UNIVERSITAT DE BARCELONA

FACULTAT DE BIOLOGIA

DEPARTAMENT DE FISIOLOGIA

CONTROL DE LA MIOGÈNESI EN PEIXOS:

FUNCIONS DE LA MIOSINA, L'IGF-II

I ELS FACTORS REGULADORS MIOGÈNICS

Tesi Doctoral

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Memòria presentada per
Marta Codina Potrony
per optar al grau de
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PUBLICACIONS

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Knockdown of *smyhc1* and blocking myosin-actin interaction with BTS (N-benzyl-p-toluene sulphonamide) disrupt myofibrillogenesis in skeletal muscle of zebrafish embryos

Development Dynamics (submitted)

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Resum

L'ensemblatge de miofibril·les requereix l'expressió coordinada de la miosina i la seva interacció amb l'actina i altres proteïnes sarcomèriques, durant la miofibril·logènesi. El múscul vermell del peix zebra (*Danio rerio*) expressa tres tipus de cadena pesada de miosina (MyHC), anomenats *smyhc1, smyhc2* i *smyhc3*, dels quals el gen *smyhc1* és la isoforma que s'expressa en primer lloc en el múscul vermell d'embrions de peix zebra.

Per tal de determinar el paper de la *smyhc1* en l'ensemblatge de les miofibril·les, hem generat un *knockdown* pel gen en múscul vermell d'embrions de peix zebra i hem analitzat el seu efecte en el procés de miofibril·logènesi *in vivo*.

El *knockdown* de l'expressió de *smyhc1* va comportar una organització deficient tant dels filaments gruixuts com dels filaments prims en el múscul vermell, mentre que es va observar poc efecte en la organització de la línia Z.

Els defectes en les miofibril·les conseqüència del *knockdown* de *smyhc1* en el múscul vermell poden ser fenocopiades tractant els embrions de peix zebra amb BTS (*N-benzyl-p-toluene sulphonamide*), un inhibidor específic de l'activitat ATPasa de la miosina, així com de la interacció miosina-actina. No obstant, al contrari que el *knockdown* de *smyhc1*, els defectes en les miofibril·les es van observar tant en el múscul vermell com en el múscul blanc dels embrions tractats amb BTS, i a més els efectes van ser reversibles després de deixar el tractament.

En conjunt, aquest estudi ha demostrat el paper essencial de la cadena pesada de la miosina tipus 1 específica de múscul vermell en la miofibril·logènesi i l'aplicació dels embrions de peix zebra com a model per a estudiar el procés de miofibril·logènesi *in vivo*.

Knockdown of *smyhc1* and blocking myosin-actin interaction with BTS (N-benzyl-p-toluene sulphonamide) disrupt myofibrillogenesis in skeletal muscles of zebrafish embryos

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Summary

Myofibril assembly requires the coordinated expression of myosin and close interaction with actin and other sarcomeric proteins during myofibrillogenesis. Zebrafish (Danio rerio) slow muscles express three types of myosin heavy chain (MyHC) genes, namely *smyhc1*, *smyhc2* and *smyhc3*. *smyhc1* is the primary isoform expressed in slow muscles of early stage zebrafish embryos. To determine the role of slow MyHC1 in myofibril assembly, we knocked down *smyhc1* expression in slow muscles of zebrafish embryos and analyzed its effect on myofibrillogenesis *in vivo*. Knockdown of *smyhc1* expression resulted in defective thick and thin filament organization in slow muscles. However, there was little effect on the Z disk organization. The myofibril defects of *smyhc1* knockdown in slow muscles could be phenocopied by treating zebrafish embryos with BTS (N-benzyl-p-toluene sulphonamide), a specific inhibitor for myosin ATPase and myosin-actin interaction. However, unlike the *smhyc1* knockdown, the myofibril defects were observed in both slow and fast muscles of BTS treated embryos and moreover the effects were reversible after removal of BTS. Together, these studies demonstrated the essential role of slow MyHC1 in myofibrillogenesis and the application of the zebrafish embryo as a model for studying myofibrillogenesis in vivo.

Short title: Skeletal muscle myofibrillogenesis

Key words: Myofibrillogenesis, myosin, sarcomere, MHC

INTRODUCTION

Muscle fibers are composed of myofibrils, one of the most complex and highly ordered macromolecular assemblies known. Each myofibril is made up of highly organized repetitive structures called sarcomeres, the basic contractile unit in skeletal and cardiac muscles. The sarcomere is mainly composed of myosin thick and actin thin filaments. Myosin and actin proteins are assembled to form the highly organized thick and thin filaments with the help of titin, nebulin, and other structural proteins in the Z disks and M bands (Squire, 1997; Gregorio et al., 1999; Clark et al., 2002; Agarkova and Perriard, 2005; Frank et al., 2006; Boateng and Goldspink, 2008). The regulatory mechanisms that lead to the formation of this highly organized structure have been extensively investigated in cell culture *in vitro*; however, the regulatory mechanism is not yet completely understood during muscle development in vivo (Epstein and Fischman, 1991; Sanger et al., 2002).

Coordinated expression and close interaction between myosin and actin are critical for the precise assembly of the highly organized myofibrils. Mutations in myosin, actin and other sarcomeric proteins often lead to defective myofibrillogenesis in skeletal and cardiac muscles. There are more than twenty different skeletal muscle diseases caused by these mutations (Laing and Nowak, 2005).

Uncovering the molecular mechanisms controlling myofibrillogenesis is critical for diagnosis and treatment of muscle diseases. Efforts to understand both normal and myopathic muscle physiology have benefited greatly from the use of animal model systems including Caenorhabditis elegans and Drosophila melanogaster. Genetic analysis demonstrates the importance of sarcomeric and associated proteins in myofibrillogenesis

(Epstein and Thomson, 1974; Epstein and Bernstein, 1992; Barral et al., 1998; Landsverk and Epstein, 2005; Moerman and Williams, 2006).

Zebrafish has recently become a powerful model system for studying gene function involved in development of skeletal and cardiac muscles (Raeker et al., 2006; Tan et al., 2006; Etard et al., 2007; Hinits and Hughes, 2007; Lieschke and Currie, 2007; Du et al., 2008; Hawkins et al., 2008). In the zebrafish embryo, two distinct classes of muscle fibers, slow and fast fibers, have been identified. Each occupies different regions of the myotome (Devoto et al., 1996). *smyhc1* represents the first MyHC gene expressed in zebrafish slow fibers in response to Hedgehog signaling (Bryson-Richardson et al., 2005; Elworthy et al., 2008). Slow muscle has been thought to express a single MyHC isotype. However, recent studies have shown that *smyhc1* resides within a tandem array of five genes that have a remarkable level of DNA sequence identity (McGuigan et al., 2004). In addition to *smyhc1*, two other MyHC genes, *smyhc2* and *smyhc3*, are expressed in a subset of zebrafish slow muscles. However, compared with *smyhc2* and *smyhc3*, *smyhc1* represents the primary MyHC gene expressed in slow muscles of zebrafish embryos (Bryson-Richardson et al., 2005; Elworthy et al., 2008).

To determine the specific role of Smyhc1 in slow muscle development, we knocked down *smyhc1* expression in zebrafish embryos and analyzed its effect on myofibrillogenesis in vivo. The *smyhc1* knockdown embryos showed a specific defect in thick and thin filament organization in slow muscles. The myofibril defects in slow muscles could be phenocopied by treating zebrafish embryos with BTS (N-benzyl-p-toluene sulphonamide), a specific inhibitor for myosin ATPase and myosin-actin interaction. However, unlike the *smhyc1* knockdown, the myofibril defects were observed

in both slow and fast muscles and moreover the effects were reversible after removal of BTS. The myofibril defects appeared to be specific for thick and thin filaments because the organization of M bands and Z disks was not affected in BTS treated embryos. Together, these studies provided a better understanding on the critical role of Smyhc1 in myofibril assembly and demonstrated the usefulness of the zebrafish embryo as a model for studying myofibrillogenesis in vivo.

