Loss of zebrafish Smyd1a interferes with myofibrillar integrity without triggering the misfolded myosin response

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ABSTRACT

Sarcomeric protein turnover needs to be tightly balanced to assure proper assembly and renewal of sarcomeric units within muscle tissues. The mechanisms regulating these fundamental processes are only poorly understood, but of great clinical importance since many cardiac and skeletal muscle diseases are associated with defective sarcomeric organization. The SET- and MYND domain containing protein 1b (Smyd1b) is known to play a crucial role in myofibrillogenesis by functionally interacting with the myosin chaperones Unc45b and Hsp90α1. In zebrafish, Smyd1b, Unc45b and Hsp90α1 are part of the misfolded myosin response (MMR), a regulatory transcriptional response that is activated by disturbed myosin homeostasis. Genome duplication in zebrafish led to a second *smyd1* gene, termed *smyd1a*. Morpholino- and CRISPR/Cas9-mediated knockdown of *smyd1a* led to significant perturbations in sarcomere structure resulting in decreased cardiac as well as skeletal muscle function. Similar to Smyd1b, we found Smyd1a to localize to the sarcomeric M-band in skeletal and cardiac muscles. Overexpression of *smyd1a* efficiently compensated for the loss of Smyd1b in *flatline* (*fla*) mutant zebrafish embryos, rescued the myopathic phenotype and suppressed the MMR in Smyd1b-deficient embryos, suggesting overlapping functions of both Smyd1 paralogs. Interestingly, Smyd1a is not transcriptionally activated in Smyd1b-deficient *fla* mutants, demonstrating lack of genetic compensation despite the functional redundancy of both zebrafish Smyd1 paralogs.

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1. Introduction

Sarcomeres are the basic functional units in striated muscles and their orchestrated assembly is fundamental for proper muscle contractions. The formation of sarcomeres, termed myofibrillogenesis, requires a controlled spatio-temporal integration of sarcomeric proteins, such as Myosin, into multiprotein complexes. In this context, Myosin assembly and its integration into sarcomeres is mediated by auxiliary proteins such as Unc45b (Unc-45 homolog B) and Hsp90α1 (heat-shock protein 90 α1) [1], [2]. Loss-of-function mutations within these genes interfere with myofibrillogenesis and lead to paralysis of zebrafish embryos [1], [2]. Interestingly, an identical phenotype was observed in zebrafish that lack the protein lysine methyl transferase (PKMT) Smyd1b, which was found to be specifically expressed in heart muscle and fast-twitch skeletal muscle cells where it physically interacts with Myosin [3]. Smyd1 was shown to form a complex with the myosin chaperones Hsp90α1 and Unc45b and thus contributes to myofibrillogenesis in cardiomyocytes and fast-twitch skeletal muscle cells [3]. In addition to their physical interaction, *hsp90α1*, *unc45b* and *smyd1b* are part of a complex gene program that is activated through defective myosin folding and assembly (termed misfolded myosin response (MMR)) leading to their massive transcriptional induction [4]. In contrast to mammals, the zebrafish harbors two *smyd1* genes, *smyd1a* and *smyd1b* that originate from genome duplication [5]. Up to now, only little is known about the physiological *in vivo* relevance of Smyd1a. Hence, to decipher the *in vivo* function of Smyd1a, we characterized Smyd1a during heart and skeletal muscle development in zebrafish. Our results demonstrated that Smyd1a,

similar to Smyd1b, localized to the sarcomeric M-band in muscle cells. Furthermore, loss of Smyd1a interfered with proper heart and skeletal muscle function *in vivo*. In addition, we showed that *smyd1a* over-expression in Smyd1b-deficient muscles efficiently repressed the transcriptional MMR and functionally compensated for the loss of Smyd1b. Interestingly, targeted inactivation of Smyd1a did not trigger the MMR, suggesting that, although functionally redundant when ectopically expressed, the biological *in vivo* roles of Smyd1a and Smyd1b are distinct.

2. Material and methods

2.1. Zebrafish strains and injection procedures

The present study was performed after appropriate institutional approvals (Tierforschungszentrum (TFZ) Ulm University, No. 0183), which conform to EU Directive 2010/63/EU. Care and breeding of zebrafish, *Danio rerio*, was conducted as described previously [6]. The following mutant alleles were used: *flatline smyd1b$^{zf340/zf340}$* [3] and *steif unc45b$^{sb60/sb60}$* [1]. Morpholino-modified antisense oligonucleotides (MOs; Gene Tools, LLC, Oregon, USA) were directed either against the translational start site and/or splice-acceptor/donor site of zebrafish *smyd1a*. As negative controls five base pair mismatch MOs were injected at the same concentration as the respective MO. All MOs were resolved in 0.2 M KCl to a final concentration of 50 μM and injections were performed into one-cell zebrafish embryos. For transgenesis, 0.2 ng reporter plasmid DNA and 0.15 ng Tol2 transposase mRNA was injected.

For rescue experiments, sense-capped mRNA of myc-tagged zebrafish *smyd1a* or *smyd1b*_tv1 was synthesized using the mMESSAGE mMACHINE system (Ambion). 3 ng of zebrafish *smyd1a* mRNA or *smyd1b*_tv1 mRNA was injected, respectively.

