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Defective sarcomere organization and reduced larval locomotion and fish survival in slow muscle heavy chain 1 (*smyhc1*) mutants

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Abstract

Zebrafish skeletal muscles are broadly divided into slow-twitch and fast-twitch muscle fibers. The slow fibers, which express a slow fiber specific Myosin heavy chain 1 (Smyhc1), are the first group of muscle fibers formed during myogenesis. To uncover Smyhc1 function in muscle growth, we generated three mutant alleles with reading frame shift mutations in the zebrafish *smyhc1* gene using CRISPR. The mutants showed shortened sarcomeres with no thick filaments and M-lines in slow fibers of the mutant embryos. However, the formation of slow muscle precursors and expression of other slow muscle genes were not affected and fast muscles appeared normal. The *smyhc1* mutant embryos and larvae showed reduced locomotion and food intake. The mutant larvae exhibited increased lethality of incomplete penetrance. Approximately 2/5 of the homozygous mutants were viable and grew into reproductive adults. These adult mutants displayed a typical pattern of slow and fast muscle fiber distribution, and regained normal slow muscle formation. Together, our studies indicate that Smyhc1 is essential for myogenesis in embryonic slow muscles, and loss of Smyhc1 results in defective sarcomere assembly, reduces larval motility and fish survival, but has no visible impact on muscle growth in juvenile and adult zebrafish that escape the larval lethality.

Keywords

smyhc1; knockout; sarcomere; slow fibers; zebrafish

Introduction

Skeletal muscles are broadly divided into two general types of muscle fibers, namely the slow-twitch and fast-twitch muscles, based on their physiological characteristics and the fiber type specific expression of *myosin heavy chain* (*MyHC*) genes. MyHC is the major determinant of contractile properties in slow and fast muscles. It has been demonstrated that

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Author Contributions

S. Li and S. Du designed research; S. Li performed research; S. Li, H. Wen and S. Du analyzed data; S. Li and S. Du wrote the paper.

different isoforms of *MyHC* genes are expressed in various muscle fiber types (1–3). In mammals, such as mice and human, slow-twitch fibers generally express MyHC-I, and fast-twitch fibers express any of the various MyHC-II isoforms (IIa, IIb, IId/x) (4, 5). In addition, there are two developmental isoforms called MyHC-embryonic and MyHC-neonatal/perinatal/fetal that are expressed during muscle development at the embryonic and neonatal stages, and transiently reactivated in adult muscles during regeneration (6–8). The functional diversity among these skeletal muscle MyHC isoforms directly contribute to the fiber type and physiological activity of various muscle types (9).

As one of most important skeletal myofibrillar proteins, myosin molecules form bipolar thick filaments which move along the thin filaments to produce contraction forces in sarcomeres, the basic contractile units in skeletal and cardiac muscle fibers (10, 11). Sarcomeres are composed of the myosin thick filaments and actin thin filaments, as well as the M- and Z-lines. The M-lines cross-link and stabilize the central portion of myosin filaments, and the Z-lines anchor the thin filaments (12–14). Although much is known about the components of sarcomeres and their structure and function, the molecular regulation of sarcomere assembly has not been well studied to date and therefore still remains enigmatic (13, 15–17).

Several models have been proposed to explain the process of sarcomere assembly (18–23). The ‘Stitching Model’ proposes that parts of a sarcomere, namely the I-Z-I complexes and bipolar myosin filaments are assembled independently and then brought together during sarcomere assembly (19). The ‘Pre-Myofibril Model’, also known as the ‘Templating Model’, argues that stress fiber-like structures containing both non-muscle and sarcomeric proteins serve as a template for the formation of sarcomeres (13, 20). This model proposes that a replacement of the non-muscle myosin filaments with muscle myosin filaments occurs during the transition from premyofibril to myofibril. A third model highlights the scaffolding role of titin that spans half the length of a sarcomere (21, 22). Titin binds to the Z-disc region with its N-terminal domain and provides anchoring at the M-line with its C-terminal region, thus asserting the interdigitation of the I-Z-I complex and myosin. Some recent studies indicate that the above different models may not be mutually exclusive, leading to the proposal of a unified model of sarcomere assembly based on the formation of muscle stress fibers and their subsequent transition into sarcomere-containing myofibrils (23).

Genetic analysis reveals that MyHC plays important roles in muscle development, growth and function. Targeted inactivation of the adult fast skeletal MyHC-IIb and IId/x genes in mice impaired animal growth and muscle contraction (24). A recent study demonstrated that MyHC-embryonic was a crucial regulator of skeletal muscle development and differentiation (25). Genetic mutations in MyHC genes have been linked to several forms of congenital myopathies in humans, such as the Trismus-pseudocamptodactyly congenital contracture syndrome caused by mutations in MyHC 8 gene, and the Freeman-Sheldon and Sheldon-Hall congenital contracture syndromes associated with genetic mutations in embryonic MyHC (MyHC3) (26–30).

Zebrafish embryos with topographically distinct slow and fast muscles fibers provide a powerful model for developmental and genetic studies of muscle development (31). Our

previous studies reported that knockdown of slow *myosin heavy chain 1* (*smyhc1*) resulted in defective sarcomere organization in zebrafish embryonic slow muscles (32, 33). However, the functional importance of *smyhc1* in fish muscle growth has not been investigated due to the lack of a *smyhc1* knockout mutant model.

To investigate the long term effect from loss of *smyhc1* in muscle development and growth, we decided to knock out the *smyhc1* gene in zebrafish. We reported here the generation of three *smyhc1* mutant alleles using CRISPR. The zebrafish *smyhc1* mutants showed a disrupted sarcomere organization with shorter sarcomere length specifically in embryonic slow muscle fibers. The mutant larvae showed reduced locomotion and increased early larval lethality of incomplete penetrance. Approximately 2/5 of the homozygous mutants were viable and grew into mature reproductive adults. Histological analysis demonstrated that the viable mutants had a typical pattern of slow and fast muscle fiber distribution and normal body weight in juvenile and adult fish. Our study indicates that *Smyhc1* is essential for myogenesis in embryonic slow muscles. Loss of *smyhc1* results in reduced motility and survival of zebrafish embryos and larvae. However, loss of *smyhc1* has no visible effect on muscle growth in juvenile and adult fish that escape the early larval lethality.

Materials and methods

Zebrafish lines and maintenance

Zebrafish were raised in the Zebrafish Facility at Institute of Marine and Environmental Technology, University of Maryland Baltimore. The zebrafish were maintained at 28°C with a photoperiod of 14h light and 10h dark. The *myom3^{annGt0067}* zebrafish line was obtained from Steve Ekker's laboratory at the Mayo Clinic (USA) which carried an insertion of red fluorescent protein gene (RFP) in the *myomesin-3* (*myom3*) gene (33, 34). Additional information can be found at <http://www.zfishbook.org/index.php?topic=GBT0067#>. All animal studies were carried out according to the guideline 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: 0516005).

DNA plasmids and construction of pTol2- α -actin: *smyhc1* transgene

The *p9.7smyhc1:gfp-I-SceI* plasmid was obtained from Dr. Stone Elworthy at the University of Sheffield (UK) (35). It contained a 9.7kb upstream sequence of zebrafish *smyhc1* gene linked with the EGFP coding sequence. The *pTol2- α -actin-EGFP* plasmid was constructed by replacing the *elongation factor 1 alpha* (*ef-1 α*) promoter in a modified pT2AL200R150G-NotI* vector (36 and Du et al., unpublished) using the muscle-specific zebrafish α -actin promoter (37).

The *pTol2- α -actin:smyhc1* transgene was constructed by cloning the full-length coding sequence of zebrafish *smyhc1* after the muscle-specific α -actin promoter. Briefly, the *pTol2- α -actin-EGFP* vector was digested with EcoR I and Not I to remove the EGFP coding sequence. The full-length coding sequence of *smyhc1* cDNA was isolated by RT-PCR using Pfu DNA polymerase and *smyhc1*-F1 and *smyhc1*-R1 in-fusion primers (Table 1). The PCR

product was cloned into the EcoR I and Not I site of the *pTol2- α -actin-EGFP* vector using the In-Fusion Cloning Kit (Takara Bio USA, Inc.). The *pTol2- α -actin:smyhc1* construct was confirmed by DNA sequencing.

Design of smyhc1 guiding RNA (sgRNA), co-injection with Cas9 and genotyping

The *smyhc1-sgRNA*, targeted to *smyhc1* exon 5 (Table 1), was synthesized *in vitro* as described (38). The *smyhc1-sgRNA* was combined with the Cas9 protein (Cat#B25640; ThermoFisher) at a final concentration of 25ng/ μ L and 250ng/ μ L, respectively. Approximately 2 nl of *smyhc1-sgRNA*/Cas9 mixture was microinjected into fertilized *myom3^{mnGt0067}* zebrafish embryos at the 1 or 2 cell stage.

