The Journal of Experimental Biology 211, 15-23
Published by The Company of Biologists 2008
doi:10.1242/jeb.012435

The *Drosophila* muscle LIM protein, Mlp84B, is essential for cardiac function

Annabelle Mery[1], Ouarda Taghli-Lamallem[1], Kathleen A. Clark[2], Mary C. Beckerle[2], Xiushan Wu[3], Karen Ocorr[1] and Rolf Bodmer[1,*]

[1]Development and Aging Program, Neuroscience, Aging and Stem Cell Research Center, Burnham Institute for Medical Research, 10901 North Torrey Pines Road, La Jolla, CA 92037, USA, [2]Hunstman Cancer Institute, University of Utah, Salt Lake City, UT 84112, USA and [3]Center for Heart Development, College of Life Science, Hunan Normal University, Changsha 410081, Hunan Province, People's Republic of China

*Author for correspondence (e-mail: rolf@burnham.org)

Accepted 16 October 2007

SUMMARY

Muscle LIM protein (MLP) is a cytoskeletal protein located at the Z-disc of sarcomeres. Mutations in the human *MLP* gene are associated with hypertrophic and dilated cardiomyopathy. MLP has been proposed to be a key player in the stretch-sensing response, but the molecular mechanisms underlying its function in normal and diseased cardiac muscle have not been established. A *Drosophila* homolog, Mlp84B, displays a similar subcellular localization at the Z-disc of sarcomeres throughout development and in the adult, suggesting *Drosophila* as a model to study MLP function. Here we employed genetic ablation and cardiac-specific RNA interference (RNAi) knockdown of *mlp84B* to investigate its role in heart function. We found that Mlp84B-deficient or heart-specific RNAi knockdown flies exhibit diastolic interval prolongation, heart rhythm abnormalities and a reduced lifespan, while showing no obvious structural phenotype. Our data demonstrate that Mlp84B is essential for normal cardiac function and establish the *Drosophila* model for the investigation of the mechanisms connecting defective cardiac Z-disc components to the development of cardiomyopathy.

Supplementary material available online at http://jeb.biologists.org/cgi/content/full/211/1/15/DC1

Key words: *Drosophila*, muscle LIM protein, cardiomyopathy, cardiac function, arrhythmia, cytoskeleton, sarcomere.

INTRODUCTION

Hypertrophic and dilated cardiomyopathies are major causes of human heart failure and mortality. Familial forms of these pathologies have led to investigation of the genetic causes, and there is a growing body of evidence that suggests a role for mutations in cardiac cytoskeletal genes (Chien, 2000; Seidman and Seidman, 2001). Cytoskeletal components located in the Z-disc have previously been recognized as critical for normal cardiac function. The Z-discs define the lateral boundaries of sarcomeres and constitute anchoring sites for actin and titin filaments, crosslinked by α-actinin. It is now well established that Z-disc components are essential for mechanical stretch sensing and sarcomere integrity (Clark et al., 2002). Loss or mutation of Z-disc proteins such as α-actinin-associated LIM protein (Pashmforoush et al., 2001), Cypher/ZASP (Vatta et al., 2003; Zhou et al., 2001), muscle LIM protein/CRP3 (Arber et al., 1997; Geier et al., 2003; Knöll et al., 2002; Mohapatra et al., 2003), titin (Gerull et al., 2002) and Tcap, also known as telethonin (Hayashi et al., 2004; Moreira et al., 2000), all lead to hypertrophic or dilated cardiomyopathy in mouse models as well as in human patients.

Loss of *Drosophila* MLP (Mlp84B) leads to developmental arrest at the pupal stage. Specifically, the muscle-dependent morphogenetic movements necessary for pupation are severely compromised in Mlp84B mutants (Clark et al., 2007). These defects may be explained by muscle weakness, and the observation that the few *mlp84B*$^{-/-}$ adult escapers fail in a flight test is consistent with this hypothesis (Clark et al., 2007). However, the cause of lethality remains unclear. Mlp84B shares many features with vertebrate MLPs including muscle-specific expression and localization at the Z-disc I-band interface (Stronach et al., 1999). Since vertebrate studies have demonstrated a critical requirement for MLP in cardiac function (Arber et al., 1997) and the MLP/CRP3 residues mutated in human patients are conserved in *Drosophila* Mlp60A and Mlp84B (Fig. 1A), we hypothesized that Mlp84B is essential for normal cardiac function in *Drosophila*. Here we show that Mlp84B is expressed in the *Drosophila* heart from late embryonic stages to adulthood and that *mlp84B*-deficient *Drosophila* display bradycardia and heart rhythm abnormalities. Thus, *Drosophila* provides a new model system in which genetic and physiological tools can be applied in combination to investigate the cardiac stretch-sensing response *in vivo*.

MATERIALS AND METHODS

Fly strains

w^{1118} flies (*Drosophila melanogaster*) were used as the wild-type control. Crosses were done at 25°C unless otherwise specified. Expression of *mlp84B* RNA interference (RNAi) was achieved using the standard Gal4/UAS system (Brand and Perrimon, 1993). The drivers used were *Daughterless (Da)-Gal4* and *tinCΔ4-Gal4* (Lo and Frasch, 2001). *UAS-mlp84B-RNAi* flies were obtained from a genome-wide RNAi collection (B. Dickson, Institute of Molecular Pathology, Vienna, Austria). The sequence of the RNAi construct was as follows: ACCGATGGCTTGACCGAGGATCA-GATCAGCGCCAACAGGCCCTTCTATAACCCGGACACCA-CGTCAATTAAGGCCCGTGACGGCGAAGGCTGTCCCCGG-TGCGGAGGAGCCGTATTCGCCGCCGAGCAACAGCTGTC-CAAGGGCAAGGTGTGGCACAAGAAGTGCTACAACTGC-

