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Functional analysis of slow myosin heavy chain 1 and myomesin-3 in sarcomere organization in zebrafish embryonic slow muscles

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Abstract

Myofibrillogenesis, the process of sarcomere formation, requires close interactions of sarcomeric proteins and various components of sarcomere structures. The myosin thick filaments and M-lines are two key components of the sarcomere. It has been suggested that myomesin proteins of M-lines interact with myosin and titin proteins and keep the thick and titin filaments in order. However, the function of myomesin in myofibrillogenesis and sarcomere organization remained largely enigmatic. No knockout or knockdown animal models have been reported to elucidate the role of myomesin in sarcomere organization *in vivo*. In this study, by using the gene-specific knockdown approach in zebrafish embryos, we carried out a loss-of-function analysis of *myomesin-3* and *slow myosin heavy chain 1* (*smyhc1*) expressed specifically in slow muscles. We demonstrated that knockdown of *smyhc1* abolished the sarcomeric localization of myomesin-3 in slow muscles. In contrast, loss of *myomesin-3* had no effect on the sarcomeric organization of thick and thin filaments as well as M- and Z-line structures. Together, these studies indicate that myosin thick filaments are required for M-line organization and M-line localization of myomesin-3. In contrast, myomesin-3 is dispensable for sarcomere organization in slow muscles.

Keywords

Myosin; Myomesin 3; M-line; Sarcomere

INTRODUCTION

Sarcomere, the basic contractile unit in skeletal and cardiac muscles, is one of the most highly ordered macromolecular assemblies in cells. Defective sarcomere organization in skeletal and cardiac muscles leads to muscular dystrophies and cardiomyopathies. Assembly of ordered sarcomere structures, also called myofibrillogenesis, requires the integration of hundreds of proteins in a sequential, regulated manner by specific protein-protein

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interactions that link various components of sarcomere structure together. The sarcomere is divided into four major compartments including the Z-line, M-line, thick and thin filaments. It has been well documented that the Z-line which anchors the actin filaments, and the M-line which is thought to cross-link the myosin filaments, play pivotal roles in sarcomere organization of thick and thin filaments (Agarkova and Perriard, 2005). Although the sarcomeric structure has been well characterized, the regulatory mechanisms of myofibrillogenesis that lead to the formation of this highly organized structure is, however, not yet completely understood.

The thick filaments in a sarcomere of skeletal muscles consist of several hundreds of myosin II molecules that contain two heavy chains with the globular head and long α-helical tail domains as well as two pairs of light chains called the essential and regulatory light chains. Myosin II molecules group together to form highly regular bipolar structures called bipolar thick filaments. The globular myosin heads are positioned on either side of the filament, and the tail regions are clustered in the middle. This geometry enables myosin II molecules in thick filaments to pull from either side, generating contractile forces. It has been reported that thick filament assembly and many aspects of myofibrillogenesis are independent of the myosin head and these processes are regulated by the myosin rod and tailpiece (Cripps et al., 1999).

The central portion of each bipolar thick filament is connected to its neighbors by proteins of the M-line. The M-line is a transverse structure in the center of the sarcomere which is thought to stabilize the thick filament lattice. The muscle-specific protein myomesin is a major candidate for the role of M-line bridges to regulate packing of thick filaments and to uniformly distribute the tension over the myosin filament lattice in the activated sarcomere (Agarkova et al., 2003). Three myomesins, namely myomesin-1, -2 and -3, have been identified in skeletal and cardiac muscles in vertebrates. Myomesin-1 is ubiquitously expressed in all types of striated muscles (Agarkova et al., 2004). Myomesin-2 (also known as M-protein), on the other hand, is predominantly expressed adult heart and fast-twitch skeletal muscles (Agarkova et al., 2004). In contrast, myomesin-3 is specifically expressed in slow muscles (Schoenauer et al., 2008). Although a proof for the essential function of myomesin awaits the generation of a knockout model, it has been shown that mice with a conditional knockout for M-band region of titin (including the myomesin binding site) resulted in muscle weakness and progressive sarcomere disassembly (Gotthardt et al., 2003; Musa et al., 2006). Moreover, it has been reported that downregulation of myomesin by siRNA interference disrupted M-band structure leading to sarcomere disorganization in cultured neonatal rat cardiomyocytes (Fukuzawa et al., 2008). A recent report showed that a myomesin mutation is associated with hypertrophic cardiomyopathy (Siegert et al., 2011). However, a direct genetic evidence of myomesin function in myofibrillogenesis and sarcomere organization is still lacking. A proof for the essential function of myomesin awaits the generation of a knockout or knockdown animal models.

In this study, we knocked down the expression of slow myosin heavy chain 1 (s*myhc1*) and *myomesin-3* expressed in slow muscles of zebrafish embryos and compared the muscle phenotypes in the sarcomere organization. We demonstrated that knockdown of *smyhc1* and *myomesin-3* had a different effect on sarcomere organization. Thick filament formation and M-line localization of myomesin-3 were completely disrupted in slow muscles of *smyhc1* knockdown zebrafish embryos. In contrast, loss of *myomesin-3* had no effect on the sarcomeric organization. Normal organization of thick and thin filaments as well as Z-lines was observed in slow muscles of *myomesin-3* knockdown embryos. Together, these studies suggest that myosin thick filaments are required for the organization of M-line structure and M-line localization of myomesin-3. In contrast, myomesin-3 is dispensable for M-line formation and sarcomere organization in slow muscles of zebrafish embryos.

Materials and methods

Zebrafish maintenance

Adult zebrafish were raised at the zebrafish facility of the Aquaculture Research Center, Institute of Marine and Environmental Technology, USA. The fish were maintained at 28°C with a photoperiod of 14 h light and 10 h dark, in 8-gallon aquaria supplied with freshwater and aeration. The *slo*^{tu44c} mutant zebrafish line was obtained from Tubingen Zebrafish Stock Center, USA (Granato et al., 1996). The *slo*^{tu44c} mutant carries a nonsense mutation in the *heat shock protein 90α1* gene (*hsp90α1)* resulting in truncated molecules missing the C-terminal domain, which is important for both homo- and heterodimerization (Hawkins et al., 2008; Ali et al., 2006). The myomesin-RFP zebrafish line [myom3(mnGt0067)] was obtained from Steve Ekker's laboratory at Mayo Clinic, USA. It carries a RFP gene trap insertion in the *myomesin-3* gene (Clark et al., 2011). Additional information can be found at http://www.zfishbook.org/index.php?topic=GBT0067#. The *Tg(ef1a:Smyd1b_tv1-GFP)* transgenic fish were generated in our laboratory (Xu and Du, unpublished).

