**Supplementary Figures:**

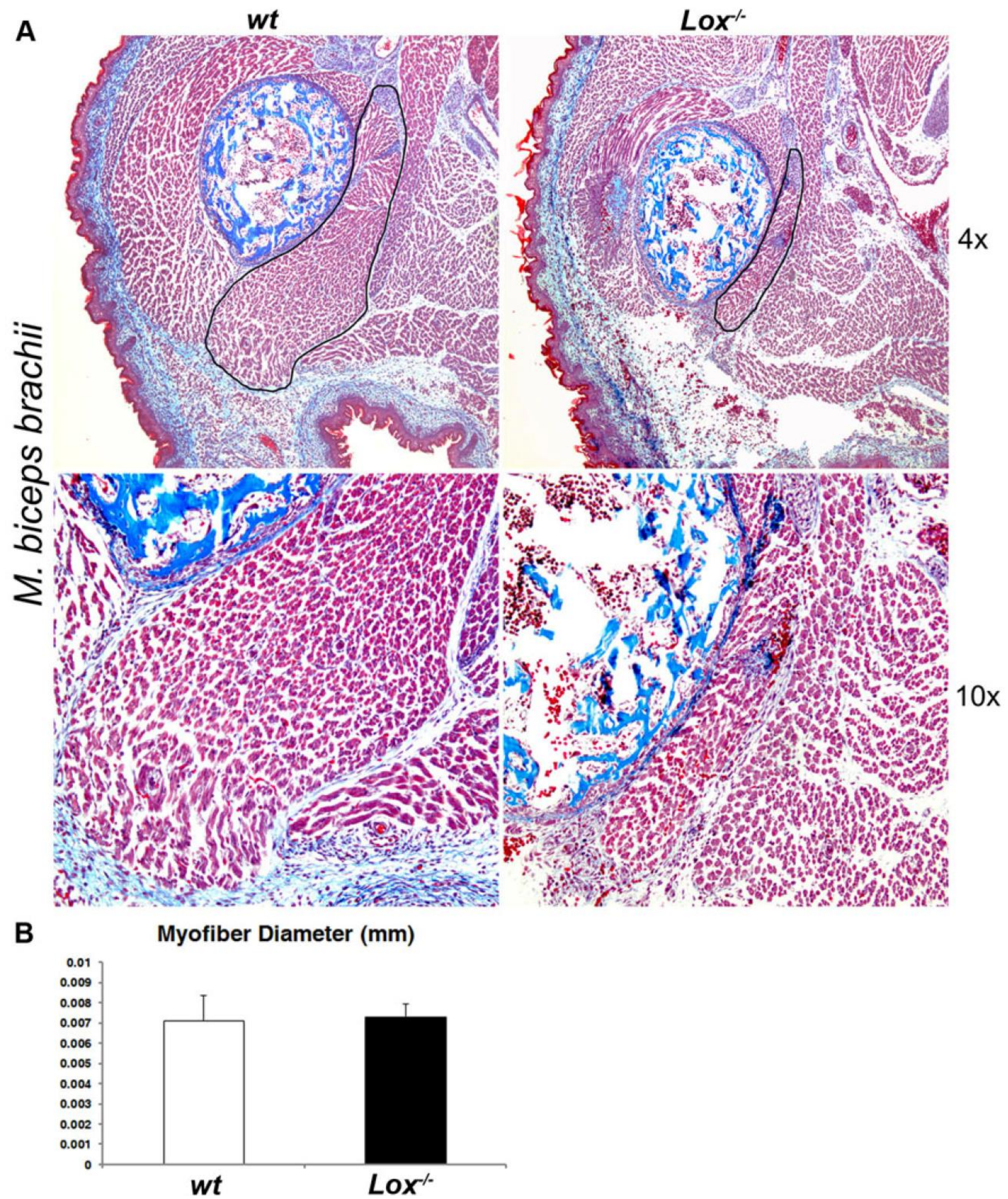


**Figure S1:** Muscles are smaller in *Lox*$^{-/-}$ mutants. Masson trichrome staining of M. Biceps brachii demonstrates smaller muscles and fewer myofibers in *Lox* mutants demonstrating phenotypes are not specific to zeugopod region (A). Myofiber diameter is not affected following *Lox* deletion (B).

