

Smyd1b is required for skeletal and cardiac muscle function in zebrafish

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ABSTRACT Smyd1b is a member of the Smyd family that is specifically expressed in skeletal and cardiac muscles. Smyd1b plays a key role in thick filament assembly during myofibrillogenesis in skeletal muscles of zebrafish embryos. To better characterize Smyd1b function and its mechanism of action in myofibrillogenesis, we analyzed the effects of *smyd1b* knockdown on myofibrillogenesis in skeletal and cardiac muscles of zebrafish embryos. The results show that knockdown of *smyd1b* causes significant disruption of myofibril organization in both skeletal and cardiac muscles of zebrafish embryos. Microarray and quantitative reverse transcription-PCR analyses show that knockdown of *smyd1b* up-regulates *heat shock protein 90* (*hsp90*) and *unc45b* gene expression. Biochemical analysis reveals that Smyd1b can be coimmunoprecipitated with heat shock protein 90 α -1 and Unc45b, two myosin chaperones expressed in muscle cells. Consistent with its potential function in myosin folding and assembly, knockdown of *smyd1b* significantly reduces myosin protein accumulation without affecting mRNA expression. This likely results from increased myosin degradation involving *unc45b* overexpression. Together these data support the idea that Smyd1b may work together with myosin chaperones to control myosin folding, degradation, and assembly into sarcomeres during myofibrillogenesis.

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INTRODUCTION

Myofibrillogenesis, the process of sarcomere assembly, is critical for muscle cell differentiation and contraction. Myofibrillogenesis involves hundreds of sarcomeric proteins assembled into a highly organized structure called the sarcomere, the basic contractile unit in striated muscles. The sarcomere is divided into four major compartments: Z-line, I-band, A-band, and M-line. The Z-line anchors the actin thin filaments of the I-band. The M-line anchors the myosin thick filaments of the A-band. The assembly and disassembly of

these multiprotein complexes follow ordered pathways that are highly regulated at the transcriptional, translational, and posttranslational levels. Disruption of these pathways leads to defective myofibril organization and skeletal and cardiac muscle diseases (Ehler and Gautel, 2008).

Recent studies show that Smyd1, a member of the Smyd family, plays a vital role in cardiogenesis and myofibrillogenesis (Gottlieb *et al.*, 2002; Tan *et al.*, 2006; Just *et al.*, 2011). Smyd1, also known as skm-Bop, is a MYND and SET domain-containing protein that is specifically expressed in skeletal and cardiac muscles (Hwang and Gottlieb, 1995, 1997). Targeted deletion of *Smyd1* in mice results in early embryonic lethality (Gottlieb *et al.*, 2002). Embryos died around embryonic day 10.5, presumably due to the disruption of right ventricle formation and cardiomyocyte maturation (Gottlieb *et al.*, 2002). The molecular mechanism by which Smyd1b functions is not well understood. Biochemical studies indicate that mouse Smyd1 and Smyd1b, one of the Smyd1 in zebrafish, can methylate histone H3 proteins *in vitro* (Tan *et al.*, 2006; Sirinupong *et al.*, 2010). However, the role of histone methylation in Smyd1b function is

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Abbreviations used: hpf, hours postfertilization; Hsp90, heat shock protein; MO, morpholino.

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controversial (Just *et al.*, 2011). In vitro studies have revealed that Smyd1 represses gene transcription in a histone deacetylase-dependent manner (Gottlieb *et al.*, 2002). Consistent with its potential function in transcriptional regulation by histone modification, Smyd1 is initially localized in the nucleus of C2C12 myoblasts (Sims *et al.*, 2002). However, it was reported that Smyd1 undergoes a nucleus-to-cytoplasm translocation during myoblast differentiation into myotubes (Sims *et al.*, 2002). Moreover, recent studies show that Smyd1b binds to myosin (Just *et al.*, 2011), suggesting that Smyd1b may have additional functions and targets in the cytoplasm. Consistent with the idea that Smyd1 may have additional targets, several recent studies demonstrated that Smyd2, another member of the Smyd family, can methylate nonhistone proteins, such as p53, RB1, and heat shock protein 90 (Hsp90; Huang *et al.*, 2006; Saddic *et al.*, 2010; Cho *et al.*, 2012; Abu-Farha *et al.*, 2011). Of interest, methylation of molecular chaperone Hsp90 by Smyd2 plays a critical role in sarcomere organization (Donlin *et al.*, 2012; Voelkel *et al.*, 2013).

A large body of evidence suggests that correct folding and assembly of myofibrillar proteins requires molecular chaperones and cochaperones (Barral and Epstein, 1999; Srikakulam and Winkelmann, 1999; Landsverk *et al.*, 2007; Kim *et al.*, 2008; Willis *et al.*, 2009). It was shown that Hsp90 and Unc45 form a complex with newly synthesized myosin proteins and play vital roles in myosin folding and assembly (Srikakulam and Winkelmann, 2004). Genetic studies in *Caenorhabditis elegans* and biochemical analyses in vitro indicate that chaperone-mediated myosin folding is an integral part of myofibril assembly (Hutagalung *et al.*, 2002). Knockdown or mutation of *hsp90α1* or *unc45* in *C. elegans* or zebrafish embryos results in complete disruption of myofibril organization (Epstein and Thomson, 1974; Barral *et al.*, 1998, 2002; Etard *et al.*, 2007; Wohlge-muth *et al.*, 2007; Du *et al.*, 2008), a phenotype very similar to *smyd1b* knockdown in zebrafish embryos (Tan *et al.*, 2006). However, the genetic and biochemical interactions among Smyd1, Hsp90α1, and Unc45b are not clear.

In this study, we characterize Smyd1b function in myofibril organization in skeletal and cardiac muscles of zebrafish embryos. We demonstrate that in addition to thick filament defects, knockdown of *smyd1b* causes significant disruption of thin and titin filaments, as well as M- and Z-lines in skeletal muscles of zebrafish embryos. Moreover, myofibril organization is also affected in cardiac muscles. Microarray and quantitative reverse transcription (qRT)-PCR analyses reveal that *smyd1b* knockdown significantly up-regulates *hsp90α1* and *unc45b* gene expression in zebrafish embryos. Biochemical analysis by coimmunoprecipitation (coIP) shows that Smyd1b can be coimmunoprecipitated with Hsp90α1 and Unc45b. Moreover, knockdown of *smyd1b* results in dramatic reduction of myosin protein accumulation in zebrafish embryos without any effect on myosin mRNA expression. Together these data support the idea that Smyd1b may work together with myosin chaperone Unc45b to control sarcomere assembly during myofibrillogenesis.

RESULTS

Knockdown of *smyd1b* expression results in significant disruption of sarcomere organization in skeletal muscles

We previously demonstrated that Smyd1b plays an important role in the thick filament assembly in slow muscles of zebrafish embryos at 24 h postfertilization (hpf; Tan *et al.*, 2006). However, a recent study showed that a loss-of-function Smyd1b mutant had a normal myofibril organization in slow muscles at 48 hpf, although the mutant embryos showed a clear fast muscle defect (Just *et al.*, 2011). To better understand the function of Smyd1b in myofibril organization in the slow muscle, we characterized thin filaments and Z-line

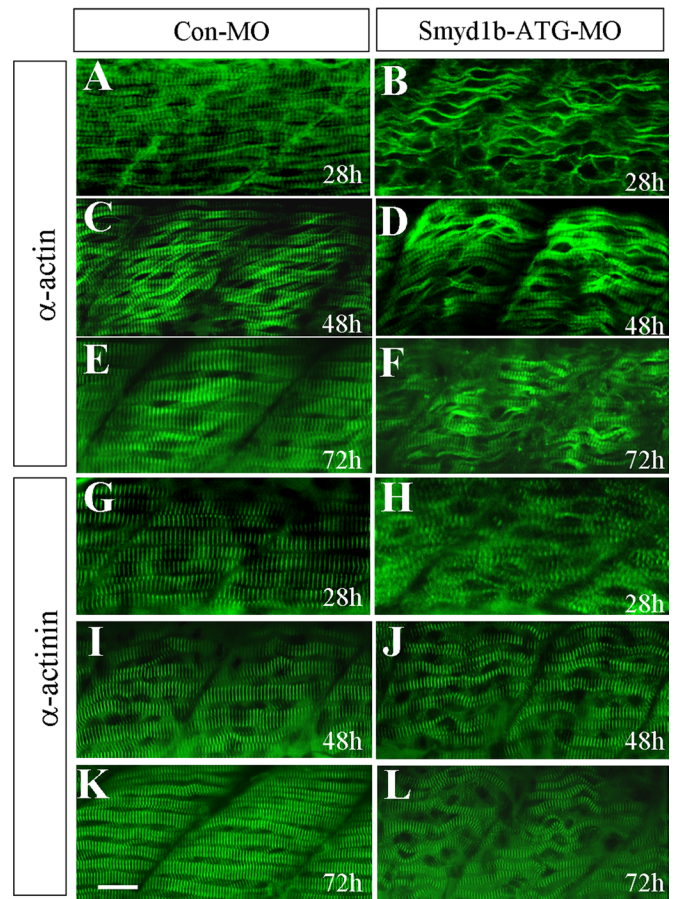


FIGURE 1: Knockdown of *smyd1b* expression results in defective thin filament and Z-line organization in slow muscles of early-stage zebrafish embryos. (A–F) Anti- α -actin antibody (Acl-20.4.2) staining shows organization of thin filaments in slow muscle fibers of control-MO (A, C, E) or Smyd1b-ATG-MO-injected (B, D, F) embryos at 28 (A, B), 48 (C, D), and 72 (E, F) hpf, respectively. (G–L) Anti- α -actinin antibody (EA-53) staining shows Z-line structure in control-MO (G, I, K) or Smyd1b ATG-MO-injected (H, J, L) embryos at 28 (G, H), 48 (I, J), and 72 (K, L) hpf, respectively. Scale bar, 25 μ m.

