The *Drosophila* muscle LIM protein, Mlp84B, cooperates with D-titin to maintain muscle structural integrity

Kathleen A. Clark[1,2,*], Jennifer M. Bland[2] and Mary C. Beckerle[1,2,3]

[1]Huntsman Cancer Institute, [2]Department of Biology and [3]Department of Oncological Sciences, University of Utah, Salt Lake City, UT 84112, USA
*Author for correspondence (e-mail: kathleen.clark@hci.utah.edu)

Accepted 24 April 2007
Journal of Cell Science 120, 2066-2077 Published by The Company of Biologists 2007
doi:10.1242/jcs.000695

Summary

Muscle LIM protein (MLP) is a cytoskeletal LIM-only protein expressed in striated muscle. Mutations in human MLP are associated with cardiomyopathy; however, the molecular mechanism by which MLP functions is not established. A *Drosophila* MLP homolog, *mlp84B*, displays many of the same features as the vertebrate protein, illustrating the utility of the fly for the study of MLP function. Animals lacking Mlp84B develop into larvae with a morphologically intact musculature, but the mutants arrest during pupation with impaired muscle function. Mlp84B displays muscle-specific expression and is a component of the Z-disc and nucleus. Preventing nuclear retention of Mlp84B does not affect its function, indicating that Mlp84B site of action is likely to be at the Z-disc. Within the Z-disc, Mlp84B is colocalized with the N-terminus of D-titin, a protein crucial for sarcomere organization and stretch mechanics. The *mlp84B* mutants phenotypically resemble weak *D-titin* mutants. Furthermore, reducing D-titin activity in the *mlp84B* background leads to pronounced enhancement of the *mlp84B* muscle defects and loss of muscle structural integrity. The genetic interactions between *mlp84B* and *D-titin* reveal a role for Mlp84B in maintaining muscle structural integrity that was not obvious from analysis of the *mlp84B* mutants themselves, and suggest Mlp84B and D-titin cooperate to stabilize muscle sarcomeres.

Supplementary material available online at
http://jcs.biologists.org/cgi/content/full/120/12/2066/DC1

Key words: LIM protein, Muscle structure/function, Cytoskeletal stabilization, *Drosophila*

Introduction

Muscle is a highly organized tissue that relies on precise, complex molecular interactions in order to perform its contractile functions. The muscle sarcomere both generates the contractile force and transmits the force to the muscle membrane. Cytoskeletal elements that link the muscle sarcomeres to the membrane also organize the receptors and ion channels that regulate muscle activity. Thus, components of the muscle sarcomere and cytoskeleton have important roles in both generating and regulating contractility. Mutations in sarcomere-associated cytoskeletal proteins are the causative agent for many skeletal and cardiac myopathies, underscoring their importance in muscle function (Seidman and Seidman, 2001; Clark et al., 2002). The identification of muscle-specific proteins, and their functional analysis by biochemical and genetic approaches, has enabled an ever more sophisticated view of muscle biology, and provided insight into the pathophysiology of several devastating myopathies.

Muscle LIM protein (MLP) was first identified as a striated muscle-specific protein upregulated during denervation of skeletal muscle (Arber et al., 1994). Genetic ablation of MLP in the mouse revealed its crucial importance in maintaining normal cardiac function. $MLP^{-/-}$ mice develop dilated cardiomyopathy, and die of heart failure (Arber et al., 1997). The $MLP^{-/-}$ mouse is now a well-established model to study the mechanisms of dilated cardiomyopathy. Mutations in human MLP are also associated with dilated cardiomyopathy and cardiac hypertrophy, highlighting the importance of elucidating MLP's mechanism of action in striated muscle (Bos et al., 2006; Geier et al., 2003; Knoll et al., 2002; Mohapatra et al., 2003; Theis et al., 2006).

Although MLP has several proposed functions (Arber et al., 1994; Arber et al., 1997; Kong et al., 1997; Flick and Konieczny, 2000; Ehler et al., 2001; Knoll et al., 2002), its function in muscle still is not well-understood. MLP is a member of the cysteine-rich protein (CRP) family, a group of cytoskeletal proteins defined by their high degree of sequence similarity, and the presence of one or more LIM domain/glycine-rich modules (Liebhaber et al., 1990; Louis et al., 1997; Weiskirchen and Gunther, 2003). The LIM domain is a double-zinc finger structure that functions as a protein docking site (Perez-Alvarado et al., 1994; Schmeichel and Beckerle, 1994; Kadrmas and Beckerle, 2004), and the glycine-rich region may also support interaction in concert with the LIM region (Harper et al., 2000). The molecular architecture of CRPs suggests roles as molecular scaffolds or linker proteins. In support of this idea, CRP family members interact directly with several cytoskeletal proteins including zyxin (Sadler et al., 1992; Louis et al., 1997), α-actinin (Pomies et al., 1997), N-RAP (Ehler et al., 2001) and Spectrin (Flick and Konieczny, 2000). Intriguingly, CRP family members also bind several transcription factors (Kong et al.,

1997; Chang et al., 2003), implicating CRP proteins as modulators of gene expression.

Because of the established physiological importance of MLP in vertebrate cardiac muscle, we have begun to address the molecular functions of CRPs using a genetic approach in *Drosophila melanogaster*. Our laboratory previously identified two genes in *Drosophila*, termed *mlp60A* and *mlp84B*, that are muscle-specific CRP family members bearing both sequence homology with vertebrate CRPs and their characteristic LIM/glycine-rich domain structure (Stronach et al., 1996). Similar to the vertebrate CRPs, Mlp84B protein is found in both the cytoplasm and nucleus during muscle differentiation (Stronach et al., 1996). In mature muscle fibers, Mlp84B is a component of the Z-disc, and also enriched at a structure analogous to the cardiac intercalated disc, known as the myotendinous junction (Stronach et al., 1999).

In this report, we describe the loss-of-function phenotype for *mlp84B* and show that Mlp84B is crucial for normal muscle function in *Drosophila*. Although *mlp84B* mutant muscles undergo apparently normal differentiation, the muscles fail over time, and animals arrest at the pupal stage. Using the *mlp84B* mutants, we tested the role of Mlp84B in the nucleus and cytoplasm, and found that nuclear Mlp84B does not have an essential function during normal fly development. We demonstrate that mutations in *D-titin* enhance *mlp84B* mutations, suggesting that the proteins cooperate in vivo. Titin has a well-established role in the assembly of muscle sarcomeres, participates in muscle mechanics as an elastic component, and more recently has been implicated as a regulator of muscle gene expression. Animals that lack Mlp84B, and have reduced D-titin activity, display a severe, synthetic disruption of muscle structure, revealing a role for Mlp84B in muscle stabilization. Our data support a model in which MLP and titin act in concert to maintain normal muscle function and structural integrity.

Results

Generation of *mlp84B* mutants

Our goal was to develop and characterize loss-of-function mutations in *mlp84B*. Previous mapping experiments placed *mlp84B* on the right arm of the third chromosome, at 84BC (Stronach et al., 1996). We identified several deficiency chromosomes in the 84BC region, including *Df(3R)dsx2M* and *Df(3R)Scx2* that remove the locus (Fig. 1A; Table 1). The published breakpoints for these deficiencies (Cavener et al.,

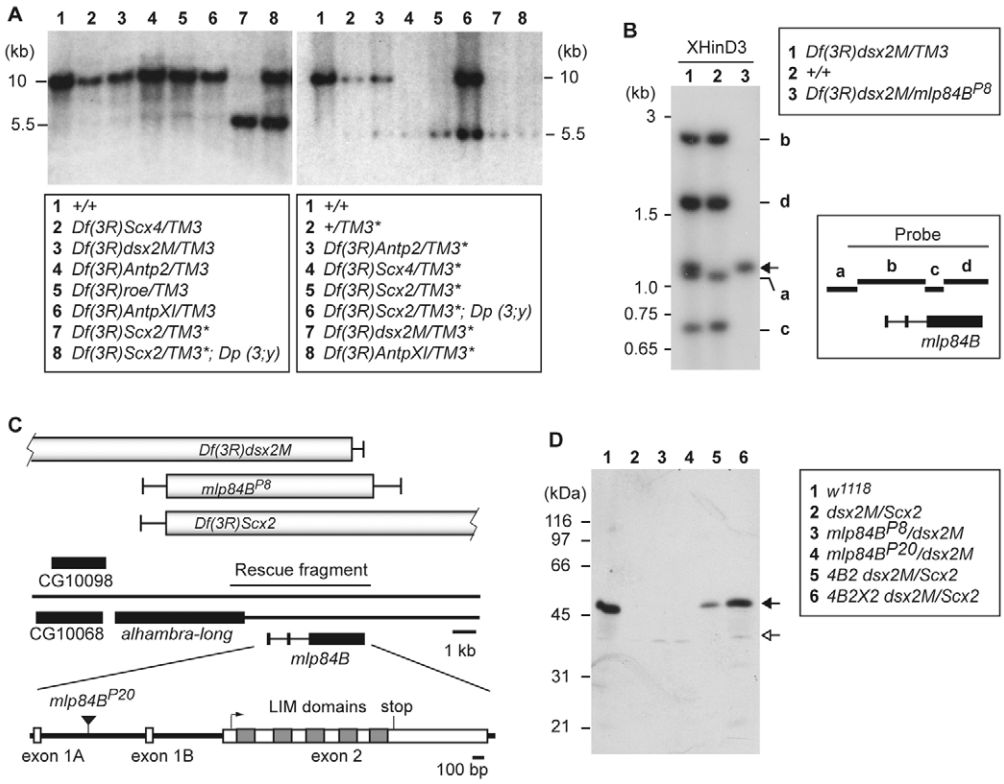

Fig. 1. Molecular genetic analysis of the *mlp84B* locus. (A) Identification of deficiencies that remove *mlp84B*. DNA from several deficiency lines in the 84B,C region was digested with *Eco*RI, and probed with the *mlp84B* cDNA. The second blot contains DNA from deficiency lines that harbor a balancer chromosome with a restriction fragment length polymorphism in the *mlp84B* region (TM3*). Loss of the larger *Eco*RI band (10 kb) indicates a deletion of *mlp84B*. (B) Southern blot analysis of the $mlp84B^{P8}$ allele showing deletion of the *mlp84B* transcription unit. The arrangement of the *Hin*dIII fragments relative to the *mlp84B* transcription unit is shown to the right [arrow indicates band contributed by the *Df(3R)dsx2M* chromosome]. (C) Molecular map of the 84C1,2 region. Deficiencies used to generate *mlp84B*-null animals are shown above the map, and the deletion in $mlp84B^{P8}$ is also shown. The *mlp84B* transcription unit is shown in detail below the map, with the position of the P-element in $mlp84B^{P20}$ indicated. (D) Western blot of protein lysates from third-instar larvae demonstrating loss of the *mlp84B* gene product in *mlp84B* mutants. 10 μg of protein was loaded per lane. Genotypes for the larvae are indicated on the right; 4B2 is the *mlp84B* transgene, and 2X4B2 indicates two copies of the transgene. The open arrow indicates a weak non-specific band detected by this antiserum.

