The myosin-interacting protein SMYD1 is essential for sarcomere organization

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Summary
Assembly, maintenance and renewal of sarcomeres require highly organized and balanced folding, transport, modification and degradation of sarcomeric proteins. However, the molecules that mediate these processes are largely unknown. Here, we isolated the zebrafish mutant flatline (fla), which shows disturbed sarcomere assembly exclusively in heart and fast-twitch skeletal muscle. By positional cloning we identified a nonsense mutation within the SET- and MYND-domain-containing protein 1 gene (smyd1) to be responsible for the fla phenotype. We found SMYD1 expression to be restricted to the heart and fast-twitch skeletal muscle cells. Within these cell types, SMYD1 localizes to both the sarcomeric M-line, where it physically associates with myosin, and the nucleus, where it supposedly represses transcription through its SET and MYND domains. However, although we found transcript levels of thick filament chaperones, such as Hsp90α1 and UNC-45b, to be severely upregulated in fla, its histone methyltransferase activity – mainly responsible for the nuclear function of SMYD1 – is dispensable for sarcomerogenesis. Accordingly, sarcomere assembly in fla mutant embryos can be reconstituted by ectopically expressing histone methyltransferase-deficient SMYD1. By contrast, ectopic expression of myosin-binding-deficient SMYD1 does not rescue fla mutants, implicating an essential role for the SMYD1–myosin interaction in cardiac and fast-twitch skeletal muscle thick filament assembly.

Key words: SMYD1, Myosin, Thick filament assembly, Zebrafish

Introduction
Coordinated synthesis, assembly and renewal of numerous contractile, structural and regulatory proteins into sarcomeric units are crucial to maintain proper heart and skeletal muscle function throughout life. However, little is known about the molecules that mediate these fundamental processes. The zebrafish is an established model organism to identify genes that are required to build and maintain the contractile muscle apparatus, thereby offering insights into the underlying molecular mechanisms and serving as a model for human muscle diseases (Rottbauer et al., 2006; Sehnert et al., 2002). Additionally, a growing body of evidence implies that correct sarcomere assembly involves several auxiliary proteins (Barral et al., 1998; Barral et al., 2002; Epstein and Thomson, 1974; Hobert et al., 1999; Miller et al., 2008; Venolia and Waterston, 1990).

Proteins containing SET [SU(VAR)3-9, Enhancer of zeste and Trithorax] and MYND (Myleoid, Nervy and DEAF-1) domains (SMYDs) are known to regulate gene transcription, mostly by mediating chromatin modifications (Rice and Allis, 2001). SET-domain-containing proteins can modulate transcription by methylating unacylated lysine residues on histone tails [histone methyltransferase (HMT) activity] (Rea et al., 2000), whereas MYND-domain-containing proteins usually repress transcription by recruiting histone deacetylases (HDACs) (Gottlieb et al., 2002). Interestingly, SMYD proteins are also known to regulate non-histone protein functions by site-specific methylation of their target proteins, such as p53 or VEGFR1 (Huang and Berger, 2008; Huang et al., 2006; Kunizaki et al., 2007). In mammals, five SMYD proteins have been identified so far (SMYD1–SMYD5) but little is known about their in vivo functions.

SMYD1 (known as mBop in mouse), is strongly expressed in cardiac and skeletal muscle cells. Its transcription in the anterior heart field is regulated by myocyte enhancer factor-2c (MEF2c), and in skeletal muscle cells by myogenic differentiation antigen 1 (MYOD) (Phan et al., 2005). In contrast to SMYD2-nullizygous mice, SMYD1-deficient mice lack proper development of the right ventricular chamber and die at embryonic day 10.5 (Gottlieb et al., 2002; Phan et al., 2005). In zebrafish, knockdown of the zebrafish SMYD1 isoforms, SMYD1a and SMYD1b by morpholino-modified antisense oligonucleotides leads to impaired heart and skeletal muscle function due to disturbed myofibril organization (Tan et al., 2006). SMYD2, although highly expressed in cardiomyocytes, was recently found to be dispensable for cardiac development because cardiac-
specific deletion of SMYD2 in mice does not interfere with normal heart morphogenesis and function (Diehl et al., 2010).

Here, in search for key regulators of cardiac and skeletal muscle function, we isolated the recessive lethal zebrafish mutant flatline (fla), which displays severe cardiac and skeletal muscle dysfunction due to impaired myofibrillogenesis. By positional cloning, we demonstrate that a nonsense mutation in SMYD1, a heart and fast-twitch muscle fiber HMT, is responsible for the fla phenotype. We find that SMYD1 localizes to the sarcomeric M-line where it interacts with myosin. Remarkably, heart and skeletal muscle function of fla embryos can be restored by ectopic expression of HMT-deficient SMYD1 but not by myosin-binding-deficient SMYD1. Taken together, our findings imply that SMYD1 plays an essential role in myosin thick filament assembly exclusively in cardiomyocytes and fast-twitch skeletal muscle cells.

