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Loss of Myomixer Results in Defective Myoblast Fusion, Impaired Muscle Growth, and Severe Myopathy in Zebrafish

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Abstract

The development and growth of fish skeletal muscles require myoblast fusion to generate multinucleated myofibers. While zebrafish fast-twitch muscle can fuse to generate multinucleated fibers, the slow-twitch muscle fibers remain mononucleated in zebrafish embryos and larvae. The mechanism underlying the fiber-type-specific control of fusion remains elusive. Recent genetic studies using mice identified a long-sought fusion factor named Myomixer. To understand whether Myomixer is involved in the fiber-type specific fusion, we analyzed the transcriptional regulation of *myomixer* expression and characterized the muscle growth phenotype upon genetic deletion of *myomixer* in zebrafish. The data revealed that overexpression of Sonic Hedgehog (Shh) drastically inhibited *myomixer* expression and blocked myoblast fusion, recapitulating the phenotype upon direct genetic deletion of *myomixer* from zebrafish. The fusion defect in *myomixer* mutant embryos could be faithfully rescued upon re-expression of zebrafish *myomixer* gene or its orthologs from shark or human. Interestingly, *myomixer* mutant fish survived to adult stage though were notably smaller than wildtype siblings. Severe myopathy accompanied by the uncontrolled

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Author Contribution SD, JS conceived the project. PW, PY and ZZ characterized the muscle defects in myomixer mutant fish. PY, ZZ, and RX generated the transgenic models used in nuclear and sarcomere analyses. RS constructed the human Myomixer expression construct. EC provided the myomixer mutant fish. All authors contributed to the data analysis and reviewed the manuscript. PW wrote the first draft and prepared figures for the manuscript. SD, PB and JZ revised the manuscript.

Statistical Analysis Statistics were done using R 4.1.2 (R Core Team 2021), the dplyr (v1.0.9; Wickham et al. 2022) and the ggplot2 packages (Wickham 2016). Tukey's multiple range tests were used to compare the difference between the WT and mutants. The differences were considered to be statistically significant when the P value was less than 0.05. Data were shown as means ± SEM.

Declarations

Conflict of Interest The authors have no relevant financial or non-financial interests to disclose. The authors have no competing interests as defined by Springer, or other interests that might be perceived to influence the results and/or discussion reported in this paper. SD serves on the editorial board of *Marine Biotechnology*.

Research Involving Human and Animal Participants 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: 0322003).

adipose infiltration was observed in both fast and slow muscle tissues of adult *myomixer* mutants. Collectively, our data highlight an indispensable role of *myomixer* gene for cell fusion during both embryonic muscle development and post-larval muscle growth.

Keywords

Myoblast fusion; Myomixer; Hedgehog; Zebrafish

Introduction

The fusion of myoblasts to create multinucleated myofibers is a key mechanism of muscle development, postnatal muscle growth, and muscle repair (Abmayr and Pavlath 2012; Chal and Pourquié 2017; Chen and Olson 2004; Sampath et al. 2018; Schejter 2016). Mononucleated myoblasts can fuse either with one another to generate myofibers de novo or with an existing myofiber for myonuclear accretion that supports muscle growth. Myoblast fusion is a dynamic and spatiotemporally controlled process that involves the migration of myogenic precursors and alignment of their membranes close to each other to establish cell–cell recognition and adhesion (Brukman et al. 2019; Krauss et al. 2017; Rochlin et al. 2010). This is followed by cytoskeleton rearrangements at the contact site, pore formation at the cell membrane, and ultimate exchange of cytoplasmic material that marks the completion of cell fusion (Kim et al. 2015; Luo et al. 2022).

Previous studies revealed the different timing of myofiber multinucleation for slow and fast muscles in zebrafish. Whereas fast muscles become multinucleated during early embryonic myogenesis (Powell and Wright 2011; Shi et al. 2018; Srinivas et al. 2007; Zhang and Roy 2017), fusion of slow muscle cells only starts during post-larval growth (Hromowyk et al. 2020). Such temporal regulation of fiber-type-specific fusion is controlled by the precision expression of fusogenic regulators (Powell and Wright 2011; Shi et al. 2017, 2018; Srinivas et al. 2007; Zhang and Roy 2017). Previous studies have demonstrated Sonic Hedgehog (Shh) signal from the notochord activates the expression of Prdm1a which induces a transcriptional program of slow myofiber identity while suppressing that of fast myofibers (Baxendale et al. 2004; Blagden et al. 1997; Coutelle et al. 2001; Currie and Ingham 1996; Du et al. 1997; Elworthy et al. 2008; Jackson and Ingham 2013; Jackson et al. 2015; Lewis et al. 1999; Roy et al. 2001; von Hofsten et al. 2008). Shh overexpression in zebrafish embryos blocks *myomaker* gene expression and myoblast fusion (Shi et al. 2018).

Myomaker and Myomixer constitute the molecular switch of myoblast fusion during myogenesis and muscle regeneration (Bi et al. 2017; Chen et al. 2020; Landemaine et al. 2014; Millay et al. 2013; Quinn et al. 2017; Shi et al. 2017, 2018; Zhang et al. 2017; Zhang and Roy 2017). Knockout of either *Myomaker* or *Myomixer* in mice resulted in defective myoblast fusion leading to perinatal lethality (Bi et al. 2017, 2018; Di Gioia et al. 2017; Millay et al. 2013, 2014; Quinn et al. 2017; Zhang et al. 2017; Zhang and Roy 2017). Similarly, knockout of *myomaker* or *myomixer* resulted in defective myoblast fusion in zebrafish embryos (Di Gioia et al. 2017; Shi et al. 2017, 2018; Zhang and Roy 2017). Interestingly, unlike *Myomaker* knockout mouse mutants, zebrafish *myomaker* mutants were

viable but showed reduced body weight and poor muscle growth (Shi et al. 2018). The consequence of *myomixer* gene deletion on fish muscle growth has yet to be characterized.

To determine the fiber-type-specific expression and Myomixer function in fish muscle development and growth, we analyzed the effect of Shh signaling on *myomixer* gene expression and characterized the muscle defects in *myomixer* mutants during development and growth. Our studies demonstrated that overexpression of Shh inhibited *myomixer* gene expression and hindered myoblast fusion in zebrafish embryos. Using transgenic reporters and models, we showed that Myomixer was critical for myoblast fusion, but not myogenesis and muscle cell differentiation. The myoblast fusion defect in *myomixer* mutant embryos could be rescued by expression of zebrafish *myomixer*, or its shark and human homologues. Homozygous *myomixer* mutant zebrafish can survive to adulthood. However, these adult mutants were smaller than wild-type sibling and showed severe myopathy in trunk skeletal muscles. Histological analysis revealed that *myomixer* mutant contained smaller fibers and increased adipocyte infiltration in their skeletal muscles. The mutant fibers contained only a few myonuclei per fiber. Collectively, data from this study demonstrate that fiber-type-specific *myomixer* expression is controlled, in part, by Hedgehog signaling, and Myomixer function is required for fish muscle growth. In addition, Myomixer function in myoblast fusion is highly conserved during evolution. Moreover, loss of *myomixer* expression or function hampers myoblast fusion and muscle growth, resulting in myopathy with increased fatty accumulation in skeletal muscles.

Results

Generation of Transgenic Fish Models for Analysis of Myoblast Fusion in *myomixer* Mutant Embryos

Previous studies have demonstrated that Myomixer plays a vital role in myoblast fusion in zebrafish embryos (Shi et al. 2017). To facilitate the phenotyping of myoblast fusion, we generated a transgenic line *pTol2-α-actin:mCherry-NLS* that expresses a nuclear targeted mCherry protein in muscle cells under the control of the muscle-specific *a*-actin promoter (Higashijima et al. 1997). First, to characterize the utility of this reporter, we transduced the plasmid into the *myomixer* mutant or wildtype (WT) embryos via DNA microinjection. Compared with WT embryos that show multinucleated myofibers clearly delineated by the expression of mCherry (Fig. 1A, C, E), muscle cells from *myomixer* mutant embryos only contain one nucleus that colocalized with the Hoechst-32258 nuclear staining signals at the center of myotome (Fig. 1B, D, F). It appeared that the fiber diameter in *myomixer* mutant was smaller compared with WT myofibers (Fig. 1A, B, E, F). Together, the proof-of-principle test validated the amenity of the *pTol2-a-actin:mCherry-NLS* reporter for enumerating myoblast fusion in vivo.

We then generated the transgenic line expressing the nuclear mCherry and a second line that stably express Lifeact-GFP which binds to and labels F-actin in muscle cells (Reischauer et al. 2014; Xu and Du 2021). The dual *Tg(Tol2-α-actin:mCherry-NLS);Tg(actc1b:Lifeact-GFP)* transgenic fish were crossed with *myomixer* mutants to produce the transgenic mutant fish. The initial characterization showed that while WT transgenic embryos contained multinucleated myofibers in their trunk muscles (Fig. 2A), the *myomixer* mutant embryos

contained only mononuclear myofibers that were fully elongated spanning the entire somite (Fig. 2B). Sarcomere organization, however, appeared normal compared with WT myofibers (Fig. 2). Collectively, Myomixer is essential for myoblast fusion in zebrafish embryos, and the *myomixer* mutant transgenic model provides a novel reliable system for analyzing myoblast fusion during muscle development and growth.