Table 1. *Drosophila* stocks used in this study

Genotype	Cytology	Description
Df(3R)Scx2, red e / TM6B, Tb Hu e	84A5-84C2	Deficiency that removes *mlp84B*
Df(3R) dsx2M / TM6B, Tb Hu e	84C1-84E1	Deficiency that removes *mlp84B*
P[w+ pCas4B2]25A; Df(3R)Scx2, red e / TM6B		*mlp84B* transgene in deficiency background
P[w+ pCas4B2]25B Df(3R)dsx2M / TM6B, Tb Hu e		*mlp84B* transgene in deficiency background
P[w+] l(3)j8c8 / TM6B, Tb Hu e	84C1	P-element transposon remobilized to generate $mlp84B^{P8}$ and $mlp84B^{P20}$
P[w+] mlp84B^{P8} / TM6B, Tb Hu e	84C1	Deletion of *mlp84B* generated by P-element remobilization
P[w+]mlp84B^{P20} / TM6B, Tb Hu e	84C1	Insertion in *mlp84B* generated by P-element remobilization
P[w+] sls^{j1D7} / TM6B, Tb Hu e	62C2	Strong loss-of-function *sls* allele
P[w+] sls^{j1D7} P[w+] mlp84B^{P8} / TM6B, Tb Hu e		
sls^{e4} / TM6B, Tb Hu e		Hypomorphic *sls* allele
sls^{s05} / TM6B, Tb Hu e		Hypomorphic *sls* allele
sls^{02001} / TM6B, Tb Hu e	62C	Hypomorphic *sls* allele

1986a; Cavener et al., 1986b; Baker and Wolfner, 1988) allowed us to refine the location of *mlp84B* to 84C1,2.

We initially created *mlp84B$^{-/-}$* animals using two *mlp84B* deficiencies, *Df(3R)dsx2M* and *Df(3R)Scx2*, whose deleted regions exhibit a relatively small overlap (~10 kb). We isolated two additional *mlp84B* mutations by P-element transposition of *l(3)j8c8*, which resides approximately 10 kb proximal to *mlp84B*. Southern blot analysis demonstrated that the entire *mlp84B* transcription unit is deleted in the *mlp84B^{P8}* allele (Fig. 1B), thus *mlp84B^{P8}* is a molecular null. By sequence analysis, we found that the *mlp84B^{P20}* allele contains a P-element insertion in an *mlp84B* intron at base-pair 493 (Fig. 1C). Both the *mlp84B* deficiencies, and the P-element alleles, fail to produce any detectable protein by western blot analysis (Fig. 1D, lanes 2-4), demonstrating that all three mutants are protein nulls.

Mlp84B is crucial for normal development

We assessed whether Mlp84B is an essential protein by determining whether *mlp84B*-deficient adults are viable. At 25°C, we fail to recover any *Df(3R)Scx2/Df(3R)dsx2M* transheterozygotes, and only a small percentage of P-element mutant hemizygotes eclose at 25°C (Fig. 2A). This lethality is due specifically to loss of Mlp84B, as viability is restored when the *mlp84B*-mutant flies also harbor one or two copies of an *mlp84B* transgene (Fig. 2A; Fig. 1D, lanes 5,6). At

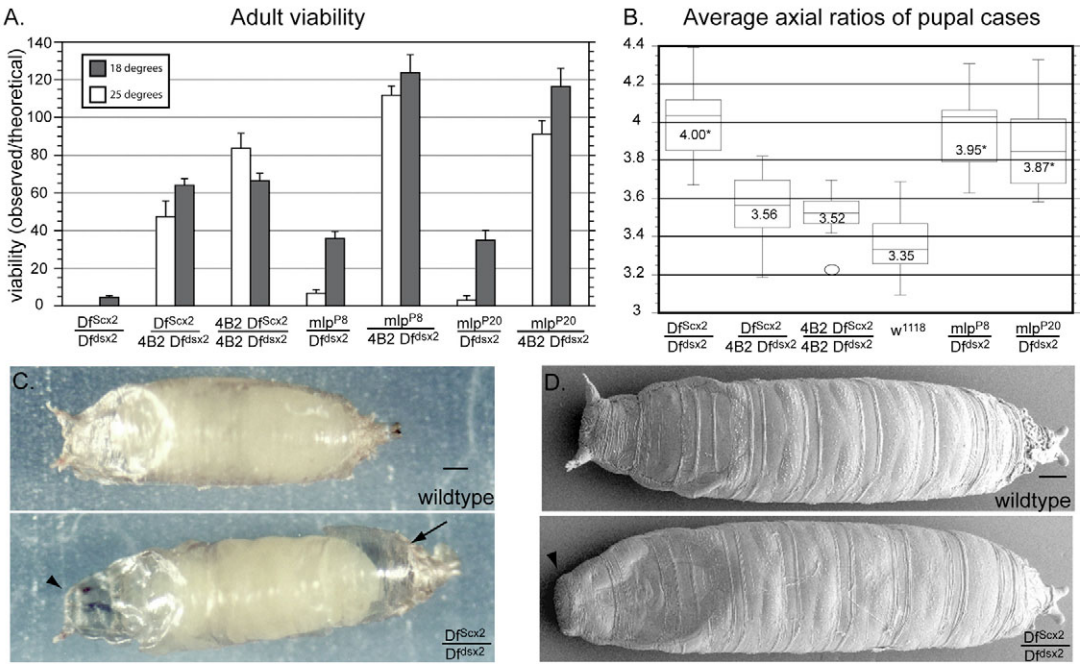

Fig. 2. Loss of Mlp84B affects muscle-driven morphogenetic processes. (A) Pupal lethality associated with loss of *mlp84B* and rescue with the *mlp84B* transgene. (B) Axial ratios for pupae of indicated genotype. [*$P>10^{-5}$, compared with two-copy rescue line (4B2 Scx2/4B2 dsx2M).] Data are displayed as a distribution of data points for each pupae, bounded by the upper quartile and lower quartile, with the median value indicated as a line in the box. Error bars show the upper and lower limits of the data, and any outliers are indicated as a circle. (C) Brightfield images of wild-type and *Df(3R)dsx2M/Df(3R)Scx2* pupae (*mlp84B$^{-/-}$*); bar, 200 μm. Anterior is to left. Arrow indicates air pocket that failed to translocate. Arrowhead shows failure in spiracle eversion. (D) Scanning electron microscopy image of wild-type and *Df(3R)dsx2M/Df(3R)Scx2* pupae; bar, 200 μm. Arrowhead shows failure in spiracle eversion.

18°C, a temperature at which only a small percentage of the transheterozygous Df flies eclose, a significant number of both P-element allele adults survive. These escaper adults are not healthy and usually die within one week of eclosion. The most likely explanation for the reduced severity of the P8 and P20 alleles relative to the transheterozygous Df combination is that the transheterozygous Df flies contain large deletions that, on their own, probably influence overall health of the fly. It is possible that other genes affected by both deficiencies (Fig. 1C) contribute to the severity of transheterozygous combination, but because we can rescue viability in a majority of these animals with an *mlp84B* transgene, we can conclude that the phenotype is because of loss of Mlp84B. In their characterization of the neighboring gene, *Alhambra*, Perrin and Durra report the generation of *mlp84B* deletions that are also lethal, consistent with our results (Perrin and Dura, 2004).

Phenotypes exhibited by Mlp84B-null animals indicate a defect in muscle function

The *mlp84B* mutants exhibit a range of phenotypes throughout the fly life cycle that indicate a requirement for Mlp84B in normal muscle function. The earliest stage at which mutants can be unambiguously distinguished from wild-type animals is during late (L3) larval development. At this stage, *mlp84B* mutants appear slightly flaccid, relative to wild type. The majority (>90%) of the *mlp84B* mutants die at the pupal stage. Although Mlp84B protein is prominent during embryonic muscle differentiation (Stronach et al., 1996), our analysis indicates that zygotic expression of Mlp84B is not essential for embryonic development. Although a maternal contribution of a gene product could mask an earlier requirement for Mlp84B, no *mlp84B* mRNA or protein is detected in newly laid embryos (Stronach et al., 1996).

Mlp84B is a muscle-specific protein, and significant muscle-dependent remodeling occurs during pupal morphogenesis (Bodenstein, 1950). For example, at pupariation, larvae shorten their bodies by contracting their body wall muscle. During morphogenesis, muscle contractions occur in a timed manner to reposition the animal within the pupal case and generate hydrostatic pressure to evert the adult structures, such as the head and appendages. All of these muscle-dependent morphogenetic movements are compromised by loss of Mlp84B. The *mlp84B* mutant pupae exhibit a larger axial ratio (length/width) than wild-type pupae, indicating failed contraction of their body wall muscle during puparium formation (Fig. 2B). The difference in axial ratio between wild-type and *mlp84B* mutant pupae is easily observed by visual inspection (Fig. 2C). Eversion of the anterior spiracles is typically defective in the transheterzygous Df *mlp84B* mutants (arrowheads in Fig. 2C,D). From examination of the *mlp84B* mutant pupae by scanning electron microscopy (Fig. 2D), we observed no gross defects in the overall shape, surface contours, or patterning of the pupal case.