**Results**

Heart and skeletal muscle function is impaired in the zebrafish mutant flatline

In search for key-regulators of cardiac and skeletal muscle function, we isolated in a large-scale ENU-mutagenesis screen, the recessive embryonic lethal zebrafish mutant flatline (fla<sup>mo1474a</sup>) (Warren and Fishman, 1998). fla mutants displayed severe cardiac and skeletal muscle dysfunction with complete penetrance in various genetic backgrounds (TL, WIK and TüAB) (Fig. 1A,B; supplementary material Fig. S1A). Usually, by 22 hours post fertilization (hpf) in wild-type embryos the heart tube is formed and a peristaltic contraction wave passes through the heart. By contrast, although the fla heart tube jogs correctly and myo- and endocardial cell layers were clearly defined in these embryos, fla mutant hearts did not display any contraction (supplementary material Fig. S1B–G; Fig. 1). Disrupted myofibrillogenesis in fla cardiomyocytes and fast-twitch skeletal muscle cells. (A,B) Lateral view of wild-type (A) and fla mutants (B) at 48 hpf. fla mutants develop pericardial edema owing to the absence of cardiac contractility. (C–H) Electron microscopic analysis of parasagittal and transversal (insets C–H) sections through cardiac and somitic muscle cells of wild-type and fla at 48 hpf. In contrast to the wild-type (C), cardiomyocytes of fla (D) completely lack sarcomeric structures. Only premature sarcomeric structures, such as stress fibers or Z-bodies can be detected in fla cardiomyocytes (D, arrowhead). Compared with wild-type (E), fla fast-twitch skeletal muscle fibers (F) fail to develop sarcomeric structures (arrowheads show Z-bodies). By contrast, myofibrillogenesis of slow-twitch skeletal muscle fibers (G,H) is not affected in fla. (I,J) fla acts cell-autonomously in cardiomyocytes and fast-twitch skeletal muscle cells. α-Actin immunostain (J) of Biotin-labeled transplanted wild-type cells (1 and 2) (I), showing normal formation of sarcomeric units in these cells. skm, skeletal muscle.
supplementary material Movie 1). To examine whether, similar to the situation in the zebrafish mutants island beat and reggae (Hassel et al., 2008; Rottbauer et al., 2001), disturbed cardiac excitation is responsible for cardiac acontractility in *fla*, we next monitored calcium transients in *fla* hearts by high-resolution fluorescent microscopy. However, similar to the situation in wild-type hearts, in *fla* hearts a calcium wave commenced in the sinus venous region, then migrated through the atrium and then the ventricle of the non-beating *fla* heart (supplementary material Fig. S1H,I; supplementary material Movie 2), indicating that the *fla* mutation does not impact on cardiac electrical impulse generation and propagation, but rather leads to electromechanical uncoupling of cardiomyocytes. In addition to their cardiac phenotype, *fla* mutants displayed severe impairment of skeletal muscle function. When touch-stimulated, *fla* mutants displayed, in contrast to wild-type embryos, only a shiver response, which does not generate enough force for the *fla* embryo to execute a ‘flight response’ (supplementary material Movie 3).

Next, to evaluate whether defective myofibrillar organization is the cause for the dysfunction of cardiac and skeletal muscles in *fla*, we assayed their ultrastructure. Usually, by 24 hpf in wild-type zebrafish, thick and thin filaments are arrayed in well-aligned overlapping filament bundles, interconnected by sarcomeric Z-discs. By contrast, in *fla* cardiomyocytes sarcomeric units were completely absent (Fig. 1C,D). Remarkably, similar to other zebrafish myofibrillogenesis mutants such as *tell tale heart, steif* or *pickwick* (Etard et al., 2007; Rottbauer et al., 2006; Xu et al., 2002), premature sarcomeric structures such as 1-Z-1 bundles could be detected in *fla* cardiomyocytes (Fig. 1D, arrowhead). By contrast, we never observed myosin bundles in *fla* mutant muscles, implying that *fla* might interfere either with early myosin assembly or stability. Similarly, in *fla* skeletal muscles we found that sarcomerogenesis was abolished in fast-twitch fibers (Fig. 1E,F), whereas sarcomeric units were properly organized in *fla* slow-twitch fibers (Fig. 1G,H).

To determine whether *fla* acts cell-autonomously or non-cell-autonomously, we generated genetic mosaics of hearts and skeletal muscle by transplanting *fla* mutant cells into wild-type embryos and vice versa. We found that, when wild-type cells contributed to mutant hearts, they contracted spontaneously and vigorously in *fla* ventricles and atria (*n*=9), whereas *fla* cardiomyocytes adjacent to the transplanted cells did not contract (supplementary material Movie 4). By contrast, *fla* cardiomyocytes that are transplanted into wild-type hearts did not contract (*n*=4) (supplementary material Movie 5). As shown by electron microscopy and immunohistological analysis, transplanted wild-type cells had proper myofibrillar architecture, including regular arrays of thick and thin myofilaments, and Z-discs as well as intercalated discs (Fig. 1J; supplementary material Fig. S2A,B), whereas surrounding fast-twitch myocytes were devoid of intact sarcomeric units. Hence, *fla* acts cell-autonomously in heart and fast-twitch skeletal muscle sarcomerogenesis.

