Supplementary material file 1

Bead implantation in chick limb buds

Heparin beads (Sigma) were soaked in 1 mg/ml of recombinant human FGF4 (R&D Systems) for 30 min on ice. Affi-Gel blue beads (Biorad) were soaked with 20 µg/ml of TGF-β2 (R&D Systems), with 10 mM of PD184352 (PD18) (Axon Medchem) or SIS3 (Merck) chemical inhibitors. FGF4 or TGFβ2 beads were grafted into the right wings of chick embryos at E3/E4 (HH19/HH21). TGFβ2+PD18 beads were grafted together in limbs of E3/E4 chick embryos. FGF4+SIS3 beads were grafted together in limbs of E3/E4 chick embryos. Embryos were harvested 4, 6 or 24 h after grafting, and grafted right and contralateral left limbs were processed for in situ hybridisation to sections or for RT-q-PCR analysis. The left wings from the same embryos were used as controls.

Production and grafting mFgf4/RCAS-expressing cells to chick limb buds

mFgf4/RCAS-expressing cells were prepared for grafting as previously described (Edom-Vovard et al., 2002). Cell pellets were grafted in the middle of the right wings of E3.5/HH21 chick embryos. The embryos were fixed 4 days after grafting at E7.5 and processed for in situ hybridisation to sections or for RT-q-PCR analysis. The left wings from the same embryos were used as controls.

Chick and mouse limb explant cultures

Limb buds were dissected from E3(HH18/19) and E5(HH25/26) chick embryos and from E9.5 mouse embryos and cultured at 37°C in 5% CO_2 in Optimem medium. For 24 h explants, limbs were embedded in collagen gel as described in (Placzek and Dale, 1999). Limb explants were treated with recombinant human TGFβ2 (R&D Systems) for 6 h or 24 h

at 20 ng/ml or with FGF4 (R&D Systems) at 200 ng/ml. The TGFβ2 signalling pathway was blocked using SB431542 (SB43, Selleck Chemicals) or SIS3 (Merck) chemical inhibitors. The MAPK ERK signalling pathway was blocked using PD184352 (PD18) chemical inhibitor (Axon Medchem). All inhibitors were diluted in DMSO (Fluka) and added to the medium at 10 µM (SB43), 20 µM (SIS3) or 3.3 µM (PD18), for 6 h or 24 h. As controls, we used media with DMSO for the chemical inhibitors and media with HCl for TGFβ2. After treatments, experimental and control explants were fixed and processed for RT-q-PCR analyses.

RNA isolation, reverse transcription and quantitative real-time PCR

Total RNAs were isolated from chick limbs, chick limb explants or mouse limb explants at different developmental stages as previously described (Havis et al., 2012). 500 ng of RNA was used as template for cDNA synthesis. Primer sequences used for RT-q-PCR are listed in supplementary material Table S1. The relative mRNA levels were calculated using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). The ΔCts were obtained from Ct normalized with chick *S17* and *GAPDH* for chick samples or with mouse *18S* and *Gapdh* mRNA levels for mouse samples. For HH18/19 and HH25/26 chick limb explants, we pooled 8 and 5 limb buds, respectively, to obtain enough material in one RNA sample. We pooled 14 E9 mouse limb buds to obtain enough material in RNA samples. Results were expressed as standard error of the mean. P values were analysed by unpaired Student's t-test using Microsoft Excel. Asterisks in figures indicate the different P values * <0.05; ** <0.01 and *** <0.001.

In situ hybridisation and Immunohistochemistry

Control or manipulated chick limbs (E3 to E9) were fixed in farnoy (60% ethanol 100, 30% formaldehyde 37% and 10% acetic acid) and processed for in situ hybridisation to

wholemounts or to 8 μm wax tissue sections with digoxigenin-labelled probes, which were detected with NBT/BCIP reagents (Havis et al., 2014). The antisense mRNA probes were used as described: ETV4 (Brent and Tabin, 2004), FGF4 (Niswander et al., 1994), mFgf4, FGF8 (Mahmood et al., 1995), SCX (Schweitzer et al., 2001), SPRY2 (Minowada et al., 1999), TGFB2 and TGFB3 (Merino et al., 1998). TNMD (chEST332f24) and THBS2 (ch972h17) are EST probes (SourceBioScience).