The *mlp84B* mutants display a striking deficiency in muscle contractility during morphogenesis (see supplementary material Movies 1, 2). At the onset of pupal morphogenesis, wild-type animals constantly contract their body wall muscle, and then evert the adult head. The *mlp84B* mutants are relatively inactive, and most arrest development at about the time of head eversion. The arrested pupae have a characteristic air pocket at the posterior end of the pupal case (see arrow in Fig. 2C). The anterior air pocket results from a failure to translocate air from the posterior- to the anterior-end of the pupal case, and thus this air pocket serves as another marker for muscle failure during pupation. The hemizygous *mlp84B* P-element mutant pupae also have a larger axial ratio (Fig. 2B), and fail to translocate the air bubble (data not shown), supporting the conclusion that the muscle defects observed in the transheterozygotes result from loss of Mlp84B.

As noted above, a small number of viable transheterozygous Df mutant adults are recovered, if the animals are reared at 18°C, although they are not healthy. We investigated whether these adult animals also show any muscle dysfunction by assessing flight muscle function (Fyrberg et al., 1990; Bernstein et al., 1993). We developed a simple flight test to evaluate flight ability. When wild-type adults are pushed off the edge of a laboratory bench, more than 90% fly before hitting the ground. None of the transheterozygous Df flies exhibited any flight ability using this assay, illustrating the complete impairment of flight. Collectively, all the phenotypes exhibited by the *mlp84B* mutants are consistent with a failure in muscle function induced by loss of Mlp84B.

Muscle fibers from *mlp84B* mutants do not display any obvious structural abnormalities

One possible explanation for the muscle failure in the *mlp84B* mutants is that muscle structure is compromised when Mlp84B is absent. Indeed, muscle fibers from the MLP$^{-/-}$ mice do display structural abnormalities (Arber et al., 1997; Ehler et al., 2001), although whether these defects are directly because of loss of MLP is not resolved (Knoll et al., 2002). We first examined the gross structure of body wall muscle from wandering third-instar larvae, just prior to puparium formation. Labeling *mlp84B* mutant larval fillets with Alexa Fluor-488-conjugated phalloidin revealed an ordered pattern of muscle fibers, which displayed a wild-type morphology (Fig. 3A,B). To examine the sarcomeric organization in more detail, we labeled muscle fibers from the *mlp84B* mutants with antibodies against α-actinin to highlight the Z-discs. At the light microscopic level, the *mlp84B* mutants did not display any disruption in Z-disc morphology or organization (Fig. 3A′,B′). Because it was possible that structural instability may only be apparent in acutely compromised muscle, we examined *mlp84B* mutant flight muscle by electron microscopy for any disruption of cytoarchitecture organization. We found that both wild-type and the *mlp84B* mutant flight muscle display a well-arrayed myofilament system and sarcomeric organization (Fig. 3C). Together, these results indicate that either loss of Mlp84B does not compromise muscle structure, or subtle structural defects exist that result in muscle failure.

Nuclear retention of Mlp84B is dispensable for normal muscle function

Both vertebrate MLP and Mlp84B are found in the nuclear and cytoplasmic compartments; thus, the crucial site of action of MLP may be in one or both of these locations. We used the transgenic tools available in the fly to generate forms of Mlp84B that are enriched in one compartment, and then tested the ability of that protein to rescue the *mlp84B* mutant flies to viability. Mlp84B::GFP is a C-terminal green fluorescent protein (GFP) fusion in the context of the genomic rescue

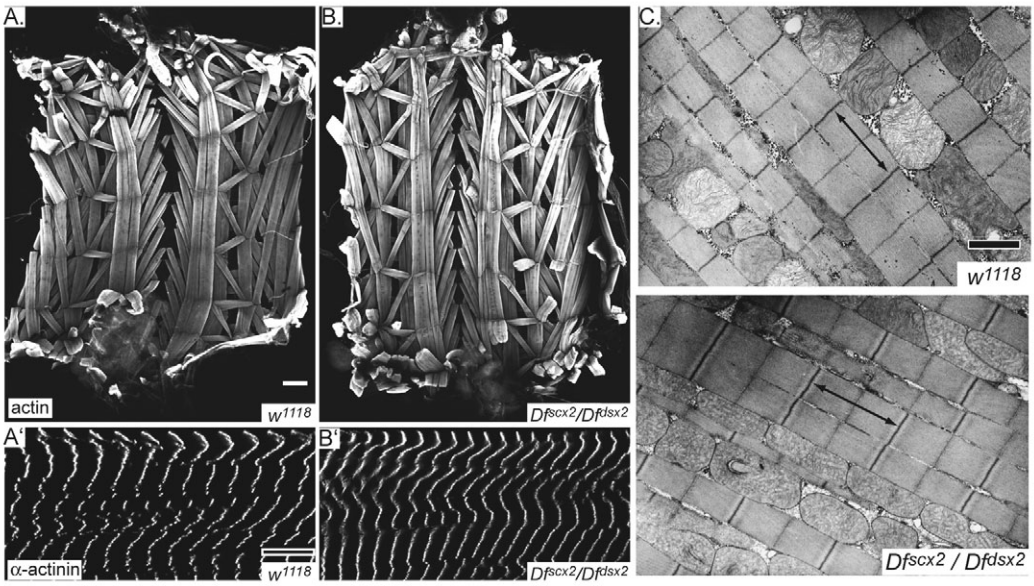

Fig. 3. Loss of Mlp84B does not grossly disrupt muscle structure. (A,A′) Wild-type third-instar muscle fillets labeled with actin (A) or α-actinin (A′). Bars, 200 μm (A); 20 μm (A′). (B,B′) Fillets from *Df(3R)dsx2M/Df(3R)Scx2* transheterozygous third-instar labeled with actin (B) or α-actinin (B′). (C) Ultrastructural analysis of flight muscle from wild-type (upper panel) and Mlp84B-deficient (lower panel) flies; bar, 10 μm. Double-headed arrows in each panel delineate one sarcomere, bounded by two Z-discs (electron-dense lines), with the M-lines in the middle (lighter line).

fragment. Mlp84B::GFP::NES is based on Mlp84B::GFP, but also contains the nuclear export sequence from PKI to promote Mlp84B::GFP nuclear exclusion. Mlp84B::GFP::NLS is a second derivative of Mlp84B::GFP, but has the large T-antigen nuclear localization sequence to promote nuclear enrichment of Mlp84B::GFP.

We first examined the localization of the three fusion proteins in an *mlp84B* mutant background to confirm that the proteins behaved as expected. The Mlp84B::GFP protein displayed strong Z-disc and faint nuclear localization in larval body wall muscle preparations (Fig. 4A). Addition of the nuclear export signal (NES) prevented nuclear accumulation of the Mlp84B::NES::GFP protein (compare Fig. 4A with Fig. 4B), whereas addition of the nuclear localization signal (NLS) sequence to Mlp84B::GFP resulted in strong nuclear and weak Z-disc localization (Fig. 4C). Addition of an NLS to vertebrate MLP results in potent myotube hypertrophy in culture, raising the possibility that our fusion proteins might behave in a

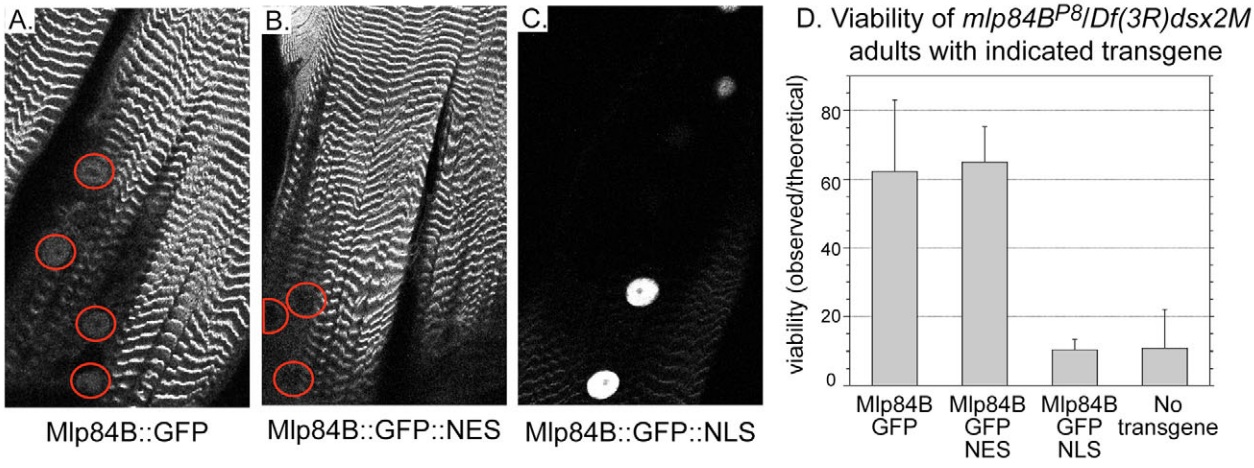

Fig. 4. Mlp84B does not have an essential nuclear function. (A-C) Two muscle fibers from transgenic third-instar larvae expressing either an (A) Mlp84B::GFP, (B) Mlp84B::GFP::NES or (C) Mlp84B::GFP::NLS fusion protein. Muscle nuclei in A and B are highlighted by red circles. Note the lack of detectable nuclear GFP signal in B relative to A and the strong nuclear signal in C. The three micrographs were recorded using the same confocal settings. (D) Quantification of rescue of the *mlp84B*^{P8} mutants by the three Mlp84B fusion proteins. The Mlp84B::GFP::NLS does not provide any appreciable rescue of pupal lethality in the *mlp84B*^{P8} mutants.