For CRISPR/Cas9 injections 400 ng/μl recombinant Cas9 protein (Eupheria GmbH, Germany) were mixed with synthetic tracrRNA (100 ng/μl) and a gene-specific crRNA (Eurofins Genomics, Germany) against *smyd1a* or *hsp90aa1*, respectively, at 50 ng/μl in 300 μM KCl. Sequences of CRISPR RNA oligonucleotides and morholinos are summarized in Table S1.

2.2. RNA extraction, cloning and quantitative real-time PCR

For RNA extraction 25 embryos were collected at 72 hpf for each sample. To extract the RNA the RNeasy® Mini Kit was used (Qiagen) according to the manufacturer's instructions. Reverse transcription was performed by using SuperScript® III Reverse Transcriptase (Life Technologies). A full length cDNA encoding zebrafish *smyd1a* and *smyd1b*_tv1 was amplified and cloned into pDONRzeo (Invitrogen). For transgenesis pDestTol2pA2 was used as backbone for multisite reaction with p3E-EGFPpA as 3′entry.

Quantitative real-time PCR was carried out according to standard protocols using SYBR-Green master mix (Roche) and a Roche LightCycler 480 II. cDNA was generated as described above from 72 hpf old embryos. To correct sample to sample variation housekeeping genes rpl13a and β-actin2 were used for normalization. All primer sequences are listed in Table S1.

2.3. Microscopy, in situ hybridization and immunostaining

Electron micrographs were obtained essentially as described previously [7]. The noninvasive birefringence analysis as well as the touch-evoke assays were carried out as described by Smith et al. [8]. For the spontaneous movement assay false-colored superimposed overviews of 24 hpf embryos were analyzed. Whole-mount RNA *in situ* hybridization was carried out essentially as described previously [6] using a full-length *smyd1b* antisense probe, as well as

antisense probes for zebrafish *smyd1a*, *vmhc*, *amhc*, *hsp90aa1* and *unc45b*. Whole mount fluorescent immunostainings were carried out as described in Inoue and Wittbrodt [9]. Fish were embedded in JB-4 (Polysciences) and 5 μm sections were cut. As antibodies we used polyclonal rabbit anti-GFP (Thermo Fisher Scientific), monoclonal mouse anti-α-actinin (Sigma Aldrich), monoclonal mouse F59 (DSHB), monoclonal mouse MF20 (DSHB) and monoclonal mouse S46 (DSHB).

2.4. Functional assessment and statistical analysis

Images were taken with an Olympus SZX 16 microscope and movies were recorded with a Leica DM IL LED microscope. The functional assessment of cardiac contractility was carried out as described [6].

For the touch evoke assay, embryos were touched by a needle tip and their flight response was analyzed. An immediate and straightforward flight was regarded as "adequate" while no, a delayed or incomplete response was regarded as "inadequate".

All results are expressed as mean ± standard deviation (S.D.) and statistical analysis were performed as indicated in the figure legends. A p-value of $P < .05$ was regarded as statistically significant.

3. Results

3.1. Smyd1a localizes to sarcomeric M-bands in zebrafish heart and fast-twitch skeletal muscle cells

In order to evaluate the expression pattern of *smyd1a*, we performed *smyd1a*-specific whole-mount *in situ* hybridizations (ISH) in zebrafish embryos. In line with a previous report [10], we found a strong and specific expression of *smyd1a* in skeletal muscle (Fig. 1A). Interestingly, our ISH analysis suggests *smyd1a* mRNA also in the embryonic zebrafish heart (Fig. 1A), which was confirmed by *smyd1a/b* specific RT-PCR analyses of heart samples (Fig. 1B). To evaluate whether *smyd1a*, similar to *smyd1b*, is differentially expressed in slow- and fast-twitch skeletal muscle cells, we assayed *smyd1a* RNA distribution within these two muscle compartments by ISH in combination with immunofluorescence staining against slow muscle myosin heavy chain. Interestingly, similar to *smyd1b* mRNA, *smyd1a* was restricted to fast-twitch skeletal muscle fibers and was absent in slow-twitch fibers (Fig. 1D). To further analyze subcellular localization of Smyd1a, we generated a transgenic line expressing *smyd1a-gfp* under control of the *unc45b^{min}* promoter that drives expression specifically in skeletal and cardiac muscle tissue (*Tg(unc45b^{min}:smyd1a-GFP)*) [11]. As reported before, expression of Smyd1b-GFP in the zebrafish line *Tg(unc45b^{min}:smyd1b-GFP)* revealed a striated pattern, which alternates with the Z-disc marker α-Actinin, demonstrating localization of Smyd1b to sarcomeric M-bands (Fig. 1E) [3], [11]. Interestingly, a clear alternating sarcomeric localization with α-Actinin was also visible for Smyd1a in our transgenic line *Tg(unc45b^{min}:smyd1b-GFP)* at 72 hpf (Fig. 1E), indicating an identical sub-cellular localization for both, Smyd1a and Smyd1b, at sarcomeric M-bands.