For genotyping, genomic DNA was isolated from whole zebrafish embryos or the head region at 48 hpf, or fin clip from juvenile and adult zebrafish, by using the alkaline method (38). A 270 bp DNA fragment covering the *smyhc1-sgRNA* target site region was amplified with PCR using *smyhc1*-P1 and *smyhc1*-P2 primers (Table 1) and used for DNA sequencing and genotyping. The PCR reaction was carried out in a 25 μ L reaction using GoTaq® DNA polymerase (Promega).

Morpholinos

The Morpholino antisense oligo against zebrafish *smyhc1* was synthesized by Gene Tools (Corvallis, OR). The *smyhc1* translation blocker (ATG-MO, Table 1) was designed to target the sequence flanking the ATG start site (32). The standard control-MO was purchased from Gene Tools. The morpholino antisense oligos were dissolved with 1 $\times$ Danieau buffer (39) to a final concentration of 0.5mM. Zebrafish embryos at the 1 or 2 cell stage were injected with approximately 2 nl (10 ng) of the ATG-MO.

smyhc1 ATG-MO: 5'-TCTAAAGTTTTACCCACTGCGGCAA-3'

Quantitative real-time PCR

Total RNA was extracted from zebrafish embryos, larvae or juveniles of wild type (WT), *smyhc1^{mb17}* or *smyhc1^{mb18}* mutants at 24 hpf (hours-post-fertilization), 3 dpf (days-post-fertilization), 10 dpf, 20 dpf, and 30 dpf using TRIzol reagent (Invitrogen). Genomic DNA was eliminated by DNase I digestion. cDNA synthesis was carried out using the First-Strand cDNA synthesis kit (ThermoFisher Scientific, K1621). Three 10-fold serial dilutions were applied to produce the standard curves of cDNA samples. PCR reaction was carried out using the standard SYBR Green PCR Master Mix (Applied Biosystems) with 1 μ L of 100x diluted cDNA template on a 7500 Fast Real-Time PCR System (Applied Biosystems). Each sample was performed in triplicate for PCR. Expression of the elongation factor 1 α (*ef-1 α*) was used as an internal control. The $2^{-\Delta\Delta CT}$ method (40) was calculated to analyze the relative levels of *smyhc1*, *smyhc2*, *smyhc3* and *myhz2* expression using the *ef-1 α* for normalization. qPCR primers were designed at the junctions of two adjacent exons to eliminate potential problems with genomic DNA contamination (Table 1). All results were expressed as mean $\pm$ SEM. Differences between WT and mutants were analyzed using a student's t-test., with aP-value of < 0.05 as the level of significance.

Whole-Mount in Situ Hybridization

The whole mount *in situ* hybridization was carried out using digoxigenin-labeled antisense probes as previously described (41). Antisense probes against zebrafish slow myosin heavy chain 1 (*smyhc1*) and fast myosin heavy chain 2 (*myhz2*) were synthesized by Sp6 RNA polymerase from Nco I or Sph I linearized *pGEM-smyhc1* or *pGEM-myhz2* plasmid, respectively (42). The *pGEM-troponin C* plasmid was digested with Spe I and transcribed with T7 RNA polymerase to synthesize the slow-specific antisense probe (32). Antisense probes of *smyhc2*, *smyhc3*, *myh6*, and *myh7* were synthesized using PCR fragments as templates. The gene specific PCR fragments were generated using specific primers (Table 1), to amplify the targeted fragments, and transcribed with T7 RNA polymerase.

Whole Mount Immunostaining

Immunostaining was carried out using whole-mount zebrafish embryos and larvae as previously described (42–44). Briefly, zebrafish embryos were anaesthetized in 0.6 mM Tricaine (pH 7.0), and then fixed in 4% paraformaldehyde in PBS for 1 hour at room temperature. The zebrafish embryos were washed in PBST for 3×15 min and then digested with 1 mg/mL collagenase for various times depending on the age of the embryos (28 hpf for 3 min, 30 hpf for 6min); or 1.5 mg/mL collagenase (48 hpf for 40 min, 72 hpf for 50min); or 2 mg/mL Proteinase K (5 dpf for 40 min, 8 dpf for 50 min), respectively. Immunostaining was performed with the following primary antibodies: anti-MyHC for slow muscles (F59 and S58, DSHB), anti-MyLC (F310, DSHB) and anti-myomesin (mMaC myomesin B4, DSHB) for fast muscles, anti-MyHC (MF20, DSHB), anti- α -actinin (clone EA-53, #A7811, Sigma, USA) for both slow and fast fibers, or anti-MyHC (S46, DSHB) for atrium of the heart. Secondary antibodies were Alexa Fluor® 488- or Alexa Fluor 555-conjugated anti-IgG secondary antibodies (A11001 and A31630, ThermoFisher Scientific). Phalloidin-TRITC (Sigma P1951, 50ng/ml in PBST) was used to stain the thin filament in slow and fast muscle fibers, and anti-Prox I antibody (MAB5654, Millipore) was used to label the nuclei of slow fibers in zebrafish embryos. After immunostaining, trunk regions were dissected from the zebrafish embryos and mounted in Vectashield (H-1000, Vector lab) for confocal microscopy (Leica SP8).

Cryostat Sectioning and Immunostaining

During immunostaining, cross sections (15 μ m thickness) of trunk muscle were generated by cryostat from WT and mutated zebrafish at 10, 30, 70, and 100 dpf. The sections were placed on slides (#1358P, Globe Scientific) and dried completely at room temperature. The tissue sections were circled with a hydrophobic pen (ImmEdge pen, Vector # H-4000), and then washed for 10 min with PBST (1x PBS with 0.4% Triton). The tissue sections were blocked with PBST containing 10% goat serum at room temperature for 40 minutes, and then incubated with the respective primary antibody overnight at 4°C. The slides were washed in PBST for 3 20 mins at room temperature, and then incubated for 1 hr with the Alexa Fluor® 488- or Alexa Fluor 555-conjugated anti-IgG secondary antibodies (A11001 and A31630, ThermoFisher Scientific). After washing with PBST for 3 × 20 mins, the tissue sections were covered with VECTASHIELD® Mounting Medium (H-1000, Vector Lab) and photographed under a Leica SP8 confocal microscope.

Morphological and histological analyses

Trunk regions were carefully dissected from 1.5-month-old zebrafish, which were anesthetized in 0.6 mM Tricaine solution (pH 7.0). The trunk tissue were fixed in Bouin's fixative buffer (#1120, RICCA) overnight at 4 °C. The samples were dehydrated with an alcohol gradient and embedded in paraffin. Afterwards, the samples were sectioned at a 5–7 µm thickness, and stained with Harris hematoxylin (HHS16, Sigma) and eosin (E511, Fisher Scientific) for photographing under a light microscope.

Larval locomotion analysis

The *smyhc^{mb18/+}* heterozygous mutant were in-crossed to generate the F2 offspring. Twenty four larvae were randomly selected from the F2 offspring and placed individually in each well of a 24-well plate. The fish larvae were kept at 28°C with a photoperiod of 14h light and 10h dark. The locomotion of each larvae was simultaneously tracked for 24 hrs with the DanioVision (Noldus Information Technology). The locomotion assays were performed on zebrafish larvae at 3, 7 and 8 dpf using a different batch of embryos each time. The fish larvae were sacrificed after the locomotion assay and genotyped individually by PCR and sequencing. Their average swimming distance was calculated for 24 hrs. All the results were expressed as mean ± SEM. The significance of mean swimming distance between WT, *smyhc^{mb18/+}*, and *smyhc^{mb18}* was analyzed using a student's t-test. A P value of < 0.05 was considered as the level of significance.

Histological analysis using transmission electron microscopy

Zebrafish embryos, larvae and juvenile were fixed in a 2% paraformaldehyde, 2.5% glutaraldehyde, 0.1 M PIPES buffer (pH 7.4) solution overnight at 4 °C. During histological analysis, the fixed samples were rinsed with 0.1 M PIPES and post-fixed in 1% osmium tetroxide and 1.5% potassium ferrocyanide in 0.1M PIPES buffer for 1 hour at 4 °C using a transmission electron microscopy (TEM). Specimen were then treated with 1% tannic acid in water for 15 min, followed by en bloc staining with 1% (w/v) uranyl acetate, and dehydration using 30%, 50%, 70%, 90%, 100% ethanol and 100% acetone in series. After dehydration, specimen were infiltrated and embedded in Araldite-Epoxy resin (Araldite, EMBED 812; Electron Microscopy Sciences, PA), following manufacturer's recommendations. Ultra-thin sections of ~70nm thickness were cut on a Leica UC6 ultramicrotome (Leica Microsystems, Inc., Bannockburn, IL), and examined in a FEI Tecnai T12 transmission electron microscope operated at 80 kV. Digital images were acquired by using an AMT bottom mount CCD camera and AMT600 software.