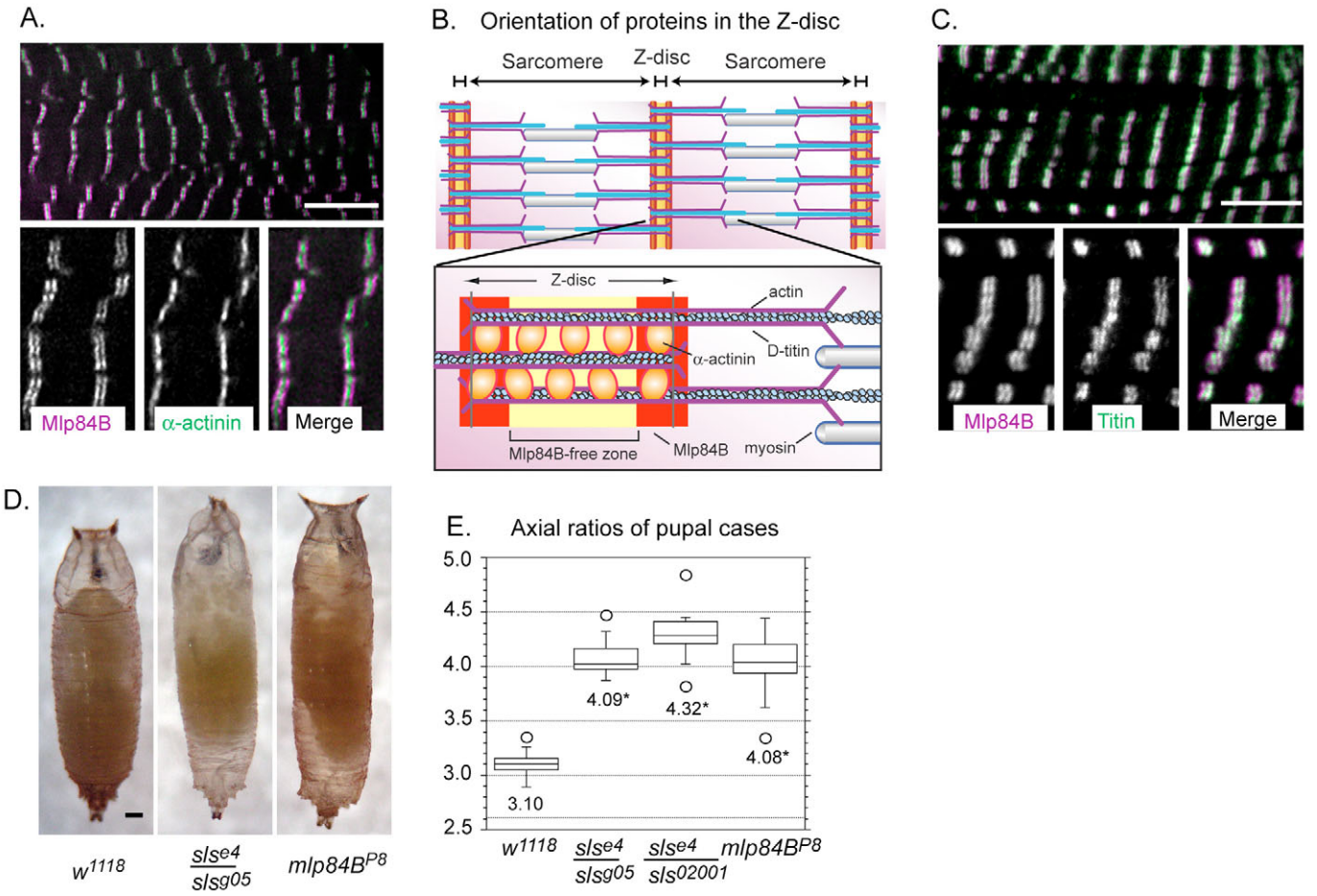

Fig. 5. Mlp84B and D-titin colocalize at the Z-disc and have similar mutant phenotypes. (A) Distribution of Mlp84B and α-actinin at the Z-disc of somatic body wall muscle; bar, 10 μm. Enlargements of two Z-bands are shown below the larger micrograph. (B) Diagram of the relative positions of Mlp84B, actin, α-actinin and D-titin in the Z-disc. (C) Colocalization of Mlp84B and D-titin in somatic muscle Z-disc; bar, 10 μm. Enlargements of two Z-bands are shown below the larger micrograph. (D) Representative pupal cases; bar, 200 μm. (E) Average axial ratios for pupae of indicated genotype (*P>10^{-6}, comparing mutant to w^{1118}).

dominant manner. However, visual inspection of myofibers from transgenic flies that also contained endogenous Mlp84B revealed no change in myofiber pattern or size in third-instar body wall muscle preparations (data not shown).

We next tested whether any of the fusion proteins could restore wild-type Mlp84B function to an *mlp84B* mutant. We found that both the Mlp84B::GFP and Mlp84B::GFP::NES fusion proteins showed comparable levels of restoration of adult viability to the *mlp84B^{P8}/Df(3R)dsx2M* flies (Fig. 4D), indicating that the Mlp84B::NES::GFP fusion protein retains the essential activities of Mlp84B. Conversely, NLS-tagged Mlp84B fails to rescue the lethal phenotype associated with *mlp84B* loss-of-function mutations, as equivalent numbers of mutant escaper adults were produced from crosses with and without the *mlp84B::GFP::NLS* transgene (Fig. 4D). NLS-tagged Mlp84B is concentrated prominently in cell nuclei, illustrating that nuclear Mlp84B is not sufficient to restore Mlp84B function in muscle. Although we cannot rule out the possibility that Mlp84B function requires transient nuclear localization, our analysis suggests that Mlp84B plays a key role at actin attachment sites in the cytoplasm.

Mlp84B and D-titin colocalize at the edge of the Z-disc

Given that our data strongly suggest that nuclear Mlp84B does not have an essential function, we focused on sarcomeric Mlp84B. In visceral muscle, Mlp84B localizes to the Z-disc boundary (Stronach et al., 1999), so we sought to better refine the subcellular distribution of Mlp84B in body wall muscle, where we see a distinct requirement for Mlp84B. By indirect immunofluorescence, Mlp84B is juxtaposed to the Z-disc in third-instar body wall muscle (Fig. 5A), and displays very limited colocalization with α-actinin. Mlp84B and α-actinin protein distributions marginally overlap at the Z-disc edge (white pixels in Fig. 5A merge), and there is a major zone devoid of Mlp84B in the center of the Z-disc (green pixels in Fig. 5A merge). Thus, Mlp84B is at the Z-disc periphery of both larval visceral and body wall muscle.

Muscle sarcomeres contain three main filament systems, the thin filaments (actin), the thick filaments (myosin) and connecting (titin) filaments (Fig. 5B). Both the actin thin filaments and titin filaments initiate at the Z-disc boundary where we see accumulation of Mlp84B. Titin is a large spring-like protein that aligns and connects the thick and thin filament

systems, providing elasticity to muscle. A *Drosophila* titin-like protein, D-titin, is encoded by the *sallimus* gene, and displays an orientation in the sarcomere similar to vertebrate titin, with its N-terminus in the Z-disc and the C-terminus associated with the thick filaments (Kulke et al., 2001). We confirmed that Mlp84B is indeed at the Z-disc periphery by double-labeling third-instar larval somatic muscle preparations with our Mlp84B serum and an N-terminal D-titin antibody (Fig. 5C). Although the Mlp84B zone (purple) is slightly wider than D-titin (green), the two signals are superimposable at the Z-disc periphery, producing a white doublet (Fig. 5C; there is also some faint non-specific D-titin signal in the I-band region produced by this antiserum). At high magnification, the coincidence of Mlp84B and D-titin is quite evident. Thus, the N-terminus of D-titin and Mlp84B colocalize at the Z-disc periphery in the muscle sarcomere.

Hypomorphic D-titin alleles show a phenotype similar to that of *mlp84B*

The presence of Mlp84B protein in the vicinity of the D-titin filament tip prompted us to look for a functional association between these two proteins. Strong loss-of-function *D-titin* mutants (*sallimus/sls*) die as embryos and display a severe disruption in muscle cytoarchitecture (Hakeda et al., 2000; Machado and Andrew, 2000; Zhang et al., 2000). Weaker *sls* mutants arrest at the pupal stage, but their detailed phenotypes have not been described. We generated different combinations of three hypomorphic *sls* alleles, and evaluated whether the mutant pupae display any phenotypes similar to *mlp84B* mutants. Both the sls^{e4}/sls^{g05} and sls^{e4}/sls^{02001} allelic combinations produce long, thin pupae, with striking similarity to what we observed for the *mlp84B* mutants (Fig. 5D). The average axial ratios for both mutant combinations was also in the range of that produced by the $mlp84B^{P8}$ mutant (Fig. 5E). Thus, perturbation of D-titin function can produce phenotypes similar to that seen for loss of Mlp84B.

Genetic interaction with D-titin reveals a role for Mlp84B in maintaining muscle structural integrity

Both the colocalization of Mlp84B and D-titin at the Z-disc, and the similarity in their mutant phenotypes, raised the possibility that these two proteins might participate in the same cellular process(es). To test this idea, we looked for modification of the *mlp84B* muscle phenotypes under conditions of decreased wild-type D-titin. We generated hemizygous *mlp84B* P-element mutants that were also heterozygous for sls^{j1D7}, a strong loss-of-function allele (Zhang et al., 2000). On their own, heterozygous sls^{j1D7} flies do not display any reduction in viability and are indistinguishable from wild-type flies. Elimination of one wild-type *sls* allele in the *mlp84B* mutant background greatly increased the adult lethality seen in the *mlp84B* P-element mutants (Fig. 6A). At 18°C, approximately 45% of the expected $mlp84B^{P8}$ hemizygotes eclose, but when these animals also carry one mutation in *sls*, less than 1% of the mutants eclose. Similar results are obtained with the $mlp84B^{P20}$ allele, confirming the specificity of the interaction (Fig. 6A).

