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***Drosophila melanogaster* Muscle LIM Protein and α -actinin function together to stabilize muscle cytoarchitecture: a potential role for Mlp84B in actin-crosslinking**

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Abstract

Stabilization of tissue architecture during development and growth is essential to maintain structural integrity. Because of its contractile nature, muscle is especially susceptible to physiological stresses, and has multiple mechanisms to maintain structural integrity. The *Drosophila melanogaster* Muscle LIM Protein, Mlp84B, participates in muscle maintenance, yet its precise mechanism of action is still controversial. Through a candidate approach, we identified α -actinin as a protein that functions with Mlp84B to ensure muscle integrity. α -actinin RNAi animals die primarily as pupae, and Mlp84B RNAi animals are adult viable. RNAi knockdown of Mlp84B and α -actinin together produces synergistic early larval lethality and destabilization of Z-line structures. We recapitulated these phenotypes using combinations of traditional loss-of-function alleles and single gene RNAi. We observe that Mlp84B induces the formation of actin-loops in muscle cell nuclei in the absence of nuclear α -actinin, suggesting Mlp84B has intrinsic actin crosslinking activity, which may complement α -actinin crosslinking activity at sites of actin filament anchorage. These results reveal a molecular mechanism for MLP stabilization of muscle, and implicate reduced actin crosslinking as the primary destabilizing defect in MLP associated cardiomyopathies. Our data support a model in which α -actinin and Mlp84B have important and overlapping functions at sites of actin filament anchorage, to preserve muscle structure and function.

Keywords

actin bundling; Z-line; Titin; Zasp; myopathy

INTRODUCTION

Muscle is a highly organized tissue that both generates and experiences mechanical force throughout its lifetime (Clark et al. 2002). During muscle contraction, force is transduced through the Z-lines, where the actin thin filaments of neighboring sarcomeres are cross-linked, to the costameres and myotendinous junctions, sites of membrane attachment for the contractile cytoskeleton. Due to the very nature of muscle, these sites of actin filament anchorage within the muscle cell must be highly stabilized to protect them from damage

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incurred during contraction and maintain structural integrity. Protein complexes present at these sites may therefore contribute to their stability, and even act to reinforce these structures during times of increased mechanical stress.

Muscle LIM protein (MLP) is a LIM-domain protein found at costameres, Z-lines and the myotendinous junction/intercalated discs in both skeletal and cardiac muscle (Arber et al. 1994; Ehler et al. 2001; Flick and Konieczny 2000). Mutations in MLP are associated with both dilated cardiomyopathy and familial hypertrophic cardiomyopathy, defining it as a protein critical for normal cardiac function in humans (Bos et al. 2006; Geier et al. 2008; Geier et al. 2003; Hershberger et al. 2008; Knoll et al. 2002; Knoll et al. 2010; Mohapatra et al. 2003; Newman et al. 2005). Genetic ablation of MLP in the mouse results in dilated cardiomyopathy (Arber et al. 1997), and further characterization of the MLP-null cardiomyocytes reveals disruption of both Z-line and intercalated disc structure (Arber et al. 1997; Ehler et al. 2001). Moreover, mechanical studies of MLP-null hearts reveal an increase in muscle compliancy before the onset of other morphological or physiological changes (Lorenzen-Schmidt et al. 2005).

While the mouse knockout studies strongly suggest that compromised structural integrity is the main contributing factor in human cardiomyopathies resulting from MLP mutations, there is still much debate about the essential functions of MLP (Boateng et al. 2009; Buyandelger et al. 2011; Gehmlich et al. 2008; Gunkel et al. 2009; Kitajima et al. 2011; Kuhn et al. 2012). For example, tissue culture studies initially characterized MLP as a MyoD cofactor important for myotube growth and terminal differentiation (Kong et al. 1997). Other analyses focused on a role for MLP in stretch sensing at the cardiomyocyte Z-disc (Heineke et al. 2005; Knoll et al. 2002). MLP is upregulated by cardiac and skeletal muscle insult (Barash et al. 2004; Heineke et al. 2005), and MLP-heterozygous mice have an impaired response to myocardial infarction (Heineke et al. 2005) suggesting involvement in muscle remodeling and repair. Serca2 levels are reduced in MLP-null cardiomyocytes (Unsold et al. 2012; Yamamoto et al. 2007), implicating impaired calcium cycling as the primary defect in the MLP knockout mice. However, not only have these physiological and molecular studies produced conflicting results (e.g. (Lorenzen-Schmidt et al. 2005; Minamisawa et al. 1999; Unsold et al. 2012)), it is difficult to discern the primary cellular defects from the secondary and downstream physiological responses to cardiomyopathy development in the MLP-null mice. Given both the association of MLP mutations with familial cardiomyopathy and the potential cardioprotective role of MLP, it is important to establish the mode of action for MLP in both normal and diseased muscle.

We previously characterized mutations in a *Drosophila* CRP/MLP family member, Mlp84B, and demonstrated that Mlp84B is essential for normal muscle function in the fly (Clark et al. 2007). Mlp84B interacts genetically with the giant connecting filament Titin, a muscle protein that also has genetic lesions associated with familial cardiomyopathies and skeletal myopathies (Gautel 2011; Kruger and Linke 2011). In a collaborative study to specifically investigate cardiac defects, we have shown that both *Mlp84B*-null flies as well as animals with a heart specific reduction of Mlp84B exhibit a prolonged diastole (Mery et al. 2008), a phenotype similar to that seen in young MLP knock-out mice before the onset of cardiac hypertrophy (Lorenzen-Schmidt et al. 2005). Mechanical studies of *Mlp84B*-null flight muscle indicate that loss of Mlp84B results in decreased muscle stiffness and power generation (Clark et al. 2011). While these physiological observations describe the muscle defects present in the *Mlp84B*-null flies, they have not yet defined the underlying molecular mechanism by which Mlp84B maintains muscle integrity and function.