structures in slow muscles of Smyd1b-knockdown embryos at different times of development. When compared with the control embryo (Figure 1, A and G), the results show that knockdown of *smyd1b* significantly disrupts the myofibril organization of thin filaments and Z-lines in slow muscles of zebrafish embryos at 28 hpf (Figure 1, B and H). However, the myofibril defects at the thin filaments and Z-lines were partially recovered in *smyd1b*-knockdown embryos at 48 and 72 hpf, although the myofibers did not appear to be straight in the knockdown embryos (Figure 1, D, F, J, and L).

To determine whether the M-line structure was also disrupted in early-stage embryos at 28 hpf and recovered at the later stages (48 and 72 hpf), we took advantage of the recently identified myomesin-3-red fluorescent protein (RFP) line generated from gene trapping (Clark *et al.*, 2011). Myomesin-3-RFP fusion protein is specifically localized to the M-lines in slow muscles of zebrafish embryos (Clark *et al.*, 2011; Xu *et al.*, 2012). The myomesin-3-RFP localization was analyzed in *smyd1b*-knockdown embryos at 28, 48 and 72 hpf (Supplemental Figure S1). Compared with the control embryos (Supplemental Figure S1, A–C), the *smyd1b*-knockdown embryos showed no sarcomeric localization of myomesin-RFP at 28 hpf (Supplemental Figure S1D).

The M-line localization of myomesin-RFP, however, became apparent at 48 and 72 hpf in the *smyd1b*-knockdown embryos (Supplemental Figure S1, E and F).

Recent studies demonstrated that zebrafish contains two *smyd1* genes, *smyd1a* and *smyd1b* (Sun *et al.*, 2008). *Smyd1a* shares high sequence similarity with *Smyd1b* and is specifically expressed in muscle cells of zebrafish embryos starting around 24 hpf (Sun *et al.*, 2008). The recovery in *smyd1b*-knockdown embryos could be due to the redundant function of *Smyd1a* in later-stage embryos (48 and 72 hpf). To test this idea, we analyzed the phenotype of single and double knockdown of *smyd1a* and *smyd1b*. The results showed that knockdown of *smyd1a* and *smyd1b* together significantly disrupted the M-line organization of myomesin-3-RFP at all three stages analyzed (28, 48, and 72 hpf; Supplemental Figure S1). In contrast, knockdown of *smyd1a* or *smyd1b* alone had little or no effect at 48 and 72 hpf (Supplemental Figure S1). Collectively these data indicate that *Smyd1a* and *Smyd1b* are required for M-line organization and myofibril assembly in slow muscles of zebrafish embryos.

Zebrafish embryos contain two distinct types of muscles, slow and fast, which are located at different regions of the myotome (Devoto *et al.*, 1996; Du *et al.*, 1997). To analyze the effects of *smyd1b* knockdown on myofibril assembly in fast muscles, we examined the organization of thick and thin filaments, as well as M- and Z-lines, in fast muscles of *smyd1b*-knockdown zebrafish embryos. The results showed that knockdown of *smyd1b* completely disrupted the organization of thick and thin filaments in fast muscles of zebrafish embryos even at late stages at 72 hpf (Figure 2, B and D). Similarly, M- and Z-lines were also disrupted (Figure 2, F and H). It has been proposed that titin, the giant protein spanning from the Z-line to the M-line, is a key regulator of myofibrillogenesis. To determine whether titin organization was affected in *smyd1b*-knockdown embryos, we carried out immunostaining with the anti-titin antibody in zebrafish embryos. The results showed that titin filaments were also disrupted in *smyd1b*-knockdown embryos (Figure 2J). Collectively these data indicate that *Smyd1b* is required for sarcomere organization in both slow and fast muscles of zebrafish embryos. However, it is not clear whether *Smyd1b* has a direct role in the global organization of sarcomere assembly. It was reported that null mutants of the *unc-54* that encodes myosin heavy chain in *C. elegans* also led to disruptions in sarcomere organization (Epstein and Thomson, 1974; MacLeod *et al.* 1977). Our recent studies show that knockdown of myosin heavy chain (MHC) not only disrupts thick filament organization, it also results in poor organization of M-lines (Xu *et al.*, 2012). It remains to be determined whether the global disruption of sarcomere organization in *smyd1b*-knockdown embryos results directly from loss of *Smyd1b* function or indirectly from disruption of myosin thick filament organization.

Smyd1b transgene rescues the skeletal muscle defect from knockdown

To confirm the specificity of the *smyd1b*-knockdown phenotype, we performed a rescue experiment using a *Tg(Smyd1b:Smyd1b^{myc})* transgenic line expressing a myc-tagged *Smyd1b* driven by its own muscle-specific promoter (Du *et al.*, 2006; Tan *et al.*, 2006). The transgene was constructed using the *Smyd1b* cDNA, which does not need splicing for expression (Supplemental Figure S2). The *Smyd1b* splicing morpholino (MO; E919-MO) was microinjected into the *Tg(Smyd1b:Smyd1b^{myc})* transgenic zebrafish embryos. Western blot showed that expression of the myc-tagged *Smyd1b* from the transgene was not affected by the E919 splicing MO (Supplemental Figure S2). The sarcomeric structures were analyzed in the E919-MO-injected embryos by immunostaining. The results showed that expression of

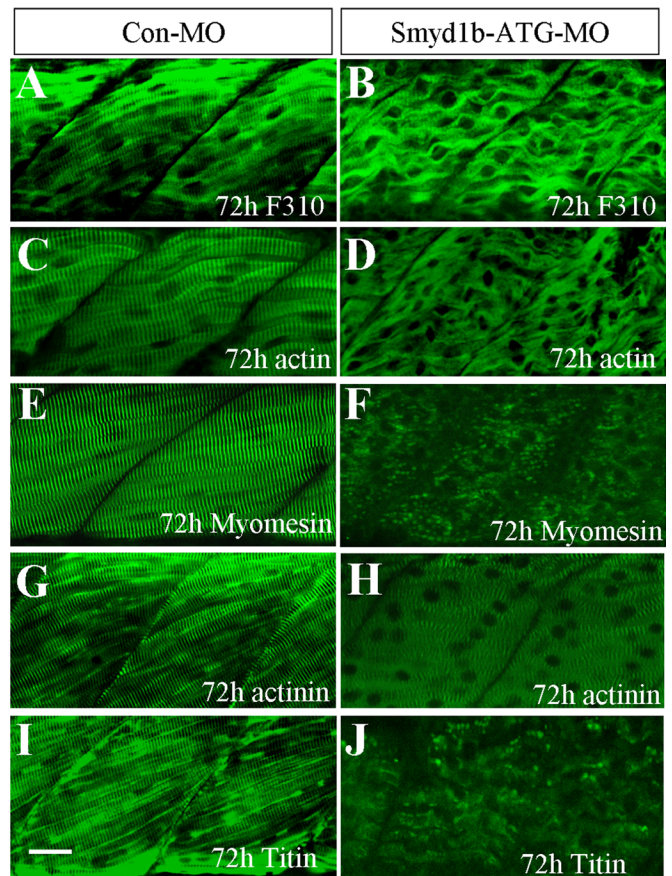


FIGURE 2: Knockdown of *smyd1b* expression results in defective sarcomere organization in fast skeletal muscles of zebrafish embryos. (A, B) Anti-MLC (F310) antibody staining (lateral view) shows thick filaments in fast muscle fibers of control (A) or *Smyd1b*-ATG-MO-injected (B) embryos at 72 hpf. (C, D) Anti- α -actin antibody (Acl-20.4.2) staining (lateral view) shows thin filaments in fast muscle fibers of control (C) or *Smyd1b*-ATG-MO-injected (D) embryos at 72 hpf. (E, F) Anti-myomesin antibody (mMaC myomesin B4) staining shows M-line structure in control-MO (E) or *Smyd1b*-ATG-MO-injected (F) embryos at 72 hpf. (G, H) Anti- α -actinin antibody (EA-53) staining shows Z-line structure in control-MO (G) or *Smyd1b*-MO-injected (H) embryos at 72 hpf. (I, J) Anti-titin (T11) antibody staining shows the sarcomeric localization of titin in fast muscle fibers in control-MO (I) or *Smyd1b* ATG-MO-injected (J) embryos at 72 hpf. Scale bar, 25 μ m.

the myc-tagged *Smyd1b* can rescue the myofibril defects in *smyd1b*-knockdown embryos (Figure 3). Compared with the nontransgenic control, transgenic embryos injected with the E919 splicing MO show normal organization of thin and titin filaments, as well as M- and Z-lines (Figure 3). Together these data confirm that muscle defects from *smyd1b* knockdown are gene specific, indicating that *Smyd1b* is essential for myofibrillogenesis in skeletal muscles.

