Supporting Information

SI Materials and Methods

Genomic DNA Isolation and Genotyping. Genotyping was performed using the AccuStart II PCR Genotyping Kit (Quantabio, 95135). Briefly, 25 μl Extraction Reagent was used to dissolve embryos by heating at 95 °C for 5 min. An equal volume of Stabilization Buffer was added after the samples cooled to room temperature. The following primers were used with 2.5 μl of extract in a 25 μl PCR reaction:

Forward: 5'-CAGAAACAGAGCGGTGACTTCATCCC-3',

Reverse: 5'-GCGAACCGATCTGTCCTCAAGTCTG-3'.

RNA Extraction and qPCR Analysis. Total RNA was extracted from a pool of 21 hpf zebrafish embryos. A total of 1 μg RNA was used for reverse transcription using the iScript™ Reverse Transcription Supermix (1708841). Gene expression was assessed using standard qPCR approaches with the KAPA SYBR FAST qPCR Master Mix (KK4605). Analysis was performed on a Step One Plus realtime PCR System (Applied Biosystems) with the following primers:

myomixer-1F: 5'-TCCGACTCTTCGGTAGTAGATT-3',

myomixer-1R: 5'-AGGATGGCTTCTTTGGACTG-3',

myomixer-2F: 5'-GACTGGGCATCTCGGAAC-3',

myomixer-2R: 5'-CCACCTTCTTGTGCATGTTAAG-3',

myomixer-3F: 5'-TCTCTGGTTGTCCGACTCTTC-3',

myomixer-3R: 5'-TGATCCGCTCCCAGATGTT-3',

myomixer-4F: 5'-GGTAGTAGATTAGCAGCGTCAG-3',

myomixer-4R: 5'-TCTTTGGACTGCTGTGAGC-3',

myomixer-5F: 5'-AACATCTGGGAGCGGATCA-3',

myomixer-5R: 5'-TGCATGTTAAGAAGGCACAGG-3',

myomaker-F: 5'-AGCACGGACTATCTGGACTA-3',

myomaker-R: 5'-GGCACGGTTTCTTGCATTAC-3',

myh3-F: 5'-GACTGAGAAGGCAGAGATTCAG-3',

myh3-R: 5'-CTCAAGCTGGACACGAAGAA-3',

myh4-F: 5'-ACTGCAGGATCTGGTTGATAAG-3',

myh4-R: 5'-AACTTGGACAGGTGAGAGTTG-3'.

The $2^{\Delta\Delta}$Ct method was used to analyze the relative changes in gene expression normalized against the 18S rRNA expression.

Generation of Myomixer Knockout C2C12 Cells. CRISPR-lentivral vectors carrying Cas9 and myomixer sgRNAs (9) were used to package lentiviruses with psPAX2 and pMD2.G vectors in 293 cells. C2C12 cells were infected by the lentiviruses for two days, followed by another two days of puromycin selection (2 μg/ml). After selection, the cells were switched to culture medium and passed for several passages to allow complete knockout of the gene. psPAX2 and pMD2.G vectors were gifts from Didier Trono (Addgene plasmid #12260 and #12259).

Membrane Fractionation. HEK293 cells were transfected with a retrovirus plasmid expressing a C-terminally Myc-tagged zebrafish myomixer. Cells were suspended in PBS by scraping off the surface of the plate with a cell scraper 48 hours post transfection. After a brief centrifuging at 300 × g for 5 minutes, the cell pellets were washed twice and membrane fractionation was performed with the Mem-PERTM Plus Membrane Protein Extraction Kit (ThermoFisher, # 89842).