3.2. Smyd1a deficiency interferes with heart and skeletal muscle function in zebrafish embryos

Next, to assess the functional *in vivo* role of Smyd1a, we depleted Smyd1a levels by the injection of Morpholino-modified antisense oligonucleotides (Morpholinos; MO) in zebrafish embryos. We used two different MOs targeting either the translational start site of *smyd1a* (MO1-*smyd1a*) or the splice donor site of exon 4 (MO2-*smyd1a*), respectively, and used corresponding mismatch MOs as specific controls (Ctrl-MO1 and Ctrl-MO2). After 48 hpf,
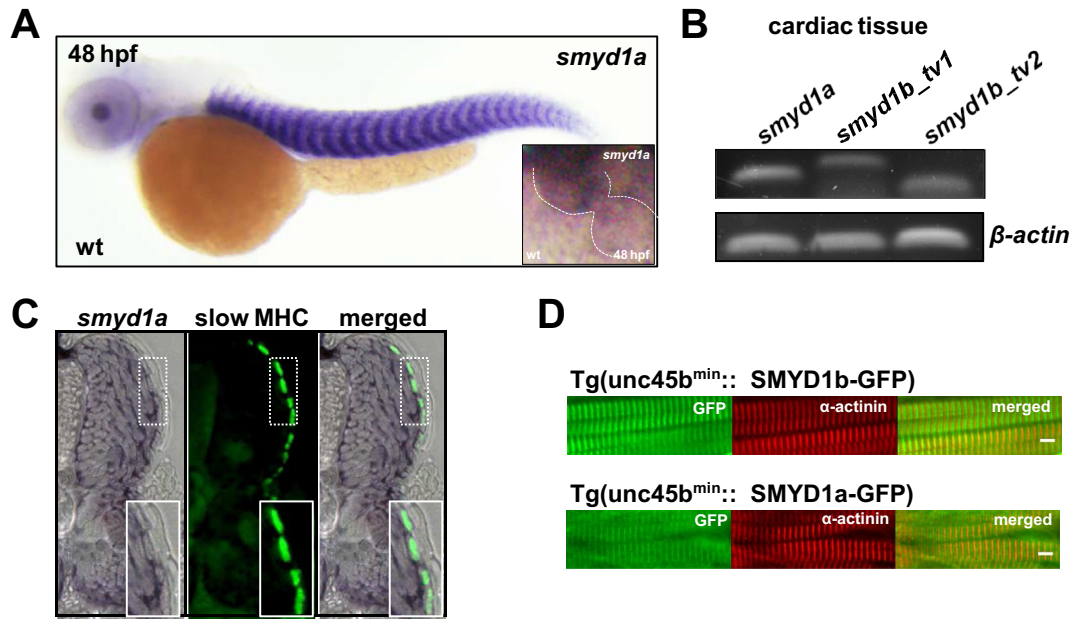
Fig. 1. Smyd1a is specifically expressed in the heart and fast-twitch skeletal muscle cells. (A, B) *smyd1a* mRNA is strongly expressed in somatic muscle cells and both heart chambers (inset) as revealed by ISH. (B) *Smyd1* specific RT-PCR confirms expression of all *smyd1* transcription variants in embryonic heart tissue. (C) *Smyd1a*-specific ISH and immunostaining of slow-twitch skeletal muscle fibers (slow MHC) in a transversal section of a zebrafish embryo. (D) Fluorescent imaging of transgenic lines carrying either a *smyd1b_tv1-gfp* fusion construct or *smyd1a-gfp* fusion construct under the muscle specific *unc45b^min*-promoter in combination with co-immunostaining against sarcomeric α-Actinin (Z-disc marker).

MO-*smyd1a* injected embryos started to develop pericardial edema (Fig. 2A, Suppl. Fig S1A) and motility defects became visible. Quantification of the morphant phenotype revealed that more than 70% of the MO1/MO2-*smyd1a*-injected zebrafish embryos showed pericardial edema formation (Suppl.Fig S1B), whereas control embryos were devoid of cardiac and skeletal muscle abnormalities. Additionally, we depleted Smyd1a by injecting a CRISPR/Cas9 ribonucleoprotein (RNP) complex, which resulted in CRISPR/Cas9-induced mutants (crispants) that resemble the *smyd1a* Morpholino knockdown phenotype (Suppl.Fig S1C). To further characterize cardiac dysfunction upon Smyd1a depletion, we measured ventricular fractional shortening (FS) of MO as well as Cas9-RNP injected embryos at 72 hpf. Smyd1a morphants and crispants showed a significantly reduced FS compared to control embryos (21 and 33% for morphants and crispants vs 66 and 73% for the respective controls) (Fig. 2B).

To proof the specificity of the splice morpholino, we co-injected MO2-*smyd1a* together with *smyd1a* mRNA or KCl (Suppl.Fig S1D). Strikingly, this co-injection strongly reduced heart failure and pericardial edema formation as well as the percentage of myopathic embryos (Suppl.Fig S2E). These data confirm the specificity and efficiency of the *smyd1a* knock-down leading to the observed striated muscle defects.