Knockdown of *smyd1b* disrupts sarcomere organization in cardiac muscles of zebrafish embryos

It was reported that targeted deletion of *smyd1* results in defective cardiomyogenesis in mouse embryos that fail to form the right ventricle (Gottlieb *et al.*, 2002). Our previous studies demonstrated that *smyd1b*-knockdown zebrafish embryos have no heartbeat and blood circulation (Tan *et al.*, 2006). To better characterize the heart defects in *smyd1b*-knockdown embryos, we analyzed heart tube

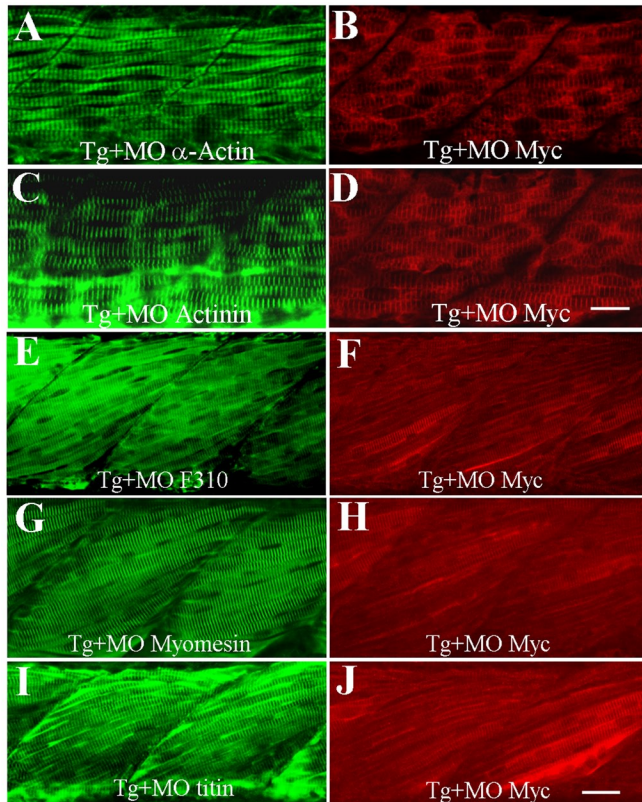


FIGURE 3: Rescue of myofibril defects by expression of myc-tagged Smyd1b. The Smyd1b E919-MO was injected into *Tg(Smyd1b:Smyd1b^{myc})* transgenic zebrafish embryos expressing a myc-tagged Smyd1b at one- to two-cell stages. Expression of myc-tagged Smyd1b proteins was analyzed by Western blot and immunostaining with anti-myc antibodies. (A, B) Anti- α -actin and anti-myc (9E10) antibody double staining shows that expression of myc-tagged Smyd1b from the transgene (B) can rescue the knockdown effect on thin filament organization (α -actin) in Smyd1b MO-injected embryos at 24 hpf (A). (C, D) Anti-actinin and anti-myc (9E10) antibody double staining shows that expression of myc-tagged Smyd1b from the transgene (D) can rescue the knockdown effect on Z-line organization (α -actinin) in Smyd1b MO-injected embryos at 24 hpf (C). (E, F) Anti-MLC (F310) and anti-myc antibody double staining shows that expression of myc-tagged Smyd1b from the *Tg(Smyd1b:Smyd1b^{myc})* transgene (F) can rescue thick filament defects in Smyd1b MO-injected embryos at 3 d postfertilization (dpf; E). (G, H) Anti-myomesin and anti-myc antibody double staining shows that expression of myc-tagged Smyd1b from the *Tg(Smyd1b:Smyd1b^{myc})* transgene (H) can rescue the disruption of the M-line in Smyd1b MO-injected embryos at 3 dpf (G). (I, J) Anti-titin and anti-myc antibody double staining shows that expression of the *Tg(Smyd1b:Smyd1b^{myc})* transgene (J) can rescue the disruption of titin assembly in Smyd1b MO-injected embryos at 3 dpf (I). Scale bars, 25 μ m.

patterning in *smyd1b*-knockdown embryos by double staining using antibodies that recognize both ventricle and atrium (MF20) or atrium specifically (S46; Stainier and Fishman, 1992). The results show that heart tube patterning is normal in *smyd1b*-knockdown embryos (Figure 4, A–F).

To further determine whether knockdown of *smyd1b* disrupts the sarcomere assembly in the cardiac muscles of zebrafish embryos, we examined thick filament and Z-line organization in cardiac muscles of *smyd1b*-knockdown embryos. Compared with the control (Figure 4G), few or no thick filaments could be detected in

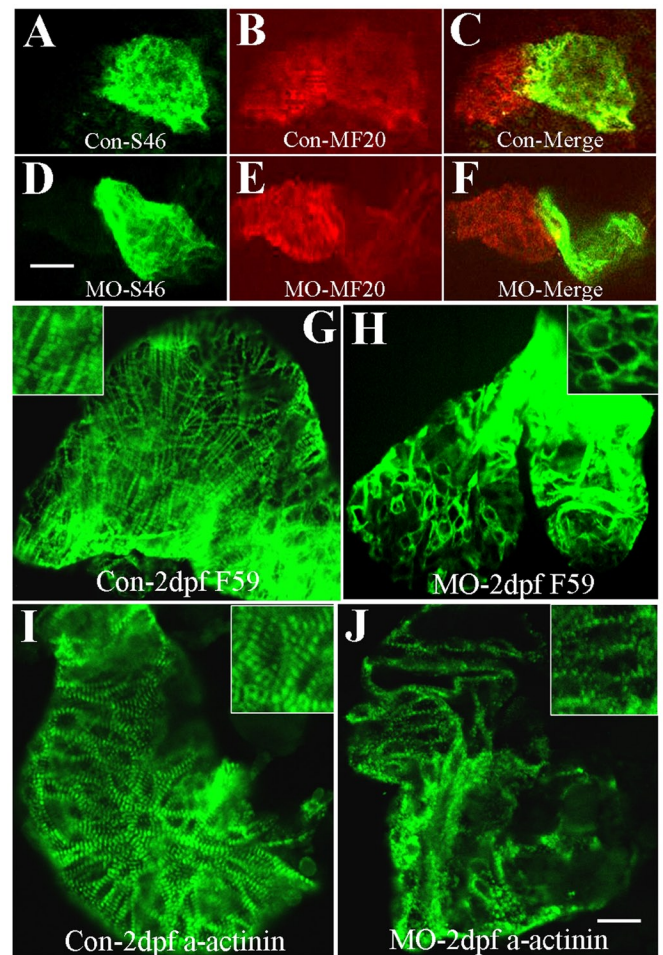


FIGURE 4: Immunostaining shows disorganized thick filaments and Z-line organization in cardiac muscles of *smyd1b*-knockdown embryos. (A–C) Double immunostaining with S46 (A) and MF20 (B) antibodies shows the atrium and ventricle formation in control-MO-injected embryos at 2 dpf. (C) Merged picture of A and B. Scale bars, 100 μ m. (D–F) Double immunostaining with S46 (D) and MF20 (E) antibodies shows the atrium and ventricle formation in Smyd1b ATG-MO-injected embryos at 2 dpf. (F) Merged picture of D and E. Scale bar, 100 μ m. (G, H) Anti-MHC (F59) antibody staining shows thick filament organization in control-MO (G) and Smyd1b ATG-MO-injected (H) embryos at 2 dpf. (I, J) Anti- α -actinin antibody staining shows Z-line structure in control-MO (I) and Smyd1b ATG-MO-injected (J) embryos at 2 dpf. Scale bar, 15 μ m.

cardiac muscles of *smyd1b*-knockdown embryos (Figure 4H). In addition, the Z-line structure was also disrupted in cardiac muscles of Smyd1b-knockdown embryos (Figure 4J). Together these studies indicate that Smyd1b is also required for myofibril organization in cardiac muscles.

