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Supplementary Material for

Control of muscle formation by the fusogenic micropeptide myomixer

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Materials and Methods

Generation of Lentivirus Library and Infection of C2C12 Cells

Mouse GeCKOv2 CRISPR knockout pooled library was a gift from Feng Zhang (Addgene plasmid # 1000000052) (*12*). For library lentivirus production, 1.8 x 10^7 Lenti-X 293T cells (Clontech, Cat. No. 632180) were placed in 15 cm tissue culture plates in 25 ml of DMEM (containing 1% penicillin/streptomycin, 10% FBS). Twelve hr later, transfection was performed using FuGENE6 (Promega, #E2692) with 16.9 μg of psPAX2 plasmid, 5.6 μg of pMD2.G plasmid and 22.5 μg of GeCKO plasmid library. Two days later, lentivirus-containing supernatants were filtered through a 0.45 μm filter and concentrated with Lenti-X Concentrator (Clontech, PT4421-2) following manufacturer's protocol. psPAX2 vector was a gift from Didier Trono (Addgene plasmid # 12260). pMD2.G vector was a gift from Didier Trono (Addgene plasmid # 12259).

A portion of the freshly made lentivirus was used to measure the titer. Briefly, C2C12 cells were infected with different volumes of viral stock for two days, followed by another two days of puromycin selection (2 μg/ml). Cell survival was measured and referred to as the infection rate. Based on the virus titer, an adjusted amount of virus stock was used to infect C2C12 cells for a target MOI of 0.2, ensuring that most cells incorporated less than 2 lentivirus particles. Two days after infection, puromycin (Invitrogen) was added at a final concentration of 2 μg/ml to select infected cells for two days. After selection, the cells were switched to 2% horse serum in DMEM (containing 1% Penicillin/Streptomycin) to differentiate for one week with fresh medium changed at day 3 of differentiation. After differentiation, the myotubes were detached from the plates by incubation for 5 minutes with 0.00625% trypsin (diluted in PBS) in 37°C, and the plates were washed gently twice with PBS, from which the detached cells were combined and centrifuged at

300 x g for 5 minutes. The myoblasts were then detached with 0.25% trypsin and washed twice with PBS to collect all the cells, which were spun down at 300 x g for 5 minutes.

Genomic DNA Purification and Library Preparation for Readout Sequencing

Genomic DNA from myoblasts and myotubes was purified using MasterPure™ DNA Purification Kit (MC85200) according to manufacturer's instructions. We performed two successive PCR reactions (18 cycles for the first and 12 cycles for the second reaction) to add barcodes, stagger and Illumina sequencing primers using Herculase II Fusion DNA Polymerase (Agilent, #600675) (*12*). PCR products were purified by Agencourt AMPure XP magnetic beads and the DNA was analyzed by Qubit Fluorometric Quantitation. The purified PCR product was sequenced on a Hi-seq 2500 with 75 bp single reads.

Sequencing Data Analysis

A reference list for GeCKO sgRNA sequences in the library was downloaded from Addgene (https://www.addgene.org/pooled-library/zhang-mouse-gecko-v2/) and demultiplexed FASTQ files were mapped to the reference file using Bowtie 2 requiring unique alignments with no mismatches. The sgRNA quantification and gene score calculation was done using sgRSEA (https://bchen4.github.io/sgrsea/).

Myomixer CRISPR sgRNA Knockout Experiments in Cultured Cells

Two guides (#1: 5'-GCTGCTGCCTGTTGCCCGCC-3', and

#2: 5'-AGGCCTCTCCAGAATCCGG-3') that target the Myomixer ORF region were individually cloned into the lenti-CRISPR v2 vector, a gift from Feng Zhang (Addgene plasmid # 52961) (*12*). Myomixer CRISPR-sgRNA and -control (empty lenti-CRISPR v2) lentivirus were made and used to infect C2C12 or primary myoblast cells followed by puromycin selection with a similar procedure performed for the lentivirus CRISPR library. After selection, the cells were switched to differentiation medium for one week followed by immunostaining, RNA, protein and genomic DNA extraction and analysis. To verify targeting of Myomixer in the C2C12 cells, genomic DNA from the mixed cell populations was amplified with the primers that amplify the targeting region (Forward: 5'-AGTTCAGGCTTCAGGTCAGAG-3', Reverse: 5'-GCTAGGGGAGTGGGAACTGT-3', PCR product size was 743 bp for non-targeted cells). PCR products were gel purified and cloned into pCRII Topo vector (ThermoFisher, K460001) and sequenced.

Quantitative Real-time PCR (qPCR)

Total RNA was extracted from cultured cells or mouse tissue with TRIZOL (Invitrogen) and cDNA was synthesized using iScript™ Reverse Transcription Supermix (1708841). Gene expression was assessed using standard qPCR approaches with KAPA SYBR FAST qPCR Master Mix (KK4605). Analysis was performed on a StepOnePlus realtime PCR System (Applied Biosystems) with the following Sybr primers:

Myomixer-F: 5'-GTTAGAACTGGTGAGCAGGAG-3',

Myomixer-R: 5'-CCATCGGGAGCAATGGAA-3'.

Myomaker-F: 5'-CCTGCTGTCTCTCCCAAG-3',

Myomaker-R: 5'-AGAACCAGTGGGTCCCTAA-3'.

Myod1-F: 5'-CCACTCCGGGACATAGACTTG-3',

Myod1-R: 5'-AAAAGCGCAGGTCTGGTGAG-3'.

Myogenin-F: 5'-GAGACATCCCCCTATTTCTACCA-3',

Myogenin-R: 5'-GCTCAGTCCGCTCATAGCC-3'.

Myh8-F: 5'-GGAGAGGATTGAGGCCCAAAA-3',

Myh8-R: 5'-CACGGTCACTTTCCCTCCATC-3'.

18S-F: 5'-ACCGCAGCTAGGAATAATGGA-3',

18S-R: 5'-GCCTCAGTTCCGAAAACCA-3'.

The 2ΔΔCt method was used to analyze the relative changes in gene expression normalized against 18S rRNA expression.

Pax7+ and Twist2+ Muscle Progenitor Cell Isolation and RNA-seq Analysis

Twist2+ muscle progenitor cells are distinct from Pax7+ satellite cells and represent a population of myogenic progenitor cells that contributes to muscle homeostasis and regeneration (*14*). Twist2+ muscle progenitor cells were freshly isolated by FACS sorting from the skeletal muscle tissues of 8-week-old Twist2-CreERT2; R26-tdTO mice 10 days post-Tamoxifen injection. Pax7+ satellite cells were isolated by FACS sorting as the integrin-7-positive and CD45/CD31/Sca1-negative population. RNA quality was verified by the Agilent 2100 Bioanalyzer and RNA-seq was performed using Illumina HiSeq 2500 by UTSW Genomics and Microarray Core Facility.

