Expression and Functional Characterization of Smyd1a in Myofibril Organization of Skeletal Muscles

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Abstract

Background: Smyd1, the founding member of the Smyd family including Smyd-1, 2, 3, 4 and 5, is a SET and MYND domain containing protein that plays a key role in myofibril assembly in skeletal and cardiac muscles. Bioinformatic analysis revealed that zebrafish genome contains two highly related smyd1 genes, smyd1a and smyd1b. Although Smyd1b function is well characterized in skeletal and cardiac muscles, the function of Smyd1a is, however, unknown.

Methodology/Principal Findings: To investigate the function of Smyd1a in muscle development, we isolated smyd1a from zebrafish, and characterized its expression and function during muscle development via gene knockdown and transgenic expression approaches. The results showed that smyd1a was strongly expressed in skeletal muscles of zebrafish embryos. Functional analysis revealed that knockdown of smyd1a alone had no significant effect on myofibril assembly in zebrafish skeletal muscles. However, knockdown of smyd1a and smyd1b together resulted in a complete disruption of myofibril organization in skeletal muscles, a phenotype stronger than knockdown of smyd1a or smyd1b alone. Moreover, ectopic expression of zebrafish smyd1a or mouse Smyd1 transgene could rescue the myofibril defects from the smyd1b knockdown in zebrafish embryos.

Conclusion/Significance: Collectively, these data indicate that Smyd1a and Smyd1b share similar biological activity in myofibril assembly in zebrafish embryos. However, Smyd1b appears to play a major role in this process.

Introduction

Members of the Smyd family are newly identified proteins that have been implicated in diverse biological functions in embryonic development and cancer [1]. Currently, five smyd genes (Smyd1, −2, −3, −4, and −5) have been identified in vertebrates based on the presence of both SET and MYND domains in their protein sequences [1]. Smyd1, also known as skm-Bop, represents the first identified member of the Smyd family [2,3]. Smyd1 is specifically expressed in skeletal and cardiac muscles and plays a key role in muscle development and embryonic survival in mice and zebrafish [4–6]. Targeted disruption of the smyd1 gene resulted in defective cardiac morphogenesis and early embryonic lethality of mouse embryos [4]. Knockdown or mutation of smyd1b gene in zebrafish led to disruption of myofibril organization in skeletal and cardiac muscles in zebrafish embryos [5,6].

The smyd1 gene is a direct downstream gene target of myogenic regulatory factors MyoD, Myogenin and Mef2 that control the muscle specific expression of smyd1 in skeletal muscles during embryogenesis and in adult muscle tissues [7–10]. A recent report showed that smyd1 expression is also regulated by serum response factor (SRF) through direct binding to the promoter region of smyd1 [11]. In addition, smyd1 gene expression can be repressed by Hepatoma-derived growth factor through interaction with a transcriptional co-repressor C-terminal binding protein (CtBP) [12]. Consistent with the idea of being a downstream factor of MyoD and Mef2, loss of Smyd1 function had no effect on myoD and myogenin gene expression and myoblast specialization in a histone deacetylase (HDAC)-dependent manner [5]. However, loss of Smyd1 function resulted in defective sarcomere organization in myofibers of skeletal and cardiac muscles, suggesting that Smyd1 is required in the late stage of muscle cell differentiation and myofiber maturation [3,6].

At present, little is known about the mechanism by which Smyd1 functions in myofibrillogenesis. In vitro studies have shown that Smyd1 has a histone methyltransferase (HMTase) activity [5,13], and could function as a transcriptional repressor in a histone deacetylase (HDAC)-dependent manner [4,14]. However, Just and colleagues reported recently that the Smyd1 mutant lacking the HMTase activity was biologically active in myofibril assembly [6], arguing against Smyd1 being a HMTase activity dependent transcriptional regulator. Interestingly, Just and colleagues showed that GST-tagged Smyd1 was capable of pulling down skeletal muscle-specific myosin heavy chain [6]. Consistent with a potential role of Smyd1 outside of the nucleus, a nuclear to
cytoplasmic translocation was observed during myoblast differentiation into myotubes [15], and Smyd1 is localized on the M-lines of sarcomeres although the biological significance of the sarcomeric localization is not clear [6,16]. Recent studies demonstrated that zebrafish genome contains two highly related smdy1 genes, smdy1a and smdy1b [17]. Most of the previous studies were focused on smdy1b, very little is known about smdy1a. It is not clear whether Smyd1 plays a similar role as Smyd1b in myofibrillogenesis. To investigate the function of Smyd1a in myofibril assembly, we isolated smdy1a from zebrafish, and characterized its expression and function during muscle development. The results showed that smdy1a was specifically expressed in skeletal muscles of zebrafish embryos. smdy1a expression came several hours later than smdy1b during myogenesis in zebrafish embryos. Functional analysis revealed that knockdown of smdy1a alone had little effect on myofibril assembly in zebrafish skeletal muscles. However, knockdown of smdy1a and smdy1b together resulted in a stronger phenotype in myofibril disorganization. Moreover, the myofibril defects from smdy1b knockdown could be rescued by an ectopic expression of the zebrafish smdy1a or mouse Smyd1 transgene. Together, these data indicate that Smyd1a and Smyd1b share similar biological activity in myofibril assembly although the function of Smyd1b appears to be more critical.