Synthesis of morpholino antisense oligos

Morpholino antisense oligos were synthesized by Gene Tools (Corvalis, OR, USA). The smhyc1-MO was targeted to the sequence flanking the ATG start codon. The *Unc-45b* and *hsp90a1* morpholino oligos were targeted to the sequence flanking the ATG start codon. The control-MO was the standard control oligo purchased from Gene Tools.

smyhc1-MO: 5′-TCTAAAGTTTTACCCACTGCGGCAA-3′

Unc-45b ATG-MO: 5′-ATCTCCAATTTCTCCCATCGTCATT-3′

hsp90a1 ATG-MO: 5′-CGACTTCTCAGGCATCTTGCTGTGT-3′.

Microinjection in zebrafish embryos

Morpholino antisense oligos were dissolved in 1x Danieau buffer to a final concentration of 0.5 mmol/L or 1 mmol/L. Approximately 1–2 nL (5 ng or 10 ng) was injected into each zebrafish embryo at the 1 or 2 cell stages.

Analysis of smyhc1 expression in wild type or smyhc1-MO injected embryos by RT-PCR

Total RNA was extracted from 50 wild type and 50 *smyhc1*-MO injected zebrafish embryos at 24 hpf. *Smyhc1* expression was determined by RT-PCR using primers myhc1-UTR and myhc1-E4-R derived from the sequence in 5′-UTR and exon-4, respectively. The PCR products were cloned into pGEM-T easy vector and sequenced.

myhc1-UTR: 5′–CAAGGTACAGAGGTCTGACAAACA-3′

myhc1-E4-R: 5′–CTATCTGACAGCATGTACTGGTA-3′

Analysis of myomesin-3 and myomesin-3- RFP expression by RT-PCR

Total RNA was extracted from 50 wild type and 50 myomesin-3 RFP homozygous zebrafish embryos at 48 hpf. Expression of *myomesin-3* and myomesin-3-RFP transcripts was determined by RT-PCR using the following primers. The PCR products were cloned into pGEM-T easy vector and sequenced.

myom3rt-fp2: 5′-AGGCAGAAATCAGAGAGCATCTGG -3′

myom3rt-rp2: 5′-CTTCCTGGTCACCTTGCACTTCAT-3′

myom3rt-fp3: 5′-ATGAAGTGCAAGGTGACCAGGAAG-3′

myom3rt-rp4: 5′-GATATACACTGGACCAGCGGAGAG-3′

myom3-fp1: 5′-TCAGGACACACACAAGCGCTCGCTGGAT-3′

myom3-rp2: 5′-AGAGACTACATCTTTATTTATCAAGCAC-3′

mRP-fp1: 5′-GAACGGCCACGAGTTCGAGATCGAG-3′

mRP-rp2: 5′-CTCGTACTGTTCCACGATGGTGTAG-3′

Whole mount in situ hybridization

Whole mount in situ hybridization was carried out using digoxigenin-labeled antisense probe in the *smyhc1* knockdown and control embryos at 24 hpf (Cordina et al., 2010). Plasmid *pGEM-stnnc* was digested with *Spe* I and transcribed with T7 RNA polymerase to synthesize the digoxigenin-labled antisense RNA probe.

Whole-mount immunostaining

Immunostaining was carried out using whole-mount zebrafish embryos (1-3 days post-fertilization) as previously described (Tan et al., 2006; Du et al., 2008). Briefly, zebrafish embryos were fixed in 4% paraformaldehyde (in PBS) for 1 h at room temperature. The fixed embryos were washed for 15 min 3 times in PBST. Three day old embryos were digested in 1 mg/mL collagenase for 75 min. Immunostaining was performed with the following primary antibodies: anti-α-actinin (clone EA-53, #A7811, Sigma,USA), anti-MyHC for slow muscles (F59, DSHB, USA), anti-myosin light chain for fast muscles (F310, DSHB), anti-MyHC (MF-20, DSHB), anti-myomesin (mMaC myomesin B4, DSHB), anti-α–actin (Ac1-20.4.2, Progen, Germany), and anti-Prox1 antibody (AB5475, Millipore, USA). Secondary antibodies were FITC or TRITC conjugates (Sigma).

RESULTS

1. Blocking splicing of smyhc1 intron 1 resulted in nuclear localization of smyhc1 mRNA transcripts

Zebrafish embryos contain two major types of skeletal muscles, slow muscle and fast muscle, based on the expression of myosin heavy chain (MyHC). Smyhc1 represents the primary MyHC expressed in slow muscles of zebrafish embryos (Fig. 1A and B) that can be labeled specifically with F59 monoclonal antibody (Devoto et al., 1996; Bryson-Richardson et al., 2005; Elworthy et al., 2008). We have previously shown that a *smyhc1*-specific morpholino (*smyhc1*-MO) targeted to the ATG start codon could knock down *smyhc1* expression and resulted in disruption of thick and thin filament organization in zebrafish slow muscles (Cordina et al., 2010). Close examination of the *smyhc1*-MO sequence revealed that the last two nucleotides (….CT), in fact, could target to the splicing acceptor (…. AG) of intron 1 in the pre-mRNA (Fig. 1C). To determine whether *smyhc1*-MO could disrupt the *smyhc1* pre-mRNA splicing, we amplified the *smyhc1* transcripts from the *smyhc1*-MO injected embryos by RT-PCR (Fig. 2A). The data showed that unlike the uninjected control which showed only one PCR product of 542 bp, two PCR products were produced in *smyhc1*-MO injected embryos (Fig. 2B). One product of 542 bp (approximately 30%) was identical to that of the uninjected control. Another product (approximately 70%) was a longer product of 702 bp (Fig. 2B). Sequence analysis confirmed that the longer PCR product of 702 bp contained the unspliced intron 1 of 160 bp, confirming that the *smyhc1*-MO could block the splicing of *smyhc1* pre-mRNA, and resulted in the generation of mis-spliced *smyhc1* transcripts.