Retroviral Expression

Myomixer coding sequence oligonucleotides were synthesized by Integrated DNA Technologies and ligated to the pMXs- Puro Retroviral Vector (Cell Biolabs, # RTV-012) (*24*) with In-Fusion® HD Cloning Plus Kit (Clontech, #638910). Myomixer$^{\Delta C}$ is a 19 amino acid deletion of the C-terminus of mouse Myomixer. After verifying the correct insertion by sequencing, the plasmids were amplified in Stable3 cells in an overnight culture, followed by plasmid preparation using NucleoBond® Xtra Maxi Columns (#740414.10). A retrovirus plasmid that expresses the N-terminus tagged Signal FLAG (SF) Myomaker was used to overexpress Myomaker (*18*), except for the 10T1/2 homologous fusion experiments, in which the un-tagged Myomaker was used. Ten micrograms of retroviral plasmid DNA were transfected using FuGENE 6 (Promega, #E2692) into Platinum-E cells (Cell Biolabs, #RV-101) which were plated on a 10 cm cell culture dish at a density of 5×10^6 cells per dish 12 hours before transfection. Two days after transfection, viral medium was filtered through a 0.45 μm cellulose syringe filter, and mixed with polybrene (Sigma) at a final concentration of 6 μg/ml. One day post-infection, the cells were washed twice with PBS and changed to fresh culture medium for 24 hours before used for mixing or differentiation experiments. For fusion experiments, virus infected C2C12 and 10T1/2 cells were mixed (5×10^4 C2C12 cells and 2×10^5 fibroblasts per well) and plated onto a 6-well plate. Twelve hours after plating, the cells were switched to myoblast differentiation medium (2% horse serum in DMEM with 1% penicillin/streptomycin) for one week with a medium change at day 3 of differentiation.

Cell Cultures and Fluorescent Protein Labelling

10T1/2 fibroblasts and C2C12 cells were maintained in 10% FBS with 1% penicillin/streptomycin in DMEM. Lentiviruses expressing GFP and retroviruses expressing mCherry were made as

described above. The packaging vector for lentiviral GFP is pLove-GFP, a gift from Miguel Ramalho-Santos (Addgene plasmid # 15949) (*25*) and retroviral mCherry is pMXs-mCherry (Cell Biolabs, #RTV-012). Twelve hours before infection, the cells were seeded in 10-cm dish at a 2 × 10^6 density. Cells were infected for two days before used for experiments.

Membrane Fractionation

Membrane fractionation was performed with the Mem-PERTM Plus Membrane Protein Extraction Kit (ThermoFisher, # 89842). Briefly, the cells were suspended in PBS by scraping off the surface of the plate with a cell scraper. After centrifuging the cell suspension at 300 × g for 5 minutes, the cell pellets were washed twice and permeabilized with constant mixing for 10 minutes at 4°C. The cytosol fraction (supernatant) was collected after 15 minutes centrifugation at 16,000 × g at 4°C. The membrane fraction (pellets) were resuspended and solubilized at 4°C for 30 minutes with constant mixing. The membrane fraction was collected as the supernatant after 16,000 × g centrifugation for 15 minutes at 4°C. The protein samples were mixed with 4x Laemmli sample buffer and analyzed by western blot analysis.

Immunoprecipitation and Western Blot Analysis

Cells were washed with and resuspended in ice-cold PBS, and lysed in 1 ml IP-A buffer (50 mM Tris, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, pH 7.4) supplemented with complete protease inhibitor (Sigma) and PhoSTOP phosphatase inhibitor (Sigma) for 15 minutes. Cell lysates were disrupted mechanically by passing them through 25G 5/8 needles 10 times and then constantly mixed for 1 hour at 4ºC. For input controls, 10% of the total cells were lysed in RIPA

(Sigma, R0278) buffer for 1 hour at 4ºC with constant mixing. Lysates were then centrifuged at 20,817 x g for 15 minutes and the pellet was discarded. Anti-FLAG M2 affinity resin (Sigma, A2220) was washed three times in 0.1M glycine-HCl (pH 3.5), followed by three times in TBS buffer wash, and equilibrated in IP-A buffer according to manufacturer's instructions. We used 100 µl resin per sample (15-cm dish at full confluency). Co-immunoprecipitation was performed in a total volume of 1.2 ml at 4 ºC under constant mixing for 16 hours. Resin was washed in high salt (50 mM Tris, 700 mM NaCl, 1 mM EDTA) buffer, followed by three washes in 50 mM Tris. Elution was performed with FLAG peptide (Sigma, F3290) at a final concentration of 200 µg/ml in IP-A buffer for 16 hours with constant mixing at 4 ºC, followed by a 2-hour incubation at room temperature under constant mixing. The elution was mixed with loading buffer and incubated at 37 ºC for 5 minutes and analyzed by SDS-PAGE western blot as described below.

Protein was isolated from cells or tissue using RIPA buffer. Protein concentrations were determined using BCA Protein Assay Reagent (ThermoFisher Scientific, 23225) followed by measurement with NanoDrop. Protein samples were mixed with 4x Laemmli sample buffer (BIO-RAD, #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), 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: Gapdh (Thermofisher, MA5-15738), N-Cadherin (Santa Cruz Biotechnology, sc7939), Insulin Receptor β (Cell Signaling Technology, #3020), EGF Receptor (Cell Signaling Technology, #2646), Tubulin (Sigma, T-6199), Myomixer (R&D Systems, #AF4580), FLAG (Sigma, F3165), Myosin (Sigma, M4273). The HRP-conjugated secondary antibodies: 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 (H

+ L)-HRP Conjugate (BIO-RAD, #170-6515) were diluted at 1:5,000 in 5% milk. Immunodetection was performed using Western Blotting Luminol Reagent (Santa Cruz Biotechnology, sc2048) or West Dura Extended Duration Substrate (Thermofisher, 34075).

Immunostaining

Cells were fixed in 4% PFA/PBS for 10 minutes, and permeabilized with 0.2% Triton X-100 in PBS and blocked with 3% BSA/PBS for 1 hour at room temperature. Cells were incubated with primary antibody overnight at 4°C, followed by incubation with Alexa Fluor conjugated secondary antibody as indicated below.

For live cell membrane-staining, cells were first washed with ice-cold PBS and blocked in 3% BSA/PBS for 15 min on ice. Primary antibody incubation was then performed using antibody against FLAG (M2 mouse anti-FLAG sigma, F3165 with a dilution of 1:500 in ice cold 1% BSA/PBS) on ice for 30 minutes. After primary antibody incubation, cells were washed with ice-cold PBS and fixed with 4% PFA/PBS at room temperature for 10 minutes. Cells were then incubated with Alexa-Fluor secondary antibody (ThermoFisher, A21422) for 30 minutes at room temperature. For Laminin staining, cells were permeabilized with 0.2% Triton X-100 in PBS and blocked with 3% BSA/PBS for 1 hour. Cells were incubated with primary antibody (rabbit anti Laminin 1:500, 1 hour at room temperature), followed by incubation with secondary antibody (ThermoFisher, A21206) and Hoechst to counterstain the nucleus.