Overexpression of Sonic Hedgehog Inhibits *myomixer* Expression and Hinders Fast Muscle Cell Fusion

In situ expression analysis revealed that *myomixer* was expressed specifically in fast muscle cells that give rise to multinucleated fast-twitching myofibers (Shi et al. 2017). No *myomixer* transcript was detected in slow muscle cells of fish embryos. The molecular regulation of *myomixer* fiber-type-specific expression is not well understood. Previous studies demonstrated that Hedgehog signaling plays an important role in slow and fast muscle specification (Lewis et al. 1999; Baxendale et al. 2004; Jackson and Ingham 2013; Jackson et al. 2015). Overexpression of Shh in zebrafish embryos can convert fast muscle to slow muscles (Currie and Igham 1996; Du et al. 1997; Shi et al. 2018). To determine whether Hedgehog signaling is involved in inhibition of *myomixer* gene expression in slow muscles, we ectopically expressed Shh in zebrafish embryos via *shh* mRNA microinjection at 1–2 cells stage. *Myomixer* mRNA expression during myogenesis was then determined by whole mount in-situ hybridization. Compared with the un-injected control (Fig. 3A), a dramatic reduction of *myomixer* mRNA expression was observed in the *shh* mRNA injected zebrafish embryos (Fig. 3B).

To determine whether overexpression of Shh in zebrafish embryos could inhibit myoblast fusion, *Shh* mRNA was injected into *pTol2-a-actin:mCherry-NLS* transgenic zebrafish embryos at 1–2 cell stages. Myonuclear numbers were then analyzed at 48 hpf. Compared with the un-injected control embryos that showed a broad distribution of nuclei within each myotome due to the presence of multinucleated myofibers (Fig. 3C), the *Shh* mRNA injected embryos displayed a central nuclear localization within each myotome (Fig. 3D). This pattern of nuclear distribution was mainly caused by the presence of mononucleated myofibers in the trunk muscle of mutant embryos. These studies indicate that overexpression of Shh in zebrafish embryos could inhibit *myomixer* gene expression and impede myoblast fusion.

Knockout of *myomixer* Does not Affect MyoD Expression and Muscle Cell Differentiation

Myoblast fusion is an important step in myogenesis and formation of multinucleated fibers during muscle cell differentiation. To test whether blocking myoblast fusion in *myomixer* mutant embryos affected myogenic regulatory gene expression and early muscle development, we analyzed the expression of *MyoD* and *Myogenin* in *myomixer* mutants. Compared with WT control (Fig. 4A, C), no significant difference of *MyoD* and *Myogenin* expression was observed in the mutant embryos (Fig. 4B, D), suggesting that loss of *myomixer* did not affect early-stage myogenic program. This is consistent with previous findings that MyoD and Myogenin directly control the expression of *Myomaker* and *Myomixer* genes at the onset of myoblast fusion (Zhang et al. 2020).

To assess the effect of *myomixer* knockout on myofiber organization, we compared the myotendinous junction (MTJ) in WT and *myomixer* mutants by immunostaining with anti-vinculin antibody. MTJ connects two adjacent muscle cells and transmit muscular forces during swimming (Charvet et al. 2011). The immunostaining results showed that similar to WT control (Fig. 4E), a characteristic V-shaped MTJ was clearly detected at the myotome boundary in the *myomixer* mutant embryos (Fig. 4F). Our early analysis using the *myomixer* mutant *Tg(actc1b:Lifeact-GFP)* transgenic model revealed highly organized sarcomeres in *myomixer* mutant myofibers (Fig. 2B), indicating a normal muscle cell differentiation. Collectively, these data demonstrate that loss of Myomixer does not affect muscle cell differentiation and fiber organization in zebrafish embryos.

Rescue of Myoblast Fusion in *myomixer* Mutant by Ectopic Expression of *myomixer* Homologues from Other Species

Myomixer homologues have been identified in multiple vertebrate species that span all the key animal groups of cyclostomes (lamprey) and gnathostomes including shark, fish, amphibian, reptile, bird and mammal (Shi et al. 2017; Zhang et al. 2021). Myomixer homologues discovered from these clades share low sequence similarity with exception to a conserved hydrophobic patch at their carboxyl termini. To assess the functional conservation, we performed a rescue experiment in *myomixer* mutant embryos by ectopic expression of zebrafish, elephant shark (*Callorhinchus milii*) or human *myomixer* open reading frames driven by the zebrafish *myomixer* promoter. These DNA constructs were individually injected into fertilized eggs from in-cross of *myomixer* heterozygous mutants. As shown in Fig. 5B, *myomixer* mutant embryos injected with *pTol2-α-actin:mCherry-NLS* alone contained exclusively single nuclear myofibers. However, the myoblast fusion defect could be partially rescued by transient expression of the zebrafish, shark, and human *Myomixer* (Fig. 5C–E). Multinucleated myofibers were clearly observed in *myomixer* mutant embryos co-injected with the *pTol2-myomixer:zMyomixer* construct and the *pTol2-a-actin:mCherry-NLS* reporter (Fig. 5C). The rescue appeared in a mosaic pattern as normally expected from gene expression via DNA injection. Compared with embryos injected with zebrafish *myomixer* construct (Fig. 5C), multinucleated fibers were clearly detected in *myomixer* mutant embryos injected with the shark (Fig. 5D) or human Myomixer expression construct (Fig. 5E). Statistical analysis showed no significant difference in the rescue activity between zebrafish, shark, and human *myomixer* genes. Collectively, these data indicate the highly conserved Myomixer function during vertebrate evolution.

Myomixer Mutant Larvae Show Normal Locomotion

To determine whether defective myoblast fusion could affect fish larval mobility, we carried out a locomotion assay using a DanioVision system. Adult *myomixer* heterozygous mutants were in-crossed to generate WT, heterozygous and homozygous mutant larvae and juveniles. Twenty-four fish larvae and juveniles were randomly selected at 5, 15 and 30 days-post-fertilization (dpf) and placed individually in a 24-well plate (2 × 12-well plate for 30 dpf assay) for simultaneous tracking of fish mobility for 24 h. Overall, the *myomixer* homozygous mutants showed normal motility compared with WT and heterozygous mutant siblings. As shown in Fig. 6, on average, the homozygous mutant larvae moved 226.6

m within the 24 h locomotion assay at 5 dpf (Fig. 6), while the *myomixer* heterozygous mutant and WT showed a mean swimming distance of 244.8 m and 256.1 m within 24 h, respectively (Fig. 6). All fish larvae regardless of the genotypes recorded a higher activity during the daytime than the nighttime (Fig. 6). Normal locomotion from all genotypes were also observed for fish larvae aged from 15 and 30 dpf (Data not shown). Together, these data indicate that loss of *myomixer* does not affect zebrafish larval motility.

Myomixer Mutants Exhibit Decreased Growth and Severe Myopathy in Adult Stage

Myoblast fusion is a key driver of post-larval muscle growth. To determine whether defective myoblast fusion in *myomixer* mutants could affect fish muscle growth, we compared fish size and weight distribution at 4 months old. Compared with the WT siblings (Fig. 7A, B), *myomixer* homozygous mutants were smaller in size for both males and females (Fig. 7A, B). On average, adult mutants weighted approximately 50% less than the WT and heterozygous siblings (Fig. 7C, D).

To further investigate the effect of *myomixer* knockout on muscle composition, we performed a histological analysis on cross sections of trunk muscles dissected from adult fish. HE staining showed a clear difference between WT and mutant skeletal muscle tissues (Fig. 8). Specifically, in contrast to the WT control that contained highly organized myofibers (Fig. 8A, B), *myomixer* mutant trunk muscles contained poorly organized smaller myofibers (Fig. 8C, D). The number of myofibers in the cross section was significantly less in homozygous mutants compared with WT controls (Fig. 8). Moreover, heavy adipose infiltrations were noticed in *myomixer* mutant trunk muscles (Fig. 8C, D). Collectively, these data indicate that Myomixer is essential for normal skeletal muscle growth and architecture in adult fish.

Myofibers of Adult *myomixer* Mutants are Smaller and Contain Few Myonuclei

To directly characterize the myofiber defects in adult *myomixer* mutants, single myofibers were isolated from trunk skeletal muscles of WT and mutant fish. The isolated myofibers were used for fiber sizes and myonuclei number analyses. The results revealed a dramatic size reduction of mutant myofibers (Fig. 9A–E). The myofiber length and width were also significantly shorter in *myomixer* mutant compared with WT siblings. On average, the WT fibers ranged from 700–1300 μm in length and 48–140 μm in width (Fig. 9F, G), whereas the size of *myomixer* mutant fibers ranged from 180-–900μm in length and 14–40 μm in width (Fig. 9F, G). In addition, the number of myonuclei was also reduced from an average of 87 nuclei per WT fiber (n = 24) to 4 myonuclei per fiber in the mutants (n = 33) (Fig. 9H). Despite of a much smaller myofiber volume (Fig. 9I), mutant myofibers showed a normal size of myonuclear domain around each nucleus (Fig. 9J). Together, these data indicate that Myomixer is required for myoblast fusion to form multinucleated myofibers during muscle growth in adult fish.

Discussion

In this study, we investigated the regulation of fiber-type-specific *myomixer* gene expression and its function in myoblast fusion, fish locomotion and muscle growth. Our studies

demonstrated that *myomixer* expression was inhibited by Shh. Ectopic expression of Shh suppressed *myomixer* expression and blocked myoblast fusion. We further showed that Myomixer function in myoblast fusion was highly conserved during evolution. The myoblast fusion defect in zebrafish *myomixer* mutant could be rescued by the expression of shark or human *Myomixer* homologous gene. Unlike *myomixer* knockout mice, zebrafish *myomixer* mutants could survive to become adult fish. However, adult *myomixer* mutants were notably smaller than wildtype siblings and showed severe myopathy with dramatic adipocyte infiltration in the trunk muscle. Single-fiber analysis demonstrated that myofibers from *myomixer* mutants were small fibers that contained only a few myonuclei per fiber. Together, these data demonstrate that fast-fiber-specific fusion involves inhibition of *myomixer* expression by Hedgehog in slow muscle cells, and the highly conserved Myomixer function in myoblast fusion is critical for post-larval fish muscle growth.