Several lines of evidence link MLP to the actin cross-linking protein α -actinin (encoded by the *Actn* gene in *Drosophila*). MLP is member of the Cysteine-Rich Proteins, LIM-domain

cytoskeletal proteins highly expressed in muscle tissue (Arber et al. 1994; Louis et al. 1997). Biochemical characterization of the CRPs demonstrates that all three vertebrate proteins (CRP1, CRP2 and CRP3/MLP) directly bind α -actinin (Louis et al. 1997); more detailed analysis of the CRP1 interaction shows a high-affinity interaction between the globular head of α -actinin and the linker region of CRP1 (Pomies et al. 1997). CRP1 and α -actinin co-distribute along stress fibers in both smooth muscle cells and fibroblasts (Pomies et al. 1997; Tran et al. 2005), while vertebrate MLP and Mlp84B overlap with α -actinin at Z-lines of striated muscle (Arber et al. 1994; Knoll et al. 2002; Stronach et al. 1999). Interestingly, α -actinin does not direct Mlp84B subcellular localization, suggesting that their partnership has other functions besides protein tethering (Stronach et al. 1999). Finally, mutations that disrupt the α -actinin/MLP interaction are associated with cardiomyopathy (Mohapatra et al. 2003). Together, these observations support the hypothesis that α -actinin and MLP/Mlp84B function together to maintain normal muscle function and might protect muscle during times of increased muscle load.

In this report, using genetic experiments, we reveal a functional interaction between Mlp84B and α -actinin in maintaining Z-line structure and muscle attachments during *Drosophila* larval development. These observations are similar to our previous studies showing a functional interaction between Mlp84B and D-titin (Clark et al. 2007), and suggest that a complex of α -actinin, D-titin and Mlp84B functions together at sites of actin filament anchorage to maintain muscle integrity. We provide evidence that Mlp84B may maintain muscle integrity by promoting strong actin crosslinks at the Z-lines and other sites of actin filament anchorage within the muscle cell. Forced nuclear accumulation of Mlp84B directs the formation of actin cables in the absence of nuclear α -actinin, suggesting that both Mlp84B and α -actinin have intrinsic actin bundling activity to stabilize muscle. Together our results define a critical, fundamental activity for Mlp84B and validate the utility of the fly system to dissect the mechanisms by which MLP promotes normal muscle function.

RESULTS

Co-reduction of Mlp84B and α -actinin results in synergistic lethality

Previously, we employed classic loss-of-function mutations in *Mlp84B* and *D-titin* to demonstrate a functional relationship between these two proteins (Clark et al. 2007). This approach was not technically feasible to examine the relationship between Mlp84B and α -actinin due to the chromosomal locations of the genes. The crosses involve the simultaneous use of first and third chromosomal balancers, which leads to chromosome instability. To circumvent this issue, we used an RNAi approach, comparing phenotypes of flies with a single RNAi knockdown of either gene product to flies in which both α -actinin and Mlp84B protein levels were reduced. This was accomplished using transgenic flies that contain either an *Mlp84B* or *Actn* hairpin RNAi construct under the control of the UAS-promoter (Dietzl et al. 2007) to reduce the expression of the corresponding gene product. To generate animals with reduced levels of both proteins, we created a recombinant fly line that contained both *Mlp84B* and *Actn* RNAi constructs on the same chromosome. Crossing the three UAS RNAi stocks to a *dMef2-Gal4* transgenic stock allowed for robust muscle-specific expression of the RNAi constructs and knockdown of their targets, as judged by western blot analysis (Figure 1A). In lysates from stage 17 embryos, we observed a significant reduction in both Mlp84B (60% reduction) and α -actinin (70% reduction) when their respective RNAs were targeted. There was no further enhancement of reduction for either protein when both RNAs were targeted in the same animal.

One of the most striking effects of Mlp84B and α -actinin co-reduction was a pronounced shift in lethal phase, compared to the individual knock-down of either α -actinin or Mlp84B (Figure 1B). The majority of *Mlp84B* RNAi animals survive to adulthood, despite the severe

reduction in Mlp84B protein, indicating that even very low levels of Mlp84B protein are sufficient to maintain basic muscle function. A majority of *Actn* RNAi animals die as pupae (Figure 1B). The *Actn* RNAi expressing animals develop more slowly than wild type controls (Figure 1C) and *Mlp84B* RNAi expressing animals (data not shown), but do eventually pupate. In contrast, over 90% of the animals with diminished expression of both *Mlp84B* and *Actn* arrest development as larvae. Most double-RNAi animals fail to thrive (Figure 1C), and die a few days post-hatching.