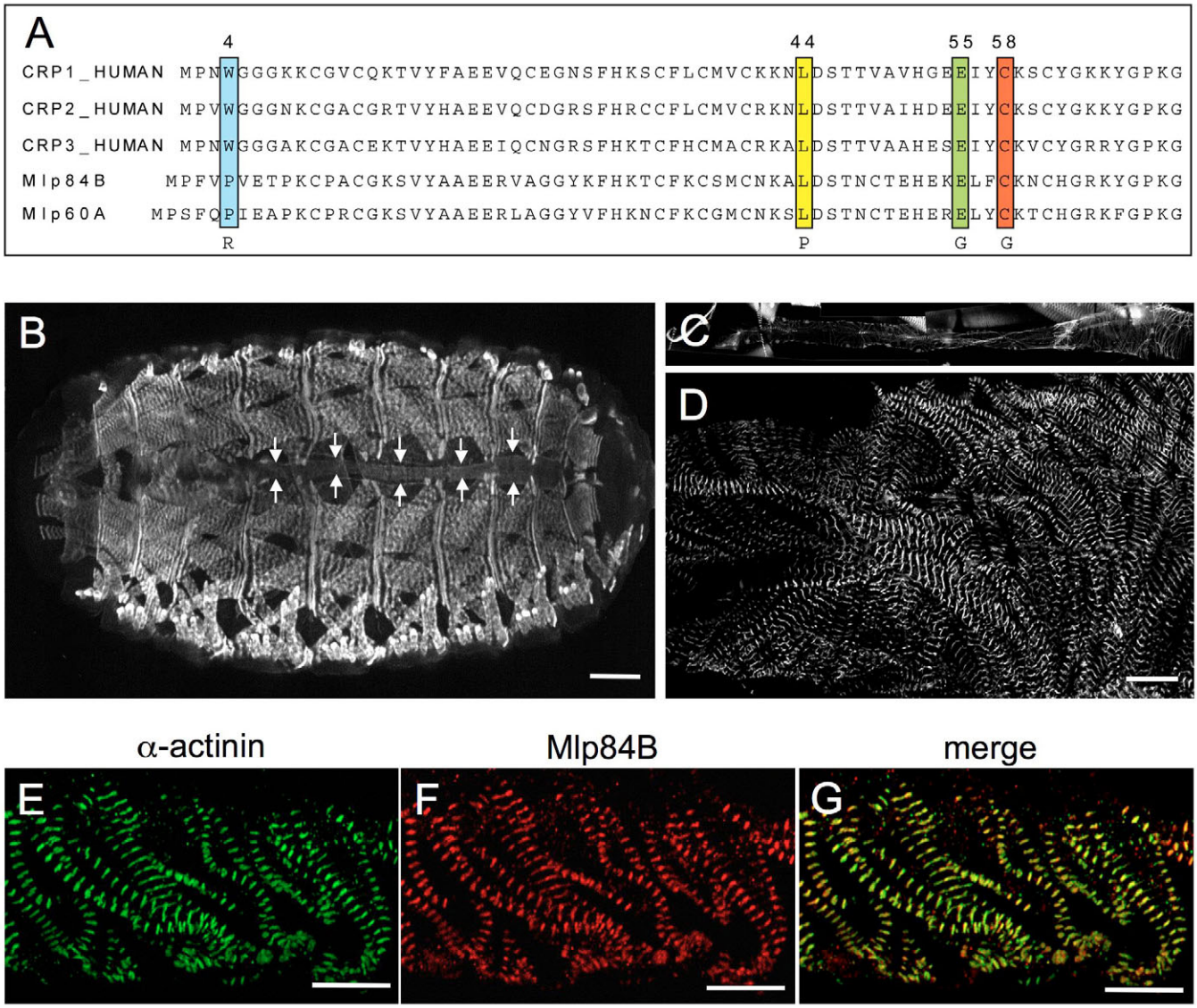

Fig. 1. Developmental cardiac expression and localization of Mlp84B. (A) Alignment of the first LIM domain glycine-rich region from human CRP1, 2 and 3 (CRP3=MLP) and Drosophila Mlp84B and Mlp60A. The first residue highlighted is a mutation associated with human dilated cardiomyopathy (see Knöll et al., 2003), while the other three highlighted residues are associated with cardiac hypertrophy (see Geier et al., 2003). (B) Stage 17 embryo stained with Mlp84B antiserum. The heart tube is delineated by arrows. (C) Image montage of a heart tube dissected from a *mlp84B::GFP* 3rd instar larva. Expression can be seen in sarcomeres as well as in cell nuclei. (D) Posterior part of a wild-type 3rd instar larva heart (segment A6) stained with Mlp84B antiserum. (E–G) Detail of the cardiac myofibrils in the larval heart showing co-localization of α-actinin and Mlp84B. All scale bars are 50 μm.

GCCGACTGCCACCGGCCATTGGACTCGGTCCTGGCCTG-CGATGGACCCGATGGCGACATCCACTGCCGCGCCTGCT-ACGGC. *mlp84B*$^{-/-}$ flies were generated by combining the *mlp84B*^{P8} P-element excision allele with the *Df(3R)dsx2M* deficiency (for details, see Clark et al., 2007). This combination results in the complete absence of Mlp84B protein (Clark et al., 2007). These flies were crossed and raised at 18ºC to optimize the yield of adult escapers. A *mlp84B* transgene was reintroduced in the *Df(3R)dsx2M* fly line to be used as a rescue of the *mlp84B* deficiency when crossed to *mlp84B*^{P8} (Clark et al., 2007). The *mlp84B-GFP* (green fluorescent protein) transgene was constructed as follows. 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. 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 to introduce a *Bam*HI site at both the 5′- and 3′-ends and allow for cloning into the *mlp84B* transgene in-frame with the *mlp84B* ORF. The *cypher-GFP* protein trap line (line G189) (Morin et al., 2001) was a gift from X. Morin to K.A.C. These flies were crossed to flies bearing the *mlp84B*^{P8} mutant allele to generate *cypher-GFP;mlp84B*^{P8}. *cypher-GFP;mlp84B*$^{-/-}$ flies were then obtained by combining *cypher-GFP;mlp84B*^{P8} *with Df(3R)dsx2M*.

RNA extraction, reverse transcription reaction and real-time quantitative PCR

Total RNA was isolated from whole flies or dissected hearts using an equal number of 1 week old males and females with TRIzol Reagent (Invitrogen, Carlsbad, CA, USA) according to standard

protocol, then DNAseI digestion was performed to ensure elimination of genomic DNA. RNA was reverse transcribed using the Superscript First Strand kit from Invitrogen and diluted 1:5 prior to real-time quantitative PCR. The nucleotide sequences of the PCR primers used were as follows: *mlp84B* forward 5′-ACGTCAATTAAGGCCCGTGAC-3′ and reverse 5′-AGGACGGCCATCTGGGAACTGG-3′; *actin79B* forward 5′-ATCCGCAAGGATCTGTATGC-3′ and reverse 5′-ACATCTGCTGGAAGGTGGAC-3′ (Akasaka et al., 2006). Real-time quantitative PCR was performed using a LightCycler (Roche Diagnostics, Indianapolis, IN, USA) rapid thermal cycler. Amplification was carried out as recommended by the manufacturer using the Light Cycler-DNA Master SYBR Green I mix containing 1 μmol l^{-1} of appropriate primer mix, added with 2 μl of cDNA. The amplification program included an initial denaturation step at 95°C for 8 min, and 45 cycles of denaturation at 95°C for 5 s, annealing at 60°C for 6 s and extension at 72°C for 15 s. Melting curves were used to determine the specificity of PCR products, which was confirmed by conventional gel electrophoresis. Relative *mlp84B* expression represents the average of triplicates normalized to *actin79B* (see Akasaka et al., 2006). Error bars represent the standard error of the mean. Student's *t*-test was used to analyze statistical significance. *P* values corresponded to two-tailed tests, and $P<0.01$ was considered statistically significant.

Lifespan analysis

Lifespan was determined by collecting a minimum of 100 male and 100 female flies for each genotype, distributed in food vials (standard yeast/molasses/cornmeal) containing 25 flies or fewer of the same sex. Flies were maintained at 25°C and transferred to a fresh vial every 2–3 days. The number of dead flies was scored at each vial change.

Immunocytochemistry and confocal imaging

Embryos were heat-fixed and processed for immunofluorescence as previously described (Clark et al., 2003). Third instar larvae were immobilized using insect pins, filleted in calcium-free Ringer solution and fixed in –20°C methanol for 10 min. Processing of the fixed fillets for immunofluorescence was done as for embryos. One week old flies were dissected to expose the heart and fixed for 30 min in 4% paraformaldehyde. The following antibodies were used at the concentrations indicated: rabbit anti-Mlp84B (B50) (Stronach et al., 1996) 1:500, rat anti-α-actinin (Technix, Cambridge, UK) 1:100 (for larval heart stainings) and mouse anti-α-actinin (a gift from J. Saide) (Saide et al., 1989) 1:20 (for adult heart stainings). Secondary antibodies were FITC-conjugated anti-mouse and Cy3-conjugated anti-rabbit IgG (Sigma, St Louis, MO, USA). Samples were mounted in Vectashield (Vector Labs, Burlingame, CA, USA). Images were acquired on an MRC 1024 SP BioRad laser point scanning confocal microscope using LaserSharp 2000 software (Bio-Rad, Hercules, CA, USA). Three to four consecutive confocal sections were acquired in the *z*-axis with a step of 2 μm and projected in ImageJ software (http://rsb.info.nih.gov/ij).

