TGFβ and FGF promote tendon progenitor fate and act downstream of muscle contraction to regulate tendon differentiation during chick limb development

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ABSTRACT

The molecular program underlying tendon development is not fully identified. Interactions with components of the musculoskeletal system are important for limb tendon formation. Limb tendons initiate their development independently of muscles, however muscles are required for further tendon differentiation. We show that both FGF/ERK MAPK and TGFβ/SMAD2/3 signalling pathways are required and sufficient for *SCX* expression in chick undifferentiated limb cells, while the FGF/ERKMAPK pathway inhibits *Scx* expression in mouse undifferentiated limb mesodermal cells. During differentiation, muscle contraction is required to maintain *SCX, TNMD* and *THBS2* expression in chick limbs. The activities of FGF/ERKMAPK and TGFβ/SMAD2/3 signalling pathways are decreased in tendons under immobilisation conditions. Application of FGF4 or TGFβ2 ligands prevents *SCX* downregulation in limbs in immobilisation conditions. TGFβ2 but not FGF4 prevent *TNMD* and *THBS2* downregulation in immobilisation conditions. We did not identify any intracellular crosstalk between both signalling pathways in their positive effect on *SCX* expression. Independently of each other, both FGF and TGFβ promote tendon commitment of limb mesodermal cells and act downstream of mechanical forces to regulate tendon differentiation during chick limb development.

INTRODUCTION

Tendon is a unique connective tissue that transmits the forces generated by muscle to bone and allows body motion. Type I collagen is the main tendon structural and functional component. The signals regulating the production and the spatial organisation of type I collagen in developing tendons are not fully identified. Moreover, because type I collagen is not specific to tendons, it is not possible to follow tendon development with collagen expression (reviewed in Gaut and Duprez, 2016). The basic helix-loop-helix (bHLH) transcription factor Scleraxis (Scx) is the unique identified early tendon marker during development. *Scx* is expressed in tendon progenitors, developing tendons and adult tendons (Schweitzer et al., 2001; Pryce et al., 2007; Mendias et al., 2012). *Scx* is not the unique master gene driving tendon development, since tendons are formed in $Scx^{-/-}$ mice, albeit displaying differentiation defects (Murchison et al., 2007). Moreover, *Col1a1* expression is downregulated in tendons of $Scx^{-/-}$ mutant mice (Murchison et al., 2007), consistent with the transcriptional regulation of the mouse *Col1a1* gene by Scx, via direct binding on *Col1a1* promoter (Lejard et al., 2007). Tenomodulin (*Tnmd)* and *Col14a1* expression is lost in developing limb tendons of $Scx^{-/-}$ mice (Murchison et al., 2007). The transmembrane glycoprotein Tnmd is considered as a late tendon marker (Jelinsky et al., 2010; Sugimoto et al., 2013) and is highly expressed in E14.5 mouse limb tendon cells (Havis et al., 2014). Thrombospondin 2 and 4 (THBS2 and THBS4) were also identified in the transcriptome of mouse limb tendon cells (Havis et al., 2014) and have been shown to be involved in tendon development in mouse, drosophila and zebrafish (Kyriakides et al., 1998; Subramanian et al., 2007; Subramanian and Schilling, 2014). Two other transcription factors are involved in tendon development, the homeobox Mkx (Mohawk) (Ito et al., 2010; Liu et al., 2010; Kimura et al., 2011) and the zinc finger transcription factor EGR1 (Early Growth Response factor1)

(Lejard et al., 2011). In contrast to *Scx*, *Mkx* and *Egr1* are not expressed during early tendon limb development and are not specific to tendons (Anderson et al., 2006; Liu et al., 2006; Ito et al., 2010; Lejard et al., 2011), but they activate *Scx* and *Tnmd* expression in various stem cell types and positively regulate type I collagen production in vivo (Ito et al., 2010; Lejard et al., 2011; Guerquin et al., 2013; Liu et al., 2015). In addition to the tendon-related transcription factors, two signalling pathways have been identified as being involved in tendon development: the Transforming Growth Factor-beta (TGFβ) and Fibroblast Growth Factor (FGF) signalling pathways (reviewed in Huang et al., 2015a; Gaut and Duprez, 2016). TGFβ signalling pathway positively regulates *Scx* expression in early E9/E10 mouse limb explants (Pryce et al., 2009; Havis et al., 2014). The TGFβ function in chick tendon development is less understood. Although TGFβ1 and 2 has been shown to increase *SCX* and *TNMD* expression in high-density cultures of chick limb cells (Lorda-Diez et al., 2009), TGFβ1 failed to activate *SCX* expression in HH20/21 chick limb explants (Lorda-Diez et al., 2010). FGF positively regulates *SCX* expression in axial and foetal limb tendons during chick development (Edom-Vovard et al., 2002; Brent et al., 2003; Brent and Tabin, 2004). In contrast to the chick model, FGF has an anti-tenogenic effect in mouse embryonic tendon cells (Brown et al., 2014) and inhibition of the ERK MAPK pathway is sufficient to increase *Scx* expression in early mouse limb explants (Havis et al., 2014). Although the above reported experimental situations differ between the chick and mouse models, it nevertheless suggests a differential *Scx* regulation by FGF between chick and mouse models.

Another important aspect of tendon development is its dependency on muscles. Axial, limb and head tendons require the presence of muscles for full development in chick, mouse and zebrafish embryos (reviewed in Gaut and Duprez, 2016). However, in the absence of muscle, *Scx* expression is normally initiated (and then lost) in limb and head regions of mouse, chick and zebrafish embryos (Schweitzer et al., 2001; Edom-Vovard et al., 2002;

Bonnin et al., 2005; Grenier et al., 2009; Chen and Galloway, 2014; Huang et al., 2015b). The muscle-dependency of *Scx* expression defines two phases for limb tendon formation, a progenitor, muscle-independent phase and a differentiation, muscle-dependent phase. This muscle dependency applies only to stylopod (arm) and zeugopod (forearm) limb tendons, since autopod (digit) tendons are dependent on cartilage in mouse embryos (Huang et al., 2015b). The molecular mechanisms underlying the muscle dependency of chick tendon development remain elusive. Although one can assume that the muscle-dependency of *Scx* expression is related to muscle activity, the requirement of mechanical forces for chick limb tendon development has not been addressed and the molecular signals downstream of muscle contraction involved in tendon differentiation have not been identified.

RESULTS

The FGF/ERK MAPK pathway activates *SCX* expression in early chick limb buds.

FGF positively regulates *SCX* expression via the ERK MAPK signalling pathway in chick somites (Brent and Tabin, 2004; Smith et al., 2005) and in foetal chick limbs (Edom-Vovard et al., 2002; Eloy-Trinquet et al., 2009). However, the role of FGF on *SCX* expression was not determined in chick limb undifferentiated cells, during the muscle-independent phase of limb tendon development. *SCX* expression is initiated in E3 (HH20) chick limb buds (Brent and Tabin, 2004). At these stages, a source of FGF is observed in the apical ectodermal ridge (Niswander et al., 1994). We implanted FGF4 beads in early chick limb buds (E3 to E4) and analysed *SCX* expression by RT-q-PCR and in situ hybridisation experiments (Fig. 1A-C). *SCX* and *COL1A2* expression was upregulated as soon as 4 h after FGF4 bead implantation; the upregulation was maintained 24 h after FGF4 bead implantation (Fig. 1A). We used *ETV4 (PEA3)* and *SPRY2* as transcriptional readouts of ERK MAPK activity (O'Hagan et al., 1996; Mason et al., 2006; Havis et al., 2014). The mRNA levels of *ETV4* and *SPRY2* were increased in FGF4-implanted limbs 4 h and 24 h after grafting (Fig. 1A) and *SPRY2* expression was activated around FGF4 beads 24 h after grafting (Fig. 1C). *TNMD* is not expressed before E5 in chick limbs (Shukunami et al., 2006) and FGF4 was not able to prematurely activate *TNMD* (data not shown). This FGF4 tenogenic effect in chick limb buds contrasted with the anti-tenogenic effect of FGF4 in mouse limb explants (Havis et al., 2014). We next performed chick limb bud explants in order to exclude differences due to different experimental designs and allow comparison between the chick and mouse models. Consistent with the in vivo FGF4 bead experiments (Fig. 1A-C), we observed that FGF4 increased the mRNA levels of *SCX* and *SPRY2* (Fig. 1D), while the blockade of ERK MAPK with the PD18 inhibitor decreased *SCX, ETV4* and *SPRY2* expression in chick limb bud explants 6 h after treatment

(Fig. 1D). In order to allow comparison, we performed equivalent mouse limb bud explant experiments and found that FGF4 significantly decreased *Scx* expression, while PD18 increased *Scx* expression in mouse limbs, 6 h after treatment (Fig. 1E), consistent with 24 h FGF4 and PD18 treatments in mouse limbs (Havis et al., 2014). We conclude that the FGF tenogenic effect observed in chick limb cells is opposite from the antitenogenic FGF effect observed in mouse limb cells.

We next tested whether the FGF4 effect on *SCX* in chick cells involved the SMAD2/3 pathway. We applied the SIS3 inhibitor in FGF4 gain-of-function experiments in chick limb buds (supplementary material Fig. S1). The blockade of SMAD2/3 did not block the positive effect of FGF4 on *SCX* expression (supplementary material Fig. S1). This result is consistent with the absence of modification of *SMAD7/Smad7* expression upon FGF/ERK MAPK manipulations, in both chick and mouse limb explants (Fig. 1D,E). Smad7 is a negative-feedback regulator that is considered as a general TGFβ/SMAD2/3 transcriptional target gene (Massague, 2012). We conclude that FGF4 positively regulates *SCX* independently of the SMAD2/3 intracellular pathway in chick limb cells.

The TGFβ/SMAD2/3 pathway activates *SCX* expression in early chick limb buds.

TGFβ2 induces *Scx* expression in E10.5 mouse limb explants (Pryce et al., 2009), while TGFβ1 does not modify *SCX* expression in E3.5 (HH20/21) chick limb explants (Lorda-Diez et al., 2010). *TGFB2* was expressed in ventral parts of E3 chick limb buds (Figure 2A), as previously described (Lorda-Diez et al., 2010). Application of TGFβ2 beads in E3/E4 (HH19/21) chick limb buds increased *SCX* expression 6 h after grafting (Fig. 2B) and the mRNA levels of *SCX* and *COL1A2* genes in grafted limbs compared to control limbs (Fig. 2C). TGFβ2 application on chick limb bud explants also increased *SCX* expression in addition to increasing *COL1A2* and *THBS2* (Fig. 2D). TGFβ2 was not able to prematurely activate

TNMD in chick limb undifferentiated cells (data not shown), as in mouse limb undifferentiated cells (Havis et al., 2014). The blockade of TGFβ receptors (SB43 inhibitor) and that of SMAD2/3 signalling pathway (SIS3 inhibitor) decreased *SCX* expression, in addition to that of *CO1A2, THBS2* and *THBS4* (Fig. 2D). Consistently, the *SMAD7* mRNA levels, the transcriptional readout of the SMAD2/3 intracellular pathway, were increased following TGFβ2 application and decreased with SB43 and SIS3 inhibitors (Fig. 2D). These results show that TGFβ2 positively regulates *SCX* expression, and that the SMAD2/3 intracellular pathway is required for *SCX* expression in early chick limb undifferentiated cells.

TGFβ is known to activate the ERK MAPK pathway as non-canonical signalling pathway (reviewed in Guo and Wang, 2009; Massague, 2012). The expression of *ETV4* and *SPRY2* was not modified upon TGFβ2 bead application (Fig. 2C). In order to confirm experimentally that the positive effect of TGFβ2 on *SCX* expression did not involve the ERK MAPK signalling pathway, we applied the PD18 inhibitor in the TGFβ2 gain-of-function experiments in chick limb buds and limb explants. The blockade of the ERK MAPK did not modify the positive effect of TGFβ2 on *SCX* expression in chick limbs (Fig. 2C) and in chick limb explants (Fig. 2D). We conclude that TGFβ2 activates *SCX* expression independently of the ERK MAPK signalling pathway in chick limb cells.

FGF4 positively regulates *TNMD* and *THBS2* expression during tendon differentiation

TNMD is considered as a late tendon marker in chick and mouse embryos, during the differentiation and muscle-dependent phase of limb tendon development (reviewed in Dex et al., 2016). *Tnmd* mutant mice display an altered structure of collagen fibrils, and reduced self-renewal and increased senescence of tendon progenitors, in post-natal tendons (Docheva et al., 2005; Alberton et al., 2015). *TNMD* was expressed in *SCX*-positive tendons in E9 chick limbs (Fig. 3A,B, arrows), but also in dermal regions (Fig. 3B, arrowhead). Retroviral mouse

Fgf4 (mFgf4/RCAS) induced ectopic *TNMD* expression in chick limbs (Figure 3C-F), in addition to activating *SCX* expression (Edom-Vovard et al., 2002). Consistently, the relative mRNA levels of *TNMD* and *SCX* tendon genes and *ETV4* were increased in mFgf4/RCAS-limbs compared to control limbs (Figure 3D). *THBS2*, another late tendon marker (Havis et al., 2014) was also upregulated in chick limbs upon retroviral Fgf4 (Fig. 3D, G-H). We conclude that FGF4 positively regulates *TNMD* and *THBS2* expression in chick limbs during the differentiation and muscle-dependent phase of limb tendon development.