Western Blot Analysis. Protein was isolated from cells using the RIPA buffer. Protein concentrations were determined using the BCA Protein Assay Reagent (ThermoFisher Scientific, 23225) followed by measurement with NanoDrop. Protein samples were mixed with 4x Laemmli sample buffer (BIORAD, #161-0747) and 20-40 µg protein was loaded and separated by Mini-PROTEAN® TGX™ Precast Gels and transferred to a polyvinylidene fluoride (PVDF) membrane (Millipore). The PVDF membrane was blocked in 5% fat-free milk for 1 hour at room temperature and then incubated with the following primary antibodies diluted in 5% milk overnight at 4°C: anti-Gapdh (Thermofisher, MA5-15738), anti-N-Cadherin (Santa Cruz Biotechnology, sc7939), anti-α-Tubulin (Sigma, T-6199), anti-myomixer (R&D Systems, #AF4580), and anti-Myc (BD Pharmingen™, 51-1485GR). The following HRP-conjugated secondary antibodies diluted at 1:5,000 in 5% milk were used: donkey anti-sheep IgG-HRP (Santa Cruz Biotechnology, sc-2473), goat anti-mouse IgG (H + L)-HRP Conjugate (BIO-RAD, #170-6516) and goat anti-rabbit IgG (H9+L)-HRP Conjugate (BIO-RAD, #170-6515). Color development was performed using

the Western Blotting Luminol Reagent (Santa Cruz Biotechnology, sc2048) or the West Dura Extended Duration Substrate (Thermofisher, 34075).

Immunofluorescent Staining. Cells were fixed in 4% PFA (diluted in PBS) for 10 minutes at room temperature, permeabilized with 0.1% Triton X-100 in PBS and blocked with 3% BSA in PBS for 1 hour at room temperature. Cells were then incubated with anti-Myosin primary antibody (Sigma, M4273) overnight at 4°C, followed by incubation with Alexa-Fluor conjugated secondary antibody (ThermoFisher, A21422) and Hoechst for 45 minutes at room temperature. Cells were washed three times (10 minutes each) with PBS with gentle shaking on a horizontal shaker. Confocal images of representative areas were taken.

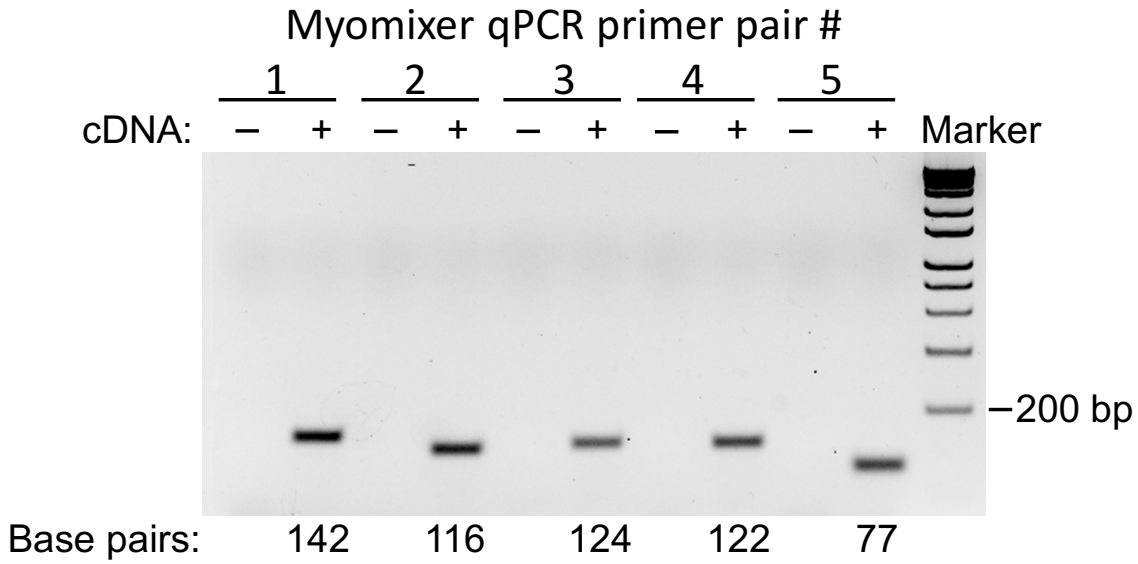

Fig. S1. Validation of the myomixer qPCR primers.