RESULTS

1. Knockdown of smyhc1 expression resulted in paralyzed zebrafish embryos at early stage.

Zebrafish embryonic muscle fibers can be divided into two major types, slow and fast, based on the expression of MyHCs and immunostaining with fiber-type specific antibodies. Recent studies have shown that multiple slow MyHC genes are expressed in slow muscle fibers during development (Elworthy et al., 2008). *smyhc1* represents the first and primary MyHC gene expressed in zebrafish slow muscles (Bryson-Richardson et al., 2005; Elworthy et al., 2008). To determine the specific role of *smyhc1* in slow muscle development, we knocked down *smyhc1* expression in zebrafish embryos. The *smyhc1* translation blocker, ATG-MO, was specifically targeted to the 25 nt sequence flanking the ATG start codon of the *smyhc1* transcripts, which shares less homology with the corresponding region of other MyHC genes (Fig. 1).

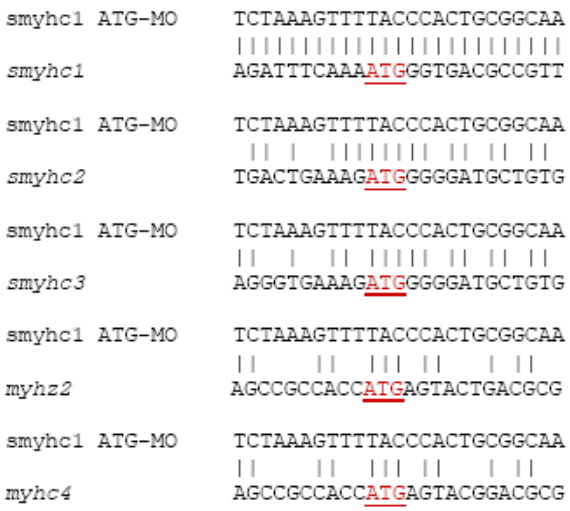

Fig. 1. Sequence comparison of smyhc1 ATG-MO target site with corresponding sequences in smyhc2, smyhc4, fmyhz2 and fmyhc4 in zebrafish. A translational morpholino (MO) antisense oligo was targeted to the flanking sequence of smyhc1 ATG start codon. It shares 50-70% identity with the corresponding sequences in zebrafish *smyhc2, smyhc4, fmyhz2* and *fmyhc4*.

The *smyhc1* ATG-MO was injected into zebrafish embryos. The injected embryos were examined morphologically for 5 days following the injection. Although the injected embryos appeared morphologically normal compared with the control (Fig. 2A-D), one striking phenotype was noted in all *smyhc1* ATG-MO injected embryos (n=369) at early stage of development (24 hpf). *smyhc1* knockdown embryos were

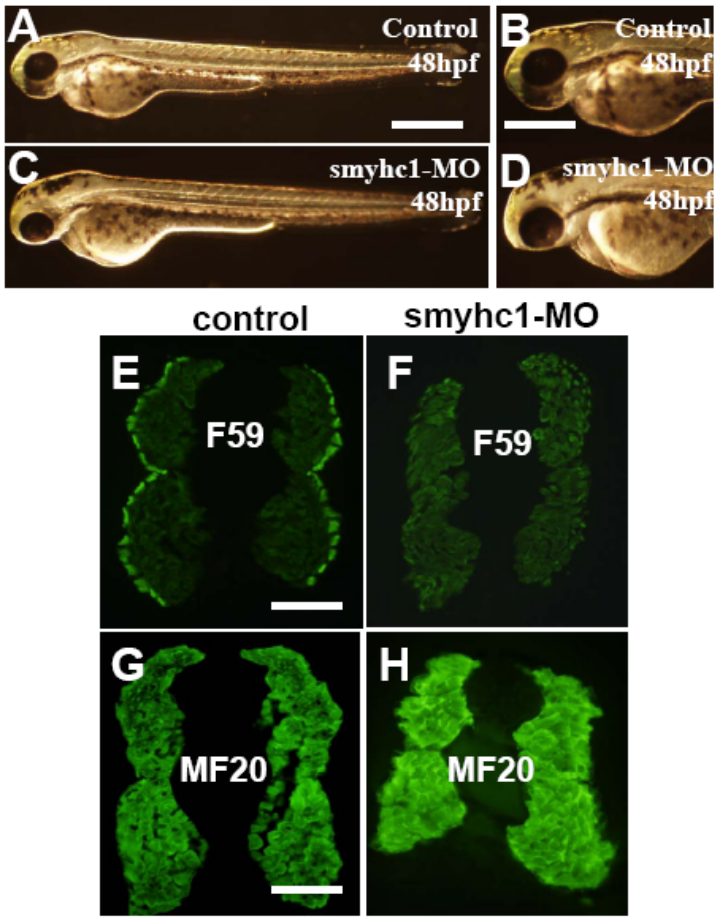

Fig. 2. Knockdown of smyhc1 expression by ATG-MO. **A-D.** Morphological comparison of control-MO (A, B) or smyhc1-ATG-MO (C, D) injected embryos at 48 hpf. **E, F.** F59 antibody immunostaining on cross-sections shows MHC expression in slow muscles of control (E) or smyhc1 ATG-MO injected embryos (F) at 48 hpf. **G, H.** MF-20 antibody staining shows MHC expression in fast muscles of control (G) or smyhc1-ATG-MO (H) injected embryos at 48 hpf. Scale bars = 200 µm in A; 120 µm in B, and 75 µm in E and G.

paralyzed, unable to show any skeletal muscle contraction in response to physical stimulation by touch. Similarly, cardiac muscle contraction appeared weaker and slower compared with control.

The paralyzed muscle defect was gradually recovered in the *smyhc1* knockdown embryos as the embryos developed further into late stages. By 48 hpf (hours post fertilization), skeletal and cardiac muscle contraction appeared in the *smyhc1* knockdown embryos. By 72 hpf, the *smyhc1* knockdown embryos showed normal skeletal and cardiac muscle contraction. This recovery is likely due to the formation of functional fast muscles at the later stage of development (48-72 hpf) in zebrafish embryos (Roy et al., 2001).

To confirm that *smyhc1* ATG-MO specifically knocked down the expression of MyHC in slow muscles, we analyzed the MyHC expression in the injected embryos by immunostaining. Immunostaining was performed using two antibodies, a slow muscle-specific MyHC antibody (F59) and a general anti-MyHC antibody (MF20). The results showed that F59 staining in superficial slow muscles was significantly reduced or completely abolished in the *smyhc1* ATG-MO injected embryos (Fig. 2F). In contrast, MyHC expression in deep myotome containing fast muscles appeared normal (Fig. 2H). Together, these data indicate that *smyhc1* ATG-MO specifically knocked down the expression of Smyhc1 protein in slow muscles but had no effect on other types of MyHCs expressed in fast muscles.

2. Knockdown of** smyhc1 **expression disrupts thick filament organization in slow muscles of zebrafish embryos.

MyHC is required for muscle cell differentiation and maturation. Knockdown of MyHC expression should not affect the early stage of myoblast specification. To test this hypothesis, *smyhc1* knockdown embryos were analyzed for myoblast specification using an early myogenic specification marker *myod*. Compared with control (Fig. 3A), similar pattern of *myod* expression was observed in *smyhc1* knockdown embryos (Fig. 3B). The two rows of *myod* expressing adaxial cells that give rise to slow muscles were clearly seen in the *smyhc1* knockdown embryos (Fig. 3B), confirming that knockdown of *smyhc1* did not alter the specification of slow muscles in zebrafish embryos.

To determine whether knocking down *smyhc1* expression might disrupt myofibril assembly in slow muscle fibers, we analyzed the organization of thick filaments in *smyhc1* knockdown embryos by immunostaining with the anti-MyHC F59 antibody. The results showed very little thick filaments in slow muscles of *smyhc1* knockdown embryos at 24 hpf (Fig. 3D). By 48 hpf, weak MHC expression and poorly organized thick filament were observed in slow muscles of *smyhc1* knockdown embryos (Fig. 3F). However, very few sarcomeres could be detected in slow muscle of *smyhc1* knockdown embryos (Fig. 3F). In contrast, injection with the control MO had no effect on the sarcomere formation and thick filament organization (Fig. 3C, E). Together, these data indicate that Smyhc1 is required for thick filament assembly in slow muscles.