RNA splicing is required for nuclear exportation. To determine whether blocking the splicing of intron 1 could interfere with the nuclear to cytoplasmic exportation of *smyhc1*

pre-mRNA, we performed *in situ* hybridization on *smyhc1*-MO injected embryos. The data showed that in contrast to the control embryos that had cytoplasmic localization of *smyhc1* mRNAs (Fig. 3A and C), blocking intron 1 splicing indeed interfered with the nuclear to cytoplasmic exportation of *smyhc1* pre-RNA (Fig. 3B and D). A clear nuclear localization of *smyhc1* pre-mRNA transcripts was observed in slow muscles of smyhc1-MO injected embryos (Fig. 3B and D). The nuclear localization of *smyhc1* RNA is consistent with the significant reduction of MyHC protein expression in *smhyc1*-MO injected zebrafish embryos (Cordina et al., 2010).

To rule out the possibility that the nuclear localization was due to disruption of thick filament organization in slow muscles of zebrafish embryos, we analyzed *smyhc1* mRNA distribution in *smyd1b* knockdown and Hsp90α1mutant embryos that also showed disorganized myosin thick filaments (Tan et al., 2006; Du et al., 2008). The data showed that unlike the *smyhc1*-MO, disruption of thick filament organization by *smyd1b* knockdown or *hsp90α1* mutation had no effect on the nuclear to cytoplasmic exportation of *smyhc1* pre-mRNA (Fig. 3G and H). Together, these data indicate that the nuclear localization was not caused by defective thick filament organization, confirming the gene-specific effect of defective splicing in nuclear localization of pre-mRNA.

2. Knockdown of smyhc1 expression resulted in defective M-line organization in slow muscles

Previous studies have demonstrated that knockdown of *smyhc1* does not affect the specification of slow muscle precursors and early formation of slow muscles in zebrafish embryos (Cordina et al., 2010). However, knockdown of *smyhc1* expression resulted in defective organization of thick and thin filaments as well Z-line structures (Cordina et al., 2010). The effect on M-line structure in slow muscles could not be analyzed due to the lack of slow muscle-specific M-line markers available in zebrafish. A recent identification of a *myomesin-3-rfp* insertion line [myom3(mnGt0067)] from gene trapping provided a unique model to visualize M-line organization in slow muscles of zebrafish embryos (Clark et al., 2011). Consistent with the slow muscle specific expression of *myomesin-3*, the myomesin-3-RFP fusion protein is specifically expressed in slow muscles of zebrafish embryos and showed the M-line localization (Clark et al., 2011; Li et al., 2011).

To test whether myosin thick filaments are required for the sarcomeric localization of myomesin, we knocked down the expression of *smyhc1* in myom3(mnGt0067) zebrafish embryos using the *smyhc1*-MO (Fig. 4A–D). The sarcomeric localization of myomesin-3-RFP was examined in the control and *smyhc1*-MO injected embryos (Fig. 4E and F). The data showed that knockdown of *smyhc1* completely diminished the M-line localization of myomesin-3-RFP (Fig. 4F). To assess whether this also affected the M-line localization of other proteins, we compared the M-line localization of Smyd1-GFP in skeletal muscles of the control and *smyhc1* knockdown zebrafish embryos. The data showed that knockdown of *smyhc1* also blocked the M-line localization of Smyd1-GFP fusion protein in slow muscles of zebrafish embryos (Fig. 4H). Together, these data demonstrated that disruption of myosin thick filaments abolished the M-line localization of myomesin-3 and Smyd1-GFP, suggesting that the thick filaments may be required for M-line organization in slow muscles of zebrafish embryos.

3. Hsp90α1 and Unc45b are required for myomesin-3-RFP M-line organization in slow muscles

It has been shown that Hsp90α1 and Unc45b are myosin chaperones that play a pivotal role in myosin folding and sarcomere assembly (Etard et al., 2007; Wohlgemuth et al., 2007; Du et al., 2008; Hawkins et al., 2008; Bernick et al., 2010). Knockdown or mutation of *hsp90α1*

or *Unc45b* leads to complete disruption of sarcomere organization in thick and thin filaments of both slow and fast muscles (Etard et al., 2007; Wohlgemuth et al., 2007; Du et al., 2008; Hawkins et al., 2008; Bernick et al., 2010). However, their effects on M-line organization have not been analyzed in slow muscles. To characterize the function of Hsp90α1 and Unc45b on M-line organization, we analyzed myomesin-3-RFP localization in *hsp90α1* or *Unc45b* knockdown embryos. The results showed that knockdown of *hsp90α1* or *Unc45b* completely abolished the sarcomeric localization of myomesin-3-RFP in slow muscles (Fig. 5B, C). Together, these data indicate that Hsp90α1 and Unc45b may play a direct role in M-line organization. Alternatively, because Hsp90α1 and Unc45b are required for myosin thick filament assembly, the disruption of myomesin-3-RFP M-line localization could result indirectly from the defective thick filament organization from *hsp90α1* and *Unc45b* knockdown.

4. Disruption of myomesin-3 locus by RFP trapping had no effect on sarcomere formation in slow muscles

The gene trap integration site in myom3(mnGt0067) fish has been recently mapped to an intron site of *myomesin-3* gene (Clark et al., 2011). It has been reported that RFP gene trap integration resulted in over 99% reduction of the normal myomesin-3 gene expression (Clark et al., 2011). To better characterize the integration site and the effect of RFP insertion on *myomesin-3* gene expression, we analyze the expression of myomesin-3-RFP chimeric mRNA transcripts by RT-PCR and its sequence. Sequence analysis revealed that the RFP integration site was located within the intron 24 of *myomesin-3* (Fig. 6A). The RFP integration resulted in the production of a myomesin-3-RFP fusion protein that includes the N-terminal 957 aa residues of myomesin-3 followed by 13 aa residues coded by part of intron 24 plus a 11 aa residues coded by sequence upstream of the RFP coding sequence in the vector, and finally the RFP sequence (Fig. 6A). The myomesin-3-RFP fusion protein lacks the C-terminal sequence of 219 aa residues.