As RNAi can have off target effects (Kulkarni et al. 2006), we used a combination of classic loss of function mutations and single RNAi to confirm the synergistic effects observed in the double Mlp84B-Actn RNAi larvae. First, we tested whether a reduction in *Mlp84B* gene product using a conventional genetic allele would exacerbate the effects of *Actn* RNAi expression. The majority of *Actn* RNAi expressing animals arrest development as pupae (Figure 1B). When we remove one functional copy of *Mlp84B* in the *Actn* RNAi expressing animals (e.g. *Actn* RNAi *Mlp84B*^{+/-null}), there is a pronounced shift in lethal phase, with most animals failing to successfully pupate (Figure 1D), just as was seen with co-reduction by RNAi.

In testing the converse, we found that expression of *Mlp84B* RNAi in animals heterozygous for a null *Actn* allele did not have an appreciable effect on viability, compared to the *Mlp84B* RNAi animals on their own. Western analysis showed that females heterozygous for the *Actn* null allele did not exhibit a significant reduction in α -actinin protein (data not shown). In an effort to find a more sensitive and viable *Actn* mutant background, we used a previously uncharacterized mutation, *Actn*^{cc1961}. The *Actn*^{cc1961} allele is a “GFP-protein trap” P-element insertion in the *Actn* coding region that results in the in-frame fusion of GFP to α -actinin (Quinones-Coello et al. 2006). Our analyses reveal that male hemizygotes are semi-viable (Figure 1E), and the surviving adults have compromised flight ability (data not shown). Western analyses of lysates from *Actn*^{cc1961} hemizygotes show a shifted band consistent with the predicted molecular mass for the α -actinin-GFP fusion protein, expressed at reduced levels as compared to wild-type endogenous α -actinin (data not shown). Thus, the *Actn*^{cc1961} flies represent a weak loss of function allele that may be sensitized to slight perturbations in α -actinin-dependent muscle processes.

We crossed the adult viable *dmef2-Gal4 UAS-Mlp84B* RNAi line with the *Actn*^{cc1961} stock to generate *Actn*^{cc1961} hemizygotes expressing *Mlp84B* RNAi. Unlike the *Actn*^{cc1961} hemizygotes on their own, *Actn*^{cc1961} hemizygotes expressing *Mlp84B* RNAi show no adult viability (Figure 1E). The *Actn*^{cc1961} hemizygotes with *Mlp84B* RNAi do pupate (data not shown), but further development is blocked shortly thereafter. Together these genetic analyses demonstrate a functional relationship between Mlp84B and α -actinin, and emphasize the utility of the fly to identify critical MLP-dependent cellular activities.

Co-reduction of Mlp84B and α -actinin severely destabilizes Z-line integrity and muscle attachment

Given the strong developmental defects described above, we postulated that the α -actinin and α -actinin/Mlp84B double knockdown larvae would both exhibit loss of muscle structural integrity, but the double-knockdown animals would have a more severe phenotype. To look at Z-line structure in newly hatched larvae, we recombined the *dMef2-Gal4* transgenic construct with the *Zasp66*^{ZCL0633} P-element insertion line which contains a GFP protein trap in a largely uncharacterized, muscle-specific PDZ protein, *Zasp66* (Quinones-Coello et al. 2006). The protein trap allowed us to visualize the Z-lines without having to dissect animals, preventing the potential introduction of additional structural defects to muscles that were not present in the intact larvae. A similar technique was used previously to screen through a large collection of RNAi stocks (including *Actn* RNAi) for

muscle phenotypes (Schnorrer et al. 2010), validating this approach. In the prior analysis, *Actn* RNAi resulted in late larval lethality and a "fading Z-line" phenotype. The phenotypes we observe are less severe (see below), likely because we performed our crosses at 25°C (versus 27°C), which should result in reduced production of the interfering RNA.

Both newly hatched (<1 day-old) wild type and *Mlp84B* RNAi expressing larvae contained well-organized Z-lines and the muscles have already begun to grow and elongate (Figure 2A,B). The Z-lines of animals expressing *Actn* RNAi are also still prominent, although the animals are not as large as the controls (Figure 2C). Animals expressing both *Mlp84B* RNAi and *Actn* RNAi display a range of phenotypes. The majority of animals had severe disruption in muscle structure and diffuse Zasp66-GFP localization, with Z-lines that were hard to discern (Figure 2D), although a minor fraction of embryos still had prominent Z-line label (Figure 2E, note the ventral orientation of this image as compared to panels A–D) indicating that Z-line maintenance, rather than formation, was compromised in the double knock-down animals.

In some double-RNAi animals, the muscle fibers appeared wispy, with a loss of defined GFP label at the muscle ends, suggestive of mechanical strain and initiation of muscle detachment (e.g. arrow in Figure 2E). As both *Mlp84B* and α -actinin are prominent at developing and mature muscle attachment sites (Stronach et al. 1996 and data not shown), we examined muscle attachment sites in 2-day old larvae to determine if there was a progressive disruption of muscle attachment in the *Actn* and *Actn/Mlp84B* RNAi-expressing animals. The Zasp66-GFP fusion protein also was enriched at muscle attachment sites and served as a morphological marker for this structure. In both wild type and *Mlp84B* RNAi expressing animals, the Zasp66-GFP signal was tightly condensed at the muscle ends, where the muscle membrane makes integrin-mediated attachments to the extracellular matrix (highlighted by arrows in Figure 2A',B'). In contrast, in both the *Actn* knockdown and the *Actn* plus *Mlp84B* double knockdown larvae, the terminal Zasp66-GFP labeling was no longer tightly localized, and some muscles appeared to pull away from the attachment sites (2C'–E'). From these observations, we conclude that concomitant decrease in *Mlp84B* and α -actinin results in a synergistic effect on both Z-line maintenance and muscle attachment integrity. *Mlp84B* and α -actinin function together in maintaining muscle integrity, and *Mlp84B* must participate in this process earlier than our previous analysis of the *Mlp84B*-null animals would have predicted (Clark et al. 2007). These data are consistent with our previous studies in which *Mlp84B*-null muscle did not display any obvious actin structural defects, but reducing *D-titin/sls* gene dosage in a *Mlp84B*-null background led to profound destabilization of muscle cytoarchitecture (Clark et al. 2007). Together, these data support a role for *Mlp84B* in stabilizing actin crosslinks at both the Z-line and muscle attachments that may be masked, in part, by the presence of α -actinin and D-titin/Sls at these sites.