Smyhc1 is specifically expressed in slow muscles of zebrafish embryos. Knockdown of smyhc1 should not affect thick filament assembly in fast muscles. To test this hypothesis, thick filament organization in fast muscles was analyzed by

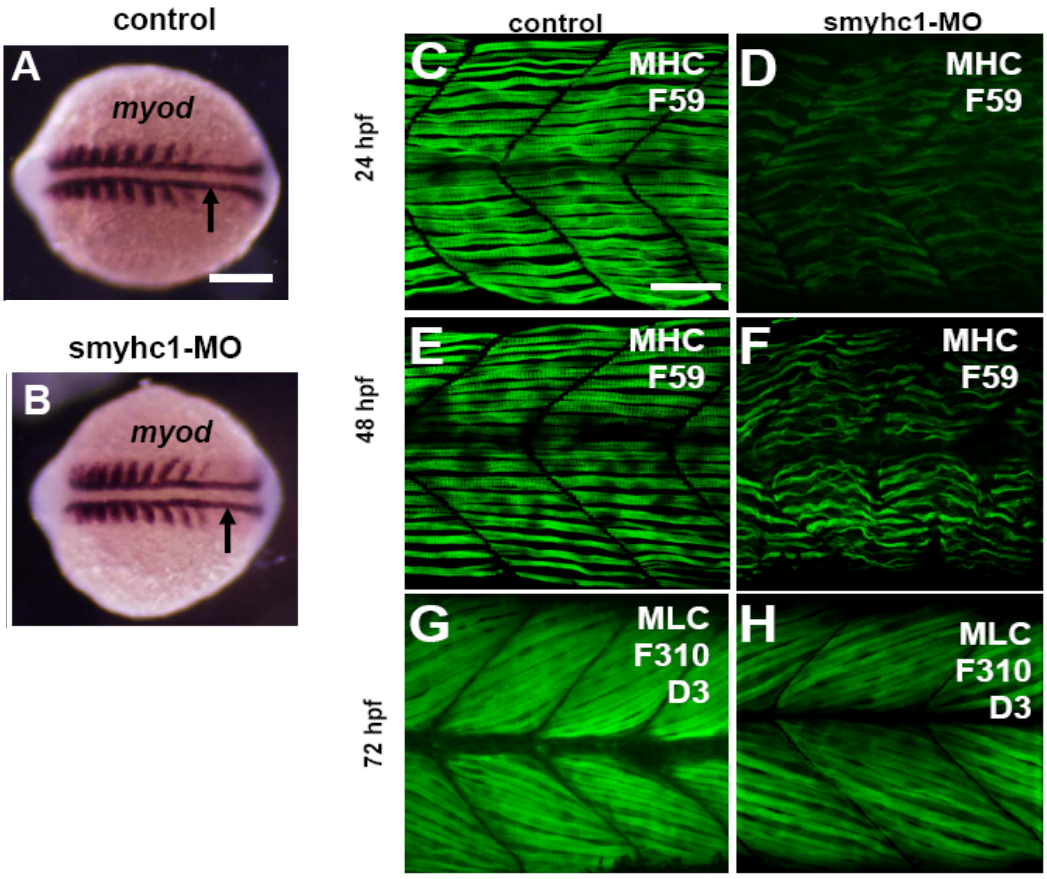

Fig. 3. Effects of smyhc1 knockdown on myod expression and thick filament organization in skeletal muscles of zebrafish embryos. **A and B.** *In situ* hybridization shows normal *myod* expression in control (A) or smyhc1-ATG-MO (B) injected embryos at 14 hpf. Adaxial cells that give rise to slow muscles are indicated by arrows. **C-F.** Anti-MHC antibody (F59) staining shows the organization of thick filaments in trunk slow muscles of control (C, E) or smyhc1-ATG-MO (D, F) injected embryos at 24 (C, D) or 48 (E, F) hpf. **G and H.** Anti-MLC antibody (F310) staining shows the organization of thick filaments in trunk fast muscles of control (G) or smyhc1-ATG-MO injected (H) embryos at 72 hpf. Scale bars = 250 µm in A; 25 µm in C.

immunostaining with the F310 monoclonal antibody, which recognizes myosin light chain specifically expressed in fast muscles. The immunostaining was carried out with the knockdown embryos at 72 hpf when functional fast fibers were well developed. Fast fibers could be easily distinguished from slow fibers by their deeper location within the myotome and myofiber projection. Unlike slow muscles that are localized in the superficial layer with a parallel projection to the midline structure, fast muscles are

helically arranged within the deeper myotome and project with a 20-30 degree angle with respect to the midline structure (Fig. 3G). Immunostaining with the F310 antibody showed that knockdown of *smyhc1* did not affect the thick filament organization in fast muscles (Fig. 3H). This is consistent with the pattern of *smyhc1* expression and the complete recovery of muscle contraction in *smyhc1* knockdown zebrafish embryos at day 3. Together, these data indicate that Smyhc1 is specifically required for thick filament assembly in slow muscles, but not in fast muscles.

3. Knockdown of** smyhc1 **expression disrupted thin filament organization in slow muscles of zebrafish embryos.

During myofibrillogenesis, actin thin filaments align around myosin filaments in a hexagonal arrangement to form the highly ordered sarcomeres. To test whether thin filament organization was affected by *smyhc1* knockdown, the *smyhc1* knockdown embryos were stained with an anti-α-actin antibody (Acl-20.4.2) at 24, 48 and 72 hpf. Compared with the control-MO injected embryos (Fig. 4A, C), *smyhc1* knockdown embryos showed disorganized thin filaments in slow muscles (Fig. 4B, D). The thin filament defects were specific to slow muscles because the organization of thin filaments appeared normal in fast muscles (Fig. 4F). Together, these data suggest that the organized assembly of thin filaments requires Smyhc1 in slow muscles.

To test whether other sarcomeric structures, such as M bands and Z disks, were affected in the *smyhc1* knockdown slow muscles, we analyzed the structure of M bands and Z disks by antibody staining. Immunostaining was performed with anti-α-actinin and anti-myomesin antibodies that specifically label the Z disks or M bands,

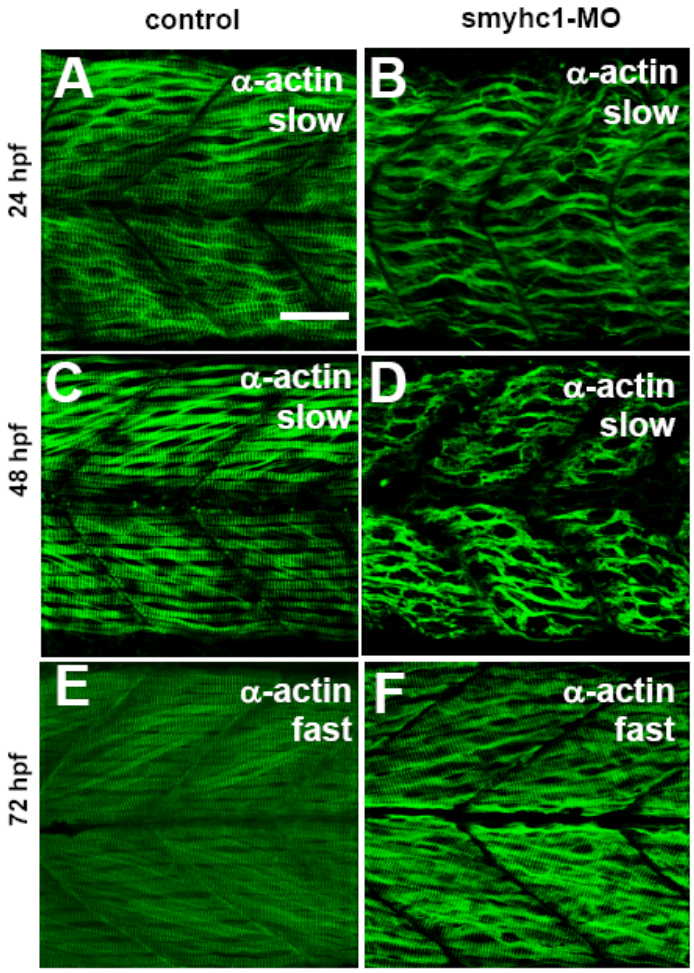

Fig. 4. Knockdown of smyhc1 expression resulted in defective thin filament organization in skeletal muscles of zebrafish embryos. **A-D.** Anti-actin antibody staining shows the organization of thin filaments in slow muscles of control (A, C) or smyhc1-ATG-MO (B, D) injected embryos at 24 (A, B) or 48 (C, D) hpf. **E, F.** Anti-actin antibody staining shows the organization of thin filaments in fast muscles of control (E) or smyhc1-ATG-MO (F) injected embryos at 72 hpf. Note, fast fibers project with a 30 degree angle with respect to the axial structure whereas slow fibers project in parallel to the axial structure. The fast fibers in F are located underneath the defective slow fibers. Scale bar = 25 μm in A.

respectively. Sarcomeric localization of α-actinin was clearly detected in both slow and fast muscles of *smyhc1* knockdown embryos (Fig. 5B, D, F), suggesting that Z disk was not affected. Moreover, organization of M bands also appeared normal in fast muscles of *smyhc1* knockdown embryos revealed by anti-myomesin antibody staining (Fig. 5H). Collectively, these results indicate that *smyhc1* is essential for thick and thin filament

assembly, but not for Z disks organization in slow muscles. Moreover, knockdown of *smyhc1* had no effect on myofibril organization in fast muscles (Fig. 5F, H).