Heart beat analysis

Image analysis of semi-intact preparations was carried out as described previously (Ocorr et al., 2007). Ten to 15 flies per genotype were anesthetized with Flynap (Carolina, Burlington, NC, USA) and dissected in an oxygenated saline solution mimicking adult hemolymph [based on previous studies (Singleton and Woodruff, 1994; Wang et al., 2003)]. The artificial hemolymph used in this study contained 108 mmol l^{-1} Na^+, 5 mmol l^{-1} K^+, 2 mmol l^{-1} Ca^{2+}, 8 mmol l^{-1} $MgCl_2$, 1 mmol l^{-1} NaH_2PO_4, 4 mmol l^{-1} $NaHCO_3$, 10 mmol l^{-1} sucrose, 5 mmol l^{-1} trehalose and 5 mmol l^{-1} Hepes (pH 7.1). All internal organs and abdominal fat were removed in order to expose the heart. Roughly equal numbers of males and females were used, except for the $mlp84B^{-/-}$ genotype, where the analyzed sample comprised three female and 13 male flies owing to the very low number of female adult escapers. Prior to imaging, dissected flies were allowed to recover for 20–30 min at room temperature in oxygenated saline solution. Sequences of cardiac contractions were recorded for 30 s (wild-type flies) or 1 min (all other genotypes) at room temperature on a Leica DM LFSA microscope (Bannockburn, IL, USA) equipped with a ×10 water-immersion lens. Movies were taken at rates of 100–200 frames s^{-1} using a fast-acquisition Hamamatsu EM-CCD digital camera (Irvine, CA, USA) controlled by SimplePCI software (Compix, Inc., Hamamatsu Corporation). M-modes were generated using a MatLab-based image analysis program written by M. Fink (M. Fink, W. Giles, R.B. and K.O., unpublished data). Briefly, a 1 pixel-wide region is defined in a single frame of the movie that encompasses both edges of the heart tube; identical regions are then cut from all of the frames in the movie and aligned horizontally, providing an edge trace that documents the movement of the heart tube edges in the *y*-axis over time in the *x*-axis. Measurements of the heart period and diastolic interval were obtained as output from the MatLab-based program. Results represent the average of the median heart period or diastolic interval in each recording. Error bars represent the standard error of the mean. Two-tailed tests were performed to determine statistical significance, and $P<0.01$ was considered statistically significant. For the histogram representation, the frequency of interval duration was plotted for all heart beats in one given genotype using a bin of 0.02 s. The cutoff was fixed at 2 s for heart period, 1.4 s for diastolic interval and 0.6 s for systolic interval. Longer intervals were pooled and represented at the cutoff value. Asystole was defined by a diastolic interval longer than 1 s, which is 3 times the average diastolic interval observed in 5 week old wild-type flies (0.33 s). Tachyarrhythmia was defined by a systolic interval longer than 0.5 s (approximately twice the average systolic interval observed in 5 week old wild-type flies, which was 0.22 s) or a diastolic interval shorter than 0.05 s (about half the shortest median diastolic interval observed in 1 week old wild-type flies, which was 0.12 s). The frequency of asystole and tachyarrhythmia was calculated by adding the asystole/tachyarrhythmia events observed in all recordings for each genotype and dividing this by the number of flies analyzed.

RESULTS

Mlp84B co-localizes with α-actinin in the developing and adult heart

To gain insight into the possible requirements for Mlp84B during development, we examined its expression at various stages. Mlp84B was undetectable in the embryonic heart until late stages, where it was less strongly expressed compared with body wall muscles (Fig. 1B). A Mlp84B::GFP fusion protein was strongly expressed in the heart tube of 3rd instar larvae (Fig. 1C) and localized to sarcomeres as well as cell nuclei. Staining with Mlp84B antiserum likewise revealed robust expression in the regularly arranged sarcomeric units constituting the cardiac myofibrils (Fig. 1D). Mlp84B was found to co-localize with α-actinin at the Z-line of cardiac sarcomeres (Fig. 1E–G).

We then examined Mlp84B expression in the adult. The adult fly heart is a tube composed of transverse or spiral muscle fibers

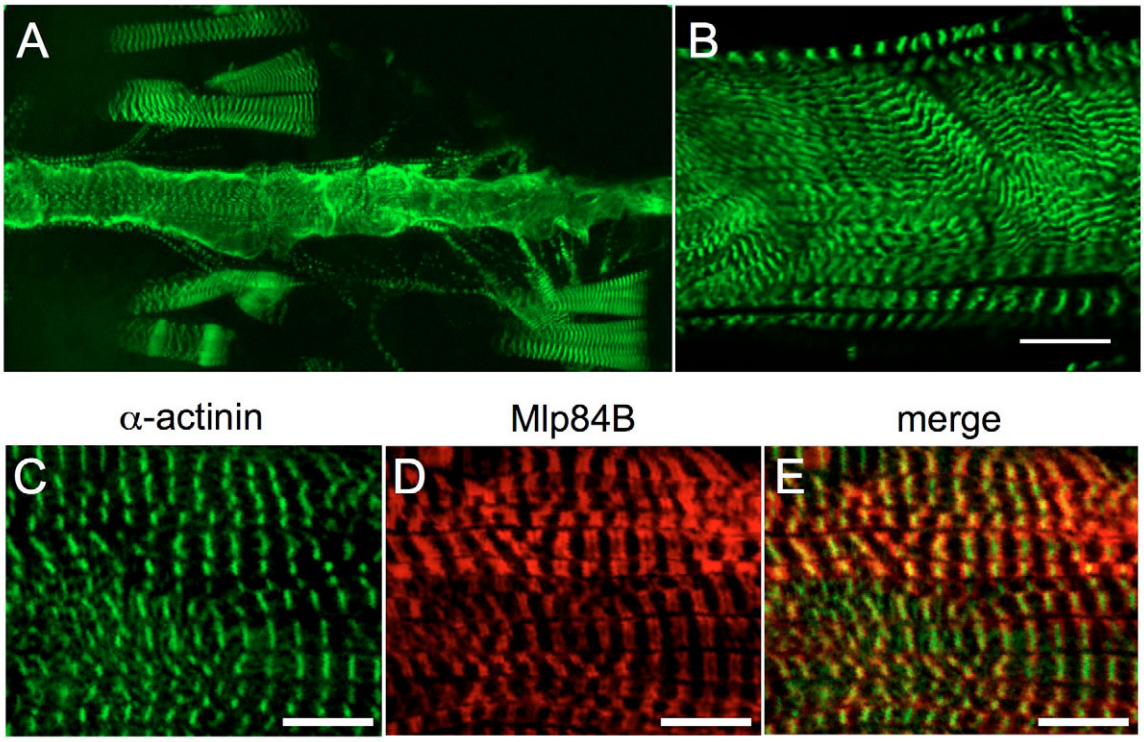

Fig. 2. Mlp84B co-localizes with α-actinin at the Z-disc of sarcomeres in the adult heart. (A) Low-magnification image of a *cypher-GFP* adult *Drosophila* heart, encompassing two abdominal segments (A3–A4; note the body wall muscles segmentally arranged on either side of the heart tube). (B) Detail of one segment of a *cypher-GFP* adult heart. Both the inner spiral and outer longitudinal myofibers are clearly visualized with the Cypher-GFP signal. The scale bar is 40 μm. (C–E) High magnification images of the longitudinal myofibrils stained with anti-α-actinin (C) and anti-Mlp84B (D) antibodies. Mlp84B forms a doublet on either side of the α-actinin band (E). The scale bar is 20 μm.

that constitute the myocardium, apposed by a layer of ventrolateral longitudinally oriented myofibrils (Fig. 2A,B) (Molina and Cripps, 2001). α-Actinin and Mlp84B immunostaining revealed co-localization at the Z-disc of sarcomeres in the longitudinal myofibrils. α-Actinin localized in a tight band (Fig. 2C) whereas Mlp84B was found in a broader domain, forming a doublet (Fig. 2D). The merged image shows that the α-actinin-positive band lies in the center of the Mlp84B doublet (Fig. 2E). This pattern is the same as that seen in larval and adult body wall muscle (Clark et al., 2007) (and data not shown) as well as murine cardiomyocytes (Arber et al., 1997).