For immunostaining of muscle sections, 8 μm raw-embedded frozen sections were fixed with 4% PFA for 10 minutes, permeabilized with 0.01% Triton X-100 for 15 min, and then sections were incubated for 1 hour with M.O.M blocking kit (Vector Laboratories) to prevent background

staining from endogenous mouse Ig. Sections were subsequently blocked in 10% normal donkey serum prepared in PBS and 0.01 % Triton X-100 for 30 minutes and incubated with sheep anti-myomixer (R&D, AP4580, 1:100 dilution) and mouse anti-MY32 primary antibodies overnight at 4°C. Primary antibodies were visualized with Alexa Fluor 568 and 488 (Life Technologies, 1:500). Nuclear counterstaining was performed with DAPI (Sigma, 1 μg/mL). The staining was visualized on a Zeiss LSM780 Confocal Microscope.

For histochemistry, mouse anti-skeletal myosin (MY-32 clone; 1:200 dilution) was used on paraffin sections in conjunction with Mouse-on-Mouse Blocking & Immunodection Kit and horseradish peroxidase (Vector Laboratories, Burlingame, CA). Bound myosin antibody was detected with diamonobenzedine chromagen (DAKO-Agilent, Carpinteria, CA) and slides counterstained with hematoxylin, according to previously described immunoperoxidase methods (*26, 27*).

Generation of Myomixer Mutant Mice

All animal procedures were approved by the Institutional Animal Care and Use Committee at the University of Texas Southwestern Medical Center. B6C3F1 mice were used as oocyte donors. Superovulated female B6C3F1 mice (6 weeks old) were mated to B6C3F1 stud males. Zygotes were harvested and kept in M16 medium (Brinster's medium for ovum culture with 100 U/ml penicillin and 50 mg/ml streptomycin) at 37°C for 1 hour. Zygotes were transferred to M2 medium (M16 medium and 20 mM HEPES). Cas9 mRNA and Myomixer sgRNAs (#1 and #2) were injected into the pronucleus and cytoplasm. Injected zygotes were cultured in M16 medium for 1 hour at 37°C and then transferred into the oviducts of pseudo-pregnant ICR female mice. E17.5 embryos were collected and processed for histology and gene expression assays. Tail genomic

DNA was extracted and used for genotyping with primers that amplify the targeting region (Forward: 5'-AGTTCAGGCTTCAGGTCAGAG-3',

Reverse: 5'-GCTAGGGGAGTGGGAACTGT-3', PCR product size is 743 bp for wild-type mouse).

Tissue Harvest and Preparation

Mouse embryos for in-situ hybridization (ISH), immunohistochemistry (IHC), and routine histology were harvested from pregnant dams, grossly imaged, and immersion fixed in 4% paraformaldehyde. Subsequent paraffin processing, embedding, sectioning and H&E staining were performed by standard procedures (*28, 29*).

RNA In Situ Hybridization

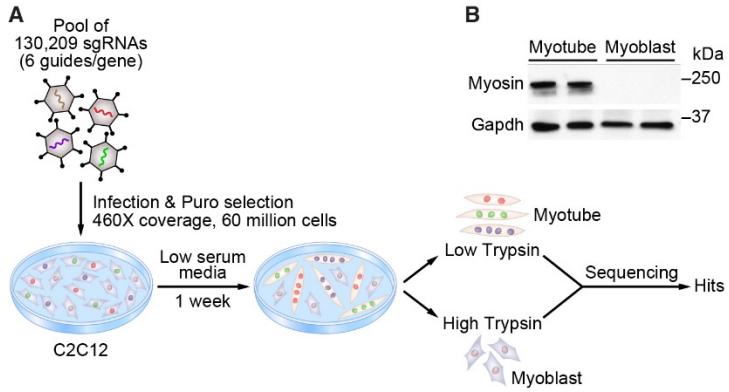
Probe template corresponding to the Myomixer ORF was cloned into pCRII-TOPO (Invitrogen, ThermoFisher, K460001). 35S-labeled sense and antisense probes were generated by Sp6 and T7 RNA polymerases, respectively, from linearized cDNA templates by in-vitro transcription using the Maxiscript kit (Ambion, Inc, Austin, TX). Radio-isotopic in-situ hybridization was performed as previously described (*30*). Signal was revealed after 14 days auto-radiographic exposure.

Microscopy

Gross embryo images were acquired on a Zeiss Stemi SV-11 stereo photo-dissection scope using an Optronics Macrofire digital CCD camera and PictureFrame 2.0 software (Optronics, Goleta,

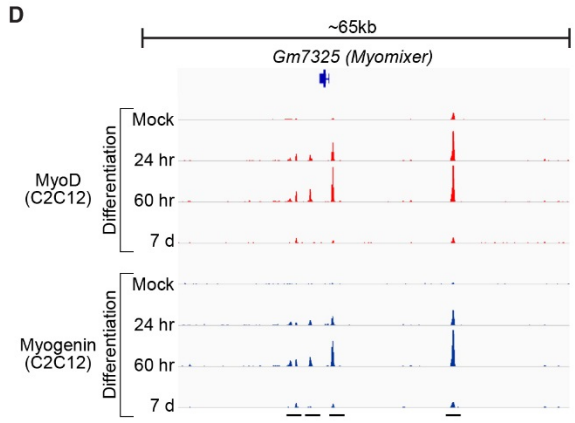
CA). Review and photography of all histologic preparations were carried out on a Nikon E600 photomicroscope equipped with bright field and incident angle dark field illumination (Nikon USA, Mellville, NY). Images were captured at 1.25 x, 4 x, 10 x (ISH), and 20 x (IHC, H&E) objective magnifications using Nikon Elements 4.20.00 software.