Results

1. Characterization of Smyd1a in Zebrafish

Sequence analysis revealed that zebrafish genome contains two highly related smdy1 genes (smdy1a and smdy1b) with similar gene structures [17]. smdy1a and smdy1b are believed to be generated by gene duplication. The zebrafish smdy1a is located on chromosome 5, whereas smdy1b is located on chromosome 8 (Figure S1). Sequence analysis revealed a strong synteny arrangement of zebrafish smdy1a gene and human smdy1 gene with the threonine synthase like 2 gene (THNSL2) and fatty acid binding protein1 gene (FABP1) in zebrafish and mouse genome (Figure S1). A similar synteny arrangement was found with the zebrafish smdy1b and fatty acid binding protein 1b like gene (fabp1b). However, no synteny arrangement was found with the smdy1b gene and threonine synthase like 2 gene on chromosome 8 in zebrafish. Sequence alignment revealed that zebrafish Smyd1a contains the highly conserved SET and MYND domains involved in protein methylation and protein-protein interactions, respectively (Figure S1). Both Smyd1a and Smyd1b share high sequence identity with Smyd1 from other vertebrate species (Figure S2), although only smdy1 gene has been identified in mice and humans.

2. Temporal and Spatial Expression of Smyd1a in Zebrafish Embryos

It has been reported previously that both smdy1a and smdy1b are specifically expressed in developing muscles of zebrafish embryos [5,9,17]. To compare their patterns of expression during embryonic development, we carried out a temporal and spatial expression analysis by RT-PCR and whole mount in situ hybridization in zebrafish embryos. Data from the expression analysis showed that smdy1a and smdy1b had different pattern of temporal expression. smdy1a was expressed several hours later than smdy1b in zebrafish embryos. smdy1b expression was first detected at 6 hours post-fertilization (hpf) with a strong expression starting around 9 hpf [5]. In contrast, smdy1a expression could not be detected until 19 hpf (Fig. 1A).

Previous studies have demonstrated that smdy1b encodes two muscle-specific isoforms of mRNA transcripts, designated as smdy1b_tv1 and smdy1b_tv2 [5,16]. smdy1b_tv1 and smdy1b_tv2 are generated by alternative splicing of the 39 bp exon 5. smdy1b_tv1 transcript contains the 39 bp sequence from exon 5, whereas smdy1b_tv2 transcript lacks this 39 bp sequence and thus is 13 aa shorter than smdy1b_tv1 [5,16]. Sequence analysis revealed that the zebrafish smdy1a gene also contained the small exon 5 of 39 bp. To test whether exon 5 could be alternatively spliced in smdy1a, we performed RT-PCR analysis using cDNA from zebrafish larvae of different stages. In contrast to smdy1b where two different isoforms were amplified (Fig. 1B), only the longer isoform of smdy1a was found to be expressed (Fig. 1C), suggesting that exon 5 in smdy1a was not alternatively spliced in zebrafish embryos.

To determine whether smdy1a expression is restricted to muscle cells, we analyzed its spatial pattern of expression by whole mount in situ hybridization. The data showed that similar to smdy1b, smdy1a is specifically expressed in skeletal muscles of zebrafish embryos (Fig. 1D–I). However, unlike smdy1b, little or no expression was detected for smdy1a transcripts in cardiac muscles (Fig. 1D, G).

3. Knockdown of smdy1a Expression in Zebrafish Embryos had Little Effect on Muscle Development

To determine whether smdy1a plays a role in muscle development, we knocked down smdy1a expression in zebrafish embryos using two smdy1a-specific anti-sense morpholino oligos, E8I8-MO and E9I9-MO. The E8I8-MO and E9I9-MO were specifically targeted to the sequences at the exon-8/intron-8, or exon-9/intron-9 junctions, respectively (Fig. 2A). RT-PCR analysis confirmed that both E8I8-MO and E9I9-MO could knock down the normal splicing of smdy1a transcripts and resulted in the production of defectively spliced smdy1a mRNA (Fig. 2B). However, the knockdown was not complete with an efficiency of approximately 50% (Fig. 2B). Sequence analyses revealed that E8I8-MO caused a defective splicing at the exon 8 and intron 8 junction, resulting in a 14 bp deletion at the end of exon 8 (Fig. 2A). This 14 deletion caused a reading frame shift, leading to the production of a mutant protein without the 105 aa C-terminal sequence. Similarly, the E9I9-MO caused a defective splicing at the end of exon 9 with a 58 bp deletion (Fig. 2A), which resulted in the production of a mutant protein without the last 63 aa C-terminal sequence.

The smdy1a knockdown embryos were examined morphologically for several days following the MO injection. Unlike the smdy1b knockdown embryos that had no skeletal and cardiac muscle contraction, the smdy1a knockdown embryos had normal skeletal and cardiac muscle contraction when observed at 24, 48 and 72 hpf. The E8I8-MO injected embryos appeared morphologically normal (Fig. 2E, F) compared with the control (Fig. 2C, D). Embryos injected with the smdy1a E9I9-MO, however, showed small head and developmental delay, suggesting some off-target toxic effect. The off-target toxic effect could be alleviated by coinjection with the p53-MO (Fig. 2G, H). To confirm that knockdown of Smyd1a did not affect early muscle development, we analyzed the specification of slow and fast muscle precursors and their subsequent migration and differentiation. Compared with control (Fig. 2I), MyoD expression appeared normal in slow and fast muscle precursors of smdy1a knockdown embryos (Fig. 2J). In addition, fiber-type-specific expression of slow or fast myosin heavy chain (MHC) genes also appeared normal (Fig. 2M, N). Slow fibers were clearly localized at the superficial layer of the myotome in smdy1a knockdown embryos (Fig. 2M), suggesting that migration of slow muscle cells was not affected by smdy1a knockdown.
Figure 1. The temporal and spatial pattern of smyd1a expression in zebrafish embryos. A. RT-PCR results show temporal expression of smyd1a in zebrafish embryos from fertilization to day 5. Elongation factor 1-alpha (ef-1α) was used as control. B. RT-PCR analysis shows the alternative splicing of smyd1b exon 5 generating two isoforms of smyd1b, smyd1b_tv1 and smyd1b_tv2. C. RT-PCR analysis shows the lack of alternative splicing of exon 5 in smyd1a in zebrafish embryos. D-I. Whole mount in situ hybridization shows the spatial pattern of smyd1a mRNA expression using a dig-labeled antisense probe. smyd1a expression was detected in skeletal muscles of zebrafish embryos at 24 (D, E, F) and 48 (G, H, I) hpf. D, G represent the side view; E, H represent the dorsal view; F, I represent the cross sections.

