Supporting Information

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SI Materials and Methods

cDNA and Real-Time PCR. For mRNA quantification in knockout mice, total RNA was isolated using TRIzol (Invitrogen) from pooled embryonic day 18.5 (E18.5) hindlimb muscles, primarily quadriceps, gastrocnemius, soleus, and tibialis anterior. Purified RNA was DNase-treated and then reverse-transcribed with random hexamers using a SuperScript III First-Strand Synthesis Kit (Invitrogen). cDNA samples were analyzed using a StepOne Real-Time PCR instrument (Applied Biosystems) with TaqMan probes for RyR1 [the skeletal muscle-specific isoform of the ryanodine receptor (RyR)] (Mm01175211_m1), voltage-dependent L type calcium channel, alpha 1S subunit (CACNA1S) (Mm00489257_m1), voltage-dependent L type calcium channel, beta 1 subunit (CACNB1) (Mm0136805_m1), and myogenin (Mm00446195_g1). SYBR Green was used for Stac (5′-TCCAGCCAACTTTGTTCAGA-3′, 5′-ATGAAGCCGTCCTGTTCTTC-3′), Stac2 (5′-AGAATGTTTGGCGATGCTGT-3′, 5′-TGCTTCTGCTCACGCCTAC-3′), and src homology 3 (SH3) and cysteine rich domain 3 (*Stac3*) (5′-CGGATTCTTCCCTCCAAACT-3′, 5′-CCACCAGCTTCATCTCCTTT-3′). Values for each sample were normalized to ribosomal RNA control probes (Invitrogen).

In Situ Hybridization. Tissues for in situ hybridization were harvested from anesthetized mice following fixation via transcardial perfusion with 4% (wt/vol) paraformaldehyde, Bouin's, or 10% (vol/vol) neutral-buffered formalin. Subsequent paraffin processing, embedding, and sectioning and histological stains were performed by standard procedures (1, 2). Probe template corresponding to the full-length coding sequence of mouse *Stac3* was PCR-amplified with the primers 5′-ATGACAGAAAAGGAAGTGGTGGA-3′ and 5′-AATCTCCTCCAGGAAGTCG-3′ and cloned into pCRII-TOPO (Invitrogen). ^{35}S-labeled probes were generated from linearized cDNA templates by in vitro transcription using a MAXIscript Kit (Ambion). Radioisotopic in situ hybridization was performed as previously described in Shelton et al. (3). Briefly, sagittal sections of E15.5 embryos and transverse thoracic sections of E14.5 embryos were deparaffinized, permeabilized, and acetylated before hybridization at 55 °C with riboprobes diluted in a mixture containing 50% (vol/vol) formamide, 0.3 M NaCl, 20 mM Tris·HCl (pH 8.0), 5 mM EDTA (pH 8.0), 10 mM $NaPO_4$ (pH 8.0), 10% (wt/vol) dextran sulfate, 1× Denhardt's, 0.5 mg/mL tRNA. Following hybridization, the sections were rinsed with increasing-stringency washes, subjected to RNase A (2 μg/mL, 30 min at 37 °C), and dehydrated before dipping in K.5 nuclear emulsion gel (Ilford). Autoradiographic exposure was 42 d. Bright-field and dark-field images were obtained for each specimen. Using ImageJ (4) for all steps, the bright-field image was converted to grayscale. For the dark-field image containing the signal, the saturation and brightness thresholds were set to capture the signal (approximately the upper third of each scale). The image was then converted to grayscale and the look-up table was converted to cyan. The resulting signal image was then subtracted from the grayscale bright-field image to yield the final image. All adjustments were made uniformly to the entire images.

Northern Blot. A Northern blot containing 20 μg total RNA per lane from 15 mouse tissues was purchased from Zyagen (MN-MT-1). [^{32}P]dCTP-labeled probe was transcribed in vitro using the full-length *Stac3* coding sequence. Following overnight hybridization at 68 °C and washing, the blot was exposed to autoradiographic film for 3 h.

Genotyping. Tail biopsies were digested overnight at 55 °C in tail digest buffer [50 mM KCl, 10 mM Tris·HCl, pH 8.3, 2.5 mM $MgCl_2$, 0.2 mg/mL gelatin, 0.45% (vol/vol) Nonidet P-40, 0.45% (vol/vol) Tween 20, 0.15 mg/mL proteinase K]. Samples were then vortexed to dissociate tissue and centrifuged (15,000 × *g*). Multiplex PCR reactions (25 μL) were prepared on ice as follows: 800 nM common primer: 5′-CTCCTCACCTGTGAGTATTTGG-3′; 400 nM wild-type primer: 5′-CTCAACCTTCCTAATGCTGTGG-3′; 200 nM knockout primer: 5′-AACTTGTTGATATCGTGGTATCGTT-3′; 1× FailSafe PCR buffer D (Epicentre); 1 μL tail digest buffer (no proteinase K); 1 μL tail lysate; 1 U Taq DNA polymerase (New England BioLabs). The thermal protocol was 94 °C for 5 min; 40 cycles of 94 °C for 15 s, 58.5 °C for 30 s, 72 °C for 15 s; 72 °C for 5 min. The wild-type allele produced a band of 507 bp and the knockout produced a band of 301 bp.