To determine the effect of RFP integration on the *myomesin-3* mRNAs expression, we carried out RT-PCR to amplify *myomesin-3* transcripts and myomesin-3-rfp fusion mRNA transcripts from total RNAs of the control or myomesin-3-RFP homozygous fish embryos as shown in Fig. 6A. The data showed a strong expression of *myomesin-3-rfp* fusion transcripts in myom3(mnGt0067) homozygous zebrafish embryos. However, no *myomesin-3* transcripts could be detected in the homozygous zebrafish embryos. This is consistent with the report that integration of the RFP trap significantly reduced the expression of the normal *myomesin-3* mRNA transcripts to less than 1% of the normal levels (Clark et al., 2011).

To determine whether disruption of *myomesin-3* expression by the RFP insertion could affect the M-line localization of myomesin-3, we analyzed the myomesin-3-RFP localization in myom3(mnGt0067) homozygous fish embryos and showed a normal M-line localization of myomesin-3-RFP in slow muscles (Fig. 7C), suggesting that the myomesin-3 N-terminal region contains the sequence required for the myomesin-3 M-line localization. To determine whether disruption of *myomesin-3* expression by the RFP insertion could affect sarcomere organization in slow muscles, we characterized the organization of thick and thin filaments and Z-lines in myom3(mnGt0067) homozygous fish embryos (Fig. 8). The results showed that blocking *myomesin-3* expression by RFP insertion had little or no effect on the sarcomere organization. Both thick and thin filaments as well as Z-lines appeared normal in slow muscles of myom3(mnGt0067) homozygous fish embryos (Fig. 8 B, E and H). Together, these data indicate that the sarcomere organization was not affected by the RFP insertion in the *myomesin-3* gene locus. This is unexpected considering that the RFP insertion in *myomesin-3* resulted in the production of a truncated myomesin-3, and a 99% reduction in the expression of wild type *myomesin-3* mRNA transcripts (Clark et al., 2011).

5. Knockdown of myomesin-3 had no effect on sarcomere organization in slow muscles of zebrafish embryos

To rule out the possibility that the myomesin-3-RFP might retain some myomesin-3 function involved in M-line organization, we performed a knockdown experiment in zebrafish embryos to directly inhibit myomesin-3 protein expression using a myomesin-3 specific translation blocker (myomesin-3 ATG-MO). The *myomesin-3* ATG-MO was injected into myom3(mnGt0067) embryos. The knockdown embryos appeared morphologically normal (Fig. 7B). The knockdown efficiency was monitored by examining myomesin3-RFP expression in the injected embryos at 48 hpf. The data showed that the myomesin-3 ATG-MO was highly effective in knocking down the expression of myomesin3-RFP (Fig. 7D). To assess the effect of myomesin-3 knockdown on M-line organization, we examined the M-line localization of Smyd1-GFP fusion protein (Xu and Du, unpublished). The data showed that M-line localization of Smyd1b-GFP was normal in myomesin-3 knockdown embryos (Fig. 7H), suggesting that the M-line structure was not disrupted by *myomesin-3* knockdown. To determine the effect of myomesin-3 knockdown on the organization of other sarcomere structures, the *myomesin-3* ATG-MO injected embryos were analyzed by immunostaining with antibodies against myosin thick filament, α-actin thin filament and α-actinin on the Z-lines. The data showed that knockdown of *myomesin-3* had no effect on thick and thin filament formation and Z-line organization (Fig. 8C, F and I). Collectively, these data indicate that myomesin-3 is dispensable for sarcomere organization in slow muscles of zebrafish embryos.

DISCUSSION

In this study, we analyzed the role of myosin heavy chain 1 and myomesin-3 in sarcomere organization in slow muscles of zebrafish embryos. We demonstrated that knockdown of *smyhc1* expression completely disrupted the sarcomeric localization of myomesin-3-RFP and Smyd1b-GFP on M-lines in skeletal muscles of zebrafish embryos. In contrast, disruption of *myomesin-3* expression by RFP insertion or knockdown had little effect on sarcomere organization in slow muscles. Together, these studies indicate that myosin thick filaments are required for M-line organization; however, myomesin-3 is dispensable for sarcomere organization and M-line formation in slow muscles.

Myosin thick filaments are required for M-line localization of myomesin-3 in slow muscles

By using the gene-specific knockdown approach, we demonstrated that myosin thick filaments are required for M-line localization of myomesin-3 and possibly M-line organization in zebrafish slow muscles. Knockdown of *smyhc1* expression completely abolished the M-line localization of myomesin-3-RFP and Smyd1b-GFP. The muscle phenotype was slow muscle specific, no disruptive effect was observed in fast muscles (Data not shown), consistent with previous findings with other sarcomere structures (Cordina et al., 2010). The *smyhc1* knockdown phenotype indicates a critical role for myosin thick filaments in the M-line organization.

Genetic and biochemical analyses have shown that chaperone-mediated myosin folding and assembly is an integral part of myofibrillogenesis during muscle development. Mutations of *Uun-45*, a myosin chaperone in *Caenorhabditis elegans*, result in paralyzed animals with severe myofibril defects in body wall muscles (Epstein et al., 1992, 1974; Barral et al., 1998; Landsverk et al., 2005; Moerman et al., 2006). Recent studies showed that Hsp90α1, which binds to UNC-45 and forms a complex with newly synthesized myosin proteins, also plays a vital role in myosin folding and myofibril assembly (Barral et al., 1998; Srikakulam et al., 2004; Etard et al., 2007; Du et al., 2008; Hawkins et al., 2008). Knockdown or mutation of *hsp90α1* or *Unc45b* resulted in defective myofibril organization in skeletal muscles (Etard et

al., 2007; Du et al., 2008; Hawkins et al., 2008). Consistent with their function in thick filament assembly and sarcomere organization, we demonstrated in this study that knockdown of *Unc45b* or *hsp90α1* completely abolished the sarcomeric localization of myomesin-3-RFP. The M-line defect in *Unc45b* and *hsp90α1* knockdown embryos could be explained by their direct function on M-line organization. Alternatively, the effect could result indirectly from the disruption of myosin thick filament organization in *Unc45b* and *hsp90α1* knockdown embryos.

