

miR-92b regulates Mef2 levels through a negative-feedback circuit during *Drosophila* muscle development

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SUMMARY

Mef2 is the key transcription factor for muscle development and differentiation in *Drosophila*. It activates hundreds of downstream target genes, including itself. Precise control of Mef2 levels is essential for muscle development as different Mef2 protein levels activate distinct sets of muscle genes, but how this is achieved remains unclear. Here, we have identified a novel heart- and muscle-specific microRNA, miR-92b, which is activated by Mef2 and subsequently downregulates Mef2 through binding to its 3'UTR, forming a negative regulatory circuit that fine-tunes the level of Mef2. Deletion of miR-92b caused abnormally high Mef2 expression, leading to muscle defects and lethality. Blocking miR-92b function using microRNA sponge techniques also increased Mef2 levels and caused muscle defects similar to those seen with the miR-92b deletion. Additionally, overexpression of miR-92b reduced Mef2 levels and caused muscle defects similar to those seen in Mef2 RNAi, and Mef2 overexpression led to reversal of these defects. Our results suggest that the negative feedback circuit between miR-92b and Mef2 efficiently maintains the stable expression of both components that is required for homeostasis during *Drosophila* muscle development.

KEY WORDS: MicroRNA, Mef2, *Drosophila*, Muscle development, miR-92b

INTRODUCTION

Myocyte enhancer factor 2 (Mef2) proteins are a family of highly conserved transcription factors that play crucial roles in the development of many tissues, particularly in all types of muscles, through activation of a wide spectrum of target genes (Potthoff and Olson, 2007). In *Drosophila*, a single *Mef2* gene is responsible for the activation of hundreds of direct targets during muscle differentiation and development (Junion et al., 2005; Sandmann et al., 2006). Aberrant levels of Mef2 cause disruption of muscle development and fail to rescue the muscle defects in *Mef2*-null mutant (Gunthorpe et al., 1999). Similar phenomena have also been reported in vertebrates, and skeletal muscle development in mice is also sensitive to the levels of myogenin (Vivian et al., 1999), a key Mef2 co-factor in vertebrate muscle differentiation (Molkentin et al., 1995). The molecular mechanism underlying these phenomena was shown in a recent study, where Mef2 activity levels differentially affected gene expression during *Drosophila* muscle development (Elgar et al., 2008). Therefore, the levels of Mef2 must be fine-tuned and maintained in an optimal range to ensure proper expression of target genes and cell differentiation both spatially and temporally.

Studies in both *Drosophila* and vertebrates have elaborated that the level of Mef2 activity can be modified by upstream activators or different transcription co-factors. In *Drosophila*, the initial phase of *Mef2* expression in the early mesoderm is activated by Twist (Cripps and Olson, 1998), and later by Tinman and the Dpp pathway during mesoderm differentiation (Cripps et al., 1999; Nguyen and Xu, 1998). At the late embryonic stage, Mef2 initiates

a positive auto-regulatory circuit through a conserved Mef2-binding site in its enhancer to sustain its own expression in all types of muscle cells (Cripps et al., 2004). Similar auto-regulatory mechanism for *Mef2* genes was also found in vertebrates. Mammalian *Mef2a* has conserved Mef2-binding sites within its enhancer through which the expression of *Mef2a* can be auto-regulated (Ramachandran et al., 2008). An identical structured regulatory region has been found in the *Mef2* gene of the cephalochordate *Amphioxus*, suggesting that this mechanism of auto-regulatory circuit is evolutionarily conserved (Ramachandran et al., 2008). Sustained auto-activation of Mef2 transcription ensures the muscle cell fate during differentiation, but it may also lead to abnormally high Mef2 accumulation without a negative regulatory mechanism to counteract the auto-activation machinery. In mammals, transcriptional co-repressors such as the Class II HDACs can repress the activity of Mef2 by directly binding to its DNA-binding domain (Lu et al., 2000; McKinsey et al., 2002a; McKinsey et al., 2002b). In *Drosophila*, the transcriptional activity of Mef2 can be downregulated by Him through the recruitment of a transcriptional co-repressor, Groucho (Liotta et al., 2007). Interestingly, the 3'UTR of mouse *Mef2a* has been found to function as a cis-acting translational repressor (Black et al., 1997), suggesting that the negative regulation through transcription co-factors might not be sufficient to counteract the auto-activation of Mef2; a novel regulatory mechanism at the translational level might be required to keep Mef2 protein levels in balance.

MicroRNAs are short non-coding RNAs that function through binding to targeting sites in the untranslated regions (UTRs) of target genes to negatively regulate their expression levels (Ambros, 2004). These characteristics make them good candidates for the negative regulation of Mef2. In general, microRNAs are expressed in the same tissues as their targets and act as 'fine tuners' and/or 'safeguards' to prevent dramatic changes in gene expression and to maintain the homeostasis of organisms (Chang et al., 2004; Chen et al., 2004; Johnston and Hobert, 2003; Karres et al., 2007; Poy et al., 2004; Sokol and Ambros, 2005; Zhao et al., 2005). Evolutionarily conserved microRNAs, such as miR-1, have been

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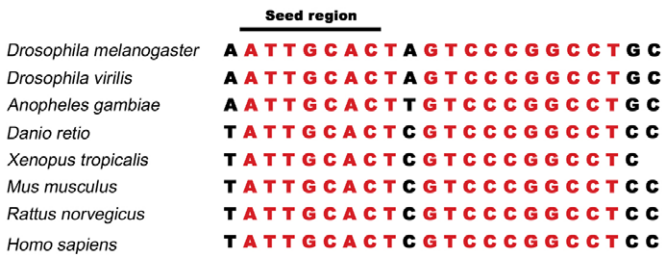


Fig. 1. miR-92b is highly conserved from *Drosophila* to human. Conserved base pairs are shown in red.

found to be specifically expressed in the heart and muscles, and are required for heart and muscle development and function in both *Drosophila* and vertebrates (Kwon et al., 2005; Zhao et al., 2005). In mammals, miR-1 was shown to repress HDAC4, which functions as a repressor for Mef2 (Chen et al., 2006). As miR-1 is activated by Mef2, this positive feedback circuit further strengthens the Mef2 activity and increases the necessity of a counteracting negative regulatory mechanism. However, a microRNA that can negatively regulate Mef2 has not been identified in *Drosophila* or vertebrates.