Myomixer Expression and Myoblast Fusion

Zebrafish slow and fast fibers differ in their fusion capability during early embryonic development. Slow muscles are single nucleated fibers, whereas fast muscles are multinucleated fibers in fish embryos and larvae. It has been suggested that distinct patterns of fusogenic gene expression might be involved in the different fusion characteristics of slow and fast muscles in zebrafish. Consistent with this idea, key fusogenic regulators such as Jam B, Kirrel, Myomaker, and Myomixer are specifically expressed in fusion competent fast muscle cells, but not slow muscle cells from early embryos (Powell and Wright 2011; Srinivas et al. 2007; Zhang and Roy 2017; Shi et al. 2017, 2018).

We showed here that overexpression of Shh, a key determinant of slow muscle identity, resulted in sharp downregulation of *myomixer* expression in zebrafish myotome, and decreased myoblast fusion. These data are consistent with our previous finding that overexpression of Shh reduced *myomaker* gene expression and abated myoblast fusion (Shi et al. 2018). Previous studies by us and others have demonstrated that Hedgehog signaling plays a vital role in slow and fast muscle specification and differentiation. Overexpression of Shh could convert fast muscle into slow fibers (Blagden et al. 1997; Coutelle et al. 2001; Currie and Ingham 1996; Du et al. 1997). Shh binds to its receptor, Patched and activates the expression of Prdm1a which induces a transcriptional program of slow myofiber identity while suppressing that of fast myofibers (Baxendale et al. 2004; Jackson and Ingham 2013; Jackson et al. 2015). Lewis et al. 1999; von Hofsten et al. 2008). Loss of Prdm1a resulted in defective slow muscle formation and ectopic expression of *myomaker* in adaxial cells, the slow muscle precursors, in *prdm1a*^{nrd} zebrafish embryos (Baxendale et al. 2004; Hromowyk et al. 2020). Collectively, these studies suggest that the fiber-type-specific *myomaker* and *myomixer* expression may determine the distinct pattern of myoblast fusion in slow and fast muscles of zebrafish embryos and larvae.

Interestingly, a recent study showed that slow muscles were able to fuse in zebrafish during late-larval stages (Hromowyk et al. 2020). The molecular mechanism underlying multinucleated slow fibers formation is not clear. Expression analysis showed that although *myomaker* does not appear to be expressed in slow muscle fibers, it is expressed in nearby cells, suggesting that slow myofiber multinucleation occurs as neighboring *myomaker*-

expressing cells fuse into differentiated *myomaker*-negative slow muscle fibers (Hromowyk et al. 2020). It remains to be determined if Myomixer is expressed in slow fibers or their neighboring cells and play a role in slow myofiber multinucleation in late larval stages.

Myomixer Gene Evolution and Conserved Function in Myoblast Fusion

A recent study revealed a complex evolutionary history of Myomaker-Myomixer axis in regulating myoblast fusion in chordates (Zhang et al. 2021). *Myomixer* appears to be an orphan gene that has evolved de novo in early vertebrates (Zhang et al. 2021). Interestingly, *myomixer* was absent from tunicates which though possess a distantly related Myomaker proteins that enabled low-grade fusion in siphon muscles. Evolutionary comparison showed that Myomixer protein sequences are moderately conserved with the exception of lamprey Myomixer which differs significantly from other vertebrate Myomixer homologues in protein length and sequences (Shi et al. 2017; Zhang et al. 2021).

Previous studies showed that shark, frog or turtle *myomixer* gene could recuse the fusion defect of *myomixer* knockout C2C12 cells in vitro (Shi et al. 2017). To assess the functional conservation of Myomixer in vivo, we performed rescue experiments in *myomixer* mutant zebrafish embryos. Our data showed that ectopic expression of shark or human *myomixer* homologues could rescue the fusion defect of zebrafish *myomixer* mutants. Collectively, these data indicate that Myomixer function is highly conserved during evolution.

Myomixer in Muscle Development, Growth and Regeneration

We showed in this study that although Myomixer is essential for myoblast fusion, it is not required for early myogenesis and muscle cell differentiation in zebrafish embryos. Loss of *myomixer* had no effect on *MyoD* and *Myogenin* gene expression and muscle cell differentiation. Functional myofibers with highly organized sarcomeres were clearly present in *myomixer* mutant embryos. In addition, homozygous *myomixer* mutant larvae showed normal locomotion behavior compared with wildtype and heterozygous siblings. Our data are consistent with previous reports that Myomaker and Myomixer act downstream of MyoD and Myogenin to promote myocyte fusion (Ganassi et al. 2018; Luo et al. 2015; Millay et al. 2014; Zhang and Roy 2017; Zhang et al. 2020).

Muscle growth depends on hyperplasia (the addition of new fibers) and hypertrophy (the growth of existing fibers). In both cases, it requires fusion of myoblasts with each other to create new multinucleated myofibers or with an existing myofiber to increase myofiber size (Chal and Pourquié 2017). Our studies here demonstrated that Myomixer is required for muscle growth in fish. Myofibers from *myomixer* mutants were significantly smaller than fibers of wildtype siblings, and the mutant fibers contained only a few myonuclei per fiber in contrast to hundreds of myonuclei found in wildtype fibers. Trunk muscle tissue was dramatically reduced in *myomixer* mutants, and moreover, significant adipocyte infiltration and severe myopathy were found in *myomixer* mutant skeletal muscles. Data from our zebrafish study are consistent with a recent finding that impaired activity of Myomixer causes myopathy resembling Carey-Fineman-Ziter syndrome (CFZS) in human (Ramirez-Martinez et al. 2022). CFZS patients display a characteristic craniofacial defect marked by bilateral facial weakness and an inability to fully abduct both eyes (Carey et

al. 1982; Carey 2004). In addition, the patients also show muscle hypoplasia in limb and leg muscles with increased adipose infiltration (Carey et al. 1982; Carey 2004). Previous studies have demonstrated that *Myomaker* hypomorphic genetic mutations are associated with CFZS in human (Di Gioia et al. 2017; Hedberg-Oldfors et al. 2018). Interestingly, similar craniofacial and trunk skeletal defects were found in *myomaker* zebrafish mutants (Di Gioia et al. 2017; Hromowyk et al. 2020; Shi et al. 2018). Collectively, these data indicate a concerted action of Myomaker and Myomixer in promoting myoblast fusion in skeletal and craniofacial muscles during muscle growth. In addition, because *myomaker* and *myomixer* zebrafish mutants show similar muscle defects as CFZS patients, they provide useful animal models for studying skeletal and craniofacial myopathies in CFZS.

The requirement of Myomaker and Myomixer in fish muscle growth is consistent with their pattern of expression and the mode of fish muscle growth. In contrast to mice, fish exhibit post-larval muscle growth through both hypertrophy and formation of new myofibers (hyperplasia). Expression analysis in seabream and trout revealed that Myomaker and Myomixer were expressed during post-larval hyperplasia with high transcript levels in fingerlings and remained readily expressed in juvenile muscles although their expression decreased in adult fish (Landemaine et al. 2019; Perello-Amoros et al. 2021a, b).

Myomaker and Myomixer are essential for muscle regeneration (Bi et al. 2018; Millay et al. 2014). Genetic deletion of *Myomaker* or *Myomixer* from satellite cells of mouse skeletal muscles abolished myoblast fusion and prevented muscle regeneration after injury (Bi et al. 2018; Millay et al. 2014). Fish muscle damage also triggers a regeneration process including new myogenesis and muscular remodeling. It is likely that Myomaker and Myomixer play critical roles in fish muscle regeneration. Perello-Amoros and colleagues showed that expression of both fusogens was upregulated during regeneration in trout and seabream with peak after 16 days post-injury (Perello-Amoros et al. 2021a, b). In zebrafish, cell proliferation and Pax7-positive satellite-like cell number are dramatically increased in adult *myomaker* mutants (Hromowyk et al. 2020).

Myomixer Function in Animal Survival

It has been reported that Myomixer is required for animal survival in mice. Knockout of *Myomixer* in mice resulted in perinatal lethality (Bi et al. 2017; Quinn et al. 2017; Zhang et al. 2017). Mouse *myomixer* mutant embryos died perinatally and no viable neonatal or adult *myomixer* mutant could be recovered (Zhang et al. 2017). The perinatal lethality of mouse mutants was caused by disruption of respiratory function (Zhang et al. 2017). Unlike mouse *myomixer* mutants, we found in this study that zebrafish *myomixer* mutants were viable and could grow into adult fish albeit of smaller sizes. The reason behind the viability of fish *myomixer* mutants is not clear. One possibility is that fish do not have the lung tissue, and thus avoiding the complication from respiratory failure in mouse *myomixer* mutants. Functional redundancy could be another possibility. It has been reported that adhesion molecules (CAMs), JamB and JamC, play a critical role in myoblast fusion in zebrafish embryos (Powell and Wright 2011). However, loss of JamB and JamC had no effect on fish muscle growth and survival (Si et al. 2019; Hromowyk et al. 2020). Interestingly, the difference in animal survival has also been observed between *myomaker* knockout zebrafish

and mice. While knockout of *myomaker* in mouse resulted in postnatal lethality (Millay et al. 2013), zebrafish mutant carrying loss of function mutations in *myomaker* could survive into adult (Shi et al. 2018). The reasons behind the survival disparities between mouse and zebrafish mutants remain to be investigated in future studies.