Reducing D-titin function in the *mlp84B* mutant background also exacerbates the pupal case elongation associated with the *mlp84B* mutants. The average axial ratio of the $sls^{J1D7/+}$ $mlp84B^{P8}$ pupae is significantly larger than the $mlp84B^{P8}$ mutants on their own (4.44 versus 3.87, $P<0.0001$), indicating a more pronounced defect in muscle contraction during pupariation (Fig. 6B). This finding is recapitulated using the $mlp84B^{P20}$ allele, where we never obtain any $sls^{J1D7/+}$ $mlp84B^{P20}$ adults (Fig. 6B). The pronounced enhancement of abnormal pupation can also be seen by visually comparing the $mlp84B^{P8}$ hemizygous pupae with, and without, reduction in D-titin (Fig. 6C).

D-titin is crucial for both the formation and maintenance of muscle sarcomeres (Hakeda et al., 2000), and therefore we examined the muscle organization of third-instar larvae (just prior to puparium formation) for structural defects. The larvae were filleted and fixed, then labeled with fluorescent phalloidin to highlight the muscle fibers. Fillets of wild-type animals display an orderly arrangement of muscle fibers in each segment, and the individual fibers have a linear appearance (Fig. 6D). Muscle fibers from $mlp84B^{P8}$ hemizygotes resemble wild-type fibers at the light microscopic level, with no apparent disruption in structure or organization (Fig. 6E). By contrast, the $mlp84B^{P8}$ hemizygous mutants that also have one mutant copy of *D-titin* show a striking array of muscle phenotypes. Most fibers are wavy and/or spindly in appearance (Fig. 6F). A significant number of fibers have torn (Fig. 6F inset), or show some degree of fraying. Intriguingly, these muscle defects resemble those seen in a weak D-titin mutant (sls^{g05}/sls^{e3}; Fig. 6G). Neither the $mlp84B^{P8}$ hemizygotes (Fig. 6E), nor the sls^{J1D7} heterozygotes on their own (Fig. 6H), display any obvious structural abnormalities at the light microscopic level, indicating that the muscle defects result from the concomitant loss of Mlp84B and reduction of D-titin.

The muscle defects seen in the $mlp84B^{P8}$ hemizygous mutants that also have one mutant copy of *D-titin* could arise from improper muscle assembly during embryogenesis or destabilization of the larval muscle as it is used. To discern between these two possibilities, we compared somatic muscle morphology in late-stage $sls^{J1D7/+}$ $mlp84B^{P8}$ embryos with stage-matched wild-type embryos. The mutant embryos contained well-formed muscle fibers that were indistinguishable from wild type (Fig. 6I). Moreover, we did not see any evidence of muscle fusion defects, which would be manifested as unfused myoblasts in the vicinity of the muscle fibers. From this we can conclude that elimination of one wild-type *sls* allele in the $mlp84B^{P8}$ hemizygous background does not affect muscle differentiation or fusion events during embryogenesis. These observations indicate that the mutant muscle must deteriorate either gradually during larval development or once the larvae begin to wander during the late third-instar stage.

Closer examination of single muscle fibers from the third-instar fillet preparations revealed a normal striated appearance in wild type and $mlp84B^{P8}$ mutants (Fig. 6J). This is consistent with our ultrastructural analysis (Fig. 3C), showing intact myofibrillar organization in the *mlp84B* mutant flight muscle. By contrast, we observed a profound loss of sarcomeric structure in muscle fibers from the $mlp84B^{P8}$ hemizygotes that are also heterozygous for sls^{j1D7} (Fig. 6J, lower panel). The micrograph in Fig. 6J highlights the loss of the striated appearance and myofiber fraying often seen in the $sls^{j1D7/+}$ $mlp84B^{P8}$ fillet preparations. The loss of striation seen in this mutant combination is not simply because of tears or other fragmentation of the myofibers, because some intact muscle

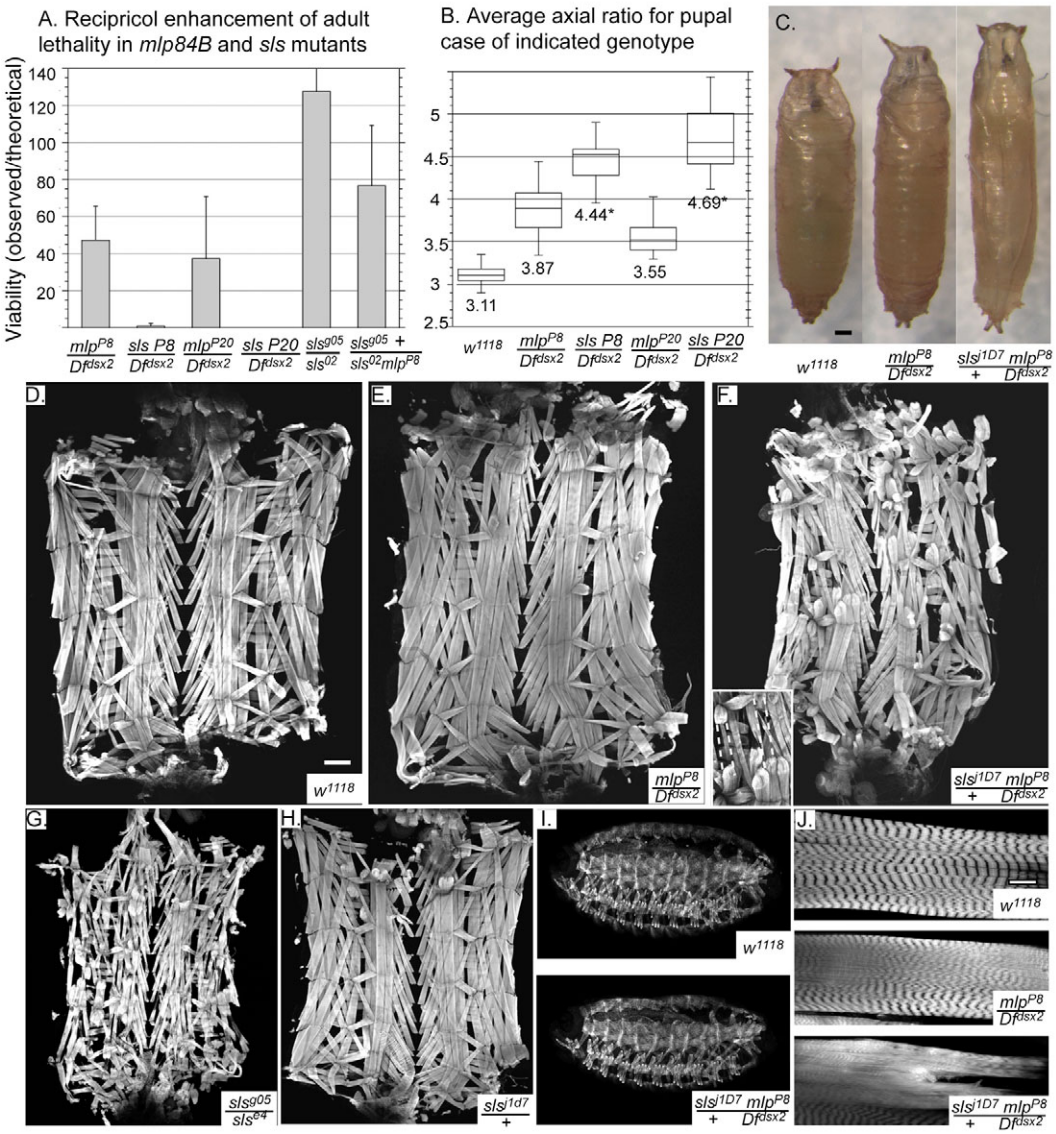

Fig. 6. Enhancement of *mlp84B* mutant phenotypes by reduction in D-titin activity. (A) Enhancement of pupal lethality at 18°C displayed by *mlp84B* P-element alleles and *sls* mutants. Numbers given are the percentage of the number of mutant adults divided by the expected number for the cross. (B) Average axial ratios for pupae of indicated genotype. (*$P>10^{-6}$, compared with the appropriate P-element allele alone.) (C) Representative pupae; bar, 200 μm. (D-H) Muscle fillets of third-instar larvae stained with Alexa Fluor-488 phalloidin. (D) Wild type; bar, 200 μm. (E) $mlp84B^{P8}/Df(3R)dsx2M$. (F) $sls^{j1D7}mlp84B^{P8}/Df(3R)dsx2M$; inset highlights torn muscle fibers. (G) sls^{g05}/sls^{e4}. (H) sls^{j1D7}/+. (I) Late-stage embryos labeled with D-titin antiserum to highlight muscle fiber morphology. (J) Single muscle fibers from third-instar larvae stained with Alexa Fluor-488 phalloidin: wild type (upper panel), $mlp84B^{P8}/Df(3R)dsx2M$ (middle panel), $sls^{j1D7}mlp84B^{P8}/Df(3R)dsx2M$ (lower panel); bar, 20 μm.

fibers also lacked the striations typical of wild-type body wall muscle (data not shown). All together, these phenotypes indicate a defect in maintaining muscle integrity, and suggest the existence of a titin-Mlp84B stabilizing complex required to maintain both sarcomeric structure and overall muscle cell integrity.

Given the striking genetic interaction between Mlp84B and titin described above, we reasoned that we might see reciprocal enhancement of *D-titin* phenotypes by concomitant reduction in *mlp84B* gene dosage. The sls^{g05}/sls^{02001} allelic combination does not affect adult viability (Fig. 6A) and does not have a significant pupariation defect, and thus represents a very weak *D-titin* mutant. We tested whether reduction of *mlp84B* gene dosage in this background would produce any lethality. Reducing Mlp84B activity in the *D-titin* mutant background led to a significant reduction in viable *D-titin* mutant adults (Fig. 6A), further indicating a functional interaction between Mlp84B and D-titin. Thus, the genetic interaction between *mlp84B* and *D-titin* is reciprocal – reducing D-titin activity in an *mlp84B* mutant background leads to a stronger phenotype and reducing Mlp84B activity in a *D-titin* mutant background similarly exacerbates the *D-titin* phenotype.