In this study, we identified an evolutionarily conserved intergenic microRNA, miR-92b, as a negative regulator of Mef2. miR-92b was specifically expressed in the heart and muscle in a pattern similar to that of Mef2. Mef2 directly activates miR-92b through three conserved Mef2-binding sites in the cis-regulatory

region of miR-92b. miR-92b in turn represses Mef2 translation through two conserved miR-92b targeting sites in its 3'UTR, forming a negative-feedback circuit that keeps Mef2 protein levels in balance. Deletion of miR-92b, or overexpression of decoy miR-92b targeting sites using microRNA sponge techniques, led to elevated Mef2 mRNA and protein expression, as well as abnormal muscle development and function. Conversely, overexpression of miR-92b reduced Mef2 levels and caused muscle attachment defects similar to the effects produced by Mef2 RNAi, while Mef2 overexpression could rescue these muscle attachment defects. Our results suggest that miR-92b plays an essential role in regulating Mef2 levels during *Drosophila* muscle development through a negative-feedback circuit with Mef2.

MATERIALS AND METHODS

***Drosophila* genetics**

All stocks were maintained and crossed at 25°C according to standard procedures. The miR-92b locus deletion was generated by using P-element insertion lines (d04814 and e00979 from the Exelixis collection at the Harvard Medical School) following reported methods (Parks et al., 2004). The miR-92b-sponge construct was generated by introducing ten repetitive microRNA complementary sequences (synthesized by GenScript) as shown in Fig. 5E separated by a four-nucleotide linker CGCG into the pUAS construct. The following fly stocks were used: miR-92b-2.7kb-GFP, miR-92b-0.8kb-GFP, miR-92b-0.8kb-mut-GFP, UAS-miR-92b, UAS-miR-92b-sp, UAS-miR-92b; UAS-Mef2, Hand-GFP (Callahan et al., 1996), Mef2-Gal4, Kr/Cyo-Mef2-Gal4-UAS-mCD8-RFP (Bloomington Stock Center) and UAS-Mef2-RNAi (VDRC, Transformant ID: 15550).

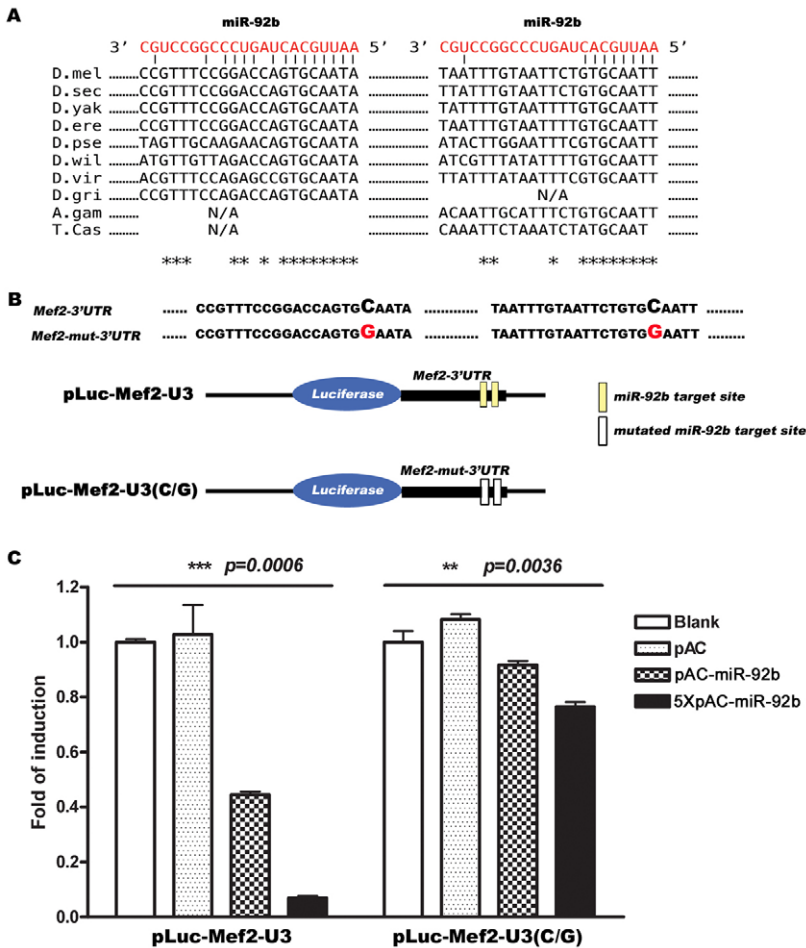


Fig. 2. miR-92b negatively regulates Mef2 through binding to conserved miR-92b targeting sites on Mef2 3'UTR. (A) miR-92b targeting sites in the 3'UTR of Mef2 gene are evolutionarily conserved. Identical nucleotides are marked with an asterisk. (B) Construction of the luciferase reporters pAc-luc-Mef2-U3 and pAc-luc-Mef2-U3 (C/G) for functional testing of Mef2 3'UTR. (C) Co-transfection of miR-92b in *Drosophila* S2 cells significantly inhibited luciferase activity regulated by Mef2 3'UTR in a dose-dependent manner. Mutation of the two miR-92b targeting sites largely abolished this inhibition. *** $P<0.005$; ** $P<0.05$; Student's t -test. Data are mean \pm s.e.m.

Immunohistochemistry and microscopy

Immunostaining of *Drosophila* embryos was performed as described previously (Kiriakidou et al., 2004), except for the actin-phalloidin staining when the embryos were exposed to methanol for as short a time as possible. The following primary antibodies were used: α -Mef2 (a gift from B. Paterson, NCI, Bethesda, MD, USA). Primary antibodies were detected with Alexa488- or Alexa633-conjugated secondary antibodies (Molecular Probes, Carlsbad, CA) as indicated. ⁴⁸⁸- and ⁵⁵⁵-phalloidin (Cytoskeleton) were used to detect actin filaments as indicated. Images were obtained with a Zeiss LSM510-Meta confocal microscope. Signal intensity was obtained from the LSM510-Meta software.

Fluorescent in situ hybridization

Fluorescent in situ hybridization was performed as described previously (Lécuyer et al., 2008) except that we used DIG-labeled-LNA-miR-92b probes (EXIQON) to detect miR-92b. Tyramide Signal Amplification (TSA; Molecular Probes) was used to detect labeled RNA probes.

Immunoblot analysis

Embryos after indicated aging time were collected from grape-juice agar plates with tap water and transfer to nylon mesh well. Embryos were washed three times with tap water before being dechorionated in 50% bleach for 2 minutes. Embryos were then washed three times with tap water to eliminate the trace bleach. To prepare the total protein sample, 50 μ l embryos were mixed with 300 μ l 2 \times Laemmli sample buffer (Sigma, USA) and heated for 5 minutes in boiling water (Wodarz, 2008). Western blot was performed as described previously (Chen et al., 2011). Briefly, proteins were resolved by SDS-PAGE (10%), transferred to a nitrocellulose membrane, and then blocked by incubation for 60 minutes at room temperature with 5% (w/v) non-fat dry milk in TBST. All the membranes were then incubated overnight at 4°C with primary antibodies diluted in TBST with 5% BSA. The membranes were then washed and incubated for 1 hour at room temperature with horseradish peroxidase-conjugated secondary antibodies (Jackson ImmunoResearch). Immune complexes were detected by enhanced chemiluminescence (Pierce). Blot images were analyzed with ImageJ.

