Supplemental Material

Supplemental Methods

Cell-free cleavage assay for testing Lmod3 TALEN activity. The following primers were used to amplify the targeted region of the Lmod3 allele:

Lmod3-Fwd: 5'-CGCATGCATGTGCATTCCTTTGTG-3'

Lmod3-Rev: 5'-TTGCTGGTAGGTGCCCGGAT-3'

Two micrograms of the resulting PCR product was combined with 3μg of left and right TALEN mRNA, 60μl of TNT T7 Quick Master Mix (Promega), and 1.5μl of 1mM methionine to a total reaction volume of approximately 75μl. Nuclease-free water was used instead of TALEN mRNA for the control reaction. The reaction was incubated at 30°C for two hours and then overnight at 30°C with 5X volume of TALEN cutting buffer (20mM Tris-HCl pH7.5, 5mM $MgCl_2$, 50mM KCl, 5% glycerol, and 0.5 mg/ml BSA) added. The following day, DNA was purified using QIAquick gel extraction columns (QIAGEN) according to manufacturer's directions. Cleaved DNA was eluted in 30μl and then ran on a standard 2% TAE agarose gel. TALEN cutting was confirmed by the presence of the lower ~200bp cleaved band.

Genotyping of* Lmod3 *KO and MCK-Lmod3 mice. *Lmod3* KO founder mice were screened by amplification of the targeted region of the Lmod3 locus using the above set of primers, followed by T7 endonuclease assay, as previously described (1, 2). Briefly, PCR amplified reactions from wild-type and TALEN-mutagenized samples were denatured and re-hybridized in order to acquire

mismatched heteroduplex DNA using the following cycles: 95^{o}C for 5 min, 95-85^{o}C at -2^{o}C/s, 85-25^{o}C at -0.1^{o}C/s. Samples were then treated with 10U of T7 Endonuclease I (New England Biolabs) at 37^{o}C for 30 minutes and ran on a 2% agarose gel. In the event of a deletion caused by non-homologous end joining repair (NHEJ), two bands were seen on the gel as opposed to a single 601bp band (Figure 2C).

Sequencing of individual PCR reactions revealed two separate deletions (Δ2 and Δ10). Following germline transmission of the alleles and generation of the Lmod3 KO mice, we devised two different genotyping strategies for the Δ2 and Δ10 KO mice.

To genotype Δ2 KO mice, primer pairs were generated that specifically recognize the mutant allele: Lmod3Δ2KOF: 5'-CGCATGCATGTGCATTCCTTTGTG-3', and Lmod3Δ2KOR: 5'-GCTTCTTGTACATTGTTGCTCCCATTTGATTCTT-3'. In addition, primer pairs were generated that specifically recognize the wild-type allele: Lmod3Δ2WTF: 5'-CGCATGCATGTGCATTCCTTTGTG-3', and Lmod3Δ2WTR: 5'-GCTTCTTGTACATTGTTGCTCCCATTTGATTCTC-3'. *Lmod3* HET mice had 601 bp bands for both pairs, whereas Δ2 KO and WT mice were only positive for the KO and WT pairs, respectively.

To genotype Δ10 KO mice, the Lmod3Δ10 F and R primers shown above were used, followed by HinfI (New England Biolabs) digestion of the PCR product. The

HinfI site was destroyed if the TALEN-mediated NHEJ caused a deletion in the cut site. Mice were considered to have a mutation if a band appeared uncut.

Genotyping of *MCK-Lmod3* Tg mice was done using the following primers for the MCK allele:

HGH-F: 5'-GTCTGACTAGGTGTCCTTCT-3',

HGH-R: 5'-CGTCCTCCTGCTGGTATAG-3'.

For genotyping of the *Lmod3-hsp68-LacZ* mice, the following primers were used for detection of the *LacZ* allele:

LacZ-F: 5'- TGAAGTGCCTCTGGATGTCGCTCCACAAGGTAAACAG-3',

LacZ-R: 5'-GGGATAGTTTTCTTGCGGCCCTAATCCGAGCCA G-3'.