Muscle contraction is required to maintain tendon marker expression in chick limb stylopod/zeugopod tendons

Scx/SCX expression is lost in stylopod/zeugopod muscleless limbs of mutant mice or experimental chick embryos (Schweitzer et al., 2001; Edom-Vovard and Duprez, 2004) defining the muscle-dependent phase of limb tendon development. In the absence of muscle activity, Scx/GFP expression is diminished but not lost in zeugopod/stylopod regions of forelimbs of E18.5 paralysed mdg mice (Huang et al., 2015b). In order to determine the importance of mechanical signals for chick limb tendon development, we blocked muscle contraction in chick embryos using the decamethonium bromide (DMB) drug. DMB acts as an acetylcholine agonist, induces depolarisation in skeletal muscles and ultimately leads to rigid muscle paralysis and to immobilised embryos (Nowlan et al., 2010). We applied DMB or control buffer in E4.5 chick embryos and analysed gene expression either by in situ hybridisation to sections and wholemounts or RT-q-PCR (Fig. 4). In the absence of muscle contraction, muscles, visualised with *MYOD* expression, were present 2 days after DMB application, but displayed splitting delay 3 days after DMB application (supplementary material, Fig. S2). As previously described, limbs of immobilised embryos were smaller compared to control limbs (Nowlan et al., 2010). During the muscle-independent phase, *SCX*

expression was not affected in chick limbs of immobilised embryos, 24 h after DMB application (Fig. 4A,B), consistent with normal *SCX* expression in muscleless limbs of E6 experimental chick embryos (Edom-Vovard et al., 2002) and E12.5 mouse Pax3 mutants (Schweitzer et al., 2001). *SCX* expression was decreased in limbs of immobilised embryos from E6.5 (Fig. 4C-F). In order to confirm the decrease of *SCX* expression observed by in situ hybridization, we compared the *SCX* mRNA levels in Paralysed limbs versus control limbs by RT-q-PCR experiments (Fig. 4G). RT-q-PCR analyses of whole forelimbs, forelimbs without digits, or digits only indicated a decrease of *SCX* expression in the absence of muscle contraction (Fig. 4G). The expression of *COL1A2* was slightly decreased in limbs of immobilised embryos (Fig. 4G), consistent with the general and non tendon-specific expression of type I collagen. The decrease of *SCX* expression was more obvious in stylopod/zeugopod regions compared to digits (Fig. 4C-F), consistent with *SCX* expression pattern in muscleless limbs of chick and mouse embryos (Schweitzer et al., 2001; Edom-Vovard et al., 2002; Bonnin et al., 2005) and with the modular development of mouse limb tendons (Huang et al., 2013; Huang et al., 2015b). Similar *SCX* downregulation was observed in stylopod/zeugopod tendons of hindlimbs in immobilised chick embryos (supplementary material Fig. S3). In situ hybridisation to forelimb sections at the levels of the zeugopod (Fig. 4H,I) and digits (Fig. 4J,K) confirmed the more pronounced decrease of *SCX* expression in zeugopod compared to digits. *SCX* was also decreased in stylopod/zeugopod tendons of forelimbs, 3 days after pancuronium bromide (PB) injection, an acetylcholine antagonist, which induced flaccid muscle paralysis (Nowlan et al., 2010) (supplementary material, Fig. S4). The expression of the late tendon markers, *TNMD* and *THBS2* was also lost in limb tendons of immobilised E7.5 embryos (Fig. 4L-O). We conclude that *SCX, TNMD* and *THBS2* expression is sensitive to mechanical signals provided by muscle contraction in stylopod/zeugopod tendons, during chick limb development.

The expression of tendon-related FGF signalling components is downregulated in paralysed limbs.

In order to determine whether FGF signalling pathway was involved in the downregulation of tendon gene expression in the absence of muscle contraction, we analysed the expression of components of FGF/ERK MAPK signalling pathway related to tendon development, in immobilised chick embryos. During the muscle-dependent phase of limb tendon development, *ETV4, SPRY1* and *SPRY2* are expressed ubiquitously in chick limbs but with a high expression at muscle and tendon interface (Eloy-Trinquet et al., 2009). *FGF4* is expressed at muscle tips close to tendons (Edom-Vovard et al., 2002), while *FGF8* is expressed in tendons close to muscles (Edom-Vovard et al., 2001). The expression of *ETV4* and *SPRY2* was dramatically decreased in limbs of immobilised chick embryos assessed by RT-q-PCR and in situ hybridisation analyses (Fig. 5A-E). The *ETV4* and *SPRY2* downregulation was more pronounced in forelimbs (digit excluded) compared to digits alone (Fig. 5A). In the absence of muscle contraction, the expression of the FGF ligands related to tendon development, *FGF4* and *FGF8,* was lost at muscle tips (Fig. 5F,G, arrows) and in tendons (Fig. 5H,I, arrows), respectively. These results showed that the expression of FGF ligands and transcriptional readouts of ERK MAPK activity was downregulated at the muscle/tendon interface in chick limbs, in the absence of muscle contraction.

FGF4 activates *SCX* expression in limbs of immobilised chick embryos

In order to determine whether FGF would rescue tendon gene expression in the absence of mechanical signals, we applied mFgf4-expressing retroviruses in chick limbs (mFgf4/RCAS) and injected DMB drug in order to prevent muscle contraction (Fig. 6A). In the absence of muscle contraction, *SCX* expression was downregulated (Figure 6C,D). mFgf4 was able to

activate ectopic *SCX* expression in limbs of immobilised embryos (Fig. 6D-F). Consistently, the relative mRNA levels of *SCX, ETV4* and *SPRY2* were significantly upregulated in mFgf4-Paralysed-limbs compared to paralysed limbs. In these experimental conditions, *TNMD* and *THBS2* expression was not changed (Fig. 6B). The relative mRNA levels of *TGFB2*, *TGFB3* and *SMAD7* genes were not changed in the presence of mFgf4 in immobilised embryos (Fig. 6B), indicating that TGFβ signalling was not modified in these experimental conditions. We performed a similar FGF rescue experiment in chick limb explants, where we considered that the E5 limb explant culture system was devoid of mechanical movements. Analysis of the relative mRNA levels in chick limb explants compared to stage-matched limbs originating from in ovo embryos showed a significant diminution of *SCX, TNMD, THBS2, ETV4*, *SPRY2* and *FGF4* gene expression (Fig. 6G), similar to that observed in immobilised chick embryos (Figs. 4,5). Consistent with the in ovo FGF rescue experiments (Figure 6A-F), the application of FGF4 recombinant in limb explant cultures induced a significant increase of the mRNA levels of *SCX, ETV4* and *SPRY2*, while not affecting *COL1A2* and *SMAD7* expression (Fig. 6H). The expression levels of *TNMD* and *THBS2* genes were not increased upon FGF4 treatment and even displayed a decrease of expression (Fig. 6H). We conclude that FGF4 activates *SCX* but not *TNMD* or *THBS2* expression in chick limbs in immobilisation conditions.

TGFβ2 maintains *SCX*, *TNMD* and *THBS2* expression in chick limbs in immobilisation conditions.

We next wanted to determine if TGFβ was also sensitive to immobilisation. Both *Tgfb2* and *Tgfb3* have been shown to be involved in mouse limb tendon development (Pryce et al., 2009). In E7.5 limbs, *TGFB2* was observed in tendons, in addition to displaying an expression in muscles (supplementary material, Fig. S5). *TGFB3* was mainly expressed in chick limb

muscles, with a faint expression in tendons (supplementary material, Fig. S5). In DMB-treated embryos, the mRNA levels of the *SMAD7* and *TGFB2* genes were decreased in paralysed limbs compared to control limbs (Fig. 7A). *TGFB2* expression was lost in limb tendons of the zeugopod regions (Fig. 7B-E, arrows), but not in digits (Fig. 7F-I) of immobilised embryos. The diminution of the relative mRNA levels of *SMAD7* and *TGFB2* genes was also observed in limb explants compared to stage-matched limbs originating from in ovo embryos (Fig. 7J). These results show that the TGFβ/SMAD2/3 signalling pathway was decreased in chick limb tendons in immobilisation conditions. Application of TGFβ2 to limb explants increased *SCX, TNMD, THBS2* and *SMAD7* expression compared to control limb explants (Fig. 7K). The expression levels of the transcriptional readouts of ERK MAPK activity were not modified upon TGFβ2 exposure. We conclude that TGFβ2 is sufficient to maintain the expression of *SCX* and the tendon differentiation markers, *TNMD* and *THBS2* in chick limbs, in immobilisation conditions.

DISCUSSION

TGFβ function in tendon development is similar in chick and mouse limbs

Our TGFβ2 gain- and loss-of-function experiments in early chick limbs and explants (Fig. 2) show that TGFβ2 is sufficient, while the SMAD2/3 intracellular pathway is required for *SCX* expression in undifferentiated limb cells. These results are fully consistent with those obtained in early mouse limb explants (Pryce et al., 2009; Havis et al., 2014). These results highlight a universal role for TGFβ in initiating the commitment of undifferentiated limb mesodermal cells towards the tendon lineage during chick and mouse development (Fig. 8). In zebrafish embryos, blocking the TGFβ pathway (using the SB431542 chemical drug) inhibits *scxa* expression (Chen and Galloway, 2014), suggesting that TGFβ is also important for the initiation of *scxa* expression in fish. The developmental TGFβ tenogenic effect is to be related to the recognized effect of TGFβ in positively regulating *Scx* expression in embryonic tendon progenitors (Brown et al., 2014) and stem cell culture systems (Pryce et al., 2009; Barsby and Guest, 2013; Goncalves et al., 2013; Guerquin et al., 2013; Havis et al., 2014). We find that the positive effect of TGFβ2 on chick limb *SCX* expression is independent of ERK MAPK signalling (Fig. 2C,D), as in mouse limb explants (Pryce et al., 2009; Havis et al., 2014).

Tnmd/TNMD is one of the tendon markers displaying the highest expression levels in E14.5 mouse Scx+ cells but is not expressed in E11.5 mouse limb bud explants (Havis et al., 2014) or E4 chick limbs (Shukunami et al., 2006) nor activated by TGFβ2 at these early stages (Havis et al., 2014). However, *TNMD* expression is activated upon TGFβ2 exposure in late chick (Fig. 8) and mouse (Havis et al., 2014) limb explants. This is consistent with previous reports showing *Tnmd* upregulation by TGFβ ligands, in 3D-culture systems of human tendon cells (Bayer et al., 2014) and of equine embryo-derived stem cells (Barsby et al., 2014), and

high density cultures of chick limb cells (Lorda-Diez et al., 2009). It is worth mentioning that TGFβ dramatically decreases *Tnmd* expression (while activating *Scx*) in 2D-culture systems of embryonic or adult mouse tendon progenitors and in mouse mesenchymal stem cells (Guerquin et al., 2013; Brown et al., 2014; Liu et al., 2015). We believe that the opposite effects of TGFβ on *Tnmd* expression are due to the different cell contact environments in 2D culture versus 3D culture systems.

FGF has a tenogenic effect in chick limb undifferentiated cells, while having an anti-tenogenic effect in mouse limb undifferentiated cells

In vivo and ex vivo experiments demonstrated that FGF activates *SCX* expression in early chick limb buds. This is consistent with FGF function in somites of chick embryos (Brent and Tabin, 2004; Smith et al., 2005). This result observed in chick embryos is opposite to those obtained in mouse limb explants, where FGF inhibits *Scx* expression and ERK MAPK inhibition activates *Scx* expression (Havis et al. 2014, Fig. 1E). The blockade of SMAD2/3 did not prevent the *SCX* activation by FGF4 in chick limbs (supplementary material Fig. S1). Moreover, *Smad7/SMAD7* expression was not modified in any of the FGF misexpression experiments (Fig. 1D,E) (Havis et al., 2014), indicating that TGFβ pathway is not involved in the positive or negative effect of FGF on *Scx/SCX* expression in chick and mouse, respectively. We believe that FGF has a tenogenic effect in chick undifferentiated limb mesodermal cells, while having and anti-tenogenic effect on mouse undifferentiated limb mesodermal cells. The reasons for the opposite effect of FGF signalling on limb mesodermal cells between the chick and mouse models remain unclear. However, these results are consistent with an absence or deleterious effect of FGF on tendon marker expression in 2D-culture systems of various stem cells, including mouse embryonic tendon progenitors (Brown et al., 2014), mouse mesenchymal stem cells (Havis et al., 2014), canine tendon fibroblasts

(Thomopoulos et al., 2010), human amniotic fluid stem cells or adipose-derived stem cells (Goncalves et al., 2013). Consistent with the FGF tenogenic function during chick tendon development, a clear beneficial FGF effect has been described during tendon repair in a chick digital tendon injury model. The FGFb ligand expression is decreased in chick tendons during the process of tendon repair (Chen et al., 2008) and ectopic application of FGF has a beneficial effect on chick tendon repair (Tang et al., 2008; Tang et al., 2014). We conclude that FGF positively regulates *SCX* in chick limb undifferentiated cells (Fig. 8).

FGF4 and TGFβ2 have a tenogenic effect, independently of each other, during chick limb development.

FGF4 and TGFβ2 activate *SCX* expression independently of each other in early chick limb buds (Fig. 2D, supplementary material Fig. S1). Although intracellular crosstalks have been identified between ERK MAPK and SMAD2/3 signalling pathways in many biological systems (reviewed in Massague, 2012), our result indicate that both signalling pathways do not interact in the activation of *SCX* in chick limb buds. The fact that two signalling pathways activate *SCX* independently of each other indicates the presence of a safety system for tendon specification in chick limbs. This safety system is classically observed during developmental processes. Another possible hypothesis could be that two pools of *SCX*-positive cells coexist within the chick limb buds, one pool being sensitive to the TGFß2/SMAD2/3 signalling pathway and another one being sensitive to the FGF ERK/MAPK signalling pathway.