β-Galactosidase Staining. Embryos were harvested and fixed in 1× PBS, 0.8% (vol/vol) formaldehyde, 0.2% (vol/vol) glutaraldehyde for 30 min to 1 h at 4 °C followed by a washing in PBS for 30 min at 4 °C. Specimens were then placed in staining solution (5 mM potassium ferrocyanide, 5 mM potassium ferricyanide, 2 mM $MgCl_2$, 1 mg/mL X-gal, 1× PBS) for several hours to overnight at 37 °C, washed the next day, and further fixed in 1× PBS with 4% (vol/vol) formaldehyde.

Muscle Electroporation and Imaging. Flexor digitorum brevis muscles (FDB) were electroporated following a published protocol (5). Following anesthesia by isoflurane, 15 μL of 2 mg/mL hyaluronidase was injected beneath the skin of the foot and footpad. One hour later the mice were anesthetized again and DNA expression vector containing a green fluorescent fusion gene was injected. The foot was then pierced with two sterile gold-plated acupuncture needles and 20 1-s pulses of 100 V were applied. Following a 3-d recovery, the mice were killed, their feet were skinned, and the unfixed FDB was examined directly in imaging buffer by two-photon laser scanning microscopy (Zeiss; LSM 780) with reverse second harmonic generation to visualize the A bands as an internal reference.

Diaphragm Immunostaining. E18.5 diaphragm muscles were fixed with 2% (vol/vol) paraformaldehyde in 0.1 M phosphate buffer (pH 7.3) overnight at 4 °C. Muscle samples were incubated with 2 nM Texas red-conjugated α-bungarotoxin (Invitrogen) for 30 min, and then with the antibody against syntaxin 1 (1:1,000) (gift from Thomas Südhof, Stanford University School of Medicine, Stanford, CA) overnight at 4 °C. Then muscle samples were incubated with fluorescein isothiocyanate-conjugated goat anti-rabbit IgG overnight at 4 °C. Muscle samples were washed with PBS and mounted in Vectashield mounting medium. Fluorescent images were acquired using a Zeiss LSM 510 Meta confocal microscope.

Electrophysiology. Electrophysiological analyses of neuromuscular synaptic and muscle activity were carried out on acutely isolated E18.5 phrenic nerve-diaphragm muscle preparations, as previously described (6). Miniature end-plate potentials and action potentials were acquired by sharp intracellular microglass electrodes via an intracellular amplifier (AxoClamp-2B; Molecular Devices), digitized with Digidata 1332 (Molecular Devices), and analyzed with pClamp 9.0 (Molecular Devices) and Mini Analysis Program (Synaptosoft). Data are presented as the mean ± SEM, and statistical differences were determined by Student's *t* test.

Skeleton Staining. Alizarin red S/alcian blue skeletal preparations were carried out using standard procedures (7). Briefly, neonatal carcasses were skinned and eviscerated. Carcasses were dehydrated in 95% (vol/vol) ethanol for several hours and then incubated in alcian blue staining solution [150 mg/L alcian blue 8GX, 20% (vol/vol) glacial acetic acid, 75% (vol/vol) ethanol] for 2 d with constant rotation. The solution was changed to fresh 95% (vol/vol) ethanol for 12 h with rotation and then to 1% (wt/vol) KOH overnight. The skeletons were transferred to alizarin red S staining solution [25 mg/L alizarin red S, 1% (wt/vol) KOH] for several hours until the bone tissue reached the desired hue. Processed specimens were stored and imaged in one part 70% (vol/vol) ethanol, one part glycerin.

Contraction Assays. The measurement of muscle contraction was carried out as described previously (8, 9). In brief, E18.5 diaphragm muscles were isolated in situ at room temperature in oxygenated Ringer's solution containing 118 mM NaCl, 4.75 mM KCl, 1.18 mM $MgSO_4$, 2.54 mM $CaCl_2$, 1.18 mM NaH_2PO_4, 10 mM glucose, and 24.8 mM $NaHCO_3$. A muscle strip cut from the right dorsal diaphragm was vertically mounted in an isometric tissue clamp of a force transducer (FORT25; WPI) suspended in a 25-mL organ bath (Myobath; WPI) filled with Ringer's solution continuously bubbled with a mixture of 95% O_2 and 5% CO_2 at 37 °C. To block neuromuscular transmission from phrenic-nerve terminals, 10 μM tubocurarine (Sigma-Aldrich) was added to the bath solution (8). Muscle contraction was elicited by field stimulation with parallel wire electrodes. Isometric contractions were evoked by a train of 50 pulses of 1-ms duration, 80 mA (A385 Stimulator; WPI) at 50 Hz delivered every 20 s. Data were acquired using a transbridge (4M; WPI) and pCLAMP 9 software (Molecular Devices) at a sampling rate of 2 kHz. Data were analyzed using Clampfit (Molecular Devices) and Origin 6 (OriginLab). After measurement of muscle contraction by field electrical stimulation, the same diaphragm muscle strip was sequentially bathed in 4-chloro-*m*-cresol (4-CMC; 1 mM; 1:500 dilution from a 0.5 M stock solution in 100% ethanol)/Ringer's solution, a high-potassium (120 mM) solution, and 4-CMC (1 mM)/Ringer's solution for 5 min each exposure. Each exposure was separated by a 5-min Ringer's solution incubation to allow the muscle to recover. The high-potassium solution was made by reducing the NaCl concentration in the Ringer's solution. Test solutions were prewarmed to 37 °C and applied by perfusion of eight times the bath volume at a flow rate of ~2.5 mL/s. Muscle contraction was recorded at a sampling rate of 2 Hz for 40 min. On completion of contraction measurement, the wet weight of the muscle strip was determined.