Cardiac *mlp84B* knockdown decreases lifespan

In order to examine heart function in *mlp84B*-deficient *Drosophila*, we expressed *mlp84B* RNAi either ubiquitously or specifically in the heart. *mlp84B* RNAi produced viable flies, probably due to incomplete knockdown. RNAi efficiency was tested in whole flies using the ubiquitous *Daughterless (Da)-Gal*4 as well as specifically in the heart using the cardiac driver *tinCΔ4-Gal4* (Lo and Frasch, 2001). In both cases, *mlp84B* relative expression was strongly decreased (by 74% and 65%, respectively, Fig. 3A,B). To determine whether *mlp84B* knockdown in the heart impairs adult viability, we conducted lifespan studies. Compared with wild-type or controls (*UAS-mlp84B-RNAi* outcrossed to wild-type), the RNAi-expressing male flies had a dramatically reduced lifespan (Fig. 3C). This finding points to a role for Mlp84B in heart performance and aging, and prompted us to examine cardiac structure and function in *mlp84B* mutants.

mlp84B-deficient flies do not show cardiac sarcomere structural defects

Body wall muscle structure is not significantly altered in $mlp84B^{-/-}$ larvae (Clark et al., 2007). Similarly, *MLP* knock-out mice show no obvious skeletal muscle phenotype, but develop severe dilated cardiomyopathy a few weeks after birth (Arber et al., 1997). We thus sought to assess cardiac myofibril cytoarchitecture in *mlp84B*-deficient 1 week old adult *Drosophila*. In $mlp84B^{-/-}$ adult hearts, anti-α-actinin staining revealed some sarcomeric disorganization in the ventral longitudinal myofibrils as evidenced by a disrupted Z-disc arrangement (Fig. 4A) compared with wild-type (Fig. 2C), including Z-disc misalignments and gaps. Normal sarcomeric architecture was restored in the $mlp84B^{-/-}$ *rescue* genotype (Fig. 4C). To visualize the inner cardiac myofibrils that were only weakly stained by the anti-α-actinin antibody, we introduced the protein trap *cypher-GFP* (Morin et al., 2001) in the $mlp84B^{-/-}$ background. Sarcomeric structure did not appear to be affected by the absence of Mlp84B in the inner, spiral muscle fibers that constitute the myocardium proper (Fig. 4E). Thus, *mlp84B* deficiency in *Drosophila* does not reflect the severe sarcomere disorganization found in $MLP^{-/-}$ murine hearts, and lethality due to the absence or down-regulation of Mlp84B cannot be readily explained by sarcomeric architecture defects.

mlp84B-deficient *Drosophila* have impaired diastolic function

For technical reasons, it is difficult to assess cardiac function in larvae. However, we were able to use an imaging-based cardiac performance assay to investigate the consequences of the absence or reduced levels of Mlp84B in the adult heart. Partial dissection

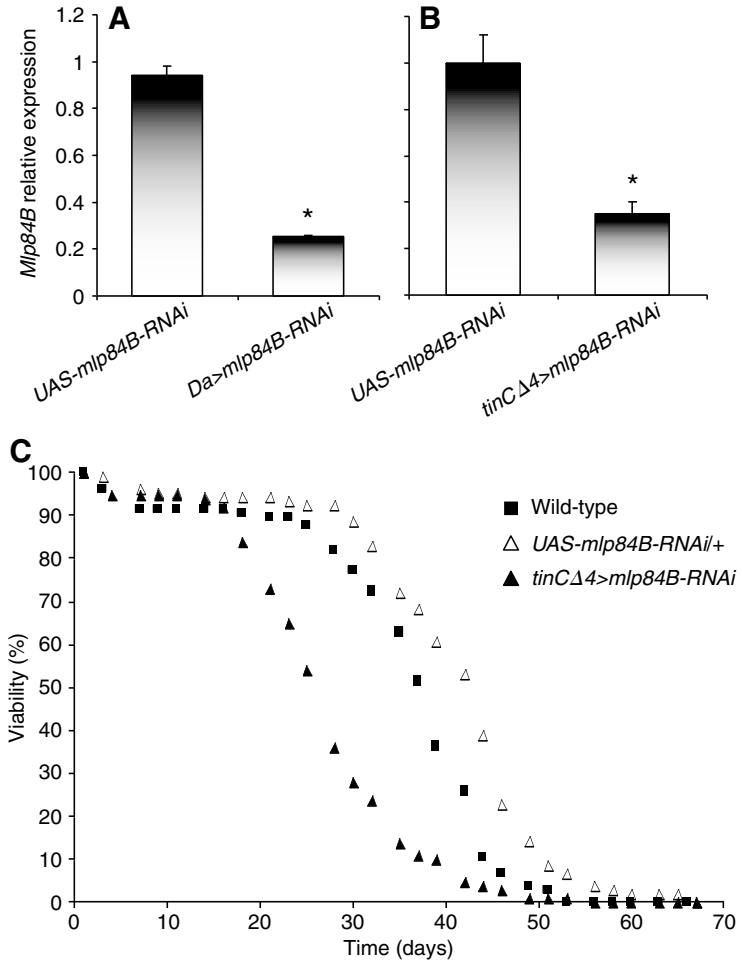

Fig. 3. Cardiac-specific *mlp84B* knockdown causes early mortality. (A,B) *mlp84B* expression level is significantly reduced in *mlp84B* RNAi-expressing *Drosophila*. A strong diminution in *mlp84B* expression level was observed using either a ubiquitous (*Daughterless-Gal4*, A) or a heart-specific (*tinCΔ4-Gal4*, B) driver. *mlp84B* expression levels were determined by performing real-time quantitative PCR on reverse-transcribed mRNA from whole fly (A) or heart only (B) and normalized to *actin79B* expression. (C) Male flies expressing *mlp84B* RNAi in the heart have a dramatically shortened lifespan compared with controls (*UAS-mlp84B-RNAi* outcrossed to wild-type). No lifespan reduction was observed in females.

of the abdomen in a solution mimicking hemolymph allows for recording of the cardiac contractions and measurement of various heartbeat parameters (Ocorr et al., 2007). We determined the heart period as well as systolic and diastolic intervals in *mlp84B*$^{-/-}$ or RNAi-expressing flies as compared with wild-type and controls (*UAS-mlp84B-RNAi* outcrossed to wild-type). Because *mlp84B*$^{-/-}$ flies have a shortened lifespan, we only measured heartbeat intervals in 1 week old flies. *Drosophila* expressing *mlp84B* RNAi in the heart were examined at 1 week and 5 weeks of age in order to uncover a potential increased deterioration of cardiac function with age. The most striking observation was an increased duration of the heart period (heart beat length consisting of systolic plus diastolic interval), and this was mainly due to a prolongation of the diastolic interval (relaxation phase; Fig. 5). In 1 week old *mlp84B*$^{-/-}$ flies, we observed a significantly longer heart period than in wild-type age-matched controls (Fig. 5A). This effect was partially reversed in the rescue line. Flies expressing *mlp84B* RNAi (*mlp84B* knockdown) in the heart were not significantly different from controls at 1 week of age (not shown); however, 5 week-old *mlp84B* knockdown flies exhibited a significantly prolonged heart period compared with controls (Fig. 5B). Significantly, the prolonged heart period seen in 5 week old *mlp84B* knockdown flies was due to an increase in the diastolic interval similar to that observed for the 1 week old *mlp84B*-deficient flies (Fig. 5C,D).

We also examined the distribution of heart periods as well as systolic and diastolic intervals, which highlights the deterioration of the heartbeat regularity (Fig. 6, and Fig. S1 in supplementary material). Both heart period and diastolic interval distributions were altered in *mlp84B*$^{-/-}$ flies (compare the position, amplitude and sharpness of the frequency peak in *mlp84B*$^{-/-}$ *versus* wild-type and rescue flies, Fig. 6A). The distribution of systolic intervals appeared to be unaffected. In 5 week old *mlp84B* RNAi-expressing flies, the frequency peak was shifted towards longer durations and the distribution of heart periods and diastolic intervals was more scattered than in age-matched wild-type and *UAS-mlp84B-RNAi* outcrossed to wild-type controls (Fig. 6B). This reveals that in addition to a decreased heart rate, diminished levels of Mlp84B resulted in a more irregular heartbeat due to an increased variability in the diastolic interval duration (see also supplementary material Fig. S1). Systolic intervals were slightly longer in 5 week old *mlp84B* RNAi-expressing flies than in controls, but this difference was not statistically significant (not shown). These data suggest that partial or total loss of Mlp84B causes a significant diastolic dysfunction.

mlp84B loss or reduced expression affects cardiac rhythmicity

As another index of heart function, we monitored *mlp84B*$^{-/-}$ mutant and *mlp84B* RNAi cardiac-knockdown flies for heart rhythm abnormalities. Specifically, we examined the occurrence of tachyarrhythmia (systolic interval longer than 0.5 s or diastolic interval shorter than 0.05 s; Fig. 7A, arrows) and the occurrence of asystole events (diastolic interval longer than 1 s; Fig. 7A, arrowheads). No tachyarrhythmia was observed in 1 week old flies of any of the genotypes. At 5 weeks, the occurrence of cardiac tachyarrhythmia was 10 times higher in 5 week old flies expressing *mlp84B* RNAi in the heart compared with wild-type and control flies (Fig. 7B). Wild-type *Drosophila* never exhibited asystoles at 1 week of age. In contrast, 1 week old *mlp84B*$^{-/-}$ flies displayed on average 4.2 events of asystole per minute (Fig. 7C). The occurrence of asystole was reduced to about half in rescue flies (Fig. 7C). Similarly, 5 week old *Drosophila* expressing *mlp84B* RNAi in the heart had a dramatically higher incidence of asystole (7.7 events per minute) compared with the same age wild-type or control flies (Fig. 7D). In summary, the observed disturbances in cardiac rhythmicity are consistent with the altered distribution of heart periods and diastolic intervals as dramatically illustrated in the histograms of Fig. 6 (see also supplementary material Fig. S1).