*flatline (fla<sup>mv-47a</sup>)* encodes zebrafish SET- and MYND-domain-containing protein 1

To reveal the ENU-induced genetic defect of *fla*, we performed a genome-wide study of microsatellite marker segregation by bulked segregant analysis and linked *fla* as inbetween the microsatellite markers Z20180 and Z65535 on chromosome 8. Genetic fine-mapping by recombination analysis of 1595 *fla* mutant embryos restricted the *fla* locus to the bacterial artificial chromosome (BAC) RPCI71-6G18. Further recombination analysis placed *Fatty-Acid-Binding protein 1b* (*FABP1b, XP_001332792*) and the SET- and MYND-domain-containing protein 1 (*smyd1, ABC54713*) within the *fla* genetic interval. By sequencing wild-type and *fla* cDNA, we found the *fla* mutation to be a thymine-to-adenine nucleotide transversion (UGU→UGA) in codon 73 of zebrafish *smyd1*, whereas in FABP1b no mutation was detected. The identified mutation was predicted to change the amino acid cysteine to a stop codon and hence to cause premature termination of translation of both zebrafish *smyd1* isoforms, *smyd1a* and *smyd1b*, at amino acid position 73 (C73stop) (Fig. 2A). To test whether the predicted truncated SMYD1 proteins were stable and thereby might have acted as dominant negatives, we generated expression plasmids of N-terminal V5-tagged wild-type or *fla* mutant SMYD1. Whereas in vitro translated wild-type SMYD1 accumulated in reticulocyte lysates, no truncated SMYD1 proteins accumulated to detectable levels (supplementary material Fig. S2C). Because mutant SMYD1 proteins were not stable, lack of SMYD1 rather than a dominant-negative effect of truncated SMYD1 appears to cause the *fla* mutant phenotype.

To evaluate whether ectopic expression of wild-type RNA encoding *smyd1* can restore *fla* heart and skeletal muscle function, we injected either 50 pg of wild-type (*smyd1b<sup>wt</sup>*) or *fla* mutant (*smyd1b<sup>fla</sup>*) mRNA into one-cell stage *fla* mutants (*fla<sup>mv-47a</sup>/*<sup>fla</sup>*→/→) derived from intercrossing *fla* heterozygous zebrafish (*n*=373). As shown in Fig. 2D, in 70±10.8% of homozygote mutant *fla* embryos heart and skeletal muscle function could be completely restored by injection of wild-type *smyd1* mRNA. Hearts of injected *fla* mutants contracted vigorously, blood circulation was established and mutant embryos displayed coordinated skeletal muscle movements (supplementary material Movie 6). Additionally, *fla* sarcomeres were restored (Fig. 2B–E). Similar effects and efficacy were observed when injecting 50 pg of *smyd1a* mRNA into one-cell stage *fla* mutant embryos (*n*=47). To validate that finding that cardiac and skeletal muscle dysfunction in *fla* mutants is indeed caused by loss of SMYD1 function, we injected morpholino-modified antisense oligonucleotides, either directed against the translational start site (MO1-*smyd1l*) or the splice donor site of exon 3 (MO2-*smyd1l*) of *smyd1l* mRNA into one-cell stage wild-type zebrafish embryos. When injected with 2.5 ng of MO1-*smyd1l* or MO2-*smyd1l*, 97±2% of the embryos (*n*=460 and 403, respectively) displayed the *fla* mutant phenotype accompanied by the absence of cardiac contractility and severe skeletal muscle dysfunction (Fig. 2F–I). As in *fla* mutants, ultrastructural analysis of MO1-*smyd1l*-injected embryos revealed disrupted sarcomerogenesis in cardiomyocytes and fast-twitch skeletal muscle cells (Fig. 2I). By contrast, injection of 5-bp mismatch control morpholinos (5mmMO) did not impact on heart and skeletal muscle function (Fig. 2H).

*smyd1* expression is restricted to nuclei and M-lines of cardiomyocytes and fast-twitch skeletal muscle cells and associates with sarcomeric myosin

From mouse and zebrafish in situ hybridization studies, *smyd1* is known to be restricted to heart and skeletal muscle tissue (Gottlieb et al., 2002; Tan et al., 2006) (Fig. 3A,B). As outlined above, *fla* mutants have sarcomerogenesis defects only in cardiac and fast-twitch skeletal muscle fibers, whereas slow-twitch fibers
are devoid of any structural or functional defects. Hence, to evaluate whether \textit{smyd1} was differentially expressed in slow- and fast-twitch skeletal muscle cells, we assayed \textit{smyd1} RNA distribution in these two muscle compartments. As shown in Fig. 3C–E, \textit{smyd1} RNA was exclusively present in the fast-twitch fiber compartment of zebrafish trunk muscles, whereas slow-twitch fibers were devoid of \textit{smyd1} RNA.