Materials and Methods

Zebrafish Lines and Maintenance

The *myomixer* mutants were generated using CRISPR-Cas9 (Shi et al. 2017). Three mutant alleles were used in this study. Two mutant alleles, *myomixer*^{sw101} and *myomixer*^{sw104}, were identified and characterized in Elizabeth Chen's laboratory at University of Texas Southwestern Medical Center. The *myomixer*^{sw101} and *myomixer*^{sw104} carry a 16 bp deletion, and 4 bp insertion, respectively. The third mutant allele *myomixer*^{mb19} was identified and characterized in Shaojun Du's laboratory at University of Maryland Baltimore. The *myomixer*^{mb19} mutant carries a 13 bp insertion. All three mutant alleles created reading frame mutations. The *myomixer*^{sw101} and *myomixer*^{mb19} mutant alleles were primarily used in this study. No visible difference was observed between these two different mutant alleles.

Zebrafish were maintained at the zebrafish facility of the Aquaculture Research Center, Institute of Marine and Environmental Technology, University of Maryland. The fish were kept in 8 gal aquaria at 28 °C with a photoperiod of 14 h light and 10 h dark. 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.

DNA Plasmids and Generation of *myomixer* Expression Constructs

The *Tg(actc1b:Lifeact-GFP)* plasmid was obtained from Didier Y. R. Stainier's laboratory at Max Planck Institute (Reischauer et al. 2014). The *pTol2-myomixer:zMyomixer* plasmid was constructed by cloning the zebrafish *myomixer* coding sequence after its own 5.1 kb promoter including the first intron sequence. Briefly, the 5.1 kb zebrafish *myomixer* promoter was amplified by PCR using Pfu DNA polymerase using zMyomixerPro-fusion-F1/R2 primers (Table 1). The PCR product was cloned into the XhoI and EcoRI sites of the *pTol2-ef1a:EGFP* vector by In-Fusion cloning. The resulting *pTol2-myomixer:EGFP* vector was then digested with BamHI and NotI to remove the EGFP coding sequence. The full-length zebrafish *myomixer* coding sequence was isolated by PCR using gene-specific primers zMymx-fusion-F1/R1 (Table 1).

The PCR product was then cloned into the BamHI and Not I site of the *pTol2-myomixer:EGFP* vector using the In-Fusion Cloning Kit (Takara Bio USA, Inc.). Similar strategy was used to clone the elephant shark Myomixer coding sequences after the zebrafish *myomixer* promoter to generate the *pTol2-myomixer:sMyomixer* construct. Myomixer coding sequence from elephant shark was amplified by PCR using the *pMXs-sMymX* vector as a template (Zhang et al. 2021). The *pTol2-myomixer:hMyomixer* construct was generated by first enzymatic digestion and release of the human Myomixer open reading frame from

a pMXs-hMymX vector (Zhang et al. 2020), and then ligation to *pTol2-myomixer:EGFP vector*.The gene-specific PCR primers for isolating shark *myomixer* coding sequences are listed in Table 1. All above constructs were confirmed by DNA sequencing.

Construction of pTol2–α–actin:mCherry–NLS Plasmid Construct

The *pTol2-α-actin:mCherry-NLS* construct was constructed by cloning the full-length coding sequence of nuclear mCherry (mCherry-NLS) 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 the *mCherry-NLS* was amplified by PCR from the mCherry-NLS-C1 (Gift from Shengyun Fan's laboratory at University of Maryland Baltimore) using *mCherry* specific primers actin:nRFP-ERI-F1/ NotI-R1 (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.).

Microinjection in Zebrafish Embryos and Generation of Transgenic Lines

For microinjection, the *pTol2-α-actin:mCherry-NLS*, *pTol2-myomixer:zMyomixer pTol2-myomixer:sMyomixer* and *pTol2-myomixer:hMyomixer* constructs were dissolved in water at 50 ng/μl. The DNA construct (not linearized) was micro-injected alone or in combination into zebrafish embryos at one-cell stage. Each embryo was injected with approximately 2 nl of the plasmid (~ 100 pg DNA). For generation of *pTol2-α-actin:mCherry-NLS* transgenic line, the DNA construct was co-injected with Tol2 mRNA (50 ng/μl) as previously described (Urasaki et al. 2006).

For Sonic hedgehog (Shh) overexpression, Shh mRNA was synthesized in vitro using the mMESSAGE mMA-CHINE T7 in vitro transcription kit (AM1344, ThermoFisher Scientific). NotI linearized plasmid containing Shh coding sequence in T7TS vector was used as template (Ekker et al. 1995). Capped Shh mRNAs were transcribed from linearized DNA template. The mRNA transcripts were purified using MEGAclear Transcription Clean-Up kit (AM1908, ThermoFisher Scientific).

DNA Isolation from Fish Embryos and Caudal Fin Clips for Genotyping

For genotyping, genomic DNA was isolated from whole zebrafish embryos or the head region of fish larvae, or fin clips from adult zebrafish using the alkaline method (Cai et al. 2019). A 387 bp DNA fragment covering the Myomixer-sgRNA target site region was amplified by PCR using the Myomixer-Seq-F2/R primer pair (Table 1). The PCR products were analyzed by EarI digestion or Sanger sequencing. Indel mutations with a 16 bp deletion or 4 bp and 13 bp insertions near the sgRNA target site abolished the EarI site in the PCR product.

Whole Mount In Situ Hybridization

Whole-mount in situ hybridization was carried out using digoxigenin-labeled RNA antisense probes as previously described (Thisse and Thisse 2008). The *myod and myogenin* probes were described in previous publications (Weinberg et al. 1996; Du et al. 2008). The DNA template for *myomixer* probe synthesis was generated by PCR using MX-ATG-F1/R-situ-T7 primers (Table 1). The amplified *myomixer* DNA fragments were transcribed with T7

RNA polymerase to generate digoxigenin-labeled antisense probes using the MEGAscript T7 Transcription kit (AM1334, ThermoFisher). The images of in situ hybridization were acquired using a Leica dissecting microscope M12 equipped with a cool CCD digital camera (DX8; Olympus, Tokyo, Japan).

Hoechst 32258 and Antibody Staining

Whole mount staining with Hoechst 32258 and anti-Vinculin antibody was carried out on zebrafish embryos between 48–96 hpf as previously described (Jiao et al. 2021). Embryos were anesthetized and fixed in 4% paraformaldehyde for 1 h at room temperature. After washing clean by PBST (PBS containing 0.1% Tween-20), the embryos were digested with collagenase (Sigma C9891) (24 hpf with 1 mg/mL collagenase for 5 min, 48 hpf with 1.5 mg/ml collagenase for 40 min, 72 hpf with 2 mg/ml collagenase for 50 min, and 96 hpf with 5 mg/ml collagenase for 1 h and treated with cold acetone (10 min) to increase the permeability. The embryos were washed with PBST for 3 times (30 min each) and stained with Hoechst 32258 (SIG-B2883) or anti-Vinculin antibody (clone 7F9; MAB3574) for 1 h, and followed by TRITC conjugated anti-mouse secondary antibody (Sigma, T-7657). After washing with PBST for 3 × 30 min, trunk regions of fish embryos were dissected and mounted in Vectashield (Vector Labs, H-1000). Then using a Leica SP8 confocal microscope for the trunk muscles photographed.

Larval Locomotion Analysis

The *myomixer* heterozygous mutant fish were in-crossed to generate offsprings. Twenty-four larvae were randomly selected from the offsprings and placed individually in each well of a 24-well plate (12-well plate for 30 dpf juvenile fish). The fish larvae were kept at 28 °C with a photoperiod of 14 h light and 10 h dark. The locomotion of each larva was simultaneously tracked for 24 h with the DanioVision (Noldus Information Technology). The locomotion assays were performed on zebrafish larvae at 5, 15 and 30 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 h. All the results were expressed as mean ± SEM. The significance of mean swimming distance between WT, *myomixer*$^{\pm}$, and *myomixer*$^{-/-}$ mutant larvae was analyzed using a student's t-test. A P value of < 0.05 was considered as the level of significance.

HE Staining of Adult Zebrafish Muscle

Adult WT and homozygous mutants (4 month-old) were euthanized in MS-222. A 5 mm trunk muscle was dissected from the region close to the dorsal fin and fixed it in Bouin's solution for 24 h. The fixed tissues were washed with distilled water for 4 h at room temperature. After dehydration in ethanol gradients and xylene, the samples were embedded in paraffin. Paraffin Sects. (7 μm thickness) were generated and used for hematoxylin and eosin (HE) staining. The imagoes were photographed using a CCD digital camera (DX8; Olympus, Tokyo, Japan) under a light-microscope.

Single Muscle Fiber Isolation and Staining

The myofibers were isolated from adult zebrafish as previously described with slight modification (Ganassi et al. 2021). Briefly, adult fishes (6 months) were euthanized in ice-cold 0.3 mg/ml tricaine solution, then the fins, internal organs, skin, and head were removed to obtain two fillets. The freshly dissected fillets were digested in 2 mg/ml Collagenase in collagenase buffer (50 mM Tricine, 10 mM $CaCl_2$ and 400 mM NaCl) for 1.5 h at room temperature. The digested fillets were rinsed with PBST and transferred into a new Petri dish with PBST solution. By using a fire-polished glass pipette (3–4 mm diameter), we gently triturated the digested fillets for 10 min to dissociate the cells and obtain single myofibers. The isolated myofibers were placed on a slide coated with 0.1% Poly-L-lysine. The isolated myofibers were fixed for 15 min in 4% PFA and permeabilized with 0.5% Triton X-100 for 10 min. The myofibers were subsequently stained with 30 ng/ml of phalloidin-TRITC conjugate (P1951, Sigma) and 1 μg/ml of Hoechst 32258 (Shi et al. 2018). Finally, the fibers were mounted in Vector shield (Vector Labs, H-1000) and photographed using a Leica SP8 confocal microscope. The fiber size and number were measured and calculated with Image Plus 6.0 at 100 × magnification.