Loss of myomesin-3 had little effects on myofibril organization

It has been suggested that myomesin plays a key role in myofibrillogenesis. Knockdown of *myomesin* by siRNA interference disrupted M-band structure leads to sarcomere disorganization in cultured neonatal rat cardiomyocytes (Fukuzawa et al., 2008). However, direct evidence from knockout or mutant models has yet to be obtained although cellular and biochemical studies have provided some indirect evidence supporting this idea. It has been shown that myomesin molecules bind with their N-terminal domains to the myosin rod, and myomesin can form antiparallel dimmers *via* their C termini, which might cross-link the neighboring thick filaments (Obermmann et al., 1997; Auerbach et al., 1999; Langer et al., 2005). In addition, myomesin can bind tightly to the titin C-terminal end extended into the M-line. The close interactions of myomesin with myosin and titin are critical for the sarcomere stabilization. Consistent with this idea, targeted homozygous C-terminal deletion of M-band titin prevents sarcomere formation in cardimyocytes (Musa et al., 2006). Moreover, M-line titin homozygous C-terminal truncations cause early-onset myopathy with fatal cardiomyopathy (Carmignac et al., 2007).

Surprisingly, data from our studies demonstrated that knockdown of *myomesin-3* expression had little or no effect on myofibril organization in slow muscles of zebrafish embryos. The M-line localization of myomesin-3-RFP and smyd1b-GFP appeared normal in homozygous myom3(mnGt0067) insertion mutant or *myomesin-3* knockdown embryos, respectively. Other sarcomeric structures, including thick and thin filaments as well as Z-lines, also appeared normal. This is in contrast to the *myosin* knockdown embryos that had little or no organized thin filaments, M- and Z-lines. Collectively, these data indicate that myomesin-3 is either not required for M-line organization in slow muscles or other *myomesin* genes expressed in slow muscles may have a redundant function that prevent the characterization of myomesin-3 knockdown effects. Consistent with this idea, our recent studies showed that zebrafish genome contains at least five *myomesin* genes (Xu and Du, unpublished).

Interestingly, we demonstrated that myomesin-3-RFP fusion protein showed a clear M-line localization in slow muscles of zebrafish embryos. It suggests that the N-terminal 957 aa residues retained in myomesin-3-RFP are sufficient to target myomesin-3-RFP onto M-lines. This is consistent with previous report that the unique myomesin N-terminal domain of approximately 138 residues is sufficient for binding with myosin in the central region of light meromyosin (Obermann et al., 1997). In addition, the three fibronectin type III repeats (My4-6) at the N-terminal region are sufficient to confer myomesin binding to a single titin immunoglobulin domain (Obermann et al., 1997). The M-line localization of myomesin-3-RFP fusion protein could be explained by its ability to bind with myosin and titin filaments.

Nuclear localization of defectively spliced *smyhc1* RNA transcripts

We demonstrated in this study that *smyhc1*-MO targeted to the ATG site and the splicing acceptor sequence (…AG) of intron 1 could block the intron 1 splicing and resulted in the nuclear retention of defectively spliced s*myhc1* pre-mRNA transcripts. This is consistent with previous reports that blocking pre-mRNA splicing by MO could lead to production of defectively spliced RNA transcripts with intron sequences that retain the RNA transcripts

within the nucleus (Yan et al., 2002; Hans et al., 2004). It has been suggested that blocking the splicing donor sequence of intron 1 could most likely result in the production of RNA transcripts with an intron insertion (Morcos, 2007). Our studies showed that targeting to the splicing acceptor of the first intron could also block intron 1 splicing and generate defectively spliced transcripts. In fact, such defective splicing is not limited to intron 1. It has been shown that MOs targeted to internal intron sequences could also produce defectively spliced mRNA transcripts that were unable to transport to the cytoplasm (Yan et al., 2002; Hans et al., 2004). Blocking the nuclear exportation of mRNA transcripts is thus a very effective approach to knock down gene expression for functional studies. In addition, blocking pre-mRNA splicing is also a powerful method to investigate the molecular regulation of pre-mRNA splicing in general, and to knock down the expression of specific isoform from alternative splicing.

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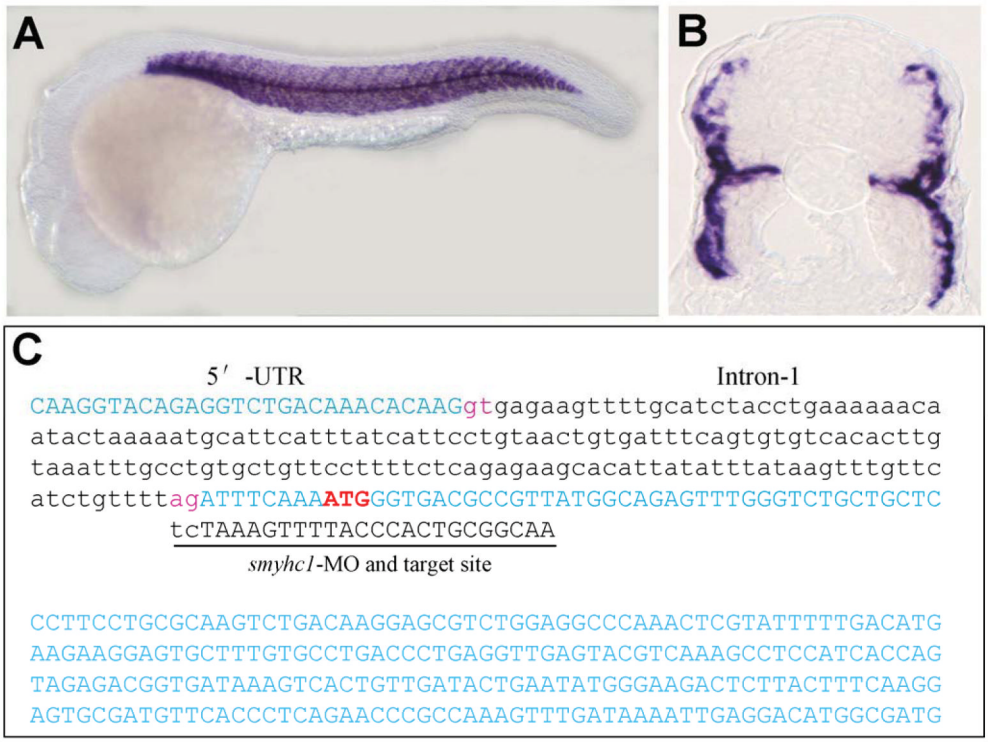