Sarcomere length measurement

The sarcomere length was measured on the EM photographs of slow and fast fibers using the Photoshop program. 20 sarcomeres each were randomly selected in slow and fast fibers of *smyhc1* mutant and WT embryos, respectively at 3 dpf. Statistical analysis by student's t-test was performed to compare the sarcomere length in slow fibers and fast myofibers in *smyhc1* mutant and WT embryos. The P value of < 0.05 was considered as the level of significance.

Analysis of food intake using fluorescence-labeled paramecia

Paramecia were collected by centrifugation at 3,000 rpm for 5 min and quantified by optical density measurement at OD500 nm as described (45). The paramecia were diluted to a density of 1000 paramecia per ml and incubated with 4-(4-(didecylamino)styryl)-*N*-methylpyridinium iodide (4-Di-10-ASP) at 25 µg/ml for 1 hr (Invitrogen, Carlsbad, CA, USA). The paramecia were then washed with water three times for 10 min each time to remove the residual 4-Di-10-ASP. Fish larvae were fed with 4-Di-10-ASP labeled or unlabeled (control) paramecia at 1000 paramecia/ml for two hours. The fish larvae were photographed immediately using a GFP2 filter on a MZ16F stereoscopic microscope (Leica Microsystems). The images were analyzed using Image J software to get the fluorescence intensity.

Results:

1. Generation of zebrafish *smyhc1* mutants by CRISPR

Our previous studies demonstrated that the knockdown of *smyhc1* by Morpholino disrupted myosin thick filament organization in zebrafish embryonic slow muscles (32, 33). To investigate the long-term effects of the loss of *smyhc1* on zebrafish muscle development and growth, we generated three mutant alleles of *smyhc1* mutants in zebrafish, *smyhc1^{mb16}*, *smyhc1^{mb17}*, and *smyhc1^{mb18}*, using CRISPR technology (Fig. 1S, Supplemental Data). Sequence analysis revealed that *smyhc1^{mb16}* carried a 1 bp deletion at position G2083 with respect to the ATG start codon (Fig. 1S, Supplemental Data). The *smyhc1^{mb17}* and *smyhc1^{mb18}* mutants carried a 1 bp (T), or a 20 bp insertion at position 2082 or 2061, respectively (Fig. 1S, Supplemental Data). All three mutations resulted in reading frame shift, leading to premature translation termination (Fig. 2S, Supplemental Data).

Nonsense-mediated mRNA decay is a commonly used cellular mechanism to eliminate mRNA transcripts containing premature translation termination codons (46–48). To determine whether nonsense-mediated mRNA decay occurred in these three mutant alleles, we carried out whole mount *in situ* hybridization analysis to compare the *smyhc1* and *myhz2* mRNA levels in *smyhc1* mutants and control zebrafish embryos (Fig. 1). A dramatic reduction of *smyhc1* mRNA transcript levels were detected in *smyhc1^{mb16}*, *smyhc1^{mb17}*, and *smyhc1^{mb18}* mutant embryos (Fig. 1C–H) compared with the WT control (Fig. 1A, B). qRT-PCR confirmed a 30 and 14 fold decrease in *smyhc1* mRNA levels in the *smyhc1^{mb17}* and *smyhc1^{mb18}* mutants, respectively (Fig. 1I). Expression of fast muscle specific *myhz2* mRNA was normal in the *smyhc1^{mb17}* and *smyhc1^{mb18}* mutants (Fig. 1I). This was supported by *in situ* hybridization (Fig. 3S, Supplemental Data). Collectively, these data indicate that the *smyhc1^{mb16}*, *smyhc1^{mb17}* and *smyhc1^{mb18}* mutant alleles likely carry loss of function mutations.

2. *smyhc1* mutation abolished slow myosin heavy chain expression and disrupted thick filament organization in slow fibers

To confirm that *smyhc1* mutations abolished Smyhc1 protein expression, we performed immunostaining using an anti-slow MyHC antibody (F59) that specifically labels the slow myosin heavy chain expressed in superficial layer of embryonic slow fibers (31, 35, 49, 50).

Compared with the WT sibling (Fig. 2A), sMyHC staining was completely eliminated in slow fibers of *smyhc1^{mb17}* and *smyhc1^{mb18}* mutant embryos at 28 hpf (Fig. 2B, C). This was further confirmed by the *smyhc1* knockdown studies (Fig. 2D).

Previous studies revealed that zebrafish contain two types of slow fibers that show distinct patterns of localization in myotome (51). The adaxial cell derived primary slow fibers form a superficial monolayer of about 20 slow-muscle fibers on the lateral surface of the myotome, whereas the secondary slow fibers are added at the dorsal and ventral region of the myotome (51). To test whether *smyhc1* mutation affected the formation of both primary and secondary slow fibers, we performed F59 immunostaining in *smyhc1^{mb17}* mutants at 48 and 72 hpf. The results showed that by 72 hpf, a few F59 positive slow myofibers were detectable in the horizontal myoseptum and dorsal/ventral regions of *smyhc1^{mb17}* myotome (Fig. 2H). This was likely due to the expression of sMyHC2 and sMyHC3 in these slow muscle cells (33, 35). However, the vast majority of the trunk region showed a lack of slow fibers (Fig. 2F and H). The loss of sMyHC expression was slow fiber type specific. Strong MyHC expression was detected in fast muscles of the *smyhc1^{mb17}* mutant embryos using the MF20 antibody (Fig. 2J). Collectively, the data indicate that the *smyhc1* mutation specifically abolishes myosin heavy chain expression in slow muscles of zebrafish embryos.

The *smyhc1* mutant had no myosin thick filaments in their slow fibers. To confirm the specificity of the *smyhc1* mutant phenotype, we carried out a rescue experiment by injecting the *pTol2- α -actin:smyhc1* transgene into *smyhc1* mutant zebrafish embryos. sMyHC1 expression and sarcomere organization were analyzed in the injected *smyhc1^{mb17}* mutant embryos through F59 immunostaining which detected Smyhc1 protein at 24 hpf. A mosaic pattern of slow fiber rescue was clearly detected in over 90% (n=55) of the injected mutant embryos (Fig. 3C), consistent with the typical mosaic pattern of gene expression via DNA injection. Double staining with Phalloidin and F59 also showed a sarcomeric thin filament organization in F59 positive slow fibers of *pTol2- α -actin:smyhc1* injected *smyhc1^{mb17}* mutant embryos at 28 hpf (Fig. 3D–F). We noted that some of the F59 positive fibers did not show the sarcomere organization instead those myofibers appeared to be multinucleated fast fibers (Fig. 3C). Collectively, the data indicate that the expression of the *smyhc1* transgene could rescue the defective sarcomere organization observed in slow fibers of *smyhc1* mutant embryos, reinforcing the specificity of the mutant phenotype.

3. Defective sarcomere organization in slow fibers but not fast fibers of *smyhc1* mutant embryos

Myosin forms the backbone of thick filaments in the sarcomeres. To determine if the loss of myosin thick filament affected the organization of other sarcomere structures, we performed double staining with phalloidin and anti-Prox1 antibody that specially label F-actin and nuclei of slow fibers, respectively. Compared to WT siblings (Fig. 4A, C), the *smyhc1^{mb17}* mutant embryos showed a significant disruption of thin filament organization in slow fibers at 22 hpf and 28 hpf (Fig. 4B and D). To assess the effect of *smyhc1* mutation on Z-line organization, the sarcomere organization of Z-lines was analyzed by anti- α -actinin immunostaining. Compared with WT siblings (Fig. 4E), the Z-line failed to align properly in

slow fibers of *smyhc1^{mb17}* mutant embryos, although Z disc-like structure was observed in slow fibers of the mutant embryos (Fig. 4F).