Knockdown of *smyd1b* results in up-regulation of *hsp90 α 1* and *unc45b* gene expression

Members of the Smyd family are methyltransferases that are able to methylate histone proteins in vitro (Hamamoto *et al.*, 2004; Brown *et al.*, 2006; Tan *et al.*, 2006; Sirinupong *et al.*, 2010). Histone methylation has been implicated in regulation of gene expression. To test whether knockdown of *smyd1b* might alter gene expression involved in myofibrillogenesis, we carried out a microarray analysis comparing the expression profile of ~20,000 genes in

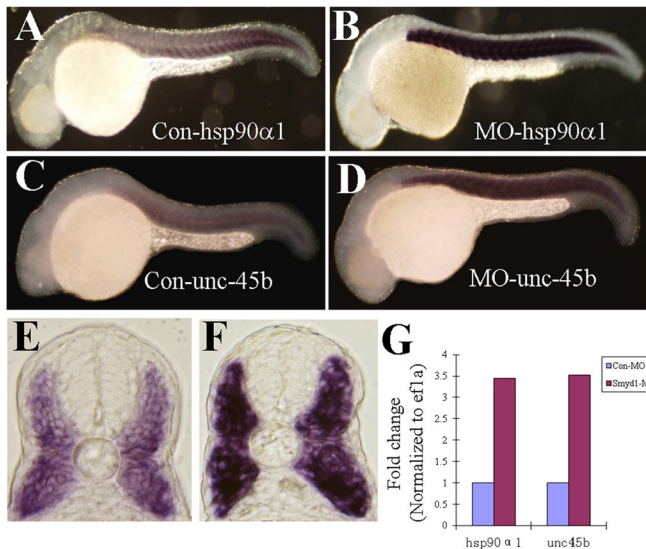


FIGURE 5: Whole-mount in situ hybridization and real-time PCR show the increased expression of *hsp90α1* and *unc45b* in *smyd1b*-knockdown embryos. (A–D) In situ hybridization (side views) shows the expression of *hsp90α1* (A, B) and *unc45b* (C, D) in control-MO (A, C) or *Smyd1b* ATG-MO-injected (B, D) embryos at 24 hpf. (E, F) Views of cross sections show the result of in situ hybridization of *unc45b* expression in control-MO (E) or *Smyd1b* ATG-MO-injected (F) embryos at 24 hpf. (G) Real-time PCR analysis of *hsp90α1* and *unc45b* expression in control-MO or *Smyd1b* ATG-MO-injected embryos at 24 hpf.

smyd1b-knockdown and control zebrafish embryos at 24 hpf. The data showed that the expression of 12 genes was up-regulated twofold to sixfold in *smyd1b*-knockdown embryos compared with the control. Strikingly, eight of them encode members of the heat shock protein family, including *hsp70*, *hsp90*, and *hsp47* (Supplemental Table S1). In addition, >200 genes were down-regulated in *smyd1b*-knockdown embryos. However, no clear trend or common pathways were noted among those down-regulated genes.

Among these up-regulated *hsp* genes, *hsp90α1* is especially interesting. *hsp90α1* is specifically expressed in muscle cells of zebrafish embryos and plays a vital role in myofibril assembly (Sass *et al.*, 1996, 1999; Du *et al.*, 2008; Hawkins *et al.*, 2008). It was shown that Hsp90 forms a complex with newly synthesized myosin protein and is involved in myosin folding and assembly (Srikakulam and Winkelmann, 2004). To validate the results from the microarray analysis, we analyzed *hsp90α1* mRNA expression in *smyd1b*-knockdown embryos by in situ hybridization and qRT-PCR. The results confirmed that knockdown of *smyd1b* significantly up-regulated *hsp90α1* gene expression (Figure 5B). qRT-PCR data indicated that the increase was approximately threefold (Figure 5G).

It was reported that Unc45 associates with Hsp90 and functions as a myosin chaperone involved in myosin folding and assembly (Barral *et al.*, 2002; Etard *et al.*, 2007). To determine whether *unc45b* expression was also up-regulated in *smyd1b*-knockdown zebrafish embryos, we conducted in situ hybridization and quantitative PCR in the *smyd1b*-knockdown embryos. The result showed that *unc45b* expression was significantly increased in *smyd1b*-knockdown embryos (Figure 5, D, F, and G). Together these results indicate that *Smyd1b* knockdown could lead to up-regulation of heat shock protein and *unc45b* gene expression in skeletal muscles of zebrafish embryos.

The up-regulation of *hsp90α1* gene expression by *smyd1b* knockdown raises the question of whether *Smyd1b* could be

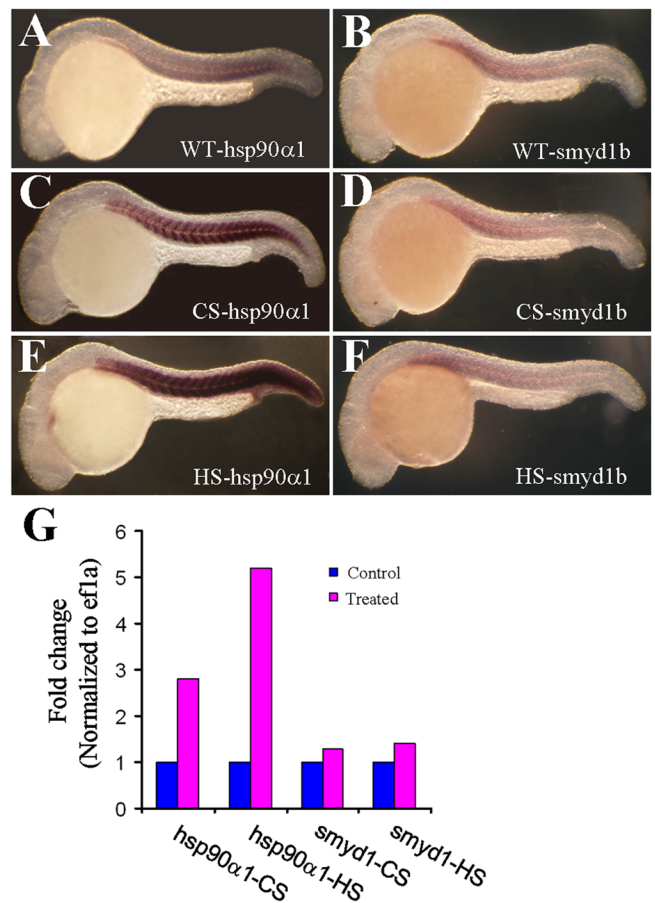


FIGURE 6: Cold and heat shock treatment has no effect on *smyd1b* gene expression in zebrafish embryos. Zebrafish embryos were subjected to cold or heat shock treatment. Expression of *hsp90α1* and *smyd1b* was analyzed in these embryos by whole-mount in situ hybridization and real time RT-PCR. (A, B) Whole-mount in situ hybridization shows the expression of *hsp90α1* (A) and *smyd1b* (B) in control embryos. (C, F) Whole-mount in situ hybridization shows the expression of *hsp90α1* (C, E) and *smyd1b* (D, F) in cold-shocked (C, D) or heat-shocked (E, F) zebrafish embryos. (G) Real time RT-PCR shows the expression of *hsp90α1* and *smyd1b* mRNA transcripts in cold-shocked (CS) or heat-shocked (HS) zebrafish embryos.

involved in stress response. To test this idea, we stressed zebrafish embryos with heat or cold shock. Expression of *hsp90α1* and *smyd1b* was subsequently determined in the heat- or cold-stressed embryos. Although both heat and cold shock treatment significantly increased *hsp90α1* expression (Figure 6, C and E), little or no change of *smyd1b* expression was detected in either heat- or cold-stressed embryos (Figure 6, D and F). This was further confirmed by qRT-PCR (Figure 6G). Together these data indicate that although knockdown of *smyd1b* induces heat shock protein gene expression, *Smyd1b* itself is not involved in cold- or heat-induced stress response.