Mlp84B and α -actinin physically associate

Consistent with a shared requirement for *Mlp84B* and α -actinin in maintaining sarcomere integrity, we previously showed that the two proteins co-distribute in larval muscle and overlap at the Z-line/I-band boundary (Figure 3A; (Clark et al. 2007; Stronach et al. 1999)). The Z-line/I-band boundary is a specialized part of the Z-line where Titin, Nebulin and the barbed ends of the thin filaments are anchored (Luther 2009). These three filament systems interact either directly or indirectly with α -actinin and the actin capping protein CapZ, and numerous studies indicate the importance for each component in maintaining sarcomere structure, suggesting that a robust, multi-protein complex in this region is critical to both maintain Z-line structure and keep the filament systems properly aligned (Papa et al. 1999; Pappas et al. 2008; Young et al. 1998). Biochemical studies of the *Mlp84B* orthologs in vertebrates (CRP1, CRP2 and CRP3/MLP) indicate a physical interaction between CRPs

and α -actinin (Louis et al. 1997), suggesting that Mlp84B may physically bind α -actinin and participate in this multi-protein complex.

We tested whether Mlp84B could also bind α -actinin, using both co-immunoprecipitation and a direct binding assay. Native Mlp84B either from whole embryos or mature muscle is largely insoluble, however Flag-tagged Mlp84B and native α -actinin co-precipitate from S2 cell lysates (Figure 3B), confirming that the proteins physically associate *in vivo*. Using a yeast two-hybrid assay, we observed reporter activity with an Mlp84B bait and α -actinin prey (Figure 3C), suggesting that the interaction *in vivo* is direct. Given that α -actinin is a major Z-line component, the physical binding of Mlp84B and α -actinin could simply provide a mechanism for localizing these proteins to muscle sarcomeres. Previous studies have shown that Mlp84B does not require α -actinin to specify its subcellular localization (Stronach et al. 1999). We show here that the converse is also true: α -actinin protein is properly localized in *Mlp84B*-null muscle (Figure 3D), suggesting that the association between Mlp84B and α -actinin does not serve to localize either protein.

Evidence for actin bundling capacity of Mlp84B

An emerging paradigm in the muscle field is the idea that LIM-domain proteins can facilitate α -actinin bundling capacity (e.g. ALP (Han and Beckerle 2009)), direct actin polymerization (e.g. Zyxin (Golsteyn et al. 1997; Smith et al. 2010)), and even possess intrinsic actin-binding activity (e.g. FHL3 (Coghill et al. 2003)). During studies aimed at characterizing potential nuclear functions for Mlp84B, we expressed a Mlp84B::GFP::NLS fusion protein using the native promoter (Clark et al. 2007). This resulted in the redistribution of Mlp84B from its primary location at the Z-line to the nucleus. Most nuclei displayed a diffuse nucleoplasmic distribution of the Mlp84B::GFP::NLS protein (highlighted by asterisk Figure 4A, also see (Clark et al. 2007)); however, a small fraction of nuclei (generally <5%) contained elaborate filaments/cables decorated with the fusion protein (arrowheads in Figure 4A). Given that vertebrate MLP and the related CRPs are reported to act as transcriptional co-activators (Chang et al. 2003; Kong et al. 1997), we first tested if the cables were DNA in nature. However, DAPI labeled nuclei did not show DNA staining in the GFP-positive cables (data not shown), indicating that the cables are not enriched for DNA. The Mlp84B::GFP::NLS-positive cables are reminiscent of nuclear actin cables formed by the over-expression of the actin bundling proteins myopodin (Weins et al. 2001) and supervillin (Wulfschlegel et al. 1999), and similar actin filaments are formed in *Drosophila* muscle nuclei expressing an N-terminal truncation of the LaminC protein (Dialynas et al. 2010). Moreover, actin cables present in nuclei expressing truncated LaminC also contain endogenous Mlp84B (Dialynas et al. 2010). Together, these observations strongly suggest that the GFP-positive cables seen upon nuclear accumulation of Mlp84B were composed of actin filaments. To confirm this, we labeled muscle preparations from Mlp84B::GFP::NLS expressing larvae with anti-actin antibody and found prominent labeling of the GFP-cables with the antibody (Figure 4B), confirming the nature of these cables as actin filaments. The nuclear actin cables also label with the F-actin marker, phalloidin (data not shown). These observations suggest that forced nuclear accumulation of Mlp84B drives actin bundling and cable formation.