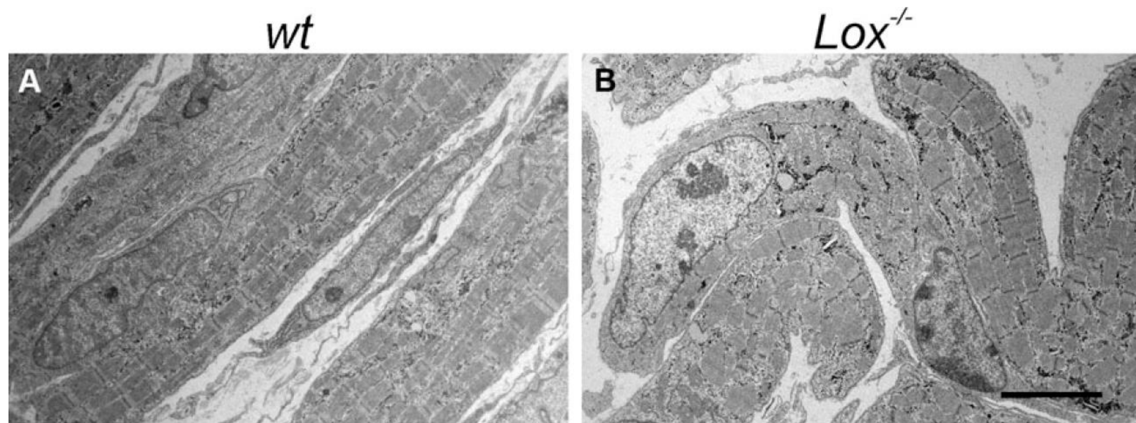


**Figure S2:** Ultrastructure of muscles is not lost in *Lox* mutants. TEM of images of muscles derived from E18.5 wild-type (*wt*; A) and *Lox* mutant (*Lox*$^{-/-}$; B) embryos. Although myofibers sometimes are curved in *Lox*$^{-/-}$ muscles, their overall sarcomeric organization is normal. Bar, 5 μm.

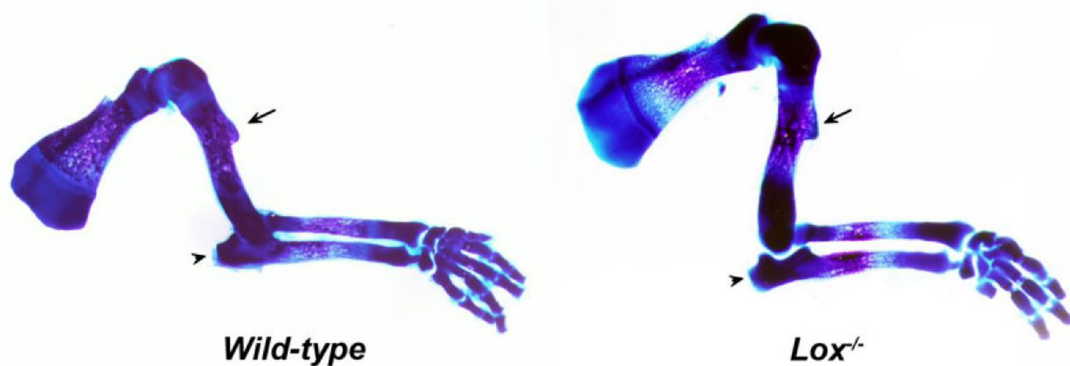


**Figure S3:** Lox does not regulate skeletal patterning. Skeletal preps of E18.5 forelimbs from *wt* (A) and *Lox*$^{-/-}$ (B) stained for alizarin red and alcian blue demonstrate the overall patterning of the skeleton is normal in Lox mutants. Note the olecranon processes (arrowheads) and deltoid tuberosities (arrows) are normally positioned and of comparable sizes in the two genotypes.

