-disc edge Lasp links the Z-disc core protein $\alpha$-actinin with thin filaments and anchors the Sallimus isoform Kettin. Sallimus forms an elastic link between the Z-discs and the ends of thick filaments similar to titin in vertebrates (Bullard et al., 2006). Our affinity purification and mass spectrometry analysis uncovered all three proteins: $\alpha$-actinin, actin, and Sallimus (Fig. 6 A).
Moreover, coimmunoprecipitation experiments with mutant Lasp proteins confirm the interaction with actin and α-actinin (Fig. 6 B). α-Actinin interacts with the LIM domain and nebulin repeats of Lasp (Fig. 6 B). In vertebrates, in vitro studies have demonstrated that the nebulin family member N-RAP also interacts with α-actinin through its LIM domain and nebulin repeats, indicating that this interaction may be conserved across evolution (Lu et al., 2003). Finally, the FRAP assay indicates that Lasp is in part tethered to the Z-disc by α-actinin (Fig. 6 E). Our mutant phenotype and the rescue and localization data also support a link of Lasp with Kettin, even though the biochemical interaction of these proteins remains to be tested. First, Lasp localizes at the Z-disc edges (Fig. 3 C).

Second, in Lasp’ mutant IFM, superresolution microscopy shows a wider spacing of Kettin I-band epitopes (Fig. 2 C), likely leading to a wider I-band as observed by EM (Fig. 2 A). Most importantly, when Lasp can no longer interact with α-actinin (Fig. 6, B and D, L.LaspΔLIM), Lasp signal is more widely spaced and colocalizes perfectly with the Kettin I-band epitope. In contrast, Lasp collapses into a single line at the Z-disc core, when the SH3 domain is deleted, which at least in vertebrate nebulin is required for interaction with titin (Witt et al., 2006; Fig. 6 D, L.LaspΔSH3). We therefore propose that Lasp ensures proper insertion of Kettin into the Z-disc. Deterioration of I-band integrity over time also indicates that Lasp plays a role in maintaining I-band structure during muscle contraction. In addition, actin binding of Lasp must also be important for I-band integrity because regulation of Z-disc width can break down completely in L.LaspY121S, which also be important for I-band integrity because regulation of filament number or spacing contribute substantially to power output. Finally, only the mutation in nebulin repeat 2 causes an abnormal widening of Z-discs, suggesting it plays a role in Z-disc termination, perhaps related to its impaired interaction with actin. We propose that vertebrate nebulin fulfills these different functions with different nebulin repeats of the same molecule. This is supported by biochemical data showing that N-terminal nebulin repeats situated in the A-band, but not C-terminal nebulin repeats, interact with myosin and regulate actomyosin ATPase activity (Root and Wang, 1994). Therefore mutations in different subsets of human nebulin repeats may give rise to different NM phenotypes.

We propose the following mechanisms for Lasp function at the A-band and the Z-disc (Fig. 8). At the A-band, Lasp nebulin repeat 2 may simultaneously interact with thin and thick filaments to regulate proper filament spacing. This likely fine-tunes proper power output because changes in filament spacing are known to affect power output in IFM (Tanner et al., 2012; Fig. 8 A’). At the Z-disc, all domains of Lasp may simultaneously interact with α-actinin, actin, and the titin family member Kettin, and these interactions ensure proper insertion of titin-like molecules at the Z-disc and thereby stabilize the entire I-band (Fig. 8 A’). In addition to the LIM domain, Lasp nebulin repeats also interact with α-actinin (Fig. 6 B), and especially nebulin repeat 1 contributes to I-band architecture (Fig. 6 C). Finally, Lasp likely also specifies the number of filaments incorporated into each myofibril through nebulin repeat 1 (Fig. 7, A and B).

As vertebrate nebulin does not have a LIM domain, the LIM domain–dependent I-band function of Lasp could be taken over by vertebrate N-RAP or Lasp-2, which contains an N-terminal LIM domain.

Because of the small size of Lasp compared with nebulin and its distinct localization, Lasp is a suitable model system to gain further insights into the in vivo function of nebulin repeats in normal physiology and disease.