Electron Microscopy. Tongues were harvested from E18.5 fetuses and fixed by immersion in 2.5% (vol/vol) glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.4) containing 50 mM $CaCl_2$, rinsed in buffer, and then postfixed in buffered 2% (wt/vol) osmium tetroxide containing 0.8% (wt/vol) potassium ferricyanide for 3 h. Tissues were rinsed with dH_2O, en bloc stained in 4% (wt/vol) uranyl acetate in 50% (vol/vol) ethanol, dehydrated with a graded series of ethanol, and embedded in EMbed 812 resin (Electron Microscopy Sciences). Thin sections were cut on a Leica Ultracut UCT ultramicrotome and then stained with 2% (wt/vol) uranyl acetate and lead citrate. Images were acquired on an FEI Tecnai G^2 Spirit electron microscope equipped with an LaB_6 source Gatan CCD camera and operated at 120 kV.

Calcium Measurements. Primary myoblasts were plated at ~90% confluence on laminin-coated 35-mm dishes and then differentiated for 6 d. Myotubes were loaded with the fluorescent calcium indicator fluo-4-AM (Invitrogen) by 30-min incubation at 37 °C with medium containing 5 μM fluo-4-AM, 0.02% (wt/vol) Pluronic F-127 (Invitrogen), and 0.1% (wt/vol) probenecid. Myotubes were subsequently washed and incubated for another 30 min at 37 °C with probenecid-containing medium. Calcium transients were recorded using a DeltaVision pDV microscope (Applied Precision) with a 20× objective, FITC filters, and a heated chamber. A total of 4,200 frames were collected at 100-ms intervals for each sample. Beginning just before imaging, medium was continuously perfused at a rate of 12.5 mL/min through a closed-bath perfusion chamber (Harvard Apparatus). The perfusion buffers were as follows: Ca^{2+}-free Ringer's solution for wash (118 mM NaCl, 4.75 mM KCl, 1.18 mM $MgSO_4$, 1.18 mM NaH_2PO_4, 10 mM glucose, 24.8 mM $NaHCO_3$); Ca^{2+}-free high-K^+ Ringer's solution (2.75 mM NaCl, 120 mM KCl); high-K^+ Ringer's solution (2.75 mM NaCl, 120 mM KCl, 2.54 mM $CaCl_2$); and Ca^{2+}-free Ringer's solution with 1 mM 4-CMC. Each treatment lasted a total of 600 frames or 1 min. *z*-axis plots were generated using ImageJ (4) by selecting the outlines of mature myotubes. *z*-axis plots for background regions were also generated and then subtracted from the myotube signal to yield the corrected value.

Primary Myoblast Cultures. Myoblasts were isolated from the forelimbs and hindlimbs of E18.5 fetuses. Briefly, skin was removed from limbs, which were then incubated at 37 °C for 4 h in tissue dissociation buffer [1× PBS, 0.05% (wt/vol) collagenase D (Roche), 2 mM $CaCl_2$, 1× penicillin/streptomycin/glutamine (Invitrogen)] with shaking at 70 rpm. The dissociated tissue solution was diluted with myoblast culture medium [Ham's F-10 (Invitrogen), 20% (vol/vol) fetal bovine serum (Invitrogen), 1× penicillin/streptomycin (Invitrogen), 2.5 ng/mL human basic fibroblast growth factor (Promega)] and pelleted by centrifugation (150 × *g*). The pellet was resuspended in myoblast growth medium, passed through a 70-μm nylon cell strainer, and preplated on a 10-cm uncoated cell-culture dish. The cells were incubated for 1.5 h at 37 °C in a humidified incubator with 5% CO_2 atmosphere. The cell suspension was then replated onto a 10-cm laminin-coated cell-culture dish. Cells were passaged by trypsinization and preplating until relatively homogeneous for myoblasts. Medium was replenished every day. For differentiation, myoblasts were plated on laminin-coated dishes at high density. When the cells were nearing confluence, the culture medium was changed to differentiation medium [DMEM (HyClone), 2% (vol/vol) heat-inactivated horse serum (Invitrogen), 1× penicillin/streptomycin/glutamine (Invitrogen)] for 5 d.