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Figure 2. Knockdown of *smyd1a* splicing by E8I8-MO and E9I9-MO. A. Location of the E8I8-MO and E9I9-MO splicing blockers at the junction of exon 8/intron 8 and exon 9/intron 9, respectively. Defective splicing using a cryptic splicing site in exon 8 in E8I8-MO injected embryos resulted in a...
deletion of 14. Defective splicing using a cryptic splicing site in exon 9 in E919-MO injected embryos resulted in a deletion of 58 bp. B. RT-PCR showing the defective splicing induced by the E818-MO or E919-MO or both MOs. Compared with the PCR results from the wild type (wt) control embryos where a single band was generated, two bands were observed in the E818-MO injected embryos. Sequence analysis revealed that the smaller band contained a 14 bp deletion at the end of exon B. The upper band represented the heteroduplex formed by the normal and defectively spliced products. Similarly, three bands were detected in E919-MO injected embryos. Sequence analysis revealed that the smaller band contained a 58 bp deletion at the end of exon 9. The middle band was the normal spliced product, whereas the upper band represented the heteroduplex formed by the normal and defectively spliced products. Two major bands were detected in E818-MO and E919-MO co-injected embryos. Sequence analysis revealed that the smaller band resulted from defective splicing. C-H. Morphology of smyda knockout embryos at 48 hpf. Zebrafish embryos injected with control MO (C, D), E818-MO (E, F) or E919-MO (G, H) at 48 hpf. I-N. Knockdown of smyda expression had no effect on myoblast specification and early differentiation in slow and fast myofibers. In situ hybridization showing normal MyoD expression in control (J, K) or smyda MO (M, N) injected embryos at 24 hpf.

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4. Knockdown of smyda and smydb together Resulted in a Stronger Muscle Phenotype in Zebrafish Embryos

To analyze the effects of smyda knockdown on sarcomere assembly in skeletal muscles, we first examined the organization of thick filaments in slow myofibers of smyda knockdown zebrafish embryos. The results showed that knockdown of smyda alone had no detectable effect on the sarcomere organization of the myosin thick filaments (Fig. 3D–F) in slow fibers of zebrafish embryos at 24, 48 and 72 hpf. The lack of muscle defect from smyda knockdown could be due to the inefficient knockdown. To determine whether increasing the efficiency of smyda knockdown could affect muscle development, we co-injected the two smyda splicing MOs into zebrafish embryos. Compared with the single MO injection, the results showed that co-injection of the two MOs increased the efficiency of smyda knockdown (Fig. 2B). However, it did not result in more defects on myofibril assembly in slow muscles (Fig. 3G–I).

To test the possibility that the lack of muscle defect from smyda knockdown was due to the redundant function from smyd1b, we carried out the double knockdown of smyda and smyd1b in zebrafish embryos. The data showed that knockdown of smyd1b alone resulted in disruption of myosin thick filament organization at 24 hpf. However, the defective thick filament organization was partially recovered in smyd1b knockdown zebrafish embryos at 48 and 72 hpf (Fig. 3K, L). Double knockdown studies showed that the partial recovery from smyd1b knockdown was diminished when smyd1b was knocked down together with smyda (Fig. 3N, O), suggesting that Smyda may have a partial function redundancy with Smyd1b in myofibril assembly.

To determine whether this was also true for other sarcomeric structures, we analyzed the α-actin thin filaments and Z-line organization in the single or double knockdown embryos. The results showed that the knockdown of smyda and smyd1b together had a stronger effect on thin filament disruption at 72 hpf (Fig. 4O) compared with the knockdown alone (Fig. 4I, L). A similar finding was also observed with the Z-line structure (Fig. 5). Collectively, these data indicate that Smyda and Smyd1b are required for myofibril organization and assembly in skeletal muscles of zebrafish embryos although Smyd1b plays a dominant role compared with Smyda.

In situ expression analysis showed that smyda was expressed in fast muscles (Fig. 1). To determine whether knockdown of smyda had any effect on fast muscles, sarcomere organization was characterized in fast muscles of smyda knockdown embryos. The results showed that knockdown of smyda alone had no effect on myofibril assembly in fast muscles (Fig. 6).