Limb tendon development relies on mechanical forces generated by muscle contraction.

Immobilisation following muscle paralysis induces a drastic diminution of *SCX*, *TNMD* and *THBS2* gene expression in stylopod/zeugopod tendons of chick limbs (Fig. 4). This shows that tendon gene expression is sensitive to mechanical signals in chick limbs. However, in the

mdg mouse model deprived of muscle activity, Scx/GFP-positive tendons are observed in stylopod/zeugopod limb regions, although they are reduced in size (Huang et al., 2015b). This difference can be first interpreted with the fact that the GFP fluorescence can be still detected even if the *Scx* promoter is no longer active or with the fact that the mouse embryos are still submitted to mechanical movements from maternal activity, whereas the pharmacologically-induced immobilisation is more drastic in chick embryos. However, an alternative and plausible explanation is that these results indicate that tendon development in chick and mouse has differential requirements for mechanical movements. It has been described in mice that muscles are required for zeugopod tendon elongation, but only tendon size and individuation depend on mechanical forces in mouse limbs (Huang et al., 2015b). The complete loss of zeugopod tendons in chick immobilized embryos show that mechanical signals are crucial for all the steps of chick zeugopod tendon differentiation. This can be correlated with the fact that tendon cells experience higher levels of mechanical signals in actively moving chick embryos in the egg compared to mouse embryos embedded in uterine membranes and with the faster development of the musculoskeletal system in chick versus mouse embryos.

Although differences exist between the mechanical signal requirement between chick and mouse tendon development, mechanical forces generated by muscle contraction are recognised as being required for skeletal system formation during chick and mouse development (reviewed in Shwartz et al., 2013). Immobilisation affects bone, cartilage and synovial joint morphogenesis, targeting general processes such as proliferation and differentiation leading to shape and size defects (Blitz et al., 2009; Kahn et al., 2009; Roddy et al., 2011a). A correlation has been established between biophysical stimuli patterns and skeletal regions affected upon immobilisation (Roddy et al., 2011b). Tendons that link muscles to bones are expected to experience high mechanical strains and are largely affected

in immobilisation conditions. The mechano-sensitivity of tendon development is maintained in adult life, since tendon cells are sensitive to mechanical signals generated by tendon loading (reviewed in Nourissat et al., 2015). *Scx* expression is downregulated in adult tendons in unloading conditions (Maeda et al., 2011), while *Scx* and *Tnmd* are activated in uploading conditions in mice (Mendias et al., 2012; Zhang and Wang, 2013). Tendon cells require appropriate mechanical signals during development and adult life.

FGF4 and TGFβ2 act downstream of mechanical signals to regulate tendon differentiation

In immobilised chick embryos, transcriptional readouts of both FGF/ERK MAPK and TGFβ/SMAD2/3 signalling pathways are downregulated in limb tendons. This shows that both pathways are sensitive to mechanical signals in chick limbs. These pathways are known to be sensitive to mechanical forces in other biological systems (Humphrey et al., 2014). Moreover, transcriptome profiling analyses have identified FGF and TGFβ signalling as being downregulated in developing humerus (limb bone) of immobilised mouse foetuses (Rolfe et al., 2014). In addition to being downregulated in tendons of immobilised embryos, both FGF4 and TGFβ2 prevent the decrease of *SCX* expression in chick limbs in immobilisation conditions. Rescue experiments with FGF4 or TGFβ2 in immobilised limbs do not activate TGFβ or FGF transcriptional readout, respectively, indicating that FGF4 and TGFβ2 act independently of each other to activate *SCX* expression during limb tendon differentiation. The ability of TGFβ2 to rescue *SCX* expression in chick limbs in immobilised embryos is reminiscent of the requirement of SMAD2/3 pathway for the *Scx* induction downstream of mechanical forces in tendon cells (Maeda et al., 2011). However, *TNMD* and *THBS2* downregulation was only prevented by TGFβ2 and not by FGF4. We hypothesise that TGFβ2 activates another signal required for *TNMD* and *THBS2* expression, which is not regulated by

FGF4, in immobilisation conditions. In the presence of muscle contraction, FGF4 activates *TNMD* and *THBS2*, while in the absence of muscle contraction FGF4 is not able to activate *TNMD* or *THBS2* expression, highlighting the independent effects of both pathways in tendon differentiation.

In summary, both FGF4 and TGFβ2 signalling molecules are involved in the commitment of undifferentiated chick limb mesodermal cells towards the tendon lineage and act downstream of mechanical forces to regulate tendon differentiation during chick limb development (Fig. 8). Both FGF4 and TGFβ2 have a tenogenic effect, independently of each other, during both muscle-independent and -dependent phases of chick limb tendon development.

MATERIAL AND METHODS

Chick and mouse embryos

Fertilized chick eggs (JA 57 strain) (Morizeau, Dangers) were incubated at 38°C. Embryos were aged according to the number of days of incubation (Embryonic day) or staged according to Hamburger and Hamilton (HH) stages (Hamburger and Hamilton, 1992). Swiss mouse embryos (Janvier) were collected after natural overnight mattings. For staging, fertilisation was considered to take place at midnight. The manipulation of chick and non-transgenic mouse embryos is not included in the guidelines of the french national ethics committee.

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

FGF4, TGFβ2, TGFβ2+PD18, FGF4+SIS3 beads or mFgf4/RCAS-expressing cells were grafted in limbs of E3/E4 chick embryos as described (Edom-Vovard et al., 2002). Embryos were harvested 4, 6 or 24 h after grafting. Detailed protocol is provided in supplementary material file 1.

Chick and mouse limb explant cultures

Chick limb explants were prepared as described in (Placzek and Dale, 1999) and treated with TGFβ2, FGF4, PD18, SB43, SIS3 or TGFβ2+PD18 as described in (Havis et al., 2014). Detailed protocol is provided in supplementary material file 1.

DMB or PB application in chick embryos

Decamethonium bromide (DMB) and Pancuronium bromide (PB) were prepared in Hank's solution, at the final concentrations of 12 mM. 100 µl of DMB or PB solution was deposed

daily with a pipetman in the amniotic fluid next to the embryos after vitelline membrane removal in E4.5, E5.5 and E6.5 chick embryos. Control embryos were injected with Hank's solution using the same daily protocol. Immobilised or control embryos were analysed at E5.5 (24 h), E6.5 (48 h) or E7.5 (72 h). Forelimbs or hindlimbs were isolated and analysed for in situ hybridisation to sections or wholemounts or for RT-q-PCR analysis. For RT-q-PCR analysis, RNAs were prepared from the whole limbs, the limbs without digits or digits alone.

RNA isolation, reverse transcription and quantitative real-time PCR

RT-q-PCR of experimental or control chick limbs, chick limb explants or mouse limb explants were performed as previously described (Havis et al., 2014). A detailed protocol is provided in supplementary material file 1.

In situ hybridisation and Immunohistochemistry

Control or manipulated chick limbs were fixed and processed for in situ hybridisation as previously described in (Havis et al., 2014). The probes were used are described in supplementary material file 1. Differentiated muscle cells were detected after in situ hybridisation with the monoclonal antibody MF20 (non diluted supernatant) developed by D.A. Fischman and obtained from the Developmental Studies Hybridoma Bank developed under the auspices of the NICHD and maintained by the University of Iowa.

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Competing interests

The authors declare no competing financial interests

Author contributions

D.D designed experiments. E.H, M-A.B, J.L, B.C and C.M performed experiments. E.H and D.D analysed the data and D.D wrote the manuscript. D.D brings the fundings. All of the authors have read and approved the final manuscript.

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Figures

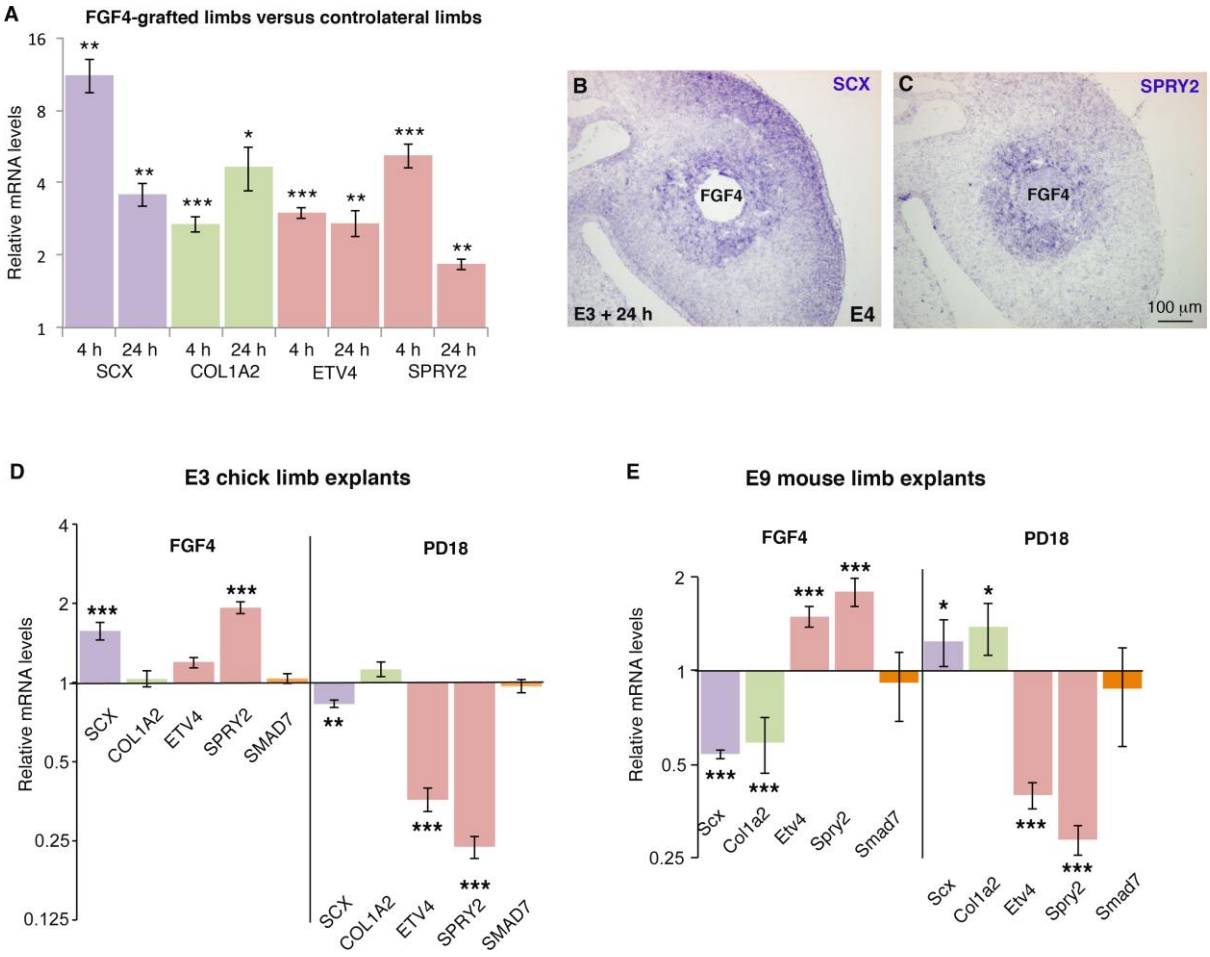

Figure 1

FGF tenogenic effect on chick limb cells. (A-C) FGF4 bead application to forelimbs of E3/E4(HH18/HH22) chick embryos. 4 h or 24 h after bead application, FGF4-grafted right limbs and control left limbs were processed for RT-q-PCR (n=6 for each time point) or for in situ hybridisation (n=3) analyses. (A) RT-q-PCR analyses of the expression levels of *SCX, COL1A2* and readouts of FGF/ERK MAPK activity, *ETV4* and *SPRY2* in FGF4-grafted right limbs, 4 h or 24 h after FGF4 application. For each gene, the mRNA levels of control left limbs were normalised to 1. (B,C) In situ hybridisation for *SCX* and *SPRY2* in E4 (E3 + 24 h) FGF4-grafted limbs. (D) E3 chick limb explant cultures. RT-q-PCR analyses of the relative

expression levels of *SCX, COL1A2*, *ETV4* and *SPRY2* and *SMAD7* in E3 chick limb explants cultured for 6 h with FGF4 (n=10) or PD18 inhibitor (n=10). (E) E9 mouse limb explant cultures. RT-q-PCR analyses of the expression levels of *Scx, Col1a2*, *Etv4* and *Spry2* and *Smad7* in E9 mouse limb explants cultured for 6 h with FGF4 (n= 5) or PD18 inhibitor (n= 5). For each gene, the mRNA levels of control limb explants were normalised to 1. P values were analysed by unpaired Student's t-test using Microsoft Excel. **P*<0.05; ***P*<0.01; ****P*<0.001; Error bars indicate s.e.m.