Western Blots. Tissue samples were snap-frozen in liquid nitrogen for storage and then dounce-homogenized in RIPA [150 mM NaCl; 1% (vol/vol) IGEPAL CA-630; 50 mM Tris-HCl, pH 8.0; 1% (wt/vol) sodium deoxycholate; 0.1% (wt/vol) sodium dodecyl sulfate; complete protease inhibitor cocktail tablet (Roche)] buffer. Cell cultures were scraped free and collected with RIPA buffer and then snap-frozen. *N*-ethylmaleimide was added at 5 mM final concentration and the samples were incubated on ice for 30 min before boiling with reducing SDS loading buffer. Samples were electrophoresed in denaturing SDS buffer on 6% polyacrylamide gels with no stacking gel for dihydropyridine receptor (DHPR) α1s and RyR1 and an Any kD gel (Bio-Rad) for α-tubulin. Gels were electroblotted onto PVDF membranes with a semidry blotter (Bio-Rad) and then blocked in 5% (wt/vol) nonfat milk in TBST [25 mM Tris-HCl, pH 7.5; 125 mM NaCl; 0.1% (vol/vol) Tween-20]. Primary antibodies were as follows: DHPR α1s (Thermo Scientific; mouse monoclonal 1A; 1:500), RyR1 (Thermo Scientific; mouse monoclonal 34C; 1:5,000), and α-tubulin (Sigma; mouse monoclonal DM1A; 1:5,000). Primary antibodies were diluted in TBST with 1% (wt/vol) nonfat milk and incubated with blots overnight at 4 °C with rotation. Blots were washed five times with TBST before addition of the secondary antibody in TBST (Bio-Rad; 1:10,000).

Blots were incubated with secondary antibody for 30 min at room temperature with vigorous shaking before five washes with TBST. Blots were dipped in West Pico chemiluminescent substrate (Thermo Scientific) and exposed to autoradiographic film.

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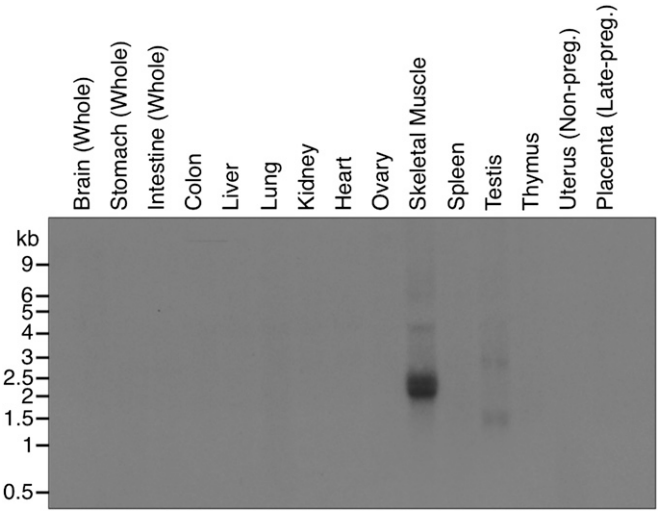


Fig. S1. Multitissue Northern blot. A multitissue Northern blot shows expression of *Stac3* mRNA exclusively in adult skeletal muscle tissue. The double bands in skeletal muscle suggest two mRNA isoforms.

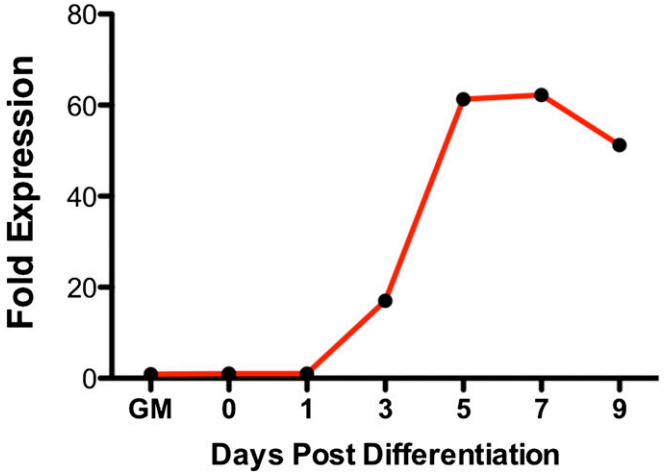


Fig. S2. *Stac3* expression during C2C12 myoblast differentiation. Over the course of differentiation, *Stac3* mRNA increases dramatically. Expression was measured by real--time PCR during differentiation of C2C12 myoblasts. The time course spans from proliferating myoblasts in growth medium (GM) to 9 d of differentiation medium. Values are fold over day 0.