DISCUSSION

Mlp84B is essential for normal heart function

The identification of the precise molecular mechanisms that link the cytoskeletal defect in the cardiac Z-disc to the development of cardiomyopathy is critical for the design of therapeutic strategies to prevent disease progression, and there is a need for models in which to study candidate genes. *Drosophila* is well suited to test

genetic interactions, but so far there has been a lack of heart function assays in this organism. A recently developed imaging assay allows for measuring contractile properties of the adult heartbeat in semi-intact adult flies (Ocorr et al., 2007). Using this technique, we examined the role of Mlp84B, the *Drosophila* counterpart of MLP, in cardiac function. *mlp84B* null mutants die at the pupal stage or as young adults, but RNAi knockdown of *mlp84B* specifically in the heart produced viable flies, allowing us to assess cardiac function (which is technically difficult in larvae and pupae). By conducting lifespan studies, we observed that male *Drosophila* with reduced cardiac expression of *mlp84B* have a shortened lifespan as compared with control flies, which is consistent with a role for Mlp84B in cardiac function. A detailed analysis of heartbeat parameters in *Drosophila* with decreased cardiac *mlp84B* expression showed a prolongation of the diastolic interval leading to a slower cardiac rhythm in both male and female flies, as well as a greatly elevated occurrence of tachyarrythmia and asystole compared with control flies. This phenotype was subtle in 1 week old *Drosophila* but significant at 5 weeks of age, suggesting an aggravation of cardiac dysfunction with muscle use and/or age. One week old *mlp84B*$^{-/-}$ flies displayed similar perturbations in diastolic function and a high incidence of asystole, which were partially rescued by reintroducing the *mlp84B* transgene in the mutant background.

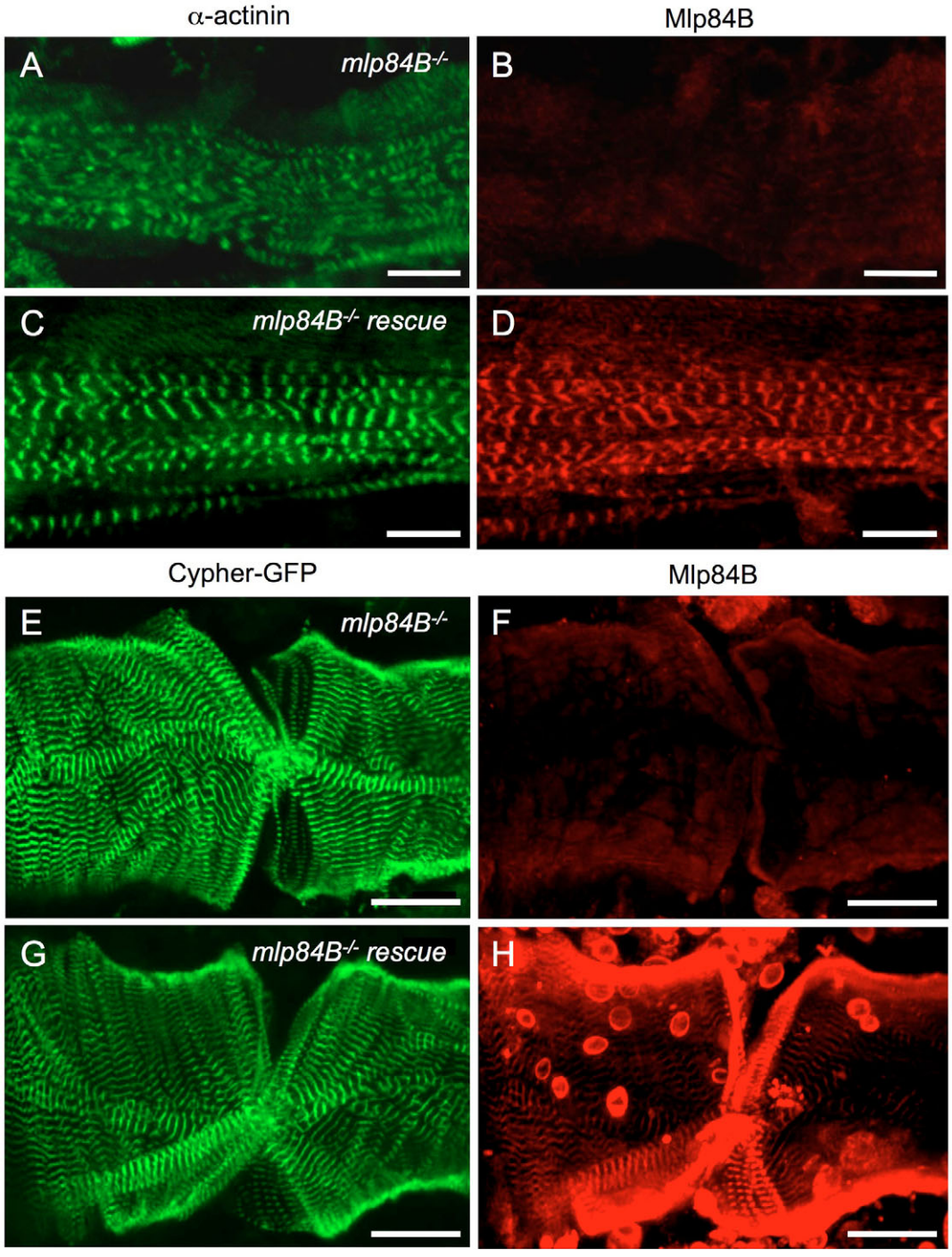

Fig. 4. Cardiac myofibril cytoarchitecture in *mlp84B*-deficient flies. *mpl84B*$^{-/-}$ (A,B) and *mlp84B*$^{-/-}$ *rescue* (C,D) adult *Drosophila* hearts were co-stained with anti-α-actinin and anti-Mlp84B antibodies. *mlp84B*$^{-/-}$ hearts show sarcomeric disorganization of the longitudinal fibers as evidenced by misalignments and gaps in α-actinin-positive bands (A). Sarcomeric structure is restored in the *mlp84B*$^{-/-}$ *rescue* line (C). Cardiac spiral myofibers were visualized in *cypher-GFP;mlp84B*$^{-/-}$ (E,F) and *cypher-GFP;mlp84B*$^{-/-}$ *rescue* (G,H) hearts. The A3 segment is shown, including the ostia in the center. No obvious structural defect was detected (E). All scale bars are 20 μm.