References

Brent, A. E. and Tabin, C. J. (2004). FGF acts directly on the somitic tendon progenitors through the Ets transcription factors Pea3 and Erm to regulate scleraxis expression. *Development* **131**, 3885-3896.

Edom-Vovard, F., Schuler, B., Bonnin, M. A., Teillet, M. A. and Duprez, D. (2002). Fgf4 positively regulates scleraxis and tenascin expression in chick limb tendons. *Dev Biol* **247**, 351-366.

Havis, E., Coumailleau, P., Bonnet, A., Bismuth, K., Bonnin, M. A., Johnson, R., Fan, C. M., Relaix, F., Shi, D. L. and Duprez, D. (2012). Sim2 prevents entry into the myogenic program by repressing MyoD transcription during limb embryonic myogenesis. *Development* **139**, 1910-1920.

Havis, E., Bonnin, M. A., Olivera-Martinez, I., Nazaret, N., Ruggiu, M., Weibel, J., Durand, C., Guerquin, M. J., Bonod-Bidaud, C., Ruggiero, F. et al. (2014). Transcriptomic analysis of mouse limb tendon cells during development. *Development* **141**, 3683-3696.

Livak, K. J. and Schmittgen, T. D. (2001). Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods* **25**, 402-408.

Mahmood, R., Bresnick, J., Hornbruch, A., Mahony, C., Morton, N., Colquhoun, K., Martin, P., Lumsden, A., Dickson, C. and Mason, I. (1995). A role for FGF-8 in the initiation and maintenance of vertebrate limb bud outgrowth. *Curr Biol* **5**, 797-806.

Merino, R., Ganan, Y., Macias, D., Economides, A. N., Sampath, K. T. and Hurle, J. M. (1998). Morphogenesis of digits in the avian limb is controlled by FGFs, TGFbetas, and noggin through BMP signaling. *Dev Biol* **200**, 35-45.

Minowada, G., Jarvis, L. A., Chi, C. L., Neubuser, A., Sun, X., Hacohen, N., Krasnow, M. A. and Martin, G. R. (1999). Vertebrate Sprouty genes are induced by FGF signaling and can cause chondrodysplasia when overexpressed. *Development* **126**, 4465-4475.

Niswander, L., Jeffrey, S., Martin, G. R. and Tickle, C. (1994). A positive feedback loop coordinates growth and patterning in the vertebrate limb. *Nature* **371**, 609-612.

Placzek, M. and Dale, K. (1999). Tissue recombinations in collagen gels. *Methods in molecular biology* **97**, 293-304.

Schweitzer, R., Chyung, J. H., Murtaugh, L. C., Brent, A. E., Rosen, V., Olson, E. N., Lassar, A. and Tabin, C. J. (2001). Analysis of the tendon cell fate using Scleraxis, a specific marker for tendons and ligaments. *Development* **128**, 3855-3866.