Smyd1 protein complex formation with Unc45b and Hsp90α1

Unc45b is a myosin chaperone that plays a pivotal role in myosin folding and assembly (Barral *et al.*, 2002; Etard *et al.*, 2007). Loss of Unc45b function results in defective myofibril organization in skeletal muscles of zebrafish embryos (Etard *et al.*, 2007; Wohlgemuth *et al.*, 2007; Hawkins *et al.*, 2008), a phenotype very

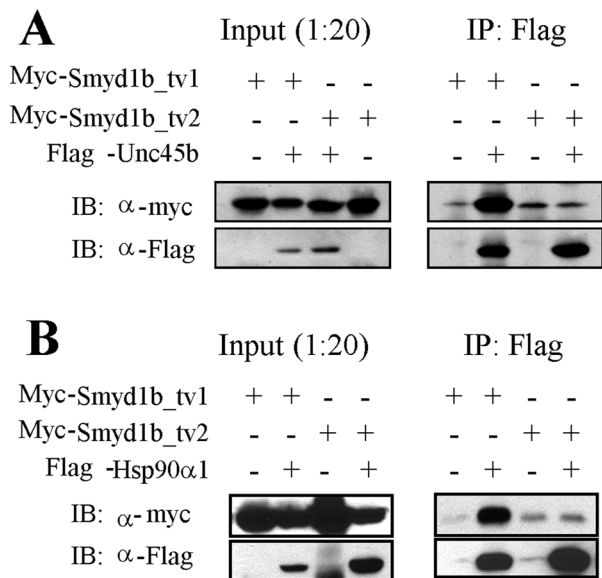


FIGURE 7: Coimmunoprecipitation shows Smyd1b-tv1, but not Smyd1b-tv2, interacts with myosin chaperone Hsp90 α 1 and Unc-45b. (A) colP shows that myc-tagged Smyd1b-tv1 can be pulled down by FLAG-tagged Unc-45b coexpressed in HEK293 cells. (B) colP shows that myc-tagged Smyd1b-tv1 can be pulled down by the FLAG-tagged Hsp90 α 1 coexpressed in HEK293 cells.

similar to *smyd1b* knockdown (Tan *et al.*, 2006). To determine whether Smyd1b might be involved in myofibrillogenesis by forming a complex with myosin chaperone Unc45b, we performed colP analysis using HEK293 cells expressing FLAG-tagged Unc45b and myc-tagged Smyd1b_tv1 or Smyd1b_tv2, encoded by two alternatively spliced isoforms of *smyd1b* (Li *et al.*, 2011). The results show that Smyd1b_tv1 can be coimmunoprecipitated with FLAG-tagged Unc45b (Figure 7A). This association appears to be isoform specific (Figure 7A). The Smyd1b_tv2 isoform shows little or no interaction with Unc45b in the colP assay (Figure 7A). The colP results suggest that Smyd1b (Smyd1b_tv1) and Unc45b coexist in a protein complex. However, it is not clear whether Smyd1b and UNC-45b actually bind to one another. Recent studies show that Unc45b and Smyd1b can bind myosin heavy chain (Barral *et al.*, 2002; Just *et al.*, 2011). One possibility is that Smyd1b and Unc45b form a protein complex mediated by myosin heavy chain.

Recent studies show that Unc45b functions as a Hsp90 α 1 co-chaperone in myosin folding and assembly (Barral *et al.*, 1998; Etard *et al.*, 2007; Du *et al.*, 2008). To determine whether Smyd1b_tv1 is also able to form a protein complex with Hsp90 α 1, we performed a similar colP assay with FLAG-tagged Hsp90 α 1. The results show that, similar to Unc45b, Smyd1b_tv1, but not Smyd1b_tv2, can be coimmunoprecipitated with FLAG-tagged Hsp90 α 1 (Figure 7B). Collectively these data indicate that Smyd1b_tv1 might associate directly or indirectly with Hsp90 α 1 and Unc45b, suggesting that they might work together to control myosin folding and assembly.

Knockdown of *smyd1b* reduces myosin protein accumulation in zebrafish embryos

Previous studies showed that loss of Hsp90 α 1 or Unc45b function significantly reduces myosin protein accumulation in zebrafish embryos (Du *et al.*, 2008; Hawkins *et al.*, 2008; Bernick *et al.*, 2010). To determine whether knockdown of *smyd1b* can result in reduction of myosin protein accumulation in zebrafish embryos, we compared

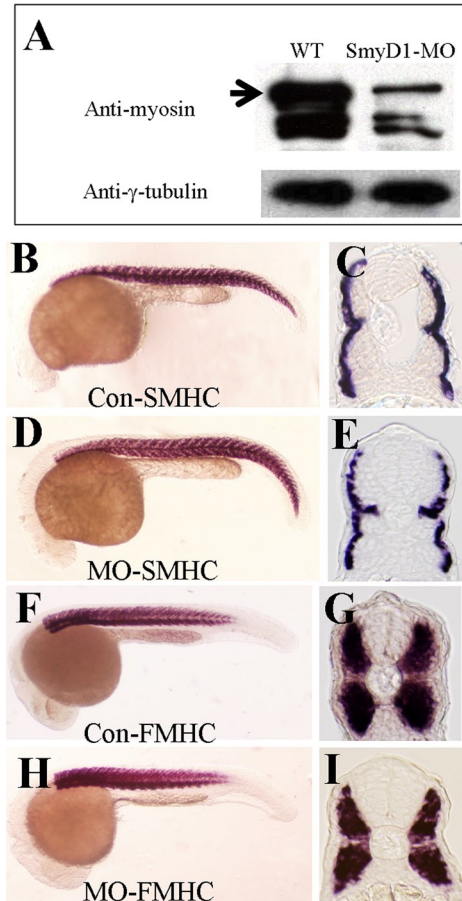


FIGURE 8: Knockdown of *smyd1b* results in decreased levels of myosin proteins but not their mRNAs. (A) Western blot using MF20 antibody shows decreased protein levels of myosin heavy chain in *smyd1b*-knockdown embryos. γ -Tubulin was used as loading control. (B–E) In situ hybridization shows the mRNA levels of slow myosin heavy chain in control-MO (B, C) or Smyd1b ATG-MO-injected (D, E) embryos at 24 hpf in different views. (F–I) In situ hybridization shows the mRNA levels of fast myosin heavy chain in control-MO (F, G) or Smyd1b ATG-MO-injected (H, I) embryos at 24 hpf in different views.

myosin protein levels in *smyd1b*-knockdown and control embryos at 24 hpf. Western blot analysis showed significant reduction of myosin heavy chain protein levels in *smyd1b*-knockdown embryos (Figure 8A). To test whether this reduced protein accumulation was due to decreased transcription of myosin gene or poor mRNA stability, we analyzed the myosin mRNA levels by in situ hybridization and quantitative RT-PCR in *smyd1b*-knockdown embryos. The result shows that there is little or no change in myosin mRNA levels in *smyd1b*-knockdown embryos compared with control (Figure 8, B–I). This was further confirmed by qRT-PCR (Supplemental Figure S3), indicating that the reduced myosin protein accumulation is not due to decreased myosin mRNA levels.

The reduced myosin accumulation in *smyd1b*-knockdown embryos is similar to previous findings in *unc45b*-knockdown embryos (Bernick *et al.*, 2010). However, knockdown of *smyd1b* resulted in increased *unc45b* mRNA expression. Previous studies in *C. elegans* and zebrafish showed that overexpression of Unc45 results in thick filament defects and decreased myosin expression (Landsverk *et al.*, 2007; Bernick *et al.*, 2010; Ni *et al.*, 2011). To better understand the

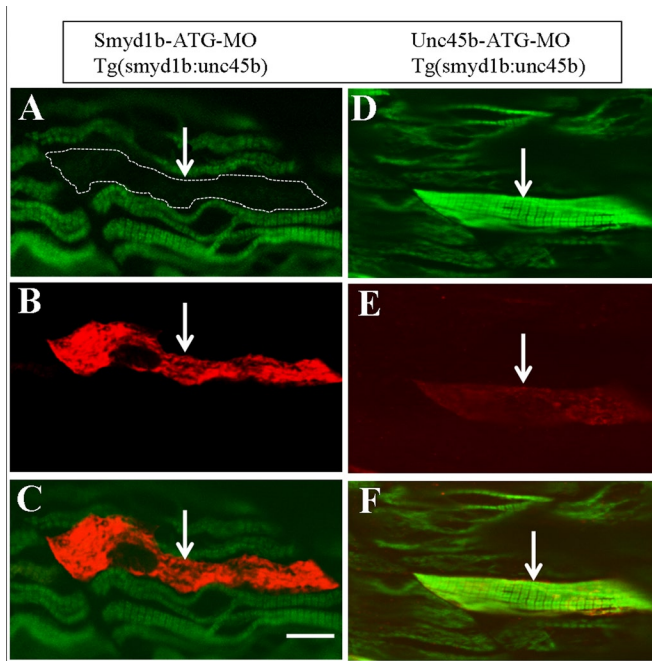


FIGURE 9: Overexpression of Unc45b increases myosin degradation in *smyd1b*-knockdown embryos. The DNA construct expressing FLAG-tagged Unc-45b under the control of *smyd1* promoter was coinjected with *Smyd1b*-ATG-MO or *Unc45b*-ATG-MO into zebrafish embryos. The effect on myosin expression and thick filament organization was analyzed by double staining with anti-FLAG (red) and anti-myosin (F59, green) antibodies at 36 hpf. (A–C) Double staining shows the expression of FLAG-tagged *Unc45b* (B) in a single fiber (indicated by an arrow) and its effect on myosin expression and thick filament organization (A, C). (C) Merged image of A and B, showing that the myofibril defect is restricted to the myofiber expressing the FLAG-tagged *Unc45b*. (D–F) Double staining shows the rescue of myosin thick filament organization in *Unc45b*-knockdown zebrafish embryos coinjected with *Unc45b*-MO and the *smyd1:unc45b^{FLAG}* DNA construct. A myofiber (arrow) expressing FLAG-tagged *Unc45b* (E) exhibited normal myosin expression and thick filament organization (D, F). Scale bar, 15 μ m.