There are two plausible mechanisms for the observed nuclear actin bundling. CRP1 is a vertebrate Mlp84B ortholog (Stronach et al. 1996), which has the capacity to directly bundle actin filaments (Jang and Greenwood 2009; Tran et al. 2005), raising the possibility that actin bundling directly by Mlp84B::GFP::NLS drives the actin cable formation detected in the nucleus. Alternatively, our observation that Mlp84B and α -actinin physically associate (Figure 3B,C) raises the possibility that Mlp84B::GFP::NLS could bring α -actinin into the nucleus, facilitating actin-bundling indirectly. To distinguish between these two alternatives, we labeled the muscle preparations from larvae expressing Mlp84B::GFP::NLS with an

antibody specific for α -actinin. While we observed strong labeling of Z-lines with the antibody, we did not detect any α -actinin signal on the nuclear actin cables (Figure 4C), indicating that Mlp84B::NLS::GFP likely does not recruit α -actinin to the nucleus to generate the actin cables. Together, these observations suggest that Mlp84B has direct actin bundling capacity, providing a mechanism of action for Mlp84B in maintenance of muscle structure.

DISCUSSION

To genetically define the molecular and cellular events in which Mlp84B participates, we employed a candidate approach, testing for a functional interaction with α -actinin. Our data lead to two main conclusions: 1) Mlp84B and α -actinin participate together in muscle stabilization, and 2) Mlp84B can direct the formation of actin filaments, likely through an intrinsic cross-linking activity. Below, we discuss possible mechanisms by which Mlp84B stabilizes muscle structure in the fly, and highlight implications of our findings in the etiology of human cardiomyopathy and muscular dystrophy.

Redundancy in sarcomere formation and maintenance

In our initial characterization of *Mlp84B* mutants, we reported that Mlp84B and Sls/D-titin function together in maintaining muscle structure (Clark et al. 2007). Now, we demonstrate a similar relationship between Mlp84B and α -actinin. In further support of the idea that there is a functional association between these proteins, mutations in both α -actinin and MLP that disrupt their association *in vitro* are associated with dilated cardiomyopathy (Gehrmlich et al. 2004; Mohapatra et al. 2003). From our analyses, we propose a model for a complex of actin, α -actinin, Mlp84B, and Sls/D-titin participating in stabilization critical for muscle integrity (Figure 5). Each protein has a different degree of contribution to stabilizing muscle, based on its individual loss-of-function phenotype. Null mutations in *Actn* and *D-titin* are embryonic lethal (Fyrberg et al. 1990; Machado and Andrew 2000; Zhang et al. 2000), whereas null mutations in *Mlp84B* are semi-viable (Clark et al. 2007). While complete loss of Mlp84B results in phenotypes that are less severe than other members of this complex, our genetic analyses indicate that Mlp84B is a central node, impacting the activity of the other complex members. Our genetic, biochemical, and cell-based analyses show that these proteins co-localize at sites of actin filament anchorage in muscle, and function together to maintain the integrity of these structures, especially at times of increased mechanical load (e.g. puparium formation).

In addition to the proteins depicted in Figure 5, several reports demonstrate that additional proteins found at sites of actin filament anchorage in muscle contribute to both the development and maintenance of muscle structure. For example, members of the Zasp/Cypher/ALP family of PDZ and LIM domain proteins co-localize with α -actinin in muscle and make critical contributions to myofibrillogenesis and muscle structural integrity (Cheng et al. 2010; Han and Beckerle 2009; Jani and Schock 2007; Pashmforoush et al. 2001; Zhou et al. 2001). Moreover, genetic studies in *Drosophila* demonstrate that α -actinin, Zasp and non-sarcomeric myosin (zipper) are interdependent for the initiation of Z-line formation. This supports a model in which α -actinin functions cooperatively with proteins in addition to Mlp84B to facilitate Z-line formation and maintenance (Rui et al. 2010). Of particular interest is that genetic lesions in α -actinin, Titin, Zasp, and MLP are all responsible for cases of familial cardiomyopathy (Bos et al. 2006; Chiu et al. 2010; Geier et al. 2008; Geier et al. 2003; Granzier and Labeit 2004; Mohapatra et al. 2003; Vatta et al. 2003; Zheng et al. 2010), implicating destabilization of actin crosslinks as a fundamental defect in these human pathologies. Of note, Steinmetz et al. recently proposed that α -actinin, MLP, Zasp and titin represent the core components of the primordial Z-line, based on their appearance during the

evolutionary development of striated muscle (Steinmetz et al. 2012), strengthening the idea that these proteins function together in Z-line assembly and maintenance.

A second D-titin-like protein in the fly, Projectin, is a Z-line associated filament with well-described contributions to muscle function and Z-line integrity (Ayme-Southgate et al. 2000; Fyrberg et al. 1992). Like D-titin, Projectin directly binds actin filaments (Podlubnaya et al. 2003), and we would expect that Projectin would also be part of the Mlp84B, D-titin, α -actinin, actin complex. Due to its location on the fourth chromosome, Projectin is not as well-characterized as D-titin/SIs, and our efforts to test for a genetic interaction with *Mlp84B* have been hampered by the genetic limitations associated with chromosome four.