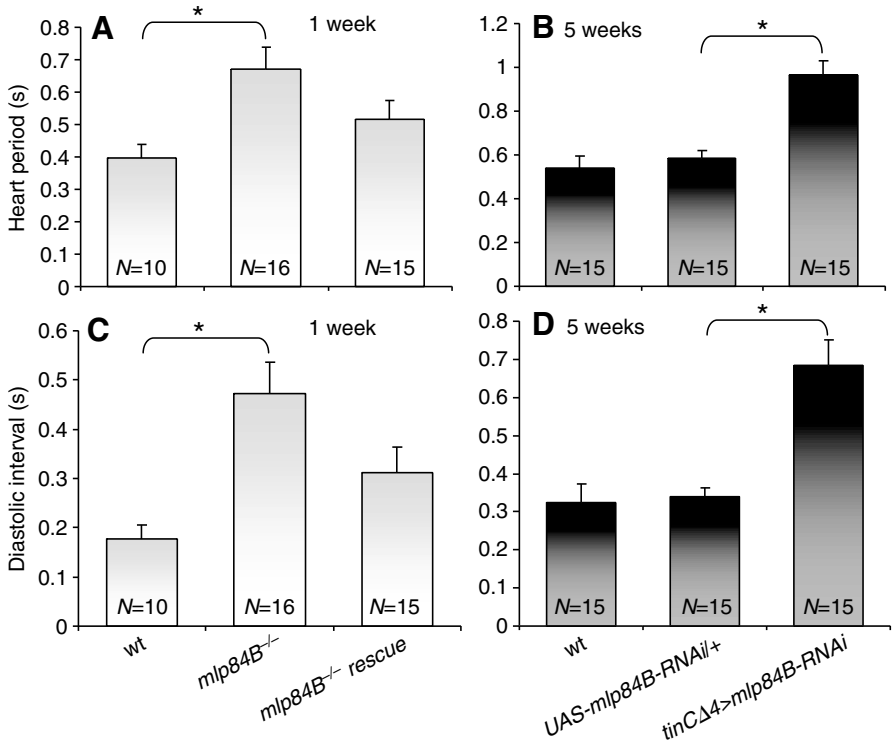

Fig. 5. Reduced levels of Mlp84B result in a slower heart beat due to prolongation of the diastolic interval. (A) Heart period is significantly longer in 1 week old *mlp84B*$^{-/-}$ flies compared with age-matched wild-type (wt) controls. Reintroduction of a *mlp84B* transgene in the *mlp84B*$^{-/-}$ background results in partial rescue. (B) Heart-specific *mlp84B* knockdown prolongs the heart period in 5 week old flies compared with age-matched wild-type or *UAS-mlp84B-RNAi* outcrossed to wild-type flies. No significant increase in heart period was observed in 1 week old *mlp84B* RNAi-expressing flies. (C,D) Diastolic interval is significantly longer in 1 week old *mlp84B*$^{-/-}$ and 5 week old *mlp84B* RNAi-expressing flies compared with corresponding controls (as in A and B). *mlp84B*$^{-/-}$ *rescue* flies show intermediate diastolic interval duration (C). No significant prolongation of the diastolic interval was detected in *mlp84B* RNAi-expressing flies at 1 week of age (not shown). See supplementary material Fig. S1 for increases in standard deviations of the heart period and diastolic interval of 1 week old *mlp84B*$^{-/-}$ flies and 5 week old *mlp84B* RNAi-expressing flies.

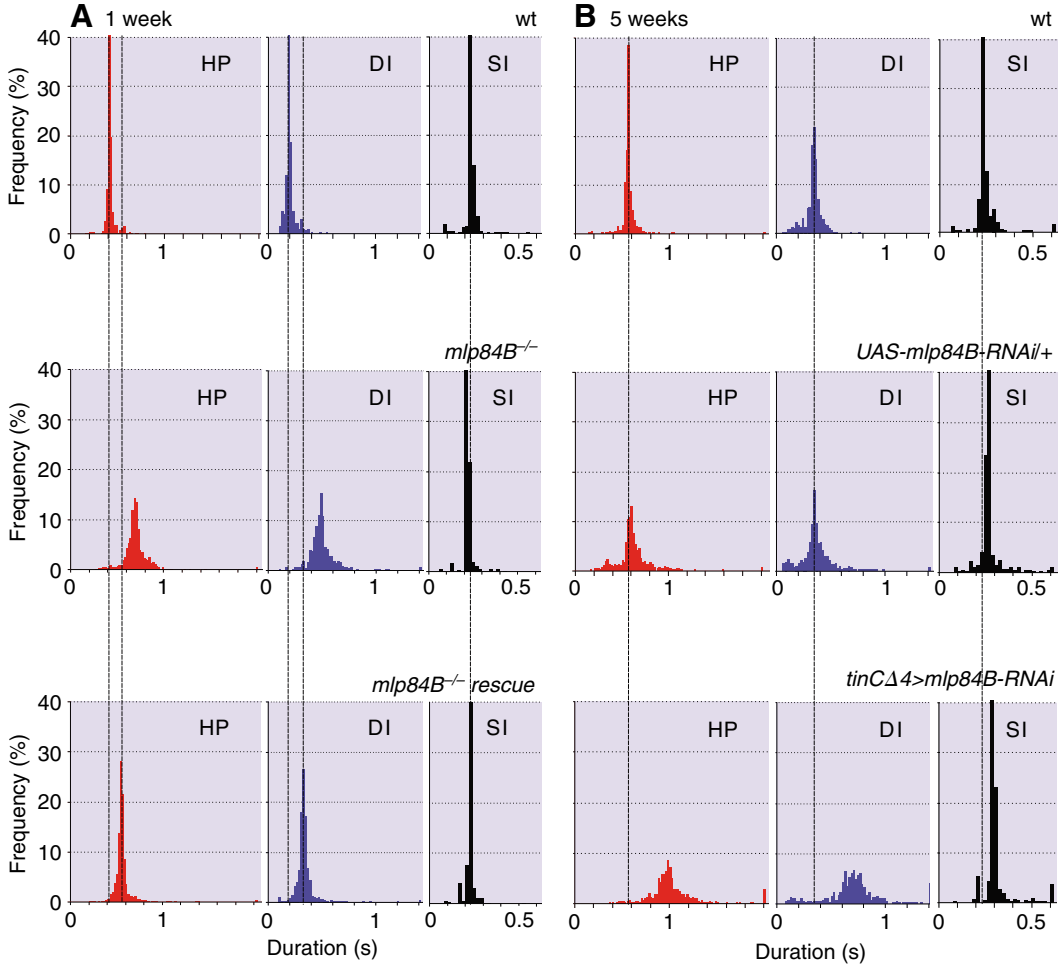

Fig. 6. Distribution of heart period and intervals is altered when *mlp84B* is absent or down-regulated in the heart. The frequency of heart period (HP), diastolic interval (DI) and systolic interval (SI) durations is represented for all heartbeats from all analyzed recordings. (A) One week old *mlp84B*$^{-/-}$ flies display globally longer and more irregular heart period and diastolic interval than wild-type or *mlp84B*$^{-/-}$ *rescue* flies. The dashed vertical lines indicate the most frequent duration for each interval in wild-type and *mlp84B*$^{-/-}$ *rescue* flies. Systolic interval appears to be unaffected. (B) Cardiac *mlp84B* mRNA knockdown results in overall longer and highly irregular heart period and diastolic interval in 5 week old flies. Systolic interval is moderately prolonged relative to 1 week old wild-type in A. The heart period and diastolic/systolic interval distributions were not obviously affected in 1 week old *mlp84B* RNAi-expressing hearts compared with corresponding controls (not shown).