DNA gel electrophoresis analysis of reverse transcription PCR products to verify the myomixer primers. cDNA was synthesized from RNA extracted from 19.5 hpf zebrafish embryos.

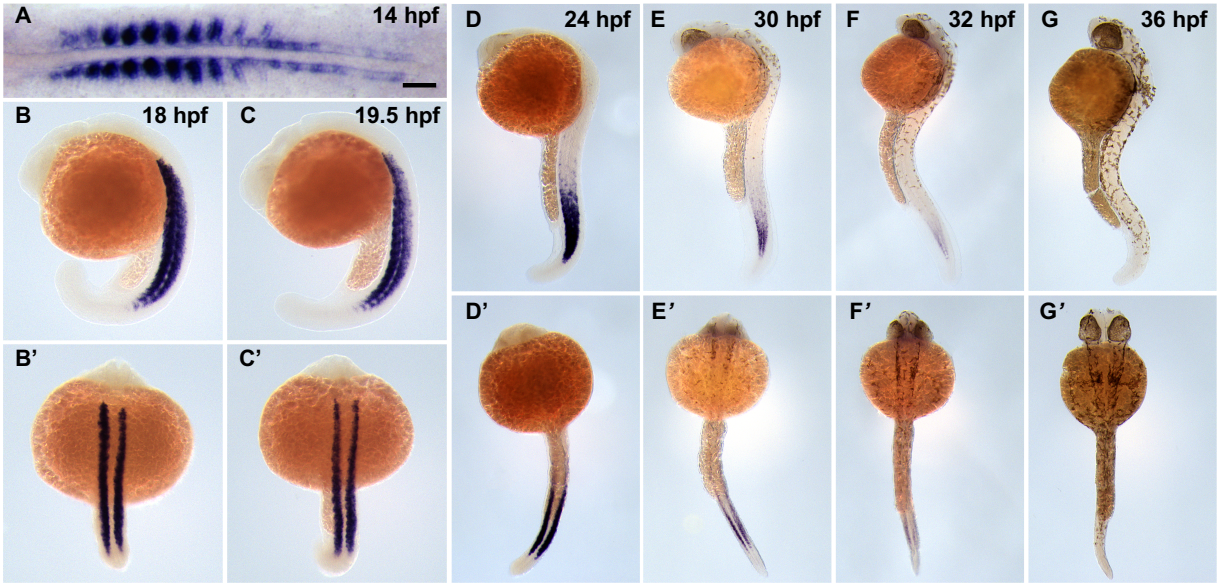

Fig. S2. In situ hybridization of myomaker during zebrafish embryogenesis. The stages of the embryos are indicated in each panel. Two views of the same embryo are shown for those older than 18 hpf. Scale bar, 50 μm.

Fig. S3. Presence of conserved putative MyoD binding sites in the myomixer promoter region.

Alignment of fugu and zebrafish myomixer genomic sequences (fugu: 2953 bp; zebrafish: 2936 bp). Identical nucleotides were marked with star (*). The predicted open reading

frame is in blue. Three consensus MyoD binding sites (CANNTG) in red are identified in the proximal promoter region of the two myomixer promoters.