5. Ectopic Expression of Zebrafish smyda or Mouse Smyd1 Transgene Could Rescue the Myofibril Defects in smyd1b Knockdown Zebrafish Embryos

It has been suggested that actinopterygian fish, such as zebrafish, had gone through one more round of whole genome duplication compared with mammals during evolution [18]. The two Smyd1 genes in zebrafish were likely generated by the gene duplication event in teleosts because mouse and human genomes contain only one Smyd1 gene. Sequence analysis revealed that zebrafish Smyda and Smyd1b share high sequence similarity with the mouse and human Smyd1 (Fig. S2). However, functional studies revealed that knockdown of smyd1a or smyd1b resulted in strong differences in muscle phenotype. Knockdown of smyd1b significantly disrupted the myofibril organization, whereas knockdown of smyd1a alone had little effect. The different severity of the knockdown phenotypes could be due to structural changes of smyda protein that made it less active compared with smyd1b. Alternatively, the different knockdown phenotype could be caused by their distinct patterns of temporal expression because smyda was expressed several hours later than smyd1b in zebrafish embryos.

To clarify these questions, we tested whether ectopic expression of smyda early in zebrafish embryos could rescue the smyd1b knockdown phenotypes. A rescue experiment was performed in the smyd1b knockdown zebrafish embryos using the smyda transgene (Smyda-zfsmyda<sup>act</sup>) directed by the smyd1b promoter. The Smyda-zfsmyda<sup>act</sup> transgene expressing a myc-tagged zebrafish Smyda was co-injected with smyd1b ATG-MO into zebrafish embryos. The smyd1b ATG-MO could specifically knock down the expression of smyd1b, however it had no effect on the expression of the zebrafish smyda<sup>act</sup> transgene because the transgene did not contain the smyd1b ATG-MO target sequence. Myofibril organization and ectopic Smyda<sup>act</sup> expression was analyzed in the co-injected zebrafish embryos by double staining with anti-MHC (F59) and an anti-myc antibodies. The data showed a clear rescue of thick filament organization in the co-injected embryos (Fig. 7A). The rescue appeared in a mosaic pattern, consistent with the pattern of gene expression through DNA injection. Double staining revealed a perfect match between the rescued myofilaments and the expression of the myc-tagged zebrafish Smyda1 (Fig. 7C, E). Collectively, these data indicate that Smyda1 could replace Smyd1b function if expressed early in muscle cells, arguing that the different severity of smyda1 and smyd1b knockdown phenotypes was likely due to distinct pattern of temporal expression.

Sequence analysis revealed that Smyda1 and Smyd1b share similar sequence identity with mouse and human Smyd1 (Fig. S2). To test whether Smyd1 function is conserved during evolution, we performed a rescue experiment in the smyd1b knockdown zebrafish embryos using the mouse smyd1<sup>act</sup> transgene directed by the zebrafish smyd1b promoter. The mouse Smyd1 transgene (smyd1<sup>act</sup>-Msmyd1<sup>act</sup>) expressing a myc-tagged mouse Smyd1 was co-injected with the
smyd1b ATG-MO into zebrafish embryos. Double staining revealed that expression of the myc-tagged mouse Smyd1 could rescue the myofibril defects from the smyd1b knockdown in a cell autonomous manner (Fig. 7B, D, F). Collectively, these data indicate that Smyd1 function in myofibril organization is likely conserved during evolution.

6. The Subcellular Localization of Zebrafish Smyd1a and Mouse Smyd1 in Myofibers

Previous studies by us and others have shown that Smyd1b is localized on the M-line of sarcomeres in both skeletal and cardiac muscles [6,16]. This was especially evident for the longer isoform Smyd1b_tv1 [16]. It is not clear whether zebrafish Smyd1a and mouse Smyd1 in skeletal muscles of zebrafish embryos. The results showed that, similar to Smyd1b, mouse Smyd1 was localized on the sarcomere of skeletal muscles (Fig. 7D). However, unlike Smyd1b, zebrafish Smyd1a showed no clear sarcomeric localization in myofibers (Fig. 7C).

To enhance the sensitivity of the detection, we generated a transgene, pTol2-Smyd1a -EGFP, expressing a GFP-tagged zebrafish Smyd1a fusion protein. The pTol2-Smyd1a-EGFP transgene was injected into zebrafish embryos. Smyd1a-EGFP expression and subcellular localization were characterized in the injected zebrafish embryos by direct observation. The results showed that Smyd1a-EGFP had a clear sarcomeric localization (Fig. 8B, E). In contrast, Smyd1a-EGFP was not localized on the sarcomeres (Fig. 8A, D). A diffuse cytosolic localization was detected for Smyd1a-EGFP in myofibers of zebrafish embryos (Fig. 8A, B), similar to EGFP control (Fig. 8C, F). Together, these data indicate
that Smyd1a and Smyd1b have distinct subcellular localization, indicating that the sarcomeric localization of Smyd1b might not be directly linked with the Smyd1 function in sarcomere organization because Smyd1a could rescue the myofibril defects from Smyd1b knockdown although Smyd1a was not localized on the sarcomere. This is consistent with previous findings that either one of the Smyd1b isoforms, Smyd1bTv1 or Smyd1bTv2, from alternative splicing was able to rescue the myofibril defects from Smyd1b knockdown although their proteins showed distinct subcellular localization [5,16].