Radioactive in situ hybridization. Radioisotopic in situ hybridization (ISH) on E10.5, E12.5 and E15.5 embryonic sections was performed as previously described (3). The following probe was used to track Lmod3 expression: CTTAAAGAGCAGAGAAAGCTGATAGCTATGTTGGAGAATGGGTTGGGGCTG CCCCCTGGGATGTGGGAGAGGTTGGGAGGACCCATGCCCGACCCCAGAAT GCAGGAGTTCTTCCAGCCTGCATCTGGGCGGCCCCTCGATGCTCAGGAAG TCCCCTTTGGTTCAAGAAAGGAAATGATCAAAAATCCGCCCCAGCCTCCACA GTGCAAGACGGACCCTGACTCCTTCAGGGTGGTGAAGCTGAAGAGAATTCA GCGCAAATCTCGAATGCCGGAAGCCAGAGAGGCACAGGAGAAAACCAACC TCAAAGATGTAATCAAAACGCTCAAGCCAGTGCCGAGAAATAGGCCGCCCC CGCTGGTGGAAATCACTCCCAGAGACCAGCTCTTGAA.

The probe was cloned into PCRII-Topo vector (Life Technologies) as per manufacturer's instructions using the following primer set and mouse skeletal muscle cDNA as a template:

L3-IS-F: 5'-CTTAAAGAGCAGAGAAAGCTGATAGCTATG-3',

L3-IS-R: 5'-TTCAAGAGCTGGTCTCTGGGA-3'.

Following cloning, probe sequence was cut out from the PCRII-Topo backbone and transcribed using the MAXIscript *in vitro* transcription kit (Life Technologies) by the T7 and SP6 promoters to generate the *anti-sense* and *sense* probes, respectively.

Northern blot analysis. Tissues were harvested from 8 week-old C3B6F1 mice and flash-frozen in liquid nitrogen. Total RNA was extracted from tissue with TRIZOL reagent (Invitrogen) according to manufacturer's instructions. The above in-situ probe sequence was used to generate probe for the Northern blot, followed by labeling with [α-^{32}P]dCTP using the RadPrime DNA Labelling System (Invitrogen), as per manufacturer's instructions. As a loading control, 28S and 18S rRNA were visualized.

Quantitative real-time PCR (qPCR). Total RNA was extracted from tissue with TRIZOL reagent (Invitrogen) according to manufacturer's instructions. cDNA was synthesized using Superscript III reverse transcriptase with random hexamer primers (Invitrogen), as per manufacturer's instructions. Gene expression was analyzed by qPCR using KAPA SYBR FAST (Kapa Biosystems) or KAPA

PROBE FAST (Kapa Biosystems) on a 7900HT Fast Real-Time PCR machine (Life Technologies). See Supplementary Table 1 for Taqman probe IDs.

The following SYBR green primers were used:

Lmod3-F: CCGCTGGTGGAAATCACTCCC

Lmod3-R: ACTCCAGCTCCTTTGGCAGTTGC

Klhl40-F: CCCAAGAACCATGTCAGTCTGGTGAC,

Klhl40-R: TCAGAGTCCAAGTGGTCAAACTGCAG;

Lmod2-F: TTGGAGAAGGAACGGCTGGG

Lmod2-R: CCTCAGAGACTTCGCTGTTGCTCTC.

qPCR for MEF2-dependency was done with cDNA from satellite cells extracted from MEF2 TKO mice as previously described (4).

Echocardiography studies. Echocardiography was performed on 12 week-old *Δ2 KO* mice and gender-matched *WT* littermates using the Vevo 2100 small animal echocardiography system (VisualSonics) and a 40-MHz transducer. Mice were gently restrained and the ultrasound probe positioned to obtain a parasternal short-axis view. The largest anteroposterior diameters in diastole and systole were assessed in at least three recorded M-mode tracings and LV fractional shortening calculated according to the formula FS(%) = [(LVIDd – LVIDs)/LVIDd] X 100. Heart rate was assessed to exclude a vagal response. A single observer analyzed the data.