Supplementary Figures

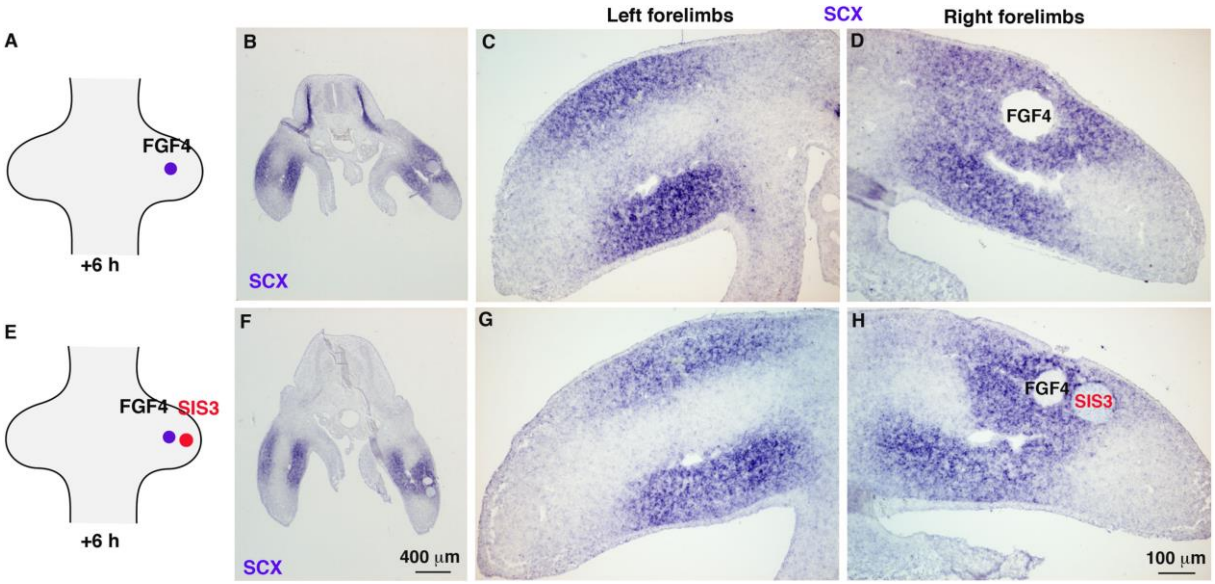

Figure S1

SMAD2/3 inhibition in FGF4 gain-of function experiments does not modify *SCX* activation by FGF4. FGF4 (A-D) or FGF4+SIS3 (E-H) beads were grafted to right forelimbs of E3/E4 (HH19/HH22) chick embryos. 6 hours after grafting, FGF4- or FGF4+SIS3-treated embryos were processed for in situ hybridisation analyses. (B-D, F-H) Transverse sections of manipulated embryos at the limb levels were hybridised with the SCX probe. FGF4 (A-D) or FGF4+SIS3 (E-H) beads activate *SCX* expression in right forelimbs (D,H) in a similar manner. This indicates that the blockade of the SMAD2/3 pathway using the SIS3 inhibitor does not modify the *SCX* induction after FGF4 application.