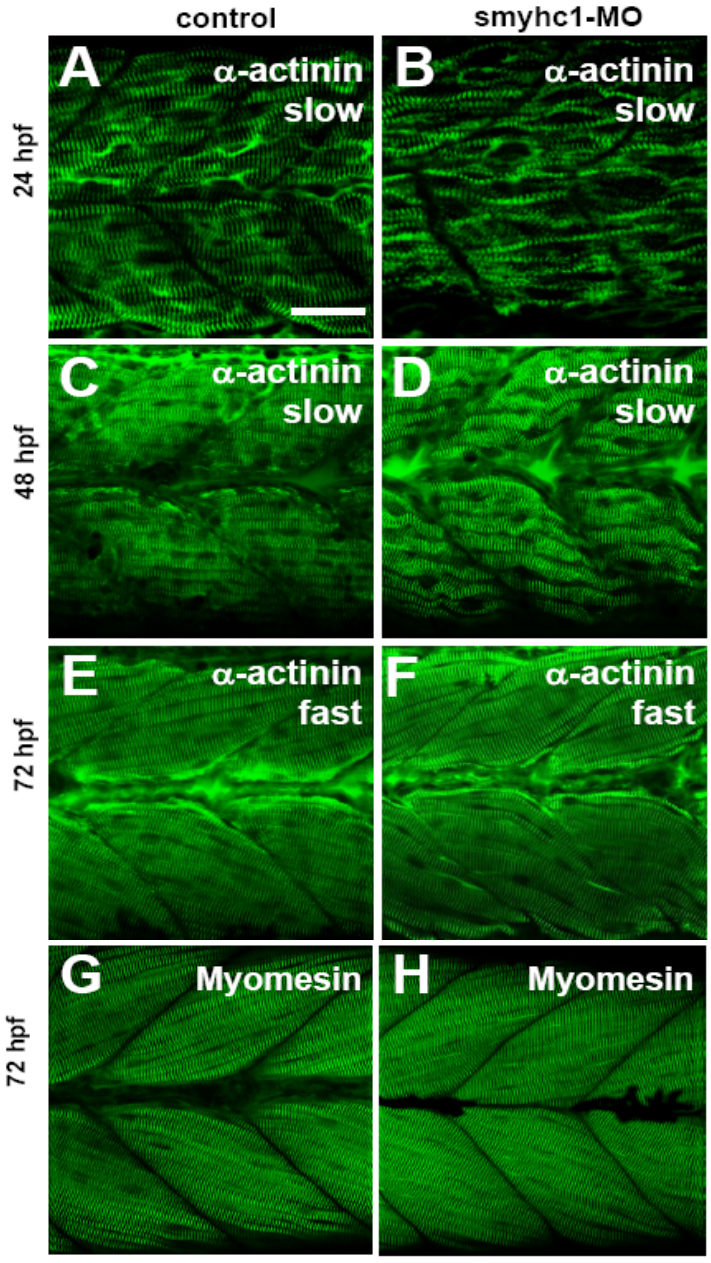

Fig. 5. Knockdown of smyhc1 expression had no effect on the organization of Z disks in skeletal muscles of zebrafish embryos. **A-D.** Anti-α-actinin antibody staining shows the organization of Z disks in slow muscles of control (A, C) or smyhc1-ATG-MO (B, D) injected embryos at 24 (A, B) or 48 (C, D) hpf. **E, F.** Anti- α -actinin antibody staining shows the organization of Z disks in fast muscles of control (E) or smyhc1-ATG-MO (F) injected embryos at 72 hpf. **G, H.** Anti- myomesin antibody staining shows the organization of M bands in fast muscles of control (G) or smyhc1-ATG-MO (H) injected embryos at 72 hpf. Scale bar = 25 μm in A.

4. Inhibition of myosin-actin interaction by BTS resulted in defective thick filament assembly in skeletal muscles of zebrafish embryos

It has been postulated that the myosin-actin interaction plays an important role in the initial phase of myofibrillogenesis. In vitro studies have demonstrated that inhibition of this myosin-actin interaction by BDM (2,3-Butanedione monoxime) and BTS remarkably suppresses the formation of cross-striated myofibrils in the cultured myofibers (Soeno et al., 1999; Ramachandran et al., 2003; Kagawa et al., 2006). Moreover, treating zebrafish embryos with BTS resulted in paralyzed embryos with a defective skeletal and cardiac muscle contraction (Dou et al., 2008). However, its effect on myofibril assembly and sarcomere formation has not been characterized in vivo.

To characterize the BTS-induced muscle defects during muscle development in vivo, we carried out a systematic analysis of BTS treatment in zebrafish embryos and characterized its effect on muscle contraction and myofibrillogenesis in skeletal muscles. A dose dependent effect was first determined by incubating zebrafish embryos with BTS at different concentrations. A clear dose-dependent effect was observed on muscle contraction (Table 1). BTS could effectively block muscle contraction at a dose of 20 μM. BTS treated embryos appeared morphologically normal, except lacking of skeletal muscle contraction (Fig. 6B). A clear edema and weak cardiac muscle contraction were detected in BTS treated embryos at 120 hpf (Fig. 6D).

To determine whether BTS could disrupt myofibril organization in skeletal muscles of zebrafish embryos, BTS treated embryos were analyzed by immunostaining with several sarcomeric specific markers including MHC, α-actinin and MLC at 36, 60 and 72 hpf. The results showed that the thick filament organization was severely

disrupted in both slow and fast muscles of BTS treated embryos (Fig. 6F, H, J). Myofibers with central nuclei, a characteristic of defective myofibers, were clearly