**Discussion**

In this study, we have characterized the muscle phenotype from knockdown of *smyd1a* or *smyd1b* alone, or together in zebrafish embryos. We demonstrated that in contrast to *smyd1b* which is absolutely required for myofibril organization in skeletal muscles, knockdown of *smyd1a* alone had very little effect on myofibril organization. However, knockdown of *smyd1a* and *smyd1b* together resulted in a stronger muscle phenotype compared with knockdown of *smyd1a* or *smyd1b* alone. We further demonstrated that the muscle defects from *smyd1b* knockdown could be rescued by the ectopic expression of the zebrafish *smyd1a* or mouse *Smyd1*, suggesting that Smyd1 function is likely to be conserved during evolution. Finally, we showed that similar to Smyd1b, mouse Smyd1 was localized on sarcomeres in skeletal myofibers. In contrast, zebrafish Smyd1a did not show any sarcomeric localization although it could rescue the Smyd1b knockdown defects.
The Unequal Role of Smyd1a and Smyd1b in Myofibril Assembly

Gene duplication plays a vital role in evolution [19]. Sequence analysis revealed that zebrafish genome contains two smyd1 genes, smyd1a and smyd1b that were likely generated by the whole genome duplication in actinopterygian fish during evolution [17,18,20]. smyd1a and smyd1b are paralogous genes that are dispersed on different chromosomes in zebrafish. Sequence analysis revealed that zebrafsh smyd1a and mouse Smyd1 showed a highly conserved synteny arrangement with the surrounding THNSL2 and FABP1 genes. The synteny arrangement with fabp1 was maintained for smyd1b in zebrafish. However, this synteny was not found between the zebrafish smyd1b and thnsl2 genes, suggesting that a genomic rearrangement or deletion might occur in this region after the whole genome duplication.

It has been suggested that after whole genome duplication, the duplicated genes could evolve in several directions without the selective pressure. Some of the duplicated genes could gain new functions, while many others turned into nonfunctional pseudogenes [19,20]. We showed in this study that both smyd1a and smyd1b are functional genes after the duplication. However, they do not play an equal role in myofibril organization. Smyd1b appears to be more critical than Smyd1a because knockdown of smyd1b gave a stronger muscle phenotype compared with smyd1a knockdown. Our data indicate that functional difference between Smyd1a and Smyd1b could be caused by their different patterns of temporal expression, different activity due to changes of protein structure, or different levels of gene expression. We showed that early ectopic expression of smyd1a by using the smyd1b promoter could rescue the myofibril defects from smyd1b knockdown, suggesting that Smyd1a could function as Smyd1b if expressed in a similar temporal and spatial pattern in zebrafish embryos. At present, it is not clear whether Smyd1a gained new functions during evolution. Knockdown of smyd1a alone failed to reveal any significant phenotype in zebrafish embryos, unless it was knocked down together with smyd1b. Together, these studies suggest that

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**Figure 5. The effect of smyd1a or smyd1b single or double knockdown on the Z-line organization in skeletal muscles.** Zebrafish embryos injected with smyd1b MO or smyd1a MO or both were fixed at 28, 48 and 72 hpf. Z-line organization was analyzed by immunostaining with anti-α-actinin antibody (EA-53), and followed by FTIC-labeled secondary antibody. The images represent side view of trunk muscles around segment 10. A–C. Lateral view of Z-line organization in skeletal muscle fibers of control-MO injected embryos at 28 (A), 48 (B) and 72 (C) hpf. D–F. Lateral view of Z-line organization in skeletal muscle fibers of smyd1a E8I8-MO injected embryos at 28 (D), 48 (E) and 72 (F) hpf. G–I. Lateral view of Z-line organization in skeletal muscle fibers of smyd1a E8I8-MO and E9I9-MO co-injected embryos at 28 (G), 48 (H) and 72 (I) hpf. J–L. Lateral view of Z-line organization in skeletal muscle fibers of smyd1b ATG-MO injected embryos at 28 (J), 48 (K) and 72 (L) hpf. M–O. Lateral view of Z-line organization in skeletal muscle fibers of smyd1a E8I8-MO and smyd1b ATG-MO co-injected embryos at 28 (M), 48 (N) and 72 (O) hpf. Scale bars: 20 μm in A–C. doi:10.1371/journal.pone.0086808.g005
Smyd1a is involved in myofibril assembly, but with a minor role in the process.

The Smyd1 Function in Myofibril Organization is Conserved during Evolution

Our studies demonstrated that Smyd1 function in myofibril organization is likely to be conserved during evolution because ectopic expression of mouse Smyd1 could rescue the muscle defects from smyd1b knockdown in zebrafish embryos. Unlike zebrafish, there is only one Smyd1 gene in mouse and human genomes. Consistent with the idea that zebrafish smyd1b is the ortholog of Smyd1 in mice, it has been reported that knockout of Smyd1 in mice resulted in early embryonic lethality from cardiomyogenesis defects [4]. A cardiac muscle defect was also observed in Smyd1b knockdown or mutant zebrafish embryos [5,6]. In addition to their functional similarity, the zebrafish Smyd1b and mouse Smyd1 share other common features in gene expression and protein localization. First, both zebrafish smyd1b and mouse smyd1 express two alternatively spliced mRNA transcripts that differ by 39 bp encoded by exon 5. Second, both mouse Smyd1 and zebrafish Smyd1b are localized on the M-lines of sarcomeres in skeletal and cardiac muscles [6], which is in contrast to the zebrafish Smyd1a that showed no sarcomeric localization.

The Sarcomeric Localization of Smyd1

The biological significance of the sarcomeric localization is not clear. It has been reported that Smyd1b could bind to myosin and showed a transient localization with thick filaments during myofibrillogenesis in zebrafish embryos [6]. However, after the completion of sarcomere organization, the Smyd1b is translocated to the M-line. Interesting, Etard and colleagues have shown that myosin chaperones Unc45b and Hsp90a could shuttle between the A band and the Z line in response to stress or damage to the myofiber [21]. It has been suggested that the sarcomeric localization of myosin chaperones and their translocation within different parts of the muscle cells could be involved in the response of muscle cells to mount efficient physiological responses to muscle stress, load requirements, and/or stretch [21]. It remains to be determined whether the sarcomeric localization of Smyd1b is involved in sarcomere remodeling and response to muscle stress.