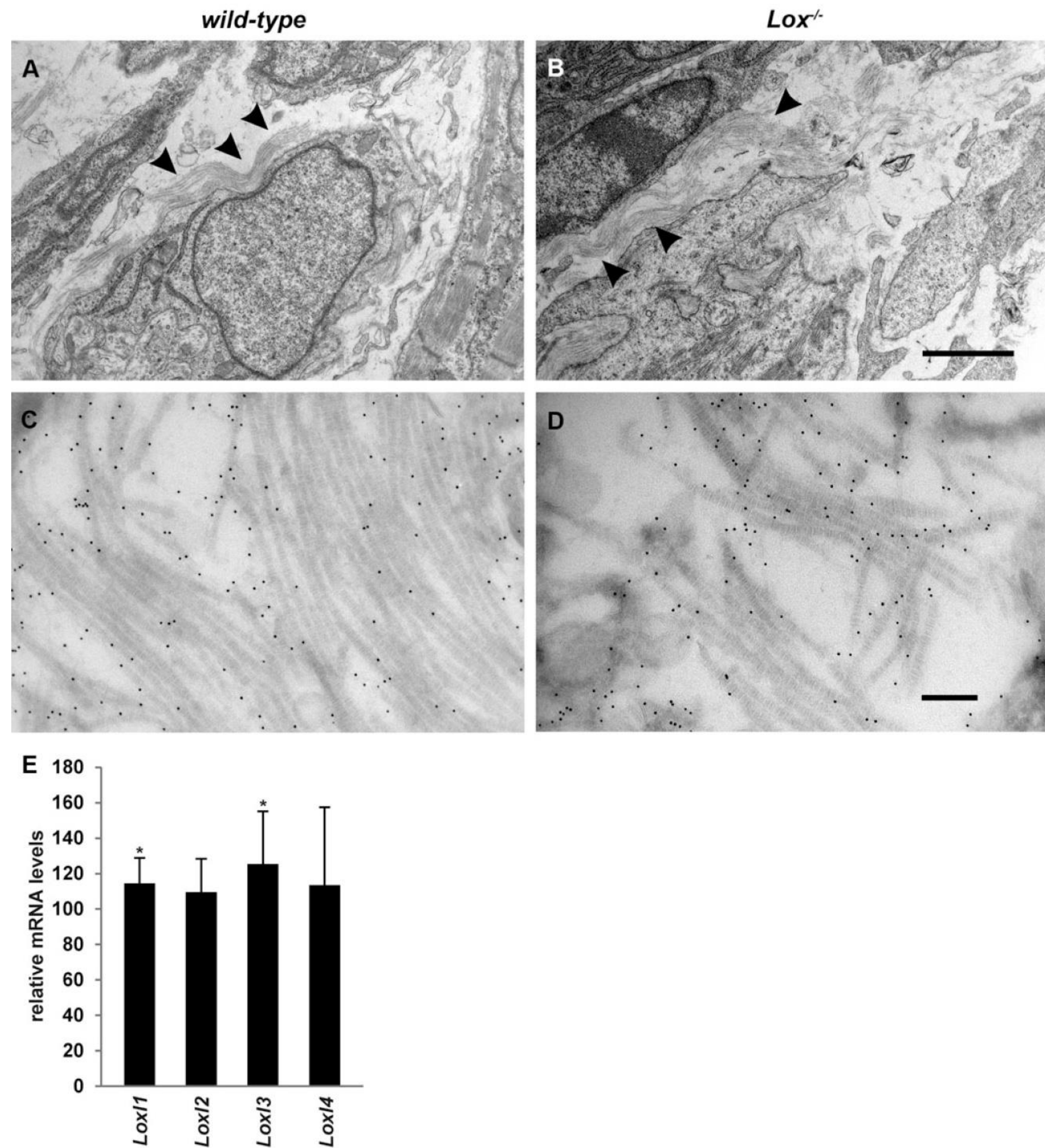


**Figure S4:** Collagen fibril organization is lost in *Lox*$^{-/-}$ muscles. TEM images derived from wild-type (A,C) and *Lox*$^{-/-}$ (B,D) of E18.5 embryos. Note that although collagen fibers are present in mutant muscles (black arrowheads, B) immuno-EM analysis using antibodies directed against ColI demonstrates collagen fibrils are disorganized in mutant muscles (C,D). Quantitative real time PCR analysis from muscle lysates for other members of the *Lox* family reveals *LoxL1* and *LoxL3* are significantly upregulated in mutants (E). Bars, 2 µm in A-B, and 200 nm in C-D.