We next explored whether the genetic interaction between *mlp84B* and *D-titin* was specific. *Samba* mutants are

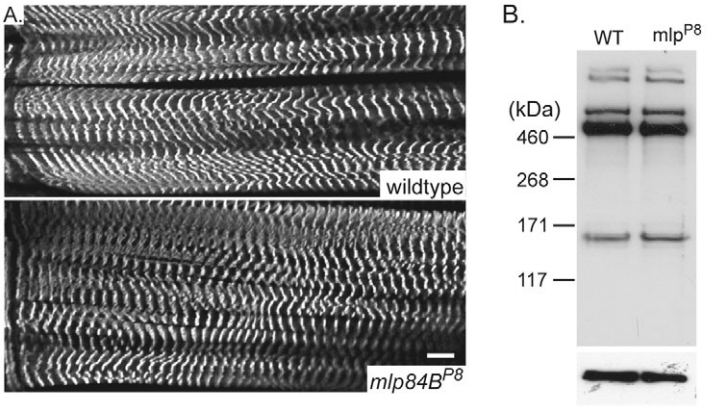

Fig. 7. Loss of Mlp84B does not affect D-titin protein localization or levels. (A) Muscle fibers from wild type (upper panel) or *mlp84B*^{P8} (lower panel) labeled with the N-terminal D-titin antibody; bar, 20 μm. (B) Western blot of protein lysates from wild-type and *mlp84B*^{P8} adults. Each lane contains protein from one-quarter of a thorax. An anti-LaminC antibody serves as a loading control (lower panel).

dominant mutations in the myosin heavy chain gene that destabilize the flight muscle, leading to severe muscle tearing (Montana and Littleton, 2004). Recessive mutations in *upheld* (*up*), which encodes Troponin T, produce a flight muscle phenotype similar to that seen in the *Samba* mutants. The heterozygous *Samba* mutant and the homozygous *up*^{101} mutant exhibit synthetic lethality with each other and display genetic interactions with other sarcomeric components (Homyk, Jr and Emerson, Jr, 1988; Homyk, Jr et al., 1980; Montana and Littleton, 2004; Nongthomba et al., 2003), making them excellent candidates to test the specificity of the *mlp84B-D-titin* interaction. *Samba*^{1}/+; *mlp84B*^{P8}/+ animals are viable and phenotypically indistinguishable from *Samba*^{1}/+ flies. In particular, whereas both *Samba*^{1} homozygous escapers and *up*^{101}/+; *Samba*^{1}/+ animals have hypercontracted femurs, the *Samba*^{1}/+; *mlp84B*^{P8}/+ adults have a normal femur, but still have an indented thorax characteristic of the *Samba*^{1}/+ flies. Consistent with the *Samba* results, we did not observe any modification of muscle phenotypes or any synthetic lethality in the *up*^{101}; *mlp84B*^{P8}/+ flies. These findings are consistent with the view that *mlp84B* displays a specific genetic interaction with *D-titin* and suggest that Mlp84B and D-titin proteins cooperate, either directly or indirectly, to maintain muscle structural integrity.

The muscle failure in the *mlp84B* mutants does not result from defects in D-titin distribution or levels

Because D-titin and Mlp84B colocalize, and both *sls* and *mlp84B* mutants display defects in muscle function, we considered the possibility that loss of Mlp84B somehow impacts D-titin function. To explore whether Mlp84B is required to stabilize D-titin protein or maintain its subcellular localization, we examined D-titin localization in the *mlp84B*$^{-/-}$ mutant muscles. No difference in localization or relative fluorescence intensity was detected in the *mlp84B*$^{-/-}$ muscle labeled with an N-terminal D-titin antiserum (Fig. 7A), indicating that D-titin is localized normally in the *mlp84B* mutant muscle. Consequently, the *mlp84B* mutant phenotype cannot be attributed simply to disturbed titin distribution.

We next investigated whether loss of Mlp84B resulted in decreased D-titin protein levels by western blot analysis on protein lysates from the *mlp84B*$^{-/-}$ adult escapers. As reported previously (Kulke et al., 2001), western immunoblot reveals the presence of at least four different D-titin isoforms in adult flight muscle (Fig. 7B), including the predominant D-titin flight muscle isoform (Kettin) at approximately 500 kDa (Kolmerer et al., 2000). No appreciable difference in the steady-state levels of any detectable D-titin isoform is observed when comparing *mlp84B* mutant and wild-type fly lysates. Thus, elimination of Mlp84B does not result in destabilization of D-titin.

Discussion

Our analysis of the *mlp84B* mutants revealed that Mlp84B is crucial for normal muscle function, and this resulting muscle failure is responsible for the pupal lethality seen in the mutants. Flies that lack Mlp84B arrest during pupal development and display feeble muscle contractions. Neither *mlp84B* homozygous mutants nor titin heterozygous mutants display any obvious morphological muscle defects, whereas muscles of *mlp84B* mutants with reduced D-titin function are greatly destabilized, revealing a role for Mlp84B in maintaining muscle integrity.

Our experimental observations support a model in which Mlp84B and D-titin are involved in the stabilization of muscle sarcomeres. First, Mlp84B and D-titin colocalize at the boundary of the Z-disc. Because D-titin spans at least half the sarcomere, it is not surprising that Mlp84B colocalizes with a region of titin. However, Mlp84B resides in a specialized region of the Z-disc where the D-titin and actin filaments originate. Given this localization, Mlp84B could cap the titin or thin filaments. Indeed, vertebrate MLP directly binds the titin-capping protein T-cap/telethonin (Gregorio et al., 1998; Knoll et al., 2002), and *Drosophila* lacks an obvious T-cap homolog. Second, the phenotypes produced by loss of Mlp84B resemble those displayed in weak loss-of-function *D-titin* mutants. Both mutants produce a long, thin pupal case that results from a failure in muscle contraction during pupariation. Although a small number of other mutants also produce this phenotype (*cryptocephalic*, *tiggrin*, *l(2)thin*, *cactus*), except for *tiggrin*, their direct link to muscle function, if any, is not established. Intriguingly, we found that a *l(2)thin* mutation does not act as a dominant enhancer of *mlp84B* (K.A.C., J.M.B. and M.C.B., unpublished), providing additional evidence for the specificity of the *mlp84B-D-titin* interaction, Finally, reducing D-titin in the *mlp84B* mutant background exacerbates the muscle defects that would be expected based on loss of Mlp84B alone. We found that the enhanced lethality of the *mlp84B* mutants that were heterozygous for a *D-titin* mutation

is not because of mislocalization or instability of D-titin. Thus, Mlp84B does not appear to be acting simply by tethering or stabilizing D-titin at the Z-disc.

How does Mlp84B contribute to the maintenance of muscle cytoarchitecture? Mlp84B could influence muscle structure because it is linked, either directly or indirectly, to actin, α-actinin and titin (Louis et al., 1997; Flick and Konieczny, 2000; Knoll et al., 2002; Grubinger and Gimona, 2004), the major structural components of the Z-line. Although we do not see any morphological abnormalities in the *mlp84B* mutant muscles on their own, reduction of D-titin in the *mlp84B* background leads to structural destabilization of the muscle. It is possible that loss of Mlp84B perturbs muscle structure in ways that are not in evidence even by electron microscopy or that loss of Mlp84B may compromise essential sarcomeric functions without severely disrupting muscle structure. Further assault on muscle integrity by reduction of D-titin levels in an *mlp84B* mutant background appears to place additional strain on the sarcomere, leading to the structural destabilization we observe in the $D\text{-}titin^{+/-}$ $mlp84B^{-/-}$ muscles.

It is also possible that Mlp84B contributes to muscle stretch mechanics and/or signaling. Murine cardiomyocytes lacking MLP have altered mechanics that are attributed to changes in muscle elasticity (Knoll et al., 2002; Omens et al., 2002). It is well-established that titin is a major contributor to muscle elasticity (reviewed by Granzier and Labeit, 2002). Alterations in the elastic properties of titin change both muscle mechanics and myofilament alignment, and thus have profound effects on muscle elasticity, function and stabilization (Hein et al., 2002). It is intriguing to speculate that Mlp84B could modulate the extensibility of D-titin, leading to changes in muscle compliancy and compromised muscle function.

Changes in muscle load lead to alterations in muscle gene expression and mechanical properties (Goldspink et al., 2002; Fluck, 2006) and it is predicted that deficits in stretch sensation or mechanotransduction would lead to contractility defects. Both MLP and titin have been implicated in the muscle response to mechanical stress. In the case of vertebrate MLP, it has been demonstrated that isolated cardiomyocytes subjected to a cyclic stretch regimen launch a specific transcriptional response that is abrogated in MLP-null cells (Knoll et al., 2002). Because of its elastic properties, titin is also proposed to be part of the stretch-sensing machinery crucial for fine-tuning cardiac performance (Miller et al., 2004). Moreover, changes in the conformation of titin lead to differential association with signaling molecules, and activation of muscle gene transcription through Serum response factor (Lange et al., 2005).