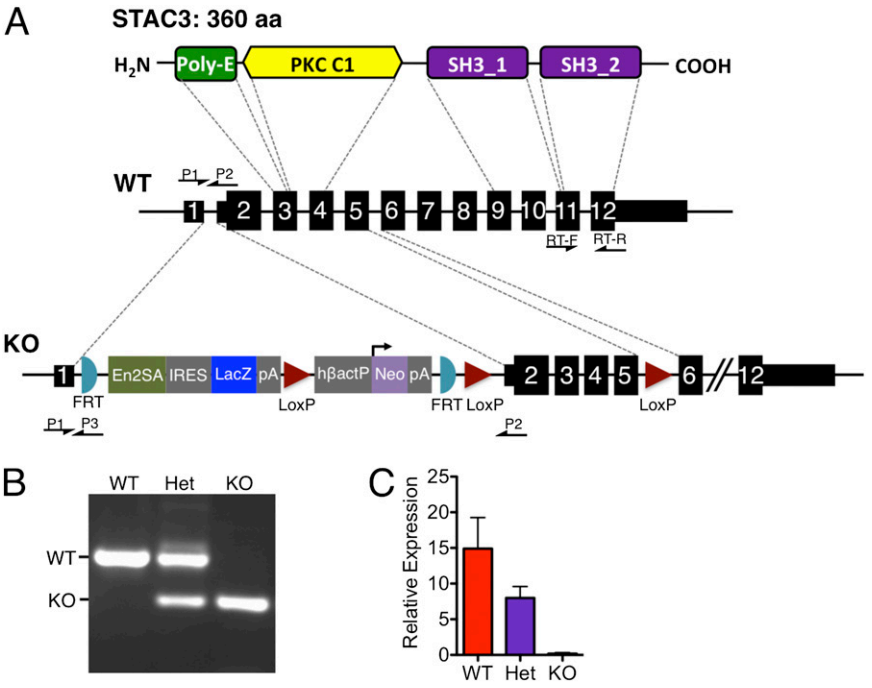


Fig. S3. Knockout mouse allele. (*A*) The National Institutes of Health Knock-Out Mouse Project "knockout first" allele for the *Stac3* locus, containing two FLP recombinase target sequences (FRT), an engrailed 2 splice acceptor (En2SA), an internal ribosome entry site (IRES), β-galactosidase (LacZ), two polyadenylation sequences (pA), three cre recombinase target sequences (LoxP), human β-actin promoter (hβactP), and a neomycin resistance gene (Neo) inserted into the first intron of the *Stac3* locus. Scale is not proportionally accurate. Poly-E, 11 consecutive glutamic acid residues; PKC C1, protein kinase C C1 domain-like; SH3, src homology 3. (*B*) Genotype-specific multiplex PCR using primers P1, P2, and P3 shows homozygosity of the knockout allele. Het, heterozygous. (*C*) Real-time PCR for *Stac3* demonstrating elimination of the transcript in E18.5 hindlimb muscles using primers RT-F and RT-R (mean ± SD; $n = 4$ for WT and KO, $n = 8$ for Het).

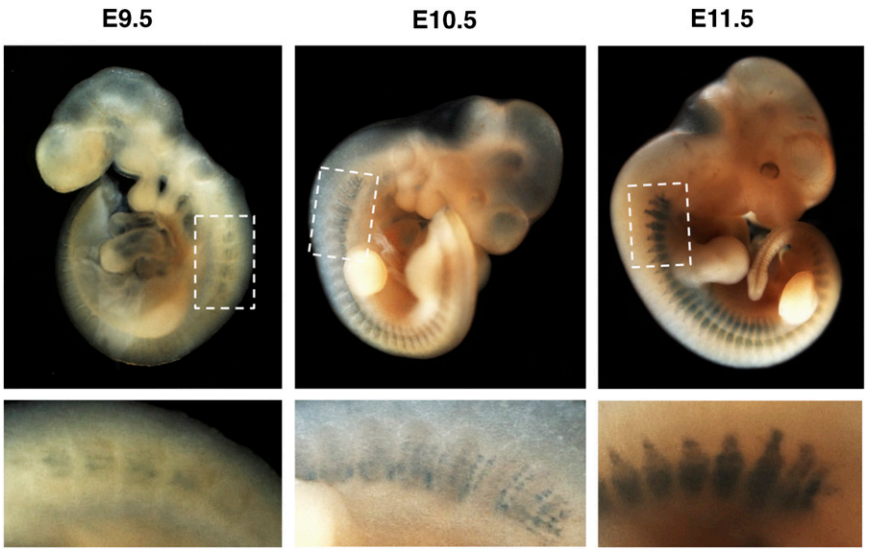


Fig. S4. Expression of the *Stac3*-LacZ knockin in embryogenesis. Expression of the *Stac3*-LacZ knockin allele is observed in somites at early stages in muscle development. Shown here are E9.5, E10.5, and E11.5. (*Lower*) Zoomed-in images from the boxed portions (*Upper*). Heterozygotes are shown.

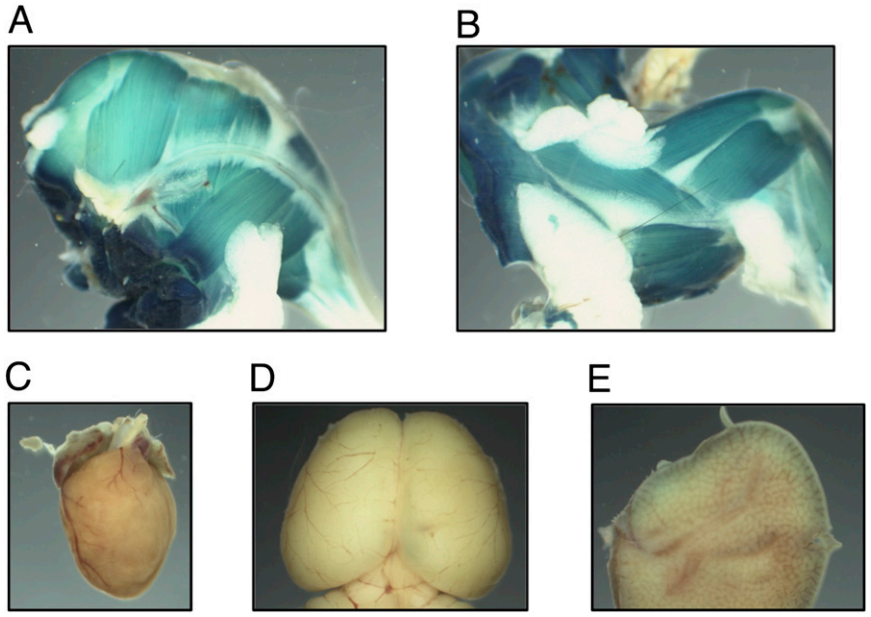


Fig. S5. Expression of the *Stac3*-LacZ knockin in postnatal mice. The *Stac3*-LacZ knockin allele is robustly expressed in postnatal skeletal muscle, but not in any other tissues. (*A*) Medial hindlimb. (*B*) Lateral hindlimb. (*C*) Heart. (*D*) Brain. (*E*) Liver. A heterozygous adult is shown.