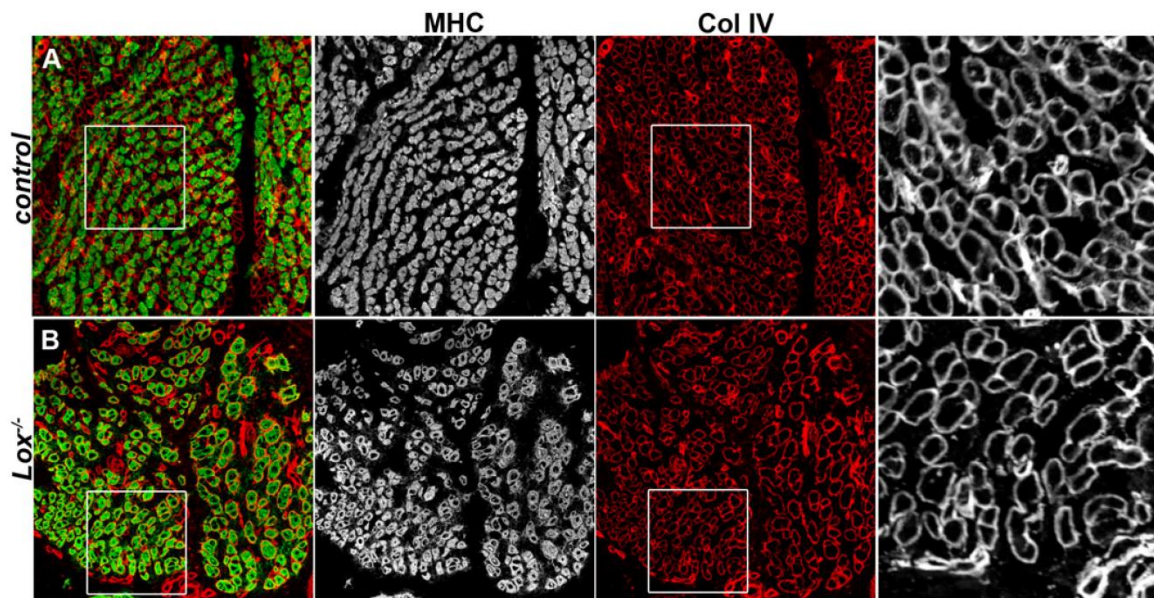


**Figure S5:** Collagen IV organization is not affected by Lox. Section immunostaining for Myosin (A4.1025; MHC) and for Collagen type IV (Col IV) on E18.5 muscle sections reveals that unlike ColI expression, that of Col IV is not disrupted in Lox mutant muscles (A,B).