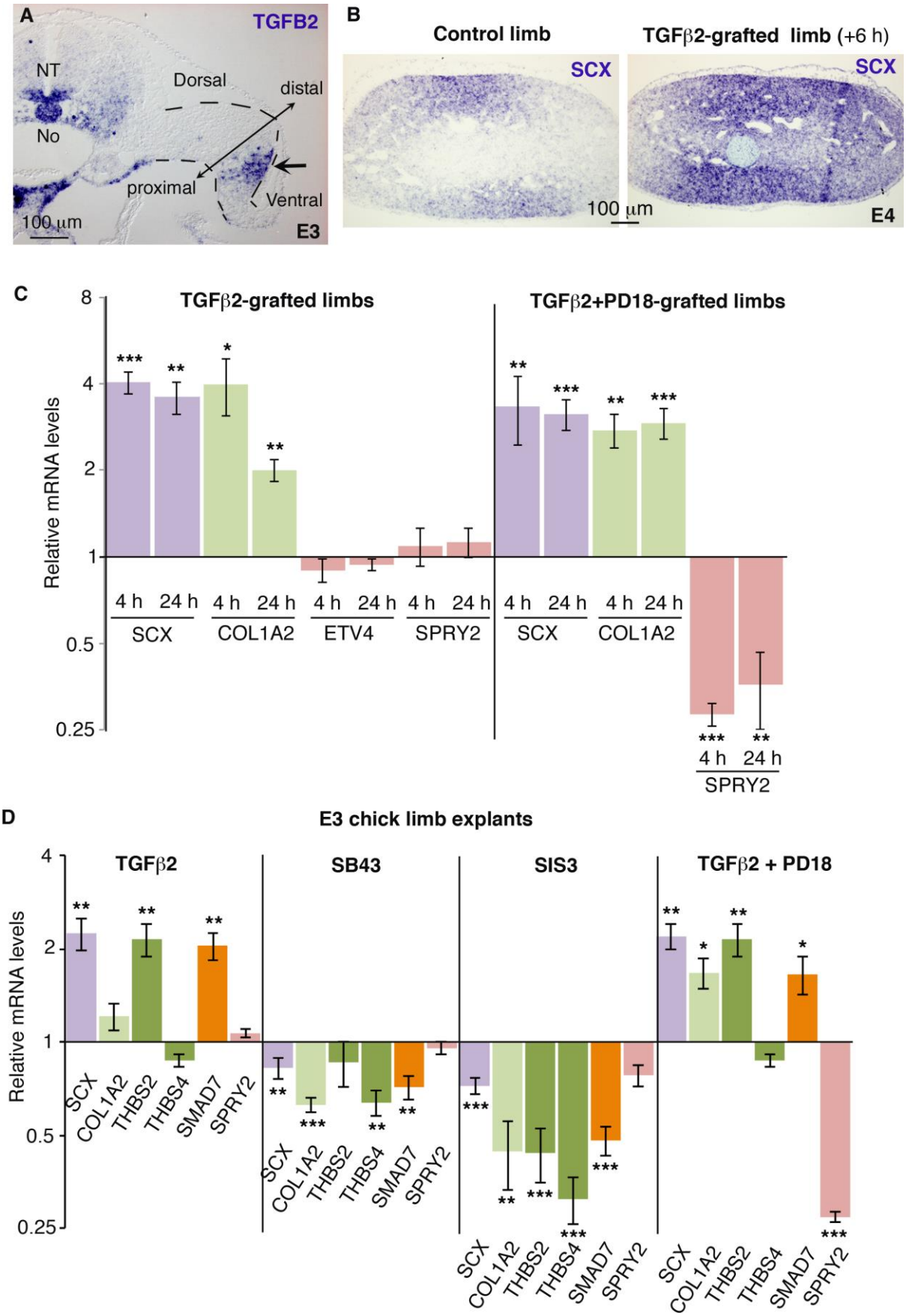

Figure 2

Involvement of the TGFβ/SMAD2/3 pathway in chick limb tendon progenitors. (A) In situ hybridisation for *TGFB2* in E3(HH19) chick embryos at the level of forelimbs. *TGFB2* was expressed in ventral limb regions (arrow), in addition to being expressed in ventral neural tube, (NT) notochord (No) and ventral aorta. Dashed lines delineate the limb bud. (B) TGFβ2 beads were grafted to forelimbs of E3.5(HH21) chick embryos. Transverse limb sections of TGFβ2-grafted right limbs and control left limbs were processed for in situ hybridisation for SCX (n=6). (C) TGFβ2 or TGFβ2+PD18 beads were grafted to forelimbs of E3/E4 (HH19/HH22) chick embryos. RT-q-PCR analyses of the expression levels of *SCX, COL1A2, ETV4* and *SPRY2* in TGFβ2- and TGFβ2+PD18–grafted right limbs, 4 h or 24 h after bead application. For each gene, the mRNA levels of control left limbs were normalised to 1. (D) RT-q-PCR analyses of mRNA levels for tendon genes and readout of signalling pathways in E3 chick limb explants cultured for 24 h with TGFβ2 (n=6), SB43 (n=7), SIS3 (n=9) or TGFβ2+PD18 (n=6). For each gene, the mRNA levels of control limb explants were normalised to 1. Error bars represent the s.e.m. P values were analysed by paired Student's t-test using Microsoft Excel. **P*<0.05; ***P*<0.01; ****P*<0.001.

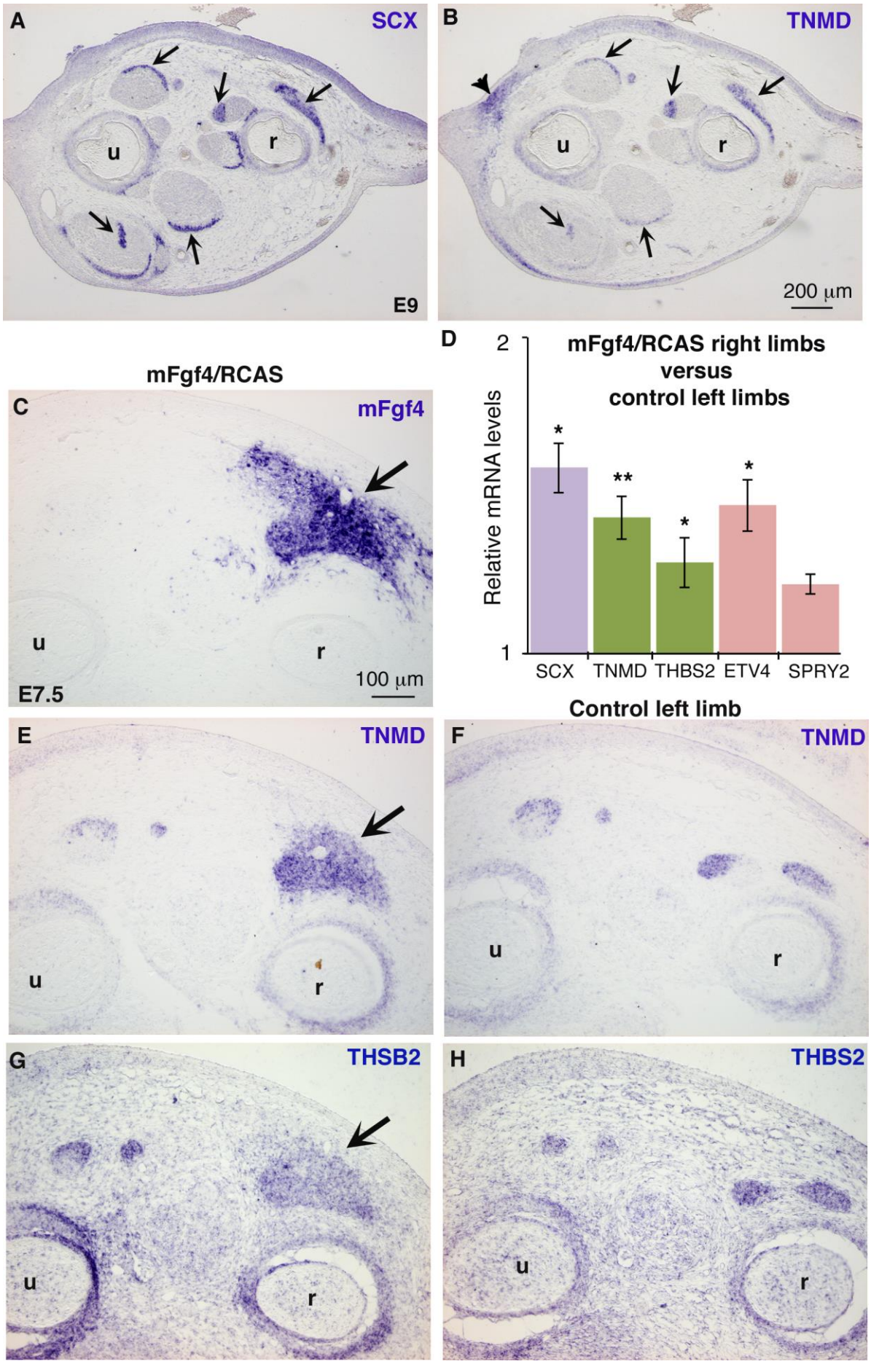

Figure 3

FGF4 positively regulates the expression of the tendon differentiation markers, *TNMD* and *THBS2*. (A,B) Adjacent transverse sections of forelimbs of E9 chick embryos were hybridised with *SCX* and *TNMD* probes. *TNMD* was expressed in *SCX*-positive tendons (arrows). *TNMD* was also expressed in the dermis (B, arrowhead). (C-H) mFgf4/RCAS-producing cells were grafted into forelimb buds of E3.5(HH21) chick embryos. Embryos (n=3) were fixed at E7.5, and grafted (C,E,G) and control (F,H) forelimbs were transversely sectioned at the level of the zeugopod. (C,E,G) Adjacent sections were hybridised with *mFgf4* to show the extent of virus spread and with *TNMD* and *THBS2* probes to show ectopic expression (E,G, arrows) in mFgf4-positive regions (C, arrow) compared to normal *TNMD* and *THBS2* expression in control left limbs (F,H, arrows). (D) RT-q-PCR analyses of mRNA levels in mFgf4/RCAS-infected limbs (4 days after grafting) (n=5). For each gene, the mRNA levels of control (left) limbs were normalised to 1. P values were analysed by paired Student's t-test using Microsoft Excel. **P*<0.05; ***P*<0.01; Error bars represent the s.e.m. u, ulna, r, radius.

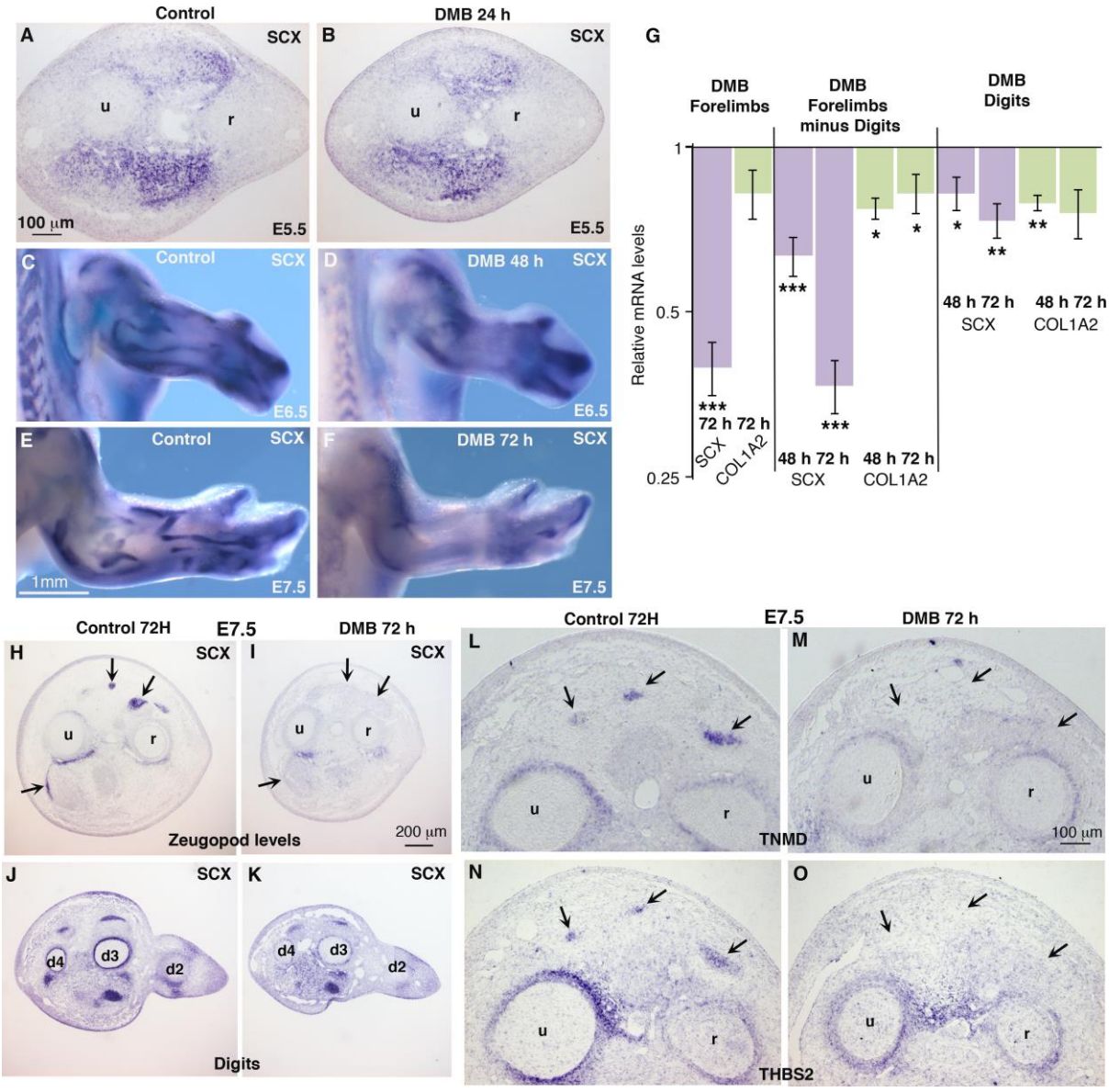

Figure 4

Muscle contraction is required to maintain *SCX* and *TNMD* expression in chick forelimbs. DMB reagent was injected into E4.5(HH24) chick embryos to induce immobilisation. Immobilised embryos were processed for in situ hybridisation (A-F,H-O) or RT-q-PCR analyses (G). (A,B) Forelimb transverse sections of 24 h control (n=2) and DMB-treated embryos (n= 4) were hybridised with SCX probe. (C-F) Forelimbs of control (n=10) and DMB-treated embryos (n=10) fixed 48 h (n=5) or 72 h (n=5) after application were hybridised with SCX probe. (G) RT-q-PCR analyses of mRNA levels for tendon genes in forelimbs (n=10), forelimbs where digits were removed (n=20) and digits (n=20) of DMB-

treated embryos, 48 h (n=10) and 72 h (n=10) after DMB application. For each gene, the mRNA levels of control limbs were normalised to 1. P values were analysed by unpaired Student's t-test using Microsoft Excel. *P<0.05; **P<0.01; ***P<0.001. Error bars represent the s.e.m. (H-O) Forelimb (H,I,L-O) and digit (J,K) transverse sections of 72 h control (H,J, L,N) and DMB-treated (I,K,M,O) embryos (n=4) were hybridised with SCX (H-K), TNMD (L,M) or THBS2 (N,O) probes. u, ulna, r, radius.