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Data Availability

All data generated or analyzed during this study are included in this published article.

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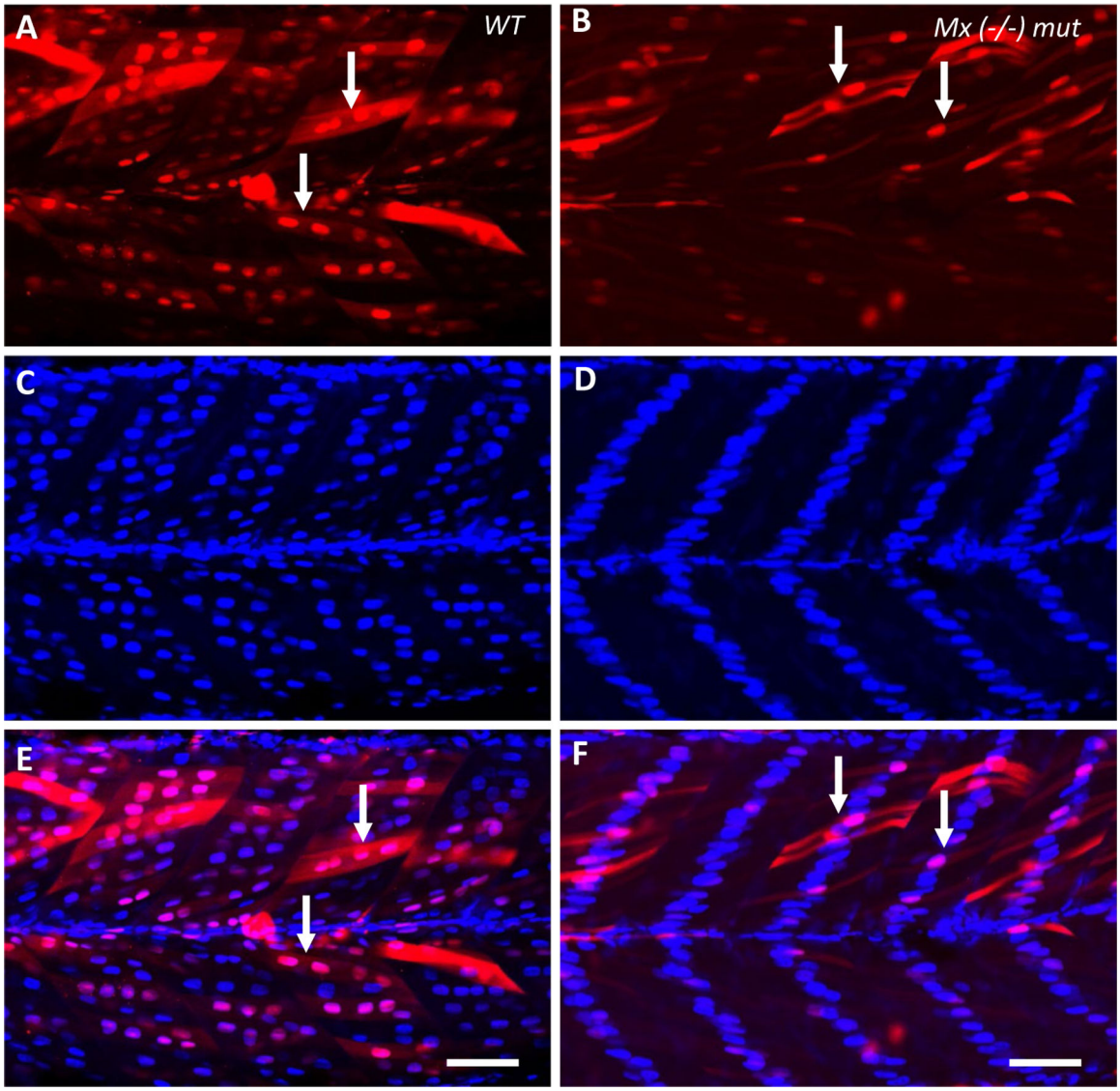

Fig. 1.

Characterization of myoblast fusion defect in *myomixer* mutant embryos using a nuclear mCherry reporter. The DNA construct, *pTol2-α-actin:mCherry-NLS*, was injected into fertilized eggs of wildtype (WT) and *myomixer* homozygous[Mx (−/−)] mutant embryos. Myoblast fusion was analyzed in the injected embryos at 48 hpf by nuclear mCherry localization (**A** and **B**; red) and nuclear labelling with Hoechst 32258 (**C** and **D**, blue). **E** and **F** are merged images of **A** and **C**, or **B** and **D**, respectively. Myotomes of WT control (**A**, **C**, **E**) contained mostly multinucleated myofibers (indicated by arrows) at 48 hpf. Myofibers in *myomixer* homozygous mutant (**B**, **D**, **F**) were mostly mononucleated fibers (indicated by arrows) with a central nuclear localization within the myotome. Scale bars (35 μm)

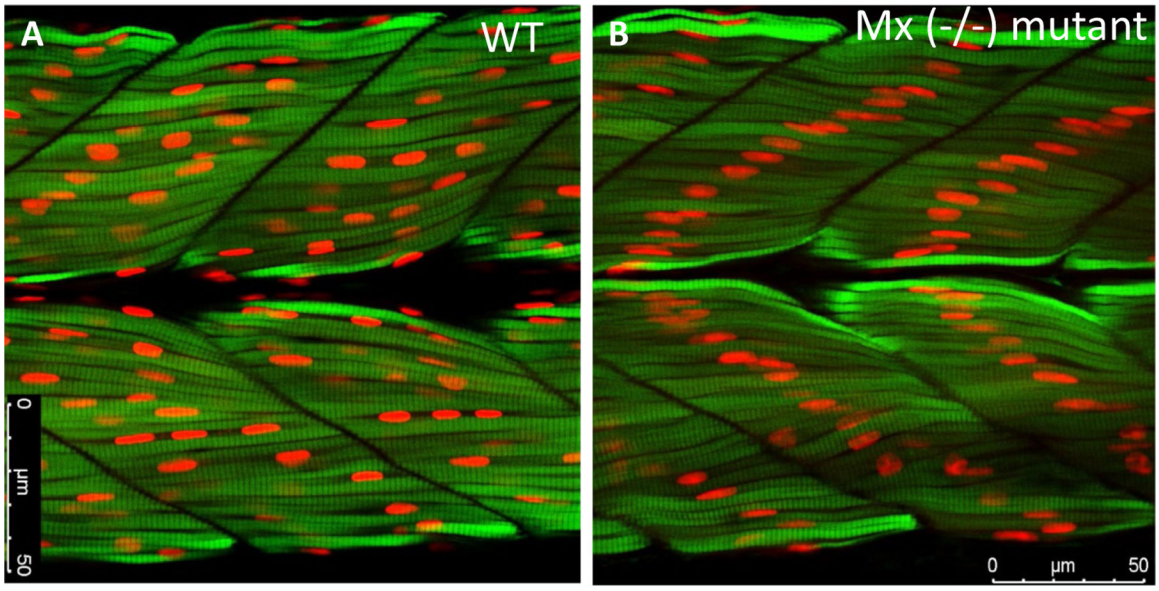

Fig. 2.

Generation of transgenic *myomixer* mutant model *expressing nuclear mCherry and Lifeact-GFP*. Transgenic zebrafish expressing the nuclear mCherry and Lifeact-GFP were generated using the *Tg(Tol2-α-actin:mCherry-NLS)* and *Tg(actc1b:Lifeact-GFP)* transgenes (**A**). The transgenic fish were subsequently crossed with *myomixer*^{sw101} mutant to generate the *Tg(Tol2-α-actin:mCherry-NLS);Tg(actc1b:Lifeact-GFP);mymx* transgenic mutant (**B**). Myonuclear localization and sarcomere organization were characterized in the WT and *myomixer* homozygous mutant transgenic embryos at 48 hpf. Compared with the multinucleated myofibers in the WT control (**A**), only mononucleated myofibers were found in the myomixer homozygous mutant (**B**). Scale bars (50 μm)