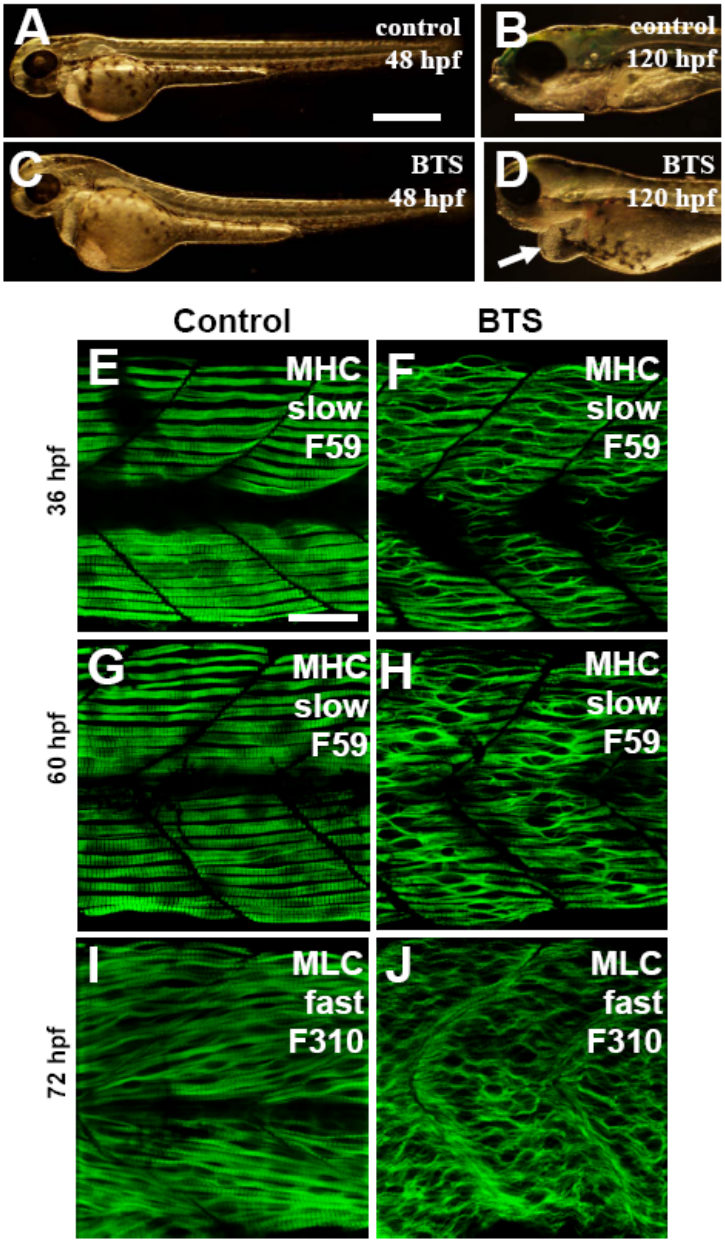

Fig. 6. BTS inhibits skeletal and cardiac muscle contraction and suppresses thick filament assembly in skeletal muscles of zebrafish embryos. **A-D.** Morphological comparison of control (A, B) or BTS treated (C, D) embryos at 48 hpf (A, C) and 120 hpf (B, D). Compared with control (B), BTS treated embryos (D) showed a clear edema (indicated by the arrow) at 120 hpf. **E-H.** Anti-MHC antibody (F59) staining shows the organization of thick filaments in slow muscles of control (E, G) or BTS treated (F, H) embryos at 36 (E, F) and 60 (G, H) hpf. **I and J.** Anti-MLC antibody (F310) staining shows the organization of thick filaments in fast muscles of control (I) or BTS treated (J) embryos at 72 hpf. Scale bars = 200 µm in A; 300 µm , and 25 µm in E.

detected in muscle fibers of BTS treated embryos (Fig. 6F, H). In contrast, incubation with DMSO had no effect on the sarcomere formation and thick filament assembly (Fig. 6E, G, I), confirming that the skeletal muscle defects were BTS specific.

5. BTS treatment results in defective thin filament organization without affecting M bands and Z disks

To test whether the thin filaments were affected by BTS treatment, BTS-treated embryos were stained with an anti-α-actin antibody. Compared with the control embryos (Fig. 7A, C, E), BTS treated embryos showed poorly organized thin filaments in both slow and fast muscles at 36, 60 and 72 hpf (Fig. 7B, D, F). This is consistent with the *smyhc1* knockdown studies, indicating that myosin-actin interaction is critical for the organized assembly of thin filaments in skeletal muscles of zebrafish embryos.

To test whether other sarcomeric structures, such as the M bands and Z disks, were also affected in BTS treated embryos, we analyzed the localization of myomesin and α-actinin, the respective M band and Z disk specific markers, by antibody staining. A clear sarcomeric localization of α-actinin was detected in both slow and fast muscles of the BTS treated embryos (Fig. 8B, D, F), suggesting that organization of Z discs was not affected. However, compared with the extended myofibers in control embryos (Fig. 8A, C, E), myofibers in BTS treated embryos appeared twisted (Fig. 8D, F). This is likely due to the secondary effect from the large central nucleus within the BTS induced defective fibers. Similar to Z disks, the organization of M bands also appeared normal in fast muscles of BTS treated embryos (Fig. 8G, H). Collectively, these results indicate that myosin-actin interaction is essential for thick and thin filament assembly in skeletal

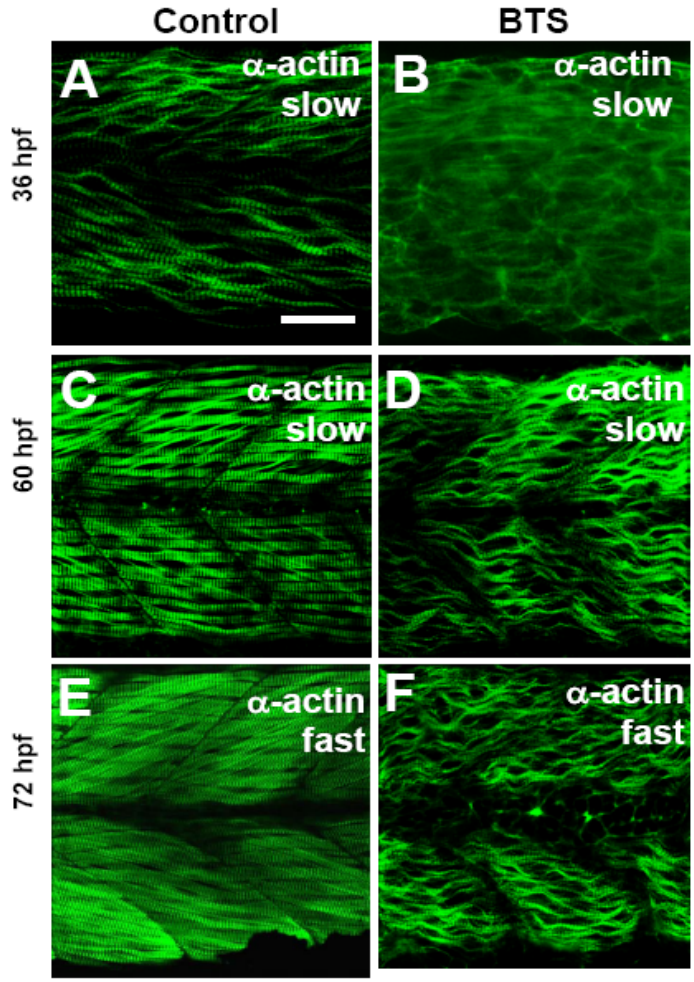

Fig. 7. BTS suppresses thin filament assembly in skeletal muscles of zebrafish embryos. **A-D.** Anti-α-actin antibody staining shows the organization of thin filaments in slow muscles of control (A, C) or BTS treated (B, D) embryos at 36 (A, B) and 60 (C, D) hpf. **E and F.** Anti- α -actin antibody staining shows the organization of thin filaments in fast muscles of control (E) or BTS treated (F) embryos at 72 hpf. Scale bar = 25 μm in A.

muscles of zebrafish embryos. Moreover, M bands and Z disks can be organized independently of thick and thin filaments.

6. The BST induces myofibril defects in organized myofiber

To determine whether disruption of myofibril organization by BTS was stage dependent, we incubated zebrafish embryos with BTS at various developmental stages from somitogenesis and myogenesis (12-72 hpf). The results showed that zebrafish