In addition to heart failure, depletion of Smyd1a also interferes with skeletal muscle function and thereby motility of injected embryos. During development, we found Smyd1a to be absent from slow-twitch skeletal muscle fibers, which are mainly used for spontaneous movement of zebrafish embryos at 24 hpf [12]. Congruously, Smyd1a depletion had no impact on the voluntary movement of embryos at 24 hpf, suggesting that slow-twitch skeletal muscle fibers develop and function independent of Smyd1a (Suppl.Fig S1F and Suppl. Fig S1G). By contrast, zebrafish embryos at 48 hpf are usually able to react to mechanical stimuli with a burst swimming mechanism that requires fast-twitch skeletal muscle contractions [12]. The mechanical stimulation of the

tail usually results in a fast and straightforward flight response of the embryo (Video S1). Using a touch-evoke response assay we found that Smyd1a depletion led to no or inadequate mobility at 48 hpf (Fig. 2C, Video S2). While over 85% of control embryos exhibited an adequate flight response only 10—35% of Smyd1a-deficient embryos were able to correctly respond to mechanical stimuli, demonstrating that Smyd1a is essential for regular fast-twitch skeletal muscle function. To investigate if impaired motility in Smyd1a morphants/crispants results from structural perturbations within skeletal muscle fibers, we next analyzed muscle birefringence in Smyd1a depleted embryos and found significantly reduced birefringence signals in muscles upon Smyd1a knockdown at 48 hpf (Fig. 2D and Suppl. Fig S2A), implying disrupted myofiber organization in Smyd1a-depleted skeletal muscles cells.

Supplementary video related to this article can be found at https://doi.org/10.1016/j.bbrc.2018.01.060.

Next, to investigate fast-twitch skeletal muscle fiber organization in Smyd1a-deficient zebrafish embryos in more detail, we performed transmission electron microscopy (TEM). TEM analysis revealed highly organized thick and thin myofilaments, interconnected by sarcomeric Z-discs in Ctrl-MO-injected zebrafish, whereas skeletal muscle cells of Smyd1a-depleted embryos showed disorganized sarcomeres (Fig. 2E). This effect was accompanied by a highly disorganized myosin array in morphant embryos (Suppl.Fig S2B). In summary, these findings suggest a functional role of Smyd1a in fast-twitch skeletal muscle fibers by contributing to muscle fiber integrity.

3.3. Ectopic expression of Smyd1a rescues Smyd1b-deficiency in fla mutant embryos

To evaluate whether transient, ectopic expression of Smyd1a is capable to reconstitute heart and skeletal muscle function in Smyd1b-deficient *fla* mutant zebrafish embryos, we injected *unc45b^min:smyd1a-gfp* DNA constructs as well as
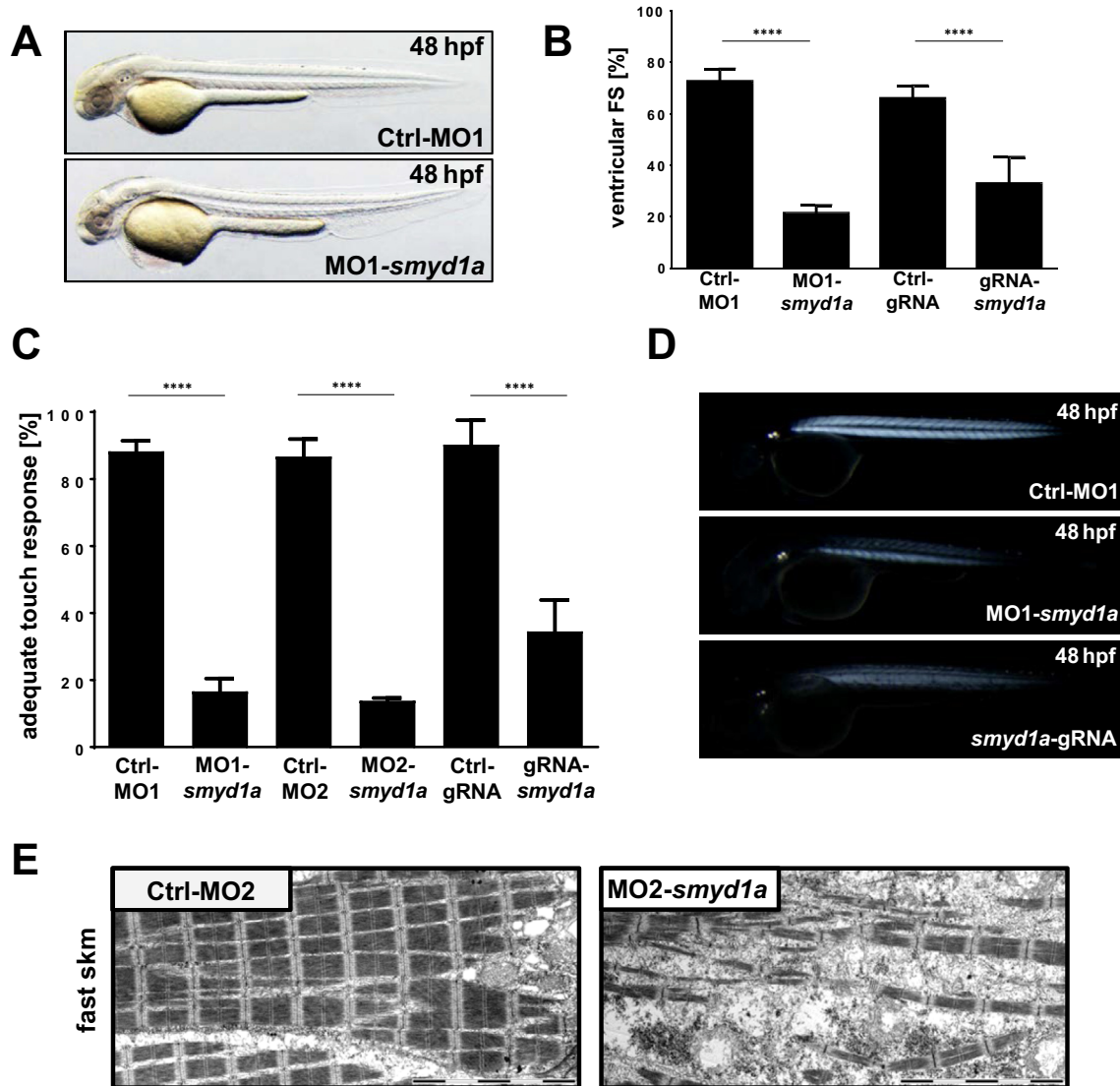
Fig. 2. Knock-down of smyd1a leads to defective skeletal muscle and heart function. (A) Zebrafish embryos at 48 hpf injected either with an morpholino against *smyd1a* (MO1-*smyd1a*) or the respective control (Ctrl-MO1). (B) Quantification of ventricular FS in % (n = 6) from A and B at 72 hpf. Shown are mean values of three independent experiments with error bars representing standard deviation (SD). Statistical analysis was performed using one-way ANOVA followed by tukey's multiple comparison analysis (****p < .0001). (C) Quantification of the touch-evoke response assay. *Smyd1a* morphants were analyzed in regard to their adequate touch responsiveness upon mechanical stimulus. Shown are mean values (+/− SD) in percent of three independent experiments. Statistical analysis was performed as in (B) (C) Birefringence images of embryos (48 hpf) injected with Ctrl-MO1, MO1-*smyd1a* or Cas9 + *smyd1a*-gRNA ribonucleoprotein (RNP) complex. (E) Electron micrographs of skeletal muscle cells of *smyd1a* morphants versus control embryos at 48 hpf.