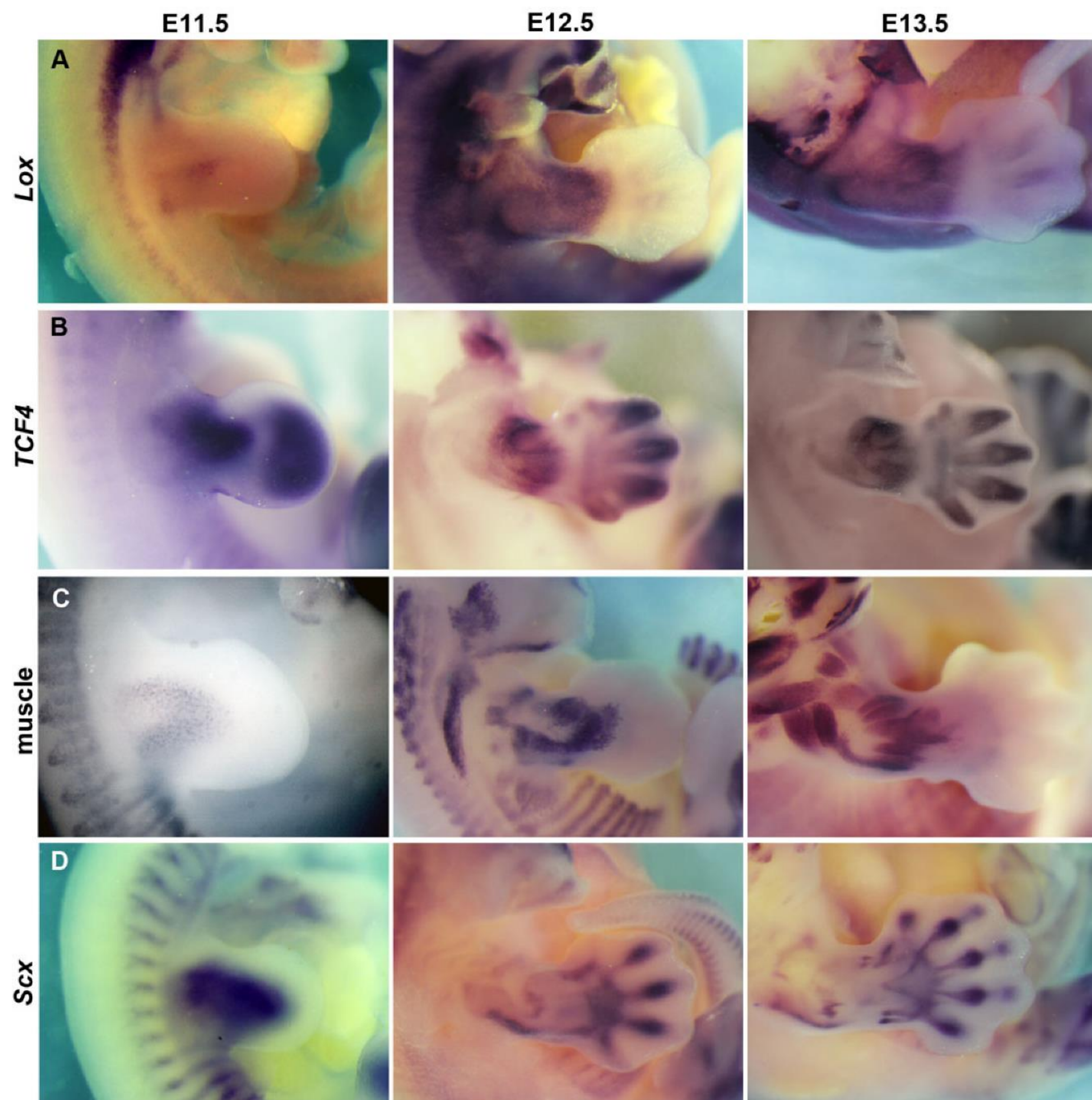


**Figure S6:** *Lox* expression in the developing limb. Whole-mount RNA *in situ* hybridization stainings for *Lox* (A), *Tcf4* marking the MCT fibroblasts (B), muscle progenitors marked by *Pax3* at E11.5 and *MyoD* at E12.5 and E13.5 (C) and *Scleraxis* (*Scx*) marking the tendon progenitors (D) at E11.5-E13.5. Note that *Lox* expression coincides with muscle development although its early expression is less well defined than that of *MyoD*.