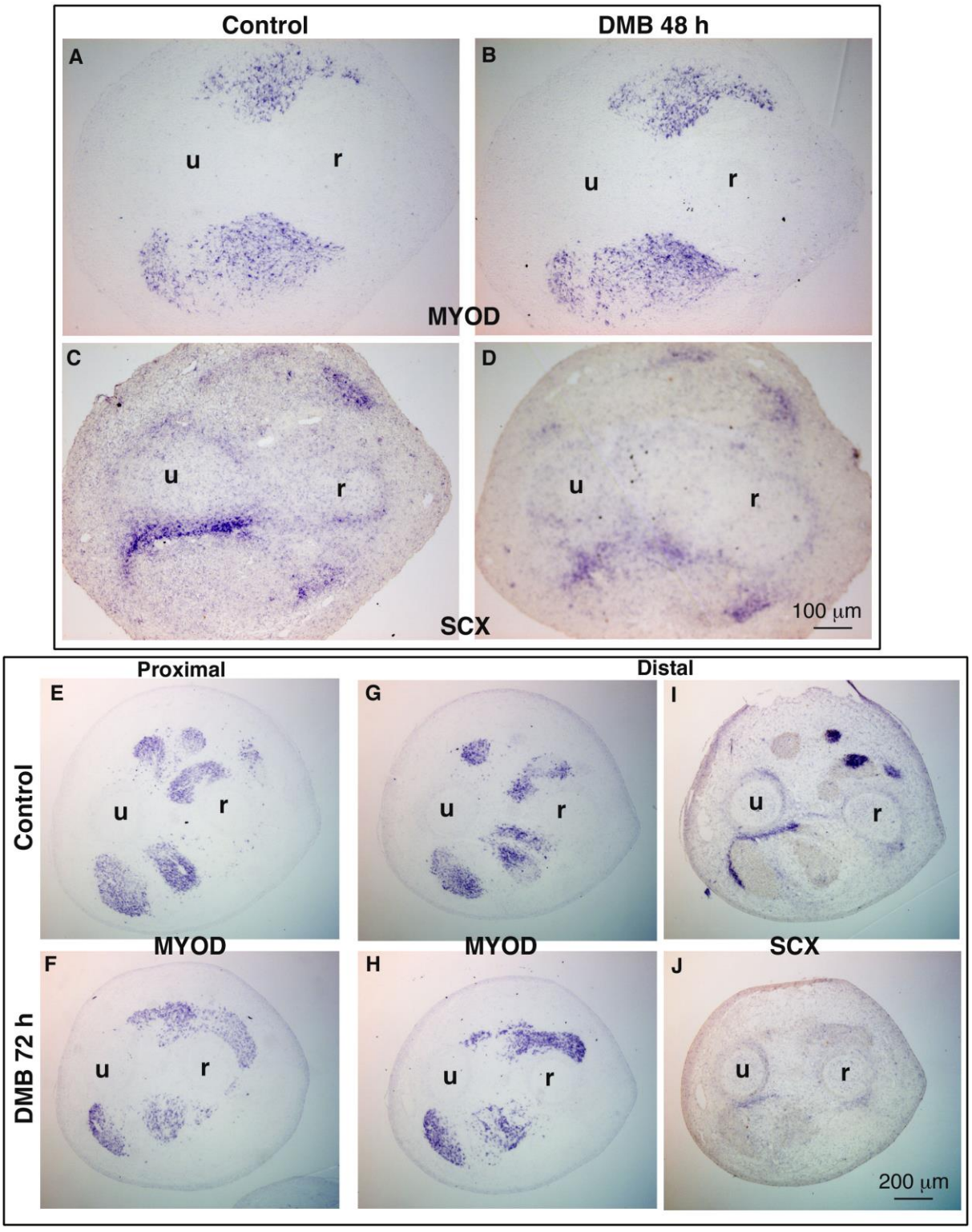

Figure S2

Inhibition of muscle contraction induces a delay of muscle development. Forelimbs of control (A,C,E,G,I) and DMB-treated (B,D,F,H,J) embryos fixed 48 h (A-D) (n=2) or 72 h (E-J) (n=3) after control or DMB application were transversely sectioned at the level of the zeugopod and hybridised with MYOD (A,B, E-H) and SCX (C,D,I,J) probes. (A,C), (B,D), (G,I), (H,J) are adjacent sections hybridized with MYOD (A,B,G,H) and SCX (C,D,I,J) probes. (A-D) 48 h after DMB injection, muscle masses visualised by *MYOD* expression did not display any obvious defect (A,B), while *SCX* expression was slightly downregulated (C,D). (E-J) 72 h after DMB injection, limb muscle development was delayed indicated by splitting defects in dorsal regions (F,H) compared to controls (E,G), at proximal (E,F) and distal (G,H) levels of zeugopod regions, while *SCX* expression was lost in zeugopod tendons (I,J). For all sections, dorsal is to the top and posterior is to the left. u, ulna, r, radius.