We have shown previously that the sarcomeric localization of Smyd1b requires Phe223 and Serine 225 [16]. Substitution of Phe223 or Ser225 with alanine significantly diminished the sarcomeric localization of Smyd1b [16]. Interestingly, the two highly conserved residues Phe223 and Ser225 are present in zebrafish Smyd1a, arguing that in addition to the Phe223 and Ser225 residues, other residues or motifs are likely to be involved in the sarcomeric localization. Sequence analysis revealed that...
Smyd1a differs from Smyd1b significantly at the C-terminal sequence (Figure S1). Compared with Smyd1a, Smyd1b has seven extra amino acids at the C-terminus including three highly conserved residues (xxLFxxK) that are present in all Smyd1 proteins that have been identified in vertebrates. It remains to be determined whether these residues may contribute to the sarcomeric localization of Smyd1b.

Materials and Methods

Ethics Statement

This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the Institutional Animal Care and Use Committee of the University of Maryland (Permit Number:0610009).

Fish Maintenance and Use

Zebrafish were maintained at 28.5 °C in 10 gallon aquarium supplied with fresh water and air at a photoperiod of 14 hours of light and 10 hours of dark at the Zebrafish Facility at Aquaculture Research Center (ARC) in Columbus Center (Baltimore). Pairs of adult male and female zebrafish were put in 1 liter tanks for natural spawning after lights were turned on in the facility. Embryos were collected and raised at 28.5 °C. To ease pain and facilitate animal handling, fish embryos over one day old were anaesthetized in 0.6 mM Tricaine that has been buffered to neutral pH around 7. The anaesthetized embryos were sacrificed quickly by keeping on ice and used directly for RNA extraction or fixation for immunostaining. All procedures were carried out in compliance with the guidelines stipulated by the Institutional Animal Care and Use Committee of the University of Maryland.

Isolation of Zebrafish smyd1a cDNA

The full-length coding sequence of zebrafish smyd1a (NM_205540) was cloned by RT-PCR using total RNA extracted from zebrafish embryos at 24–72 hpf. The PCR was carried with the Advantage PCR kit using the following primers: zfsmyd1amyc-P1 (5′–AGCATGACCGTGGAGAAGACGGAC-3′) and zfsmyd1amyc-P2 (5′–GTGTTCTATGGCTTGTCTGGACTT-3′) derived from DNA sequences near the start codon and stop codon.
respectively. The PCR product was cloned into pGEM-T vector. The resulting plasmid was named pGEM-T-zf\textit{smyd1a}.

**Analysis of smyd1a and Smd1b Expression in Zebrafish Embryos by RT-PCR**

To analyze the temporal pattern of \textit{smyd1a} expression during development, total RNA was extracted from zebrafish embryos at 20 min, 3h, 6h, 9h, 12h, 16h, 1d, 2d, 3d, 4d and 5d post fertilization. \textit{smyd1a} expression was determined by RT-PCR using \textit{zf\textit{smyd1a}}-P1 and \textit{zf\textit{smyd1a}}-P2 primers. To characterize the expression of tv1 and tv2 isoforms from alternative splicing of exon 5, RT-PCR was carried out using two new sets of primers \textit{smyd1a}-tv-P1/P2 or \textit{smyd1b}-tv-P1/P2, respectively. PCR using these primers produced small PCR fragments covering the alternatively spliced exon 5.

\textit{smyd1a}-tv-P1: 5'\textendash GCTTCAGATAATCCACATACGC-3'.
\textit{smyd1a}-tv-P2: 5'\textendash CATAACTGACGGTCAGCTCCTGT-3'.
\textit{smyd1b}-tv-P1: 5'\textendash TCATGGTGAGCGATCAGCGCGGC-3'.
\textit{smyd1b}-tv-P2: 5'\textendash ACGTTCAGATAATCCACATACGC-3'.

**Synthesis of Morpholino Antisense Oligos**

Morpholino antisense oligos were synthesized by Gene Tools. The \textit{smyd1b} ATG-MO was based on the target sequence surrounding the ATG start codon. The two \textit{smyd1a} splicing blockers (E8I8-MO, E9I9-MO) were based on the sequence at the exon-8/intron-8 or exon-9/intron-9 junction, respectively. The standard control MO from Gene Tools was used as control.

\textit{smyd1b} ATG-MO: 5'\textendash ACTTCCAAACTCCATTCTGTGATC-3'.
\textit{smyd1a} E8I8-MO: 5'\textendash ATATCGCAACACTCACATG-TATCCA-3'.
\textit{smyd1a} E9I9-MO: 5'\textendash GGTGTACACTGAGGTCAGACGTCA-3'.
\textit{smyd1a} EGFP control: 5'\textendash GGTTGTACACGTCA-3'.
\textit{zf\textit{smyd1a}} EGFP control: 5'\textendash GGTTGTACACGTCA-3'.

**Figure 8. Characterization of the Smyd1a subcellular localization using the Smyd1a-EGFP fusion protein.** DNA constructs expressing the zebrafish Smyd1a-EGFP or Smyd1b-EGFP fusion proteins or EGFP control were injected into zebrafish embryos. Their expression and localization were determined in myofibers of the injected zebrafish embryos at 27 (A, B, C) and 48 (D, E, F) hpf. A and D represent Smyd1b-EGFP; B and E represent Smyd1a-EGFP; C and F represent EGFP control. Scale bars: 6 μm in B; 10 μm in E.