interaction between *Smyd1b* and *Unc45b* in myosin folding and degradation, we directly expressed *Unc45b* in *smyd1b*-knockdown embryos by coinjecting a *Tg(smyd1b:Unc45b^{FLAG})* transgene encoding a FLAG-tagged *Unc45b* with the *Smyd1b* ATG-MO into zebrafish embryos. A typical mosaic pattern of *Unc45b* expression was detected in the injected embryos (Figure 9). Consistent with the idea that overexpression of *Unc45b* is detrimental to myosin thick filament organization, our data show that myofibers with ectopic *Unc45b* expression show no or little myosin expression and thick filament organization compared with neighboring myofibers without ectopic *Unc45b* expression (Figure 9, A–C). To test that FLAG-tagged *Unc45b* is bioactive, we coinjected the *Tg(smyd1b:Unc45b^{FLAG})* transgene with the *Unc45b*-ATG-MO into zebrafish embryos. In contrast to the effects in the *smyd1b*-knockdown embryos, ectopic expression of FLAG-tagged *Unc45b* rescued the thick filament defects in *unc45b*-knockdown embryos, suggesting that the FLAG-tagged *Unc45b* is biologically active (Figure 9, D–F). Collectively, these data suggest that *Smyd1b* and *Unc45b* might work together to control myosin folding and degradation. Moreover, up-regulation of *unc45b* expression might be involved in myosin degradation in *smyd1b*-knockdown embryos.

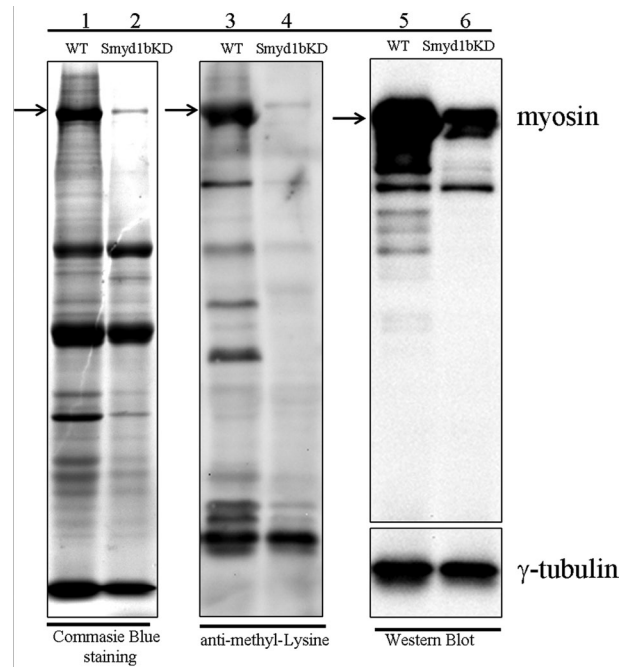


FIGURE 10: Analysis of protein methylation in *smyd1b*-knockdown embryos by Western blot. Lanes 1 and 2, Coomassie blue staining showing protein expression in wild-type (WT) control and *smyd1b*-knockdown embryos at 72 hpf. Lanes 3 and 4, protein methylation in WT control and *smyd1b*-knockdown embryos at 72 hpf by Western blot using anti-methyl lysine antibody. Lanes 5 and 6, myosin heavy chain protein levels in WT control and *smyd1b*-knockdown embryos at 72 hpf by Western blot using MF-20 antibody. γ -Tubulin was probed as a loading control.

Protein methylation was suggested to play important roles in protein stabilization/destabilization (Yang *et al.*, 2009; Egorova *et al.*, 2010; Zhang *et al.*, 2012). Several sarcomere proteins, such as myosin, actin, and creatine kinase, are methylated at their lysine residues (Tong and Elzinga, 1983; Iwabata *et al.*, 2005). To test whether myosin heavy chain is methylated in zebrafish embryos, we carried out a Western blot analysis using anti-methyl lysine and anti-myosin antibodies. The data showed that several proteins, including myosin heavy chain, are indeed methylated in zebrafish embryos (Figure 10). Consistent with the reduced levels of myosin protein accumulation in *smyd1b*-knockdown embryos, the levels of methylated myosin were also significantly reduced in *Smyd1b*-knockdown zebrafish embryos (Figure 10). Together these data indicate that myosin heavy chain proteins are methylated at lysine residues in zebrafish embryos. The significant reduction of myosin accumulation in *smyd1b*-knockdown may be directly or indirectly linked to myosin methylation, which may play an important role in protein stability and assembly into sarcomeres.

DISCUSSION

In this study, we characterized the myofibril defects in skeletal and cardiac muscles of *smyd1b*-knockdown zebrafish embryos. We demonstrated that *Smyd1b* is required for myofibril organization in both skeletal and cardiac muscles. Knockdown of *smyd1b* expression resulted in complete disorganization of all key sarcomeric structures, including thick, thin, and titin filaments, as well as M- and Z-lines in skeletal and cardiac muscles of early-stage zebrafish embryos. We further demonstrated that knockdown of *smyd1b* resulted

in up-regulation of *unc45b* and *hsp90α1* gene expression, two important myosin chaperones involved in myosin folding and sarcomere assembly. Biochemical analysis by colP revealed that Smyd1b_tv1 might associate directly or indirectly with Unc45b and Hsp90α1 in protein complexes. Moreover, knockdown of *smyd1b* expression resulted in a significant reduction of myosin methylation and protein accumulation without any significant effect on their mRNA expression. Together these data support the idea that Smyd1b is required for myofibril assembly in all striated muscles, and Smyd1b might work together with myosin chaperones to control sarcomere assembly during myofibrillogenesis.

Smyd1b is required for myofibril organization in slow muscles of early-stage zebrafish embryos

We previously demonstrated that Smyd1b is required for thick filament organization in slow muscles of zebrafish embryos (Tan *et al.*, 2006). However, a recent report suggests that Smyd1b is not required for sarcomere organization in slow muscle of zebrafish embryos (Just *et al.*, 2011). To test the hypothesis that the discrepancy between these two studies could be caused by different timing of the phenotypic analyses, we analyzed the sarcomere organization in slow muscles of *Smyd1b*-knockdown embryos at various stages during development. Consistent with this idea, we demonstrated that Smyd1b is required for myofibril assembly in slow muscles of early stage zebrafish embryos but becomes dispensable in late-stage embryos in slow muscles. This is likely due to the expression of another *smyd1b*-related gene, *smyd1a*, in muscle cells of zebrafish embryos starting around 24 hpf (Sun *et al.*, 2008). Collectively data from these studies also suggest that Smyd1a and Smyd1b may not play an equal role in myofibrillogenesis. Smyd1b appears to play a more critical role than Smyd1a in sarcomere organization, especially in fast muscles and early-stage slow muscles of zebrafish embryos.

In addition to the thick filament defects, we showed that knockdown of *smyd1b* also disrupted the organization of thin and titin filaments, as well as of M-lines. This raises the question of whether M- and Z-line defects result directly from *smyd1b* knockdown or a secondary effect from myosin degradation and thick filament disorganization. Our recent studies from direct myosin knockdown in zebrafish embryos are consistent with a secondary effect (Codina *et al.*, 2010; Xu *et al.*, 2012). We showed that knockdown of myosin heavy chain 1 (*myhc1*) resulted in sarcomeric defects in both thick and thin filaments, as well as in M- and Z-lines (Codina *et al.*, 2010; Xu *et al.*, 2012). Together these studies indicate that the muscle phenotype from *smyd1b* knockdown could be due, at least in part, to disruption of myosin folding and assembly.