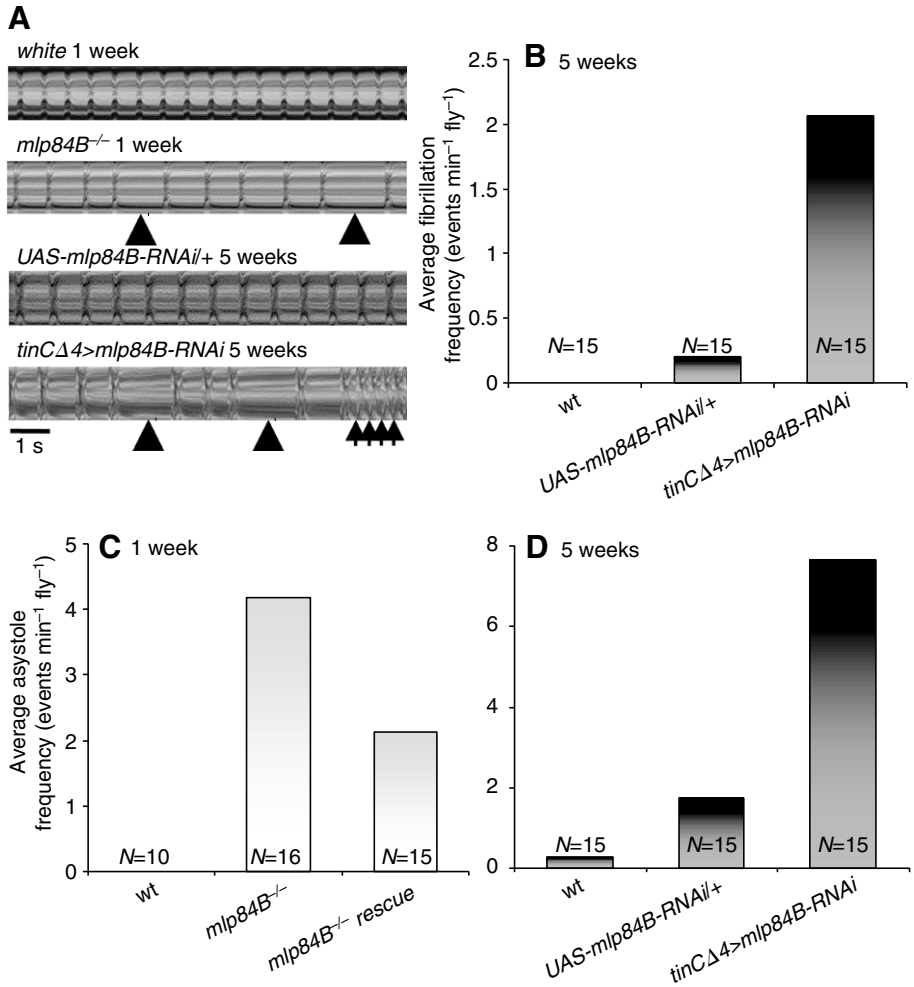

Fig. 7. Loss or cardiac knockdown of *mlp84B* results in heart rhythm abnormalities. (A) M-mode representations of heart contractions recorded in wild-type, *mlp84B*$^{-/-}$, *UAS-mlp84B-RNAi/+* and *tinCΔ4>mlp84B RNAi* flies. Asystole and tachyarrhythmia are shown by arrowheads and arrows, respectively. (B) The frequency of fibrillation is higher in 5 week old *mlp84B* RNAi-expressing flies than in age-matched *UAS-mlp84B-RNAi/+* or wild-type control flies. (C) Asystole is observed in 1 week old *mlp84B*$^{-/-}$ flies but not wild-type flies. The occurrence of asystole is reduced when the *mlp84B* transgene is reintroduced in the *mlp84B*$^{-/-}$ background. (D) Five week old *Drosophila* expressing *mlp84B* RNAi in the heart show a high rate of asystole compared with wild-type or *UAS-mlp84B-RNAi/+* flies.

Taken together, these data establish an essential role for Mlp84B in heart function.

Drosophila as a model of MLP deficiency

In mice, deletion of the *MLP* gene induces dilated cardiomyopathy and heart failure that develops after 4 weeks of postnatal life. Among other defects, *MLP*$^{-/-}$ animals display severe sarcomeric disarray (Arber et al., 1997), a slower heart rate, and diastolic dysfunction defined by slower relaxation time (Minamisawa et al., 1999). When examining heart function in adult flies with no or reduced *mlp84B* expression, we found a reduction in heart rate that was the result of a significantly prolonged diastolic interval, which is reminiscent of the mouse phenotype. Although we do not know the reason for the additional abnormalities in myofiber morphology in mutant mouse hearts, a possible clue may be that the mouse heart beats much faster than the fly heart (10 *versus* 2 beats s^{-1}, respectively), making it more prone to structural defects. Another explanation could be that various secondary pathomechanisms such as upregulation of proteases and tissue remodeling contribute to myofibrillar disarray in *MLP*$^{-/-}$ mice and human patients with mutated *MLP*. The heart rhythm defects observed in *mlp84B* null or *mlp84B* knockdown flies may actually represent the primary defect caused by the absence of MLP, making *Drosophila* a useful model to investigate the primary mechanisms of the disease without the bias of the secondary remodeling seen in mice.

We also examined the heart rhythm in aging flies with reduced cardiac *mlp84B* expression. Remarkably, we observed that both *mlp84B* null and *mlp84B* knockdown flies have a high incidence of asystole and that *mlp84B* knockdown also causes cardiac tachyarrhythmia in 5 week old flies. These anomalies could be interpreted as manifestations of diastolic dysfunction, perhaps because reduced levels of Mlp84B impair stretch sensing at the Z-disc of cardiac myofibrils, which may result in a failure to maintain a constant relaxation time. Consequently, the diastolic interval duration can be either prolonged or reduced, which is detected as asystole and tachyarrythmia in our analysis. It would be interesting to determine whether muscle tension is also altered in *mlp84B* mutants.

The severe structural sarcomeric defects characteristic of the *MLP*$^{-/-}$ murine heart were not observed in flies. It is possible that in *Drosophila* other proteins, such as Mlp60A (Stronach et al., 1996), compensate for Mlp84B deficiency to maintain sarcomere integrity. Another feature of *MLP*$^{-/-}$ mice is a dramatically enlarged heart as compared with wild-type (Arber et al., 1997). We measured systolic and diastolic diameters in *mlp84B*$^{-/-}$ or *mlp84B* RNAi-expressing *Drosophila versus* control animals and did not observe any significant increase at the ages examined. It might be that the dilatation phenotype develops in older *mlp84B*-deficient flies, which would need to be investigated with conditional mutants, since the partial heart-specific RNAi knockdown does not seem to be sufficient. Cardiac chamber dilatation in mutant flies has previously been reported in flies with mutations in the *troponin I* and *tropomyosin* genes or expressing a mutated *δ-sarcoglycan* human gene in the heart (Wolf et al., 2006). Deletion of the *MLP* gene in mice also results in impaired systolic function, characterized by a severe decrease in fractional shortening (Arber et al., 1997). In our *mlp84B* mutants, we did not detect any significant change in fractional shortening. However, it should be noted that young *MLP*$^{-/-}$ mice that have not yet developed cardiomyopathy display alterations in passive myocardial properties and relaxation time, but heart diameter and systolic characteristics are normal at that stage, including fractional shortening (Lorenzen-Schmidt et al., 2005).

Candidate partners of Z-disc-associated proteins in the stretch-sensing response

Based on the phenotype of young *MLP*$^{-/-}$ mice, it has been proposed that the progression to heart failure in the MLP deficiency model may be driven by diastolic dysfunction and abnormal passive properties rather than systolic dysfunction (Lorenzen-Schmidt et al., 2005). MLP interacts with Tcap, and mutations in the Tcap binding region of *MLP* have been found in a subset of patients with dilated cardiomyopathy (Knöll et al., 2002). Interestingly, Tcap also associates with the cytoplasmic domain of the stretch-dependent potassium channel β-subunit minK (Furukawa et al., 2001). The diastolic interval defects that we observed in Mlp84B-deficient flies may thus be due to impaired stretch-dependent signaling on this channel, in turn affecting the delayed rectifier potassium current $I(K_s)$. Another candidate for linking MLP to the cardiomyopathy phenotype is the giant protein Titin, whose interaction with Tcap is required for sarcomeric integrity (Gregorio et al., 1998), suggesting an important role of the Z-disc complex titin–Tcap–MLP in mechanical signaling and regulation of passive myocardial properties. Titin may play a key role in this complex, since it contributes over 80% of passive force during stretch (Wu et al., 2000) and appears to be involved in mechanical signaling (Granzier and Labeit, 2004). In support of this hypothesis, *titin* mutations have been reported to be associated with a dilated cardiomyopathy phenotype in zebrafish (Xu et al., 2002) and humans (Gerull et al., 2002). In *Drosophila*, the counterpart of the *titin* gene is *sallimus* (*sls*), also referred to as *D-titin* or *kettin*. Loss-of-function *sls* mutants die as late embryos with severely disrupted muscle organization (Hakeda et al., 2000; Machado and Andrew, 2000; Zhang et al., 2000). *mlp84B*$^{-/-}$ larvae that are also heterozygous for a *sls* mutation display a dramatic loss of sarcomeric integrity whereas *mlp84B*$^{-/-}$ or *sls*$^{+/-}$ mutants show no visible muscle structure phenotype, demonstrating a genetic interaction (Clark et al., 2007). It will be interesting to investigate the role of *sls* in *Drosophila* heart function, as a functional MLP–titin interaction may be conserved from flies to humans.