unc45b^{min}:smyd1b-gfp constructs as positive control into homozygous *fla* mutant embryos. At 72 hpf, we analyzed GFP-fluorescence in skeletal muscle cells in combination with immunostainings against α-Actinin (Fig. 3A). As expected, ectopic expression of Smyd1b-GFP led to the restoration of sarcomeric structures in fast-twitch skeletal muscle fibers, whereas adjacent GFP-negative muscle cells still displayed disorganized sarcomeres (Fig. 3A). Interestingly, by expressing Smyd1a-GFP in *fla* mutant muscle cells, we were also able to efficiently and cell-autonomously rescue sarcomeric structures of single fast-twitch muscle cells (Fig. 3A).

Next, to analyze whether strong and homogeneous-systemic over-expression of Smyd1a is able to restore heart and skeletal muscle function in *fla* mutant embryos, we microinjected *smyd1a* mRNA. By the injection of *smyd1b* mRNA as a positive control, 88.60% of homozygous *fla* mutant embryos exhibited restored skeletal muscle function as well as a rigorously beating heart (Fig. 3B). Strikingly, we found that injection of *smyd1a* mRNA in *fla*

mutant embryos also efficiently rescued the *fla* phenotype with 79.97% (Fig. 3B). Measuring ventricular FS of *smyd1a* and *smyd1b* mRNA injected *fla* mutants showed a clear reconstitution of cardiac contractility compared to control-injected embryos (Fig. 3C). These data demonstrate that ectopic expression of Smyd1a is capable of compensating for the loss of Smyd1b function *in vivo*. Interestingly, although ectopic expression of Smyd1a is able to compensate for the loss of Smyd1b function, Smyd1a was not found to be transcriptionally induced in homozygous *fla* mutant embryos as revealed by *in situ* hybridization against *smyd1a* and supported by qPCR analysis (Fig. 3D and E). These data suggest the lack of genetic compensation in *fla* and explain the severity of the striated muscle defects in *fla* mutant embryos.