Protein complex formation with Unc45b and Hsp90α1

We showed by colP assay that the longer isoform of Smyd1b (*Smyd1b_tv1*) could be coimmunoprecipitated with myosin chaperone Unc45b and Hsp90α1 expressed in HEK293 cells. The colP results could only suggest that Smyd1b (*Smyd1b_tv1*) coexists with Unc45b or Hsp90α1 in a protein complex. They do not reveal whether Smyd1b actually binds to UNC-45b and Hsp90α1. Future pull-down studies using purified proteins will be required to demonstrate direct physical binding between Smyd1b and UNC-45b or Hsp90α1 proteins. Regardless of whether *Smyd1b_tv1* can bind directly or indirectly with Unc45b and Hsp90α1, several pieces of evidence suggest that *Smyd1b_tv1* may work together with Unc45b and Hsp90α1 to control myosin folding and sarcomere assembly during myofibrillogenesis. First, genetic analysis shows that knockdown or mutation of *unc45b* or Hsp90α1 results in similar muscle defects as *Smyd1b* knockdown in zebrafish embryos (Etard *et al.*,

2007; Wohlgemuth *et al.*, 2007; Du *et al.*, 2008; Hawkins *et al.*, 2008). Second, biochemical analysis reveals that knockdown or mutation of *smyd1b* results in significant reduction of myosin protein accumulation with no effect on mRNA levels, a phenotype very similar to that observed in *Unc45b*- or *Hsp90α1*-knockdown zebrafish embryos (Etard *et al.*, 2007; Wohlgemuth *et al.*, 2007; Du *et al.*, 2008; Hawkins *et al.*, 2008).

Up-regulation of *hsp90α1* and *unc45b* gene expression in *Smyd1b*-knockdown embryos

In this study, we showed that knockdown of *Smyd1b* resulted in up-regulation of *hsp90α1* and *unc45b* gene expression in zebrafish embryos. Up-regulation of *hsp90α1* and *unc45b* gene expression appears to be a common response in zebrafish embryos with myofibril disorganization. Disruption of myofibril organization by loss of *Unc45b* function also leads to up-regulation of *hsp90α1* expression (Etard *et al.*, 2007), and up-regulation of *unc45b* expression was noted in *hsp90α1*-mutant or -knockdown zebrafish embryos (Etard *et al.*, 2007; Du *et al.*, 2008; Hawkins *et al.*, 2008). The mechanism underlying this up-regulation of gene expression is not clear. Disruption of myosin folding and assembly by *smyd1b* knockdown likely increases misfolding of myosin and other sarcomere proteins in muscle cells. The up-regulation of *hsp90α1* and *unc45b* expression could result from increasing demand for refolding and degradation of misfolded sarcomeric proteins in *smyd1b*-knockdown embryos. Consistent with this, we found that other stress response genes, such as *hsp70* and *hsp47*, are also up-regulated in *smyd1b*-knockdown zebrafish embryos (Supplemental Table S1).

Recent studies show that overexpression of *Unc45* results in thick filament defects and decreased myosin expression in *C. elegans* and zebrafish (Landsverk *et al.*, 2007; Bernick *et al.*, 2010; Ni *et al.*, 2011). Consistent with the idea that up-regulation of *unc45* expression could be involved in myosin degradation in *smyd1b*-knockdown embryos, we demonstrated in this study that ectopic expression of *Unc45b* in *smyd1b*-knockdown embryos further enhanced myosin degradation and resulted in little or no thick filament organization compared with *smyd1b* knockdown alone. The molecular mechanism by which *Unc45b* regulates myosin degradation is not clear. It has been suggested that *Unc45* functions as a myosin chaperone that regulates myosin accumulation and assembly by linkage to the ubiquitin proteasome system for degradation (Landsverk *et al.*, 2007). Structural and functional analyses show that *Unc45* contains an N-terminal TPR domain involved in interaction with Hsp90 and a C-terminal UCS domain required for interaction with myosin (Barral *et al.*, 2002). Deletion of the C-terminal UCS domain abolished the disruptive effect of *Unc45b* overexpression in myosin thick filament organization. In contrast, deletion of the N-terminal TPR domain required for binding with Hsp90 had no effect (Bernick *et al.*, 2010; Ni *et al.*, 2011).

***Smyd1b* functions in cardiac muscle myofibrillogenesis**

In this study we showed that knockdown of *smyd1b* does not affect heart tube patterning in zebrafish embryos. Heart tube with clear atrium and ventricle structures was observed in *smyd1b*-knockdown zebrafish embryos, consistent with a report by Just *et al.* (2011). The lack of heart tube patterning defects in fish embryos differs from the mouse *smyd1b*-knockout phenotype, in which the right ventricle fails to form in mouse embryos (Gottlieb *et al.*, 2002). The reason for the observed discrepancy between these two studies is not clear. It could be due to the different heart patterning between fish and mammals. In contrast to mammalian heart, which has two separate ventricles, fish heart has only one ventricle.

Our studies, however, demonstrated that Smyd1b plays an important role in myofibril assembly in cardiac muscles. Knockdown of *smyd1b* resulted in disorganization of sarcomeric structures in heart muscle cells. Both myosin thick filaments and Z-lines were disrupted in cardiac muscles of *smyd1b*-knockdown zebrafish embryos. This is consistent with a report on *smyd1b*-mutant zebrafish embryos (Just *et al.*, 2011). The myofibril defects in cardiac muscles resemble the skeletal muscle defects in *smyd1b*-knockdown embryos. Collectively these studies indicate that Smyd1b plays an important role in myofibril assembly in both skeletal and cardiac muscles.

Smyd1b and protein methylation

Previous studies showed that Smyd1b is a lysine methyltransferase that can methylate histone 3 *in vitro* (Tan *et al.*, 2006). Recent studies (Just *et al.*, 2011), however, demonstrated that the histone methyltransferase activity is not required for Smyd1b function in myofibrillogenesis. It has been increasingly recognized that members of the Smyd family are able to methylate nonhistone proteins. For example, Smyd2 can methylate Hsp90 and plays a critical role in sarcomere organization (Abu-Farha *et al.*, 2011; Donlin *et al.*, 2012; Voelkel *et al.*, 2013). The *in vivo* targets of Smyd1 remain are not known. Yeast two hybrid and colP analyses identified several tentative Smyd1-binding proteins, including skNAC, Mical2b, helicase, and FKBP8 (Park *et al.*, 2010). It remains to be determined whether any of these proteins are methylation targets of Smyd1.

In this study, we analyzed the overall protein methylation in zebrafish embryos and showed that myosin heavy chain is methylated in zebrafish embryos. In addition, we found that the myosin methylation was significantly decreased in *smyd1b*-knockdown embryos. The significant reduction of myosin methylation could be due to the increased myosin protein degradation in *smyd1b*-knockdown embryos. Conversely, methylation of myosin by Smyd1b may be required for its folding and stability, and, hence, knockdown of *smyd1b* expression may result in poor methylation of myosin proteins, leading to increased degradation. Protein methylation has been suggested to play important roles in protein stabilization and destabilization (Yang *et al.*, 2009; Egorova *et al.*, 2010; Zhang *et al.*, 2012). Because methylation and ubiquitination both occur at lysine residues, methylation of lysine residues could prevent them from ubiquitination. It has been suggested that methylation is a protein stable mark (Byvoet *et al.*, 1972; Duerre and Lee, 1974; Rice and Allis, 2001). Given all of these considerations, one potential mechanism is that myosin methylation by Smyd1 may prevent myosin from ubiquitination, which leads to protein degradation. Consistent with the idea that myosin methylation may control its satiability, it was shown that myosin methylation markedly increased its ATPase activity *in vitro* (White and Rayment, 1993). Given that Smyd1b is a myosin-binding protein (Just *et al.*, 2011), we speculate that Smyd1b may function as a muscle-specific methyltransferase involved in myosin methylation, which plays a critical role in myofibril assembly.

MATERIALS AND METHODS

Zebrafish lines and maintenance

Mature zebrafish were raised at the Zebrafish Facility of the Aquaculture Research Center, Institute of Marine and Environmental Technology (Baltimore, MD). 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 myomesin-RFP zebrafish line (GBT0067) was obtained from Stephen Ekker's laboratory at

the Mayo Clinic (Rochester, MN). It carries the RFP insertion in the myomesin 3 gene (Clark *et al.*, 2011; Xu *et al.*, 2012; for additional information see www.zfishbook.org/index.php?topic=GBT0067#).