Plasmid constructs. Plasmid constructs were made as follows: Lmod3 enhancer fragments were PCR-isolated from mouse genomic DNA template and inserted into the the pGL3-Basic vector or upstream of the hsp-LacZ vector minimal promoter using KpnI/XhoI sites, using the following primer sets:

LMOD3 -1690F: 5'-ACGGGGTACCTGCAATTGCTCTCAACAGGGA-3',

LMOD3-1069F:

5'-ACGGGGTACCGTTTAACTTACTGTCTCAAGACTATCCTGTCATGC-3',

LMOD3-526F: 5'-ACGGGGTACCGGACGACGCTTCTATTCCAGGAGAAACT-3',

LMOD3-258F: 5'-ACGGGGTACCTGTAGTAGTTCCACAACTTGACTCTGCC-3',

LMOD3proR:

5'-TCTACCGCTCGAGAGTTTACTTTTTTTCTATCTGTTTCTAGATCACCAG-3'.

Mutations were introduced to the Lmod3 promoter using following primers:

L3-SRFmut-F:

5'-TGTGGAACTACTACATGAGTGCGTTTTTTGCAACTTGAGTCATCTGGAC-3',

SRFmut-R:

5'-GTCCAGATGACTCAAGTTGCAAAAAACGCACTCATGTAGTAGTTCCACA-3',

MEF2mut-F:

5'-GCTCAGCTCATGTGGGATCTACCCTAAGCAAGGTTTTCTGGGCAGA-3',

MEF2mut-R:

5'-TCTGCCCAGAAAACCTTGCTTAGGGTAGATCCCACATGAGCTGAGC -3',

MEF2mut-nonTATA-F:

5-GCTCATGTGGGATCTATATTGGGCAAGGTTTTCTGGGCAGAG-3',

MEF2mut-nonTATA-R:

5’-CTCTGCCCAGAAAACCTTGCCCAATATAGATCCCACATGAGC-3'.

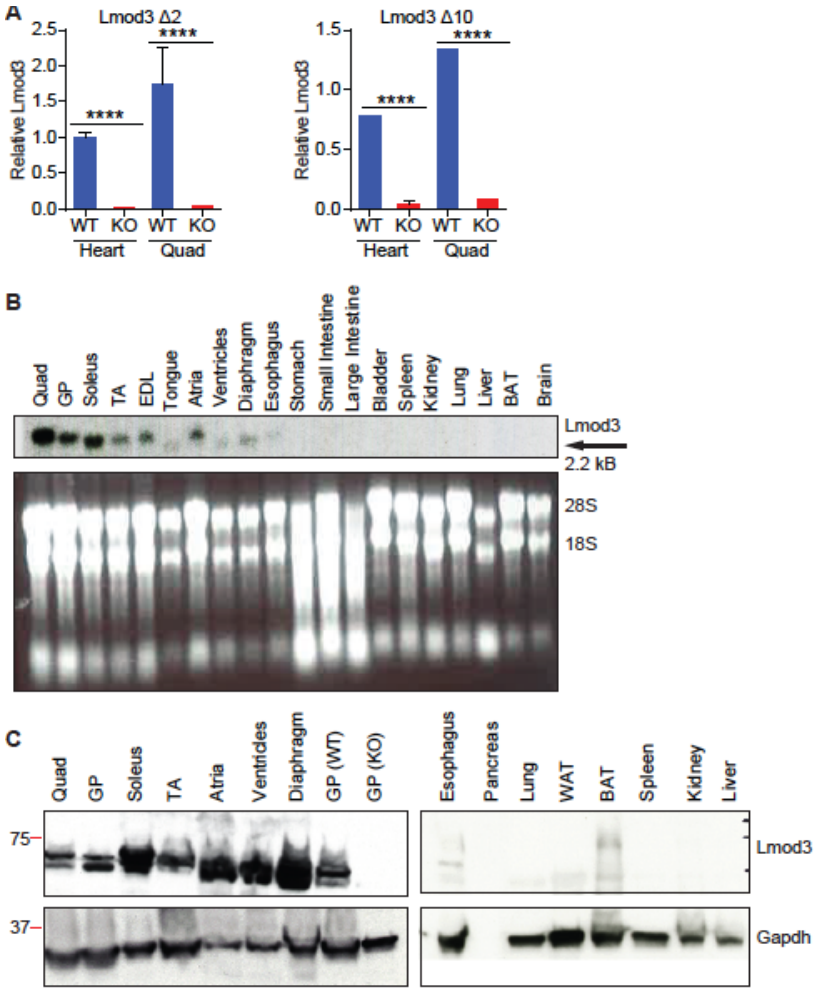
Extraction of muscle tissue and western blot analysis. Flash-frozen muscle tissues were dissociated in FLAG lysis buffer with 1% Triton X-100, cOmplete mini EDTA protease inhibitor cocktail, PhosSTOP phosphatase inhibitor cocktail, and 6M urea using a mini pestle. Tissue lysate was centrifuged at 14,000rpm for 15 minutes at 4°C and the supernatant containing protein was transferred to a new tube. Protein was mixed with one volume of 2X Laemmli buffer with 5% β-mercaptoethanol and SDS-PAGE electrophoresis was performed following 5 minutes of sample incubation at 100°C. All protein was transferred to Immobilon-P PVDF membrane (EMD Millipore) using a Mini Trans-Blot cell (Bio-Rad). All antibodies were diluted in 5% non-fat milk in TBS/0.1% Tween-20. To analyze Lmod3, a 1:10,000 dilution of rabbit anti-Lmod3 (ProteinTech, cat no.14948-1-AP) was used with a 1:10,000 dilution of goat anti-rabbit HRP conjugated secondary antibody (Bio-Rad). All primary antibodies were incubated with blots overnight at 4°C, while secondary antibodies were incubated for 30 minutes at room temperature.