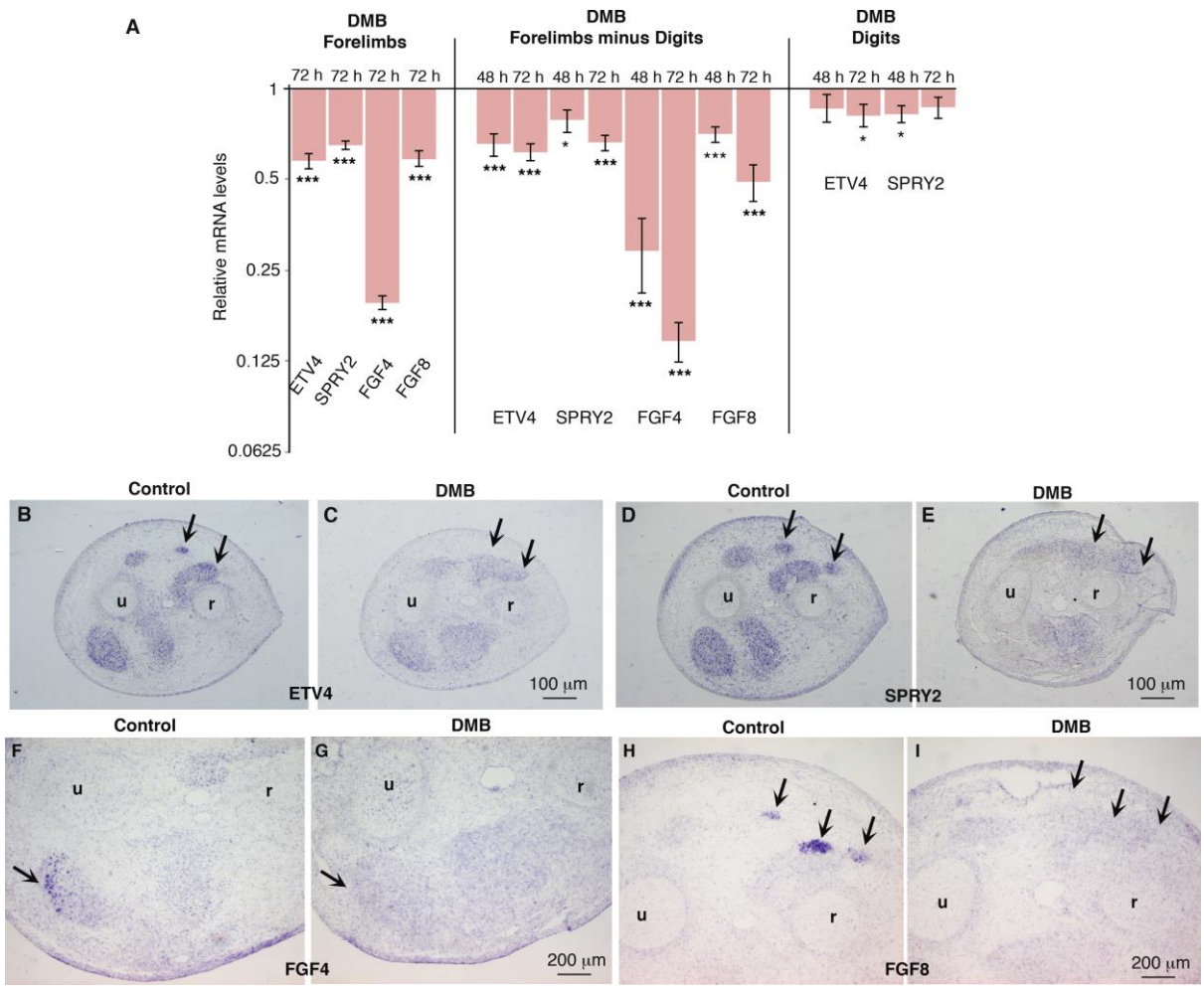

Figure 5

Muscle contraction is required to maintain active FGF/ERK MAPK signalling in chick limb tendons. (A) RT-q-PCR analyses of mRNA levels for *ETV4, SPRY2, FGF4* and *FGF8* in forelimbs (n=9), forelimbs (digits excluded) (n=19) and digits (n=20) of DMB-treated embryos, 48 h (n=20) and 72 h (n=19) after DMB application. For each gene, the mRNA levels of control limbs were normalised to 1. P values were analysed by unpaired Student's t-test using Microsoft Excel. **P*<0.05; ****P*<0.001; Error bars indicate s.e.m. (B-I) Forelimb transverse sections at the level of the zeugopod of 72 h control (B,D,F,H) and DMB-treated (C,E,G,I) embryos were hybridised with ETV4 (B,C), SPRY2 (D,E), FGF4 (F,G) and FGF8 (H,I) probes (n=3). Arrows point gene expression in control limbs (B,D,F,H) and loss of gene expression in DMB-limbs (C,E,G,I). u, ulna, r, radius.

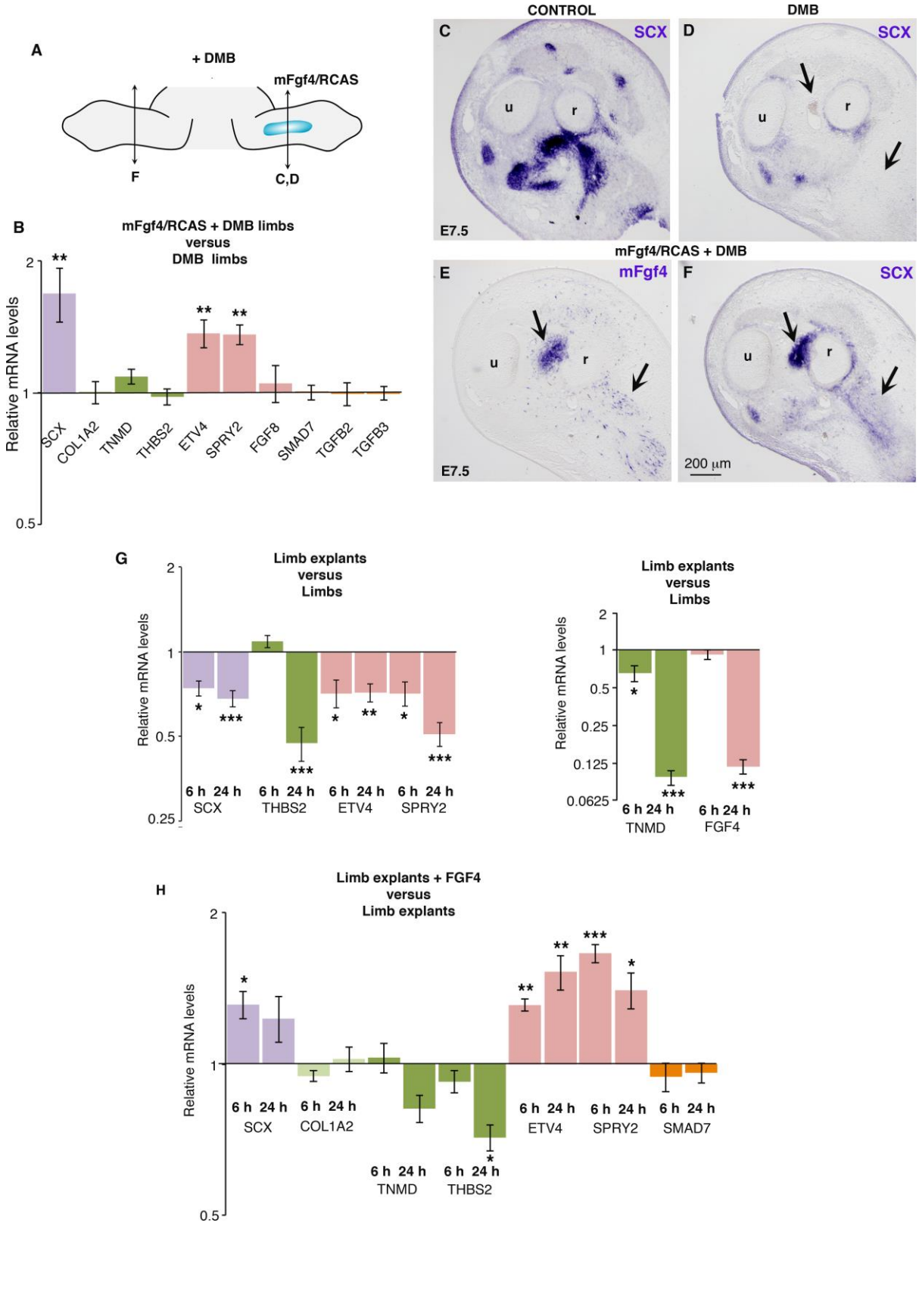

Figure 6

FGF4 induces *SCX* expression in limbs of immobilised embryos. (A) mFgf4/RCAS producing cells were grafted into right forelimbs of E3.5 chick embryos. These embryos were then treated with DMB at E4.5 and fixed 3 days after DMB application at E7.5. (B, D-F) The manipulated embryos were either processed for RT-q-PCR analysis (B) (n=7) or for in situ hybridisation to limb sections (D-F) (n=3). (B) RT-q-PCR analyses of mRNA levels for components of tendon genes, of the FGF/ERK and of the TGF-β/SMAD2/3 signalling pathway*s* in mFgf4/RCAS-forelimbs of DMB- treated embryos. For each gene, the mRNA levels of contralateral limbs (DMB-treated only) were normalised to 1. (D-F) Transverse sections of right mFgf4/RCAS forelimbs (E,F) and left forelimbs (D) of DMB- treated embryos were hybridised with mFgf4 (E) and SCX (D,F). (C) In situ hybridisation to E7.5 control limbs with SCX probe. u, ulna, r, radius. (G) The mRNA levels for *SCX, ETV4*, *SPRY2, TNMD, THBS2* and *FGF4* were compared by RT-q-PCR analysis in E5 (HH25/26) limb explants cultured for 6 h (n=5) and 24 h (n=5) versus limbs (n=10) of stage-matched embryos. (H) RT-q-PCR analyses of mRNA levels in E5 limb explants cultured for 6 h (n=7) and 24 h (n=5) in the presence or absence of FGF4. For each gene, the mRNA levels of E5 limb explants cultured for 6 and 24 h with no FGF4 were normalised to 1. P values were analysed by unpaired Student's t-test using Microsoft Excel. **P*<0.05; ***P*<0.01; ****P*<0.001; Error bars indicate s.e.m.