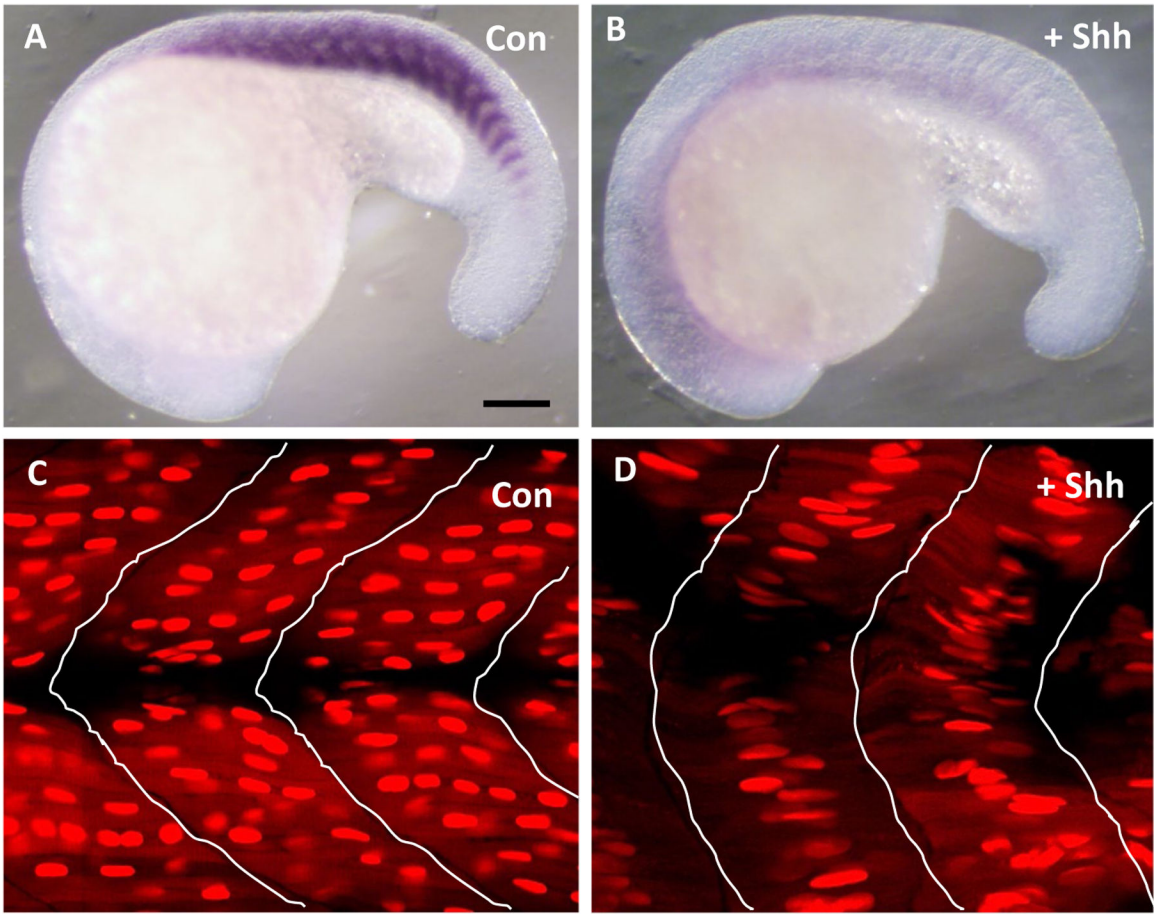

Fig. 3.

Shh overexpression inhibits myomixer expression and myoblast fusion. Whole mount in situ hybridization shows the expression of myomixer in un-injected control (**A**), or Shh mRNA injected (**B**) embryos at 20 hpf. Myonuclei distribution in myotomes of un-injected control (**C**), or Shh (**D**) injected *Tg(Tol2-α-actin:mCherry-NLS)* transgenic embryos at 48 hpf. These images were taken from representative embryos from approximately 30 embryos for each group. Scale bars: 120 μm in A, and 30 μm in C

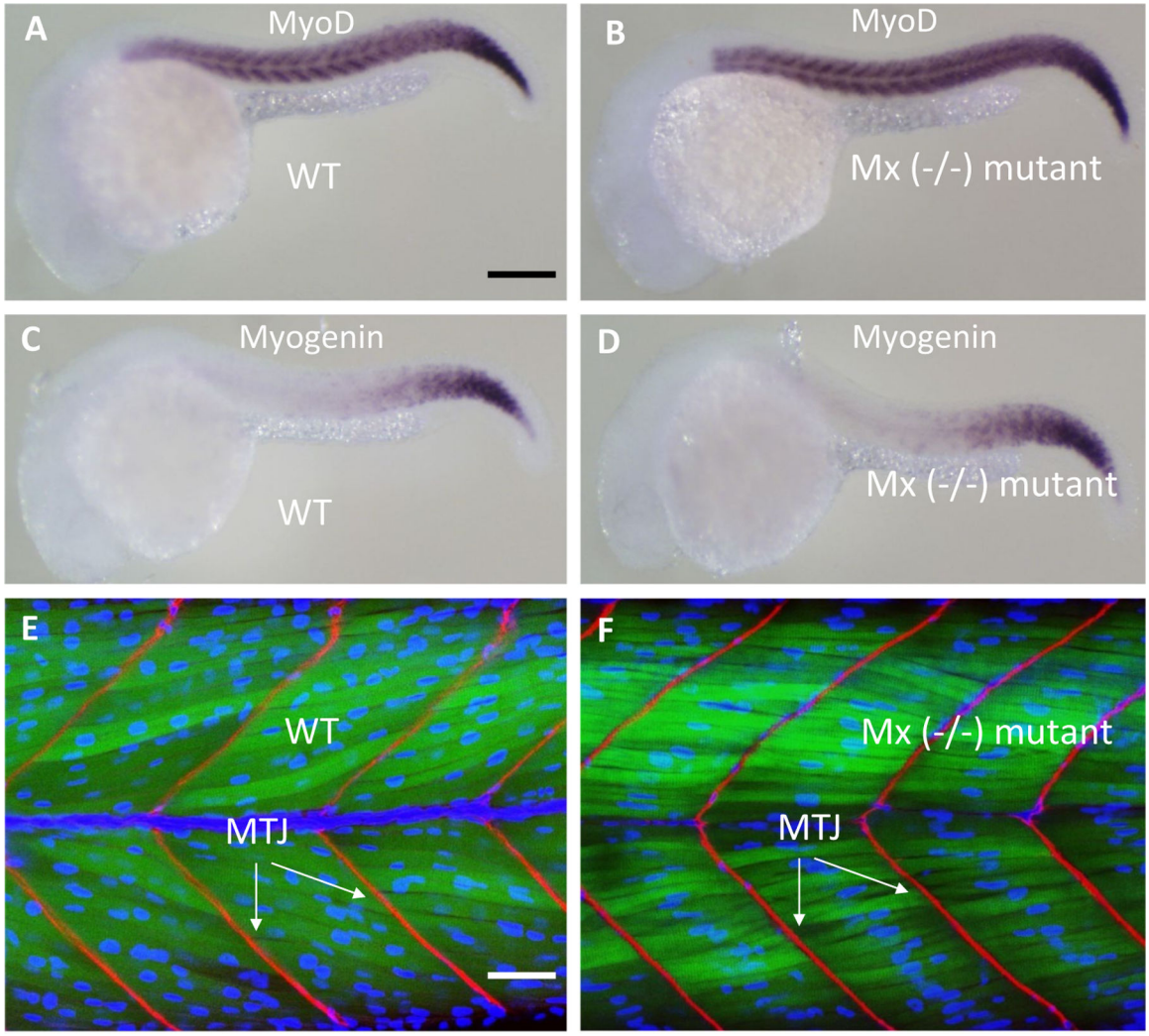

Fig. 4.

Myomixer knockout does not affect early myogenesis and formation of myotendinous junction. Whole mount in situ hybridization shows the expression of MyoD and Myogenin in WT (**A**, **C**) and *myomixer* homozygous mutant (**B**, **D**) embryos at 24 hpf. Immunostaining with anti-Vinculin antibody shows the myotendinous junctions (MTJ) in WT (**E**) and *myoixer* homozygous mutant (**F**) embryos at 96 hpf. The green background is from the expression of the *Tg(actc1b:Lifeact-GFP)* transgene in myofibers, while the blue staining represent myonuclei labeled with Hoechst 32258. These images were taken from representative embryos from approximately 40 embryos for each group. Scale bars: 120 μm in A, and 25 μm in E

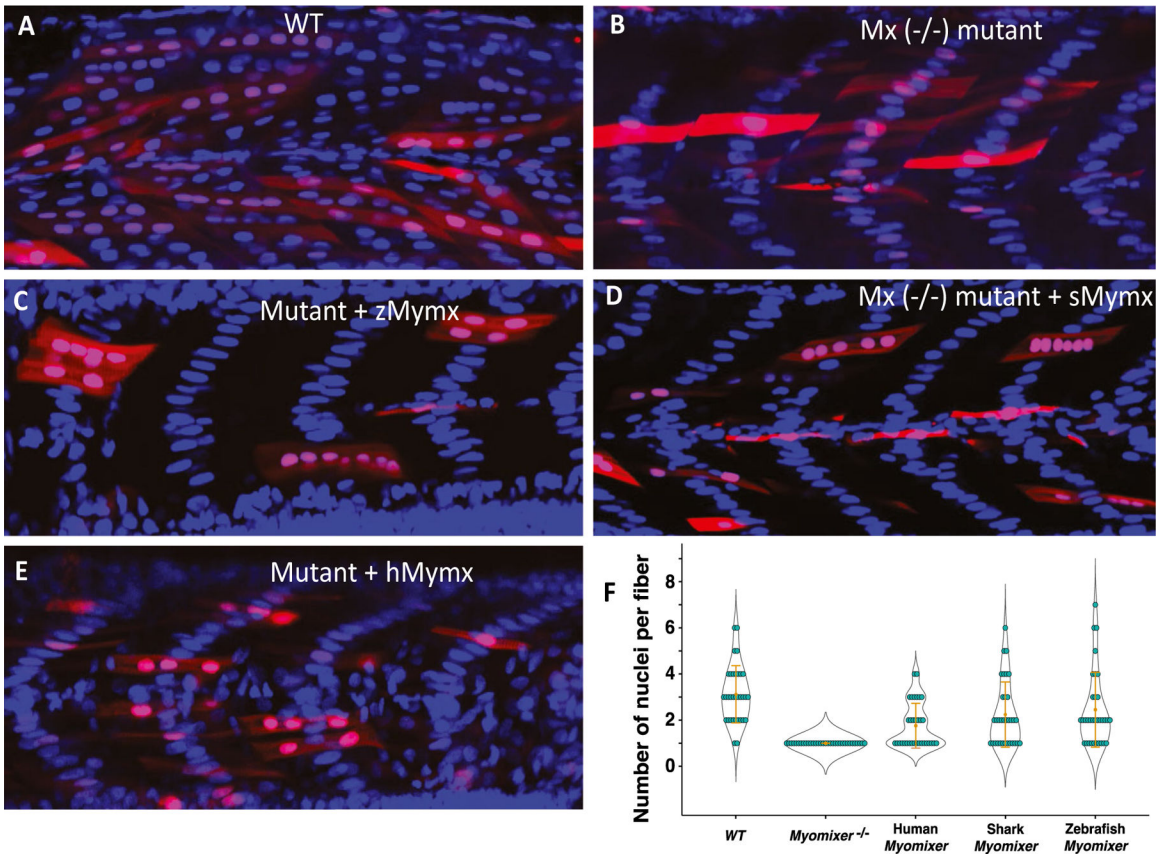

Fig. 5.