The myosin thick filaments are anchored at the M-lines in the middle of the sarcomere. To determine if M-lines were also disrupted in *smyhc1* mutants, we took advantage of the generation of *smyhc1^{mb17}* mutant in *myom3^{mn0067Gt}* zebrafish, which expressed a myomesin-3-RFP (Myom3-RFP) fusion protein localized specifically at the M-line of slow fibers (33). The *myom3^{mn0067Gt}; smyhc1^{mb17/+}* mutants were in-crossed to generate the *myom3^{mn0067Gt}* WT or *myom3^{mn0067Gt}; smyhc1^{mb17}* homozygous mutants. Myom3-RFP expression and M-line localization were analyzed in slow fibers by direct observation under confocal microscopy. The results showed that in contrast with *myom3^{mn0067Gt}* WT that showed a clear Myom3-RFP localization at the M-lines (Fig. 4G), *myom3^{mn0067Gt}; smyhc1^{mb17}* homozygous mutant exhibited a complete loss of Myom3-RFP localization in slow muscles (Fig. 4H). Together, these data indicate that *smyhc1* is essential for the sarcomere M-line organization in slow muscles.

To assess whether the *smyhc1* mutation affected the fast muscle organization, we analyzed key sarcomere structures in fast muscles by staining with phalloidin and various sarcomere specific antibodies against MyHC, MyLC, α -actinin and Myomesin. The results showed that the loss of *smyhc1* had no visible effect on myofibril organization in fast muscle fibers of *smyhc1^{mb17}* mutant embryos (Fig. 5A–J).

To validate the results from the immunostaining and to better define the slow fiber defects, we performed subcellular-structural analysis using transmission electron microscopy (TEM) on WT and *smyhc1^{mb17}* mutant larvae at 3 dpf. The data revealed that slow fibers contained poorly organized sarcomeres. Compared with the WT control (Fig. 5K–M), there were no thick filaments and M-line structures in slow fibers of the *smyhc1^{mb17}* mutants (Fig. 5N and P). However, thin filaments and Z-lines appeared less disrupted (Fig. 5O). Strikingly, in contrast to the average sarcomere size of 1.6 μ m in WT slow fibers (n=20), the distance between two Z-lines was reduced to approximately 1.0 μ m in the *smyhc1^{mb17}* mutant slow fibers (n=20) (Fig. 5R). This sarcomere defect was slow fiber specific. Sarcomere organization was normal in fast fibers of the mutant embryo (Fig. 5O). Collectively, these results showed that *smyhc1* was specifically required in slow muscles of zebrafish embryos, and knockout of *smyhc1* shortened the sarcomere length in slow fibers, but had no effect on sarcomere organization in fast fibers.

4. Knockout of *smyhc1* resulted in no effect on slow muscle precursor formation and fiber survival

Slow fibers are derived from adaxial cells that specifically express *smyhc1* at early stages of myogenesis. The adaxial cells originally located next to the notochord migrate laterally to the region underneath the skin to form the superficial layer of slow fibers (31). To test whether the loss of *smyhc1* had a negative impact on slow muscle specification and adaxial cell migration, we analyzed the formation of slow muscle precursors by F59 immunostaining and *Myod* mRNA *in situ* hybridization (Fig. 4S, Supplemental Data). The data showed that although F59 staining was not detected in *smyhc1^{mb17}* mutant embryos (Fig. 4S, Supplemental Data), *MyoD* positive adaxial cells were clearly visible as two rows

adjacent to the notochord in the *smyhc1^{mb17}* mutant embryos (Fig. 4S, Supplemental Data). The existence of slow muscle cells was further confirmed *in situ* by using another slow muscle specific marker, *Troponin C* (Fig. 4S, Supplemental Data). Compared with the WT controls (Fig. 4S, Supplemental Data), the expression of slow muscle specific *Troponin C* showed little or no change in the *smyhc1^{mb17}* mutant embryos (Fig. 4S, Supplemental Data). Troponin C positive slow fibers were clearly detected at the superficial layer of muscle fibers in the *smyhc1^{mb17}* mutants (Fig. 4S, Supplemental Data). Collectively, the results indicate that the knockout of *smyhc1* does not affect the initial formation of slow muscle precursors and their migration into the superficial layer to become embryonic slow muscle cells.

To determine whether loss of *smyhc1* affected the survival of defective slow fibers, we analyzed the defective slow fibers in *smyhc1^{mb17}* mutant embryos by Phalloidin staining. The data showed that although sarcomere organization was completely disrupted in slow fibers, the defective fibers were clearly observed in the *smyhc1^{mb17}* mutant zebrafish larvae at 5 and 8 dpf (Fig. 6E and G). To further verify the presence of defective slow fibers in the *smyhc1^{mb17}* mutants, we injected DNA constructs (*p9.7smyhc1:gfp-I-SceI*) expressing GFP specifically in slow fiber into *myom3^{mn0067Gt}; smyhc1^{mb17}* homozygous mutant embryos. GFP expression was clearly detected in some slow muscle fibers of the injected mutant embryos at 1, 6, 10, and 20 dpf (Fig. 6I–P). The mosaic pattern of GFP expression is consistent with typical gene expression via DNA injection. We noted that the GFP expressing slow fibers appeared in different shapes in WT and *smyhc1^{mb17}* mutant embryos (Fig. 6I–P). The GFP positive slow fibers appeared straight with even size diameters in WT embryos at 6 and 10 dpf (Fig. 6J and L). In contrast, the GFP positive slow fibers in mutant embryos were skinny with uneven sized diameters (Fig. 6M–O). However, by 20 dpf, slow fibers appeared in normal shape with clear sarcomere organization in *smyhc1^{mb17}* mutant embryos (Fig. 6P).

The appearance of normal slow fibers at 20 dpf could come from the recovery of original defective slow fibers or de novo formation of new slow fibers. To clarify this question, we performed the cell tracking experiment in live embryos using the *smyhc1*:GFP expression as a cell marker. The *p9.7smyhc1:gfp-I-SceI* construct was injected into *myom3^{mn0067Gt} WT* or *myom3^{mn0067Gt}; smyhc1^{mb18}* homozygous mutant embryos at 1–2 cell stages. Slow fibers marked with GFP were identified and tracked in WT or mutant live fish embryos at 14, 16 and 18 dpf. In WT embryos, the GFP positive slow fiber clearly contained the organized sarcomeres at 14 dpf (Fig. 7A–C). In contrast, the GFP fiber in mutant embryo had no sarcomere structure at 14 and 16 dpf (Fig. 7D, E). However, cell tracking results showed that the same defective slow fiber without M-line organization at 14 and 16 dpf clearly formed sarcomeres at 18 dpf (Fig. 7F). Together, these data indicate that although loss of *smyhc1* disrupts sarcomere organization in slow fibers, it does not affect the survival of the defective slow fibers in *smyhc1^{mb17}* mutant embryos. Moreover, the defective fibers can recover at later stages.

5. Reduced motility in *smyhc1* mutant zebrafish embryos and larvae

To determine whether the loss of *smyhc1* affected fish mobility, we carried out a locomotion assay using the DanioVision. The *smyhc1^{mb18/+}* heterozygous mutants were in-crossed to

generate WT, *smyhc*^{*I*^{*mb18*^{+/+}}} heterozygous and *smyhc*^{*I*^{*mb18*}} homozygous mutant embryos. Twenty-four fish larvae were randomly selected at 8 dpf and cultured individually in a 24-well plate for simultaneous tracking of fish mobility for 24 hours under the DanioVision. Eight larvae had a significantly reduced locomotion during the 24 hrs observation (Fig. 8A). Genotyping analysis revealed that 6 of these 8 larvae were *smyhc*^{*I*^{*mb18*}} homozygous mutants (Fig. 8A). Overall, the *smyhc*^{*I*^{*mb18*}} homozygous mutants showed reduced motility, with an average of 89.97 m moved within the 24 hours locomotion assay ($P < 0.0001$) (Fig. 8B), while the *smyhc*^{*I*^{*mb18*^{+/+}}} heterozygous mutant and WT showed a mean swimming distance of 202.3 m and 197.9 m within 24 hrs, respectively (Fig. 8B). Interestingly, both WT and *smyhc*^{*I*^{*mb18*^{+/+}}} were more active during the daytime and less active during the nighttime, whereas the *smyhc*^{*I*^{*mb18*}} remained inactive throughout the day (Fig. 8C).

To assess whether the motility defect occurred at the early stages, we next performed the locomotion assay at 3 dpf. The results showed that the *smyhc*^{*I*^{*mb18*}} homozygous mutant embryos had a significantly lower swimming distance (92.01 m) compared with WT (107.30 m) or heterozygous mutant (103.20 m) within the 24 hrs analyzed (Fig. 8B). Together, these data indicate that loss function of *smyhc*1 results in reduced motility of mutant zebrafish embryos and larvae.