Supplemental Tables

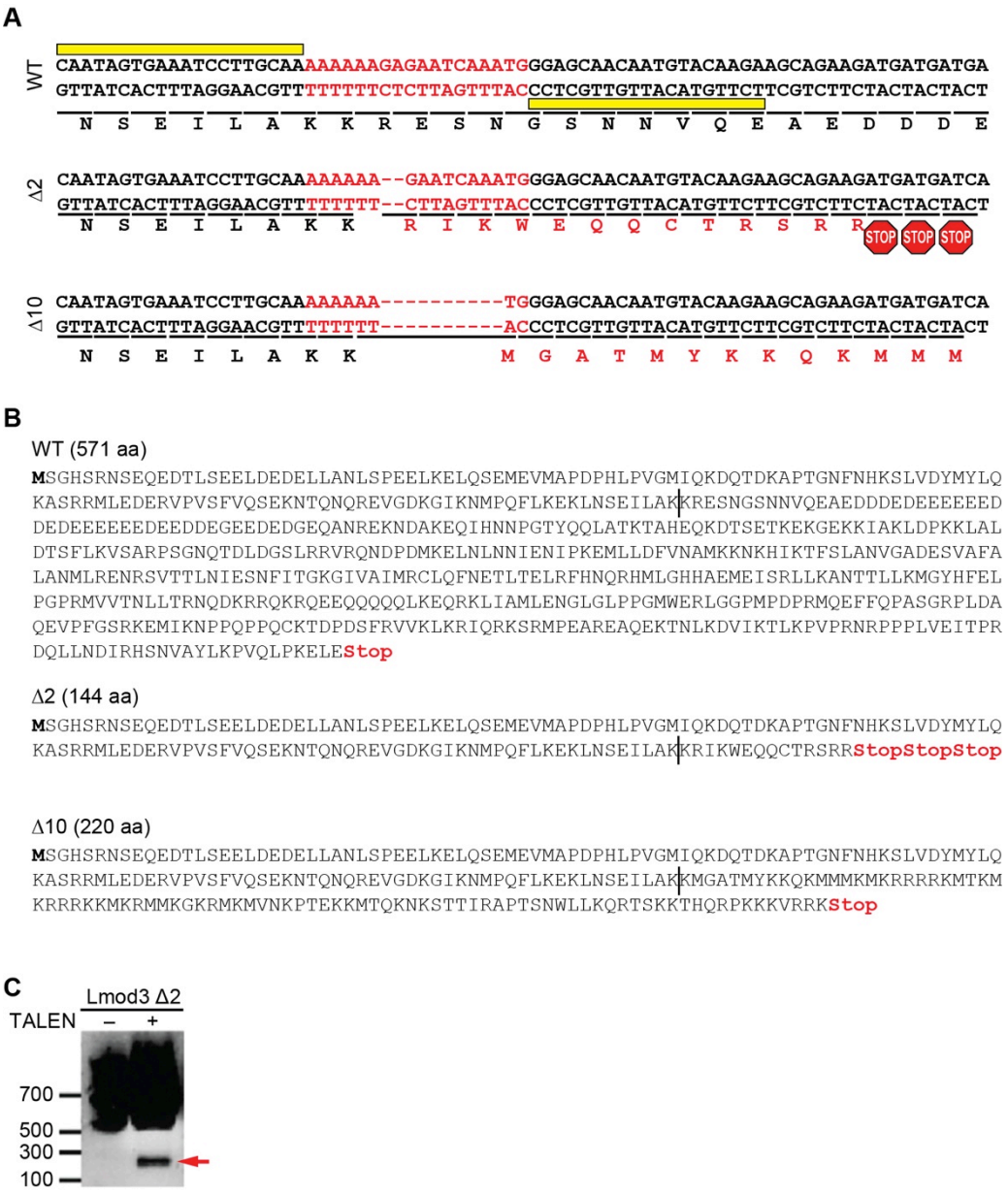
Supplemental Table 1. Taqman probes used in quantitative real-time PCR analysis.