Recent data have also suggested a role for MLP in muscle repair. Murine MLP mRNA levels are dramatically increased during eccentric contraction, a physiological state in which muscles are stretched while activated (Barash et al., 2004). This type of muscle use results in structural damage, which is subsequently repaired, resulting in muscle hypertrophy. $MLP^{-/-}$ mice show a slight lag in recovery of muscle function after eccentric exercise, as compared with their littermates (Barash et al., 2005). A muscle damage response may exist in *Drosophila* and involve the fly MLP homologs. Specific mutations in myosin heavy chain, troponins, actin and other contractile proteins can lead to hypercontraction of the flight muscle (Nongthomba et al., 2003). These mutant flight muscles experience a lot of strain and usually tear, resulting in muscle damage (Naimi et al., 2001; Nongthomba et al., 2003; Montana and Littleton, 2004). Transcriptional profiling of two hypercontraction mutants revealed an upregulation of several actin-associated muscle proteins, suggesting the existence of a cytoskeletal remodeling program in injured flight muscle, perhaps similar to the remodeling program in damaged cardiomyocytes (Montana and Littleton, 2006). Intriguingly, *mlp84B*, *mlp60A* and *D-titin* transcripts are upregulated in these mutants, suggesting that the *mlp* and *D-titin* promoters are coresponsive to an undefined muscle damage signal. Mlp84B and D-titin are positioned to act together, either as part of the repair machinery or to stabilize the muscle during the repair process. A role for Mlp84B and D-titin in the muscle damage response and/or muscle repair is consistent with the phenotypes we observe in the mutant animals. Our findings suggest a new explanation for the mechanism by which mutations in human MLP might contribute to cardiac hypertrophy (Geier et al., 2003) or dilated cardiomyopathy (Knoll et al., 2002). We predict that further studies of Mlp84B in *Drosophila* should translate into our understanding of vertebrate MLP, and provide much-needed insight into pathology of cardiac disease.

Materials and Methods

Drosophila stocks and genetics

All stocks were reared under standard laboratory conditions at 25°C, with exceptions noted in the text. Strain w^{1118} served as the wild-type control in all experiments, except where noted. Generation of transgenic flies was done using established techniques (Spradling, 1986). Lethal-phase analysis of the *mlp84B* mutants was performed by collection of embryos from GFP-balanced parental lines, which were then placed on fresh apple juice plates, and animals were assessed daily for any lethality. GFP was used to distinguish mutants (GFP–) from their balanced siblings (GFP+). Adult viability was calculated as the percentage of the number of mutant adults divided by the expected number for the cross (total number of adults/3). Some of the rescued lines gave higher than expected numbers of rescued mutant adults, presumably because of their ability to out compete their balanced siblings for survival. At least 10 independent crosses were performed for each genotype, with the total number of adults scored in excess of 1000.

Antibody production, affinity purification and immunodetection of proteins

A rabbit polyclonal antiserum (B54) was generated against a 20 amino acid peptide (CLRENGDVPSVRNEARLEPR) corresponding to residues (91-109) of the predicted Mlp84B protein. The serum was affinity purified against the peptide using standard procedures (Pierce). Fillet and fixation of third-instar larvae were performed as described in Stronach et al. (Stronach et al., 1999); fillets depicted in Fig. 4 were fixed in –20°C methanol for 10 minutes. The following antibodies were used at the concentrations indicated: mouse anti-LaminC (LCD 28.26) (Riemer et al., 1995) (Developmental Studies Hybridoma Bank) 1:20; rabbit anti-Mlp84B (B50) (Stronach et al., 1996) 1:500; rabbit anti-Mlp84B (B54) (this study) 1:100; rabbit anti-FLAG (B43) (in-house) 1:80; rat anti-α-actinin (Technix) 1:10,000; rat anti-D-titin (Machado et al., 1998) 1:5000. Fluorescently labeled secondary antibodies were purchased from Molecular Probes and used at 1:200. Fluorescent images were obtained from an Olympus FV300 confocal microscope and processed using Adobe Photoshop 7.0.

Pupal morphogenesis analysis

Still images of pupae were collected on an Olympus SZX12 dissection microscope with an Olympus C-5050Z camera and processed with ImageJ and Photoshop 7.0. Axial ratios were calculated by measuring the length of the pupae and dividing by the width (measured at the exact middle of the pupae), with n>20. Statistical analysis of the axial ratios was performed using KaleidaGraph (Synergy Software). Scanning electron microscopy of pupae was performed on an S2460N Hitachi scanning electron microscope. Video analysis of developing pupae was performed using an SZX12 dissection microscope with an Olympus OLY-105 video camera and captured directly to videotape. Videotape was digitized and edited using iMovie.

Molecular biology

The *mlp84B* transgene consists of a 7 kb genomic fragment containing the entire *mlp84B* transcription unit, plus 2 kb of upstream sequence and 1.5 kb of downstream sequence. The DNA outside the *mlp84B* transcription unit is not predicted to encode for any other protein, thus the transgene should provide back functional Mlp84B protein, and no other gene product. Mlp84B protein produced from the transgene displays the same spatial and temporal expression as the native protein (data not shown) (Stronach et al., 1996). To generate the GFP transgenes, a *Bam*HI site was engineered just before the stop codon of the Mlp84B open reading frame (ORF). The GFP ORF was amplified by PCR using primers with *Bam*HI sites in the 5′-end to allow for cloning into the introduced *Bam*HI site in the *mlp84B* transgene.

Transmission electron microscopy

Fixation and preparation of thin sections of *Drosophila* thorax for electron microscopy were performed essentially as described in Cripps et al. (Cripps et al., 1999). Four-day-old adults were used. Embedded thorax was stained en bloc in uranyl acetate, and thin sections were post-stained with uranyl acetate and Reynold's lead citrate. Images from a Hitachi H-7100 were captured to film. Individual pictures were scanned and processed in Photoshop 7.0 for assembly into figures.

We thank Deborah Andrews for the D-titin antiserum and the Bloomington Stock Center provided fly strains. We are especially grateful to Patricia Renfranz for her early participation on this project, and Diana Lim for assistance with figure preparation. Indirect immunofluorescent images were obtained at the University of Utah School of Medicine Cell Imaging Facility. We thank the University of Utah electron microscopy facility, and Nancy Chandler and Joel Mancuso for assistance with electron microscopy. This work was funded by an NIH grant (HL60591-08) to M.C.B., and grants from the American Heart Association and Muscular Dystrophy Association to K.A.C.

References

Arber, S., Halder, G. and Caroni, P. (1994). Muscle LIM protein, a novel essential regulator of myogenesis, promotes myogenic differentiation. *Cell* **79**, 221-231.

Arber, S., Hunter, J. J., Ross, J., Jr, Hongo, M., Sansig, G., Borg, J., Perriard, J. C., Chien, K. R. and Caroni, P. (1997). MLP-deficient mice exhibit a disruption of cardiac cytoarchitectural organization, dilated cardiomyopathy, and heart failure. *Cell* **88**, 393-403.

Baker, B. S. and Wolfner, M. F. (1988). A molecular analysis of doublesex, a bifunctional gene that controls both male and female sexual differentiation in Drosophila melanogaster. *Genes Dev.* **2**, 477-489.

Barash, I. A., Mathew, L., Ryan, A. F., Chen, J. and Lieber, R. L. (2004). Rapid muscle-specific gene expression changes after a single bout of eccentric contractions in the mouse. *Am. J. Physiol. Cell Physiol.* **286**, C355-C364.

Barash, I. A., Mathew, L., Lahey, M., Greaser, M. L. and Lieber, R. L. (2005). Muscle LIM protein plays both structural and functional roles in skeletal muscle. *Am. J. Physiol. Cell Physiol.* **289**, C1312-C1320.

Bernstein, S. I., O'Donnell, P. T. and Cripps, R. M. (1993). Molecular genetic analysis of muscle development, structure, and function in Drosophila. *Int. Rev. Cytol.* **143**, 63-152.

Bodenstein, D. (1950). The postembryonic development of Drosophila. In *Biology of Drosophila* (ed. M. Demerec), pp. 275-367. New York: John Wiley.

Bos, J. M., Poley, R. N., Ny, M., Tester, D. J., Xu, X., Vatta, M., Towbin, J. A., Gersh, B. J., Ommen, S. R. and Ackerman, M. J. (2006). Genotype-phenotype relationships involving hypertrophic cardiomyopathy-associated mutations in titin, muscle LIM protein, and telethonin. *Mol. Genet. Metab.* **88**, 78-85.

Cavener, D., Corbett, G., Cox, D. and Whetten, R. (1986a). Isolation of the eclosion gene cluster and the developmental expression of the Gld gene in Drosophila melanogaster. *EMBO J.* **5**, 2939-2948.

Cavener, D. R., Otteson, D. C. and Kaufman, T. C. (1986b). A rehabilitation of the genetic map of the 84B-D region in Drosophila melanogaster. *Genetics* **114**, 111-123.

Chang, D. F., Belaguli, N. S., Iyer, D., Roberts, W. B., Wu, S. P., Dong, X. R., Marx, J. G., Moore, M. S., Beckerle, M. C., Majesky, M. W. et al. (2003). Cysteine-rich LIM-only proteins CRP1 and CRP2 are potent smooth muscle differentiation cofactors. *Dev. Cell* **4**, 107-118.

Clark, K. A., McElhinny, A. S., Beckerle, M. C. and Gregorio, C. C. (2002). Striated muscle cytoarchitecture: an intricate web of form and function. *Annu. Rev. Cell Dev. Biol.* **18**, 637-706.

Cripps, R. M., Suggs, J. A. and Bernstein, S. I. (1999). Assembly of thick filaments and myofibrils occurs in the absence of the myosin head. *EMBO J.* **18**, 1793-1804.

Ehler, E., Horowits, R., Zuppinger, C., Price, R. L., Perriard, E., Leu, M., Caroni, P., Sussman, M., Eppenberger, H. M. and Perriard, J. C. (2001). Alterations at the intercalated disk associated with the absence of muscle LIM protein. *J. Cell Biol.* **153**, 763-772.

Flick, M. J. and Konieczny, S. F. (2000). The muscle regulatory and structural protein MLP is a cytoskeletal binding partner of betaI-spectrin. *J. Cell Sci.* **113**, 1553-1564.

Fluck, M. (2006). Functional, structural and molecular plasticity of mammalian skeletal muscle in response to exercise stimuli. *J. Exp. Biol.* **209**, 2239-2248.

Fyrberg, E., Kelly, M., Ball, E., Fyrberg, C. and Reedy, M. C. (1990). Molecular genetics of Drosophila alpha-actinin: mutant alleles disrupt Z disc integrity and muscle insertions. *J. Cell Biol.* **110**, 1999-2011.