Mlp84B as an actin-crosslinking protein

Efforts to directly test purified Mlp84B for actin crosslinking activity have been hampered by insolubility issues with the protein. However, several lines of evidence support the idea that Mlp84B directly crosslinks actin filaments. The most compelling evidence is our own observation that forced nuclear accumulation of Mlp84B results in the formation of nuclear actin cables that also contain Mlp84B itself. We do not detect α -actinin in these structures although it is still a formal possibility that our antibody cannot bind to α -actinin in the cables, or that Mlp84B recruits another actin-crosslinking protein to direct cable assembly. This phenomenon is recapitulated in the nuclear cables seen in muscles expressing an N-terminal truncation of Lamin C, which also contain endogenous Mlp84B and F-actin (Dialynas et al. 2010). Additionally, Mlp84B is a *Drosophila* ortholog of the CRPs (Louis et al. 1997; Stronach et al. 1996), muscle-enriched LIM domain proteins with established actin binding and bundling activity (Grubinger and Gimona 2004; Kihara et al. 2011; Tran et al. 2005). Key residues critical for CRP1 actin-bundling activity are conserved between vertebrate MLP and Mlp84B (data not shown). Moreover, related LIM proteins in *Dictyostelium* and plants also possess actin-binding and/or bundling activity (Khurana et al. 2002; Thomas et al. 2007), suggesting that acting bundling may be a conserved feature of CRP-related proteins.

Phenotypes associated with loss of Mlp84B are consistent with a disruption of an actin cross-linking function. Weakened actin crosslinks at the Z-line and muscle-membrane attachments would account for the following: 1) increased compliancy and decreased muscle stiffness exhibited by *Mlp84B* mutant flight muscle (Clark et al. 2011), 2) decreased force propagation and decreased power in the *Mlp84B* null muscle (Clark et al. 2011), 3) increased compliancy and prolonged diastole in the *Mlp84B* mutant hearts (Mery et al. 2008), and 4) lack of robust muscle contraction necessary for pupariation (Clark et al. 2007). In summary, all the observed phenotypes in the *Mlp84B* null flies can be explained by weakened actin crosslinks, suggesting this is the primary cellular defect caused by loss of Mlp84B. This conclusion does not exclude the possibility that Mlp84B or MLP make additional contributions to muscle that do not involve actin-crosslinking. Indeed, the modular nature of these proteins suggests that they have multiple functions, but our evidence supports actin bundling as a key activity for Mlp84B in maintaining muscle integrity.

Implications for cardiomyopathy and skeletal myopathies

A potential actin cross-linking activity for *Drosophila* Mlp84B has direct implications for vertebrate MLP function in both normal and pathologic muscle. Mutations in vertebrate MLP are associated with several cases of familial cardiac hypertrophy (Bos et al. 2006; Geier et al. 2008; Geier et al. 2003; Knoll et al. 2002). Mice devoid of MLP develop dilated cardiomyopathy (Arber et al. 1997), and mice heterozygous for mutations in MLP are more susceptible to myocardial infarction (Heineke et al. 2005). Both the intact hearts and isolated cardiomyocytes from MLP-null mice have been subjected to extensive analysis, and many

defects are associated with loss of MLP, including calcium handling (Unsold et al. 2012), Calcineurin signaling (Heineke et al. 2005), intercalated disc structure (Ehler et al. 2001), stretch signaling (Knoll et al. 2002), increased compliancy and prolonged diastole (Lorenzen-Schmidt et al. 2005). It is intriguing to speculate that a critical, primary defect in the MLP-null cardiomyocytes is structural due to weakened actin crosslinks at the Z-lines and other sites of actin filament anchorage. If so, at least some of the pathophysiological phenotypes associated with MLP mutations may be downstream effects that develop as a response to suboptimal structural integrity.

In addition to ensuring integrity in muscle subject to a normal range of mechanical stress, MLP may also participate in the repair response in damaged muscle. MLP levels dramatically increase when skeletal muscle is subject to damage from eccentric contractions (Barash et al. 2004; Kostek et al. 2007), during remodeling/fiber type switching (de Lange et al. 2004; Ebert et al. 2010; Lehnert et al. 2006; Schneider et al. 1999), and in several muscular dystrophies (Marotta et al. 2009; Sanoudou et al. 2006; Winokur et al. 2003). Cardiac muscle damage also leads to MLP upregulation (Donker et al. 2007; Wilding et al. 2006). Notably, both α -actinin3 and MLP deficiencies negatively impact recovery from eccentric contractions (Barash et al. 2005; Vincent et al. 2010). These observations suggest that the upregulation of MLP in damaged muscle may be a critical response, either to strengthen the cell during times of increased mechanical load, or to stabilize the cell infrastructure during repair. In the fly, there is a modest increase in *Mlp84B* RNA levels in muscle damaged from genetically-induced hypercontraction (Montana and Littleton 2006), but no corresponding increase in protein (data not shown). However, *Mlp60A*, the other MLP family member in *Drosophila*, shows a robust increase at both the RNA and protein levels in the hypercontraction mutants (Montana and Littleton 2006); (data not shown). This suggests that the MLP-dependent signaling mechanisms involved in responding to muscle damage are evolutionarily conserved and supports further investigation of MLP family members in *Drosophila*.