6. *smyhc*1 mutants exhibited larval lethality of incomplete penetrance

To determine whether *smyhc*1 mutation affected fish survival, we genotyped all adult fish generated from various in-crosses of the three mutant alleles. We found that the homozygous mutants represented only 4–14% of the offspring generated from these in-crosses of *smyhc*1 heterozygous mutants (Table 2). Statistical analysis revealed that the size of the homozygous population was significantly less than the expected 25% from the Mendelian inheritance. To investigate when mutant fish might die during development and growth, we carried out a temporal death curve analysis (Fig. 8D). F3 mutant embryos were generated from the *smyhc*^{*I*^{*mb17*^{+/+}}} heterozygous in-cross, 180 healthy embryos were randomly selected and divided in three tanks with 60 embryos each. The numbers of dead fish were recorded every day from 1–30 dpf, and all the dead fish in tank #2 were genotyped by PCR and sequencing. The data showed that the homozygous *smyhc*1 mutant larvae began to die as early as 7dpf (Fig. 8D). Among the 11 dead fish larvae found in tank #2, nine of them were identified as *smyhc*^{*I*^{*mb17*}} homozygous mutants (Fig. 8D). These fish appeared to be skinny compared with the rest of the fish in the same tank (data was not shown).

Given that the homozygous mutant larvae showed decreased motility and skinny morphology, we tested whether the mutant larvae might have abnormal eating behavior. We performed a food-intake assay using fluorescent labeled paramecia (Fig. 9A). The results showed that feeding fluorescent labeled paramecia resulted in a bright belly in most of the WT or *smyhc*^{*I*^{*mb17*^{+/+}}} heterozygous mutant larvae at 7dpf (Fig. 9B). In contrast, out of the 16 *smyhc*^{*I*^{*mb18*}} homozygous mutants, two third of them (n=11) had little or no fluorescent signal at the belly region (Fig. 9B). The average fluorescence intensity of *smyhc*^{*I*^{*mb18*}} was 460 AU (arbitrary unit), significantly lower than the 1614 AU and 1428 AU detected in the WT and *smyhc*^{*I*^{*mb18*^{+/+}}} mutant, respectively (Fig. 9B). The reduced food intake was not caused by any defect in the jaw muscle because normal muscle structure and sarcomere

organization were observed in the *smyhc1^{mb17}* jaw muscles compared with WT control (Fig. 5S, Supplemental Data). The normal muscle structure was likely due to the expression of *smyhc2* in the jaw muscle (Fig. 5S, Supplemental Data). Together, these results indicate that decreased food intake likely caused the increased larval lethality in some of the homozygous mutant larvae.

7. Slow fibers were recovered in *smyhc1* mutants of juvenile and adult zebrafish

To assess whether *smyhc1* mutation had any effect on fish growth and muscle structure, we performed a histological analysis of their muscles and compared the body weight of adult WT and *smyhc1^{mb17}* mutant fish. Weight and histological analyses showed no significant difference between *smyhc1^{mb17}* mutants and WT sibling ($P > 0.05$, Fig. 10A–E). Furthermore, immunostaining revealed a well-organized slow and fast muscle patterning in *smyhc1^{mb17}* mutants at 30 dpf, similar to WT control (Fig. 10F–I). This was further confirmed by *in situ* hybridization using slow and fast muscle-specific probes (Fig. 10K–N). Together, these data indicate that loss of *smyhc1* does not affect the slow and fast muscle patterning and muscle growth in adult mutants.

To characterize the timing of slow muscle recovery, we performed a time course analysis by F59 staining at various life stages, (10, 30, 70, and 100 dpf). At 10 dpf, majority of the trunk region showed a lack of F59 staining in the mutant larvae (Fig. 11E). However, slow muscles appeared normal in the trunk region of *smyhc1^{mb17}* mutants at 30, 70, and 100 dpf (Fig. 11F–H), which were indistinguishable from the WT control (Fig. 11B–D). To assert these findings, we analyzed the fish muscles using another slow fiber specific antibody (S58) that recognizes myosin heavy chain in slow muscles (31, 35). Similar to the results of F59 staining, slow fibers were clearly detected in *smyhc1^{mb17}* mutants at 30, 70 and 100 dpf by S58 antibody (Fig. 6S, Supplemental Data). To assess whether the slow muscle recovery correlated with the expression of the other two *myhc* genes, *smyhc2* and *smyhc3*, we analyzed their mRNA expression by RT-PCR and *in situ* hybridization. The results showed that *smyhc2* expression was significantly elevated at 20 and 30 dpf (Fig. 11I). Analysis by *in situ* hybridization further confirmed the slow fiber specific expression of *smyhc2* and *smyhc3* in *smyhc1^{mb17}* mutants at 30 dpf similar to WT control (Fig. 11J–M).

To determine whether the reappeared slow fibers were functional with organized sarcomeres in the *smyhc1* mutant, we analyzed the M-line organization based on the Myom-3-RFP localization in slow fibers of *myom3^{mn0067Gt}; smyhc1^{mb17}* mutants at 1, 4, 8, 12, 18, 20 and 30 dpf. As shown in Fig. 12, no M-line organization of Myom-3-RFP was detected in the mutant larvae at 1, 4, and 8 dpf (Fig. 12H, I, J). However, Myom-3-RFP signal was detected as small dots in myofibers of mutant embryos 12 dpf (Fig. 12K). By 18 dpf, a mosaic pattern of normal slow fibers with clear Myom-3-RFP M-line localization was observed (Fig. 12L). By 20 dpf, a clear sarcomeric M-line organization was detected in all slow fibers of the *myom3^{mn0067Gt}; smyhc1^{mb17}* mutant larvae (Fig. 12M), suggesting that functional slow fibers reappeared in the mutant at the later stages.

To assess the slow fiber recovery, we conducted the TEM analysis comparing the subcellular structure of slow fibers in WT and *smyhc1^{mb17}* mutant larvae at 10 and 20 dpf. The results showed that slow fibers contained poorly organized short sarcomeres with no thick filaments

and M-line structures in *smyhc1^{mb17}* mutants at 10 dpf (Fig. 13B). However, by 20 dpf, sarcomeres with thick filaments and normal sizes were detected in *smyhc1^{mb17}* mutant slow muscle fibers (Fig. 13D). Collectively, these studies demonstrate that although Smyhc1 is required for the formation of embryonic slow fibers, loss of *smyhc1* has no effect on slow muscle development and growth in juvenile and adult fish. Moreover, functional slow fibers could reappear in juvenile and adult *smyhc1* mutant zebrafish.

Discussion

In this study, we characterized the muscle defects and recovery in *smyhc1* mutants. We demonstrated that targeted mutation of *smyhc1* abolished sarcomere organization in slow muscles of zebrafish embryos, while the formation of slow muscle precursors and their subsequent elongation into myotubes and myofibers were not affected. The *smyhc1* mutant larvae showed reduced locomotion, poor food intake and larval lethality at an incomplete penetrance. Approximately 2/5 of the homozygous mutants were viable and grew into reproductive adults. These adult *smyhc1* zebrafish mutant displayed a typical pattern of slow and fast muscle distribution and had similar body weight compared with WT sibling. Together, our studies indicate that Smyhc1 is essential for myogenesis and sarcomere organization in embryonic slow muscles, and plays a critical role in larval locomotion and survival. However, it is dispensable for muscle growth and slow fiber formation in juvenile and adult muscles.

1. Defective myofibril organization in *smyhc1* mutants

Myosin, a key component of the sarcomere, plays a vital role in myofibril organization and muscle contraction. We demonstrated in this study that *smyhc1* is essential for myofibril organization in slow myofibers. Loss of *smyhc1* did not affect the specification of slow muscle precursors and their migration into the superficial layer to form the elongated slow fibers. However, the slow fibers had defective sarcomeres without thick filaments and M-lines. The Z-disc like structure and thin filaments appeared less affected in the *smyhc1* mutant embryos. These data support a close interaction between thick filaments and M-line organization and the theory that z-discs and thin filaments could be formed without the myosin thick filaments and M-lines. This is consistent with the premyofibril model in that minisarcomeres consisting of α -actinin containing z-discs, muscle actin, and non-muscle myosin could be formed early during myofibrillogenesis prior to the integration of muscle myosin and titin to form nascent myofibrils with beaded Z-bands (13, 20, 52). Furthermore, our data are also in line with the I-Z-I model in the independent assembly of I-Z-I bodies and bipolar thick filaments (19).