3.4. Loss of Smyd1a does not trigger the misfolded myosin response

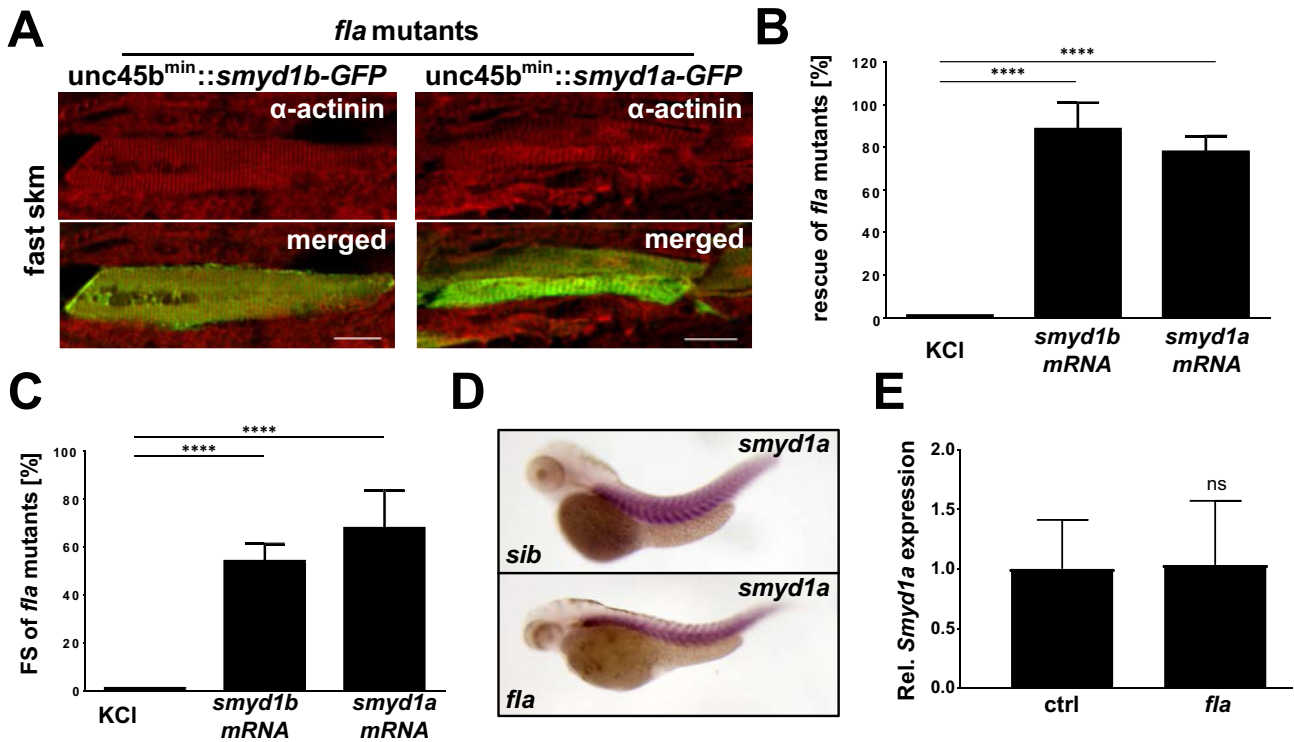Loss of Smyd1b function leads to defective myosin folding and

Fig. 3. Smyd1a is required for proper sarcomere organization and can compensate for the loss of Smyd1b. (A) Co-immunostaining of plasmid (*unc45b^min:smyd1b_tv1-gfp*- or with *unc45b^min:smyd1a-gfp*) injected *fla* mutants with sarcomeric α-Actinin. [Scale bar: 20 μm]. (B) *Flatline* embryos were injected with either KCl (control), *smyd1b_tv1* or *smyd1a* mRNA. At 72 hpf *flatline* mutants were identified and evaluated in regard to restored cardiac contractility. Shown are the mean percentages (+/− SD) of rescued *fla* embryos (n = 80) of four independent experiments. Statistical analysis was performed using one-way ANOVA (****p < .0001). (C) Measurement and statistical analysis as in (B) of ventricular FS of rescued *fla* mutants by injection of *smyd1a*- or *smyd1b*-mRNA at 72 hpf. Data are mean values ± SD. (D) *Smyd1a* specific ISH in *fla* mutants compared to control siblings (sib). (E) qRT-PCR analysis of *smyd1a* expression in control versus *fla* embryos.

assembly and consequently its degradation [3]. Defective myofibril formation due to impaired myosin folding and assembly results in a response that was described as the misfolded myosin response (MMR) [4]. MMR is characterized by the transcriptional up-regulation of the myosin chaperones Hsp90α1 and Unc45b as well as Smyd1b (Suppl.Fig S2C) [3], [4].

To define whether Smyd1a, similar to Smyd1b, is also part of the complex transcriptional response induced by misfolded myosin, we first analyzed the expression of Smyd1a in Hsp90α1-depleted crispants or in the *unc45b* mutant *steif*. In contrast to *smyd1b*, *smyd1a* transcription was neither induced in *hsp90aa1* crispants (Fig. 4A) nor in *steif* (Fig. 4B) as well as *flatline* as shown before (Fig. 3D), suggesting that *smyd1a* expression is not regulated in response to misfolded myosin. Vice versa, to prove whether loss of Smyd1a results in MMR, we evaluated the expression of *smyd1b*, *unc45b* and *hsp90aa1* in Smyd1a-deficient embryos. We found that all three MMR-responsive genes, *smyd1b*, *unc45b* and *hsp90aa1*, were not induced upon *smyd1a* knockdown (Fig. 4C). These findings demonstrate that, in contrast to Smyd1b, Smyd1a is not involved in the transcriptional response associated with misfolded myosin and that loss of Smyd1a does not trigger the MMR.

Finally, we evaluated whether ectopic expression of Smyd1a suppresses MMR in homozygous *fla* mutant zebrafish embryos. Similar to the microinjection of *smyd1b* mRNA into *fla* mutant embryos, ectopic Smyd1a expression efficiently represses the transcriptional response caused by misfolded myosin. *Unc45b* as well as *hsp90aa1* expression was normalized by the injection of both, Smyd1a and Smyd1b, whereas injection of KCl into homozygous *fla* mutant zebrafish embryos did not suppress the MMR (Fig. 4D).