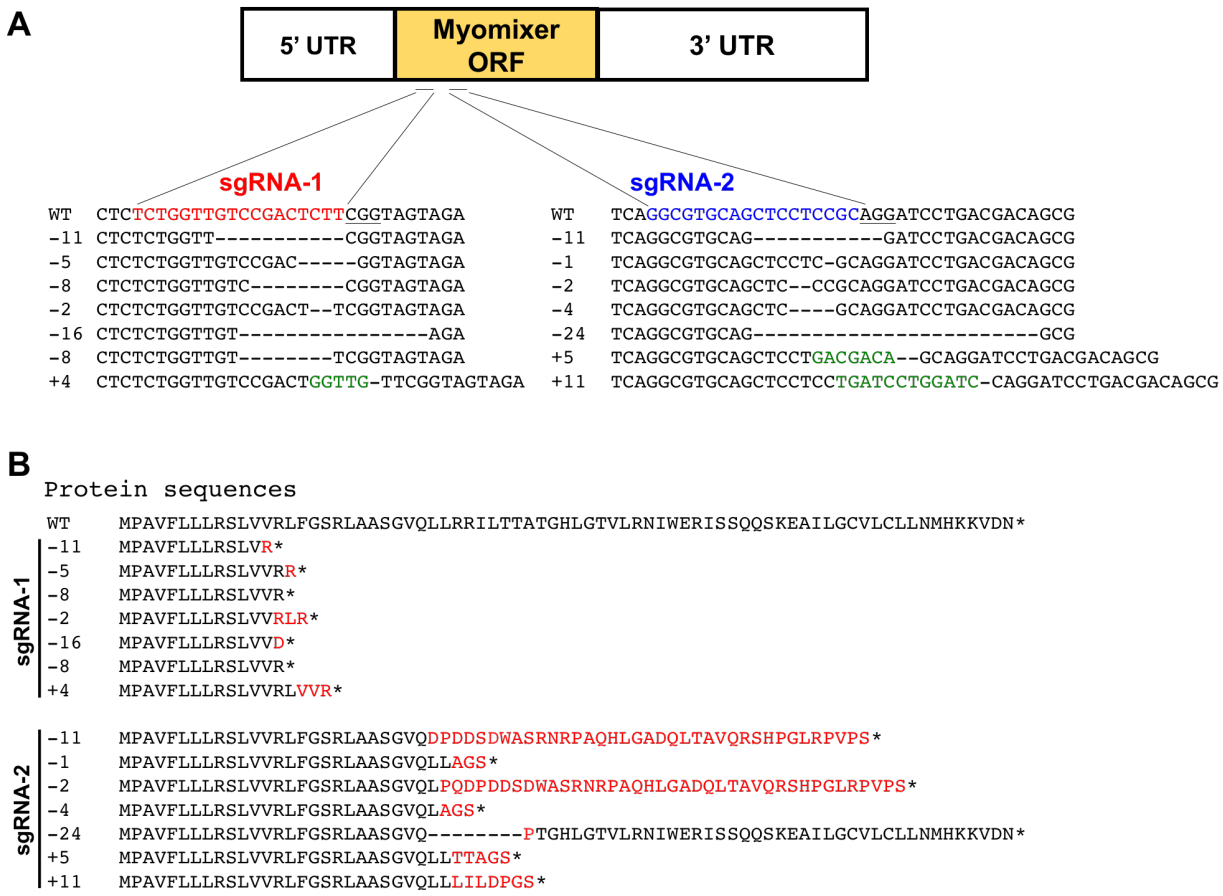

Fig. S4. Cas9/CRISPR mutagenesis of myomixer.

(*A*) DNA sequences of the PCR products amplified from wild-type and F0 zebrafish. Two sgRNAs (sgRNA-1 in red and sgRNA-2 in blue) were injected individually with Cas9 and both generated indels efficiently. The protospacer adjacent motif (PAM) sequence is underlined. Green letters denote the nucleotides inserted through non-homologous end joining (NHEJ). (*B*) Protein sequences of wild-type and mutated myomixer ORFs encoded by the myomixer sequences shown in (*A*). Amino acids marked in red represent mutated sequences. * represents stop codon.