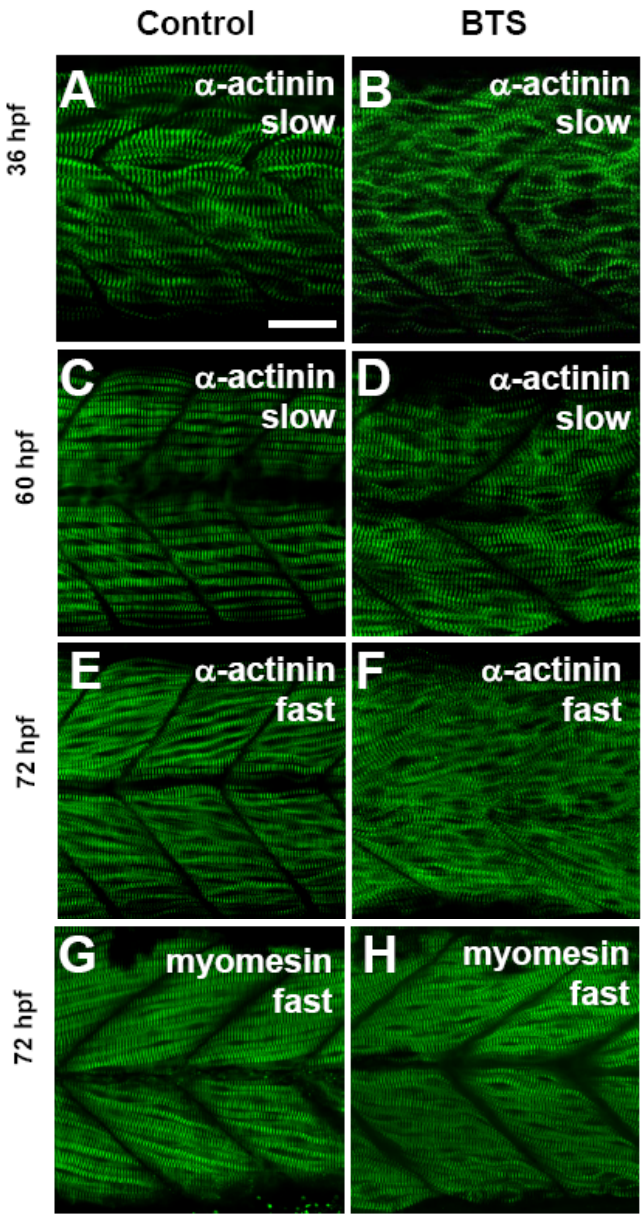

Fig. 8. BTS treatment had no effect on Z disk and M band organization in skeletal muscles of zebrafish embryos. **A-F,** Anti-α-actinin antibody staining shows the Z disk organization in slow (A-D) or fast (E, F) muscles of control (A, C, E) or BTS treated (B, D, F) embryos at 36 (A, B), 60 (C, D), or 72 hpf (E, F). **G, H.** Anti-myomesin antibody staining shows the Z disk organization in fast muscles of control (G) or BTS treated (H) embryos at 72 hpf. Scale bar = 25 μm in A.

embryos of all stages were highly sensitive to BTS-induced paralysis, regardless of the developmental stage tested. BST treatment was able to paralyze fish embryos having

normal muscle contraction before the treatment, suggesting that BST can disrupt the sarcomeric structure in myofibers with organized myofibrils. To test this hypothesis, we treated zebrafish larvae with BTS at 36 hpf, well after the formation of organized slow myofibers in zebrafish embryos (Fig. 9A). The zebrafish embryos were treated with BTS for 2, 4 and 8 hours and then analyzed by immunostaining with anti-MyHC antibody (F59). The results showed that 8 hours of BTS treatment was sufficient to induce significant disruption of thick filament organization in slow fibers (Fig. 9D).

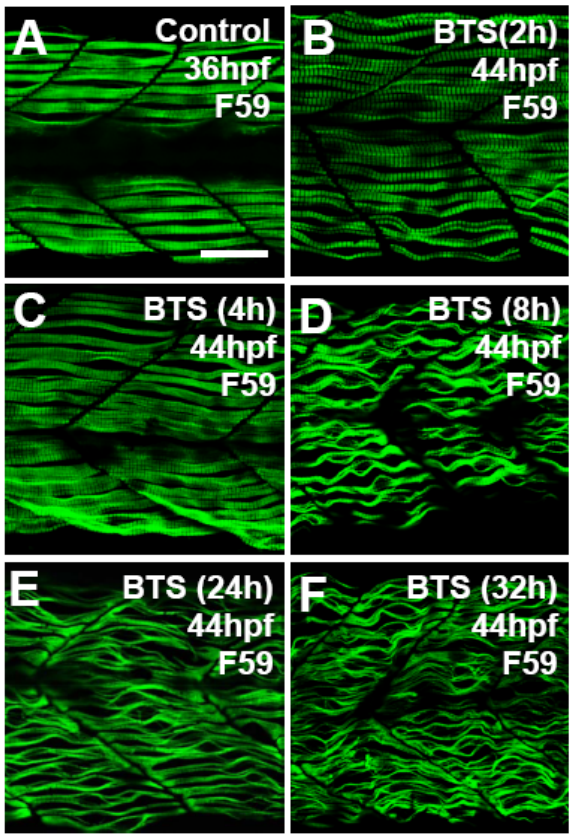

Fig. 9. BTS disrupts thick filament organization in muscle fibers in zebrafish embryos. **A.** Anti-MHC antibody (F59) staining shows thick filament organization in slow muscles of control zebrafish embryos at 36 hpf, before the BTS treatment. **B-D.** F59 staining shows thick filament organization in zebrafish slow muscles after treating with BTS (50 μM) for 2h (B), 4h (C) and 8h (D). 8 hours of BTS treatment disrupts the thick filament organization (D). **E and F.** F59 staining shows disorganized thick filaments in zebrafish embryos with early and longer BTS treatment. E, 24 hours BTS treatment starting at 20 hpf. F, 32 hours BTS treatment starting at 12 hpf. Scale bar = 25 μm in A.

However, treating zebrafish embryos with BTS at earlier stages (12 or 20 hpf) and a longer treatment (24 or 32 hours) appeared to have a stronger effect on the myofibril disruption (Fig. 9E, F). Together, these studies demonstrated that BTS not only affects the myofibril assembly in young myofibers undergoing myofibrillogenesis, it could also disrupt the myofibril organization in functional mature myofibers.

7. ***The BTS induced muscle defects are reversible***

It has been shown that BTS binds to myosin S1 and weakens the affinity of myosin for actin, leading to dissociation of myosin in the presence of ADP (Cheung et al., 2002). Consequently, BTS causes a decrease in actin-activated myosin ATPase and in muscle-tension development (Cheung et al., 2002; Shaw et al., 2003). Previous studies have shown that BTS reversibly suppresses force production in skinned skeletal muscle fibers from rabbit (Cheung et al., 2002), suggesting that the effect of BTS on myofibrillogenesis is likely to be reversible *in vivo*. To test this hypothesis, we treated zebrafish embryos continuously with BTS for 8 hrs between 12-20 hpf (6-22 somites) and then transferred the embryos to fresh fish water without BTS for recovery for 16 hours. Muscle contraction was closely examined in these embryos after the removal of BTS. The zebrafish larvae showed a significant improvement in muscle contraction 8 hours after recovery (data not shown).

To characterize the muscle recovery at the cellular levels, we analyzed the sarcomeric structure of skeletal muscles before and after the recovery by immunostaining. The results revealed that myofibril organization was significantly disrupted by the BTS treatment before the recovery (Fig. 10A). However, after 16

hours recovery, myofibril organization was dramatically improved in the BTS treated embryos. Organized sarcomeres were clearly observed in the recovered fish embryos (Fig. 10C). Together, these results showed that the BTS induced myofibril defects are reversible in zebrafish embryos, indicating that temporally BTS treatment does not cause permanent damage to the developing muscles in zebrafish embryos, and the inhibitory effect of BTS on myofibrillogenesis is unlikely due to cytotoxic effects.

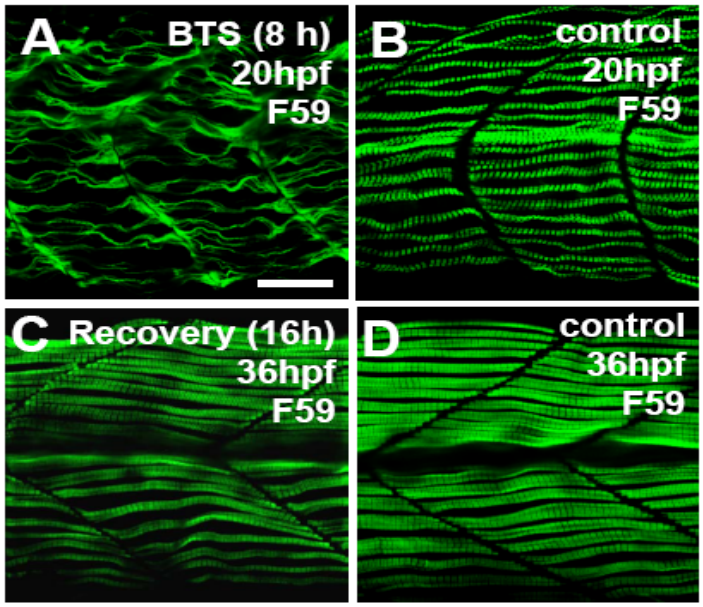

Fig. 10. BTS induced myofibril defects are reversible in zebrafish embryos. **A.** Anti-MHC antibody (F59) staining shows thick filament organization in slow muscles of zebrafish embryos incubated with BTS between 12-20 hpf, and followed immediately with F59 staining at 20 hpf. A thick filament defect was observed in the BTS treated group. **B.** Control zebrafish embryos of the same stage (20 hpf) shows organized thick filaments at 20 hpf. **C.** F59 staining shows the recovery from BTS treatment after embryos being transferred to fresh fish water for 16 hours at 36 hpf. **D.** A control embryo of the same stage (36 hpf) as in C without prior BTS treatment. Scale bar = 25 μm in A.