Utilizing *Mlp84B* to further elucidate mechanisms of muscle stabilization

The redundancy inherent in the protein complexes that anchor actin filaments in muscle strongly suggests that additional proteins will be involved in this process. Modification of *Mlp84B* phenotypes provides a robust assay to screen for novel proteins that participate in the stabilization of muscle in an unbiased and high-throughput manner. Our success with testing known mutants (*D-titin* and *Actn*) as modifiers of *Mlp84B* phenotypes indicates that screening for novel proteins should be productive. Indeed, in a pilot modifier screen using chemical mutagenesis, we isolated 5 enhancers that exacerbate the pupal elongation and/or larval lethality of *Mlp84B* null mutants and 2 suppressors that rescue pupal shape and/or allow for an increased frequency of adult escapers (data not shown). Three of the enhancers are new *sls/D-titin* alleles, both validating this approach and providing evidence that there are other novel, undefined *Mlp84B* modifiers that may participate in muscle stabilization. Together, our observations reinforce the conclusion that *Drosophila* is a relevant system to study MLP function and further define molecular pathways important for maintenance of muscle structural integrity and response to damage.

MATERIALS AND METHODS

Drosophila stocks and reagents

All stocks were reared under standard laboratory conditions at 25°C. The following stocks were used: P[*w+* *dMef2-Gal4*], from Alan Michelson; *UAS-Mlp84B* RNAi (18593 and 18594) and *UAS-Actn* RNAi (7760 and 7761), from the Vienna *Drosophila* RNAi Center; *P[PTT-GC]Actn^{cc1961}/FM7* and *P[PTT-GA]Zasp66^{ZCL0663}* from Lynn Cooley; *Mlp84B^{P8}*

(*Mlp84B*-null), *Df(3R)dsx2M*, *Df(3R)Scx2*, *P[w⁺ Mlp84B::NLS::GFP]* are previously described (Clark et al. 2007). *w¹¹¹⁸* served as the wild type control in all experiments, except where noted.

Western analysis and immunoprecipitation

Western analysis of protein lysates was performed essentially as previously described (Clark et al. 2007). Fifty stage 17 embryos of a given genotype were homogenized directly in 1X Lammeli sample buffer and 10 embryo equivalents were loaded per lane. The following antibodies were used: rat anti- α -actinin antibody at 1:50 (Babraham Institute, UK); rabbit anti-Mlp84B at 1:10,000; (Stronach et al. 1996), mouse anti-tubulin at 1:10,000 (Developmental Studies Hybridoma Bank), and mouse anti-Flag at 1:1000 (Sigma). Quantification of western blots was performed using Image J.

For immunoprecipitations, S2 cells were transfected with pMT-Mlp84B-Flag (Flag-tagged Mlp84B (Stronach et al. 1996) subcloned into pMT/V5/HisA (Invitrogen)). S2 cell lysates were prepared in lysis buffer (50 mM Tris-HCl, pH 7.9, 150 mM NaCl, 0.1% Triton X-100) plus protease inhibitors, and were incubated with anti-Flag M2 agarose (Sigma), then boiled in 2x Laemmli sample buffer followed by western blotting.

Lethal phase analyses

A comprehensive lethal phase analysis was performed for the RNAi-crosses as follows: 1) Males with the UAS-RNAi construct were crossed to *P[PTT-GA]Zasp66^{ZCL0663}; P[w⁺ dMef2-Gal4]* virgin females. 2) For each cross, 100 eggs were collected from a 1 hour timed collection and aged 24 hours to assay hatch rates (eggs containing segmented embryos were considered fertilized; all others were not included in counts). Embryos that did not hatch within 48 hr were considered dead; 3) Hatched larvae were counted and placed in standard rearing vials supplemented with wet yeast paste; 4) Pupae were counted 36 hours after first observed pupation to determine larval survival. A minimum of 5 crosses/egg collections was performed for each knockdown (>500 embryos). To generate *Actn* RNAi expressing animals with one null allele of *Mlp84B* (Figure 1D), the *Mlp84B^{P8}* mutation was recombined with *P[w⁺ dMef2-Gal4]*, and the recombinant line (balanced with TM6B) was subsequently crossed to UAS-*Actn* RNAi. One hundred hatched larvae were collected from each cross, and the percent viable pupae was calculated as the number of non-Tubby pupae divided by 50. For crosses to assess adult viability of the *Actn^{cc1961}* mutation (Figure 1E), mutant males were identified by red eyes or by α -actinin-GFP expression. Progeny from crosses depicted in Figure 1E were identified using appropriate markers, and observed male viability was determined as the number of males of that genotype divided by the total number of male progeny of that cross.

Immunofluorescent imaging

For GFP-imaging of intact larvae, larvae were quickly heat-fixed, placed in 50% glycerol and imaged using an Olympus FV1000 confocal with a 20X objective (1 day old larvae) or 60X/water (~36 hr larvae). Fillets for nuclear actin imaging were prepared and labeled as described previously (Clark et al. 2007). Antibodies used for immunofluorescence were mouse anti-actin at 1:5000 (C4, Millipore); rat anti- α -actinin at 1:50 (Babraham Institute, UK); Alexa-633 Goat anti-Rat at 1:5000 (Molecular Probes); Alexa-595 Goat anti-mouse at 1:5000 (Molecular Probes).

Larval size analysis

Larvae were quickly heat-fixed and aligned on a glass slide for imaging. Still images of larvae and pupae were collected on an Olympus SZX12 dissection microscope with an Olympus C-5050Z camera and processed with ImageJ and Photoshop 12.0.

Yeast two-hybrid

A cDNA encoding the full-length Mlp84B was cloned into in pGBD-C1 (James et al. 1996) and the full-length α -actinin and laminin preys are in pACT2. The yeast host strain, PJ69-2a, was transformed with bait and prey, and then reporter activity was assayed as described previously (James et al. 1996; Kadrmas et al. 2004).