Luciferase assay

S2 cells transfected with pMef2-U3-luciferase or pMef2-U3(C/G)-luciferase reporter gene with or without pAc-miR-92b vector were cultured in plates for 24 hours before assayed for the luciferase activity. Equal cell numbers were collected for each assay and the luciferase activity was measured by a luminometer using Luciferase Assay System (Promega).

RT-PCR

Embryos from 2-hour collections were aged for 14 hours at 25°C (or 10 hours at 29°C if indicated) and individually inspected and sorted to ensure that all were at the correct stage. Homozygous mutant embryos were chosen based on the absence of YFP fluorescence in the balancer chromosome. Embryos were collected and rinsed with PBS and then dechorionated in 30% bleach, followed by homogenization and total RNA isolation using Trizol reagent (Invitrogen). First-strand cDNA was primed with oligo-dT using the SuperScript First-strain cDNA synthesis kit (Invitrogen). Primers for RT-PCR are as follows: rp-49-sense, 5'-TACAGGCCCAAGATCGTGAA-3'; rp-49-antisense, 5'-ACCGT-TGGGGTTGGTGGAG-3'; Mef2-sense, 5'-CAACGACAGAGCCAG-ACAT-3'; Mef2-antisense, 5'-TGCCAGACGCACATCC-3'. Images were analyzed with ImageJ.

Body wall contraction assay

Adults were allowed to lay eggs on grape juice agar plates for 2 hour. After aging at indicated temperature for 16 hours, 1st instar larvae were picked and placed on a new grape juice agar plate. For a single larva, the number of body wall contractions (BWCs) that occurred in three different 30-second periods were recorded, averaged and converted to BWCs per minute.

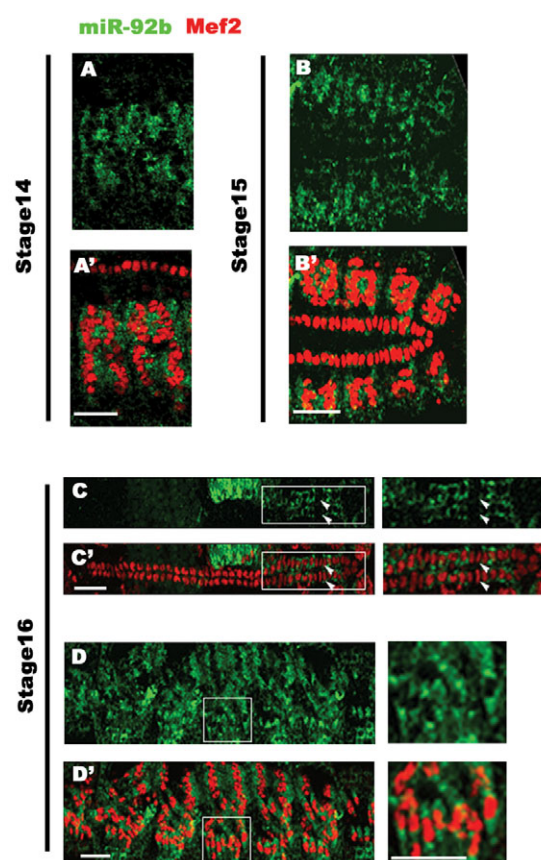


Fig. 3. miR-92b is a heart- and muscle-specific microRNA. (A-D') Confocal images of miR-92b in situ (green) and anti-Mef2 antibody (red) in wild-type embryos. (A,A') Lateral view of stage 14 embryo showed the initial expression of miR-92b in the somatic muscles. (B,B') Dorsal view of stage 15 embryo showed miR-92b expression in cardioblasts and somatic muscles. (C,C') Dorsal view of stage 16 embryo showed miR-92b expression in a subset of cardioblasts. Enlarged area shows posterior part of the heart (arrowheads indicate one pair of ostia). (D,D') Lateral view of stage 16 embryo showed miR-92b expression in somatic muscles. Enlarged area showed lateral transversal muscles of one hemi-segment. Scale bars: 25 μ m.

Livability assay

To test the effect in lethality of deletion miR-92b and expression miR-92b-sp, we set up three cages of Δ miR-92b/Tm6B-dfd-YFP flies, three cages for miR-92b-2.7kb-GFP; Δ miR-92b/Tm6B-dfd-YFP flies, and other three cages of Kr/Cyo-mCD8RFP-Mef2-Gal4>UAS-miR-92b-sp. Flies were allowed to lay eggs on grape juice agar plates for 2 hour and aging at 25°C (Δ miR-92b groups) or at 29°C (miR-92b-sp group) for indicated time period. Then the number of living homozygous and heterozygous Δ miR-92b larvae (Δ miR-92b groups), as well as that of RFP-positive and -negative larvae (miR-92b-sp group) on each plates were counted and converted to the livability.

RESULTS

Identification of miR-92b as a potential Mef2 regulator

Several computational methods have been developed to predict microRNA targeting sites in animals (Enright et al., 2003; Jones-Rhoades and Bartel, 2004; Kiriakidou et al., 2004). Using the online targeting prediction databases generated based on these methods, we searched the 3'UTR of the *Drosophila* Mef2 and found putative targeting sites for 35 microRNAs (Betel et al., 2010;

Lewis et al., 2005; Maragkakis et al., 2011; Ruby et al., 2007). Among these, 17 microRNAs have targeting sites that are conserved in 12 *Drosophila* species. Using in situ hybridization combined with previous published microRNA expression pattern data (Aboobaker et al., 2005; Aravin et al., 2003; Leaman et al., 2005), we found that only miR-92b (among these 17 microRNAs) was specifically expressed in the developing mesoderm when Mef2 becomes auto-activated at stages 15-16, suggesting that miR-92b is a possible regulator of Mef2.

miR-92b negatively regulates Mef2 levels by binding to Mef2 3'UTR in vitro

miR-92b is highly conserved from *Drosophila* to human (Fig. 1). There are two putative evolutionarily conserved miR-92b targeting sites in the 3'UTR of *Mef2* in all 12 *Drosophila* species, including distantly related *D. willistoni* and *D. virilis* (Fig. 2A). At least one miR-92b targeting site in *Mef2* 3'UTR can also be found in other insect species, including the mosquito *Anopheles gambiae* (*A. gam*) and the beetle *Tribolium castaneum* (*T. cas*) (Fig. 2A), suggesting that the binding of miR-92b to *Mef2* 3'UTR is evolutionarily conserved and functionally important. To test the function of these miR-92b targeting sites in *Mef2* 3'UTR, we generated a reporter construct with the full-length *Mef2* 3'UTR attached to the downstream *Firefly* luciferase gene driven by an Actin5C promoter (pAc-Luc-Mef2-U3, Fig. 2B). In parallel, we also generated a reporter construct in which the two miR-92b targeting sites in *Mef2* 3'UTR were mutated [pAc-Luc-Mef2-U3(C/G), Fig. 2B]. We found that the luciferase activity of the first reporter was dramatically inhibited in