Geier, C., Perrot, A., Ozcelik, C., Binner, P., Counsell, D., Hoffmann, K., Pilz, B., Martiniak, Y., Gehmlich, K., van der Ven, P. F. et al. (2003). Mutations in the human muscle LIM protein gene in families with hypertrophic cardiomyopathy. *Circulation* **107**, 1390-1395.

Goldspink, G., Williams, P. and Simpson, H. (2002). Gene expression in response to muscle stretch. *Clin. Orthop. Relat. Res.* S146-S152.

Granzier, H. and Labeit, S. (2002). Cardiac titin: an adjustable multi-functional spring. *J. Physiol.* **541**, 335-342.

Gregorio, C. C., Trombitas, K., Centner, T., Kolmerer, B., Stier, G., Kunke, K., Suzuki, K., Obermayr, F., Herrmann, B., Granzier, H. et al. (1998). The NH2 terminus of titin spans the Z-disc: its interaction with a novel 19-kD ligand (T-cap) is required for sarcomeric integrity. *J. Cell Biol.* **143**, 1013-1027.

Grubinger, M. and Gimona, M. (2004). CRP2 is an autonomous actin-binding protein. *FEBS Lett.* **557**, 88-92.

Hakeda, S., Endo, S. and Saigo, K. (2000). Requirements of Kettin, a giant muscle protein highly conserved in overall structure in evolution, for normal muscle function, viability, and flight activity of Drosophila. *J. Cell Biol.* **148**, 101-114.

Harper, B. D., Beckerle, M. C. and Pomies, P. (2000). Fine mapping of the alpha-actinin binding site within cysteine-rich protein. *Biochem. J.* **350**, 269-274.

Hein, S., Gaasch, W. H. and Schaper, J. (2002). Giant molecule titin and myocardial stiffness. *Circulation* **106**, 1302-1304.

Homyk, T., Jr and Emerson, C. P., Jr (1988). Functional interactions between unlinked muscle genes within haploinsufficient regions of the Drosophila genome. *Genetics* **119**, 105-121.

Homyk, T., Jr, Szidonya, J. and Suzuki, D. T. (1980). Behavioral mutants of Drosophila melanogaster. III. Isolation and mapping of mutations by direct visual observations of behavioral phenotypes. *Mol. Gen. Genet.* **177**, 553-565.

Kadrmas, J. L. and Beckerle, M. C. (2004). The LIM domain: from the cytoskeleton to the nucleus. *Nat. Rev. Mol. Cell Biol.* **5**, 920-931.

Knoll, R., Hoshijima, M., Hoffman, H. M., Person, V., Lorenzen-Schmidt, I., Bang, M. L., Hayashi, T., Shiga, N., Yasukawa, H., Schaper, W. et al. (2002). The cardiac mechanical stretch sensor machinery involves a Z disc complex that is defective in a subset of human dilated cardiomyopathy. *Cell* **111**, 943-955.

Kolmerer, B., Clayton, J., Benes, V., Allen, T., Ferguson, C., Leonard, K., Weber, U., Knekt, M., Ansorge, W., Labeit, S. et al. (2000). Sequence and expression of the kettin gene in Drosophila melanogaster and Caenorhabditis elegans. *J. Mol. Biol.* **296**, 435-448.

Kong, Y., Flick, M. J., Kudla, A. J. and Konieczny, S. F. (1997). Muscle LIM protein promotes myogenesis by enhancing the activity of MyoD. *Mol. Cell. Biol.* **17**, 4750-4760.

Kulke, M., Neagoe, C., Kolmerer, B., Minajeva, A., Hinssen, H., Bullard, B. and Linke, W. A. (2001). Kettin, a major source of myofibrillar stiffness in Drosophila indirect flight muscle. *J. Cell Biol.* **154**, 1045-1057.

Lange, S., Xiang, F., Yakovenko, A., Vihola, A., Hackman, P., Rostkova, E., Kristensen, J., Brandmeier, B., Franzen, G., Hedberg, B. et al. (2005). The kinase domain of titin controls muscle gene expression and protein turnover. *Science* **308**, 1599-1603.

Liebhaber, S. A., Emery, J. G., Urbanek, M., Wang, X. K. and Cooke, N. E. (1990). Characterization of a human cDNA encoding a widely expressed and highly conserved cysteine-rich protein with an unusual zinc-finger motif. *Nucleic Acids Res.* **18**, 3871-3879.

Louis, H. A., Pino, J. D., Schmeichel, K. L., Pomies, P. and Beckerle, M. C. (1997). Comparison of three members of the cysteine-rich protein family reveals functional conservation and divergent patterns of gene expression. *J. Biol. Chem.* **272**, 27484-27491.

Machado, C. and Andrew, D. J. (2000). D-Titin: a giant protein with dual roles in chromosomes and muscles. *J. Cell Biol.* **151**, 639-652.

Machado, C., Sunkel, C. E. and Andrew, D. J. (1998). Human autoantibodies reveal titin as a chromosomal protein. *J. Cell Biol.* **141**, 321-333.

Miller, M. K., Granzier, H., Ehler, E. and Gregorio, C. C. (2004). The sensitive giant: the role of titin-based stretch sensing complexes in the heart. *Trends Cell Biol.* **14**, 119-126.

Mohapatra, B., Jimenez, S., Lin, J. H., Bowles, K. R., Coveler, K. J., Marx, J. G., Chrisco, M. A., Murphy, R. T., Lurie, P. R., Schwartz, R. J. et al. (2003). Mutations in the muscle LIM protein and alpha-actinin-2 genes in dilated cardiomyopathy and endocardial fibroelastosis. *Mol. Genet. Metab.* **80**, 207-215.

Montana, E. S. and Littleton, J. T. (2004). Characterization of a hypercontraction-induced myopathy in Drosophila caused by mutations in Mhc. *J. Cell Biol.* **164**, 1045-1054.

Montana, E. S. and Littleton, J. T. (2006). Expression profiling of a hypercontraction-induced myopathy in Drosophila suggests a compensatory cytoskeletal remodeling response. *J. Biol. Chem.* **281**, 8100-8109.

Naimi, B., Harrison, A., Cummins, M., Nongthomba, U., Clark, S., Canal, I., Ferrus, A. and Sparrow, J. C. (2001). A tropomyosin-2 mutation suppresses a troponin I myopathy in Drosophila. *Mol. Biol. Cell* **12**, 1529-1539.

Nongthomba, U., Cummins, M., Clark, S., Vigoreaux, J. O. and Sparrow, J. C. (2003). Suppression of muscle hypercontraction by mutations in the myosin heavy chain gene of Drosophila melanogaster. *Genetics* **164**, 209-222.

Omens, J. H., Usyk, T. P., Li, Z. and McCulloch, A. D. (2002). Muscle LIM protein

deficiency leads to alterations in passive ventricular mechanics. *Am. J. Physiol. Heart Circ. Physiol.* **282**, H680-H687.

Perez-Alvarado, G. C., Miles, C., Michelsen, J. W., Louis, H. A., Winge, D. R., Beckerle, M. C. and Summers, M. F. (1994). Structure of the carboxy-terminal LIM domain from the cysteine rich protein CRP. *Nat. Struct. Biol.* **1**, 388-398.

Perrin, L. and Dura, J. M. (2004). Molecular genetics of the Alhambra (Drosophila AF10) complex locus of Drosophila. *Mol. Genet. Genomics* **272**, 156-161.

Pomies, P., Louis, H. A. and Beckerle, M. C. (1997). CRP1, a LIM domain protein implicated in muscle differentiation, interacts with alpha-actinin. *J. Cell Biol.* **139**, 157-168.

Riemer, D., Stuurman, N., Berrios, M., Hunter, C., Fisher, P. A. and Weber, K. (1995). Expression of Drosophila lamin C is developmentally regulated: analogies with vertebrate A-type lamins. *J. Cell Sci.* **108**, 3189-3198.

Sadler, I., Crawford, A. W., Michelsen, J. W. and Beckerle, M. C. (1992). Zyxin and cCRP: two interactive LIM domain proteins associated with the cytoskeleton. *J. Cell Biol.* **119**, 1573-1587.

Schmeichel, K. L. and Beckerle, M. C. (1994). The LIM domain is a modular protein-binding interface. *Cell* **79**, 211-219.

Seidman, J. G. and Seidman, C. (2001). The genetic basis for cardiomyopathy: from mutation identification to mechanistic paradigms. *Cell* **104**, 557-567.

Spradling, A. C. (1986). P element-mediated transformation. In *Drosophila: A Practical Approach* (ed. D. B. Roberts), pp. 175-199. Oxford: IRL Press.

Stronach, B. E., Siegrist, S. E. and Beckerle, M. C. (1996). Two muscle-specific LIM proteins in Drosophila. *J. Cell Biol.* **134**, 1179-1195.

Stronach, B. E., Renfranz, P. J., Lilly, B. and Beckerle, M. C. (1999). Muscle LIM proteins are associated with muscle sarcomeres and require dMEF2 for their expression during Drosophila myogenesis. *Mol. Biol. Cell* **10**, 2329-2342.

Theis, J. L., Bos, J. M., Bartleson, V. B., Will, M. L., Binder, J., Vatta, M., Towbin, J. A., Gersh, B. J., Ommen, S. R. and Ackerman, M. J. (2006). Echocardiographic-determined septal morphology in Z-disc hypertrophic cardiomyopathy. *Biochem. Biophys. Res. Commun.* **351**, 896-902.

Weiskirchen, R. and Gunther, K. (2003). The CRP/MLP/TLP family of LIM domain proteins: acting by connecting. *BioEssays* **25**, 152-162.

Zhang, Y., Featherstone, D., Davis, W., Rushton, E. and Broadie, K. (2000). Drosophila D-titin is required for myoblast fusion and skeletal muscle striation. *J. Cell Sci.* **113**, 3103-3115.