To evaluate, where SMYD1 protein localizes within heart and skeletal muscle cells, we performed immunohistological stainings using a SMYD1-specific antibody. As expected for proteins with HMT activity, SMYD1 localized in the nuclei of cardiomyocytes (Fig. 3F–H). Furthermore, we found SMYD1 to be distributed in a striped pattern within the cytoplasm, implicating association with sarcomeric proteins. As shown by co-immunostaining of SMYD1 and myomesin in murine heart, SMYD1 localized at the M-line – the anchorage point of myosin – of muscle cells (Fig. 3I, J). To assess whether SMYD1 directly interacted with myosin at the M-line, we performed in vitro pull-down assays using GST-tagged SMYD1b and sarcomeric myosin. As shown in Fig. 3K, SMYD1b was indeed capable of specifically binding myosin. To narrow down the location of the SMYD1–myosin interaction site, we generated distinct domain-specific deletion variants of SMYD1b and again tested their ability to interact with myosin (Fig. 3K). Interestingly, only full-length SMYD1b and the C-terminal deletion variant SMYD1b\textsuperscript{391del}, but not SMYD1b\textsuperscript{278del} and SMYD1b\textsuperscript{89del}, were capable of binding myosin, implicating an essential role of amino acids 278–391 of SMYD1b for the physical interaction with myosin.

To investigate whether, similar to other myosin-interacting proteins, such as UNC-45b or Hsp90a, intracellular localization of SMYD1 is dynamic, we tracked the localization of transiently overexpressed SMYD1a in a model of decelerated myofibrillogenesis in zebrafish muscle cells (Etard et al., 2008). Therefore, we constructed a SMYD1a–GFP chimeric gene, co-injected the construct with low levels of UNC-45b-specific morpholinos, to slow down myofibril formation in fertilized zebrafish eggs, and followed the localization of the
Fig. 3. SMYD1 is expressed in the heart and fast-twitch skeletal muscle cells, and interacts with myosin at the sarcomeric M-line. (A,B) smydl RNA is strongly expressed in somitic muscle cells (A) and both heart chambers (B). (C–E) smydl-specific in situ hybridization, in combination with an immunostain of slow-twitch skeletal muscle fibers (F59, green) of transversal sections through the somitic muscles of a wild-type (wt) embryo. smydl is expressed in fast-twitch and is absent in slow-twitch skeletal muscle cells. (F–H) Immunohistochemistry of murine heart sections reveals that SMYD1 protein (green) localizes to the sarcomere and the nucleus of cardiomyocytes. Cell nuclei are counterstained with DAPI (blue). (I,J) Co-immunostains of SMYD1 and α-Actinin (I), as well as SMYD1 and myosin (J), in murine heart sections reveal localization of SMYD1 to sarcomeric M-lines. (K) The scheme outlines different SMYD1b deletion variants that are used for the myosin in vitro pulldown assay (turquoise, pre-set and post-set domain; blue, MYND domain; purple, C-terminus). SMYD1b89del-GST (lane 1) and SMYD1b91del_GST (lane 4) proteins interact with myosin from rabbit muscle in an in vitro pulldown assay, whereas SMYD1b99del_GST (lane 2) and SMYD1b278del_GST (lane 3) do not bind myosin. GST (lane 5) and UNC-45b (lane 6) are used as negative and positive myosin-binding controls, respectively.

fusión protein by confocal microscopy. Remarkably, we found that SMYD1a–GFP protein accumulates in a double-banded pattern characteristic of the myosin in the A-band at 48 hpf (Fig. 4A). Furthermore, we show by co-immunostaining with antibodies directed against α-actinin that SMYD1a–GFP had shifted back to the sarcomeric M-line at 72 hpf (Fig. 4B–E), demonstrating that SMYD1a colocalizes with myosin at the sarcomeric A-band during myofibrillogenesis and associates with the M-line when myofilbril assembly is completed. These findings suggest that SMYD1 plays a crucial role during early myosin assembly.

**SMYD1, Hsp90a1 and UNC-45b are co-regulated during sarcomere assembly in zebrafish**

SMYD1 is known to regulate gene transcription by methylating histones. Hence, we wondered whether altered transcription of sarcomeric constituents was responsible for defective sarcomerogenesis in fla mutants. Interestingly, we find that mRNA levels of various myofilibrillar components, such as the thin filament components α-tropomyosin (tpm4), tropomin T (tmnt), the thick filament components cardiac myosin light chain 2 (cmlc2), cardiac myosin light chain 1 (cmlc1), ventricular-specific myosin heavy chain (vmhc) and atrial myosin heavy chain (amhc) and the elastic filament titin are not differentially regulated in fla embryos (Fig. 5A; supplementary material Fig. S3A), indicating that altered transcription of sarcomeric components does not account for disturbed sarcomerogenesis in fla heart and skeletal muscle. Next, to identify transcriptional targets of SMYD1 that might account for defective sarcomerogenesis in fla, we assayed transcriptional profiles of 36-hour-old fla mutants and wild-type littermates using cDNA expression arrays. Interestingly, we find that transcripts encoding muscle chaperones and co-chaperones such as heat shock protein 90-alpha1 (hsp90a1) and unc-45b, which are known to be crucial for regular sarcomere assembly, were strongly upregulated in fla (up to 8-fold) (supplementary material Fig. S3B). These findings were confirmed both by qRT-PCR (relative expression of hsp90a1=3.8±1.2 and unc-45b=3.5±1.1; n=5, P<0.01) and in situ hybridization (Fig. 5B–G). As revealed by western blot analyses, Hsp90a protein levels were also severely increased in fla mutants compared with those in wild-type littermates (supplementary material Fig. S3C). To assess whether UNC-45b protein levels were also increased in fla, we tested four different UNC-45b-specific antibodies, but unfortunately none of these antibodies was functional in western blot analyses using zebrafish protein lysates. To assess whether upregulation of molecular chaperones in fla cardiomyocytes and fast-twitch skeletal muscle cells was induced by cellular stress evoked by defective sarcomerogenesis, we evaluated the expression of Hsp90a1 in the zebrafish cardiac myofibrillogenesis mutants silent heart (sih) (Troponin T deficiency) and tell tale heart (tel) (MLC2 deficiency) (Rottbauer et al., 2006; Sehner et al., 2002). Interestingly, although similar to fla mutants, no sarcomeric units were built in cardiomyocytes of sih and tel mutants, hsp90a1 was not up-regulated in these mutants (Fig. 5H–K), indicating that the induction is not a common mechanism in response to defective sarcomere assembly.