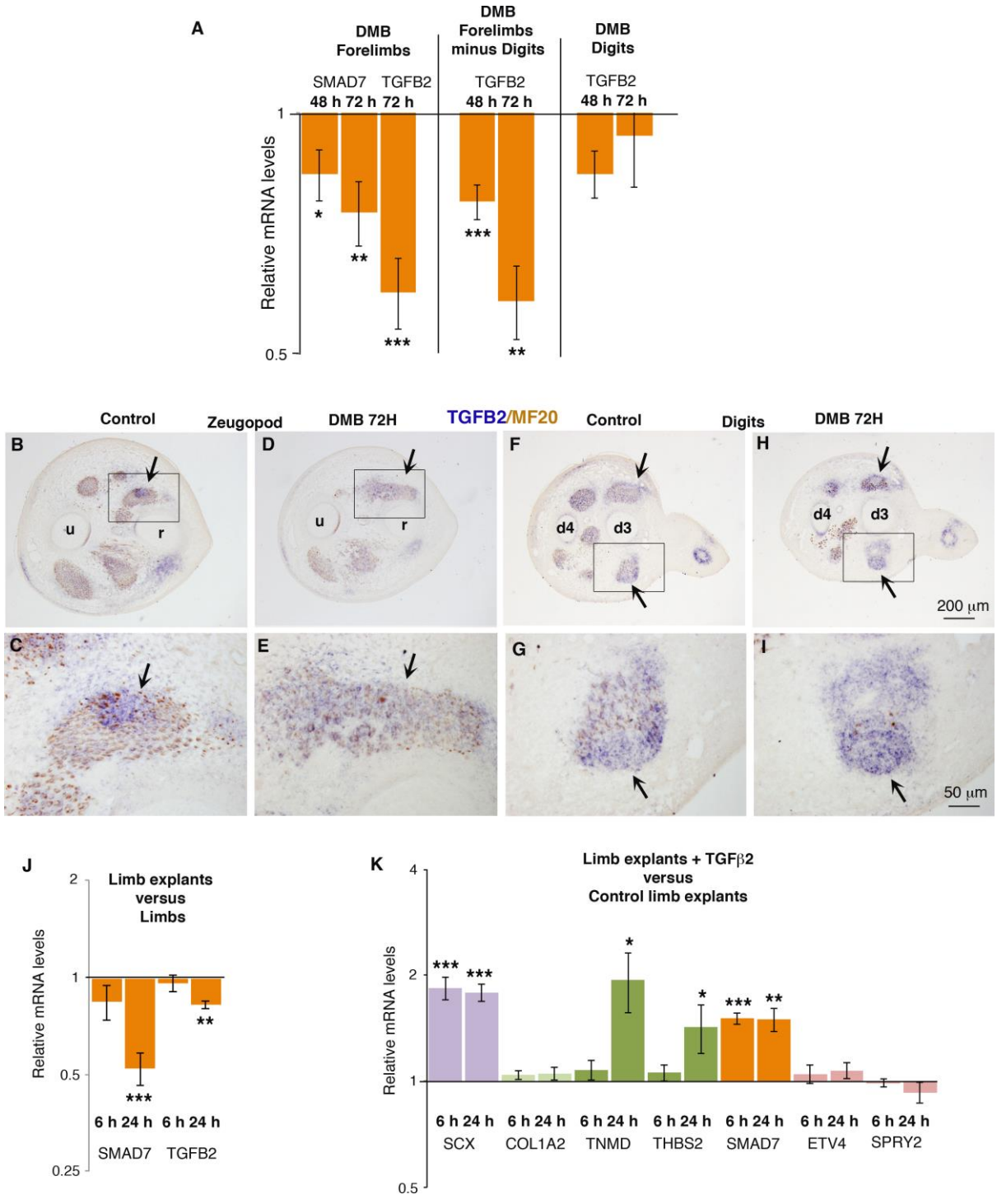

Figure 7

TGFβ2 prevents *SCX* downregulation in limb explants. (A-I) DMB application into E4.5 chick embryos. E7.5 immobilised embryos were either processed for RT-q-PCR analysis (n=10) or in situ hybridisation to limb sections (n=3). (A) RT-q-PCR analyses for components of the TGFβ pathway, in paralysed limbs. For each gene, the mRNA levels of limbs of control embryos were normalised to 1. (B-I) Transverse limb sections at the zeugopod (B-E) and digit (F-I) levels of immobilised embryos (D,E,H,I) or control embryos (B,C,F,G) were hybridised with the TGFB2 probe and then immunostained with MF20 antibody. (C,E,G,I) are high magnifications of squared regions of panels (B,D,F,H). (B-I) Arrows point to zeugopod (B-E) or digit (F-I) tendons in control or DMB embryos. u, ulna, r, radius. (J) The mRNA levels for *SMAD7* and *TGFB2* were compared by RT-q-PCR analysis in E5 (HH25/26) limb explants (n=10) cultured for 6 h (n=5) and 24 h (n=5) versus limbs of stage-matched embryos. (K) RT-q-PCR analyses of mRNA levels in E5 limb explants (n=15) cultured for 6 h (n=8) and 24 h (n=7) in the presence or absence of TGF-β2. For each gene, the mRNA levels of E5 limb explants cultured for 6 h and 24 h with no TGF-β2 were normalised to 1. P values were analysed by unpaired Student's t-test using Microsoft Excel. **P*<0.05; ***P*<0.01; ****P*<0.001; Error bars indicate s.e.m.

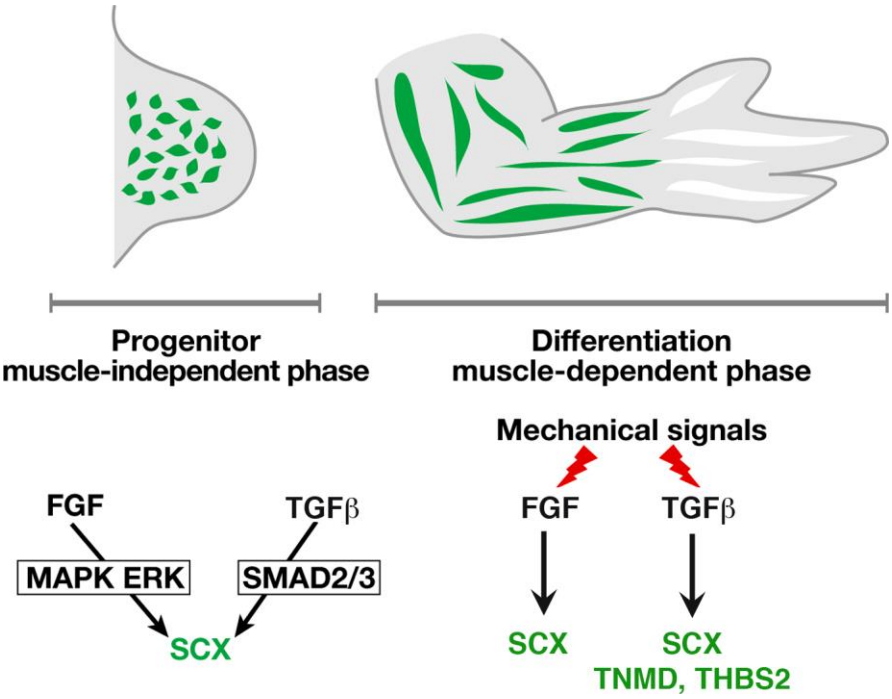

Figure 8

FGF4 and TGFβ2 involvement in stylopod/zeugopod limb tendons during chick development

During the progenitor stage that is independent of muscle, FGF or TGFβ ligand, independently of each other, has a tenogenic effect on limb mesodermal undifferentiated cells. Moreover, the MAPK ERK pathway and SMAD2/3 intracellular pathways are both required for *SCX* expression in early chick limbs. During the differentiation step that is dependent of muscle, muscle contraction is required for FGF/MAK ERK and TGFβ/SMAD2/3 activity at muscle/tendon interface. Downstream of mechanical forces, FGF4 and TGFβ2 positively regulate *SCX* expression, while TGFβ2 (but not FGF4) regulates *TNMD* and *THBS2* expression in limb tendons.

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

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Supplementary Figures

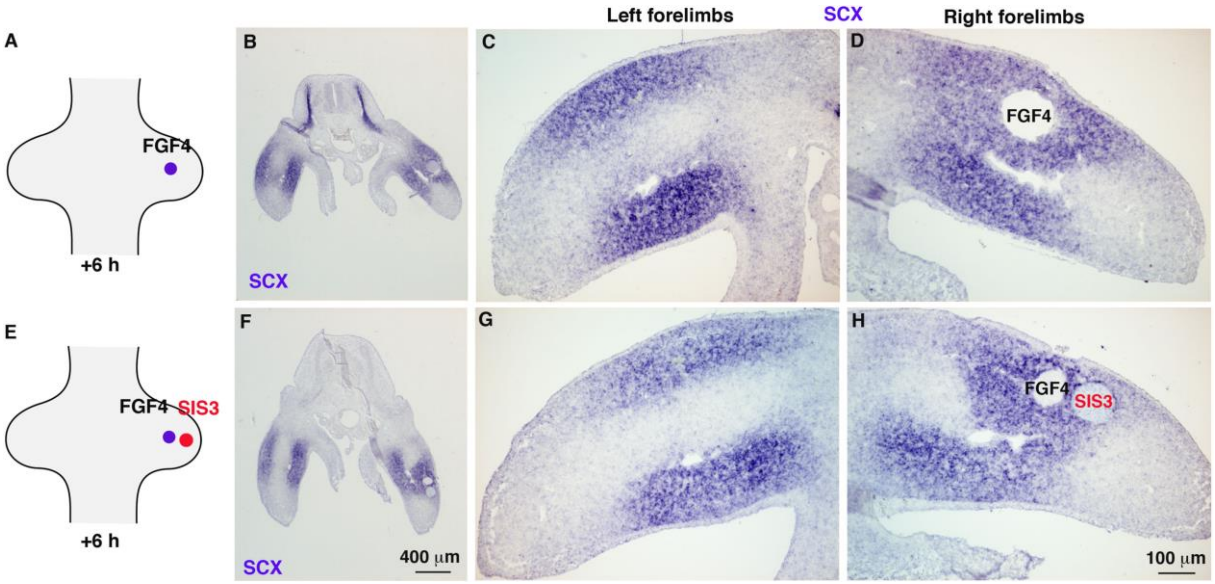

Figure S1

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.

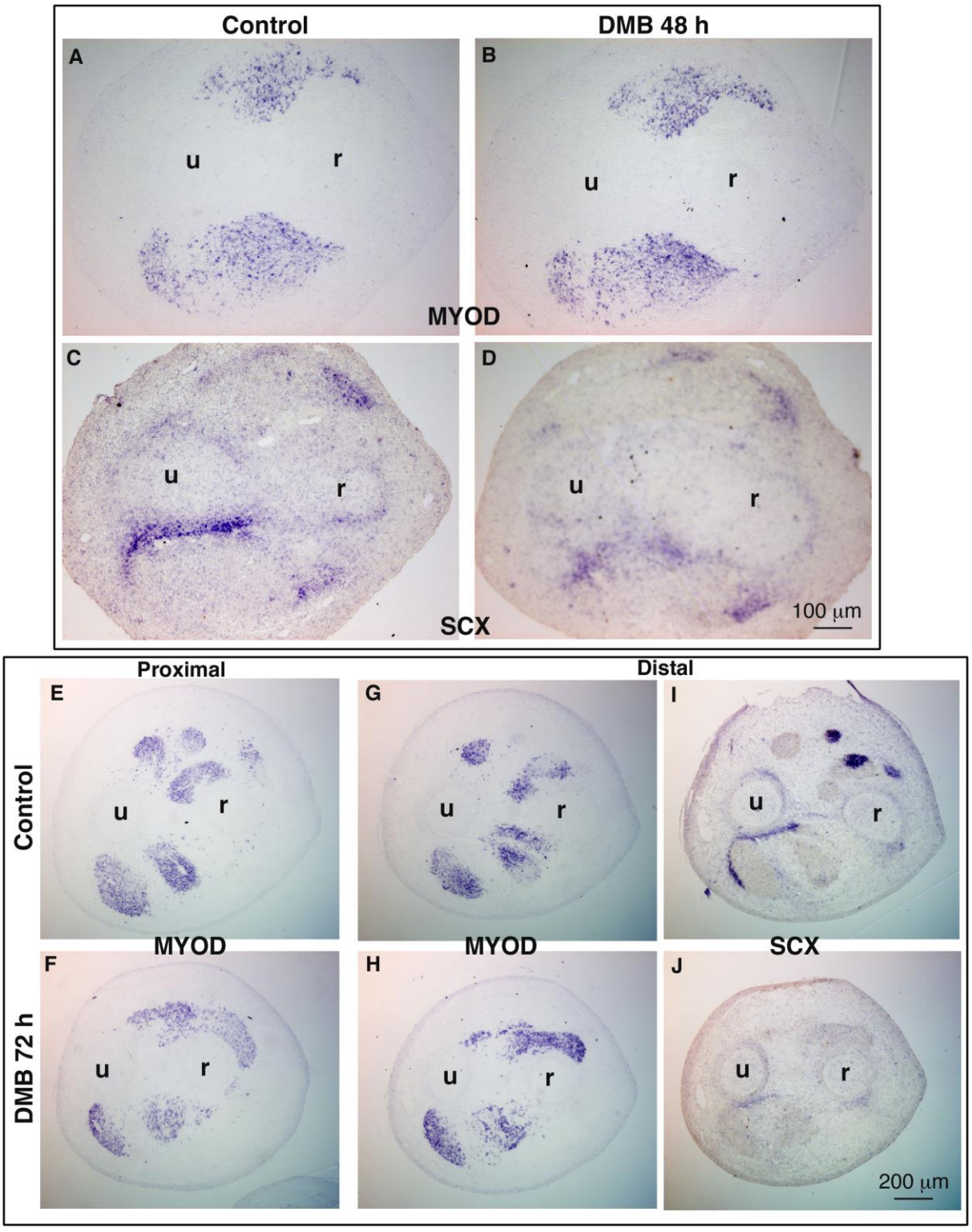

Figure S2

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.