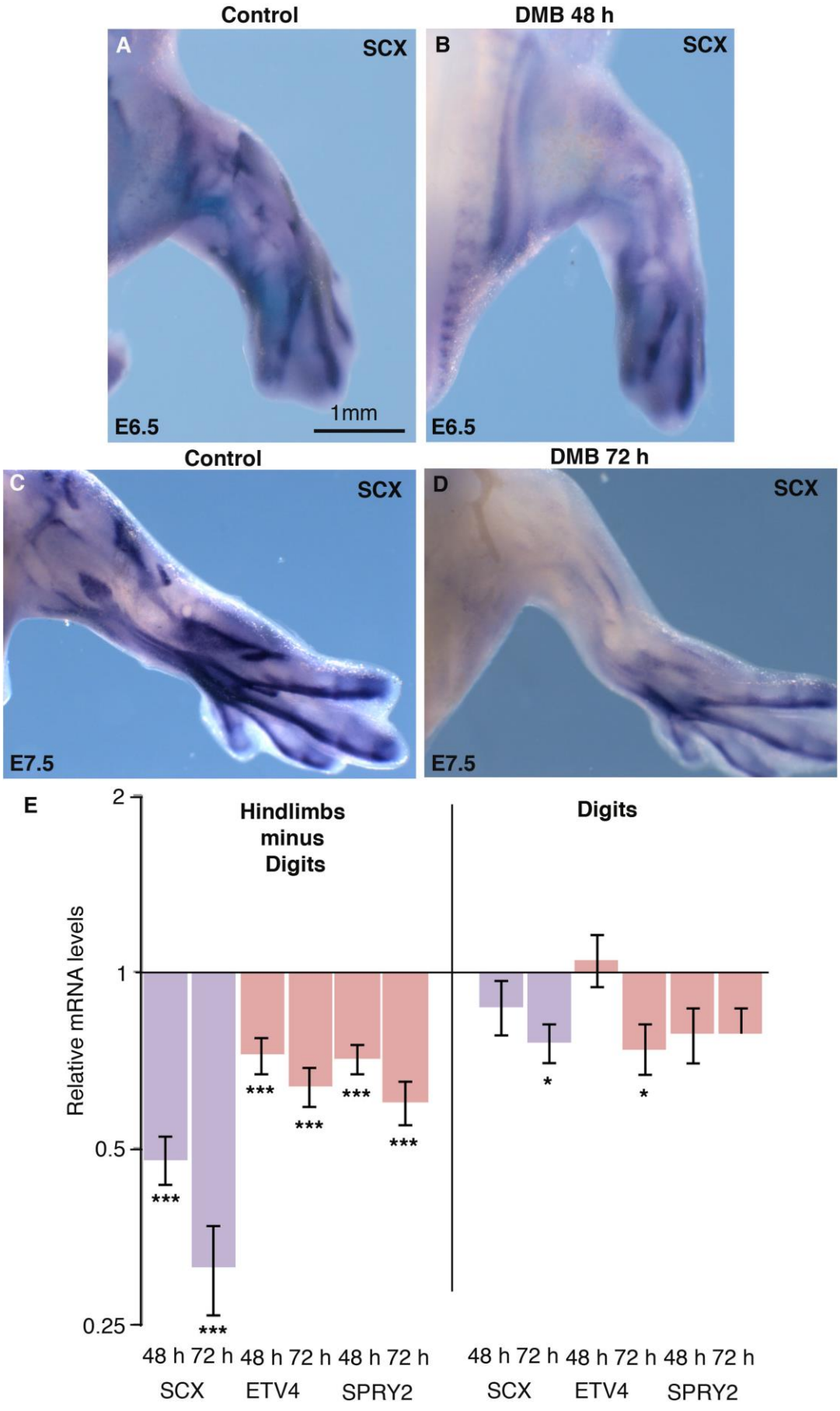

Figure S3

Muscle contraction is required to maintain *SCX* expression in stylopod/zeugopod tendons in chick hindlimbs. (A-D) Hindlimbs of control (A,C) and DMB-treated (B,D) embryos fixed 48 h (A,B) (n=6) or 72 h (C,D) (n=5) after control solution or DMB application were hybridised with *SCX* probe. (E) RT-q-PCR analyses of mRNA levels for *SCX*, *ETV4* and *SPRY2* genes in hindlimbs where digits were removed and in digits of DMB-treated embryos (n=15), 48 h (n=10) and 72 h (n=5) after DMB application. For each gene, the mRNA levels of control limbs were normalised to 1. *SCX* expression was decreased in stylopod and zeugopod regions of hindlimbs of DMB-treated embryos assessed by in situ hybridisation and RT-q-PCR analyses. P values were analysed by two-tail and unpaired Student's t-test using Microsoft Excel. **P*<0.05; ****P*<0.001; Error bars indicate s.e.m.