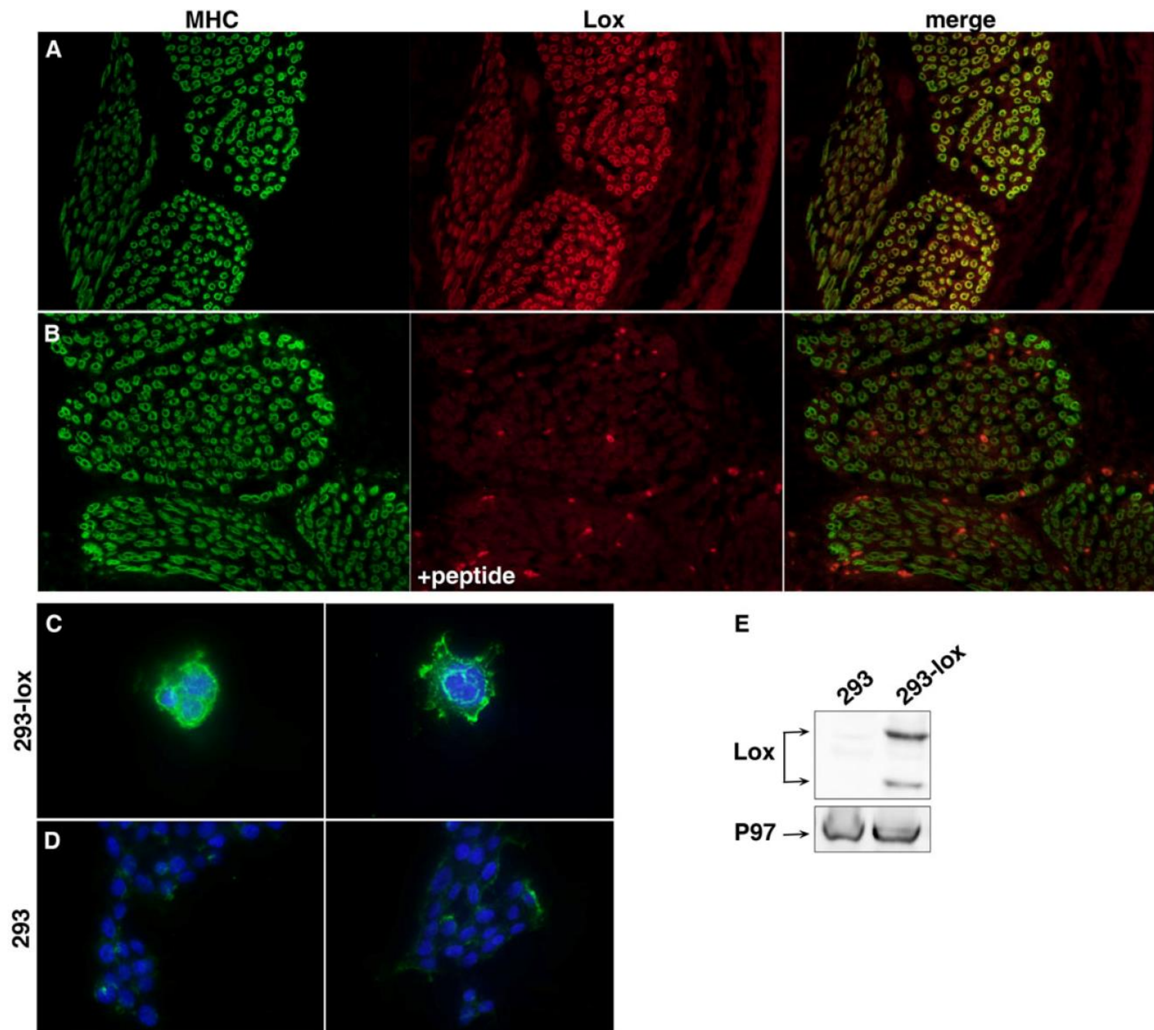


**Figure S7:** Lox antibody specificity. Lox antibody was raised against the EDTSCDYGYHRRFA peptide. Antibody specificity was tested using immunohistochemistry on paraffin sections (A,B), immunocytochemistry (C,D) and western blots (E). E15.5 sections were stained with anti-MHC (A4.1025, green) and anti-Lox (red) which was either pre-incubated with the peptide it was raised against or in blocking solution. Note that incubation with the peptide completely blocked staining suggesting the antibody specifically identifies this peptide (A,B). Mock or Lox transfected HEK 293 cells were stained for DAPI and Lox (green) (C,D) or lysed for a western blot analysis (E). Note the specific augmentation of Lox expression in cells over-expressing Lox.

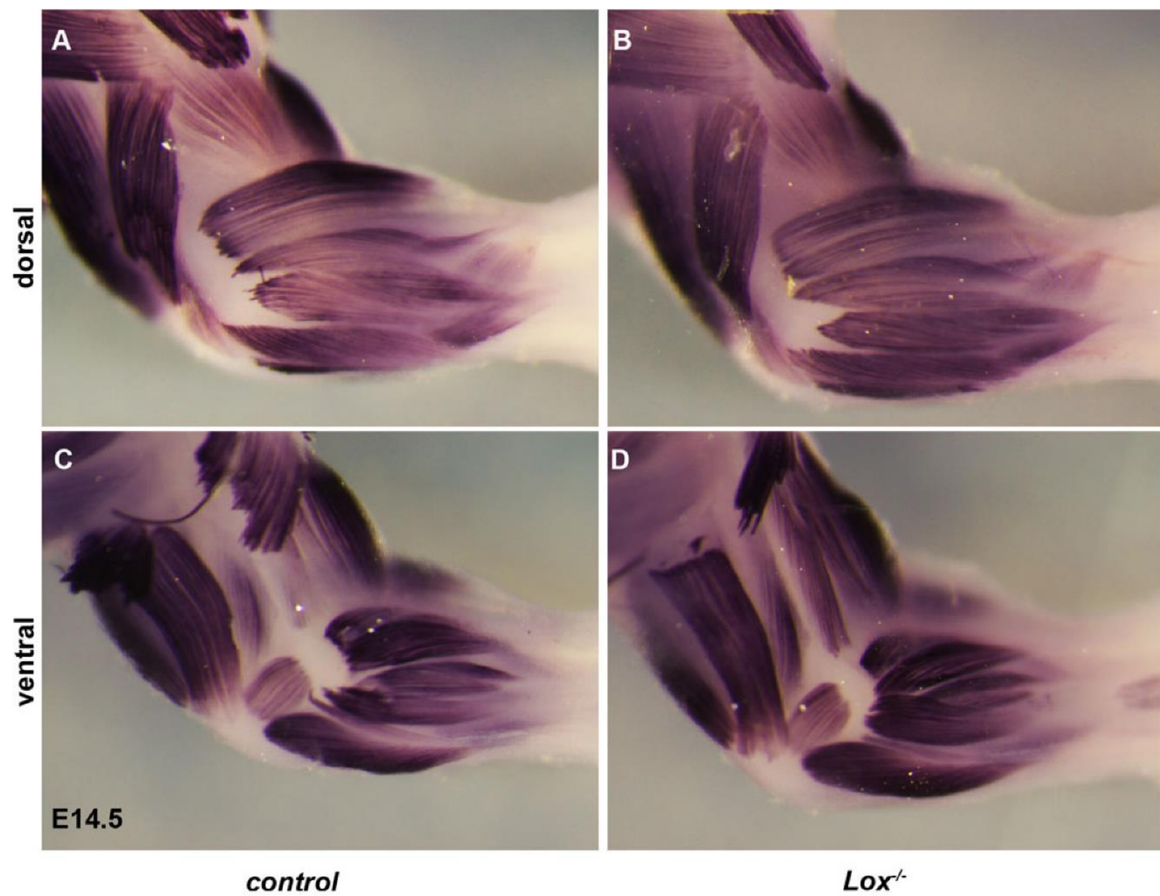


**Figure S8:** Muscle patterning defects and reduced myofiber amounts are not observed in *Lox* mutant limbs at E14.5. Whole-mount MHC (My32) of wild-type (A,C) and *Lox*$^{-/-}$ (B,D) demonstrates normal MHC expression at this stage.