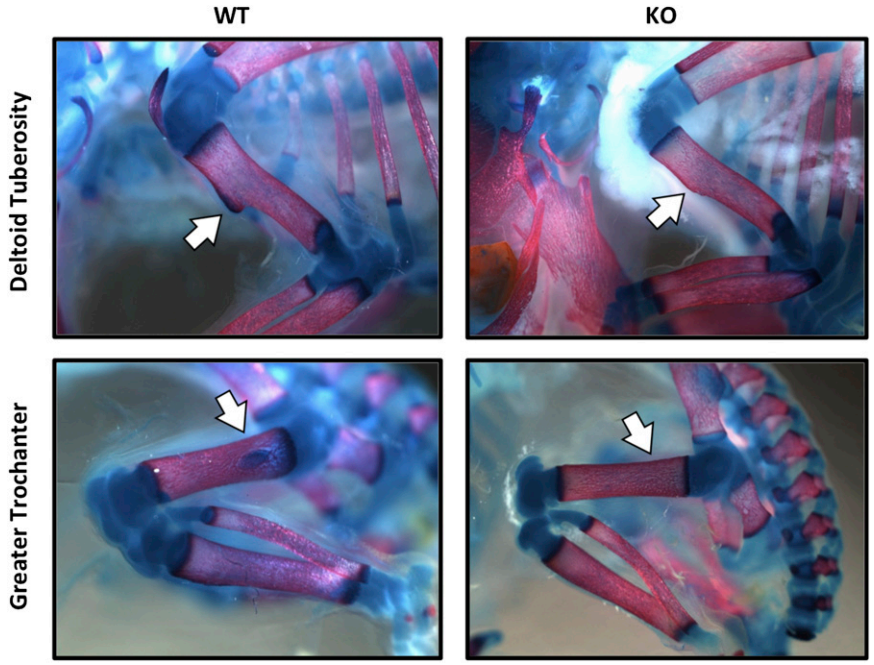


Fig. S6. Decreased bone development at muscle insertion sites. The deltoid tuberosity (arrows in *Upper*) and greater trochanter (arrows in *Lower*) of the femur are severely underdeveloped in KO mice, suggesting lack of fetal muscle contraction. Remaining cervical and scapular fat tissue is also visible in the knockouts.