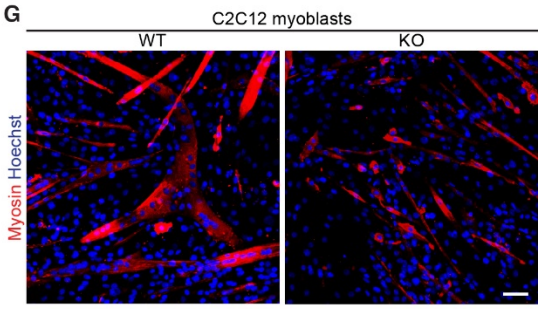
Supplemental Figures



C

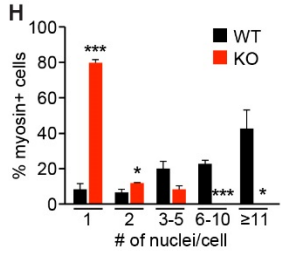
		CRISPR information		Expression data		
Gene	Full name	Score	P value	C2C12	Twist2+	Pax7+
Klhl30	kelch-like 30	3.7	0.002	1.8	13.0	11.2
Gm7325	predicted gene 7325	3.1	0.006	51.0	109.6	15.0
Filip1	filamin A interacting protein 1	2.6	0.016	20.1	122.5	20.0
Dio2	deiodinase, iodothyronine, type II	2.8	0.012	1.5	12.1	3.9
Adamtsl5	ADAMTS-like 5	2.7	0.014	2.2	12.9	8.7

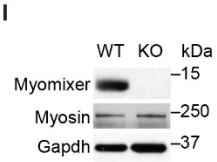




F

Sample ID	Sequence across the deletion	Deletion (bp)	Cut by guide	Mutation
D09	CTGCCTGTTG.....CCGCCTGGCC	1	Guide1	Frame shift after V21
G09	CTGCCTGTT.....GCCTGGCCCG	4	Guide1	Frame shift after V21
H08	CTGCCTGTTG.....CCGCCTGGCC	1	Guide1	Frame shift after V21
H09	GCCAGCAACA.....GATTCTGGAG	7	Guide2	Frame shift after Q60
C09	CTGTTGCCCG.....ATTCTGGAGA	122	Guide1 and 2	Deletes R23-E67 and frame shift





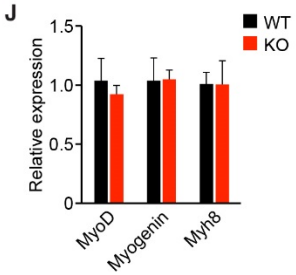


Figure S1. CRISPR screening strategy and validation of Myomixer single guide (sg) RNAs.

(**A**) Schematic of the genome-wide CRISPR loss-of-function screen. C2C12 myoblasts were infected with recombinant lentiviruses expressing Cas9 and sgRNAs. Addition of puromycin selects for cells that have genome integration of viral DNA, thereafter stably expressing Cas9 and sgRNA to knockout its target gene. Cells were switched to differentiation media (low serum) for one week. Trypsin digestion was used to separate myotubes from myoblasts. Sequencing identified the screen "hits". If the gene is essential for differentiation or fusion of C2C12 myoblasts, loss-of-function of this gene will block myotube formation and these altered cells will appear in the myoblast population. The ratio of the sgRNAs identified in myoblasts versus myotubes reflects the function of its target gene in myoblast differentiation or fusion. (**B**) Western blot analysis using MY32 antibody shows enrichment of myosin heavy chain expression in trypsin separated myotubes (low trypsin) compared to myoblasts (high trypsin). (**C**) List of selected genes ("hits") identified in the screen. Hits (enrichment score > 2.5) were compared to genes up-regulated (>2.5-fold) during differentiation of Pax7+ satellite cells and Twist2+ myogenic progenitors, as well as genes up-regulated (>1.5-fold) during C2C12 differentiation (GSE4694). (**D**) Analysis of chromatin immunoprecipitation sequencing data (GSM915183, GSM915185, GSM915186, GSM915165, GSM915159, GSM915163, GSM915166, GSM915164) to show occupancy dynamics of MyoD and Myogenin on the *Myomixer* promoter during C2C12 differentiation. (**E**) The *Myomixer* gene spans three exons with the ORF confined to exon 3. Positions of sgRNAs and primers for genotype analysis are shown. (**F**) Sequencing of genomic PCR fragments from C2C12 cells following disruption of Myomixer by CRISPR. (**G**) Immunostaining of WT and Myomixer KO C2C12 cells with MY32 (myosin heavy chain) antibody and Hoechst stain shows requirement of Myomixer for fusion. Scale bar, 50 μm. (**H**) Quantification of nuclei in WT and Myomixer KO C2C12 cells (N = 3). (**I**) Western blot showing Myomixer, Myosin and Gapdh expression in WT and Myomixer KO C2C12 cells. (**J**) RNA expression of the indicated transcripts in WT and Myomixer KO C2C12 cells detected by qPCR. *P < 0.05, ***P < 0.001, Student's t test. Data are mean +/- SEM.

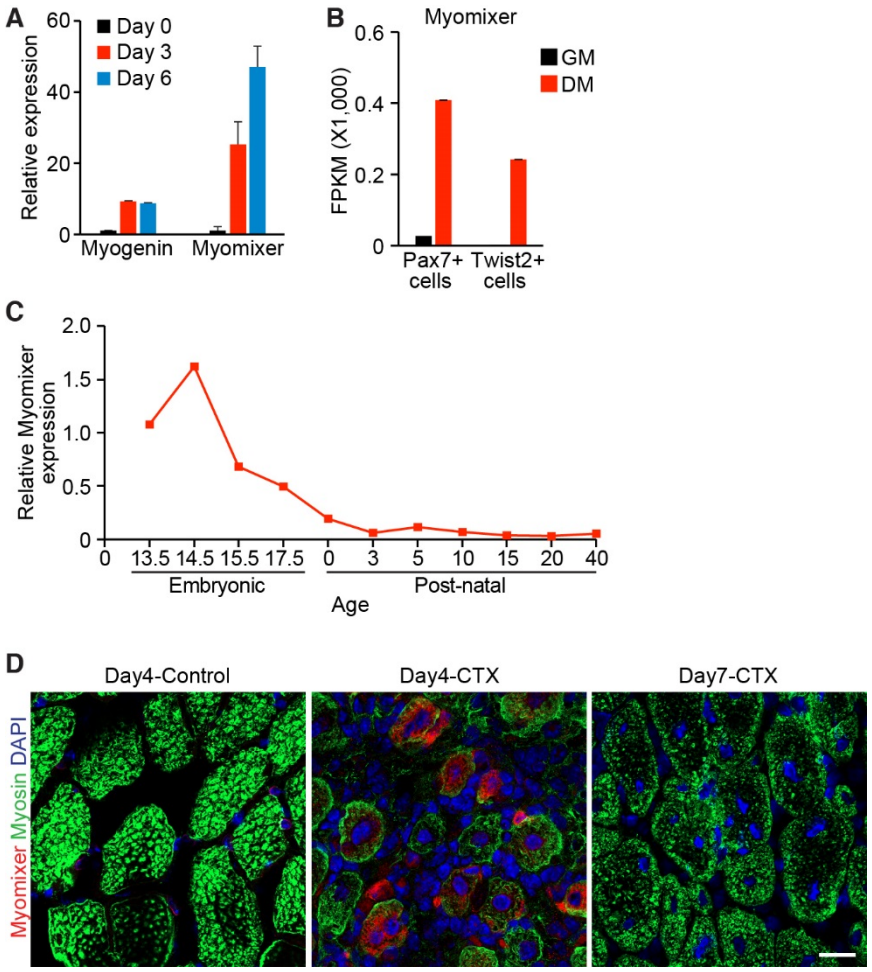
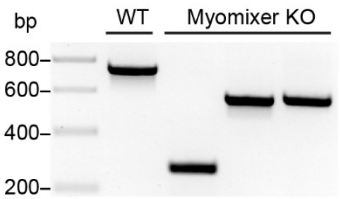