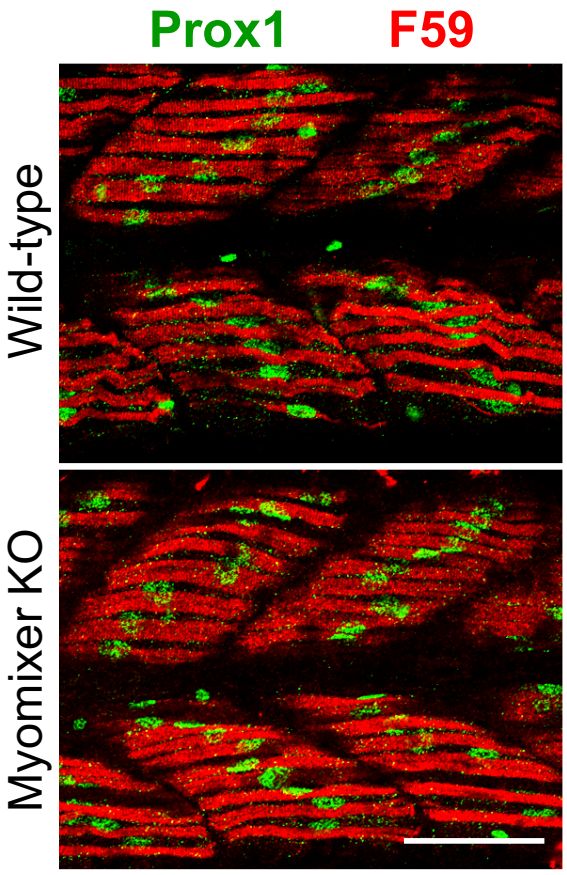

Fig. S5. Inactivation of myomixer does not affect slow muscle formation.

Confocal images of 48-hpf wild-type and myomixer KO embryos stained with anti-slow muscle nuclei (Prox1) and anti-slow muscle Myosin (F59) antibodies. Scale bars, 50 μm.

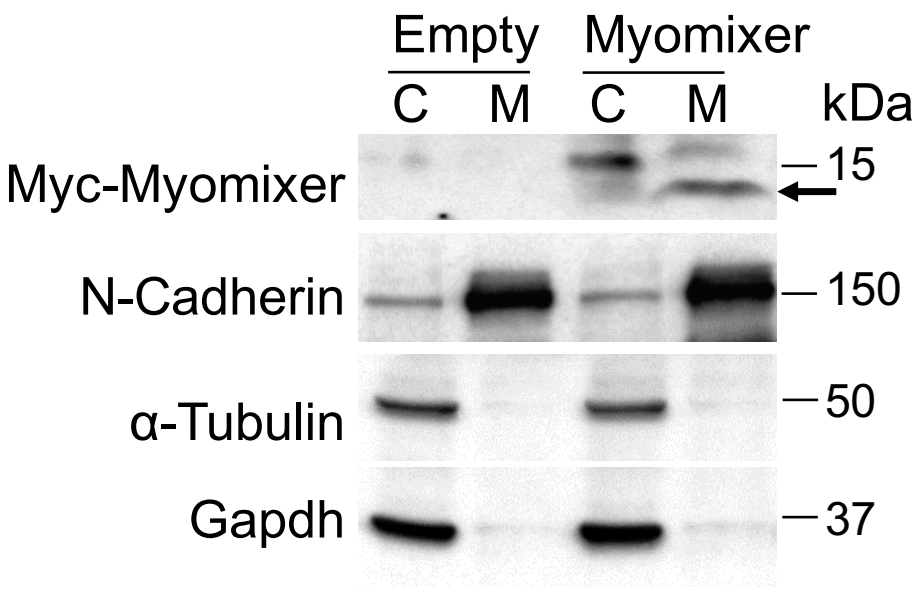

Fig. S6. Zebrafish myomixer is present in the membrane fraction.

Western blot analysis of cytosolic (C) and membrane (M) fractions of C-terminally Myc-tagged zebrafish myomixer expressed in HEK293 cells. N-Cadherin was used as a positive control for the membrane fraction. α-Tubulin and Gapdh were used as positive controls for the cytosolic fraction.