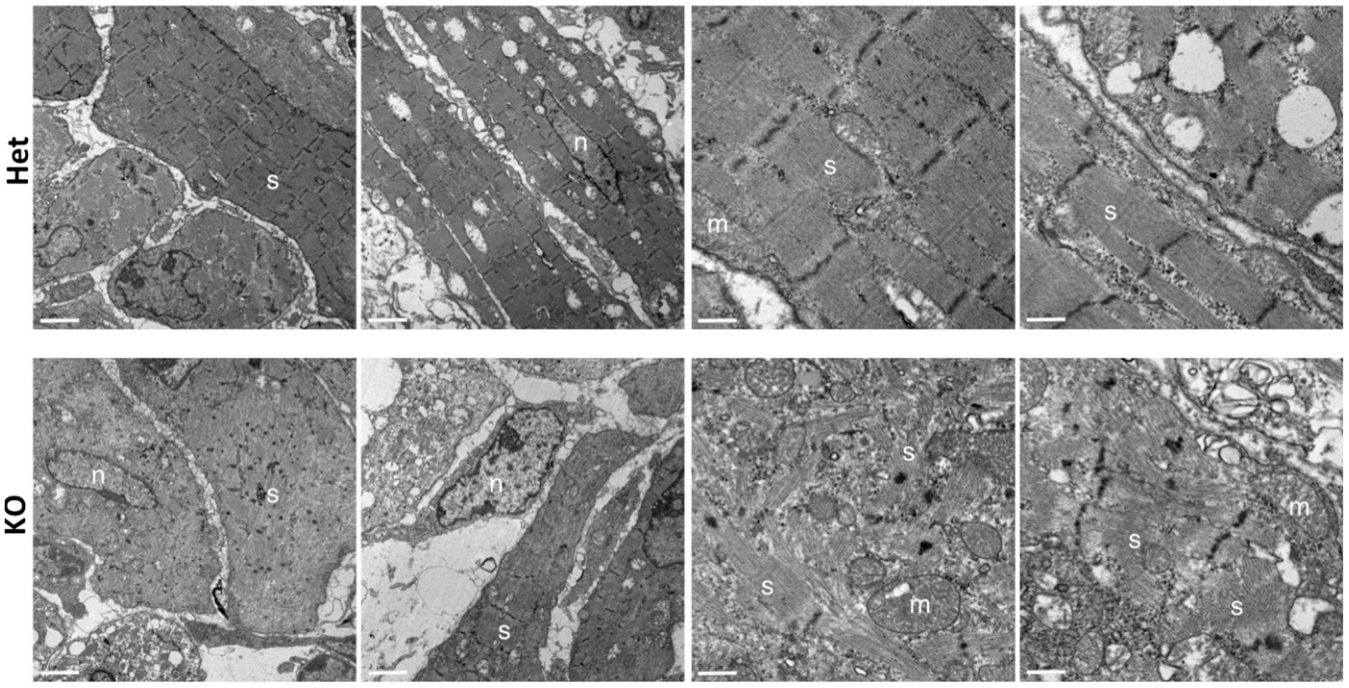


Fig. S7. Transmission electron microscopy of E18.5 tongues. Transmission electron microscopy images of tongues from *Stac3* Het and KO E18.5 mice shows the presence of sarcomeres in KO muscle, but with marked disorganization compared with a heterozygous littermate. [Scale bars, 2 μm (*Left* two images), 0.5 μm (*Right* two images).] m, mitochondrion; n, nucleus; s, sarcomere.

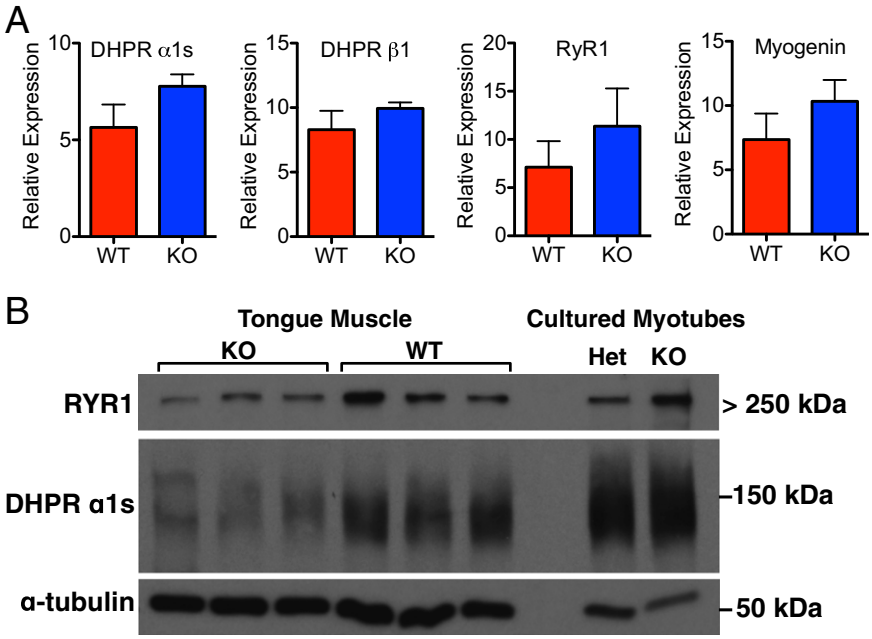


Fig. S8. Expression of known excitation–contraction (EC) coupling components. (*A*) mRNA expression of the EC coupling components DHPR α1s and β1 subunits and RyR1 is normal in E18.5 hindlimb muscles, as well as the skeletal muscle master transcription factor myogenin (mean ± SD; $n = 4$ for each genotype). (*B*) Protein expression of DHPR α1s and RyR1 by Western blot shows a reduction in E18.5 tongues but not in cultured myotubes (*Far Right*).

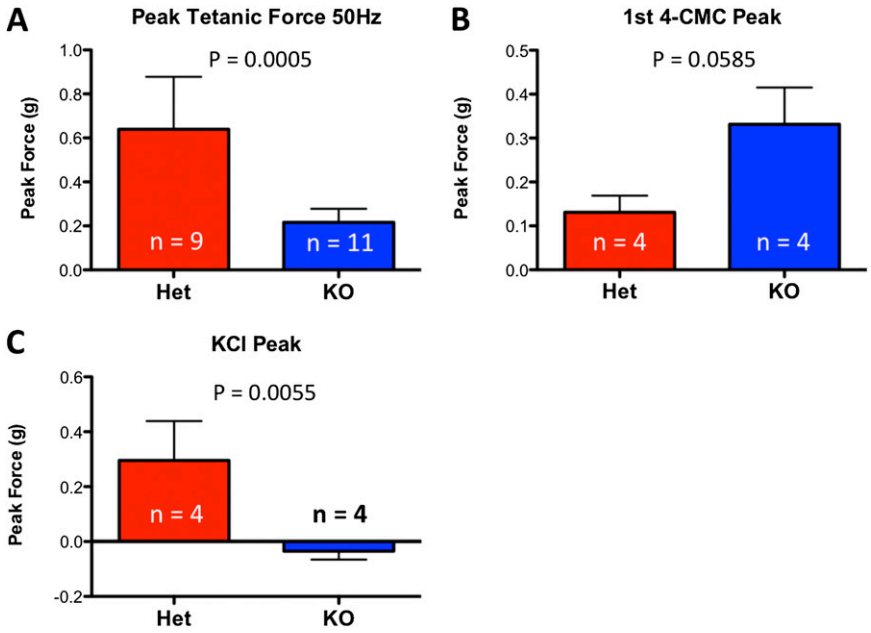
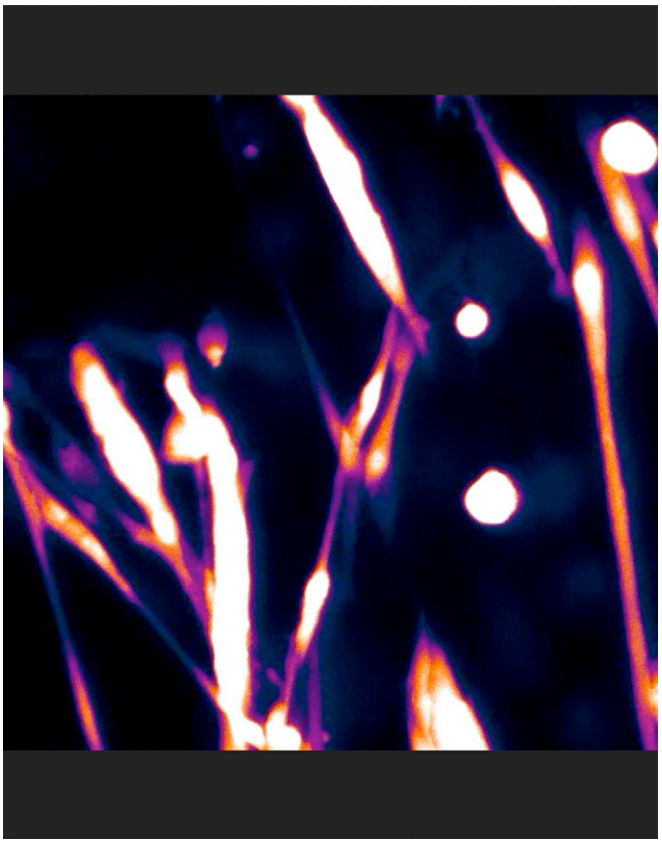