ID#	Gene Symbol	Gene Name
Mm00808218_g1	Acta1	actin, alpha 1, skeletal muscle
Mm00725412_s1	Acta2	actin, alpha 2, smooth muscle
Mm00786736_s1	Cfl1	cofilin 1, non-muscle
Mm00802455_m1	Des	desmin
Mm00441922_m1	Fbxo32	F-box only protein 32
Mm01340842_m1	Mef2c	myocyte enhancer factor 2C
Mm00461840_m1	Mkl1	MKL (megakaryoblastic leukemia)/myocardin-like 1, MRTF-A
Mm00463877_m1	Mkl2	MKL/myocardin-like 2, MRTF-B
Mm01332489_g1	Myh1	myosin, heavy polypeptide 1, skeletal muscle, adult
Mm01332564_m1	Myh2	myosin, heavy polypeptide 2, skeletal muscle, adult
Mm01299731_m1	Myh3	myosin, heavy polypeptide 3, skeletal muscle, embryonic
Mm01332518_m1	Myh4	myosin, heavy polypeptide 4, skeletal muscle
Mm01319006_g1	Myh7	myosin,heavy polypeptide 7,cardiac muscle
Mm00440378_m1	Myl4	myosin, light polypeptide 4
Mm01203489_g1	Myod1	myogenic differentiation 1
Mm00446195_g1	Myog	myogenin
Mm00834079_m1	Pax7	paired box gene 7
Mm00450821_m1	Pfn2	profilin 2
Mm00481536_m1	Sln	sarcolipin
Mm00600378_m1	Tpm1	tropomyosin
Mm01185221_m1	Trim63	tripartite motif-containing 63