Drosophila S2 cells co-transfected with pAc-miR-92b (Fig. 2C). This inhibition was dose dependent and could be enhanced using higher levels of miR-92b (Fig. 2C). Mutation of the miR-92b targeting sites in the *Mef2* 3'UTR largely abolished this inhibition (Fig. 2C), suggesting that miR-92b inhibits Mef2 expression through binding to the two conserved miR-92b targeting sites.

miR-92b is expressed in a similar pattern to that of Mef2

To investigate the spatial and temporal expression patterns of miR-92b, we performed fluorescent in situ hybridization using the locked nucleic acid (LNA) probe against miR-92b combined with Mef2 antibody staining. miR-92b was first detected in the somatic mesoderm and colocalized with Mef2 at stage 14 (Fig. 3A,A'), and then appeared in the cardioblasts at stage 15 (Fig. 3B,B'). By stage 16, miR-92b could be found in all somatic muscle cells, as well as a subset of cardioblasts (Fig. 3C,D'). In the heart, miR-92b was detected only in the posterior segments of cardioblasts with the exception of the *seven-up*-positive ostia cells (arrowheads indicate one pair of ostia cells in Fig. 3C,C'). miR-92b was detected in all somatic muscles in a pattern overlapping with Mef2 (Fig. 3D,D').

Mef2 activates miR-92b by direct binding to the miR-92b heart and muscle enhancer

In order to map the cis-regulatory region of miR-92b, we examined the enhancer activity of the entire 2.7 kb genomic region containing miR-92b (Fig. 4A). Embryos with the 2.7 kb enhancer-driven nuclear GFP showed heart- and muscle-specific expression patterns

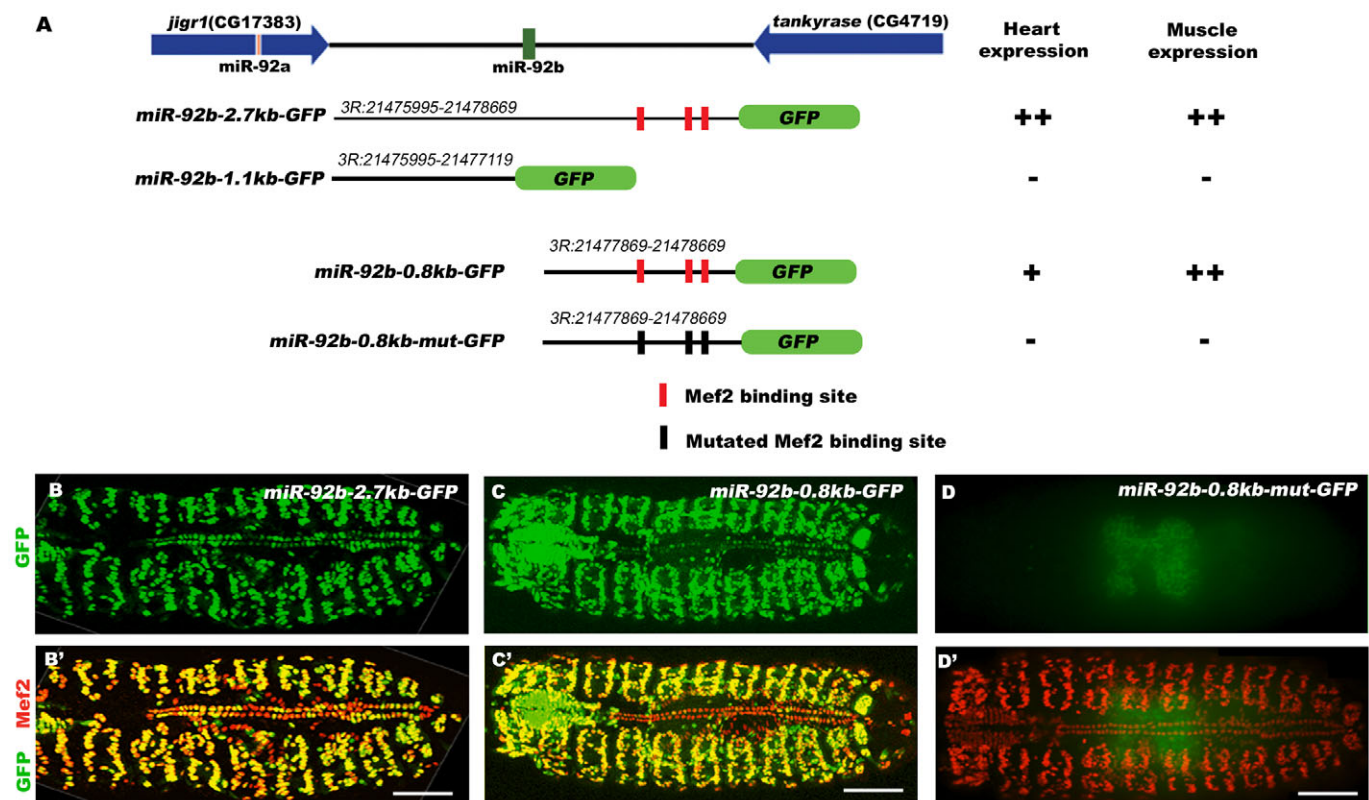


Fig. 4. miR-92b is directly activated by Mef2 through conserved Mef2-binding sites. (A) Cis-regulatory analysis of miR-92b. Levels of reporter expression are indicated by ++ (strong expression), + (weak expression) and - (no expression). (B-D') Confocal images of nuclear GFP reporter (green) and anti-Mef2 antibody (red) in stage 16 embryos of the 2.7 kb miR-92b enhancer (B,B'), 0.8 kb miR-92b enhancer (C,C') and the 0.8 kb miR-92b enhancer with Mef2-binding site mutated (D,D'). Scale bars: 50 μm.

similar to the miR-92b expression pattern we observed with in situ hybridization (Fig. 4B,B'). The expression pattern of this 2.7 kb enhancer also completely overlapped with that of Mef2 (Fig. 4B,B').

We further narrowed down the miR-92b enhancer region to a 0.8 kb fragment 3' to miR-92b, and identified three putative Mef2-binding sites in this region (Fig. 4A), which also are highly conserved among the 12 *Drosophila* species. Transgenic flies carrying the miR-92b-0.8kb-GFP reporter gene showed an expression pattern similar to the 2.7 kb miR-92b enhancer and to the miR-92b in situ hybridization results (Fig. 4C,C'; supplementary material Fig. S1). Moreover, mutation of the three conserved Mef2-

binding sites in the 0.8 kb miR-92b enhancer (Fig. 4A) completely abolished the activity of this enhancer (Fig. 4D,D'), suggesting that Mef2 directly activates miR-92b through these binding sites.