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Morpholino and DNA Microinjection in Zebrafish Embryos

Morpholino antisense oligos were dissolved in 1x Danieau buffer [22] to a final concentration of 0.5 mM. Approximately 1–2 nl (3–10 ng) of MO was injected into each embryo at 1 or 2 cell stages. For co-injection with transgenes, equal volumes of MO (1 mM) and DNA construct (100 μg/ml) was mixed and 1–2 nl was microinjected into each embryo at 1 or 2 cell stages. All the microinjection experiments and subsequent analyses were carried out at least three times with 100–150 embryos per sample in each experiment.

Analysis of Smyd1a mRNA Splicing in MO Injected Embryos by RT-PCR

To determine the efficacy of E8I8-MO and E9I9-MO splicing blocker on smyd1a transcript splicing, total RNA was extracted from E8I8-MO or E9I9-MO single injection or E8I8-MO and E9I9-MO co-injected embryos at 48 hpf. smyd1a transcripts were amplified using smyd1a-E7-F1 and smyd1a-P2 primers or smyd1a-E7/B-F1 and smyd1a-P2 primers. The smyd1a-E7-F1 primer (5′- CTTCCAGGCTCTGGTAGAAGATTGAA-3′) was derived from the exon7. The smyd1a-E7/B-F1 primer (5′-ACCTCCATGAGGTGATCAGATG-3′) was derived from the junction of the exon7 and exon 8 sequences, while the smyd1a-P2 primer (5′ -TCATGCTTGTATGCACCTTG-3′) was from the antisense sequence near the stop codon. The PCR products were cloned into pGEM-T Easy vector for sequencing analysis.

Construction of Zebrafish smyd1b-zfsmyd1amyc Transgene

To generate the smyd1b-zfsmyd1amyc transgene for muscle specific expression of myc-tagged zebrafish Smyd1, the coding sequence of the zebrafish smyd1a was amplified with pfu DNA polymerase using the pGEM-T-zf(BglII) site was introduced at the mRNA 5′-end of the transcript together with the zfEGFP construct. A BglII site was introduced at the 5′ and 3′ ends of the smyd1a coding sequence via the respective PCR primers. The PCR products were digested with BglII and then cloned into the compatible BamHI site of the T2A200R150G vector. The DNA sequence at the smyd1a and EGFP junction was confirmed by sequencing.

Whole Mount in Situ Hybridization and Immunostaining

Whole mount in situ hybridization was carried out using digoxigenin-labeled antisense probes as previously described [24–26]. The pGEM-T-Zsmyd1a plasmid was digested with Ncol and transcribed with Sp6 RNA polymerase to synthesize digoxigenin-labeled antisense RNA probes. Immunostaining was carried out using whole mount zebrafish embryos (1–3 dpf) as previously described [5,27]. 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 (0.5% paraformaldehyde in 1x PBS). Embryos of 48 and 72 hpf were digested with 1 mg/ml collagenase for 45 and 75 minutes, respectively. In situ hybridization for MyoD, slow and fast myosin heavy chains were carried out using the respective digoxigenin-labeled probes as described previously [28].

Immunostaining was performed with the following primary antibodies: anti-α-actinin (clone EA-53, #A7811, Sigma), anti-MHC for slow muscles (F59, DSHB), anti-actin (Ac1–20.4.2, Progen), and anti-myc tag (71D10, Cell Signaling). Secondary antibodies were FITC or TRIC-conjugates (Sigma). The images were acquired using an upright microscope (Zeiss, Oberkochen, Germany) equipped with a confocal image analyzer (BIO-RAD Radiance 2100 Imaging Systems, Hercules, CA).

Supporting Information

Figure S1 Structure comparison of zebrafish Smyd1a and Smyd1b. A. The synteny arrangement of zebrafish smyd1b and mouse Smyd1 with fatty acid binding protein 1b (fabp1b) and thronine synthase like 2 (thrs2) genes. B. The diagrams of zebrafish smyd1a genomic and protein structures. C and D. Sequence comparison of functional MYND (C) and SET (D) domains in Smyd1a and Smyd1b with other vertebrate Smyd1 and Smyd3 proteins. The identically conserved residues in the MYND and SET domains are indicated by the asterisk (*). The similar amino acid residues are indicated by = . ZF, zebrafish; Fu, fugu; Ch, chicken; Mo, mouse; Hu, human. (PDF)

Figure S2 Sequence alignment of vertebrate Smyd1 proteins. Sequence comparison of zebrafish Smyd1a, Smyd1b, chicken Smyd1, mouse Smyd1 and human Smyd1 proteins. (PDF)
Author Contributions

Conceived and designed the experiments: JG JL BL SD. Performed the experiments: JG JL BL SD. Analyzed the data: JG EY JZ SD. Wrote the paper: SD.