Molecular chaperones, such as Hsp90a1 and UNC-45b, are essential for proper folding of thick filament myosin during myofibrillogenesis. Very recently, it has been shown that overexpression of UNC-45b in zebrafish muscle cells leads to impaired sarcomere organization (Bernick et al., 2010). Thus, to investigate whether downregulation of UNC-45b in fla mutant embryos might rescue myofibrillogenesis, we injected ‘sub-effective’ doses (i.e. doses unable to induce a muscle phenotype in wild-type embryos) of UNC-45b-specific morpholinos into homozygous fla mutant embryos and evaluated heart and skeletal muscle function through high-resolution video microscopy.
Interestingly, we found that knocking down UNC-45b in \textit{fla} mutant embryos did not reconstitute heart and skeletal muscle function ($n=67$), implying that overexpression of UNC-45b is not the molecular cause of the observed muscle phenotype in \textit{fla}. In the zebrafish mutants \textit{akineto} and \textit{steif}, which are deficient in the myosin chaperones \textit{hsp90a1} and \textit{unc-45b}, respectively, \textit{hsp90a1} and \textit{unc-45b} mRNA levels are strongly elevated (Etard et al., 2007; Hawkins et al., 2008). Hence, to evaluate whether SMYD1 was also upregulated in \textit{hsp90a1-} and \textit{unc-45b}-deficient zebrafish embryos, we assayed SMYD1 transcript levels by antisense RNA in situ hybridization. We found strong upregulation of \textit{smyd1} in \textit{hsp90a1-} and \textit{unc-45b}-deficient embryos (Fig. 6A–D). Surprisingly, we also found that the \textit{smyd1} transcript levels were substantially upregulated in \textit{fla} heart and skeletal muscle cells (Fig. 6E,F), suggesting that SMYD1, \textit{hsp90a1} and \textit{unc-45b} are co-regulated during zebrafish sarcomerogenesis.

Loss of \textit{hsp90a1} or \textit{unc-45b} function results in the destabilization and degradation of their client protein myosin (Barral et al., 1998; Hawkins et al., 2008; Landsverk et al., 2007). Strikingly, and in contrast to their unchanged transcript levels, protein levels of sarcomeric myosins were severely downregulated in \textit{fla}, implying that loss of SMYD1 function, similar to the situation in \textit{hsp90a1-} and \textit{unc-45b}-deficient embryos, leads to the destabilization and subsequent degradation of myosin proteins in \textit{fla} mutants (Fig. 6G; supplementary material Fig. S3D).

\textbf{SMYD1 HMT activity is dispensable for thick filament assembly in vivo}

In addition to its localization at the M-line of heart and fast-twitch skeletal muscle cells, SMYD1 is present in the cell nucleus where it is known to regulate gene transcription through...
methylating histones. To assess whether the HMT activity was indispensable for thick filament assembly in *fla* heart and fast-twitch skeletal muscles or whether the interaction of SMYD1 and myosin was pivotal for regular sarcomerogenesis, we first generated a construct that expressed HMT-deficient SMYD1b (SMYD1bY247F) (Brown et al., 2006), injected the mRNA into *fla* mutants and evaluated its capability to reconstitute heart and skeletal muscle function and structure. Strikingly, we found that 50 pg of *smyd1bY247F* mRNA efficiently rescued the *fla* phenotype (61.2 ± 7.0%; n = 47, three experiments) (Fig. 7A,E). Hearts of *fla* mutants injected with *smyd1bY247F* mRNA contracted vigorously and mutant embryos displayed coordinated skeletal muscle movements (supplementary material Movie 7). Accordingly, we found reconstitution of sarcomerogenesis in fast-twitch skeletal muscle cells (Fig. 7C), demonstrating that SMYD1 HMT activity is dispensable for thick filament assembly. By contrast, overexpression of SMYD1b278del (50 pg per embryo), a SMYD1 deletion variant where the SET and MYND domain is preserved but the myosin interaction site is absent, in *fla* mutants did not reconstitute heart and skeletal muscle function and structure (n = 31, three experiments) (Fig. 7B,F; supplementary material Movie 8). Additionally, no sarcomeres were built in *fla* embryos injected with *smyd1b278del* mRNA (Fig. 7D), indicating that the SMYD1–myosin interaction is pivotal for sarcomere assembly in zebrafish heart and fast-twitch skeletal muscle cells.