4. Discussion

A precise and adequate spatiotemporal regulation of muscle protein homeostasis is required for the regular function of heart and skeletal muscles. One important sarcomeric protein involved in sarcomerogenesis is Smyd1. In mice, a global Smyd1 knock-out results in severe developmental heart defects leading to embryonic lethality [13]. Additionally, conditional Smyd1 deficiency in skeletal myocytes led to severe muscle weakness, myofiber hypotrophy and myofibrillar disarray, substantiating the essential role of Smyd1 in skeletal muscle cells [14]. Due to a genome duplication event, the zebrafish has two Smyd1 genes and one of the two paralogues, Smyd1b, was shown to be crucial for heart and fast-twitch muscle structure and function [3], [5]. In the present work, we investigated the functional role of the second zebrafish *smyd1* gene, *smyd1a*, and found that targeted inactivation of *smyd1a* also interfered with the structured organization of sarcomeres thus influencing heart and skeletal muscle function. Additionally, although ectopic expression of Smyd1a was found to be capable of compensating for the loss of Smyd1b, genetic compensation by the *in vivo* transcriptional induction of Smyd1a is completely lacking in Smyd1b-depleted embryos.

Gao and coworkers found zebrafish *smyd1a* expressed in skeletal muscle cells and report a rather cytoplasmic localization in skeletal muscle cells [10]. Interestingly, we found here that although weakly expressed, Smyd1a, similar to zebrafish Smyd1b, was also present in the heart. Additionally, we were able to demonstrate that *smyd1a* was restricted to fast-twitch skeletal muscle fibers in zebrafish. These findings clearly demonstrate that both zebrafish Smyd1 paralogues, Smyd1a and Smyd1b, are co-
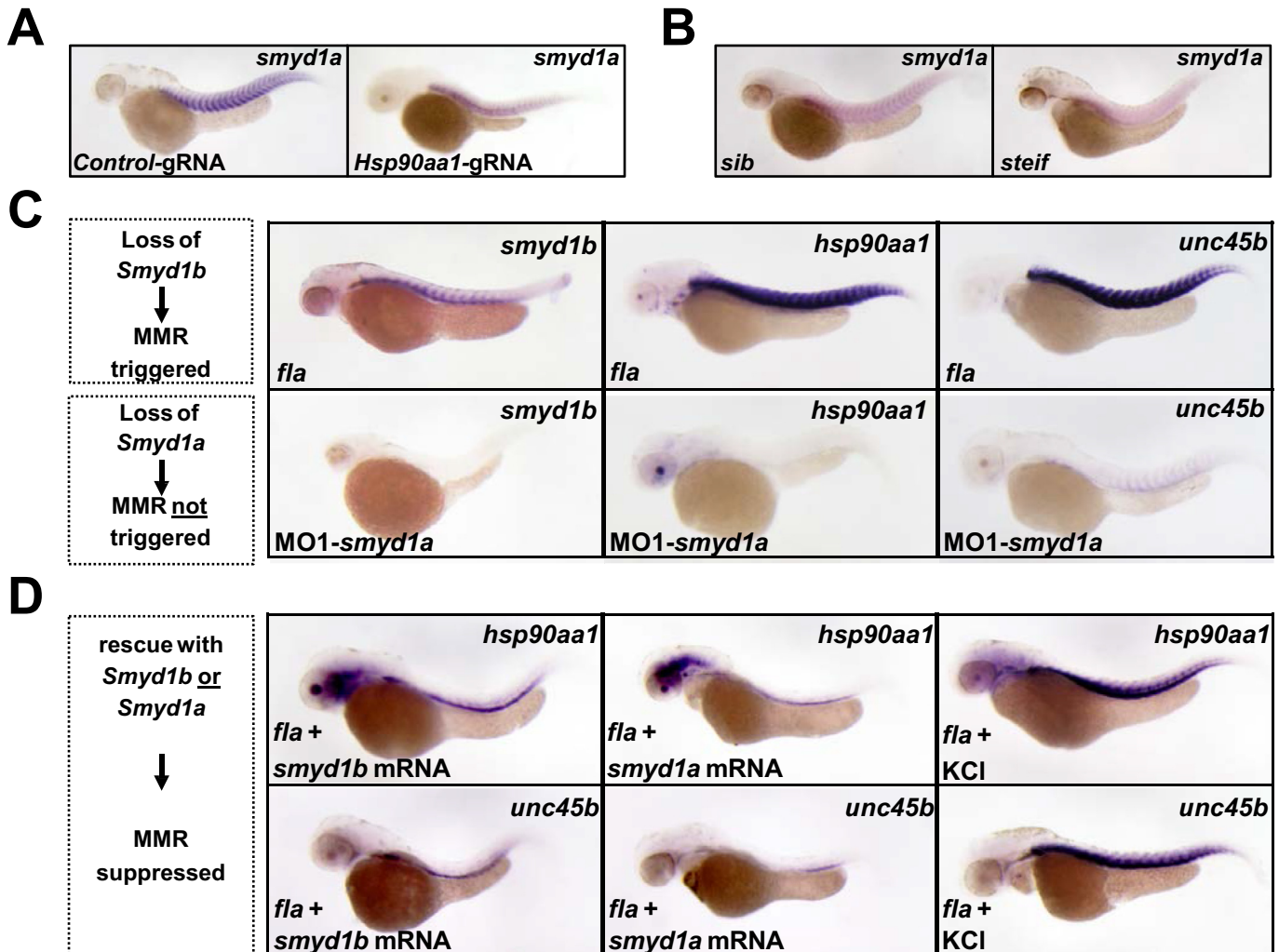
Fig. 4. Loss of Smyd1a does not trigger the misfolded myosin response. (A) ISH analysis of *smyd1a* expression in *unc45b* mutant embryos (*steif*) or embryos injected with a Cas9-protein/*hsp90aa1*-gRNA complex, respectively. (B) mRNA levels of the muscle chaperones *unc45b* and *hsp90aa1* are strongly upregulated in muscle cells of *fla* mutants, whereas their expression is not affected by loss of *smyd1a*. (C) Rescue of *fla* mutants by injection of either *smyd1a*- or *smyd1b*-mRNA.