8. Disruption of myofibril assembly by BTS does not increases hsp90α1 expression in zebrafish embryos

It has been reported that disruption of myosin folding and assembly by Unc45b mutation (or knockdown) resulted in increased *hsp90α* gene expression in zebrafish

embryos (Etard et al., 2007). UNC-45b is a myosin chaperone that plays an important role in myosin folding and assembly. The upregulation of *hsp90a* expression is considered as a typical stress response to increased misfolding of myosin protein. It is, however, not clear whether disruption of sarcomere assembly could also lead to a stress response in zebrafish embryos.

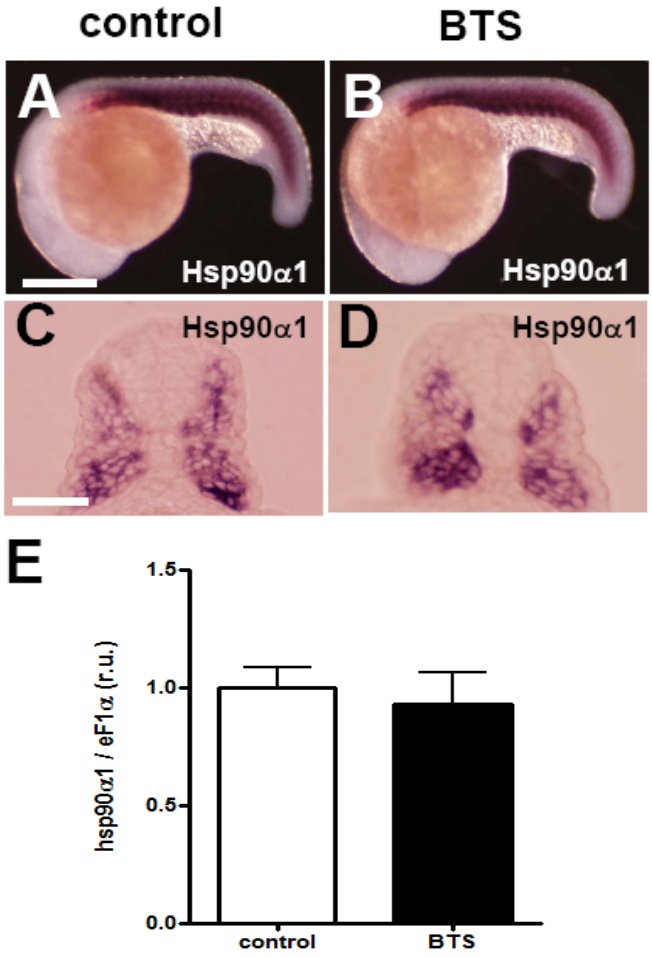

Fig. 11. Disruption of myofibril assembly by BTS does not alter Hsp90α1 gene expression in zebrafish embryos. **A-D,** Whole mount in situ hybridization shows Hsp90α1 expression in control (A, C) and BTS treated (B, D) embryos at 23 hpf. Side views (A, B) or cross-sections (C, D). Scale bars = 150 µm in A; 75 µm in C. **E.** Analysis of *hsp90α* gene expression in BTS and wild type embryos by real time PCR. The relative levels of gene expression were normalized to the housekeeping gene *ef-1α* and expression was compared to normal levels in the control embryos using the delta delta Ct method. Experiments were performed in triplicate and each reaction was run in duplicate. A two-tailed Student's t-test was used in statistic analysis.

To test this idea, we analyzed *hsp90α1* gene expression in BTS treated zebrafish embryos that have disrupted myofibril organization. *Hsp90α* expression was analyzed in BTS treated zebrafish embryos by whole mount *in situ* hybridization and real time PCR. The results showed that expression of *hsp90α* was not altered in BTS treated zebrafish embryos (Fig. 11B, D). Real time PCR further confirmed the results from the in situ hybridization analyses (Fig. 11E). Collectively, these data indicate that blocking myofibril assembly by BTS does not lead to upregulation of *hsp90α* gene expression in zebrafish embryos.

DISCUSSION

In this study, we analyzed the role of Smyhc1 in myofibril assembly in skeletal muscles of zebrafish embryos. We demonstrated that knockdown of *smyhc1* expression in zebrafish embryos resulted in defective organization of thick and thin filaments in slow muscles. Similarly, blocking myosin-actin interaction with BTS disrupted the organized assembly of thick and thin filaments in both slow and fast skeletal muscles, resulting in paralysis. However, the organization of M bands and Z disks was not affected in skeletal muscles of *smyhc1* knockdown or BTS treated embryos. Moreover, the myofibril defects induced by BTS were reversible after the removal of BTS. Together, these studies provide important insights into the role of MyHC in myofibrillogenesis and highlight the usefulness of the zebrafish embryo as a model to study myofibrillogenesis *in vivo*.

Function of Smyhc1 in myofibrillogenesis in slow muscles

Zebrafish muscle fibers can be broadly classified into two major types, slow and fast, based on the differences in contraction speeds, metabolic activities and the expression of MyHC genes. It has been postulated that zebrafish slow muscles express only a single MyHC isotype. However, recent studies indicate that zebrafish slow muscles express at least three types of MyHC isoforms during development. Smyhc1 represents the first and predominant MyHC isotype expressed in zebrafish embryonic slow muscles. By using the gene-specific knockdown approach, we demonstrated that Smyhc1 plays a key role in myofibrillogenesis of slow muscles. In slow muscles of *smyhc1* knockdown embryos, the organized thick and thin filaments were clearly missing or severely suppressed. However, knockdown of *smyhc1* had little effect on

the development of fast muscles. The fiber-type specific phenotype is consistent with the temporal and spatial pattern of *smyhc1* expression in zebrafish embryos. *smyhc1* represents the primary MyHC gene expressed in slow muscles of zebrafish embryos. Its expression starts at 10 somite stage (14 hpf), well before the expression of *smyhc2* and *smyhc3* could be detected in the secondary slow muscles at 72 and 96 hpf (Bryson-Richardson et al., 2005; Elworthy et al., 2008).

Results from the *smyhc1* knockdown studies clearly demonstrate the critical role of MyHC in organized assembly of thin filaments in slow muscles of zebrafish embryos. This is consistent with previous studies showing that myosin filaments function as an accelerator for actin polymerization in vitro, and actin-myosin interaction plays an important role in the organization of actin and myosin filaments into the highly organized sarcomeres (Hayashi et al., 1977). This is also consistent with studies in C. elegans and D. melanogaster that established a clear correlation between MyHC gene mutation and skeletal muscle disease among invertebrates (Dibb et al., 1985; Chun and Falkenthal, 1988). MyHC mutation disrupts myofibrillar assembly of thick filament in the indirect flight muscle (Chun and Falkenthal, 1988).

We showed that knockdown of *smyhc1* in zebrafish embryos resulted in restricted myofibril defects in thick and thin filaments. M band and Z disk organization was not affected in skeletal muscles of *smyhc1* knockdown zebrafish embryos. The data are consistent with previous finding in D. melanogaster showing that MyHC gene mutation disrupts thick and thin filament assembly in flight muscles, with little effect on Z disk organization (Chun and Falkenthal, 1988). Results from our studies are also consistent with early report that the basic framework is not affected when both thick and

thin filaments are removed (Funatsu et al., 1990; Funatsu et al., 1993). Together, these studies support the idea that the basic framework of the sarcomere consisting of the Z disks and M bands is established in conjunction with giant proteins titin, nebulin and obscurin, prior to the attachment of thick and thin filaments (van der Ven et al., 2000; Kontrogianni-Konstantopoulos et al., 2006; Witt et al., 2006).