Figure S2. Gene expression during C2C12 cell differentiation, tongue muscle development and muscle regeneration. (**A**) RNA was isolated from C2C12 cells differentiated at the indicated time and Myogenin or Myomixer expression was measured by qPCR. (N = 3). (**B**) RNA-seq data of Myomixer in Pax7+ and Twist2+ skeletal muscle progenitors in growth medium (GM) and 2 days after switching to differentiation medium (DM). FPKM: fragments per kilobase of transcript per million mapped reads. (**C**) Expression of Myomixer transcripts during tongue muscle development detected by qPCR (N = 4). (**D**) Cross-section of CTX-injured muscles at day 4 and day 7 showing Myomixer (red) and myosin (MY32: green) immunostaining. Scale bar, 20 µm. Data are mean +/- SEM.

A



B

Sample ID	Sequence across the deletion	Deletion (bp)	Cut by guide	Mutation
A21	GGTTCTGGAAGA.....GGACCACTCC	470	Guide1 and 2	Deletes entire mixer ORF
A28	AACAGCCACC.....AGTGGGCGAA	167	Guide1 and 2	Frame shift after V21
B12	CTGCTGCCTG.....GAGGAAGGAG	163	Guide1 and 2	Frame shift after P20

Figure S3. Genotyping of genomic DNA from Myomixer KO embryos. (**A**) Myomixer KO embryos at E17.5 were isolated and the genotype was determined from tail DNA by PCR genotyping. WT band is 743 bp. The same pair of primers was used for genotyping as in fig. S1E. (**B**) PCR products from genotyping were sequenced and showed different types of deletions in the Myomixer sgRNA targeting region.

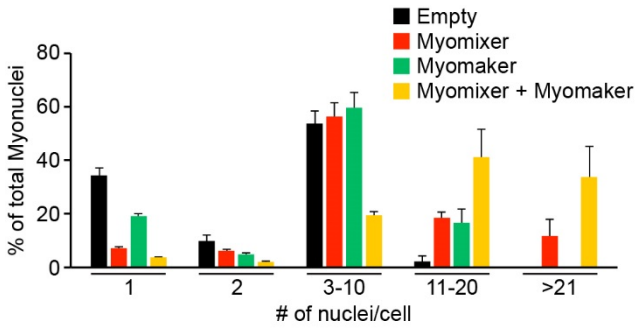
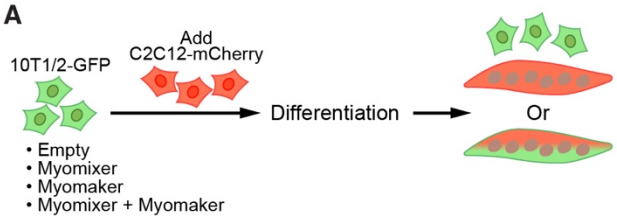
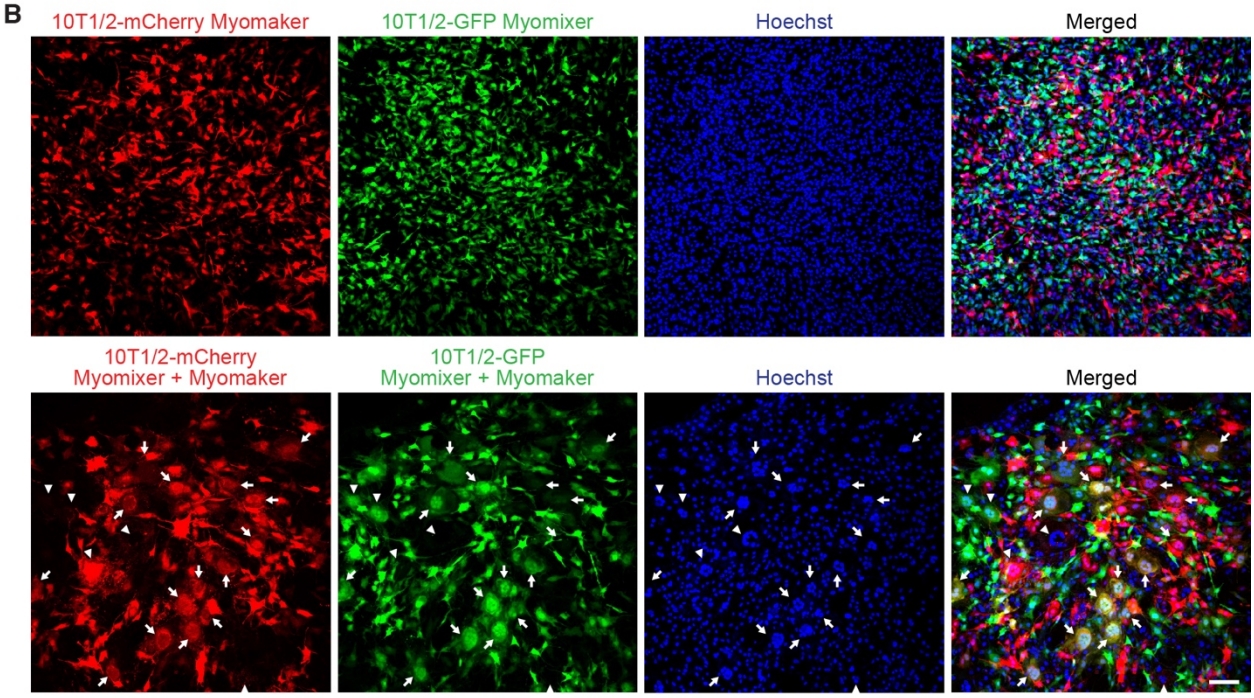
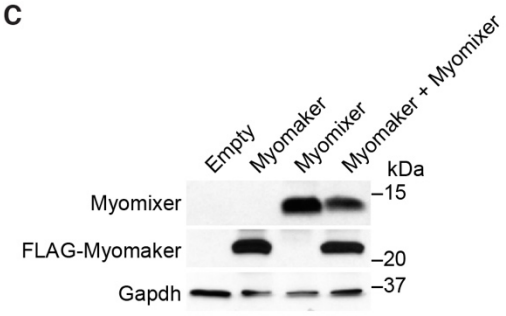


Figure S4. Myomixer synergizes with Myomaker to promote C2C12 cell fusion.
Myonuclei (nuclei inside Myosin+ C2C12 cells) were counted from the images shown in Fig. 4A (N = 3). Data are mean +/- SEM.