A recent study demonstrated that loss-of-function mutations in *myo18b* resulted in defective myofibril assembly in fast muscles of zebrafish embryos (53; 54). *myo18b* codes for an unconventional myosin protein and is mainly expressed in skeletal and cardiac muscles. The *myo18b* mutant, also known as *frozen* and *schalafrig* mutant, showed impaired motility. Unlike the *frozen* mutant, the *smyhc1* mutants were motile and could swim with reduced locomotion. This could be explained by slow fibers being a minor population of skeletal muscles in zebrafish larvae compared with fast fibers which constitute approximately 80–

90% of the total myotomal muscles in zebrafish. The *smyhc1* mutants had normal fast myofibers because *smyhc1* is specifically expressed in slow fibers and loss of *smyhc1* only caused a slow-fiber specific defect. In contrast, *frozen* mutant carried a genetic mutation in *myo18b* gene which is strongly expressed in fast muscles, thus it is anticipated that *frozen* mutant could have a stronger muscle phenotype compared with *smyhc1* mutants.

2. Reduced survival of *smyhc1* mutant

The *smyhc1* mutants showed larval lethality of incomplete penetrance. The cause for the larval lethality is not well understood. The incomplete penetrant lethality of *smyhc1* mutants differs dramatically from the 100% embryonic lethality found in *frozen* mutants (53, 54). The difference could be explained by the strong expression of *myo18b* in zebrafish embryonic hearts and the cardiac muscle defects in *frozen* mutants. It has been reported that inactivation of *Myhc* resulted in cardiomyopathy leading to embryonic lethality (55, 56). Mutations in *MYH7*, a gene encoding a myosin heavy chain beta (MHC- β) isoform expressed in the heart and slow-twitch skeletal muscles (type I fibers), are associated with hypertrophic cardiomyopathy and dilated cardiomyopathy (57–59). However, we showed by immunostaining that cardiac muscles with highly organized sarcomere structure were clearly formed in *smyhc1* mutant larvae (Fig. 7S and 8S, Supplemental data), contradicting the theory that a heart defect was the likely cause of increased lethality in *smyhc1* mutants. Instead, our data revealed that majority of the *smyhc1* mutant larvae showed a poor food-intake of paramecia. This is likely caused by their reduced motility that is critical for catching paramecia.

3. Slow muscle recovery in *smyhc1* adult mutant

Our studies revealed that approximately 2/5 of the *smyhc1* homozygous mutants could grow into reproductive adults. This is consistent with a recent finding that mouse MyHC-embryonic mutants are viable and able to reproduce (25). We demonstrated that the viable *smyhc1* zebrafish mutants showed no visible difference in body weight and muscle structure compared with WT juvenile and adult zebrafish. Immunostaining revealed a similar pattern of slow fiber distribution in *smyhc1* mutants and WT sibling, suggesting a complete recovery of slow muscles in *smyhc1* mutants. This differs from previous findings in humans carrying genetic mutations in *MHY7* or *MYH2* that are specific markers for type 1 and type 2A muscle fibers, respectively. Humans carrying mutations in these myosins have a particular loss of type 1 or type 2 muscle fibers (60 – 63).

The recovery of slow muscles of *smyhc1* mutant fish might be explained by our finding that *smyhc1* mutation did not affect the initial formation of slow muscle precursors and the survival of defective slow fibers in *smyhc1* mutant embryos and larvae. However, this could not explain the reactivation of slow MyHC protein expression in slow muscles of *smyhc1* mutants. We believe that genetic redundancy might be behind the recovery of slow fibers in *smyhc1* mutants. Elworthy and colleagues have reported that zebrafish contains three *myhc* genes that are specifically expressed in slow fibers of zebrafish at various stages (35). Although *smyhc1* is the predominant MyHC expressed in embryonic and early larval slow muscle cells, *smyhc1* expression gradually disappeared by 42 dpf, and was replaced by *smyhc2* and *smyhc3* which are the major forms expressed in adult slow muscles (35, 49).

The appearance of slow muscles in *smyhc1* adult mutants could be explained by the expression of *smyhc2* and *smyhc3* in secondary fibers of juvenile and adult zebrafish.

Previous studies have demonstrated that the embryonic MyHC (MYH3) is a key regulator of myogenic differentiation during embryonic, fetal and neonatal myogenesis (25). Myofibers expressing MYH3 are primary fibers provide the templates upon which secondary fibers muscles will be built during muscle growth. Mutations in MyHC-embryonic cause autosomal dominant distal arthrogryposis, the most severe form of which is Freeman-Sheldon Syndrome (FSS). FSS exhibited over contraction of the hands, feet, and orofacial muscles, and other joints of the body (29). We demonstrated here that loss of embryonic *smyhc1* impaired the differentiation of primary slow fibers in early stage embryos and larvae, but did not affect the formation of slow fibers in juvenile and adult skeletal muscles. Our finding does not contradict with the previous findings in mice and humans for one of two possible reasons. First, the zebrafish *smyhc1* gene is not the homologue of MYH3 in mice and human. Second, in zebrafish *smyhc1* mutants, the primary embryonic slow muscle cells did not die although they had defective sarcomeres. The defective primary slow fibers might be sufficient to provide the templates for the formation of secondary slow fibers during muscle growth in juveniles and adult skeletal muscles.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Nonstandard Abbreviations:

CRISPR	Clustered Regularly Interspaced Short Palindromic Repeats
hpf	hour post fertilization
dpf	day post fertilization
MyHC	myosin heavy chain
MyLC	myosin light chain

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Fig. 1. Expression analysis of *smyhc1* and *myhz2* mRNAs in *smyhc1* mutant zebrafish embryos. *In situ* hybridization using a dig-labeled *smyhc1* specific antisense probe showing the *smyhc1* transcripts in WT control (A and B), *smyhc1^{mb16}* (C and D), *smyhc1^{mb17}* (E and F), or *smyhc1^{mb18}* (G and H) mutant zebrafish embryos at 24 hpf. A, C, E, and G are side views with inserted cross sections. B, D, F and H are dorsal views. qRT-PCR analysis of *smyhc1* and *myhz2* mRNA expression in WT control, *smyhc1^{mb16}* and *smyhc1^{mb17}* mutant zebrafish embryos at 24 hpf (I). *ef1a* was used as an internal reference gene. Error bars are $\pm$ SEM, Student's t test, * means $P < 0.05$. Scale bar = 100 μ m.

Fig. 2. Immunostaining shows slow and fast MyHC expression in WT control and *smyhc1mb18* mutant zebrafish embryos.

A-D. Slow fiber specific F59 antibody staining of WT control (A), *smyhc1mb17* (B) and *smyhc1mb18* (C) mutants or *smyhc1*-MO knockdown (D) embryos at 28 hpf. E-H. F59 antibody staining shows slow MyHC expression in WT control (E, G) and *smyhc1mb17* mutant (F, H) at 48 and 72 hpf, respectively. Note, a few F59 positive slow fibers (indicated by arrows) were observed in the horizontal myoseptum and ventral myotome of *smyhc1mb17* mutant at 72 hpf (H). I and J. Fast fiber specific MF20 antibody staining shows the expression of MyHC in fast muscle of WT control (I) and *smyhc1mb17* mutant (J) embryos at 72 hpf. Scale bar = 25 μ m.

Fig. 3. Ectopic expression of smyhc1 transgene in *smyhc1^{mb17}* mutant embryos.

The DNA construct (*pTol2-α-actin:smyhc1*) expressing the zebrafish slow MyHC1 was microinjected into *smyhc1^{mb17}* mutant embryos at 1–2 cell stages. The slow MyHC1 expression and thick filament organization were analyzed by F59 antibody staining in WT control (A), un-injected *smyhc1^{mb17}* mutant (B) and *pTol2-α-actin:smyhc1* injected *smyhc1^{mb17}* mutant (C) at 24 hpf. White arrows indicate multinucleated fast fibers with ectopic expression of the *pTol2-α-actin:smyhc1* transgene. D-F, double staining with F59 and Phalloidin in *pTol2-α-actin:smyhc1* injected *smyhc1^{mb17}* mutant shows the thick (E) and thin (F) filament organization in the rescued slow fibers. Scale bars = 25 μm.

Fig. 4. Defective sarcomere organization in slow fibers of *smyhc1mb17* mutant embryos.

The sarcomere organization was analyzed in WT control and *smyhc1mb17* mutant embryos by various single and double staining. A and B. Double staining with anti-Prox-1 antibody (Green) and Phalloidin (Red) shows slow fibers of WT control (A) and *smyhc1mb17* mutant (B) zebrafish at 24 hpf. C and D. Phalloidin staining shows α -actin thin filaments in WT control (C) and *smyhc1mb17* mutant (D) embryos at 24 hpf. E and F. Anti-actinin antibody staining shows the Z-lines in WT control (E) and *smyhc1mb17* mutant (F) embryos at 24 hpf. G and H. RFP expression and M-line organization in *myom3^{mnGt0067}* WT or *myom3^{mnGt0067}; smyhc1mb17* mutant (H) embryos at 48 hpf. Scale bars = 25 μ m.