Fig. 1. Expression of *smyhc1* in zebrafish slow muscles and target sequence of smyhc1-MO

A and **B:** *In situ* hybridization shows the slow muscle specific expression of *smyhc1* in wild type zebrafish embryos at 24 hpf. **A**, side view; **B**, view of cross section. **C:** DNA sequence shows the smyhc1-MO targeted site at the ATG start codon and the intron 1 splicing acceptor. The 5′-UTR, intron 1 and partial coding sequence at the 5′ end are shown.

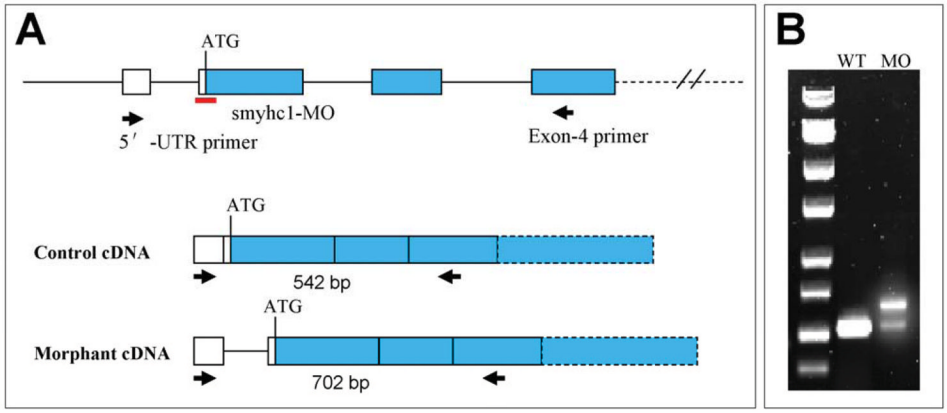

Fig. 2. Defective splicing of *smyhc1* pre-mRNA in *smyhc1*-MO injected zebrafish embryos

A: diagram shows the gene structure of smyhc1 at the 5′-UTR. The ATG start codon, MO target site, and PCR primers are indicated. **B:** RT-PCR analysis of *smyhc1* expression in the control and *smyhc1*-MO injected embryos at 24 hpf. Compared with the control, two PCR products were amplified in the *smyhc1*-MO injected embryos.

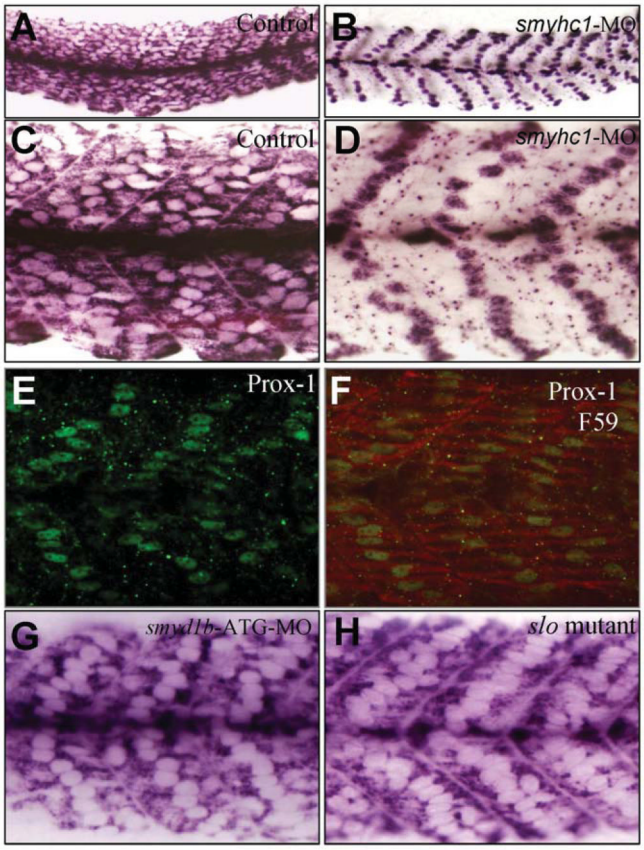

Fig. 3. The nuclear localization of *smyhc1* pre-mRNA in *smyhc1* ATG-MO injected embryos

A–D: *in situ* hybridization shows *smyhc1* mRNA localization in the control (**A**, **C**) or *smyhc1*-MO (**B**, **D**) injected embryos at 24 hpf. Nuclear localization was observed in the *smyhc1*-MO injected embryos (**B**, **D**). **E** and **F:** nucleus localization in slow muscles revealed by immunostaining with anti-Prox1 antibody (**E**), together with F59 antibody (**F**) for slow muscles in wild type embryo at 24 hpf. **G** and **H:** *in situ* hybridization shows the cytosolic localization of *smyhc1* mRNA in *smyd1* knockdown (**G**) or *Hsp90α1*(*slo*) mutant (**H**) embryos at 24 hpf.