Supplemental Figures

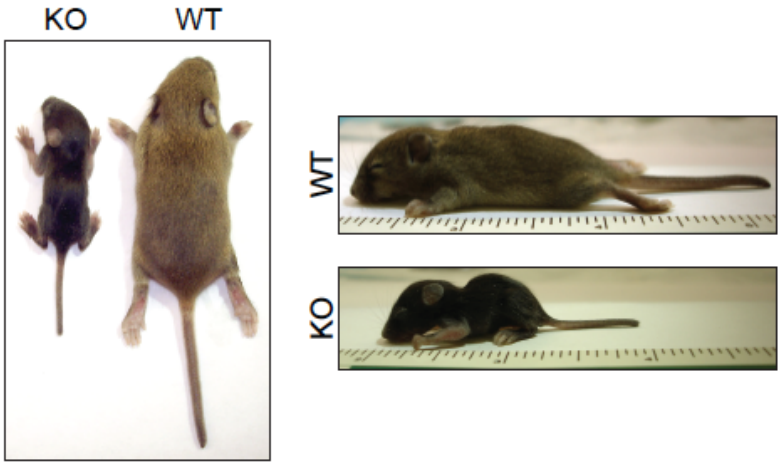


Supplemental Figure 1. Expression analysis of Lmod3. (A) Lmod3 expression was assessed by quantitative realtime PCR analysis of RNA isolated from heart and quadriceps (quad) from Δ2 KO and Δ10 KO mice. Experiments were performed in triplicate with three biological replicates and expression was normalized to 18S rRNA. ****p<0.001. (B) Northern blot analysis of tissues harvested from 8 week-old C3B6F1 mice shows muscle and heart-specific expression of Lmod3. 28S and 18S rRNA stained with ethidium bromide are shown as loading control. (C) Western blot analysis of tissues harvested from 8 week-old C3B6F1 mice confirms muscle and heart-specific expression of *Lmod3*. 100 µg of protein was loaded into each well and GAPDH was used as a loading control.

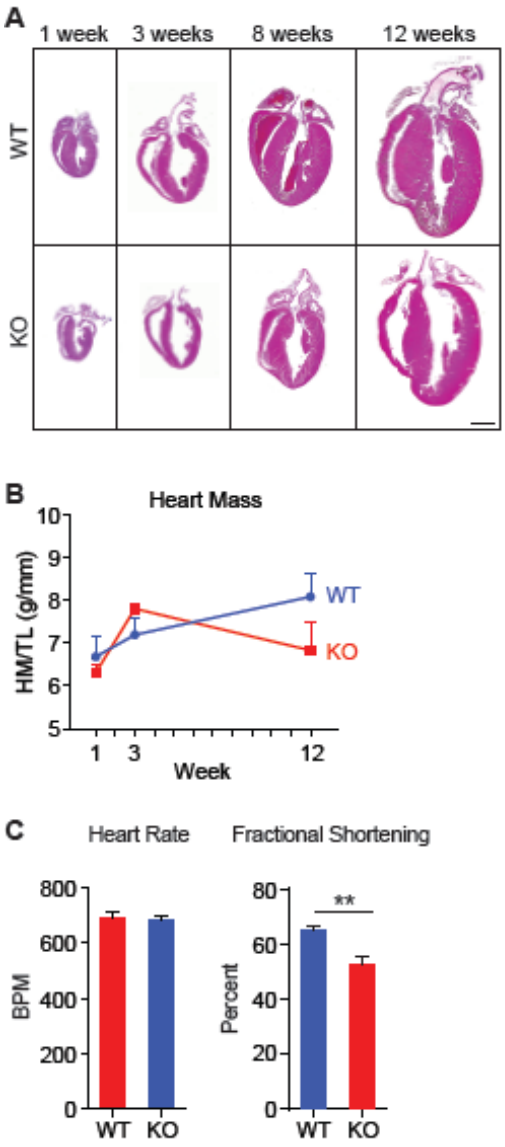


Supplemental Figure 2. Frameshift deletions by TALEN mutagenesis lead to truncated transcripts and complete loss of Lmod3 protein. (A) Both frameshift deletions (Δ2 and Δ10) introduce a premature termination codon into the Lmod3 locus. The yellow bars indicate the TALEN pair. The DNA sequence in red is the spacer region where the TALEN-mediated double strand break occurs. Red amino acid sequences indicate the protein sequence of the shifted frame. (B) Truncated protein products that would theoretically be synthesized in the Δ2 and Δ10 Lmod3 mutant mice. Vertical lines in protein sequence indicate

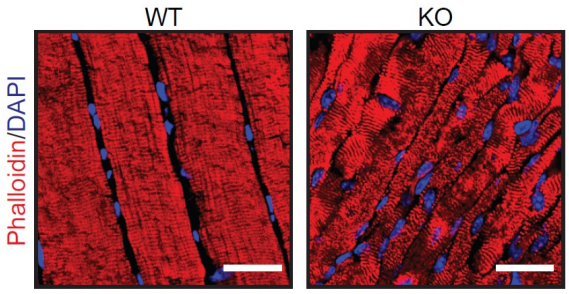
the frameshift. (C) PCR-amplified Lmod3 target region was treated with TALEN mRNA (+) or nuclease-free water (-). The cleavage product (200 bp lower band denoted with red arrow) is observed in the targeted sample.