Fig. 5. Sarcomere organization in slow and fast muscle fibers of WT and *smyhc1* mutant embryos.

The sarcomere organization in fast muscle fibers was analyzed in WT control (A, C, E, G, I) and *smyhc1^{mb17}* mutant (B, D, F, H, J) embryos by various types of immunostaining. These include immunostaining with MF20 (A and B), F310 (C and D), anti-α-Actinin (E and F) or anti-Myomesin (G and H) antibodies, or Phalloidin (I and J) that show the sarcomere structures of thick filaments (A-D), Z-lines (E, F), M-lines (G, H) and thin filaments (I, J), respectively. Scale bars = 25 μm in A-I. TEM analysis of sarcomere structure in WT and *smyhc1^{mb17}* mutant embryos (K-R). TEM images of slow and fast muscle fibers in WT control (K-M) and *smyhc1^{mb17}* mutant (N-P) trunk muscles at 3 dpf. K. A representative image containing the slow (black frame) and fast (white frame) muscle fibers in WT control. L and M. Enlarged images from the framed fast (L) and slow (M) muscle fibers in K. N-P. A representative image containing the slow (black frame) and fast (white frame) muscle fibers in *smyhc1^{mb17}* mutant trunk muscles. O and P. Enlarged images from the framed fast (O) and slow (P) muscle fibers in N. While normal sarcomeres were observed in fast muscle fibers (O), defective sarcomeres with reduced length and absence of I-band and M-lines were observed in slow muscle fibers (P) of the mutant embryos. Scale bars = 500 nm in K-P. R: Statistical analysis of sarcomere length comparison in fast and slow muscle fibers of WT and mutant embryos. The length of randomly selected 20 sarcomeres in slow and fast fibers of WT and mutant embryos were measured from Z-disc to Z-disc. The average sarcomere length of mutant slow fibers (n=20) was 1.0 μm. Compared to WT slow fibers (n=20) with average sarcomere length of 1.6 μm, the sarcomere size was significantly reduced in the mutant slow fibers based on the Student's t test (p<0.0001). Error bars are ±SEM.

Fig. 6. The presence of defective slow fibers in *smyhclmb17* mutant trunk muscles.

A-H. Phalloidin staining shows thin filament organization in WT control (A-D) and *smyhclmb17* mutants (E-H) at 5 and 8 dpf, respectively. A, E, C and G. Side views of thin filaments in slow fibers of WT (A and C) and mutant (E, and G) embryos. B, F, D and H. Side views of thin filaments in fast fibers of WT (B and D) and mutant (F, and H) embryos. The white and yellow arrows indicate slow and fast fibers, respectively. I-P. The DNA construct (*p9.7smyhcl:gfp-I-SceI*) was microinjected into *myom^{3mnGt0067}* WT or *myom^{3mnGt0067}; smyhclmb17* mutant zebrafish embryos at 1–2 cell stages. GFP expression slow fibers were observed in the injected WT (I-L) or mutant (M-P) embryos at 1, 6, 10 and 20 dpf. GFP positive slow fibers were detected in the injected *myom^{3mnGt0067}; smyhclmb17* mutant embryos. However, the slow fibers in mutant embryos showed an abnormal shape (N and O) compared with GFP positive slow fibers in the *myom^{3mnGt0067}* WT (J and K) embryos at 6 and 10 dpf. The GFP positive slow fibers appeared normal in *myom^{3mnGt0067}; smyhclmb17* mutant at 20 dpf (P). Scale bars = 50 μm (A) and 15 μm (I).

Fig. 7. Cell tracking analysis showed the recovery of M-line organization in slow fibers of *smyhc1* mutant fish at later larval stage.

The *p9.7smyhc1:gfp-I-SceI* construct expressing GFP specifically in slow fibers was injected into *myom3^{mn0067Gt} WT* (A-C) or *myom3^{mn0067Gt}; smyhc1^{mb18}* homozygous mutant (D-F) embryos at 1–2 cell stages. GFP positive slow fibers were identified and tracked in the live fish embryos at 14, 16 and 18 dpf. The M-line localization of myom-3-RFP was used to determine the sarcomere organization in the tracked myofibers. A-C. A GFP positive slow fiber in WT embryos was tracked from 14 to 18 dpf. High magnification showed the M-line organization in the same fiber in aa, bb and cc. D-F. A GFP positive slow fiber in *smyhc1* mutant larvae was tracked from 14 to 18 dpf. High magnification showed the gradual appearance of M-line organization in the same slow fiber in f and ff at 18 dpf.

Fig. 8. The *smyhc1* mutant fish larvae displayed hypoactive locomotion and increased larval lethality of incomplete penetrance.

The locomotion activity of 24 randomly selected healthy fish larvae from *smyhc1^{mb18/+}* in-cross was analyzed at 8 dpf using the DanioVisio. The locomotion track was recorded for each individual larvae for 24 hrs. The genotype of each larvae was determined after the locomotion assay. A. A representative track of 10 minutes recording was shown. B. The fish were grouped according to their genotypes, and the average swimming distance of each fish was plotted. C. The moving distance was every hour within the 24 hr period with daytime and night time indicated. Error bars are $\pm$ SEM. Total fish, n=24. Student's t test. **** means P value < 0.0001. To study the larval lethality of *smyhc1* mutants, 180 fish embryos from *smyhc1^{mb17/+}* in-cross were divided into three tanks with 60 fish per tank at 24 hpf. The fish death was recorded every day from 1–30 dpf (D). The genotype of all the dead fish found in tank #2 was determined. The results showed that 9 out of 11 dead fish were homozygote (D). The two unidentified dead fish was due to failure in isolating DNA for PCR and genotyping.

Fig. 9. Analysis of food intake in *smyhc1* mutant larvae

A. 4-Di-10-ASP was used to label paramecia for food intake assay. 4-Di-10-ASP was added to paramecia culture. The paramecia were collected by centrifugation and used to feed 7 dpf fish larvae for 2 hrs. Compared with the control larvae fed with non-labeled paramecia, fish larvae fed with 4-Di-10-ASP labeled paramecia showed clear a stomach with fluorescent signal.

B. Image J analysis comparing the fluorescent intensity of ingested paramecia in WT, *smyhc1^{mb17/+}* heterozygous and *smyhc1^{mb17}* homozygous mutant larvae. Statistical analysis revealed a significant reduction of food intake in the homozygous mutants. Error bars are $\pm$ SEM. Total fish, n=83. Student's t test. ** means P value < 0.01, *** means P value < 0.005. Representative images of WT and *smyhc1^{mb17}* homozygous mutant larvae are shown. Scale bars = 1 mm.

Fig. 10. The *smyhc1* mutant adult zebrafish displayed normal muscle fiber structure and body weight.

A-D. Morphological and muscle structure comparison between WT and *smyhc1* mutant fish at 45 dpf and 7 mpf. The skeletal muscle structure was analyzed by HE staining in cross sections of trunk muscles from WT control (A) and *smyhc1^{mb17}* mutant (B) fish at 45 dpf. No significant difference was detected at the morphological and structural levels between WT (A, C) and *smyhc1^{mb17}* mutant (B, D) zebrafish. E. Body weight (g) comparison between WT control, *smyhc1^{mb18/+}* heterozygous and *smyhc1^{mb18}* homozygous mutant at 7-month old. No significant difference in body weight was found among these three groups ($p < 0.05$). Error bars are $\pm$ SEM. Wild type, $n=16$; Heterozygote, $n=46$; Homozygote, $n=10$; Student's *t* test. To investigate the slow and fast muscle fibers distribution in *smyhc1* mutants, the muscle cross sections were analyzed by immunostaining and *in situ* hybridization using fiber type specific markers (F-N). Immunostaining with fast muscle-specific anti-myosin light chain antibody (F310) showed the similar pattern of fast muscle distribution in WT (F) and *smyhc1^{mb17}* mutant (G) fish at 30 dpf. Moreover, immunostaining with slow muscle-specific anti-myosin antibody (F59) showed the similar pattern of slow fiber distribution in WT (H) and *smyhc1^{mb17}* mutant (I) fish at 30 dpf. *In situ* hybridization using fast muscle-specific *myosin heavy chain (fmyhz)* anti-sense probe showed similar pattern of expression in fast muscles of WT (K) and *smyhc1^{mb17}* mutant (L) fish at 30 dpf. *In situ* hybridization using slow muscle-specific *Troponin C* anti-sense probe showed similar pattern of expression in slow muscles of WT (M) and *smyhc1^{mb17}* mutant (N) fish at 30 dpf. Scale bars = 250 μ m in B, 1.3 cm in D, 150 μ m in F, K and M.