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Figure 1. Mlp84B and α -actinin functionally interact. (A) Western blot analysis of relative protein levels in stage 17 embryos, with UAS-RNAi constructs indicated. All UAS-RNAi lines were expressed with a *dMef2-Gal4* muscle specific driver. Extent of knockdown, as compared to the wild type control, was 70% for α -actinin and 60% for Mlp84B. Note similar reduction in protein levels from single RNAi knockdown and *Actn* + *Mlp84B* double knockdown embryos. (B) Quantification of temporal lethality of RNAi-expressing animals during development. Bar for each RNAi combination indicates endpoint percent survival for each stage; for example 69% of the wildtype (WT) embryos survived to adulthood, 9.5% died as pupae, 17% died as larvae and 4.5% died as embryos. Any lethality in the WT control was

due to experimental manipulation of samples and represents “baseline” survival for the experiment. A minimum of 500 animals was scored for each line. (C) Micrographs of 3 day old larvae expressing indicated RNAi constructs. Each panel is the same magnification and scale bar = 0.5 mm. (D) Decrease in *Mlp84B* gene dosage reduces the extent of pupation with *Actn* RNAi. Pupation data were calculated from number of *Actn* RNAi pupae observed divided by total number of pupae produced in cross. Note that *Mlp84B^{P8}* is a null allele. (E) Analysis of *Actn^{cc1961}* adult viability in combination with *Mlp84B* RNAi. Data is expressed as percentage of observed genotypes for all male progeny of a given cross, compared with expected distribution of genotypes. The *Mlp84B* RNAi crosses are both designed to generate *Actn^{cc1961}* *Mlp84B* RNAi progeny, but contain different first and third chromosomes to assess contributions of the TM6B balancer chromosome to *Actn^{cc1961}* viability. Note that no *Actn^{cc1961}* *Mlp84B* RNAi adults were recovered from either cross.

Figure 2.

Concomitant reduction of Mlp84B and α -actinin severely compromises Z-line integrity and stability of muscle attachment sites. (A–E) Micrographs of 1 day old larvae expressing Zasp66::GFP highlight muscle sarcomeres. Note similar size of WT (A) and *Mlp84B* RNAi-expressing larvae (B). The *Actn* RNAi larvae (C) is smaller than both WT and *Mlp84B* RNAi expressing age-matched larvae, but has Z-line label with the GFP protein trap. Two representative *Mlp84B Actn* double RNAi larvae (D,E) are shown to indicate the range of phenotypes seen. Panel D demonstrates reduction in Z-line label seen in the double knockdown animals while larvae in E still retains Z-line label. Arrow in E highlights muscle with signs of strain, described in text. Larvae in A–D are presented in a lateral view, with

anterior left, while E is a ventral view. Scale bar in A = 50 μm . (A'-E') Micrographs of muscle attachment sites in 3-day-old larvae. Arrows in each panel indicate muscle-muscle attachments labeled with Zasp::GFP. Note loss of tightly compacted label at muscle ends in C' and D,' and partial detachment of muscles in C'-E'. A detaching muscle is boxed in E'. Scale bar in A' = 5 μm .

Figure 3.

Mlp84B and α -actinin co-localize and physically interact. (A) Section of muscle from third instar larvae labeled with antibodies as shown. Note codistribution of Mlp84B and α -actinin signals in merged panel and overlap of signals at the Z-line/I-band boundary. (B) Western blots of lysates and immunoprecipitated complexes from S2 cells; samples that express Flag-tagged Mlp84B are indicated above the blot. Lanes 3 and 4 are Flag-immunoprecipitates from S2 lines either not transfected, or expressing the Flag-tagged Mlp84B, respectively. Endogenous α -actinin is only pulled down in the precipitate in the presence of the Flag-tagged Mlp84B. (C) Yeast two-hybrid analyses show that an Mlp84B bait and α -actinin prey have reporter activity together, but do not activate reporter activity

when combined with VP16 prey or Lamin bait, respectively. (D) α -actinin protein is properly localized in both wild type and *Mlp84B*-null muscle. Panels both show muscle from 3rd instar larvae labeled with α -actinin antibody. Scale bar is 10 μ m.

Figure 4.

Nuclear Mlp84B drives actin bundling and cable formation. (A) Section of muscle from third instar larvae that expresses Mlp84B::NLS::GFP. Some nuclei contain diffusely localized Mlp84B::NLS::GFP (asterisk), while others have Mlp84B::NLS::GFP-positive filaments (arrowheads) Scale bar = 20 μ M. (B) Mlp84B::NLS::GFP-positive filaments co-label with actin antibody. Scale bar in merged image = 20 μ m. (C) Muscle fillet from 3rd instar larvae expressing Mlp84B::NLS::GFP. GFP-positive nuclear cable (upper panel) does not label with α -actinin antibody, although there is prominent Z-line labeling with the α -actinin antibody (middle panel). Scale bar in merged image = 40 μ m.

Figure 5.

A complex of α -actinin, Mlp84B and SIs/D-titin functions to maintain actin crosslinks. Lines connecting each protein reflect both strength of actin crosslinks and evidence for a physical and/or genetic interaction. Question mark indicates presumed binding between Mlp84B and actin inferred from our data. Size of protein node indicates relative amount in gene product for each of the situations diagrammed.