We are grateful to Barry Dickson and Georg Dietzl (Institute of Molecular Pathology, Vienna, Austria) for the gift of the *UAS-mlp84B-RNAi Drosophila* line and to Judith Saide for providing the anti-α-actinin antibody. Laurent Perrin is acknowledged for critical reading of the manuscript. This work was supported by grants from the National Institutes of Health (NHLBI and NIA) to R.B.

REFERENCES

Akasaka, T., Klinedinst, S., Ocorr, K., Bustamante, E. L., Kim, S. K. and Bodmer, R. (2006). The ATP-sensitive potassium (KATP) channel-encoded dSUR gene is required for Drosophila heart function and is regulated by tinman. *Proc. Natl. Acad. Sci. USA* **103**, 11999-12004.

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.

Brand, A. H. and Perrimon, N. (1993). Targeted gene expression as a means of altering cell fates and generating dominant phenotypes. *Development* **118**, 401-415.

Chien, K. R. (2000). Genomic circuits and the integrative biology of cardiac diseases. *Nature* **407**, 227-232.

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.

Clark, K. A., McGrail, M. and Beckerle, M. C. (2003). Analysis of PINCH function in Drosophila demonstrates its requirement in integrin-dependent cellular processes. *Development* **130**, 2611-2621.

Clark, K. A., Bland, J. M. and Beckerle, M. C. (2007). The Drosophila muscle LIM protein, Mlp84B, cooperates with D-titin to maintain muscle structural integrity. *J. Cell Sci.* **120**, 2066-2077.

Furukawa, T., Ono, Y., Tsuchiya, H., Katayama, Y., Bag, M. L., Labeit, D., Labeit, S., Inagaki, N. and Gregorio, C. C. (2001). Specific interaction of the potassium channel beta-subunit minK with the sarcomeric protein T-cap suggests a T-tubule-myofibril linking system. *J. Mol. Biol.* **313**, 775-784.

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

Gerull, B., Gramlich, M., Atherton, J., McNabb, M., Trombitás, K., Sasse-Klassen, S., Seidman, J. G., Seidman, C., Granzier, H., Labeit. S. et al. (2002). Mutations of TTN, encoding the giant muscle filament titin, cause familial dilated cardiomyopathy. *Nat. Genet.* **30**, 201-204.

Granzier, H. L. and Labeit, S. (2004). The giant protein titin: a major player in myocardial mechanics, signaling, and disease. *Circ. Res.* **94**, 284-295.

Gregorio, C. C., Trombitás, 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.

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.

Hayashi, T., Arimura, T., Itoh-Satoh, M., Ueda, K., Hohda, S., Inagaki, N., Takahashi, M., Hori, H., Yasunami, M., Nishi, H. et al. (2004). Tcap gene mutations in hypertrophic cardiomyopathy and dilated cardiomyopathy. *J. Am. Coll. Cardiol.* **44**, 2192-2201.

Knöll, 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.

Lo, P. C. and Frasch, M. (2001). A role for the COUP-TF-related gene seven-up in the diversification of cardioblast identities in the dorsal vessel of Drosophila. *Mech. Dev.* **104**, 49-60.

Lorenzen-Schmidt, I., Stuyvers, B. D., ter Keurs, H. E., Date, M. O., Hoshijima, M., Chien, K. R., McCulloch, A. D. and Omens, J. H. (2005). Young MLP deficient mice show diastolic dysfunction before the onset of dilated cardiomyopathy. *J. Mol. Cell. Cardiol.* **39**, 241-250.

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.

Minamisawa, S., Hoshijima, M., Chu, G., Ward, C. A., Frank, K., Gu, Y., Martone, M. E., Wang, Y., Ross, J., Jr, Kranias, E. G. et al. (1999). Chronic phospholamban-sarcoplasmic reticulum calcium ATPase interaction is the critical calcium cycling defect in dilated cardiomyopathy. *Cell* **99**, 313-322.

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.

Molina, M. R. and Cripps, R. M. (2001). Ostia, the inflow tracts of the *Drosophila* heart, develop from a genetically distinct subset of cardial cells. *Mech. Dev.* **109**, 51-59.

Moreira, E. S., Wiltshire, T. J., Faulkner, G., Nilforoushan, A., Vainzof, M., Suzuki, O. T., Valle, G., Reeves, R., Zatz, M., Passos-Bueno, M. R. et al. (2000). Limb-girdle muscular dystrophy type 2G is caused by mutations in the gene encoding the sarcomeric protein telethonin. *Nat. Genet.* **24**, 163-166.

Morin, X., Daneman, R., Zavortink, M. and Chia, W. (2001). A protein trap strategy to detect GFP-tagged proteins expressed from their endogenous loci in *Drosophila*. *Proc. Natl. Acad. Sci. USA* **98**, 15050-15055.

Ocorr, K., Reeves, N., Wessells, R., Fink, M., Chen, H.-S. V., Akasaka, T., Yasuda, S., Metzger, J. M., Giles, W., Posakony, J. W. et al. (2007). KNCQ potassium channel mutations cause cardiac arrhythmias in *Drosophila* that mimic the effects of aging. *Proc. Natl. Acad. Sci. USA* **104**, 3943-3948.

Pashmforoush, M., Pomies, P., Peterson, K., Kubalak, S., Ross, J., Hefti, A., Aebi, U., Beckerle, M. and Chien, K. R. (2001). Adult mice deficient in actinin-associated LIM-domain protein reveal a developmental pathway for right ventricular cardiomyopathy. *Nat. Med.* **7**, 591-597.

Saide, J. D., Chin-Bow, S., Hogan-Sheldon, J., Busquets-Turner, L., Vigoreaux, J. O., Valgeirsdottir, K. and Pardue, M. L. (1989). Characterization of components of Z-bands in the fibrillar flight muscle of Drosophila melanogaster. *J. Cell Biol.* **109**, 2157-2167.

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

Singleton, K. and Woodruff, R. I. (1994). The osmolarity of adult Drosophila hemolymph and its effect on oocyte-nurse cell electrical polarity. *Dev. Biol.* **161**, 154-167.

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.

Vatta, M., Mohapatra, B., Jimenez, S., Sanchez, X., Faulkner, G., Perles, Z., Sinagra, G., Lin, J.-H., Vu, T. M., Zhou, Q. et al. (2003). Mutations in Cypher/ZASP in patients with dilated cardiomyopathy and left ventricular non-compaction. *J. Am. Coll. Cardiol.* **42**, 2014-2027.

Wang, J. W., Wong, A. M., Flores, J., Vosshall, L. B. and Axel, R. (2003). Two-photon calcium imaging reveals an odor-evoked map of activity in the fly brain. *Cell* **112**, 271-282.

Wolf, M. J., Amrein, H., Izatt, J. A., Choma, M. A., Reedy, M. C. and Rockman, H. A. (2006). Drosophila as a model for the identification of genes causing adult human heart disease. *Proc. Natl. Acad. Sci. USA* **103**, 1394-1399.

Wu, Y., Cazorla, O., Labeit, D., Labeit, S. and Granzier, H. (2000). Changes in titin and collagen underlie diastolic stiffness diversity of cardiac muscle. *J. Mol. Cell. Cardiol.* **32**, 2151-2162.

Xu, X., Meiler, S. E., Zhong, T. P., Mohideen, M., Crossley, D. A., Burggren, W. W. and Fishman, M. C. (2002). Cardiomyopathy in zebrafish due to mutation in an alternatively spliced exon of titin. *Nat. Genet.* **30**, 205-209.

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.

Zhou, Q., Chu, P. H., Huang, C., Cheng, C. F., Martone, M. E., Knoll, G., Shelton, G. D., Evans, S. and Chen, J. (2001). Ablation of Cypher, a PDZ-LIM domain Z-line protein, causes a severe form of congenital myopathy. *J. Cell Biol.* **155**, 605-612.