**Discussion**

Although timely and spatially coordinated assembly of a large number of striated muscle proteins is crucial for regular heart and skeletal muscle function, so far surprisingly little is known about the molecular control mechanisms that guarantee proper shuttling, processing and assembly of sarcomeric components. Here, we demonstrate that SMYD1 is crucial in orchestrating thick filament assembly in cardiomyocytes and fast-twitch skeletal muscle cells in zebrafish.

SMYD1 is exclusively expressed in cardiomyocytes and skeletal muscle cells in mice and zebrafish (Gottlieb et al., 2002; Phan et al., 2005; Tan et al., 2006). Targeted deletion of *smyd1* in mice results in embryonic lethality at ~E10.5, most probably owing to reduced levels of Hand2 in the precardiac mesoderm, leading to impaired development of the right ventricular chamber and left ventricular contractile failure (Gottlieb et al., 2002). Both murine and zebrafish SMYD1 can act as HMTs (Gottlieb et al., 2002; Tan et al., 2006). Accordingly, localization of SMYD1 in the nucleus is indispensable for its function as HMT. Interestingly, as well as its expression in the nucleus we find significant amounts of SMYD1 localizes to the sarcomeric M-line of cardiac myocytes. Furthermore, in a model of decelerated myofibrilllogenesis (Etard et al., 2008), we find that the subcellular localization of SMYD1 is highly dynamic. We show that SMYD1 first associates with the A-band, where unfolded Myosin resides. Later on, SMYD1 shuttles to the sarcomeric M-line when myofibrilllogenesis is completed. These results are supported by our finding that SMYD1 physically interacts with sarcomeric myosin, implying that there is an active role of SMYD1 for the assembly of myosin filaments. However, several muscle proteins such as Muscle Lim Proteins (MLPs), the Nuclear Factor Of Activated T Cells 3 (NF-AT3), Core-binding factor β (CBFβ) and Muscle-Specific Ring Finger Proteins (MURFs) show a similar intracellular distribution, with binding to specific sarcomeric sites, such as the M-line, I-band or Z-disc, and also having a functional role within the nucleus. Interestingly,
upon defined stimuli, such as biomechanical stress or denervation, all these proteins then translocate to the cell nucleus to impact on gene transcription (Ecarnot-Laubriet et al., 2000; McElhinny et al., 2002; Meder et al., 2010; Nicol et al., 2000). Similarly, SMYD1 binding to the M-line might also serve as a buffer to maintain balanced levels of nuclear SMYD1 activity and to guarantee immediate crosstalk between sarcomeres and the nucleus. Interestingly, SMYD2, another member of the SMYD protein family, was found to methylate not only histones but also non-histone proteins, such as p53 (Huang et al., 2006). Furthermore, SMYD3 was found to dimethylate Vascular Endothelial Growth Factor Receptor 1 (VEGFR1), which leads to an enhanced kinase activity of VEGFR1 (Kunizaki et al., 2007). Similarly, SMYD1 might also function as a methyltransferase with M-line proteins as a substrate. In this context, it is interesting that modification of myosin through methylation has been known about for more than 30 years but the functional role of this modification and the proteins that transfer methyl groups to myosin are still unknown (Huszar and Elzinga, 1972).

We find that SMYD1 is expressed only in cardiomyocytes and fast-twitch skeletal muscle cells but not slow-twitch skeletal muscle cells. These findings are in contrast to recent observations using a smydl-GFP transgenic zebrafish line suggesting that SMYD1 is expressed in the heart and both skeletal muscle fiber types, with a stronger signal intensity in the slow-twitch fiber population (Du et al., 2006). These contradictory findings might be explained by the complex orchestration of regulatory signals within a distinct promoter region. In the context of the smydl promoter, it is probable that important regulatory elements or sites of selective DNA methylation, restricting the expression of SMYD1 to cardiomyocytes and fast-twitch skeletal muscle cells, are not included in the smydl-GFP transgenic promoter construct used during the generation of the transgene. Our findings are also supported by the fact that defective sarcomerogenesis is only present in the heart and fast-twitch skeletal muscles, but not slow-twitch muscle fibers. Nevertheless, it will be interesting to dissect the regulatory elements excluding SMYD1 from the slow-twitch fiber cell population.

To our knowledge, SMYD1 is the first protein that controls myofibrillar assembly exclusively in fast-twitch skeletal muscle cells and cardiomyocytes. Recently, two proteins have been found to control slow-twitch skeletal muscle cell differentiation and myofiber arrangement without an effect on cardiomyocytes and fast-twitch fibers. By characterizing the zebrafish mutant u-boot (ubo), the SET-domain-containing protein Blimp-1 was found to be essential for slow-twitch fiber specification downstream of Hedgehog (Hh) signaling (Baxendale et al., 2004). Consequently, the homeobox gene proxl was shown to act downstream of Blimp-1 to regulate exclusively slow-twitch myofibrillar assembly (Roy et al., 2001).