Mosaic rescue of myoblast fusion in *myomixer* mutant by ectopic expression of *myomixer* homologues from shark and human. A and B, WT (**A**) and homozygous mutant (**B**) embryos injected with the *pTol2-α-actin:mCherry-NLS* reporter construct alone. C-E, embryos injected with DNA constructs expressing zebrafish (zMymx; **C**), shark (sMymx; **D**) or human (hMymx; **E**) Myomixer. Scale bar (25 μm). Statistical analysis was performed to compare the numbers of nuclei in myofibers from multiple embryos for each group (n=33). Significant difference (P value < 0.005) was detected between mutant embryos injected with the *pTol2-α-actin:mCherry-NLS* reporter construct and embryos co-injected with the reporter and DNA construct expressing zebrafish, shark or human *myomixer* gene. Error bar are ± SD

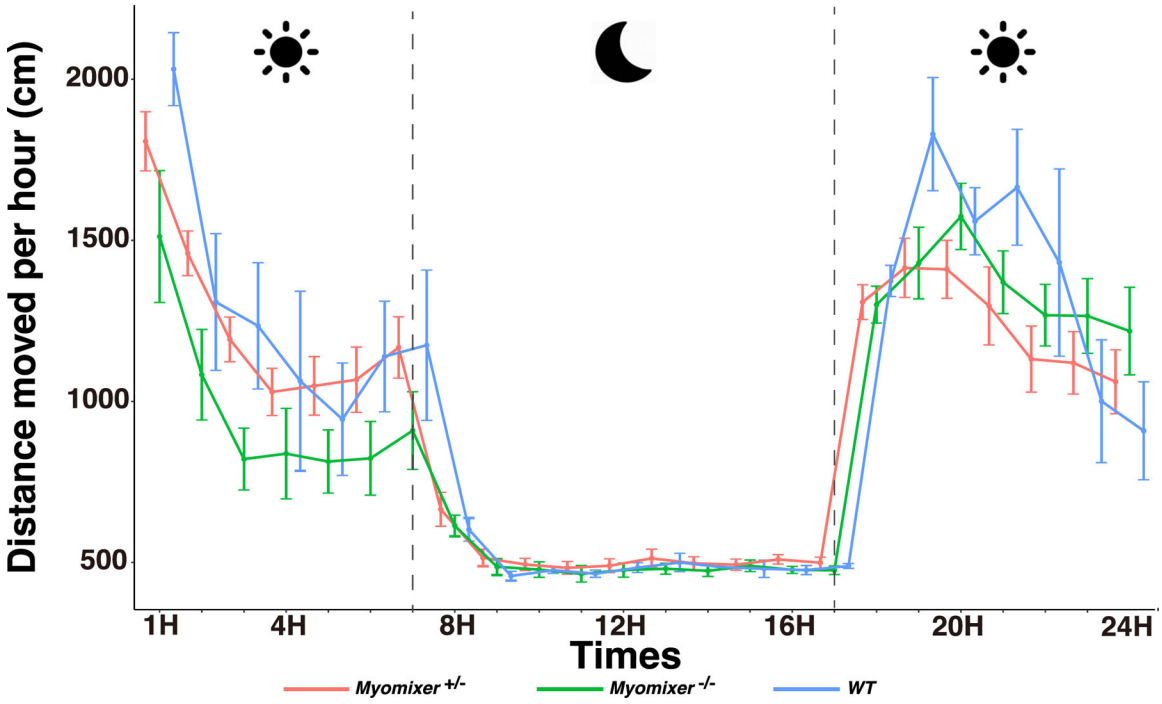

Fig. 6.

Larval locomotion analysis using DanioVision. The locomotion assays were performed on zebrafish larvae at 5 dpf on DanioVision. Individual fish larvae (n=24) of WT, *myomixer* heterozygous and homozygous mutants were placed in a 24 well plate and kept at 28°C in a DanioVision machine with a photoperiod of 14h light and 10h dark. The average swimming distance for each fish larvae was calculated for 24 hrs. The moving distance represents distance moved for each hour within the 24-hours period analyzed. The daytime and nighttime are indicated. Error bars are ± SEM

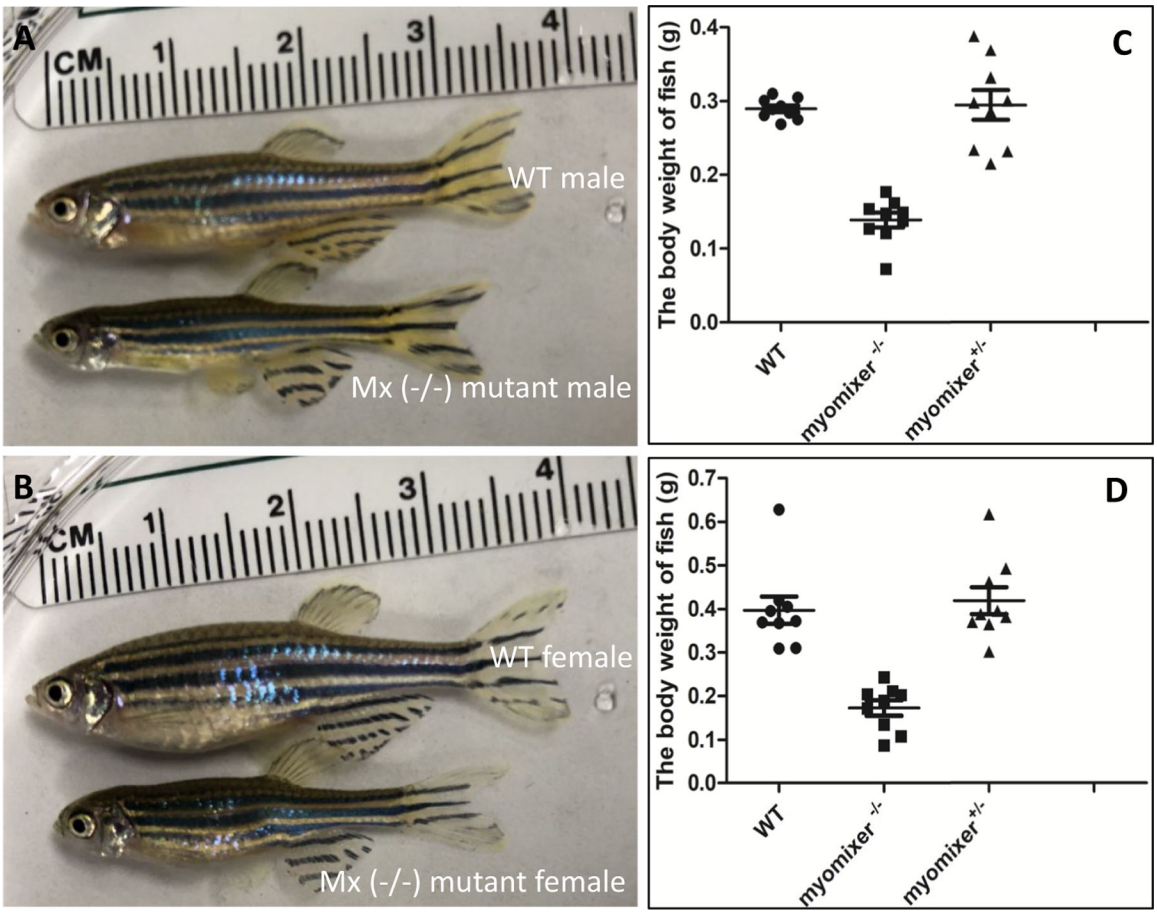

Fig. 7.