Morpholino and DNA microinjection into zebrafish embryos

Morpholino antisense oligos against zebrafish Smyd1a and Smyd1b were synthesized by Gene Tools (Philomath, OR). The Smyd1b translation blocker (Smyd1b ATG-MO) was based on the antisense sequence near the ATG start site. The Smyd1b splicing blocker (Smyd1b E9I9-MO) was based on the antisense sequence at the exon 9/intron 9 junction. The Smyd1a splicing blockers (Smyd1b E8I8-MO and Smyd1a E9I9-MO) were based on the antisense sequence at the exon 8/intron 8 and exon 9/intron 9 junctions, respectively. The standard control MO from Gene Tools was used as control. Morpholino antisense oligos were dissolved in Danieau buffer (Nasevicius and Ekker, 2000) to a final concentration of 0.5 and 1 mM. Zebrafish embryos were injected at the one- or two-cell stage with 2 nl of MO as described (Tan *et al.*, 2006). DNA microinjection was carried out as described (Du *et al.*, 1997).

Smyd1b ATG-MO: 5'-ACTTCCACAACTCCATTCTGGATC-3'

Smyd1b E9I9-MO: 5'-CGTCACCTCTAGGTCTTTAGTGATG-3'

Smyd1a E8I8-MO: 5'-ATATCGCAACACTCACATGTATCCA-3'

Smyd1a E9I9-MO: 5'-GGTTGTACCTCCAGGTCTCTGCTGA-3'

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

Tg(Smyd1b-Smyd1b) transgenic zebrafish and Tg(Smyd1b:Unc45b^{FLAG}) transgene

The *Tg(Smyd1b:Smyd1b-tv1^{myc})mb6* and *Tg(Smyd1b:Smyd1b-tv2^{myc})mb7* transgenic zebrafish lines were generated as described previously (Tan *et al.*, 2006). *Tg(Smyd1b:Smyd1b-tv1^{myc})* and *Tg(Smyd1b:Smyd1b-tv2^{myc})* minigenes were constructed by using cDNA encoding the myc-tagged Smyd1b-tv1 or Smyd1b-tv2 cloned after the 5.3-kb zebrafish *smyd1b* promoter and its 5' flanking sequence (Du *et al.*, 2006; Tan *et al.*, 2006). The *Tg(Smyd1b:Unc45b^{FLAG})* transgene was constructed by using cDNA encoding the FLAG-tagged zebrafish Unc45b cloned after the 5.3-kb zebrafish *smyd1b* promoter and its 5' flanking sequence (Bernick *et al.*, 2010).

Immunostaining of whole-mount fish embryos

Immunostaining was carried out using whole-mount zebrafish embryos (24–72 hpf) as previously described (Tan *et al.*, 2006), with the following antibodies: anti-myosin heavy chain for slow muscle (F59; Developmental Studies Hybridoma Bank [DSHB], Iowa City, IA), anti-myosin heavy chain (MF20; DSHB), anti-myosin heavy chain (S46; DSHB), anti- α -actinin (clone EA-53, #A7811; Sigma, St. Louis, MO), anti-MHC for fast muscles (F310; DSHB), anti-myomesin (mMaC myomesin B4; DSHB), anti- α -actin (Ac1-20.4.2; Progen, Heidelberg, Germany), and anti-titin (clone T11, #T9030; Sigma). Secondary antibodies were fluorescein isothiocyanate or tetramethylrhodamine isothiocyanate conjugates (Sigma).

Analysis of protein expression by Western blot

Wild-type or MO-injected zebrafish embryos were dechorinated manually at 24 or 72 hpf. The embryos (50 embryos each group) were washed with 1 ml of phosphate-buffered saline (PBS) and crushed gently to remove the yolk by pipetting with a glass pipette in 0.5 ml of PBS. The embryos were collected by a quick spin at 3000 rpm for 1 min. The embryo extract was washed once with 0.5 ml of PBS and solubilized in 100 μ l of 2 \times SDS loading buffer (0.125 M Tris-Cl, pH 6.8, 4% SDS, 20% glycerol, 0.2 M dithiothreitol,

0.02% bromophenol blue) containing 1 mM phenylmethylsulfonyl fluoride and protease inhibitor (P8340; Sigma) by passage through a 21-gauge needle 10–15 times. The proteins were denatured by boiling for 3 min and analyzed on a 7.5% SDS–PAGE gel. Proteins from approximately five embryos were loaded on each lane of the SDS–PAGE gel. Proteins from the gel were transferred onto a polyvinylidene fluoride membrane (Immobilion-P; Millipore, Billerica, MA) by electrophoresis. Immunodetection of MHC, γ -tubulin, or myc-tagged proteins was carried out with their primary antibodies and followed by corresponding peroxidase-conjugated secondary antibodies. For Western blot to detect proteins with methylated lysine residues, the horseradish peroxidase-conjugated anti-methylated lysine antibody (TF1213; Enzo Life Sciences, Farmingdale, NY) was used. SuperSignal Western Blot Enhancer Kit (46640; Thermo Scientific, Waltham, MA) was used to enhance the sensitivity of the Western blot analysis according to the manufacturer's instructions. All blots were developed using the Pierce ECL Western Blotting Substrate (Thermo Scientific).

Real-time PCR analysis

Real-time PCR was carried out using 7500 Fast Real-Time PCR System (Applied Biosystems, Carlsbad, CA). The PCR was carried out using standard SYBR Green PCR Master Mix (Applied Biosystems). Standard curves of cDNA samples were constructed using 10-fold serial dilutions. The relative levels of gene expression were compared based on the normalized value of the endogenous control elongation factor 1- α (*ef-1 α*). Real-time PCR was carried out using the following primers:

zfhsp90 α 1-P6: agccagacttcggtgaatcaa
 zfhsp90 α 1-P7: ttctctgttttctaagttaa
 zfmyh2-P4: gctcacctaccagactgagga
 zfmyh2-P5: actcagcaatatcagcagct
 zfsmyhc1-P4: gctcacctaccagactgagga
 zfsmyhc1-P5: catctgttgacctgagattca
 zfunc45b-P4: gctgcaaggaggccaagaca
 zfunc45b-P5: gatcatcagcatccagcatgt
 zfef1a-P3: cttcaacgctcaggtcatcat
 zfef1a-P4: acagcaaagcgaccaagagga
 zSmyd1b-RT-F: atctgaacgtgtctgcaga
 Smyd1b-P6: tcttcggcacttgactcatcc

Whole-mount in situ hybridization

Whole-mount in situ hybridization was carried out using digoxigenin-labeled antisense probes as previously described (Du and Dienhart, 2001). The plasmid *Hsp90 α 1-P* was digested with *Nco*I and transcribed with Sp6 RNA polymerase to synthesize digoxigenin-labeled antisense RNA probe. Antisense probes against zebrafish slow myosin heavy chain 1 (*smyhc1*) or fast muscle myosin heavy chain 2 (*myh2*) were synthesized by Sp6 RNA polymerase from *Nco*I or *Sph*I linearized *pGEM-smyhc1* or *pGEM-myh2* plasmid, respectively. The zebrafish *unc-45b* antisense probe was synthesized with Sp6 RNA polymerase from plasmid *pGEM-unc45b* linearized with *Bam*HI.

Immunoprecipitation and immunoblotting

HEK293 cells were seeded at 2.5×10^5 per well in six-well plates 1 d before the transfection. Four micrograms of each indicated plasmid was transiently transfected by calcium phosphate precipitation.

Cells were harvested 24 h after the transfection and lysed in 1 \times cell lysis buffer (10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM ethylene glycol tetraacetic acid, and 0.2% Nonidet P-40) with 1 \times protease inhibitor mixture (P2714; Sigma). For IP, 300 μ g of total protein was incubated with 30 μ l of prewashed Anti-FLAG M2 Affinity Gel (Sigma A2220) at 4°C for 2 h in a total volume of 600 μ l containing 4% glycerol in 1 \times cell lysis buffer. The beads were washed three times in 1 \times cell lysis buffer before processing for SDS/PAGE and immunoblotting.

Heat shock and cold shock treatment of zebrafish embryos

For the heat shock experiment, 50 wild-type zebrafish embryos at 24 hpf were treated for 15 min from 28.5 to 38°C, then 38°C for 30 min, and then another 15 min from 38 to 28.5°C. For the cold shock experiment, 50 wild-type zebrafish embryos at 24 hpf were incubated for 30 min at 4°C. For real-time PCR, total RNA of the heat-shocked or cold-shocked embryos, as well of control embryos, was extracted by using TRIzol reagent (Invitrogen, Carlsbad, CA). cDNA was synthesized using the first-strand cDNA synthesis kit (Life Sciences). For in situ hybridization, the heat-shocked, cold-shocked, and wild-type embryos were fixed in 4% paraformaldehyde. Whole-mount in situ hybridization was performed with digoxigenin-labeled antisense probes as described (Tan *et al.*, 2006).

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