Whereas mRNA levels of the main sarcomeric components, such as myosins, are unaffected by loss of SMYD1 function, protein levels of these myofibrillar constituents are severely downregulated in cardiomyocytes and fast-twitch skeletal muscle cells. As shown recently, a loss-of-function mutation in the muscle chaperone Hsp90a1 in the zebrafish mutant akineto leads to severe defects in skeletal muscle sarcomerogenesis due to impaired thick filament assembly (Hawkins et al., 2008). Remarkably, and similar to the situation in fla, myosin protein levels are severely reduced in this mutant. Another muscle chaperone, SMYD1, interacts with Hsp90a1 to guide the assembly of myosins into sarcomeric units (Srikakulam and Winkelmann, 1999). UNC-45b-deficient zebrafish embryos and worms have strongly disorganized sarcomeres, although initial Z-body formation and the basal organization of thick filaments seems not to be altered, implicating a rather restricted role of UNC-45b in the integration of thick filaments into sarcomeres (Barral et al., 2002; Etard et al., 2007). Strikingly, not only the loss but also elevated levels of UNC-45b result in inhibition of sarcomere assembly in worms and zebrafish, indicating that muscle chaperone levels have to be precisely balanced for unconstrained sarcomerogenesis and myosin stability (Bernick et al., 2010; Hoppe et al., 2004; Janiesch et al., 2007; Landsverk et al., 2007). We find UNC-45b expression levels to be severely upregulated in fla. Interestingly, a mild knockdown of UNC-45b in fla mutant embryos does not reconstitute heart and skeletal muscle function, implying that overexpression of UNC-45b is not the molecular cause of the observed muscle phenotype in fla.

SMYD1 is known to regulate target gene transcription by methylating histones in the nucleus. We find here that SMYD1 also exhibits nucleus-independent functions, such as interaction with sarcomeric myosin proteins. Remarkably, we find that SMYD1 HMT activity is dispensable for sarcomerogenesis in fla because thick filament assembly is restored by ectopic expression of HMT-deficient SMYD1. By contrast, myosin-binding-deficient SMYD1 does not reconstitute heart and fast-twitch skeletal muscle function, demonstrating that the SMYD1–myosin interaction is pivotal for proper thick filament assembly. These findings suggest that SMYD1, similar to Hsp90a1 or UNC-45b, also plays a role in myosin processing or chaperoning during myofibrillogenesis (Etard et al., 2007; Hawkins et al., 2008; Landsverk et al., 2007).

Chaperones and associated proteins are increasingly recognized as important regulators that balance the dynamics of sarcomeric unit synthesis and degradation in the heart and skeletal muscles. However, the mechanisms by which these fundamental processes are controlled are only poorly understood. In fla, we now show for the first time that SMYD1 is a binding partner of myosin and that this interaction is crucial for thick filament assembly in heart and fast-twitch skeletal muscle cells. Strategies that target these mechanisms might ultimately lead to novel treatment concepts for skeletal myopathies and heart muscle diseases.

Materials and Methods

Cell transplantation, injection procedures and virtual M-mode measurement

Care and breeding of zebrafish Danio rerio was as described previously (Rottbauer et al., 2005). The present study was performed after securing appropriate institutional approvals. It conforms to the Guide for the Care and Use of Laboratory Animals published by the ‘US National Institutes of Health’ (NIH Publication No. 85–23, revised 1996). Pictures and movies were recorded at 72 hpf. To inhibit pigmentation, 0.003% 1-phenyl-2-thiourea was added to the embryo medium (Bendig et al., 2006a). Cell transplantation was performed and analyzed essentially as described previously (Rottbauer et al., 2002). Embryos used for transplantation were produced by intercrossing fla–/– fish, thus yielding a wild-type to mutant embryo ratio of 3:1. 3% tetramethylrhodamin dextran and 3% biotin dextran (Molecular Probes, Invitrogen) were mixed in 0.2 M KCl and microinjected into donor embryos at the one-cell stage. Labeled donor cells were transplanted at the early stage of embryo and embryos were assessed by fluorescence microscopy at 24 and 48 hpf. To confirm the integration of labeled donor cells into accep tor embryo heart and skeletal muscles, whole embryos were stained with anti-biotin antibodies (DAB), fixed with 4% paraformaldehyde, embedded in JB-4 and sectioned. Labeled donor embryos were genotyped using polymorphic markers flanking the fla gene. Morpholinos were directed against a splice donor site (MO2-zmydl, 5'-GGACGTTGACGTACACGCAGGA-3') and the translational start site of smydl (MO1-zmydl, 5'-AAAAACTTCCACAAACTCTATTCTG-
Two smyd1-specific 5 bp mismatch control morpholinos (MOI-smymdl1mm,
5'-AAAAGTGGCAGAGATTGACTTGTG-3' and MO2-smymdl1mm, 5'-
GGACCTTGACCTGACCCCCCGAGA-3') (GENETOOLS, LLC) were
injected at the same concentration as a negative control. UNC-45b-specific
morpholinos were directed against the translational start site of zebrafish
unc-45b (Etard et al., 2007). Sense-capped RNA was synthesized using the
mMESSAGE-mMACHINE system (Ambion) from pCS2 (Etard et al., 2005). All
error bars represent standard deviations (s.d.). To analyze contractile force of wild-
type and mutant hearts, we undertook M-mode measurements with the help of the
zebraFS software application (http://www.benegfx.de) (Meder et al., 2009).