Deletion of miR-92b led to increased Mef2 levels and muscle defects

To address the function of miR-92b in heart and muscle development, we generated a miR-92b deletion allele using FRT-mediated deletion strategy (Parks et al., 2004) (Fig. 5A). Deletion of miR-92b was verified by genomic PCR (supplementary material Fig. S2). The homozygous miR-92b deletion mutant (Δ miR-92b) is lethal

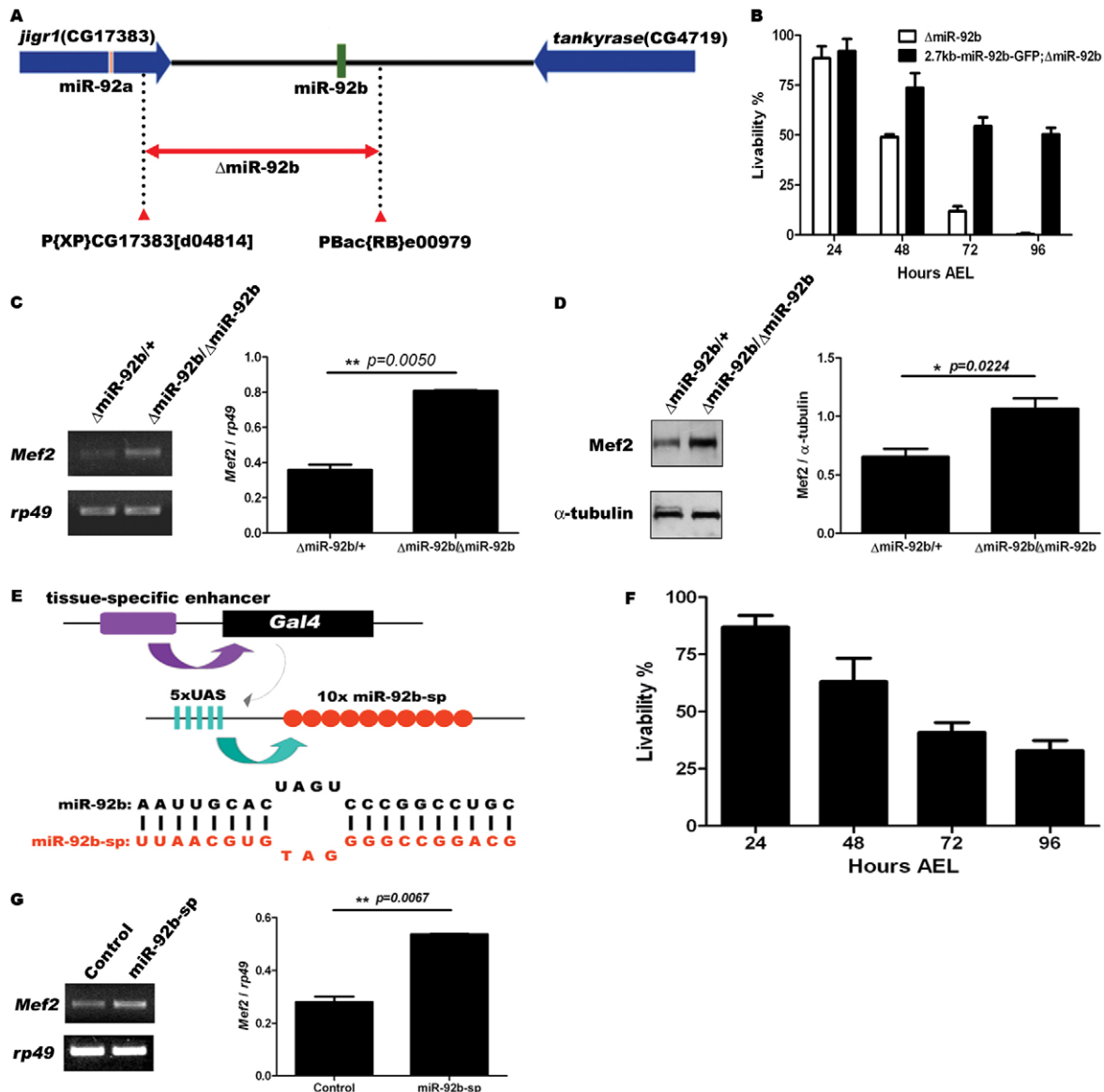


Fig. 5. miR-92b negatively regulates Mef2 levels in vivo. (A) Generation of the miR-92b deletion allele using Exelixis insertion lines. (B) Homozygous Δ miR-92b larvae died within 96 hours of egg laying (AEL) during 1st to 2nd instar stage. Reintroducing miR-92b into Δ miR-92b background could partially rescue the larval lethality. (C,D) Increased *Mef2* mRNA (C) and protein (D) levels were shown in stage16 Δ miR-92b embryos. *rp49* (*RpL32* – FlyBase) (C) and α -tubulin (D) were used as controls. Densitometric quantifications show the ratio of *Mef2* to *rp49* (C) or *Mef2* to α -tubulin (D). (E) The miR-92b-sponge cassette contains 10 copies of decoy miR-92b target sites in a UAS-containing vector. Transgenic flies with miR-92b-sp can be crossed to tissue-specific Gal4 lines and in turn induce the tissue-specific blocking of miR-92b in vivo. (F) Mesoderm-specific expression of miR-92b sponge partially induced larval lethality. (G) Targeted expression of miR-92b-sponge in muscles increased *Mef2* mRNA level. *rp49* was used as a control. Densitometric quantifications show the ratio of *Mef2* to *rp49*. Data are mean \pm s.e.m.

at larval stage (Fig. 5B). To test the effect of miR-92b on Mef2 levels, we examined both the mRNA and the protein levels of Mef2 in stage 16 homozygous Δ miR-92b embryos. Compared with their heterozygous siblings, Δ miR-92b homozygous embryos showed significantly increased mRNA and protein levels of Mef2 (Fig. 5C,D), indicating that miR-92b is essential in restricting the Mef2 expression level to an appropriate range during muscle development.