Adult mutants also displayed the typical slow and fast muscle fiber structures by HE staining, and the body weight of mutant fish and control fish had no difference. This may help to explain that why mutant fish can survive without *smyhc1*. Bar=1cm

Fig. 11. Expression of slow MyHC protein and slow muscle recovery in *smyhc1* mutant. Immunostaining with F59 antibody shows the expression of slow MyHC on cross sections of trunk muscles from WT (A-D) and *smyhc1^{mb17}* mutant (E-H) zebrafish at 10, 30, 70 and 100 dpf (Dorsal to the top). The expression of slow MyHC was detected in the lateral superficial slow muscles of the *smyhc1^{mb17}* mutant at 30 dpf (F), and the myoseptum region between the dorsal and ventral muscle mass (G and H). Scale bars = 50 μ m in A, 150 μ m in B, 350 μ m in C and 500 μ m in D. Analysis of *smyhc2* and *smyhc3* mRNA expression in *smyhc1* mutants (I-M). I. qRT-PCR analysis of *smyhc1*, *smyhc2* and *smyhc3* mRNA expression in WT and *smyhc1^{mb17}* at 3, 10, 20, and 30 dpf, using *ef1a* as reference gene. Error bars are $\pm$ SEM, Student's t test. * means P value < 0.05. J-M. *In situ* hybridization

shows *smych2* (J and K) and *smyhc3* (L and M) expression in WT control (J and L) and *smyhc*^{*J^{mb17}*} mutant (K and M) trunk muscle at 30 dpf. Scale Bars =100 μ m in J, L.

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Fig. 12. The recovery of sarcomere organization in slow fibers of *smyhc1* mutant.

Myom3-RFP expression and M-line organization in *myom3^{mnGt0067}* WT (A-E) or *myom3^{mnGt0067}; smyhc1^{mb17}* mutant (F-J) were analyzed at 1, 4, 8, 12, 18, 20 and 30 dpf. M-line localization of Myom3-RFP was observed in *myom3^{mnGt0067}; smyhc1^{mb17}* mutant at 30 dpf. Scale bars = 20 μ m.

Fig. 13. Comparison of ultrastructure in slow and fast muscles of *smyhc1* mutants at 10 and 20 dpf.

TEM images were obtained from slow and fast muscles of control and *smyhc1*^{mb17} mutant fish at 10 dpf (A-D) and 20 dpf (E-H).

A-D. Ultrastructure in slow muscles of WT (A, C) and mutant (B, D) fish larvae at 10 (A, B) and 20 (C, D) dpf. The defective sarcomere organization in slow fibers of *smyhc1* mutant larvae was improved at 20 dpf (D) compared with the 10 dpf (B).

E-H. Ultrastructure in fast muscles of WT (E, G) and mutant (F, H) fish larvae at 10 (E, F) and 20 (G, H) dpf. No significant difference was detected.

I and J: Sarcomere length was measured in slow and fast muscle fibers of WT and *smyhc1*^{mb17} at 10 dpf (I) and 20 dpf (J), respectively. The length of the sarcomere, measured from Z-disc to Z-disc. There was a significant reduction in sarcomere length in slow fibers of the mutant at 10 dpf, but not at the 20 dpf. Error bars are $\pm$ SEM. Total sarcomeres, $n=20$, Student's t test. Scale bars = 1.2 μ m

Table 1:

Sequences of PCR primers and sgRNA target site

sgRNA or Primers	Sequences
smyhc1-sgRNA target	5'-GGCTGACAGCATGTACTGGT AGG -3'
Smyhc1-P1	5'-ACCTATTCAGGACTGTTCTGTGTCA-3'
Smyhc1-P2	5'-GTGATGAGGACGGACTGGTTCTCTC-3'
smyhc1- In-Fusion-F1	5'-ACCGCGGTGGCGGCCTTACTCTTCATCATGTCCCTTTCTTGG-3'
<i>smyhc1</i> - In-Fusion-R1	5'-TTTTGGCAAAGAATTACCATGGGTGACGCCGTTATGGCAGAGT-3'
zfmyhz2-P4-qPCR	5'-GCTCACCTACCAGACTGAGGA-3'
zfmyhz2-P5-qPCR	5'-ACTCAGCAATATCAGCAGCT-3'
zfsmyhc1-P4-qPCR	5'-GCTCACCTACCAGACTGAGGA-3'
zfsmyhc1-P5-qPCR	5'-CATCTTGTTGACCTGAGATTCA-3'
zfef1a-P3-qPCR	5'-CTTCAACGCTCAGGTCATCAT-3'
zfef1a-P4-qPCR	5'-ACAGCAAAGCGACCAAGAGGA-3'
Smyhc1-F-qPCR	5'-TGAAGAGGCTGAGGAACAGG-3'
Smyhc1-R-qPCR	5'-AGAACCAGTACTTGAACATGGC-3'
Smyhc2-F-qPCR	5'-AGGCTGAGGAACAGGCCAAT-3'
Smyhc2-R-qPCR	5'-AGAAAGCACCACAGCAATCC-3'
Smyhc3-F-qPCR	5'-AGGAACAGGCCAATTCTAACCT-3'
Smyhc3-R-qPCR	5'-AGAGTTAGAGAGATGGGAGAGC-3'
Smyhc2-F-situ	5'-GCAAGCTCACATCATTGTACCATCT-3'
Smyhc2-R-situ-T7	5'-GATCACTAATACGACTCACTATAGGCCTCCAGACGCTCCTTATCTGATT-3'
Smyhc3-F-situ	5'-GTGTCATCGTTACCTTTCTTTTTGA-3'
Smyhc3-R-situ-T7	5'-GATCACTAATACGACTCACTATAGGAGGTACGGAGCCGAGCTCCAAAC-3'
MYH6-F-situ	5'-ACGTGGGGACAGTTTCCTCAACT-3'
MYH6-R-situ-T7	5'-GATCACTAATACGACTCACTATAGGATGCGGCGTATCTCTCCTTTAGAT-3'
MYH7-F-situ	5'-AGACAAGTCGGACGTTTCATCTG-3'
MYH7-R-situ-T7	5'-GATCACTAATACGACTCACTATAGGGGTGAATGTCAGCTTCCTTTA-3'

Table 2.

The homozygous population showed comparatively lower survival rate

	Genotypes	Obtained	Expected Mendelian Inheritance
<i>smyhc</i> ^{<i>I</i>^{<i>mb16</i>^{<i>+/+</i>}}} × <i>smyhc</i> ^{<i>I</i>^{<i>mb16</i>^{<i>+/+</i>}}} (n=28)	WT	32%	25%
	<i>smyhc</i> ^{<i>I</i>^{<i>mb16</i>^{<i>+/+</i>}}}	64%	50%
	<i>smyhc</i> ^{<i>I</i>^{<i>mb16</i>}}	4%	25%
<i>smyhc</i> ^{<i>I</i>^{<i>mb17</i>^{<i>+/+</i>}}} × <i>smyhc</i> ^{<i>I</i>^{<i>mb17</i>^{<i>+/+</i>}}} (n=77)	WT	34%	25%
	<i>smyhc</i> ^{<i>I</i>^{<i>mb17</i>^{<i>+/+</i>}}}	55%	50%
	<i>smyhc</i> ^{<i>I</i>^{<i>mb17</i>}}	12%	25%
<i>smyhc</i> ^{<i>I</i>^{<i>mb17</i>^{<i>+/+</i>}}} × <i>smyhc</i> ^{<i>I</i>^{<i>mb18</i>^{<i>+/+</i>}}} (n=16)	WT	19%	25%
	<i>smyhc</i> ^{<i>I</i>^{<i>mb17</i>^{<i>+/+</i>}}}	38%	25%
	<i>smyhc</i> ^{<i>I</i>^{<i>mb18</i>^{<i>+/+</i>}}}	38%	25%
	<i>smyhc</i> ^{<i>I</i>^{<i>mb17</i>^{<i>mb18</i>}}}	6%	25%
<i>smyhc</i> ^{<i>I</i>^{<i>mb18</i>^{<i>+/+</i>}}} × <i>smyhc</i> ^{<i>I</i>^{<i>mb18</i>^{<i>+/+</i>}}} (n=72)	WT	22%	25%
	<i>smyhc</i> ^{<i>I</i>^{<i>mb18</i>^{<i>+/+</i>}}}	64%	50%
	<i>smyhc</i> ^{<i>I</i>^{<i>mb18</i>}}	14%	25%

The adult zebrafish from heterozygote in-cross were genotyped, the results showed that the homozygous population were much smaller than expected 25% Mendelian inheritance.