Transmission electron microscopy, in situ hybridization, immunostaining
and immunoblotting

Electron micrographs were obtained essentially as described previously (Rottbauer
et al., 2001). Whole-mount RNA in situ hybridization was carried out essentially as
described previously (Rottbauer et al., 2005) using a full-length smyd1 antisense
probe, as well as antisense probes for zebrafish mlf2, vmhc, amhc, titin, tnm4, tnt,
hsp90a1 and unc-45b. Immunostaining of zebrafish embryos fixed in Dent’s fix
were carried out with antibodies against slow muscle myosin heavy chains (FS9)
(DSHB) and Nexlin (BD Biosciences). Embryos were embedded in JB-4, 5-

dehydrated and embedded in paraffin. Sections were cut at 4 µm, mounted on
slides, and stained with hematoxylin and eosin. Immunoblotting, proteins were
separated by SDS-PAGE and transferred onto nitrocellulose membranes. Immunoblotting
procedures were carried out essentially as described (Kubicek, 2005). Antibodies to
zmyd1, Mph20, Titin, Myosin Heavy Chain, Hsp90a, Actinin and β-actin (Sigma) and
signals detected by chemiluminescence (polycyclic anti-rabbit-IgG antibody conjugated
to horseradish peroxidase).

Genetic mapping, positional cloning and mutation detection

DNA from 24 flmnlc2 mutant and 24 wild-type embryos was pooled and bulked
segregation analysis was performed as described (Rottbauer et al., 2005). RNA from
flmnlc2 mutant and wild-type embryos was isolated using TRIzol (Life
Technologies) and reverse transcribed. Eight independent smydl cDNA clones from
mutant and wild-type embryos were sequenced. Genomic DNA from flmnlc2 mutant and wild-type embryos was sequenced around the point-mutation.

Microarray and quantitative real-time PCR

For microarray analysis 25 flmnlc2 mutant and 25 wild-type embryos were collected at
36 hpf, and RNA was isolated with TRIzol reagent. A clean-up of the RNA was
performed by using the RNAeasy Kit (Qiagen). Microarray analysis was performed by
Atlas Bioslabs (Berlin) using a zebrafish-specific Affymetrix chip. Quantitative real-time PCR was carried out according to standard protocols with the
SYBR-Green method using an ABI 7000. All error bars represent standard deviations
(s.d.).

Expression and purification of recombinant SMYD1 and UNC-45b

smyd1 variants and unc-45b were cloned into the pDEST24 and/or pcDNA3.1/
v5-DEST vector (Invitrogen). GST-tagged SMYD1 and UNC-45b fusion proteins were
expressed in Escherichia coli BL21AI by inducing bacteria with 0.1% arabinose overnight at
30°C. Bacteria were harvested by centrifugation and resuspended in lysis buffer [150 mM NaCl, 50 mM Tris-HCl pH 7.4, 5 mM EDTA at pH 7.4, 1% Triton X-100 and protease inhibitors (Complete, Roche)].
Bacterial cells were disrupted by sonication, and cell debris was removed by centrifugation (14,000 g for 30 minutes at 4°C). GST fusion proteins were purified with glutathione–Sepharose matrix (Amersham). For pull-down analyses, myosin
isolated from rabbit muscle (Fluka, 70045) was incubated at room temperature for
1 hour with the glutathione–Sepharose matrix and GST–SMYD1 complex. After
washing and boiling for 10 minutes at 95°C, the proteins were separated by SDS
page, transferred onto a PVDF membrane and probed with antibodies against muscle
myosin heavy chain (MF20) (DSHB). In vitro translation of wild-type and
mutant zebrafish 9-smyd1, flmnlc2 and mutant and wild-type embryos was isolated using TRIzol (Life
Technologies). In vitro translation reactions were performed with cell lysates and
the glutathione–Sepharose matrix. After
incubation at room temperature for
30 minutes at 4°C), GST fusion proteins were purified

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SMYD1 controls thick filament assembly 3135

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M-line and thick filament structure and may have nuclear functions via its interaction with glucocorticoid modulatory element binding protein-1. J. Cell Biol. 157, 125-136.


20 somites

A

24hpf

wild-type

flatline

B cmic2

C 

endo myo

D cmic2

E endo myo

F

G

H

I

200

210

220

230

190

195

200

205

210

ve fluorescence

ventricle atrium

125ms

125ms

relative fluorescence

relative fluorescence

wt flañt

wt flañt
transplanted cell

C

V5-SMYD1a wt
V5-SMYD1a fla
V5-SMYD1b wt
V5-SMYD1b fla

anti-V5