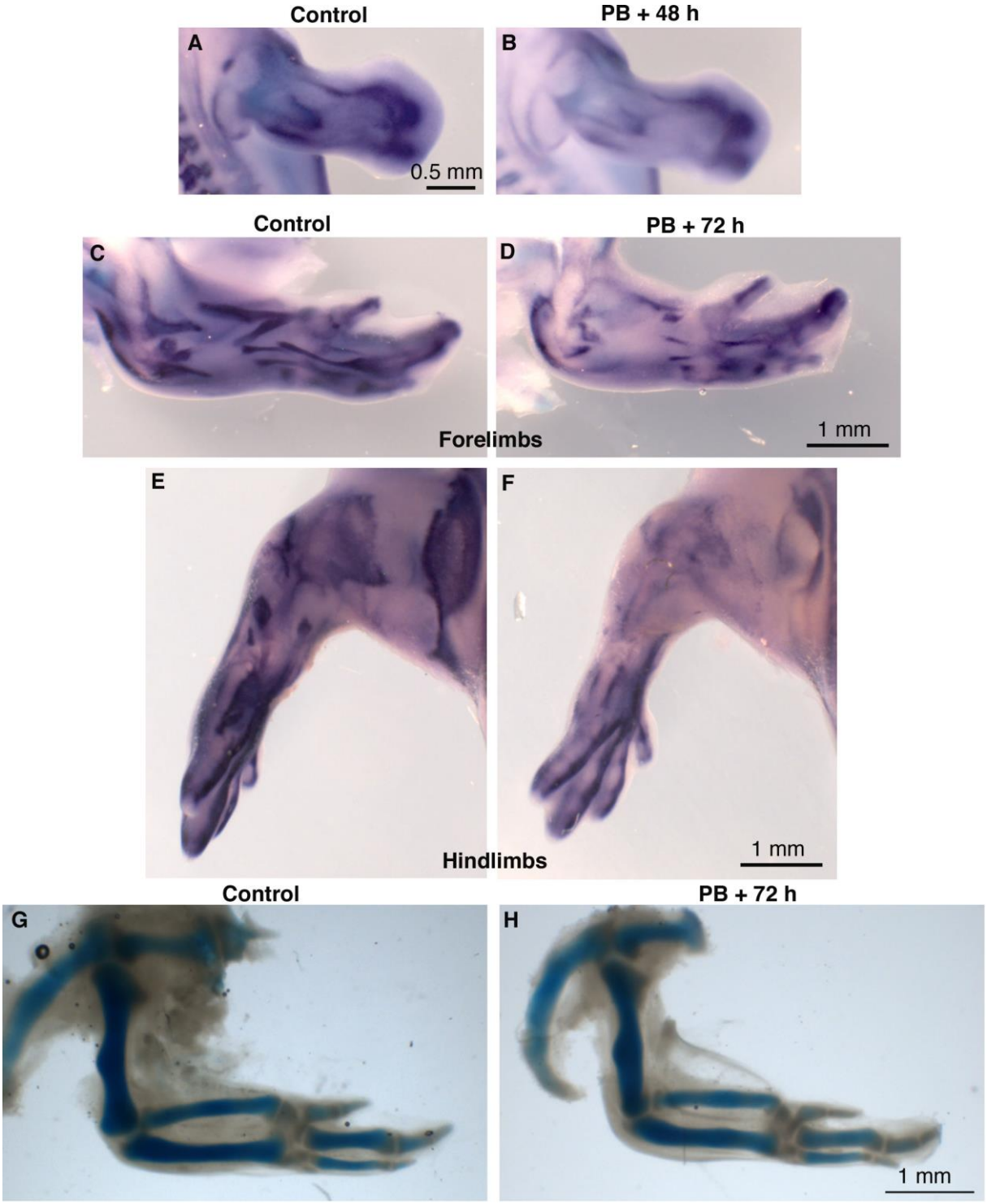

Figure S4

***SCX* expression is downregulated in zeugopod forelimb regions following flaccid paralysis in PB-treated embryos.** (A-F) Limbs of control (A,C,E) and PB-treated (B,D,F) embryos fixed 48 h (A,B) or 72 h (C-F) after control solution or PB application were hybridised with *SCX* probe. (A,B) 48 h after PB application, *SCX* expression was slightly downregulated in PB-treated embryos (n=4). (C-F) 72 h after PB application, *SCX* expression was downregulated in stylopod/zeugopod regions of forelimbs (D) and hindlimbs (F) compared to control limbs (n=4) (C,E). (G,H) Muscle paralysis did not show any obvious cartilage modification (H) compared to control limbs (G).