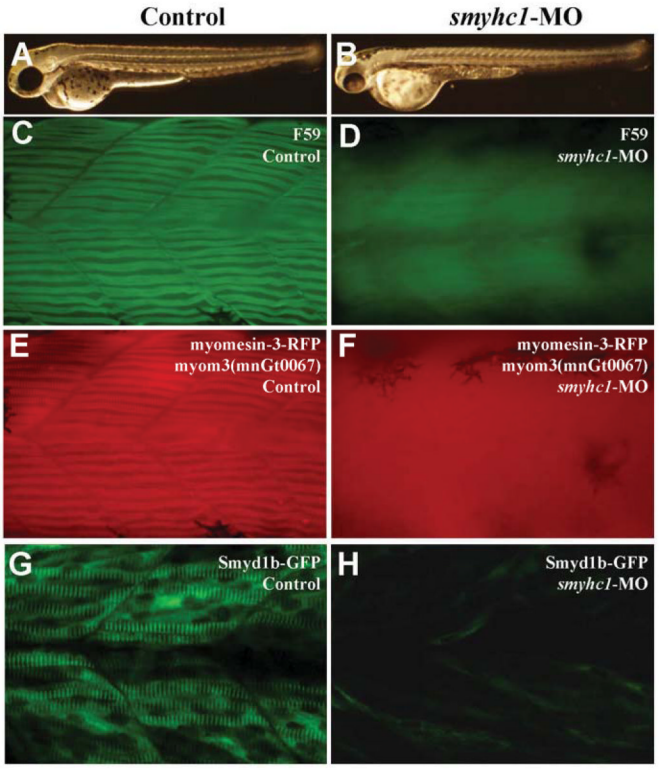

Fig. 4. Effects of *smyhc1* knockdown on the M-line localization of myomesin-3-RFP and smyd1b-GFP in zebrafish embryos

A and **B:** morphological comparison of the control (**A**) and *smyhc1*-MO injected (**B**) embryos at 48 hpf. **C** and **D:** immunostaining with antibody (F59) shows the Smyhc1 expression and thick filament organization in the control (**C**) and *smyhc1*-MO injected (**D**) embryos at 24 hpf. **E** and **F:** sarcomeric localization of myomesin-3-RFP in the control (**E**) or *smyhc1*-MO injected (**F**) zebrafish embryos at 24 hpf. **G** and **H:** sarcomeric localization of Smyd1b-GFP in the control (**G**) or *smyhc1*-MO injected (**H**) zebrafish embryos at 24 hpf

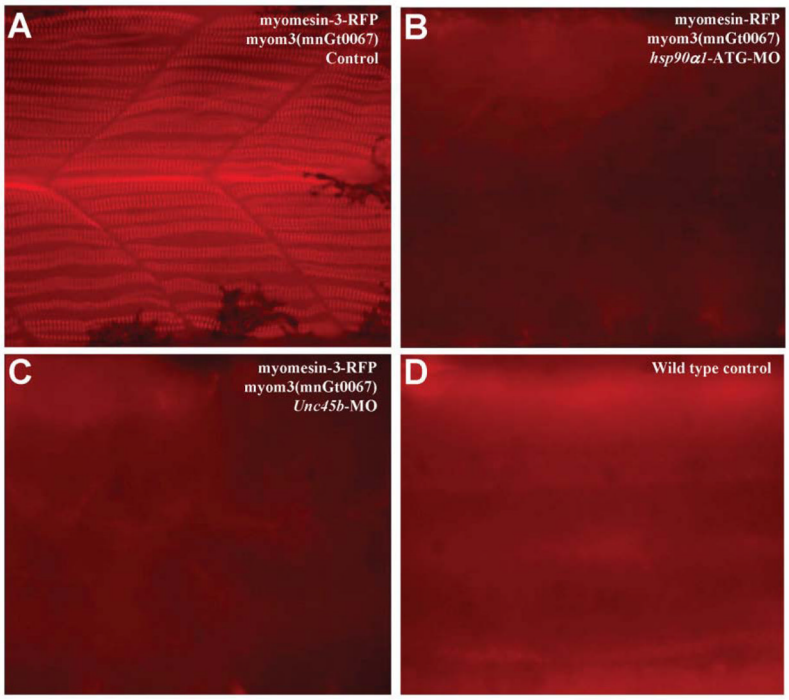

Fig. 5. Effects of *hsp90α1* and *Unc45b* knockdown on the sarcomeric localization of myomesin-3-RFP in slow muscles of zebrafish embryos

Sarcomeric localization of myomesin-3-RFP in slow muscles of myom3(mnGt0067) fish embryos (**A**), *hsp90α1* (**B**) or *Unc45b* (**C**) knockdown myom3(mnGt0067) embryos, or wild type control embryos (D) at 48 hpf.

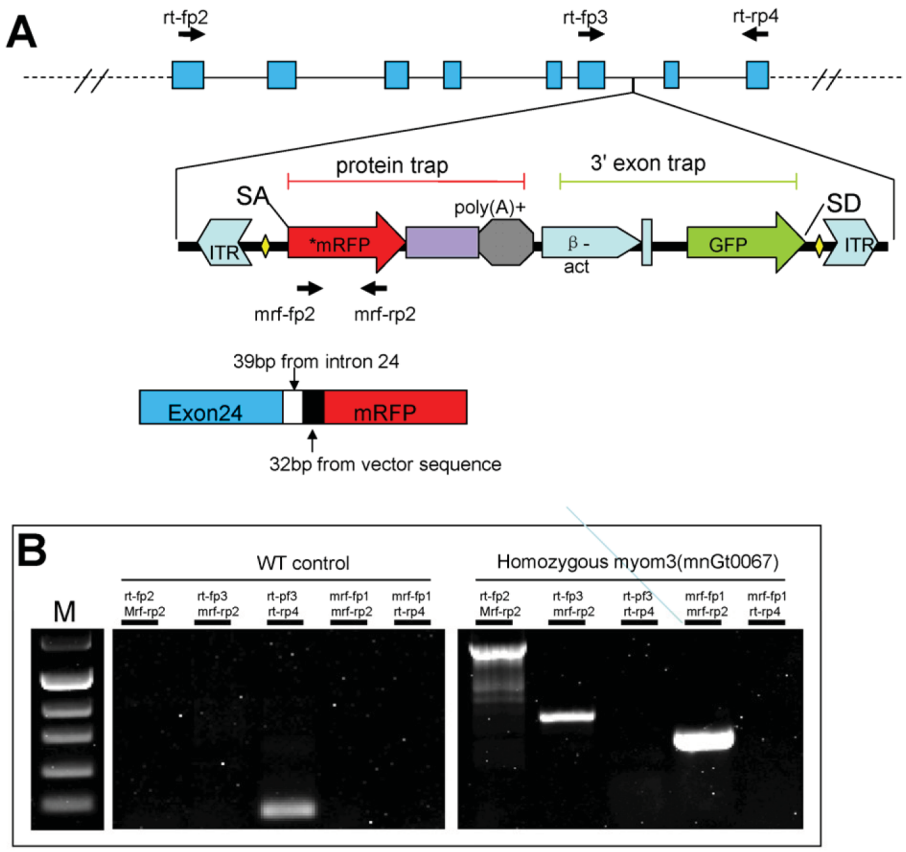

Fig. 6. Diagram shows the gene trap integration in *myomesin-3* gene and PCR strategy for analyzing the expression of myomesin-3 and myomesin-3-RFP mRNA transcripts