D

Cell added	Cell infected	Retroviral expression: Myomixer	Retroviral expression: Myomaker	Fusion	
	C2C12	–	–	+	
	C2C12	–	+	++	Homologous Fusion
	C2C12	+	–	++	
	C2C12	+	+	++++++	
	10T1/2	–	+	–	
	10T1/2	+	–	–	Homologous Fusion
	10T1/2	+	+	+	
C2C12 + 10T1/2		–	–	–	
C2C12 + 10T1/2		–	+	++	Heterologous Fusion
C2C12 + 10T1/2		+	–	–	
C2C12 + 10T1/2		+	+	++++++	

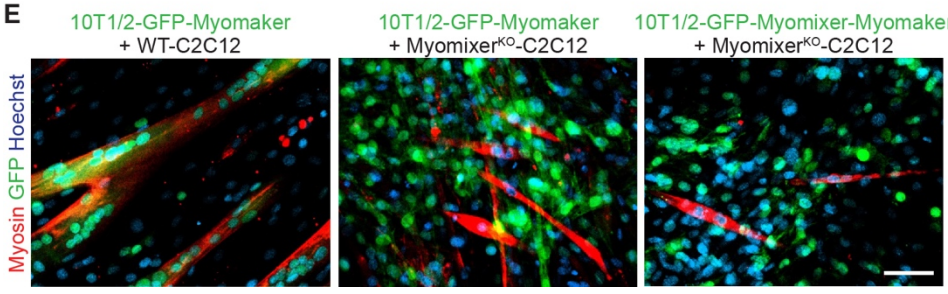


Figure S5. Effects of Myomixer expression on cell fusion. (**A**) GFP-labelled 10T1/2 fibroblasts infected with retroviruses expressing Myomaker, Myomixer or both. Two days after infection, cells were mixed with mCherry labeled C2C12 cells and differentiated for one week. (**B**) Fluorescence images of GFP, mCherry and Hoechst to counterstain nucleus. Arrows point to multi-nucleated GFP+/mCherry+ cells. Arrowheads point to other multi-nucleated cells that arise from fusion of unlabeled cells, GFP+/GFP+ or mCherry+/mCherry+. Scale bar, 50 μm. (**C**) Expression of Myomixer and FLAG-Myomaker in 10T1/2 fibroblasts detected by western blot analysis. (**D**) Summary of fusogenic activities of Myomixer and Myomaker in different cell types. For heterologous fusion, Myomixer and/or Myomaker were retrovirally expressed in 10T1/2 fibroblasts and mixed with C2C12 cells. (**E**) Fluorescence images of GFP and MY32 (myosin) immunostaining (red) of mixed cultures of C2C12 cells (WT or Myomixer KO) with 10T1/2-GFP fibroblasts infected with retroviruses expressing Myomaker and/or Myomixer, following one week of differentiation. Nuclei were counterstained with Hoechst. Scale bar, 50 μm.

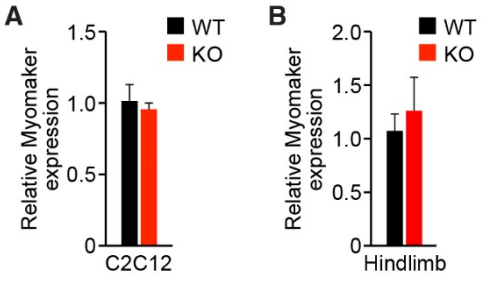


Figure S6. Measuring Myomaker expression. Using qPCR, Myomaker expression was measured in (**A**) WT and Myomixer KO C2C12 cells (N = 3) and (**B**) WT and Myomixer KO E17.5 mouse hindlimb (N = 6 for WT and 9 for KO). Data are mean +/- SEM.