BTS disrupts thick and thin filament organization

We demonstrated that treating zebrafish embryos with BTS, a specific inhibitor for myosin ATPase and myosin-actin interaction, disrupts myofibril organization in both slow and fast muscles in zebrafish embryos, resulting in paralyzed fish larvae. Results from our in vivo studies are consistent with previous finding in primary cell cultures showing that BTS suppress the formation of thick and thin filaments in muscle cells in culture (Soeno et al., 1999; Ramachandran et al., 2003; Kagawa et al., 2006). Moreover, we demonstrated that BST could paralyze fish embryos that had normal muscle contraction before the treatment, suggesting that BST is capable of disrupting organized sarcomeres in mature myofibers with functional myofibrils. This is consistent with the mechanistic action of BTS. Cheung et al. (2002) have shown that BTS binds to and inhibits skeletal myosin S1 ATPase activity. BTS binding weakens the affinity of myosin for actin, leading to dissociation of myosin in the presence of ADP (Cheung et al., 2002; Shaw et al., 2003). BTS is thus not only effective in suppressing the myofibril assembly in young myofibers undergoing myofibrillogenesis, it could also disrupt the organized myofibril filaments in functional myofibers.

Data from our studies provide new evidence that ATPase activity of MHC head is required for myofibril assembly. This is consistent with studies from C. elegans that attest to an important function for myosin head in thick filament assembly. Mutations in functionally important domains of the myosin head, including the binding sites for ATP and actin strongly interfere with assembly of MyHC into thick filaments in C. elegans (Bejsovec and Anderson, 1988). Thus, although the myosin rod is capable of assembly in vitro into thick filament-like structures (Lowey et al., 1969), the function of the myosin head may be required *in vivo* for normal filament formation.

Disruption of myofibril assembly by BTS does not result in an upregulation of hsp90α gene expression

It has been demonstrated that disruption of myosin folding and assembly by knockdown or mutation of myosin chaperone Unc45b resulted in a stress response and the upregulation of *hsp90α* gene expression (Etard et al., 2007). In contrast to Unc45b knockdown, we demonstrated that disruption of thick filament assembly by BTS does not result in an upregulation of *hsp90α* gene expression. The difference in heat shock response could be due to the distinct mechanism behind BTS induced myofibril defects and Unc45b knockdown. Unc45b is a myosin chaperone required in myosin protein folding. Knockdown of Unc45b will likely result in increased misfolding of myosin proteins that can lead to heat shock response and upregulation of *hsp90α* gene expression. In contrast, BTS inhibits MyHC ATPase activity and blocks myosin-actin interaction (Cheung et al., 2002). Blocking myosin assembly by BTS may not interfere with the folding of myosin proteins, and thus no heat shock response. It should be noted that the

different response in *hsp90α* gene expression could also be caused by the distinct phenotype from Unc45b mutation and BTS treatment. Although similar myofibril defects were observed in thick and thin filaments, distinct effects were observed with respect to the Z disks and M bands in BTS treated and *unc45b* mutant embryos. Unc45b mutation disrupted the organization of Z disks (Etard et al., 2007), whereas BTS treatment had little effect on their organization. It remains to be determined whether disruption of Z disk organization may trigger a stress response and the upregulation of *hsp90α* gene expression.

MATERIALS AND METHODS:

Synthesis of morpholino antisense oligos

Morpholino antisense oligos were synthesized by Gene Tools (Carvalis, OR). The *smhyc1* translation blocker (ATG-MO) was targeted to sequence flanking the ATG start codon. The control MO was the standard control oligo purchased from Gene Tools. Smyhc1 ATG-MO: 5'- TCTAAAGTTTTACCCACTGCGGCAA- 3'.

Microinjection in zebrafish embryos

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

BTS treatment and recovery

BTS (Kontrogianni-Konstantopoulos et al., S949760, Sigma) stock solution (50 mM) was prepared in dimethylsulfoxide (DMSO). For dose-dependent analysis, zebrafish embryos were incubated with BTS in fish water containing different concentrations of BTS (0.16 μM, 0.8 μM, 4 μM, 20 μM, 100 μM). The treatment started at the 6 somite stage (12 hpf) and stopped 30 hours after the treatment.

For analysis of BTS induced myofibril defects, zebrafish embryos (100 each dish) were incubated with 50 μM of BTS. The treatment started at the 6 somite stage (12 hpf) and stopped and terminated at 36, 60 and 72 hpf by fixing in 4% paraformaldehyde and used for antibody staining.

For the recovery experiments, the fish embryos (100 each group) were first treated with BTS (50 μM) for 8 hours between 12-20 hpf (6-22 somites). Thirty embryos from each group including the untreated control were fixed immediately after the treatment and used to determine the myofibril organization before the recovery. The rest of the embryos were transferred to fresh fish water and allowed to recover for 16 hours. The embryos were fixed in 4% paraformaldehyde immediately after the recovery period and used for immunostaining with F59 antibody.

For BTS treatment with old embryos that have functional myofibers, BTS (50 μM) was added to three groups of fish embryos at 36, 40 and 42 hpf, respectively. The embryos were incubated with BTS for 8, 4 and 2 hours, respectively, and then fixed in 4% paraformaldehyde following the treatment and used for immunostaining with F59 antibody. In another set of experiments, BTS treatment started at 12 or 20 hpf and lasted for 32 and 24 hours, respectively.

Whole mount immunostaining

Immunostaining was carried out using whole mount zebrafish embryos (1-3 days pots fertilization) as previously described (Tan et al., 2006; Du et al., 2008). Briefly, zebrafish embryos were fixed in 4% paraformaldehyde (in PBS) for 1 hour at room temperature. The fixed embryos were washed for 15 minutes 3 times in PBST. Three day old embryos were digested in 1 mg/ml collagenase for 75 minutes. Immunostaining was performed with the following primary antibodies: anti-α-actinin (clone EA-53, #A7811, Sigma), anti-MyHC for slow muscles (F59, DSHB), anti-myosin light chain

for fast muscles (F310, DSHB), anti-MyHC (MF-20, DSHB), anti-myomesin (mMaC myomesin B4, DSHB), and anti-actin (Ac1-20.4.2, Progen). Secondary antibodies were FITC conjugates (Sigma).

Analysis of gene expression by real time PCR and whole mount in situ hybridization

For in situ hybridization and real time RT-PCR analyses, the zebrafish embryos were treated with 50 μM of BTS for 11 hours starting at 6 somite stage. Half of the treated fish embryos (50) were fixed at 23 hpf (24 somites) for in situ hybridization. Whole mount in situ hybridization was carried out using digoxigenin-labeled antisense probes as previously described (Du et al., 2008). Antisense probe against zebrafish hsp90α1 was synthesized by Sp6 RNA polymerase from NcoI linearized pGEM-Hsp90α1-P plasmid. The zebrafish unc-45b antisense probe was synthesized with Sp6 RNA polymerase from plasmid pGEM-unc45b linearized with BamHI.

The rest of the embryos (50) were used for RNA extraction and real time RT-PCR analysis. Total RNA was extracted from the control or BTS treated zebrafish embryos at 23 hpf using TRIzol reagent (Invitrogen). cDNA synthesis was carried out with the First-Strand cDNA synthesis kit (Life Sciences) using 4 μg of total RNA as template. Real time PCR was carried out using 7500 Fast Real-Time PCR System (Applied Biosystems). The PCR reaction was carried out using the standard SYBR Green PCR Mater Mix (Applied Biosystems). Standard curves of cDNA samples were constructed using 10 fold serial dilutions, and PCR efficiencies for each gene were determined with the slope of a liner regression model. The relative levels of gene expression were normalized to the housekeeping gene *ef-1α* and expression was

compared to normal levels in the control embryos using the delta delta Ct method (Livak and Schmittgen, 2001). Experiments were performed in triplicate and each reaction was run in duplicate. To establish differences between means, a two-tailed Student's t-test was used. Variables were considered significantly different at $P<0.05$. Real time PCR was carried out using the following primers designed at the junctions of two adjacent exons to eliminate potential problem with genomic DNA contamination.

zfhsp90α1-P6:agccagacttcggtgaatcaa

zfhsp90α1-P7: ttctctctgtttctcaatgtaaa

zfunc45b-P4: gctgcaaggaggtccaagaca

zfunc45b-P5: gatcatcagcatccagcatgt

zfef1α-P3: cttcaacgctcaggtcatcat

zfef1α-P4: acagcaaagcgaccaagagga

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