Myomixer mutants show reduced growth and body weight. Photographs of WT and *myomixer* homozygous mutant representative male (**A**) and female (**B**) fish at 120 dpf. (**C**) and (**D**). Body weight distribution and comparison among WT, heterozygous and homozygous mutant siblings

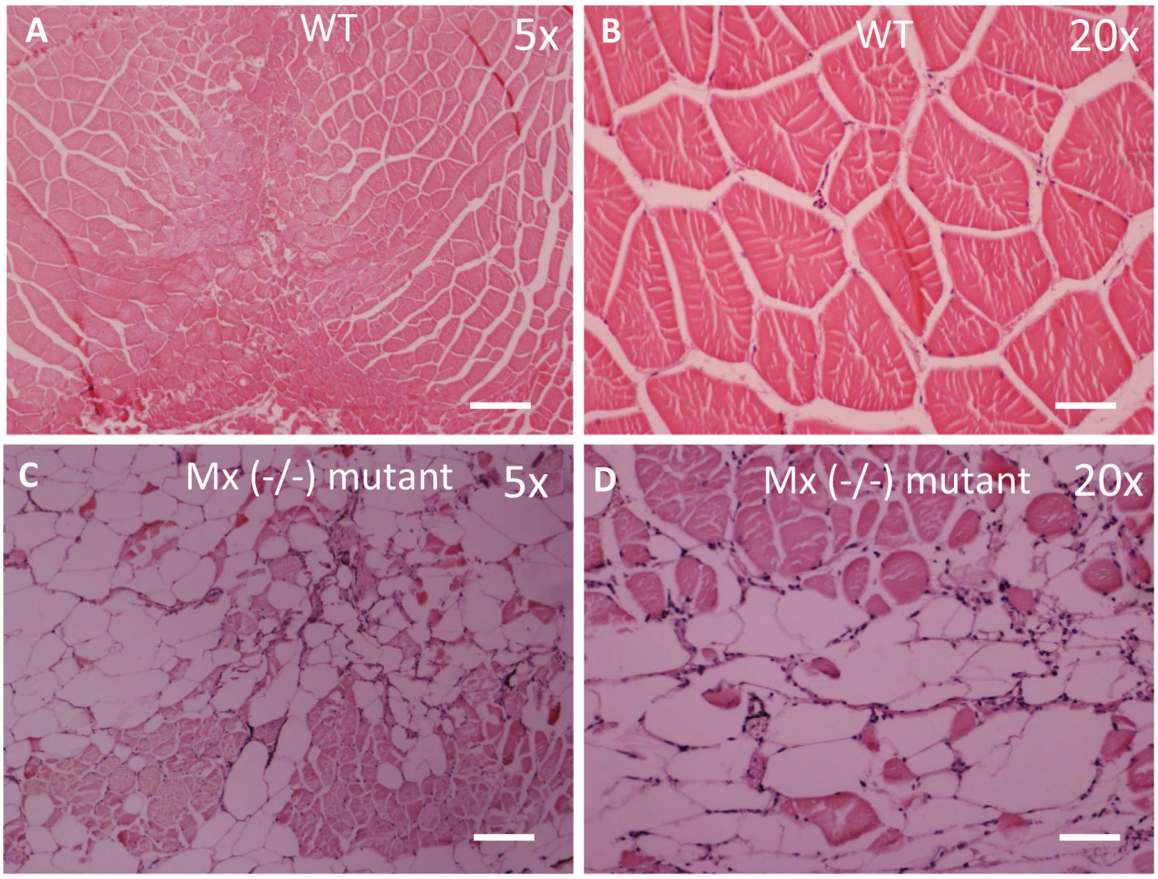

Fig. 8.

Myomixer mutants show severe myopathy in trunk skeletal muscle with increased adipocyte infiltration. HE staining of cross sections from trunk skeletal muscles in WT control (**A**, **B**) and *myomixer* homozygous mutant (**C**, **D**) fish at 120 dpf. Dramatic adipocyte infiltration was observed in the trunk skeletal muscle of the mutant fish. The pictures were taken using both 5x and 20x lenses as indicated. Scale bars: 160 μm in A and B, 40 μm in C and D

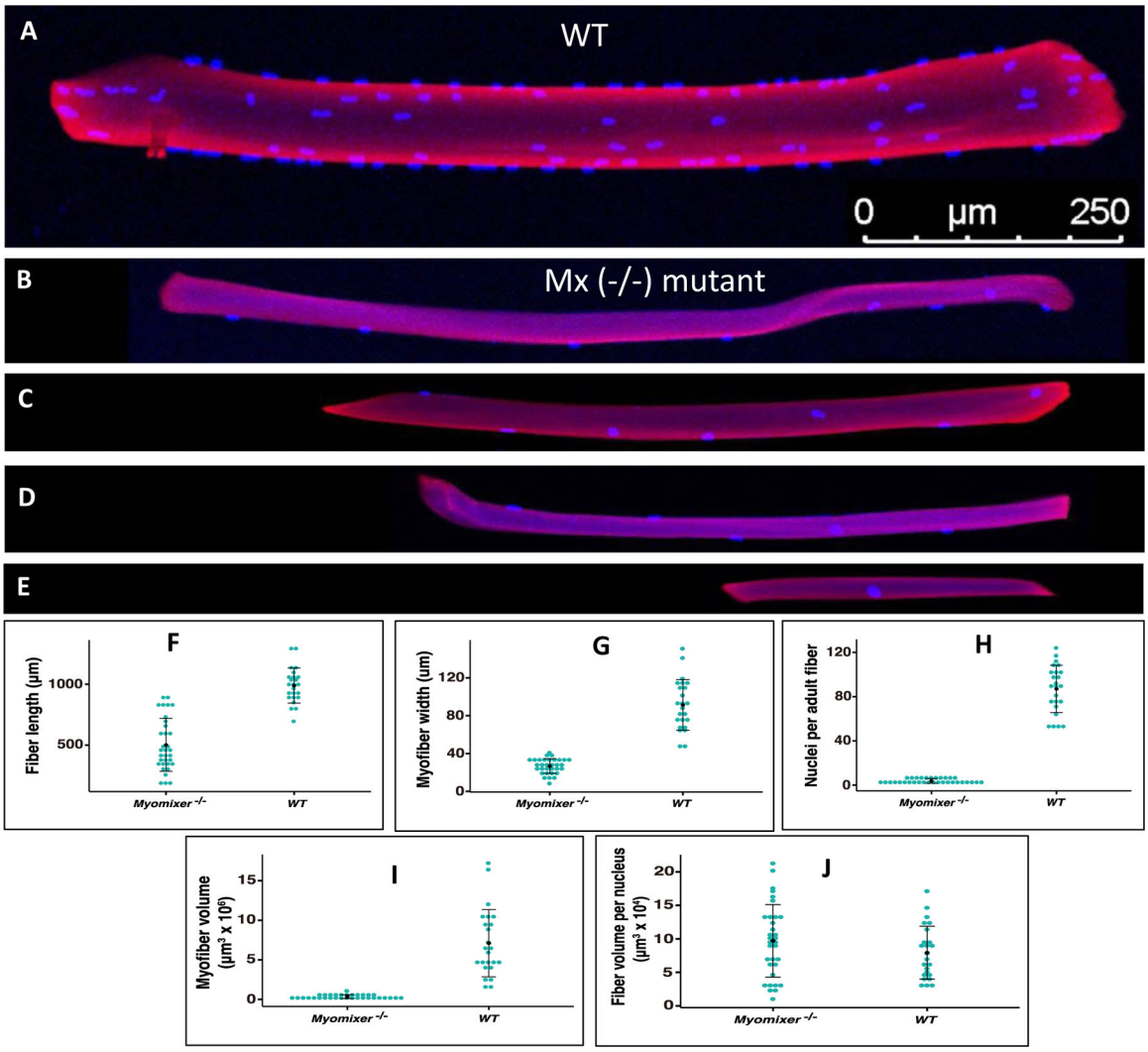

Fig. 9.

Single-fiber analysis of trunk skeletal muscles from WT and *myomixer* mutants. Single fibers were isolated from trunk skeletal muscles of WT and *myomixer* homozygous mutant at 120 dpf. The isolated fibers were stained with Hoechst 32258 and phalloidin-TRIC, and photographed by confocal microcopy. Representative WT myofiber (**A**) and *myomixer* mutant fibers of various sizes (**B-E**) are shown. Scale bar (250 μm). Twenty-four WT fibers and 33 mutant fibers were randomly selected for the analysis. Myofiber length (**F**), fiber width (**G**), number of nuclei per fiber (**H**), fiber volume (**I**) and volume per nucleus (**J**) were determined for each fiber analyzed. Significant differences were found between WT and homozygous mutant myofibers with respect to fiber length (**F**), width (**G**), the number of nuclei per fiber (**H**), and fiber volume (**I**), except the fiber volume per nucleus (**J**)

Table 1

Sequences of PCR primers

sgRNA or Primers	Sequences
zMyomixerPro-fusion-F1	5’- TTGGGCCCGGCTCGAGCATCTCTCTCTTTCAAGTTCAGAGCA -3’
zMyomixerPro-fusion-R2	5’- ATCCGTCGAGGAATTCGTCTGAGAGAGAGAGAGAGAGAGAAAGAG -3’
zMymx-fusion-F1	5’- ATTCCTCGACGGATCACCATGCCAGCCGTTTTCCTCTTGCTGC -3’
zMymx-fusion-R1	5’- ACCGCGGTGGCGGCCTCAGTTGTCCACCTTCTTGTGCATG -3’
sMymx-fusion-F1	5’ - ATTCCTCGACGGATCCACCATGTGCGCTCTACTATGTCTACGTG -3’
sMymx-fusion-R1	5’- ACCGCGGTGGCGGCCTCATTTGTCTGTGTCCCTTTTGTGC - 3’
actin:nRFP-ERI-F1	5’- TTTTGGCAAAGAATTACCGGTCGCCACCATGGTGAGCAAG - 3’
actin:nRFP-NotI-R1	5’- ACCGCGGTGGCGGCCTCAGTTATCTAGATCCGGTGGATCC - 3’
Myomixer-Seq-F2	5’- CTCAGTCATGCCAGCCGTTTTCCTC - 3’
Myomixer Seq-R	5’- GCGAACCGATCTGTCCTCAAGTCTG - 3’
MX-ATG-F1	5’- ATGCCAGCCGTTTTCCTCTTGCTGC -3’
MX-R-situ-T7	5’- GATCACTAATACGACTCACTATAGGTCAGTTGTCCACCTTCTTGTGCATG -3’

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