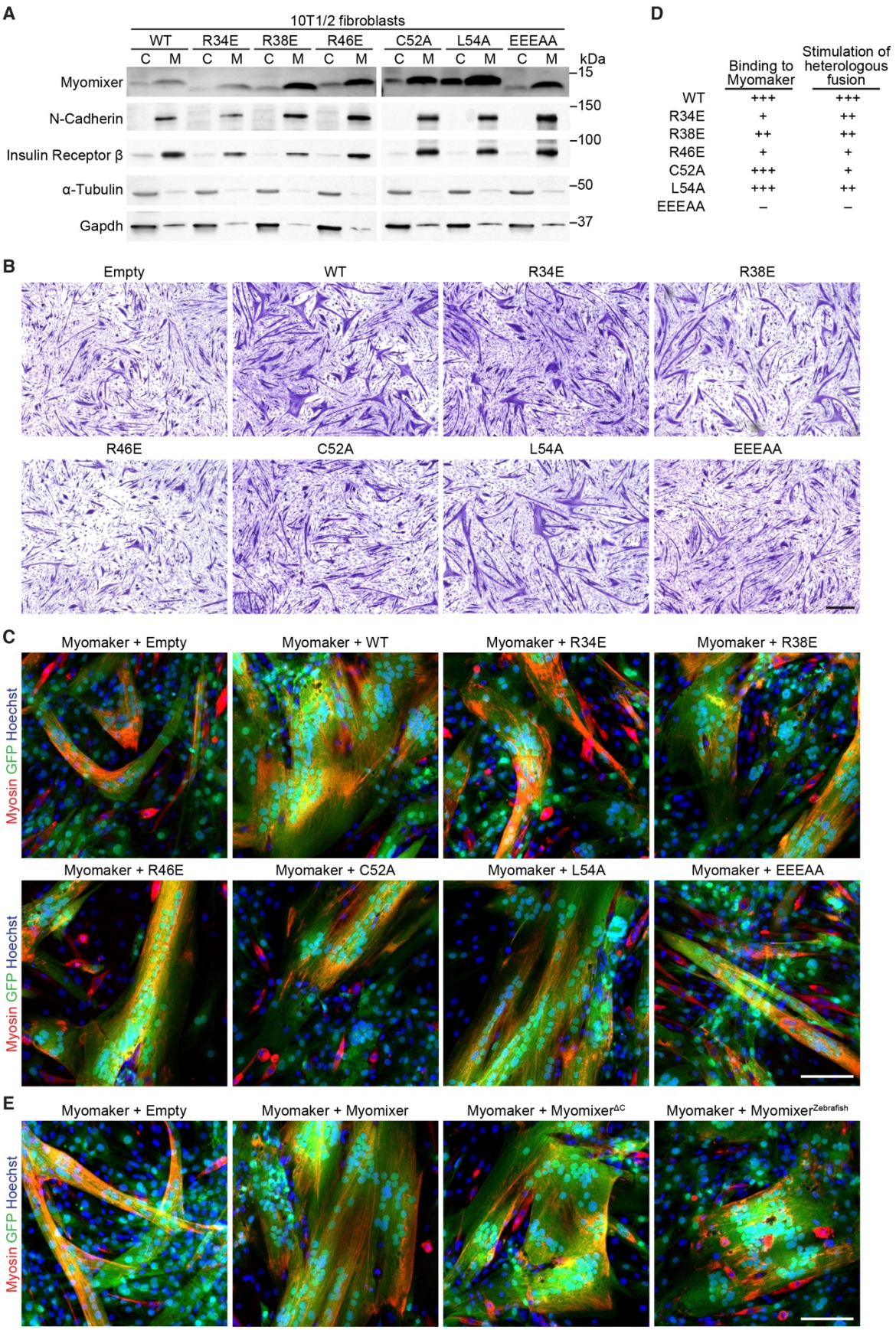

A
10T1/2 fibroblasts
WT
R34E
R38E
R46E
C52A
L54A
EEEAA
C M C M C M C M C M C M C M
kDa
Myomixer
N-Cadherin
Insulin Receptor β
α-Tubulin
Gapdh
15
150
100
50
37
D
Binding to Myomaker
Stimulation of heterologous fusion
WT +++ +++
R34E + ++
R38E ++ ++
R46E + +
C52A +++ +
L54A +++ ++
EEEAA – –
B
Empty
WT
R34E
R38E
R46E
C52A
L54A
EEEAA
C
Myomaker + Empty
Myomaker + WT
Myomaker + R34E
Myomaker + R38E
Myomaker + R46E
Myomaker + C52A
Myomaker + L54A
Myomaker + EEEAA
Myosin GFP Hoechst
E
Myomaker + Empty
Myomaker + Myomixer
Myomaker + Myomixer^ΔC
Myomaker + Myomixer^Zebrafish
Myosin GFP Hoechst

Figure S7. Effects of mutations on Myomixer membrane localization and cell fusion.

(A) Western blot analysis of cytosolic (C) and membrane (M) fractions of retrovirus-Myomixer infected 10T1/2 cells. α-Tubulin and Gapdh blots were used as positive controls of cytosolic proteins. Insulin receptor β and N-Cadherin blots were used as positive controls of membrane proteins. (**B**) Crystal violet blue staining of the C2C12 cells that overexpressed Myomaker together with different forms of Myomixer as indicated. Cells were differentiated for one week. Scale bar, 500 μm. (**C and E**) Fluorescence images of GFP and MY32 (myosin) immunostaining (red) of mixed cultures of C2C12 cells with 10T1/2-GFP fibroblasts infected with retroviruses expressing Myomaker together with different forms of Myomixer, following one week of differentiation. Nuclei were counterstained with Hoechst. Scale bar, 50 μm. (**D**) A summary of the effects of different mutations on membrane localization and function of Myomixer.

References and Notes

1. C. F. Bentzinger, Y. X. Wang, M. A. Rudnicki, Building muscle: Molecular regulation of myogenesis. *Cold Spring Harb. Perspect. Biol.* **4**, a008342 (2012). doi:10.1101/cshperspect.a008342 Medline

2. M. Buckingham, P. W. J. Rigby, Gene regulatory networks and transcriptional mechanisms that control myogenesis. *Dev. Cell* **28**, 225–238 (2014). doi:10.1016/j.devcel.2013.12.020 Medline

3. K. Rochlin, S. Yu, S. Roy, M. K. Baylies, Myoblast fusion: When it takes more to make one. *Dev. Biol.* **341**, 66–83 (2010). doi:10.1016/j.ydbio.2009.10.024 Medline

4. A. R. Demonbreun, B. H. Biersmith, E. M. McNally, Membrane fusion in muscle development and repair. *Semin. Cell Dev. Biol.* **45**, 48–56 (2015). doi:10.1016/j.semcdb.2015.10.026 Medline

5. R. S. Krauss, G. A. Joseph, A. J. Goel, Keep your friends close: Cell-cell contact and skeletal myogenesis. *Cold Spring Harb. Perspect. Biol.* **9**, a029298 (2017). doi:10.1101/cshperspect.a029298 Medline

6. A. Simionescu, G. K. Pavlath, Molecular mechanisms of myoblast fusion across species. *Adv. Exp. Med. Biol.* **713**, 113–135 (2011). doi:10.1007/978-94-007-0763-4_8 Medline

7. S. M. Abmayr, G. K. Pavlath, Myoblast fusion: Lessons from flies and mice. *Development* **139**, 641–656 (2012). doi:10.1242/dev.068353 Medline

8. H. Yin, F. Price, M. A. Rudnicki, Satellite cells and the muscle stem cell niche. *Physiol. Rev.* **93**, 23–67 (2013). doi:10.1152/physrev.00043.2011 Medline

9. J. D. Doles, B. B. Olwin, Muscle stem cells on the edge. *Curr. Opin. Genet. Dev.* **34**, 24–28 (2015). doi:10.1016/j.gde.2015.06.006 Medline

10. A. S. Brack, T. A. Rando, Tissue-specific stem cells: Lessons from the skeletal muscle satellite cell. *Cell Stem Cell* **10**, 504–514 (2012). doi:10.1016/j.stem.2012.04.001 Medline

11. E. H. Chen, E. N. Olson, Unveiling the mechanisms of cell-cell fusion. *Science* **308**, 369–373 (2005). doi:10.1126/science.1104799 Medline

12. N. E. Sanjana, O. Shalem, F. Zhang, Improved vectors and genome-wide libraries for CRISPR screening. *Nat. Methods* **11**, 783–784 (2014). doi:10.1038/nmeth.3047 Medline

13. I. H. B. Chen, M. Huber, T. Guan, A. Bubeck, L. Gerace, Nuclear envelope transmembrane proteins (NETs) that are up-regulated during myogenesis. *BMC Cell Biol.* **7**, 38 (2006). doi:10.1186/1471-2121-7-38 Medline

14. N. Liu, G. A. Garry, S. Li, S. Bezprozvannaya, E. Sanchez-Ortiz, B. Chen, J. M. Shelton, P. Jaichander, R. Bassel-Duby, E. N. Olson, A Twist2-dependent progenitor cell contributes to adult skeletal muscle. *Nat. Cell Biol.* **19**, 202–213 (2017). doi:10.1038/ncb3477 Medline

15. F. Yue, Y. Cheng, A. Breschi, J. Vierstra, W. Wu, T. Ryba, R. Sandstrom, Z. Ma, C. Davis, B. D. Pope, Y. Shen, D. D. Pervouchine, S. Djebali, R. E. Thurman, R. Kaul, E. Rynes, A. Kirilusha, G. K. Marinov, B. A. Williams, D. Trout, H. Amrhein, K. Fisher-Aylor, I.