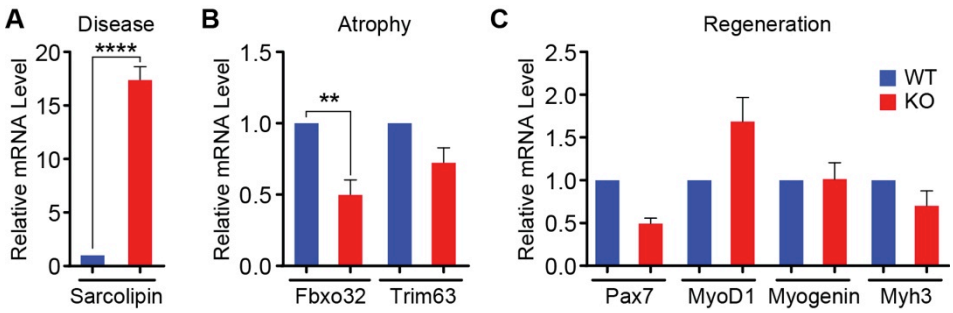
Supplemental Figure 3. Extreme failure to thrive in Lmod3 KO mice. A case of extreme failure to thrive is shown from an Lmod3 KO mouse and its WT littermate at 12 days of age.



Supplemental Figure 4. Lmod3 KO mice have a smaller heart size and minimal impairment in cardiac function. (A) H&E analysis of heart sections show that Δ2 KO mice have considerably smaller hearts compared to their wild-type litter mates, starting as early as one week of age. Scale bar: 1 mm. (B) The difference in heart size is not significant when normalized to the tibia length of the animals. (HM/TL: heart mass/ tibia length). (C) Heart rate and fractional shortening of 12 week-old KO mice as determined by echocardiography. **p<0.01. Data are presented as mean ± SEM. n=6 mice/group.