A:. The RFP gene trap is integrated at the intron 24 of *myomesin-3*. A cDNA fragment covering the junction site of myomesin-3 and RFP fusion was amplified by RT-PCR. Additional sequence of 39 bp from intron 24 and 32 bp from the vector sequence upstream of RFP was found in the myomesin-3-RFP fusion transcript. *myomesin-3* GenBank accession No. XM_001921030. **B:** RT-PCR results show the expression of *myomesin-3* mRNA transcripts or myomesin-3-RFP fusion products in wild type or homozygous myom3(mnGt0067) fish embryos.

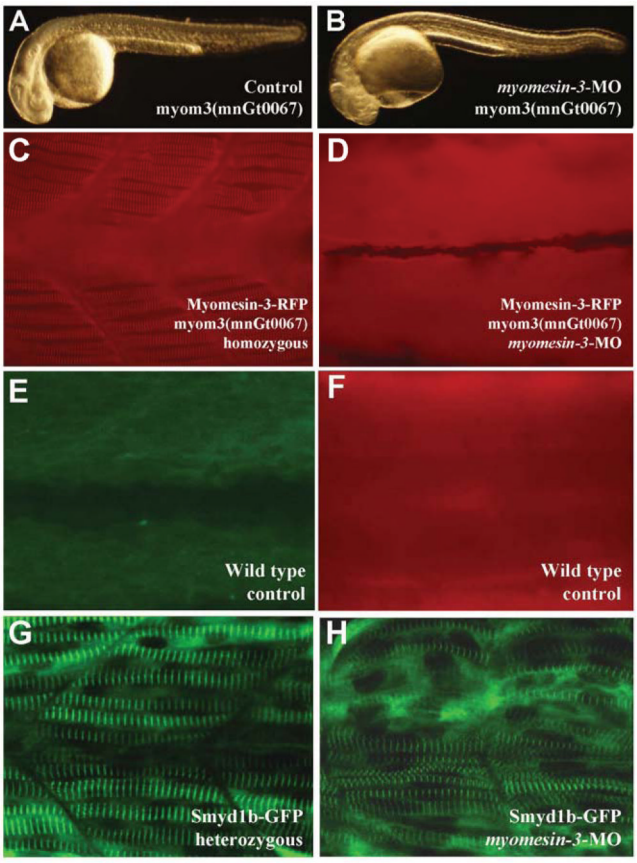

Fig. 7. The effect of RFP gene trap insertion and *myomesin-3* knockdown on M-line organization in slow muscles of zebrafish embryos

A and **B:** morphological comparison of the control (A) and *myomein-3* ATG-MO injected (**B**) embryos at 24 hpf. **C** and **D:** the M-line localization of myomesin-3-RFP in myom3(mnGt0067) homozygous control (**C**) or *myomesin-3* knockdown (**D**) embryos at 48 hpf. **E** and **F:** negative controls of wild type embryos for GFP (**E**) and RFP (**F**) at 28 hpf and 48 hpf, respectively. **G** and **H:** sarcomeric localization of Smyd1b-GFP at the M-lines in Smyd1b-GFP heterozygous control (G) or *myomesin-3* knockdown (**H**) zebrafish embryos at 28 hpf.

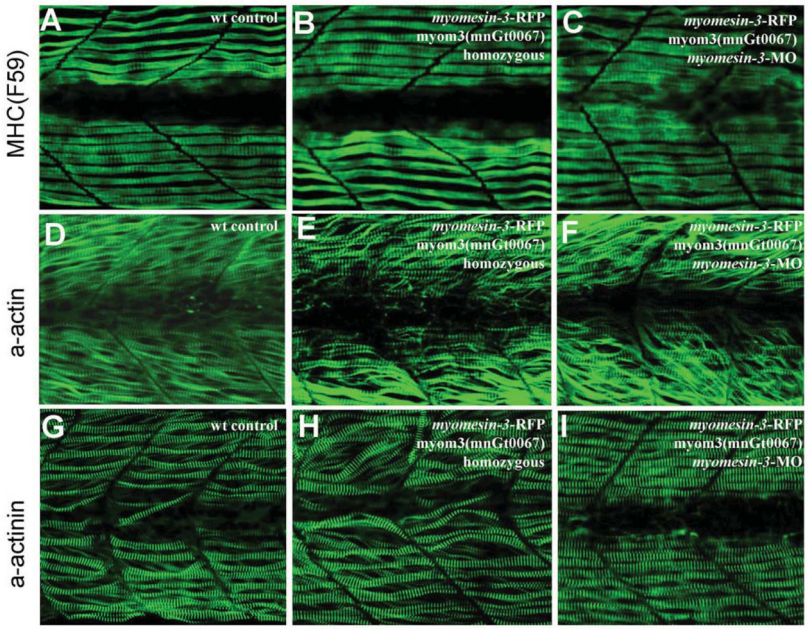

Fig. 8. The sarcomere organization in slow muscles of homozygous myomesin-3-RFP and *myomesin-3* knockdown zebrafish embryos

A–C: anti-MyHC antibody (F59) staining shows the thick filament organization in slow muscles of the control (**A**), homozygous myom3(mnGt0067) (**B**), or myomesin-3 knockdown (**C**) embryos at 28 hpf. **D–F:** α-actin immunostaining shows the organization of thin filaments in slow muscles of the control (**D**), homozygous myom3(mnGt0067) (**E**), or *myomesin-3* knockdown (**F**) embryos at 28 hpf. **G–I:** α-actinin immunostaining shows the organization of Z-lines in slow muscles of the control (**G**), homozygous myomesin-3-RFP (H), or *myomesin-3* knockdown (**I**) embryos at 28 hpf.