References

1. Leinhart K, Brown M (2011) SET/MYND Lysine Methyltransferases Regulate Gene Expression and Protein Activity. Genes 2: 210–218.
2. Hwang I, Gottlieb PD (1995) Bop: a new T-cell-restricted gene located upstream of and opposite to mouse CD8b. Immunogenetics 42: 355–361.
3. Hwang I, Gottlieb PD (1997) The Bop gene adjacent to the mouse CD8b gene encodes distinct zinc-finger proteins expressed in CTLs and in muscle. Journal of Immunology 158: 1163–1174.
4. Gottlieb PD, Pierce SA, Sims RJ, Yamagishi H, Weihe EK, et al. (2002) Bop encodes a muscle-restricted protein containing MYND and SET domains and is essential for cardiac differentiation and morphogenesis. Nature Genetics 31: 25–32.
5. Tan X, Rodlant J, Li H, DeDeyne P, Du SJ (2006) SmyD1, a Histone Methyltransferase, Is Required for Myofibril Organization and Muscle Contraction in Zebrafish Embryos. Proceedings of the National Academy of Sciences of the United States of America 103: 2713–2718.
6. Just S, Meder B, Berger IM, Etard C, Trano N, et al. (2011) The myosin-interacting protein SMYD1 is essential for sarcomere organization. Journal of Cell Science 124: 3127–3136.
7. Blais A, Tsikitis M, Acosta-Alvear D, Sharan R, Kluger Y, et al. (2005) An initial blueprint for myogenic differentiation. Genes & Development 19: 553–569.
8. Phan D, Rasmussen TL, Nakagawa O, McAnally J, Gottlieb PD, et al. (2005) BOP, a regulator of right ventricular heart development, is a direct transcriptional target of MEF2C in the developing heart. Development 132: 2669–2678.
9. Du SJ, Rodlant J, Tan X (2006) Muscle-specific expression of the smyd1 gene is controlled by its 5.3-kb promoter and 5'-flanking sequence in zebrafish embryos. Developmental Dynamics 235: 3306–3315.
10. Park CY, Pierce SA, von Drehle M, Ivey KN, Morgan JA, et al. (2010) aKNAc, a Smyd1-interacting transcription factor, is involved in cardiac development and skeletal muscle growth and regeneration. Proceedings of the National Academy of Sciences of the United States of America 107: 20736–20735.
11. Li D, Niu Z, Yu W, Qian Y, Wang Q, et al. (2009) SMYD1, the myogenic activator, is a direct target of serum response factor and myogenin. Nucleic Acids Research 37: 7059–7071.
12. Yang J, Everett AD (2007) Hepatoma-derived growth factor binds DNA through the N-terminal PWWP domain. BMC Molecular Biology 8: 101.
13. Sirinupong N, Brunzelle J, Ye J, Pirzada A, Nico L, et al. (2010) Crystal structure of cardiac-specific histone methyltransferase SmyD1 reveals unusual active site architecture. Journal of Biological Chemistry 285: 40635–40644.
14. Costantini DL, Arruda EP, Agarwal P, Ken KH, Zhu Y, et al. (2005) The homeodomain transcription factor Irx5 establishes the mouse cardiac ventricular repolarization gradient. Cell. 123: 347–38.
15. Sims RJ, 3rd, Weihe EK, Zhu L, O’Malley S, Harris JV, et al. (2002) m-Bop, a repressor protein essential for cardiogenesis, interacts with skNAC, a heart- and muscle-specific transcription factor. Journal of Biological Chemistry 277: 26524–26529.
16. Li H, Xu J, Bian YH, Rodlant P, Shen T, et al. (2011) Smyd1b, tvl, a key regulator of sarcomere assembly, is localized on the M-line of skeletal muscle fibers. PLoS ONE 6: e26524.
17. Sun XJ, Xu PF, Zhou T, Hu M, Fu CT, et al. (2006) Genome-wide survey and developmental expression mapping of zebrafish SET domain-containing genes. PLoS ONE 3: e1499.
18. Amores A, Force A, Yan YL, Joy L, Amemiya C, et al. (1998) Zebrafish box clusters and vertebrate genome evolution. Science 282: 1711–1714.
19. Ohno S, Wolf U, Adan NB (1968) Evolution from fish to mammals by genome duplication. Hereditas 59: 169–187.
20. Meyer A, Scharlt M (1999) Gene and genome duplications in vertebrates: the one-to-four (→to-eight in fish) rule and the evolution of novel gene functions. Current Opinion in Cell Biology 11: 699–704.
21. Etard C, Roostalu U, Sirinupong N (2008) Shuttling of the chaperones Ucn45b and Hsp90α between the A band and the Z line of the myofibril. J Cell Biol 180: 1163–75.
22. Nasrécim C, Elker SC (2009) Effective targeted gene ‘knockdown’ in zebrafish. Nature Genetics 41: 216–220.
23. Urasaki A, Mervan G, Kawakami K (2006) Functional dissection of the Tol2 transposable element identified the minimal cis-sequence and a highly repetitive sequence in the subterminal region essential for transposition. Genetics 174: 639–649.
24. Thié C, Thié B (2008) High-resolution in situ hybridization to whole-mount zebrafish embryos. Nature Protocols 3: 59–69.
25. Zhang J, Fu G, Chu W, Chen J, Liu Z, et al. (2009) cDNA cloning and expression analysis of the myosin heavy chain (MYH) gene of the mandarin fish Siniperca chuatsi. Marine Biotechnology 13: 151–162.
26. Zhang G, Chu W, Hu S, Meng T, Pan L, et al. (2011) Identification and analysis of muscle-related protein isoforms expressed in the white muscle of the mandarin fish (Siniperca chuatsi). Marine Biotechnology 13: 151–162.
27. Costarelli M, Li J, Guaniero J, Kao JP, Du SJ (2010) Loss of Smyd1 between fish and mammals studies. PLoS ONE 5: e10027.
28. Du SJ, Li H, Bian Y, Zhong Y (2008) Heat-shock protein 90alpha1 is required for organized myofibril assembly in skeletal muscles of zebrafish embryos. Proc Natl Acad Sci U S A 105: 554–559.