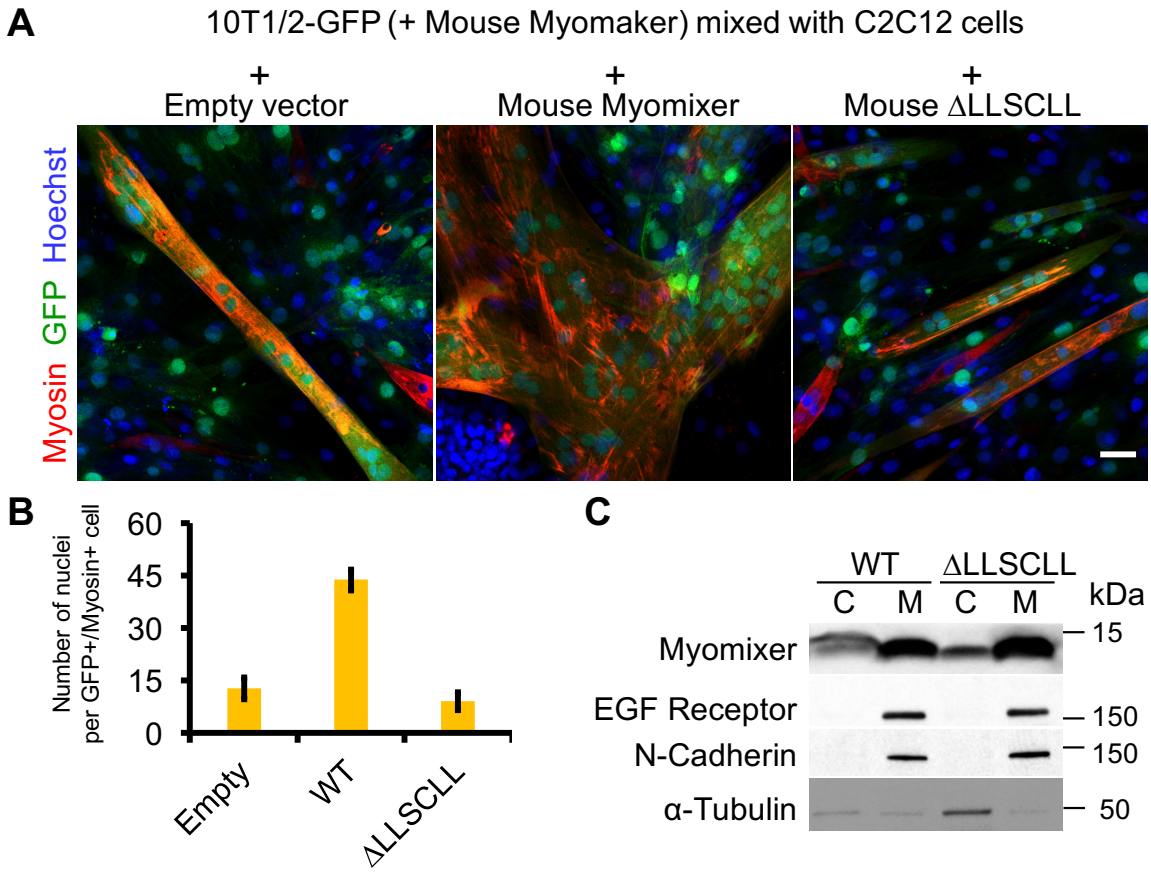

Fig. S7. Functional analysis of mouse myomixer mutant in 10T1/2-C2C12 heterologous fusion assay.

(*A*) 10T1/2-GFP fibroblasts were infected by retroviruses expressing mouse myomaker with or without wild-type or mutant mouse myomixer, and mixed with C2C12 cells for heterologous fusion following one-week of differentiation. Cells were stained with anti-Myosin and Hoechst. Scale bar, 50 μm. (*B*) Western blot of cytosolic (C) and membrane (M) fractions of mouse wild-type and mutant myomixer (ΔLLSCLL) in retrovirus infected 10T1/2 fibroblasts. EGF receptor and N-Cadherin were used as positive controls for membrane proteins, and α-Tubulin was used as a positive control for cytosolic proteins.