Materials and methods

Fly stocks and genetics

UAS-Lasp (amino acids 1–657 corresponding to Lasp-PB in FlyBase), UAS-S.Lasp (amino acids 1–504 corresponding to Lasp-PA in FlyBase), UAS-LaspGFP, UAS-LaspΔLIM (deletion from T4 to H100), UAS-LaspΔNEB (deletion from A106 to K171), UAS-LaspΔSH3 (stop at D507), UAS-L.LaspΔSH3, and UAS-L.LaspΔLIM transgenic lines were generated by P-element transformation. The following stocks were used (see FlyBase for complete description): ry506 Act88F KM88es (provided by A. Cammarato, Department of Neurology, University of California, San Francisco, CA).
Behavioral assays

For the crawling assay, third instar mutant larvae were identified by the absence of the GFP balancer. Five larvae of the desired genotype were placed on a 5-cm fresh apple juice plate marked with zones of five concentric circles (Fig. S1 D) and zonal location of each larva was recorded after 60 s. These measurements were performed in triplicate for each group of five, and data for 12 independent sets (60 animals coming from three different crosses) were collected.

Flight and climbing assays were performed with 3- to 5-d-old or 15- to 18-d-old flies at RT. For flight tests, flies were released in sets of 10 animals from the center of a flight chamber. Light was placed at the top of the flight chamber to attract flies and only flies flying up were considered to be able to fly. For climbing assays, 10 adult flies were transferred to an empty vial and lightly tapped to the bottom. The number of flies that climbed to a height of 7 cm within 10 s was recorded. Data for five independent sets of 10 flies each (50 animals coming from three different crosses) were collected for both assays. In all assays, Mef2-Gal4 flies were used as wild-type controls.

The relative distribution of animals at the end of the motility, flight, or climbing assay was plotted in GraphPad Prism (GraphPad Software), using the mean percentage of animals present in each zone or not flying or climbing.

Immunostainings

We used the following primary antibodies for immunofluorescent stainings of IFMs: rat anti-α-actinin MAC276, rat anti-Kettin Klgl16 MAC155,
Gray intensities for bleached Z-disc, unbleached Z-disc, and background regions were determined manually for each image in a time series. Normalized intensity was obtained using the following equation: \( I_{\text{norm}} = (I_{\text{bleach}} - I_{\text{background}})/(I_{\text{bleach}} - I_{\text{unbleach}}) \). Finally, five independent recovery normalized datasets (bleaching not below 80%) were averaged using GraphPad Prism software. The fitted curve as well as the mobile fraction was calculated by the software. Images were acquired with RT with Metamorph software (Molecular Devices) on a Quantix WaveFX spinning-disk confocal system with an EM-CCD camera (Hamamatsu) on an inverted microscope (DMi6000B; Leica) using a 63x, 1.40-0.6 HCX Plan Apochromat oil immersion objective.

Immunoprecipitation and binding assays

For the affinity purification, 200 adult fly thoraces expressing UAS-LaspgFP with Mef2-Gal4 were cut in half and incubated overnight in lysis buffer [50 mM Tris-HCl, pH 8, 150 mM NaCl, 1 mM EDTA, 0.5% Triton X-100, and complete EDTA-free protease inhibitor cocktail (Roche)] plus 50% glycerol. As control, flies expressing UAS-2xGFP with twi-Gal4 in mesodermal tissues were treated identically. They were then homogenized in lysis buffer and protein extracts were incubated with prewashed GFP beads (GFP-Trap M, ChromoTek) for 2 h at 4°C. After incubation, the beads were washed three times with wash buffer [50 mM Tris-HCl, pH 8, 150 mM NaCl, 0.1% Triton X-100, and complete EDTA-free protease inhibitor cocktail]. Bound proteins were eluted by boiling in 2x SDS sample buffer and sent for mass spectrometry (Institute for Research in Immunology and Cancer Proteomics Center, Université de Montréal).