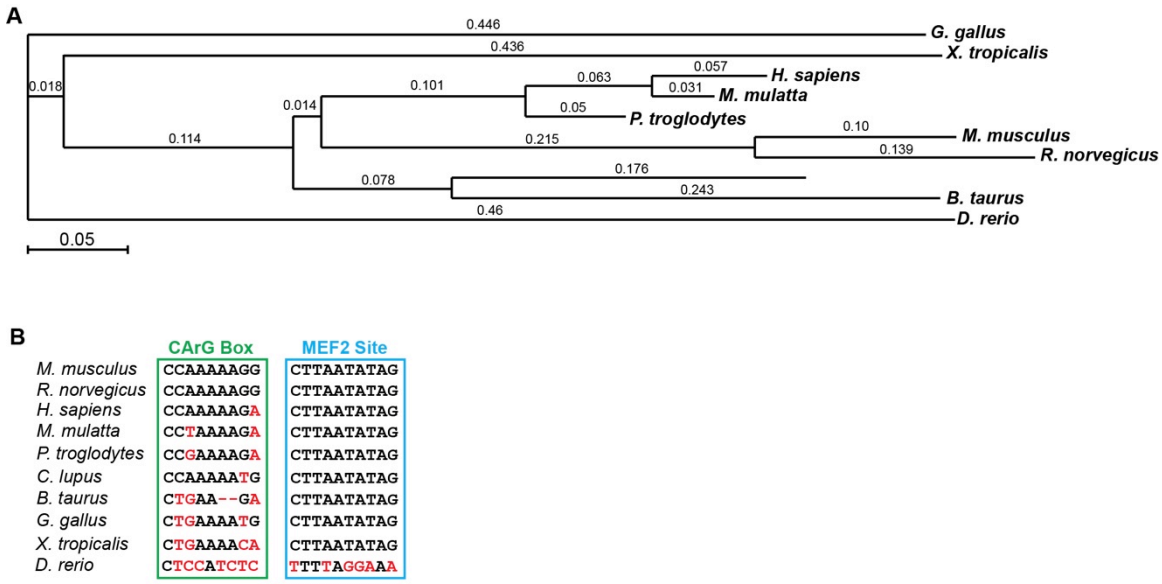


Supplemental Figure 5. Phalloidin staining of longitudinal muscle sections. Tibialis anterior muscle was dissected from 3 week-old Δ2 KO and WT mice. Rhodamine conjugated phalloidin (red) and DAPI (blue) immunostaining of longitudinal myofibers shows disorganization of sarcomeres and disruption of thin filament architecture in the KO. Scale bars: 20 μm.

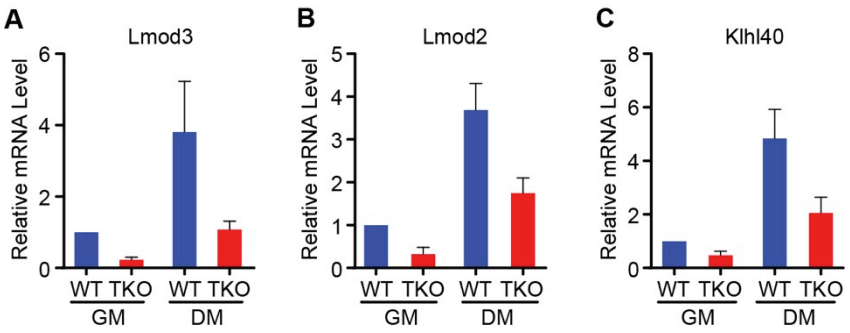


Supplemental Figure 6. Gene expression analysis of *Lmod3* KO mice.

RNA was isolated from skeletal muscle dissected from 3 week-old Δ2 KO and WT mice. Quantitative real-time PCR analysis was conducted with Taqman probes for muscle disease (A), atrophy (B) and regeneration (C) genes. Experiments were performed in triplicate with three biological replicates and expression was normalized to 18S rRNA. **p<0.01. ****p<0.001.



Supplemental Figure 7. Sequence conservation of MEF2 and SRF binding sites of the *Lmod3* promoter. (A) ClustalW multiple sequence alignment of the Lmod3 genomic sequence from fish to human. (B) The MEF2 site is perfectly conserved across all species except for zebrafish. The CArG box is partially conserved. Alignments and taxonomy map were generated using MacVector v13.0.7.



Supplemental Figure 8. Lmod3, Lmod2 and Klhl40 are MEF2-dependent.

Quantitative real-time PCR analysis was performed to assess (A) *Lmod3,* (B) *Lmod2* and (C) *Klhl40* gene expression in WT and MEF2 TKO satellite cells cultured in either growth media (GM, 10% FBS in DMEM) or differentiation media (DM, 2% horse serum in DMEM). DM data shown were collected at day 3. Experiments were performed in triplicate with three biological replicates and expression was normalized to 18S rRNA.

Supplemental References Cited

1. Kim HJ, Lee HJ, Kim H, Cho SW, and Kim JS. Targeted genome editing in human cells with zinc finger nucleases constructed via modular assembly. *Genome Res.* 2009;19(7):1279-88.
2. Wefers B, Panda SK, Ortiz O, Brandl C, Hensler S, Hansen J, Wurst W, and Kuhn R. Generation of targeted mouse mutants by embryo microinjection of TALEN mRNA. *Nat Protoc.* 2013;8(12):2355-79.
3. Shelton JM, Lee MH, Richardson JA, and Patel SB. Microsomal triglyceride transfer protein expression during mouse development. *J Lipid Res.* 2000;41(4):532-7.
4. Liu N, Nelson BR, Bezprozvannaya S, Shelton JM, Richardson JA, Bassel-Duby R, and Olson EN. Requirement of MEF2A, C, and D for skeletal muscle regeneration. *Proc Natl Acad Sci U S A.* 2014;111(11):4109-14.