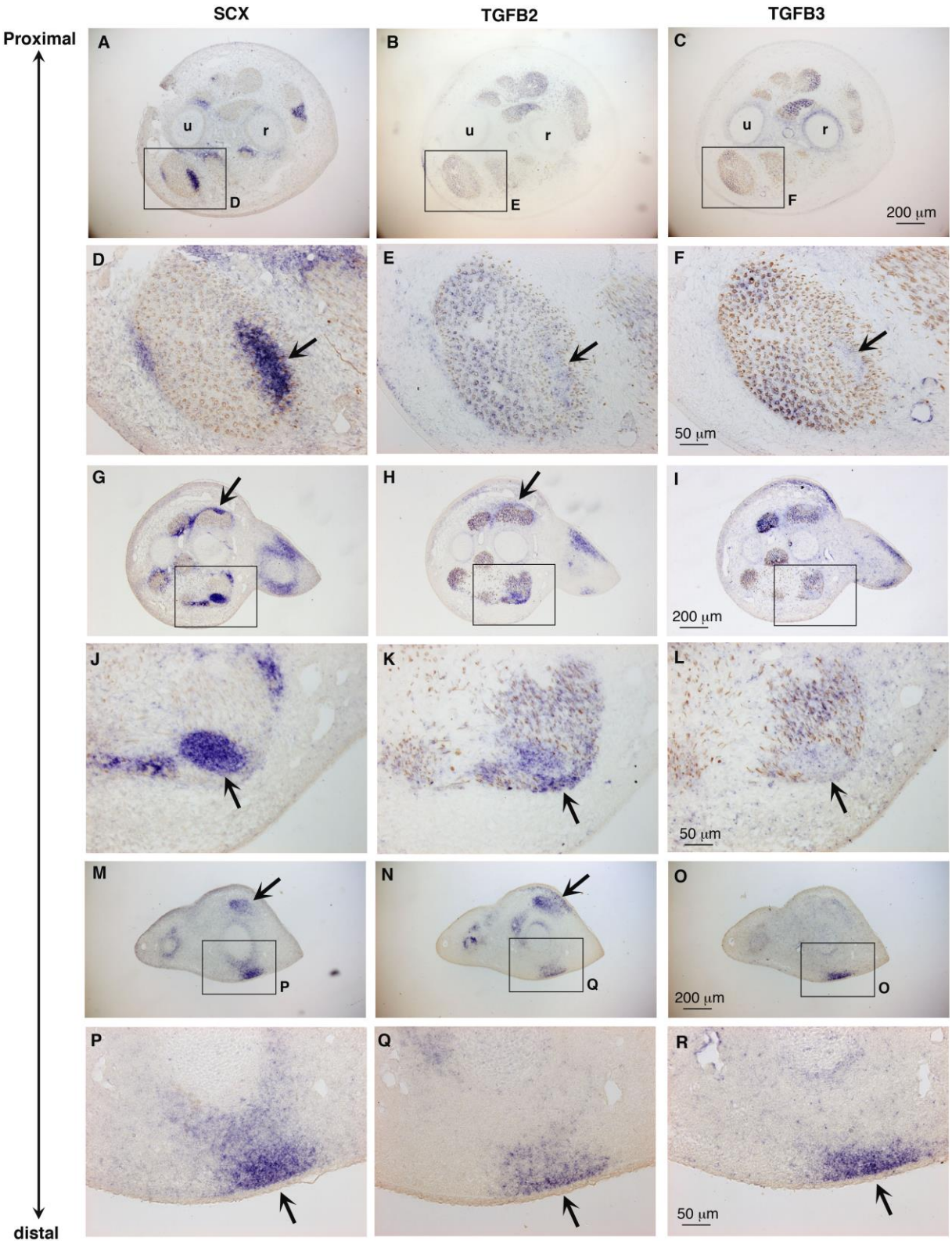

Figure S5

Endogenous *TGFB2* and *TGFB3* expression in forelimbs of E7.5 chick embryos. Adjacent transverse forelimb sections of E7.5 embryos at the levels of the zeugopod (A-F), digits (G-L) and digit tips (M-R) were hybridised with the SCX (A,D,G,J,M,P), TGFB2 (B,E,H,K,N,Q) and TGFB3 (C,F,I,L,O,R) probes. (A-C), (G-I) and (M-O) are adjacent sections. (D-F), (J-L) and (P-R) are high magnifications of (A-C), (G-I) and (M-O) panels, respectively. *TGFB2* was expressed in muscles but also in tendons (E,H,K,N, arrows) based on *SCX* expression on adjacent sections (D,G,J,M, arrows). *TGFB3* displayed a strong expression in muscles but also a faint expression in tendons (C,F,I,L, arrows). At digit tips, *TGFB2* (N,Q) and *TGFB3* (O,R) were observed in *SCX* expression domain (M,P) underneath the ectoderm (P-R, arrows). All forelimb sections are dorsal to the top and posterior to the left. u, ulna, r, radius.