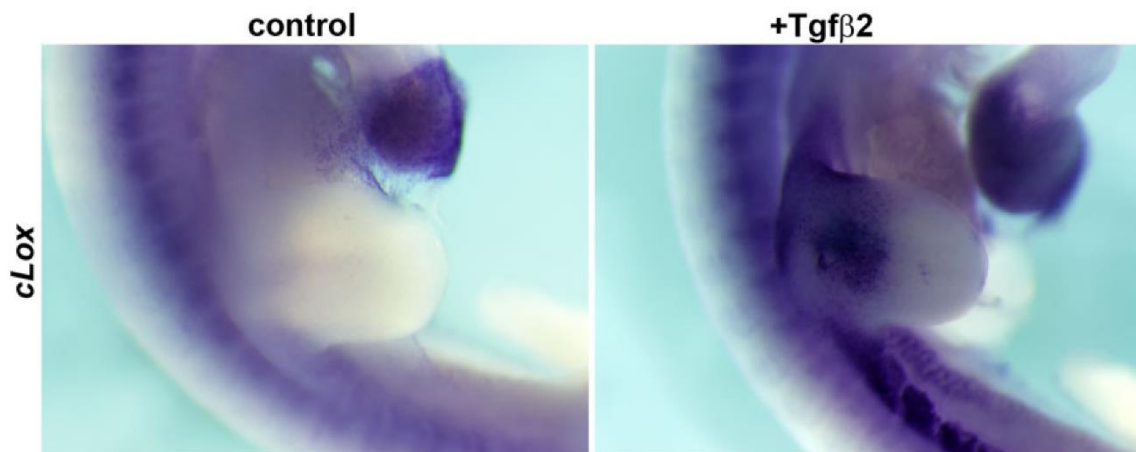


**Figure S9:** TGF-β regulates *Lox* expression. Beads soaked with PBS (control) implanted into chick limb bud do not induce *Lox* expression whereas those implanted with TGF-β2 (or TGF-β1) lead to strong ectopic *Lox* expression.

**Supplemental materials and methods:**

***In situ* hybridization:** Whole-mount RNA *in situ* hybridizations were carried out according to standard procedures, essentially as previously described (Riddle et al., 1993) on at least 3 mutant embryos at each stage. Most probes used were previously described: *MyoD* (Davis et al., 1987), *Scx* (Schweitzer et al., 2001), *Tcf4* (Kardon et al., 2003), *Myogenin* (Yee and Rigby, 1993). Mouse *Lox* probe was generated from an IMAGE clone (2655752, Source BioScience).

**Immunohistochemistry**: section immunohistochemistry was performed essentially as previously described (Hasson et al., 2010). The following antibodies were used: anti-Pax7 [1:10; Developmental Studies Hybridoma Bank (DHSB)]; anti-Laminin (1:200; Sigma); anti-Myosin (A4.1025, 1:10; DSHB]; anti-Myosin (My32; 1:800; Sigma); ColI (1:200; Abcam); ColIV (1:200; Abcam); anti-Tcf4 (1:200; Millipore); anti-Tcf4 (1:100; Cell Signaling); anti-pSMAD2 (3108; Cell Signaling; 1:100); several anti-Lox antibodies were used which gave similar results (1:200 from Abcam, Novus Biologicals, LSBio and generated by GenScript). Anti-My32 (1:800; Sigma) whole-mount immunohistochemistry was carried out essentially as described in (Hasson et al., 2010).

**Isolation of mouse embryonic fibroblasts (MEFs):** E14.5 embryos were decapitated and sliced into pieces. Pieces were incubated with trypsin for 15 min. Tissues were pipetted through and more trypsin were added and incubation was continued for 5 min. Cell and tissue mix was first washed with Dulbecco's modified Eagle's medium (Invitrogen Corp.) containing 20% fetal bovine serum (v/v), 5 mM L-glutamine, 1% nonessential amino acids, 2x penicillin and streptomycin (PS), 250 μg/ml amphotericin B and then plated in the same medium. Cultures of fibroblast that formed around tissue pieces were trypsinized, passaged, expanded and genotyped. 2x PS was used within first two cell divisions and 20% FBS was gradually decreased to 10%. MEFs were used up to a maximum of 9 passages.

**Phosphorylated SMAD Western blot:** MEFs were grown in medium described above. Cells were incubated with different concentration of mouse recombinant TGF-β1 (7666-MB-005, R&B Systems) for different periods of time and then lysed with a buffer (50 mM HEPES, 150 mM NaCl, 1 mM EDTA, 10 % glycerol, 0.1 % Tween 20, 1 % NP-40, 2.5 mM EGTA) on ice for 20 min. The supernatant protein concentrations were determined using the Bradford technique (Bio-Rad protein assay). Supernatants were resolved by SDS-PAGE and blotted onto Immobilon-P membranes (Millipore). Western blots were blocked with Tris-bufferd saline with 5% nonfat dry milk and probed with the following primary antibodies: Phospo-Smad2 (3108; Cell Signaling), Smad2 (5339; Cell Signaling) and anti-α-tubulin (B-6199;

Sigma-Aldrich). Bound antibodies were detected with horseradish peroxidase-conjugated secondary antibodies (Dako) and ECL detection reagents (Thermo Scientific and Millipore).