Fig. S9. Quantification of contraction assays. (*A*) Peak force in response to tetanic stimulus in E18.5 diaphragms is significantly reduced in STAC3 KO mice. (*B*) Peak force in response to the first 4-CMC application (a representative trace is shown in Fig. 5*B*) is comparable in Het and KO diaphragms, but (*C*) is significantly reduced in KOs in response to KCl application. All values are weight-normalized means ± SD; *P* values were calculated using Student's unpaired *t* test.

Table S1. ***Stac3***$^{-/-}$ **mice are born in expected ratios**

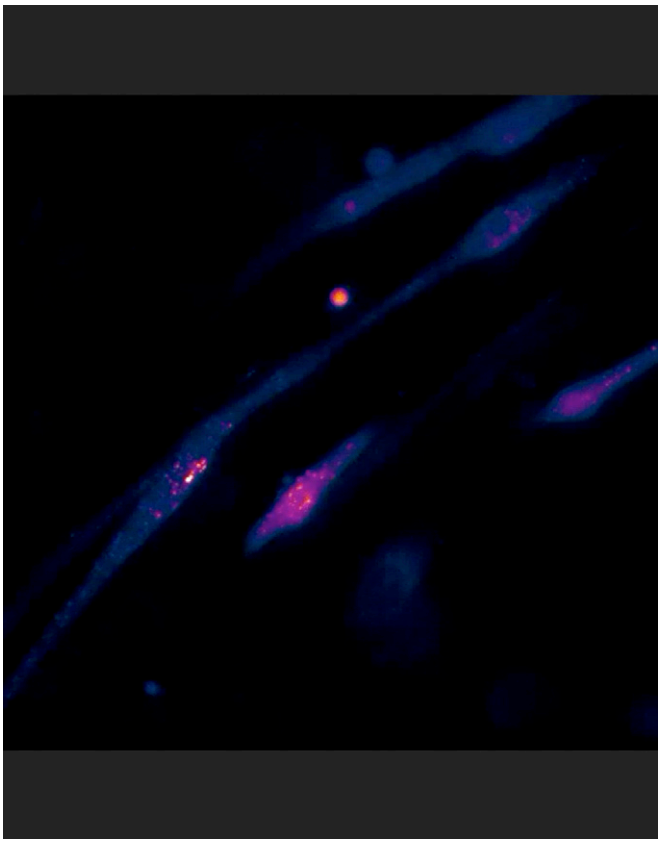
E18.5	+/+, *n* (%)	+/–, *n* (%)	–/–, *n* (%)
Observed	14 (30)	17 (37)	15 (33)
Expected	11.5 (25)	23 (50)	11.5 (25)

Mice resulting from *Stac3*$^{+/-}$ crosses were born in the expected Mendelian ratios. χ^2 two-tailed *P* value = 0.2045.



Movie S1. Fluo-4-AM loaded WT myotubes. WT myotubes were loaded with the fluorescent Ca^{2+} indicator, fluo-4-AM, and imaged during depolarization with KCl, which results in a massive release of calcium. Pseudocolored with the Gem LUT in ImageJ.

Movie S1



Movie S2. Fluo-4-AM loaded KO myotubes. KO myotubes were loaded with the fluorescent Ca^{2+} indicator, fluo-4-AM, and imaged during depolarization with KCl. 4-CMC was then added to the myotubes which resulted in SR Ca^{2+} release. Pseudocolored with the Gem LUT in ImageJ.

Movie S2