For immunoprecipitation, 150 adult fly thoraces were cut in half and incubated overnight in lysis buffer [50 mM Tris-HCl, pH 8, 150 mM NaCl, 1 mM EDTA, and 0.5% Triton X-100] and complete EDTA-free protease inhibitor cocktail plus 50% glycerol. Then, the eluates were incubated with prewashed Ni-NTA agarose beads (Qiagen) for 2 h at 4°C. After incubation the beads were washed three times with wash buffer [50 mM Tris-HCl, pH 8, 150 mM NaCl, 0.1% Triton X-100, and complete EDTA-free protease inhibitor cocktail], and bound proteins were eluted in wash buffer containing 200 µg/ml of 3x Flag peptide (Sigma-Aldrich) for 1 h at 4°C. Then, the eluates were incubated with prewashed Ni-NTA agarose beads (Qiagen) for 2 h at 4°C. After incubation the beads were washed three times with wash buffer and bound proteins were eluted by boiling in 2x SDS sample buffer and loaded on an 8% acrylamide gel. The following antibodies were used: rat anti-α-actinin MAC276, rat anti-actin antibody M65237, rat anti-mysin MAC147 at 1:2000 (Babraham Bioscience Technologies), and mouse anti-Flag antibody at 1:5000 (Sigma-Aldrich). The immunoreaction was visualized with ECL (GE Healthcare).

Binding of L.Lasp to thin filaments was measured by a co-sedimentation assay as described previously (Burkart et al., 2007). In brief, IFM were dissected in relaxing solution with 0.5% Triton X-100 and protease inhibitors (Roche) from Act88F/88 mutants, which lack thin filaments. IFM were separated into single myofibrils by pipetting and washed three times. Myofibrils were then homogenized in lysis buffer [10 mM Tris-HCl, pH 8, 50 mM NaCl, 2 mM MgCl₂, and 2 mM DTT]. Soluble HisLasp protein was purified from the lysate of sonicated cells on a Ni-NTA agarose column (GE Healthcare). The homogenate was incubated with or without 10 µM of purified HisLasp for 1 h at RT under agitation. Centrifugation for 30 min at 30,000 rpm in an Optima Ultracentrifuge (Beckman Coulter) was used to separate pellet and supernatant. Samples were boiled in 2x SDS sample buffer, equally loaded on an 8% acrylamide gel and stained with Coomassie brilliant blue.

FRAP analysis

Heterozygous mutant females for α-actinin (Actn14<sup>−/−</sup>), were crossed to the recombinant line Mef2-Gal4, UAS-LaspgFP. Eggs were collected for 4 h on an apple juice plate and aged for 3 d at 25°C. Male third instar larvae, carrying the Actn14<sup>−/−</sup> mutation and expressing LasspgFP, were selected and anesthetized for 15 min in Kwan Long Medicated Oil (25% menthol), 15% methyl salicylate, 10% camphor, 10% eucalyptus oil, 7% lavender oil, and 0.5% chloroform. Larvae were then mounted in between the coverslip and the objective with mineral oil. Heart beating was verified before acquisition to ensure larvae were alive during the recording. Fluorescence recovery was recorded for 3 (LasspgFP) or 10 min (Zasp52GFP) with a 491-nm laser at low power, imaging every 2 or 10 s. Three larvae from different crosses (at least nine different animals for each genotype) were analyzed and five independent recordings were done for each. Data were analyzed using ImageJ software.

EM

Thoraces were treated with 5 mM MOPS, pH 6.8, 150 mM KCl, 5 mM EGTA, 5 mM ATP, and 1% Triton X-100 for 2 h at 4°C, followed by overnight incubation in the same buffer without Triton X-100 but 50% glycerol. This was repeated for a second time. Samples were then washed in rigor solution [5 mM MOPS, pH 6.8, 40 mM KCl, 5 mM EGTA, 5 mM MgCl₂, and 5 mM Na₃H]. Flies were transferred to a solution (20 mM MOPS, pH 6.8, 2 mM EGTA, 5 mM MgCl₂, and 5 mM Na₃H) for 2 h at 4°C. Samples were washed in washing solution, and then in 0.1 mM sodium phosphate, followed by a postfixation in ice-cold 1% osmium tetroxide in 0.1 M sodium phosphate for 1 h at RT. Samples were washed in water and stained with 2% uranyl acetate for 1 h at 4°C. Finally, samples were washed in water, dehydrated in an ethano- ethanol series (50%, 70%, 80%, 90%, and 100%), and then embedded in epoxy propane, before being embedded into epon-815 (Electron Microscopy...
Online supplemental material