**Luciferase Analysis:** The TGF-β pathway analysis was performed using Cignal SMAD Reporter Assay Kit (CCS-017L; Qiagen). Briefly, 30 000 cells were plated into 12-well plates and next day cells were transfected with pathway reporters using FuGENE HD transfection reagent (Promega) according to manufacturer's protocol. Recombinant TGF-β was added to samples for the designated time. Cells were lysed on the following day and luciferase measured with Dual-Luciferase Reporter Assay System (Promega).

**Collagen measurements:** The amount of collagen within *wt* and *Lox*$^{-/-}$ E18.5 forelimb muscles was measured using the hydroxyproline assay while following the user's manual (Chondrex). Briefly, subsequent to tissue hydrolysis in 10N HCl at 120C for 24 hours hydroxyproline levels were measured. Based on that, collagen levels which contain 13.5% hydroxyproline, were calculated.

**TGF-β activation:** Affi-gel beads (153-7301; Bio-Rad) were washed in PBS and incubated for 1 hr with TGF-β1 and TGF-β2 (R&D Systems) on ice. Beads were then implanted *in ovo* in lateral plate mesoderm and early limb bud of HH St. 18-19 and embryos were harvested 24-48 hrs later.

**Lox knockdown using shRNA:** HEK293-T cells were co-transfected with 8µg of the Lox MISSION shRNA lentiviral plasmid (SHCLNG-NM_010728) or with a shRNA control plasmid (SHC003), with lenti packaging PAX2 vector and with VSVG envelope vector using Lipofectamine-2000 reagent according to the vendor's instructions. The cells were then incubated in 37°C for 16-24 hours. The conditioned medium containing viruses, was collected and filtered through 0.45µm filters. C2C12 cells were incubated with the virus-containing medium in the presence of Polybrene (Sigma H9268, 8µg/ml) for 16 hours. After the first incubation, cells were washed twice with PBS and incubated with fresh growth medium with puromycin (2µg/ml).

**Histology of skeletal elements:**

Newborn pups were skinned and eviscerated and then stained for 24-48 hours in 0.06% Alizarin red S and 0.014% Alcian blue (final concentrations). Skeletons were then cleared in KOH followed by glycerol for imaging.

**Table S1:** qRT-PCR oligos

| Binding site in template | Primer sequences (5’ to 3’) | Product size (bp) |
|---|---|---|
| Tgfβ1, forward<br>Tgfβ1, reserve | GAGCCCGAAGCGGACTACTA<br>TGGTTTTCTCATAGATGGCGTTG | 82 |
| Tgfβ2, forward<br>Tgfβ2, reserve | AGTTTACACTGCCCCTGCTG<br>AGAGGTGCCATCAATACCTGC | 105 |
| Tgfβ3, forward<br>Tgfβ3, reserve | CACCACAACCCACACCTGAT<br>CAGGTTGCGGAAGCAGTAAT | 117 |
| Ctgf, forward<br>Ctgf, reserve | GGGCCTCTTCTGCGATTTC<br>ATCCAGGCAAGTGCATTGGTA | 151 |
| α-Sma, forward<br>α-Sma, reserve | GTCCCAGACATCAGGGAGTAA<br>TCGGATACTTCAGCGTCAGGA | 102 |
| Col1a2, forward<br>Col1a2, reserve | AAGGGTGCTACTGGACTCCC<br>TTGTTACCGGATTCTCCTTTGG | 155 |
| Col3a1, forward<br>Col3a1, reserve | CTGTAACATGGAAACTGGGGAAA<br>CCATAGCTGAACTGAAAACCACC | 144 |

**Table S2: TGFβ inhibition rescues *Lox*$^{-/-}$ muscle patterning defects**

* EDC and ECR in mutants were considered as the muscles at the location of their

| | **Wild-type** | ***Lox*$^{-/-}$** | ***Lox*$^{-/-}$ + TGFβ inhibitor** |
|---|---|---|---|
| **EDC* length** | 100±10.4 (n=6) | 76.6±33.4 (n=6) | 92.7±15.7 (n=8) |
| **ECR* length** | 100±9.5 (n=6) | 79.8±34 (n=6) | 85.6±27.7 (n=8) |

normal counterparts.

## Supplementary References:

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