Antoshechkin, G. DeSalvo, L.-H. See, M. Fastuca, J. Drenkow, C. Zaleski, A. Dobin, P. Prieto, J. Lagarde, G. Bussotti, A. Tanzer, O. Denas, K. Li, M. A. Bender, M. Zhang, R. Byron, M. T. Groudine, D. McCleary, L. Pham, Z. Ye, S. Kuan, L. Edsall, Y.-C. Wu, M. D. Rasmussen, M. S. Bansal, M. Kellis, C. A. Keller, C. S. Morrissey, T. Mishra, D. Jain, N. Dogan, R. S. Harris, P. Cayting, T. Kawli, A. P. Boyle, G. Euskirchen, A. Kundaje, S. Lin, Y. Lin, C. Jansen, V. S. Malladi, M. S. Cline, D. T. Erickson, V. M. Kirkup, K. Learned, C. A. Sloan, K. R. Rosenbloom, B. Lacerda de Sousa, K. Beal, M. Pignatelli, P. Flicek, J. Lian, T. Kahveci, D. Lee, W. J. Kent, M. Ramalho Santos, J. Herrero, C. Notredame, A. Johnson, S. Vong, K. Lee, D. Bates, F. Neri, M. Diegel, T. Canfield, P. J. Sabo, M. S. Wilken, T. A. Reh, E. Giste, A. Shafer, T. Kutyavin, E. Haugen, D. Dunn, A. P. Reynolds, S. Neph, R. Humbert, R. S. Hansen, M. De Bruijn, L. Selleri, A. Rudensky, S. Josefowicz, R. Samstein, E. E. Eichler, S. H. Orkin, D. Levasseur, T. Papayannopoulou, K.-H. Chang, A. Skoultchi, S. Gosh, C. Disteche, P. Treuting, Y. Wang, M. J. Weiss, G. A. Blobel, X. Cao, S. Zhong, T. Wang, P. J. Good, R. F. Lowdon, L. B. Adams, X.-Q. Zhou, M. J. Pazin, E. A. Feingold, B. Wold, J. Taylor, A. Mortazavi, S. M. Weissman, J. A. Stamatoyannopoulos, M. P. Snyder, R. Guigo, T. R. Gingeras, D. M. Gilbert, R. C. Hardison, M. A. Beer, B. Ren, Mouse ENCODE Consortium, A comparative encyclopedia of DNA elements in the mouse genome. *Nature* **515**, 355–364 (2014). doi:10.1038/nature13992 Medline

16. A. P. Fong, Z. Yao, J. W. Zhong, Y. Cao, W. L. Ruzzo, R. C. Gentleman, S. J. Tapscott, Genetic and epigenetic determinants of neurogenesis and myogenesis. *Dev. Cell* **22**, 721–735 (2012). doi:10.1016/j.devcel.2012.01.015 Medline

17. Y. M. Chen, Z. W. Du, Z. Yao, Molecular cloning and functional analysis of ESGP, an embryonic stem cell and germ cell specific protein. *Acta Biochim. Biophys. Sin. (Shanghai)* **37**, 789–796 (2005). doi:10.1111/j.1745-7270.2005.00120.x Medline

18. D. P. Millay, D. G. Gamage, M. E. Quinn, Y.-L. Min, Y. Mitani, R. Bassel-Duby, E. N. Olson, Structure-function analysis of myomaker domains required for myoblast fusion. *Proc. Natl. Acad. Sci. U.S.A.* **113**, 2116–2121 (2016). doi:10.1073/pnas.1600101113 Medline

19. D. P. Millay, J. R. O'Rourke, L. B. Sutherland, S. Bezprozvannaya, J. M. Shelton, R. Bassel-Duby, E. N. Olson, Myomaker is a membrane activator of myoblast fusion and muscle formation. *Nature* **499**, 301–305 (2013). doi:10.1038/nature12343 Medline

20. B. R. Nelson, D. M. Anderson, E. N. Olson, Small open reading frames pack a big punch in cardiac calcium regulation. *Circ. Res.* **114**, 18–20 (2014). doi:10.1161/CIRCRESAHA.113.302716 Medline

21. D. M. Anderson, K. M. Anderson, C.-L. Chang, C. A. Makarewich, B. R. Nelson, J. R. McAnally, P. Kasaragod, J. M. Shelton, J. Liou, R. Bassel-Duby, E. N. Olson, A micropeptide encoded by a putative long noncoding RNA regulates muscle performance. *Cell* **160**, 595–606 (2015). doi:10.1016/j.cell.2015.01.009 Medline

22. D. M. Anderson, C. A. Makarewich, K. M. Anderson, J. M. Shelton, S. Bezprozvannaya, R. Bassel-Duby, E. N. Olson, Widespread control of calcium signaling by a family of SERCA-inhibiting micropeptides. *Sci. Signal.* **9**, ra119 (2016). doi:10.1126/scisignal.aaj1460 Medline

23. B. R. Nelson, C. A. Makarewich, D. M. Anderson, B. R. Winders, C. D. Troupes, F. Wu, A. L. Reese, J. R. McAnally, X. Chen, E. T. Kavalali, S. C. Cannon, S. R. Houser, R. Bassel-Duby, E. N. Olson, A peptide encoded by a transcript annotated as long noncoding RNA enhances SERCA activity in muscle. *Science* **351**, 271–275 (2016). doi:10.1126/science.aad4076 Medline

24. T. Kitamura, Y. Koshino, F. Shibata, T. Oki, H. Nakajima, T. Nosaka, H. Kumagai, Retrovirus-mediated gene transfer and expression cloning: Powerful tools in functional genomics. *Exp. Hematol.* **31**, 1007–1014 (2003). doi:10.1016/S0301-472X(03)00260-1 Medline

25. R. Blelloch, M. Venere, J. Yen, M. Ramalho-Santos, Generation of induced pluripotent stem cells in the absence of drug selection. *Cell Stem Cell* **1**, 245–247 (2007). doi:10.1016/j.stem.2007.08.008 Medline

26. P. Cianga, C. Medesan, J. A. Richardson, V. Ghetie, E. S. Ward, Identification and function of neonatal Fc receptor in mammary gland of lactating mice. *Eur. J. Immunol.* **29**, 2515–2523 (1999). doi:10.1002/(SICI)1521-4141(199908)29:08<2515:AID-IMMU2515>3.0.CO;2-D Medline

27. J. Borvak, J. Richardson, C. Medesan, F. Antohe, C. Radu, M. Simionescu, V. Ghetie, E. S. Ward, Functional expression of the MHC class I-related receptor, FcRn, in endothelial cells of mice. *Int. Immunol.* **10**, 1289–1298 (1998). doi:10.1093/intimm/10.9.1289 Medline

28. D. C. Shehan, B. B. Hrapchak, *Theory and Practice of Histotechnology* (Battelle Press, ed. 2, 1980).

29. A. E. Woods, R. C. Ellis, *Laboratory Histopathology, A Complete Reference* (Churchill Livingstone, 1996).

30. J. M. Shelton, M. H. Lee, J. A. Richardson, S. B. Patel, Microsomal triglyceride transfer protein expression during mouse development. *J. Lipid Res.* **41**, 532–537 (2000). Medline