expressed in the heart and fast-twitch skeletal muscle cells. In fact, similar expression pattern of *smyd1a* was also observed for other teleost fish such as the Chinese Perch [15]. The transgenic expression of a Smyd1a-GFP fusion protein in zebrafish embryos using the established skeletal and cardiac muscle tissue specific *unc45b* promoter [11] revealed a clear sarcomeric pattern in striated muscle tissue, which alternates with α-Actinin, suggesting Smyd1a localizing to the sarcomeric M-band *in vivo* similar to Smyd1b. The recruitment of Smyd1b to the M-line was shown to be highly dependent on Phe223 and Ser225 within its sequence [16]. These residues are also present in the amino acid sequence of Smyd1a, suggesting a similar mechanism for its recruitment to the sarcomeric M-line.

By using MO- and CRISPR/Cas9-mediated knockdown of Smyd1a in zebrafish embryos, we found Smyd1a to have a functional role in both, fast-twitch skeletal and cardiac muscle. Loss of Smyd1a led to an impaired sarcomeric organization resulting in decreased heart contractility and a defective touch-evoke response. The fact that this touch-evoke response is dependent on fast-twitch muscle function and that *smyd1a* expression was only observed in this muscle fiber type suggests a functional role of Smyd1a in the function of this type of muscle fibers.

To assess whether Smyd1a is able to take-over Smyd1b functions, we performed rescue experiments in Symd1b-deficient *fla* mutant zebrafish embryos. Indeed, ectopic expression of *smyd1a* by mRNA injection restored the *fla* heart and skeletal muscle phenotype to a similar extent as *smyd1b* mRNA injection. Very recently, genetic compensation was reported in zebrafish as an efficient mechanism to protect an organism against otherwise deleterious loss of gene functions [17]. Interestingly, in *smyd1b*-deficient *fla* mutant embryos *smyd1a* was not found to be upregulated, demonstrating that genetic compensation is absent in *fla* although Smyd1a principally is capable of functionally compensating for the loss of Smyd1b.

Myosin folding and assembly is mediated by auxiliary proteins such as Unc45b, Hsp90α1 or Smyd1b and loss of any of these proteins interferes with Myosin assembly leading to the paralysis of zebrafish embryos [1] [2], [3]. Additionally, loss-of-function of any of these myosin chaperoning proteins and thereby defective myosin folding and assembly cause their own transcriptional induction, a molecular process named misfolded myosin response (MMR) [4]. Remarkably, for Smyd1a such an intrinsic upregulation was not observed in Smyd1b-deficient, Unc45b-deficient or Hsp90α1-deficient zebrafish, suggesting that Smyd1a is not part of

the transcriptional cascade during the MMR. Vice versa, Smyd1a depletion was not sufficient to induce MMR, implying that Smyd1a is not essential for myosin folding and assembly or that Smyd1b in Smyd1a-deficient embryos resumes important biological functions. Further studies will be necessary to investigate the functional and physiological relevance of this discrepancy. It might be possible that Smyd1a expression is controlled via different mechanisms, for example mechanical overload or hypoxia. In this context, it is already known that Smyd1, together with its interaction partner skNAC (muscle-restricted isoform of the DNA binding protein nascent polypeptide-associated complex) has a crucial role in the response to oxidative and ER stress in the developing mammalian heart [18]. Thus, in zebrafish it might be possible that Smyd1a exhibits the main PKMT activity whereas Smyd1b is mainly responsible for sarcomere organization. This hypothesis is in line with the fact, that *fla* mutants can be rescued with a PKMT-deficient version of Smyd1b, but not a variant that cannot bind to myosin [3]. Further analysis of Smyd1a versus Smyd1b expression under different developmental stages and environmental conditions will contribute in more detail to the understanding of common and distinct features of these Smyd1 paralogues in zebrafish. These investigations will further help to clarify also the mode of action of mammalian Smyd1 during myofibril organization and muscle function.

Acknowledgement

Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.bbrc.2018.01.060.

Transparency document

Transparency document related to this article can be found online at https://doi.org/10.1016/j.bbrc.2018.01.060.

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