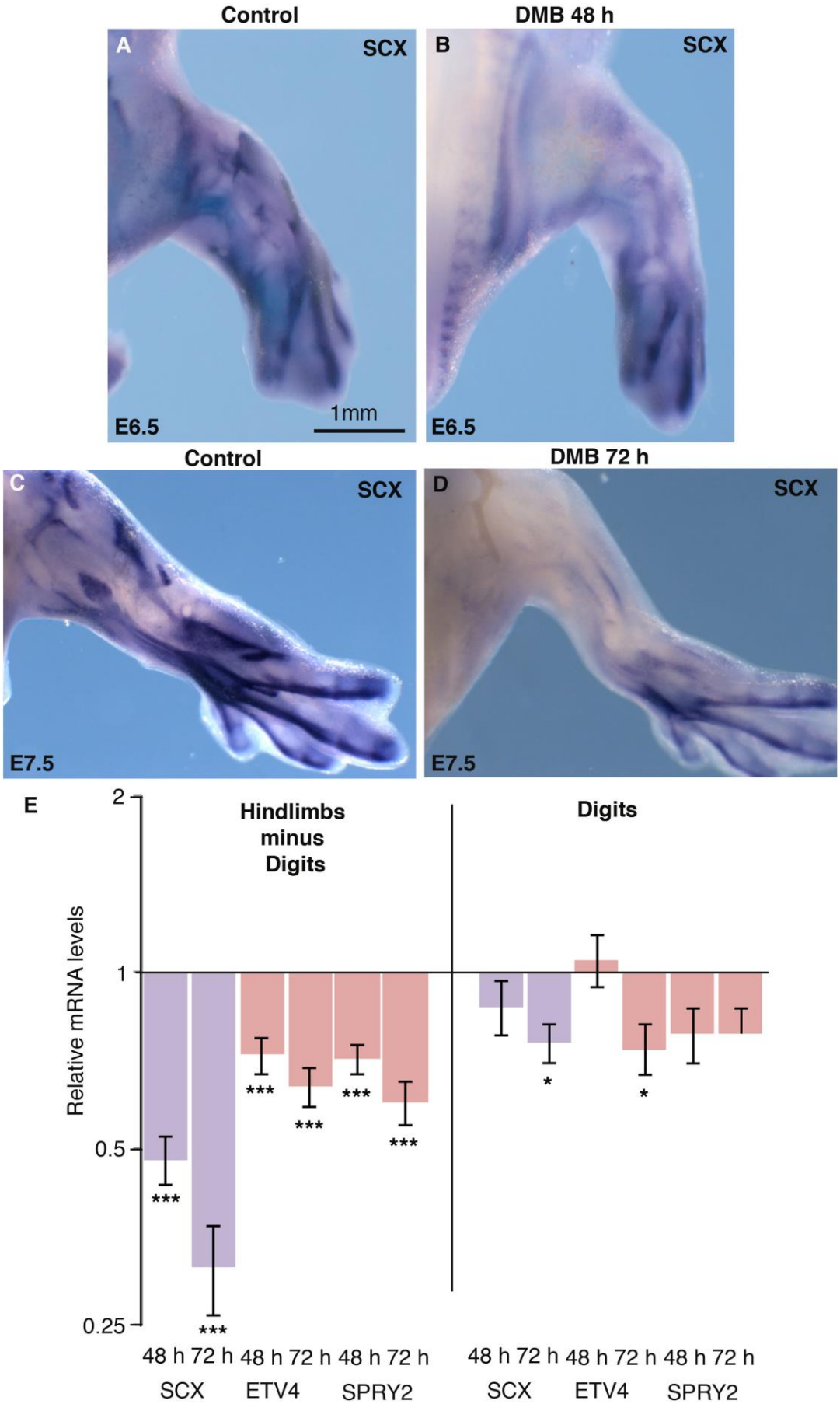

Figure S3

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.

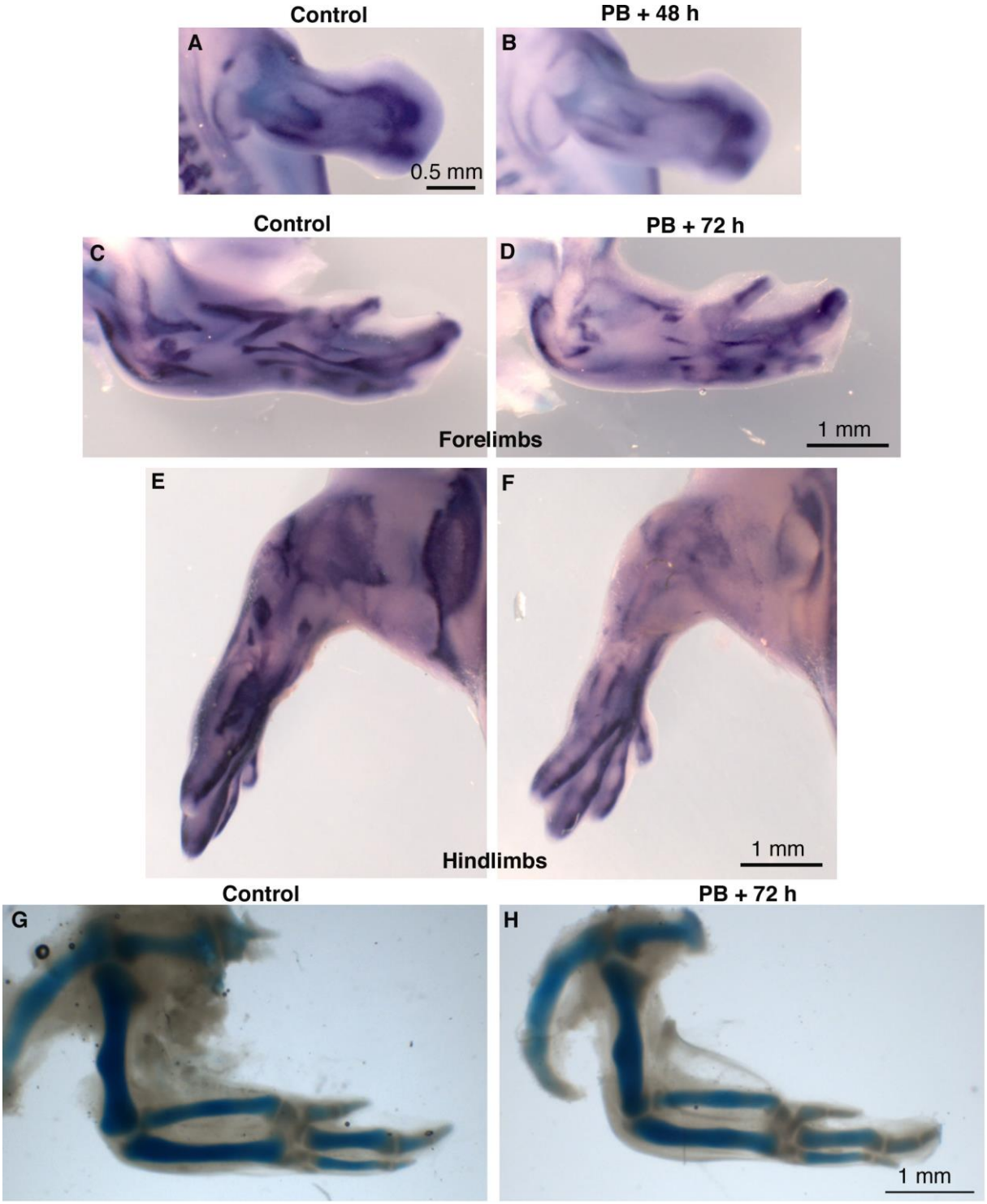

Figure S4

***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).

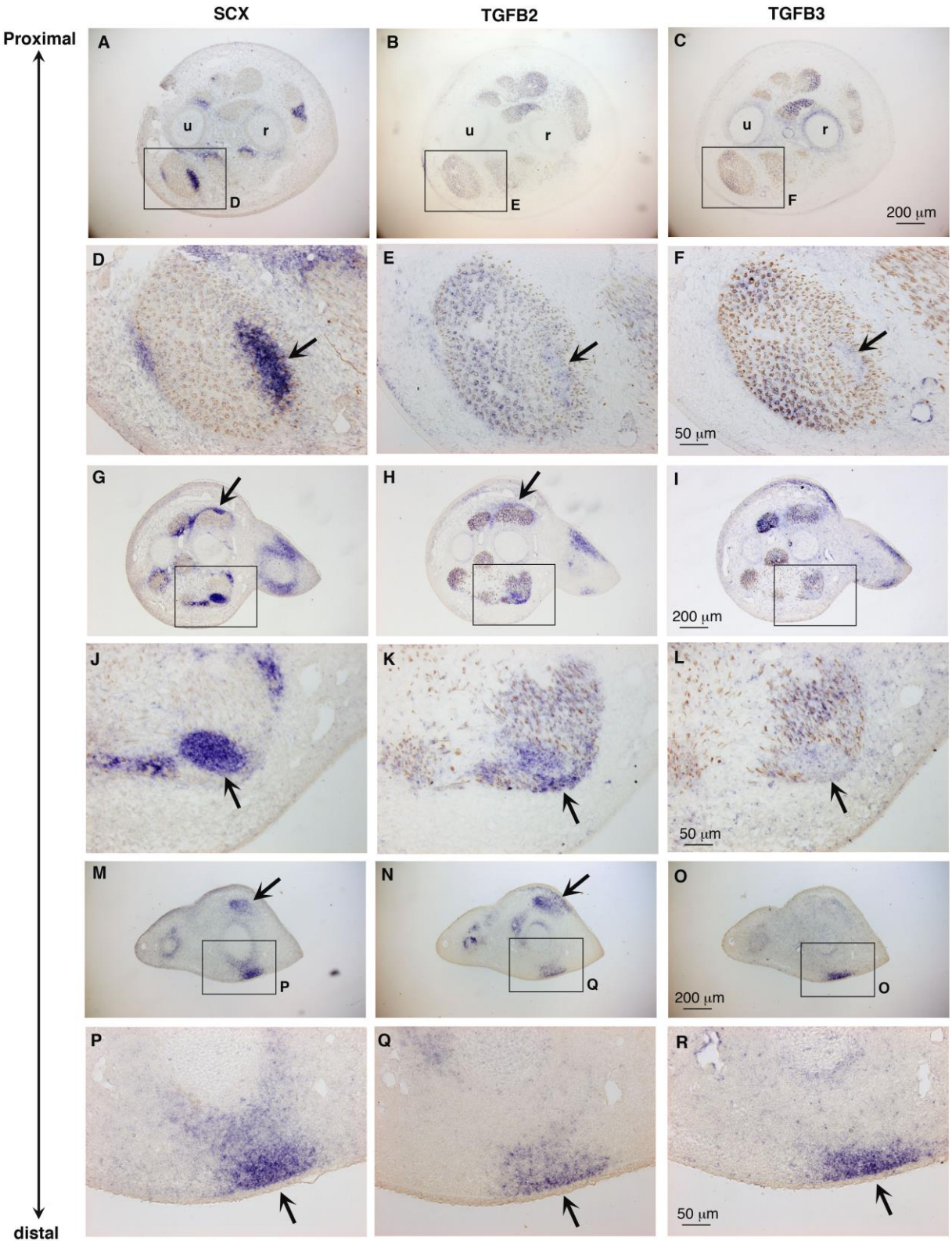

Figure S5

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.

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Supplementary Figures

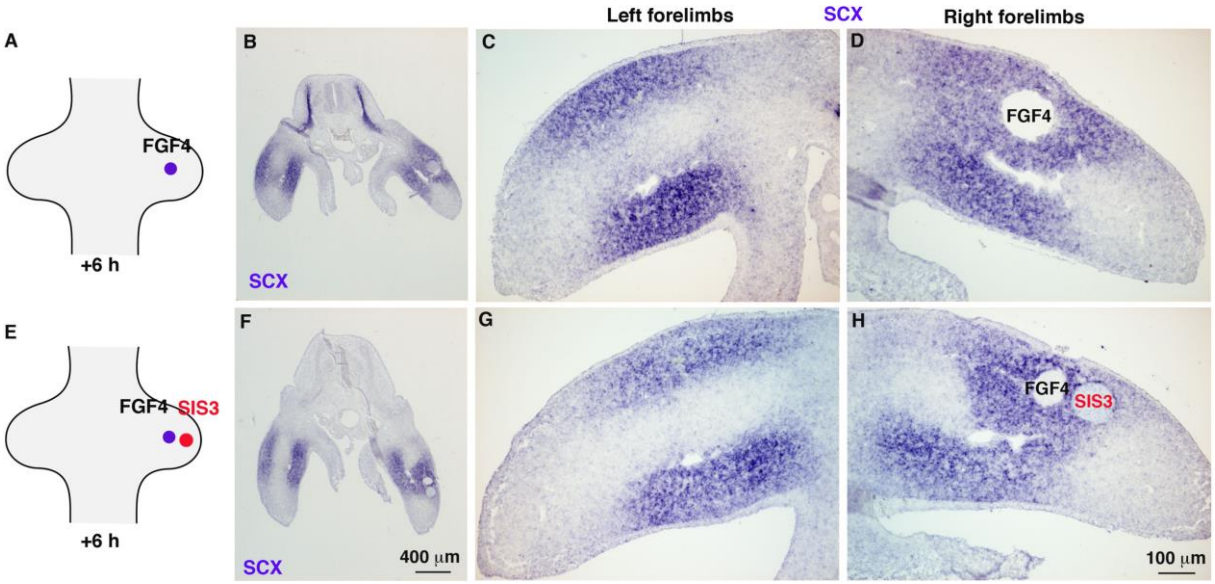

Figure S1

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.