The miR-92b deletion allele we generated also affected another gene called *jigr1* (CG17383), which is located upstream of miR-92b (Fig. 5A; supplementary material Fig. S2). To address the possibility that the defects in the Δ miR-92b allele were caused by mutations of other genes, we put the miR-92b back into the Δ miR-92b background using the miR-92b-2.7kb-GFP transgene, as this transgene contains all the cis-regulatory information as well as

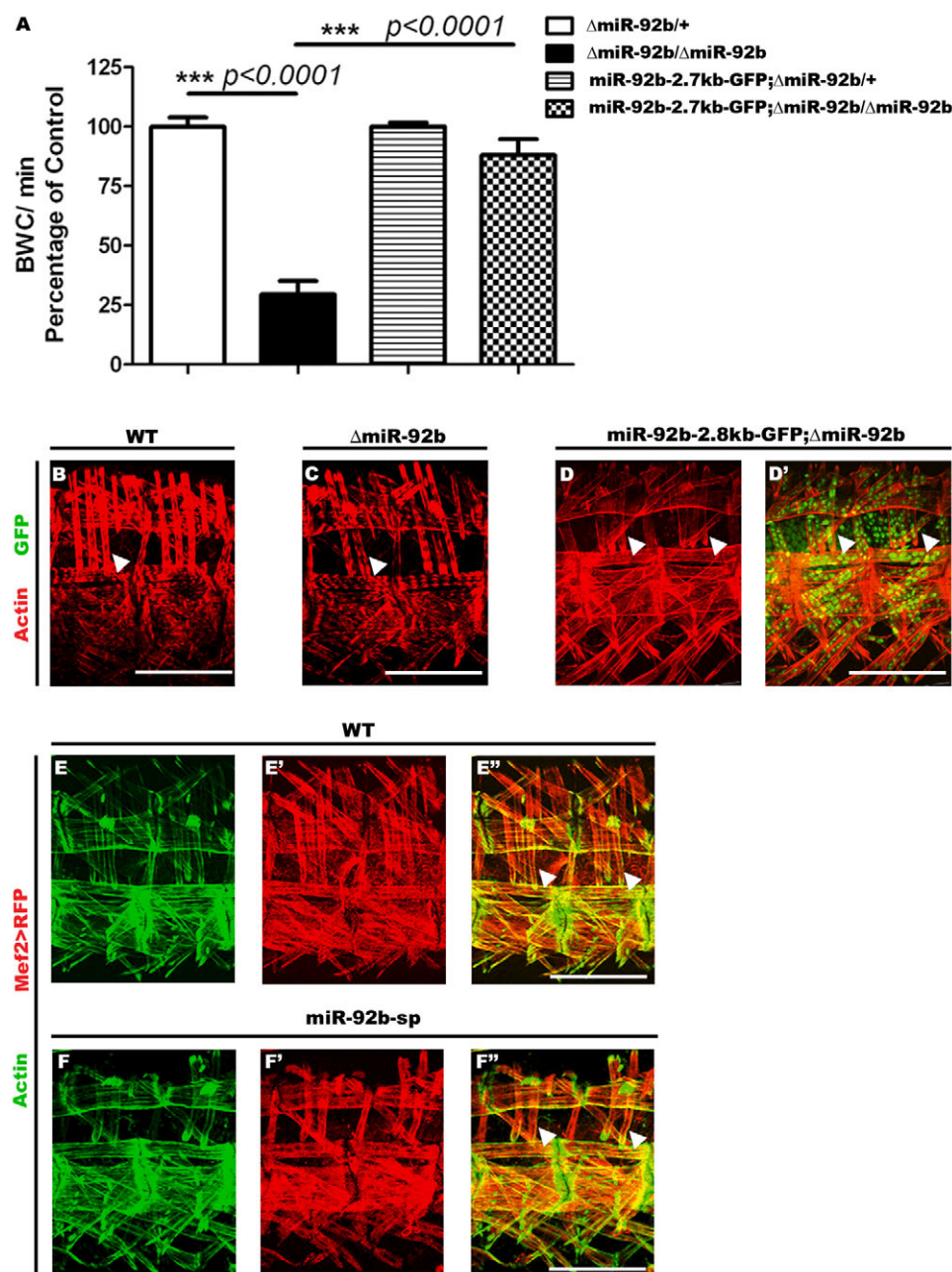


Fig. 6. miR-92b is required for muscle development and function. (A) Homozygous Δ miR-92b larvae showed reduced body wall contraction (BWC) compared with their heterozygous siblings. Reintroducing miR-92b into the Δ miR-92b background could rescue the BWC defects caused by deficiency of miR-92b ($n=10$; *** $P < 0.0001$ Student's t -test). Data are mean \pm s.e.m. (B-D') Confocal images of somatic muscles stained with phalloidin (red) in stage 16 embryos. (B,C) The lateral transversal muscles (indicated by arrowheads) were often missing in Δ miR-92b embryos (70.5%, $n=44$) (C) when compared with the wide-type embryos (B). (D,D') Reintroducing miR-92b back into Δ miR-92b background rescued the missing lateral transversal muscle defects (88%, $n=25$). (E-F'') Overexpression of miR-92b-sponge using Mef2-Gal4 (F-F'') also caused loss of the lateral transversal muscles (indicated by arrowheads) (38.9%, $n=36$) compared with wild-type embryos (E-E''). Somatic muscles in stage 16 embryos were shown by both phalloidin-staining (green) and UAS-RFP (red) driven by Mef2-Gal4. Scale bars: 50 μ m.

miR-92b itself (Fig. 5B). We found that 52.2% of homozygous Δ miR-92b flies ($n=92$) could be rescued and lived to adulthood, indicating that the observed phenotypes and lethality of Δ miR-92b allele were mainly caused by the lack of miR-92b.

Blocking the access of miR-92b to its targeting sites increased Mef2 levels

To further investigate the precise spatiotemporal requirement of miR-92b, we used the microRNA sponge technology (Loya et al., 2009) to generate a UAS-miR-92b-sponge transgene, which contains 10 repetitive sequences complementary to miR-92b with mismatches at the position 9-12 for enhanced stability (Ebert et al., 2007) (Fig. 5E). Blocking the miR-92b function using Mef2-Gal4 driven UAS-miR-92b-sp caused partial lethality at larval stage (Fig. 5F, aging at 29°C) and increased Mef2 mRNA levels similar to the Δ miR-92b allele (Fig. 5G).

These results indicate that the miR-92b sponge could efficiently compete with the endogenous miR-92b targeting sites, including the ones in *Mef2* 3'UTR, and subsequently release the inhibition of Mef2 expression caused by miR-92b.

miR-92b is required for muscle development and function

Homozygous Δ miR-92b embryos survived to early larval stage but showed sluggish movement when compared with their heterozygous siblings, suggesting muscle functional defects. We performed a body wall contraction (BWC) assay on 1st-instar Δ miR-92b larvae to analyze quantitatively their muscle functional defects (Sokol and Ambros, 2005). Δ miR-92b homozygous larvae showed significantly reduced BWCs per minute compared with their heterozygous siblings (Fig. 6A), suggesting that miR-92b was required for normal larval locomotion.