Fig. S1 shows the Lasp1 mutant phenotype in BWm and TDT muscles. Fig. S2 shows EM sections of wild-type and Lasp1 mutant IFM at different ages and confocal images of IFM thin filaments of 18-d-old flies. Fig. S3 shows anti-Lasp antibody staining of wild-type BWm, TDT, and IFM muscles. Fig. S4 shows the rescue of larval Lasp1 phenotypes with different mutant Lasp proteins. Fig. S5 is a time-lapse series of FRAP experiment of LaspGFP in wild-type versus Actin5C mutant larvae. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.201401094/DC1.

We thank L. Zhou for the initial observation of shorter sarcomere length in Lasp1 mutants; A. Cammarota, B. Bullard, and A. Ephrussi for materials; and F. Fagotto and P. Lasko for comments on the manuscript. We thank J. Lacoste and B. Kuster-Schock (Cell Imaging and Analysis Network facility) for help with confocal and superresolution microscopy, J. Mui at Facility for Electron Microscopy Research for help with EM, B. Hu for embryo injections, and E. Bonneil for mass spectrometry.

This work was supported by operating grant MOP-93727 from the Canadian Institutes of Health Research. The authors declare no competing financial interests.

Submitted: 21 January 2014
Accepted: 15 July 2014

References


Figure S1. Lasp<sup>1</sup> mutant BWM and TDT muscles have shorter sarcomere and thin filament length. (A) Wild type (top) and Lasp<sup>1</sup> mutant (bottom) of VL3 muscle in larval BWM stained with phalloidin (magenta) to label actin thin filaments and with anti-Kettin (blue) to mark Z-discs (arrowheads). Staining reveals no overt defects. (B) Graphs represent actin and Kettin grayscale intensity across the dashed line on the corresponding top panel. Lasp<sup>1</sup> mutants (pink line) have shorter sarcomeres and thin filaments compared with wild type (blue line). (C) Quantification of sarcomere and thin filament length from wild-type and Lasp<sup>1</sup> mutant muscles. Reexpressing L.Lasp with Mef2-Gal4 rescues sarcomere length and partially rescues thin filament length. (D) Larval motility assay using a 3-cm agar plate divided into zones as shown, with larvae placed in zone 1. The average percentage of larvae in each zone at the end of the assay is plotted. Lasp<sup>1</sup> mutants have crawling defects that are rescued by reexpressing L.Lasp with Mef2-Gal4. (E) TDT muscles of wild type (top) and Lasp<sup>1</sup> mutant (bottom) stained with phalloidin (magenta) to visualize thin filaments and anti-Kettin (blue) to mark Z-discs (arrowheads). Staining reveals no overt defects. (F) Quantification of sarcomere and thin filament lengths from wild type and Lasp<sup>1</sup> mutants. Note the decrease in both sarcomere and thin filament length in Lasp<sup>1</sup> compared with wild type. Reexpressing L.Lasp with Mef2-Gal4 rescues both sarcomere and thin filament length. (G) Graphs represent actin and Kettin grayscale intensity across the dashed line on the corresponding top panel. Lasp<sup>1</sup> mutants (pink line) have shorter sarcomeres and thin filaments compared with wild type (blue line). (H) Climbing assay demonstrates that Lasp<sup>1</sup> mutant flies have climbing defects that can be rescued by reexpressing L.Lasp. Error bars indicate SD. Bars, 5 µm. ***, P < 0.0001; *, P < 0.01. Data shown are from larvae or 3-d-old adults.
Figure S2. EM sections and confocal microscopy of IFM myofibrils. (A and B) EM longitudinal section of single IFM myofibrils from 3-d-old adults. (A) Wild type. (B) Lasp\(^1\) mutant. Sarcomeres and thin filaments are shorter, but no gross structural defects are visible in Lasp\(^1\) mutants. Close-up view shows less electron-dense material at A-I junction in 30% of Lasp\(^1\) mutant sarcomeres (n = 40). (C and D) EM cross sections of IFM myofibrils from 18-d-old adults reveal that wild-type myofibrils (C) are smaller than Lasp\(^1\) mutant myofibrils (D). High magnification view of cross section shows no major defects in the hexagonal arrangement of thin and thick filaments. However, filament spacing is increased (Lasp\(^1\) mutant pink hexagon) compared with the wildtype blue hexagon. Bars: (left) 500 nm; (right) 100 nm. (E) Quantification of cross-sectional area, actin and myosin filaments (Fts) per myofibril, and myosin filament (MF) density. Notably, Lasp\(^1\) mutant myofibrils are larger in cross section and the density of thick filaments is lower, showing that filament spacing is wider. n = 10 myofibrils from five different flies. (F) Confocal images of wild type (top) and Lasp\(^1\) mutant IFM (bottom) of 18-d-old adults stained with phalloidin (magenta) to label actin thin filaments and anti-Kettin (blue) to mark Z-discs. Actin thin filament packing is irregular in Lasp\(^1\) mutants. Bar, 5 µm.
Figure S3. **Lasp antibody staining is identical to LLaspGFP localization.** Confocal images of wild-type (top) and \(Lasp^1\) mutant (bottom) IFM myofibrils (A), TDT (B), and BWM (C) stained with phalloidin (magenta) to visualize thin filaments, anti-Kettin antibody (blue) to mark Z-discs (open arrowheads), and anti-Lasp antibody (green). Lasp localizes to Z-discs in IFM, whereas it also localizes at the A-band (brackets) in TDT and BWM. Western blotting demonstrates the absence of Lasp in \(Lasp^1\) mutants. In wild-type IFM (A), TDT (B), and BWM (C), the antibody recognizes the two longest isoforms of Lasp (71.9 and 74.2 kD predicted weight). Closed arrowheads indicate the M-line. Bars, 5 µm. Data shown are from larvae or 3-d-old adults.