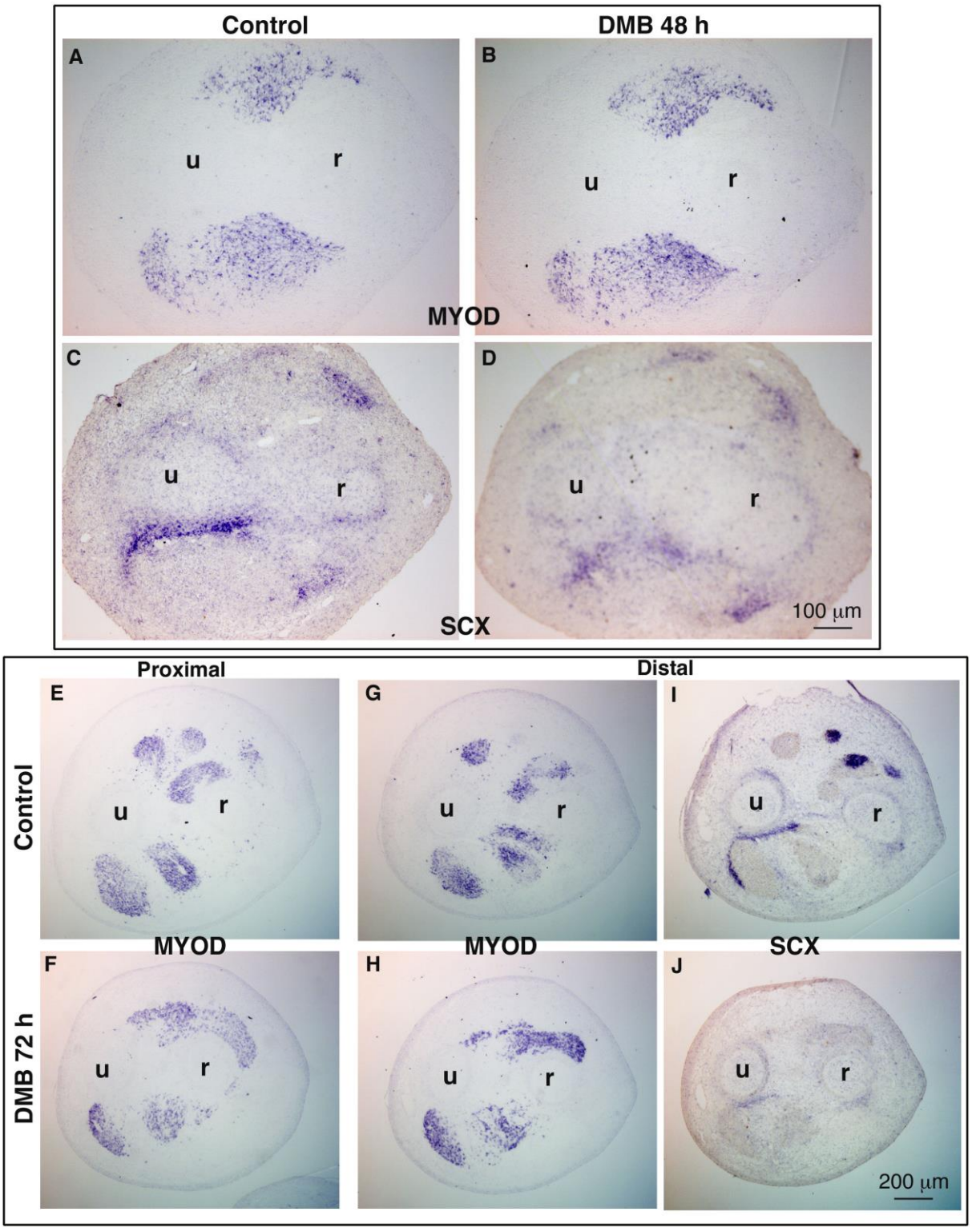

Figure S2

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.

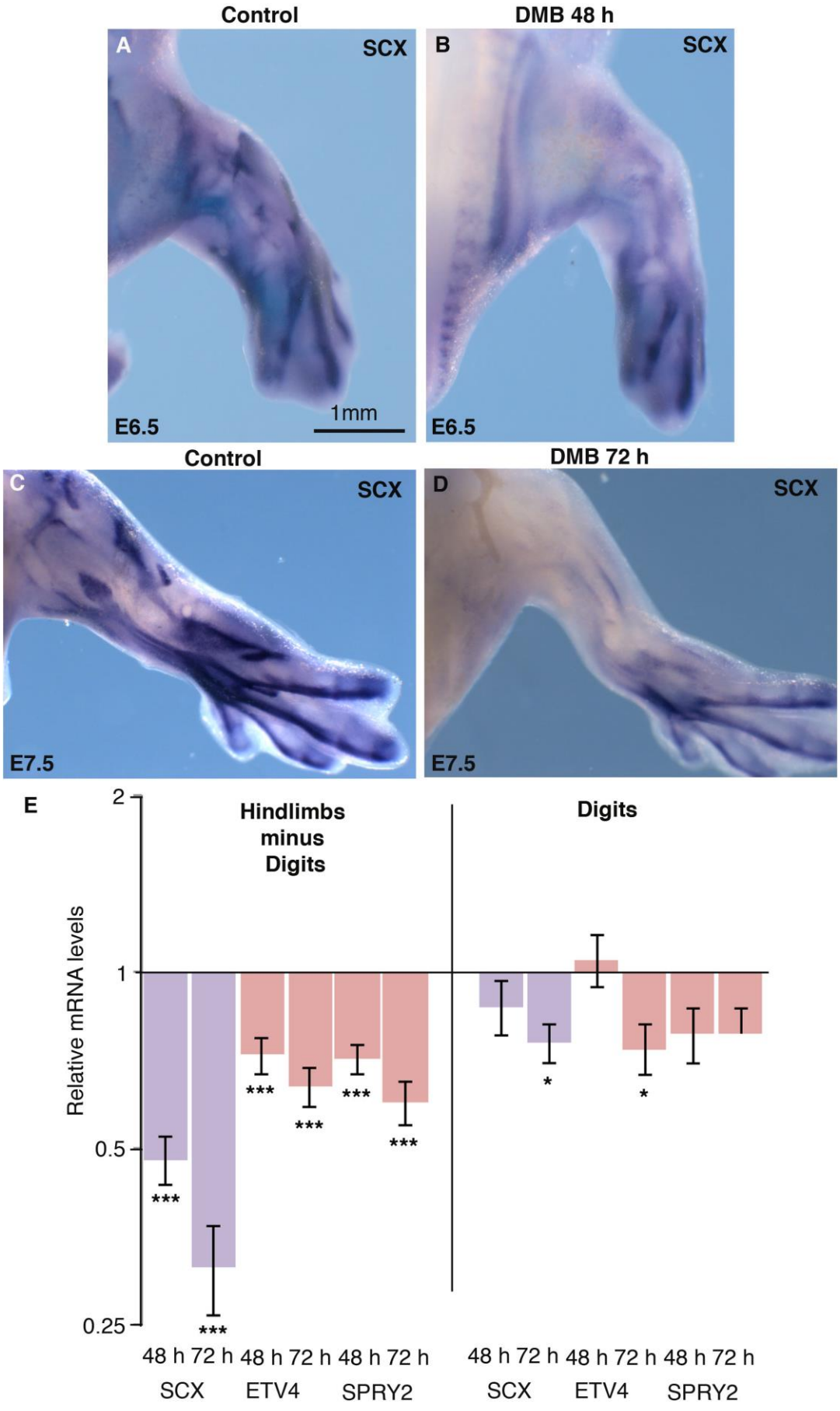

Figure S3

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.

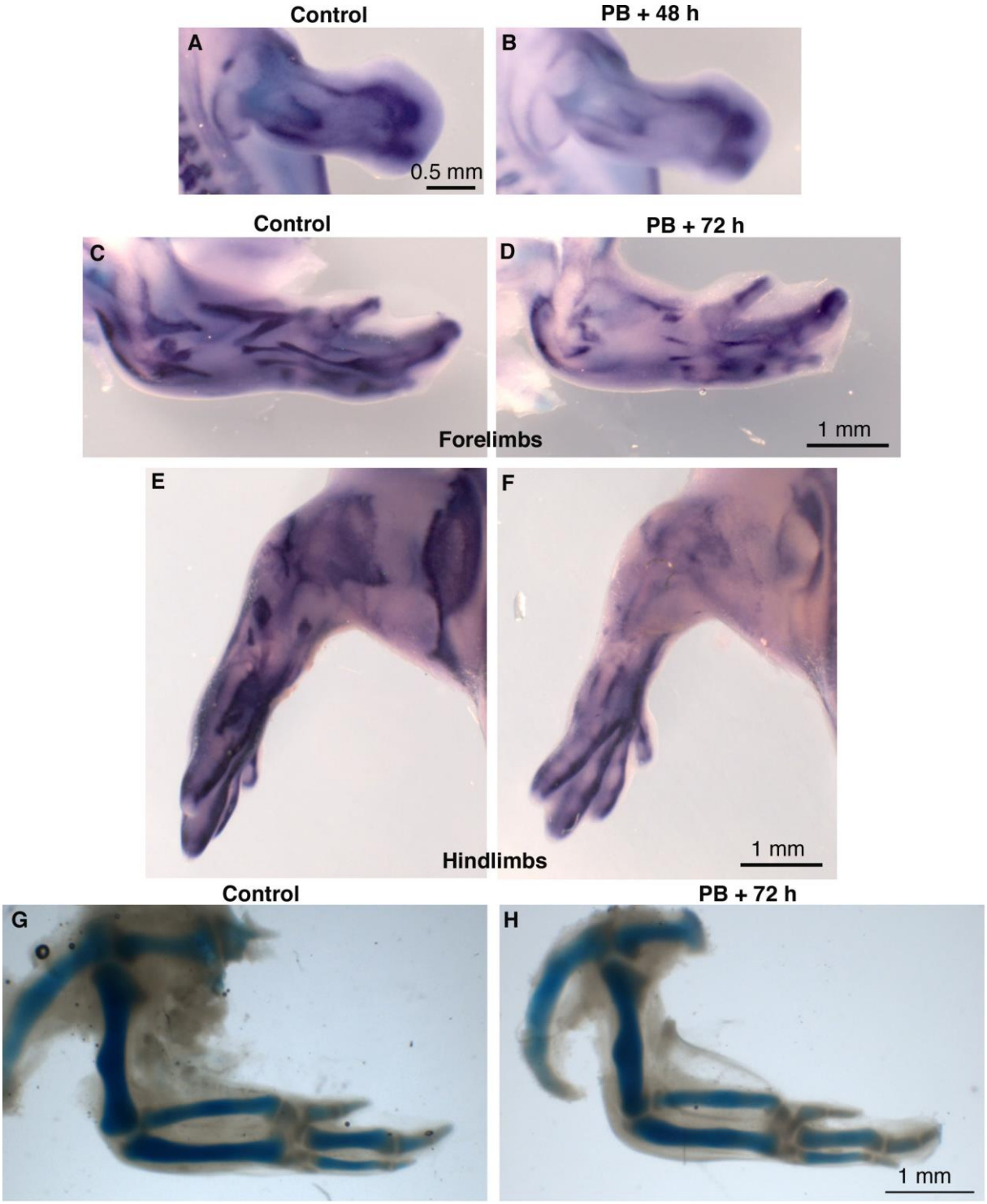

Figure S4

***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).

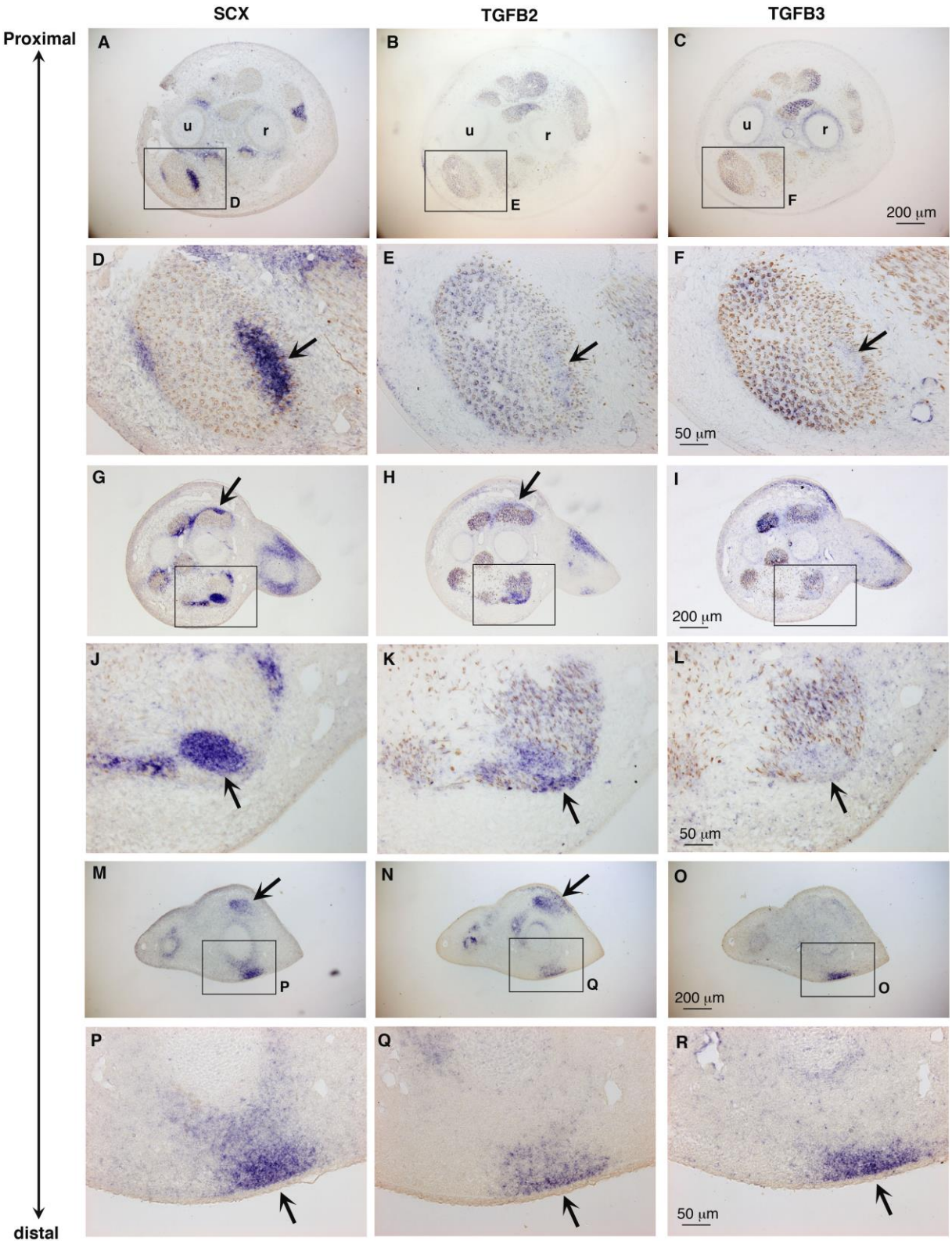

Figure S5

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.