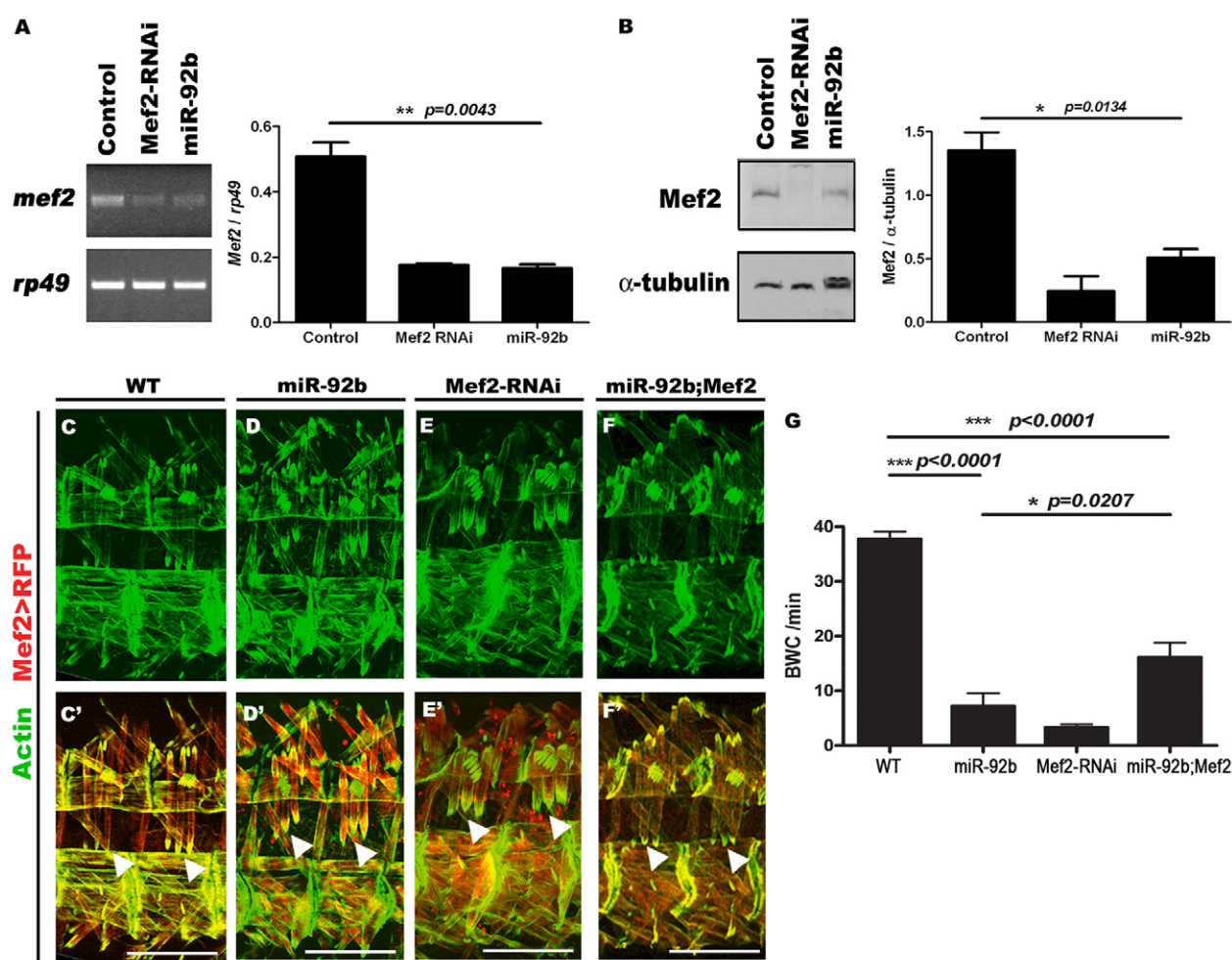


Fig. 7. Ectopic miR-92b in muscles reduced Mef2 levels and led to muscle extension and attachment defects. (A) RT-PCR using stage 16 embryos with Mef2-Gal4 driven miR-92b overexpression showed that ectopic miR-92b led to reduced *Mef2* mRNA levels similar to the effect of Mef2 RNAi knock down. *rp49* (*RpL32* – FlyBase) is used as a loading control. Densitometric quantifications show the ratio of *Mef2* to *rp49*. (B) Western blot using Mef2 antibody and stage 16 embryos showed that ectopic miR-92b led to reduced Mef2 protein levels, similar to the effect of Mef2-RNAi knock down. α -Tubulin is used as a loading control. Densitometric quantifications show the ratio of Mef2 to α -tubulin. (C–F) Somatic muscles of stage 16 embryos labeled with phalloidin (green) and UAS-RFP (red) driven by Mef2-Gal4. (D,D') Ectopic miR-92b led to muscle extension and attachment defects (indicated by arrowheads), a phenotype also observed in embryos with Mef2-RNAi (E,E'). These muscle defects caused by ectopic miR-92b were rescued by Mef2 overexpression (F,F'). (G) Body wall contraction (BWC) assays showed impaired muscle function in early larvae with ectopic miR-92b expression or Mef2 RNAi. The reduced BWC can be partially rescued by simultaneous Mef2 overexpression ($n=10$; *** $P<0.0001$; * $P<0.05$ Student's *t*-test). Data are mean \pm s.e.m. Scale bars: 50 μ m.

As embryonic somatic musculature gives rise to larval body wall muscle, we wondered whether defects could also be seen in embryonic musculature of Δ miR-92b embryos. Using fluorescent-labeled phalloidin, which recognizes filamentous actin and highlights the muscle structure, we found that although the overall muscle structure was not dramatically affected in Δ miR-92b embryos, lateral transversal muscle fibers were often missing (arrowheads in Fig. 6B) (70.5%, $n=44$). When we reintroduced miR-92b into the Δ miR-92b background using miR-92b-2.7kb-GFP transgene, both BWC defects of 1st instar larvae (Fig. 6A) and embryonic musculature defects (arrowheads in Fig. 6D,D') (88%, $n=25$) could be rescued. Similar muscle defects were found in embryos with miR-92b-sponge overexpression (38.9%, $n=36$) (arrowheads indicate missing muscle fibers in Fig. 6F-F'), suggesting that miR-92b was required for the formation of normal embryonic musculature.

miR-92b repressed Mef2 expression in vivo in a dose-dependent manner

To further test the inhibitory function of miR-92b on Mef2 expression and its biological function, we generated a UAS-miR-92b transgenic line for a gain-of-function assays. Overexpression of miR-92b using a muscle-specific driver resulted in a dramatic reduction of *Mef2* mRNA (Fig. 7A) and protein levels (Fig. 7B), suggesting that ectopic miR-92b could repress Mef2 expression in vivo.