Figure S4. **Nebulin repeats have different functions in larval BWM.** (A) Dot plot summarizes the ability of different Lasp proteins to rescue sarcomere length of \(Lasp^1\) mutant BWM. LLasp and LLasp\(^{Y86S}\) rescue sarcomere length almost fully and LLasp\(^{LIM}\) slightly less. LLasp\(^{NEB12}\), LLasp\(^{SH3}\), and LLasp\(^{Y121S}\) cannot rescue sarcomere length. (B) Crawling assay monitoring larval motility in five zones on an agar plate. LLasp, LLasp\(^{LIM}\), and LLasp\(^{Y86S}\) rescue larval crawling ability similarly well. LLasp\(^{NEB12}\), LLasp\(^{SH3}\), and LLasp\(^{Y121S}\) cannot rescue larval crawling ability. ***, \(P < 0.0001\); ns, no significant difference. \(n\) refers to sarcomeres measured or larvae tested for each genotype.
Figure S5. **Lasp is stabilized at the Z-disc by α-actinin.** (A) L.LaspGFP localization in wild-type and Actn14 null mutant background in larval BWM. Muscles are stained with phalloidin (magenta) to visualize thin filaments and with anti–α-actinin (blue) to mark Z-discs and confirm that α-actinin is absent in Actn14 null mutants. L.LaspGFP localization is slightly different in the absence of α-actinin: the GFP signal appears more diffuse across the Z-disc and throughout the I-band. Note that the muscles are also smaller in Actn14 null mutants. (B) Western blotting confirms the absence of α-actinin in Actn14 mutants. Actin is used as a loading control. (C) Time points shown in seconds of a time-lapse series to compare L.LaspGFP dynamics in wild type (top) or Actn14 mutant (bottom) of larval BWM. White squares show FRAP zone before and after laser bleaching. Fluorescence recovery is faster in Actn14 mutant. Bars, 5 µm. MG4, Mef2-Gal4.