To test the effect of ectopic miR-92b on muscle development, we examined the muscle structure in stage 16 embryos with UAS-miR-92b driven by Mef2-Gal4 (aging at 29°C, Fig. 7D,D'). We found that the lateral transversal muscles of stage 16 embryos with ectopic mesodermal expression of miR-92b always failed to reach the muscle attachment sites (MASSs) adjacent to the dorsal border of the lateral longitudinal muscle (arrowheads in Fig. 7D,D', compared with wild type in Fig. 7C,C' with arrowheads pointing to the MASSs). Interestingly, similar muscle extension and attachment defects were found in stage 16 embryos when Mef2 was knocked down using UAS-Mef2-RNAi (aging at 25°C, Fig. 7E,E'). These results indicate that normal Mef2 levels were necessary for muscle extension and attachment, and ectopic miR-92b could reduce Mef2 levels below the required threshold. Mef2 overexpression could rescue the lateral longitudinal muscle extension and attachment defects caused by ectopic miR-92b (Fig. 7F,F'). The ectopic miR-92b-induced muscle functional defect could also be partially rescued by Mef2 overexpression (Fig. 7G). These rescue experiments suggest that one essential role of miR-92b is to regulate Mef2 levels.

DISCUSSION

A precise control of Mef2 level is important for muscle development and function, but how this is achieved was unclear. In this study, we identified a novel negative-feedback circuit between miR-92b and Mef2, which is essential for the maintenance of homeostasis in muscle development through precise regulation of Mef2 level in muscle cells. This is the first example of a direct negative-feedback loop between a transcription factor and a microRNA in *Drosophila*. Mef2 activates the expression of miR-92b through three conserved Mef2-binding sites located in the miR-92b cis-regulatory region. Mature miR-92b in turn represses Mef2 expression via two conserved miR-92b targeting sites in the *mef2* 3'UTR (Fig. 8). This negative-feedback circuit counteracts the Mef2 auto-activation machinery to maintain Mef2 levels in an appropriate range for muscle development.

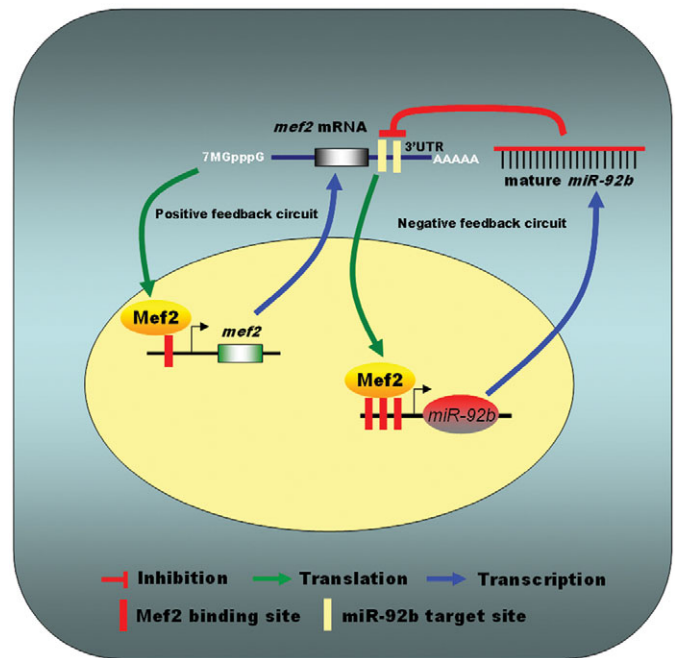


Fig. 8. A model of the regulatory feedback loop between miR-92b and Mef2. To balance its self-activation circuit, Mef2 activates miR-92b expression through direct binding on the miR-92b enhancer. Matured miR-92b binds to the Mef2 3'UTR to reduce the expression level of Mef2, which in turn keeps miR-92b at a low expression level. This negative-feedback loop efficiently keeps both Mef2 and miR-92b levels within the normal range.

It has been shown that different muscle genes require different levels of Mef2 activity to be turned on (Elgar et al., 2008), and these levels might contribute to the differentiation of various muscle cell types. The regulatory circuit between miR-92b and Mef2 provides an additional layer of regulation of Mef2 activity, previously undescribed. It is worth noting that Mef2 is not the only target for miR-92b, and the phenotypes we observed from the miR-92b loss- and gain-of-function studies are not solely caused by Mef2 level changes. When comparing the muscle phenotypes of miR-92b deletion, miR-92b sponge and the Mef2 overexpression, we found that the muscle defects in Mef2 overexpression embryos varied a lot from embryo to embryo, including cell shape changes, missing muscle fiber, duplication of muscle fibers, etc. (Z.C., Y.Z. and Z.H., unpublished). These observations suggest that the overexpression of Mef2 affects muscle development in a unique way when compared with the miR-92b deletion or miR-92b sponge overexpression. These results indicate that miR-92b has other target genes involved in muscle development besides Mef2. Using a combination of different microRNA targeting site prediction databases, we have found over 100 possible target genes for miR-92b and have confirmed several of them using luciferase reporter assays (S.L., Z.C. and Z.H., unpublished). Therefore, the level of miR-92b is also important and needs to be precisely controlled. The negative-feedback circuit between miR-92b and Mef2 provides an efficient mechanism to keep the level of both components stable in order to maintain the homeostasis of muscle development.

miR-92b is highly conserved from *Drosophila* to human, but its biological functions during development have not been studied in vertebrates. Significant changes in miR-92b expression levels have been observed in the process of neointima formation in a rat model

of vascular injury (Ji et al., 2007), in individuals with heart failure (Goren et al., 2012), and in individuals with a cardiac rehabilitation following surgical coronary revascularization (Taurino et al., 2010), indicating that miR-92b is involved in visceral and cardiac muscle differentiation or regeneration. As Mef2 genes are also involved in these processes, it is likely that miR-92b may play a conserved role in regulating Mef2 levels in mammals. Interestingly, we found putative miR-92b targeting sites in the 3'UTRs of *Mef2a*, *Mef2c* and *Mef2d* in both mouse and human, indicating that the inhibitory role of miR-92b on Mef2 could be evolutionarily conserved from *Drosophila* to humans. A direct negative-feedback circuit between Serum Response Factor (SRF) and miR-133 (Liu et al., 2008; Zhao et al., 2005) has been identified in mouse during smooth muscle development. The discovery of a similar direct negative-feedback circuit between Mef2 and miR-92b in *Drosophila* suggests that negative-feedback circuits between transcription factors and microRNAs could be broadly present as an evolutionarily conserved regulatory mechanism during development.

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Author contributions

Z.C. and Z.H. designed the research; Z.C., S.L. and Y.Z. performed the research; Z.C. and Z.H. analyzed the data; Z.C. and Z.H. wrote the paper.

Competing interests statement

The authors declare no competing financial interests.

Supplementary material

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