

MUSCULOSKELETAL INTEGRATION AT THE WRIST UNDERLIES MODULAR DEVELOPMENT OF LIMB TENDONS

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SUMMARY

The long tendons of the limb extend from muscles that reside in the zeugopod (arm/leg) to their skeletal insertions in the autopod (paw). How these connections are established along the length of the limb remains unknown. In this study, we show that limb tendons are formed in modular units that combine to form a functional contiguous structure; in muscle-less limbs, tendons develop in the autopod but do not extend into the zeugopod, and in the absence of limb cartilage, the zeugopod segments of tendons develop despite the absence of tendons in the autopod. Analyses of cell lineage and proliferation further indicate that distinct mechanisms govern growth of autopod and zeugopod tendon segments. To elucidate the integration of these autopod and zeugopod developmental programs, we reexamined early tendon development. At E12.5, muscles extend across the full length of a very short zeugopod and connect through short anlagen of tendon progenitors at the presumptive wrist to their respective autopod tendon segment, thereby initiating musculoskeletal integration. Zeugopod tendon segments are subsequently generated by proximal elongation of the wrist tendon anlagen, in parallel with skeletal growth, underscoring the dependence of zeugopod tendon development on muscles for tendon anchoring. Moreover, a subset of extensor tendons initially form as fused structures, due to initial attachment of their respective wrist tendon anlage to multiple muscles. Subsequent individuation of these tendons depends on muscle activity. Collectively, these results establish an integrated model for limb tendon development that we propose as a framework for future analyses of tendon and musculoskeletal phenotypes.

INTRODUCTION

The musculoskeletal system is an efficient assembly of tissues that coordinate to enable movement and provide structural stability (Benjamin and Ralphs, 2000). Functionality of the musculoskeletal system is determined by the precise connection of each muscle to its skeletal insertion, yet little is known about the initial establishment of these connections or how the development and growth of the musculoskeletal tissues is integrated. The exquisite coordination of muscles, tendons and cartilage from early stages of development suggests that these tissues may be coordinated and interdependent during induction, differentiation and growth of the musculoskeletal system.

In the developing embryo, the induction of axial tendons in the tenogenic compartment, the syndetome, was shown to depend on signals emanating from the adjacent myotome, as disruption of myotome formation in chick and mouse embryos resulted in failure of tendon progenitor induction (Brent et al., 2005; Brent and Tabin, 2004; Chen and Galloway, 2014). In contrast, induction of cranial tendon progenitors was not disrupted in the absence of muscle, but subsequent differentiation of cranial tendons failed in muscle-less mutants (Chen and Galloway, 2014; Grenier et al., 2009; Grifone et al., 2008).

Studies of interdependence in the musculoskeletal system of developing limbs uncovered an additional level of complexity during tendon development. While early chick experiments concluded that muscle and tendon formed autonomously in the limb (Brand et al., 1985; Chevallier et al., 1977; Shellswell and Wolpert, 1977), a later more comprehensive study identified surprising divergence in the development of distal and proximal limb tendons (Kardon, 1998). Like cranial tendons, induction of limb tendon progenitors was not dependent on muscle. However, while, tendon differentiation was not perturbed in the

autopod of muscle-less limbs, there was an early loss of zeugopod tendons, and no differentiated tendons were ever observed in the zeugopod of muscle-less limbs (Kardon, 1998). The muscle dependency of limb tendons was also evaluated in the mouse using the *Splotch* (*Sp*) mutant, in which disruption of the *Pax3* gene results in failure of myoblast migration, thus generating muscle-less limbs (Bober et al., 1994). Similar to chick embryos, in muscle-less, *Sp* embryos, limb tendon progenitor induction was not affected but tendon progenitors subsequently failed to differentiate (Bonnin et al., 2005; Schweitzer et al., 2001). It was therefore suggested that mouse tendon development can be divided into two stages: a progenitor, muscle-independent stage followed by a differentiated, muscle-dependent stage (Bonnin et al., 2005; Tozer and Duprez, 2005). However, early lethality of *Sp* mutant embryos restricted the scope of this analysis, underscoring a need for analysis of tendon development in other mutants in which limb muscle development is perturbed.

To date, much of the work on interdependency during musculoskeletal development has focused on the effects of muscle loss, and cartilage dependency in tendon development has received considerably less attention. A few studies have suggested that the tendon and cartilage cell fates represent alternative lineages, derived from a common tendo-chondro progenitor population and that inhibition of chondrogenesis may drive these progenitors toward a tendon cell fate (Brent et al., 2003; Brent et al., 2005; Lorda-Diez et al., 2009). This assertion was also supported by recent data showing that the tendon and cartilage cells comprising the entheses, the skeletal insertions of tendons, are derived from a pool of *Sox9* and *Scx* co-expressing progenitors that then differentiate toward their respective lineages (Blitz et al., 2013; Sugimoto et al., 2013). There is however, also some evidence that cartilage may play a direct role in tendon induction. An elegant experiment in chick demonstrated that induction of an ectopic digit by removal of interdigital ectoderm resulted in formation of accompanying tendons (Hurle et al., 1989).

To address the open questions in the roles of muscle and skeleton in limb tendon development, we analyzed the tendon phenotype of mouse mutants that affect muscle or cartilage development. We find that limb tendons are regulated in a modular fashion, with separate and independent developmental programs for the zeugopod (arm) and autopod (paw) tendon segments, which depend on signals from muscle and cartilage, respectively. Collectively, these results establish a foundation for an integrated model of limb tendon induction, differentiation and growth.

RESULTS

Differentiation and maintenance of autopod tendon segments is independent of muscle

Movement of the mouse paw is largely controlled by extrinsic muscles that reside in the zeugopod. Muscle forces are transmitted by long extensor and flexor tendons that extend across the wrist and insert in the autopod (Fig. 1A) (Watson et al., 2009). To test the requirement for muscle in limb tendon formation, we used *Splotch delayed* (Sp^d) mice (Vogan et al., 1993). In contrast with embryonic lethality before E14.5 of *Pax3* loss of function mutations (*Sp*) that were used in previous studies of muscle dependency for tendons (Bober et al., 1994), Sp^d is a point mutation in *Pax3* that also disrupts myoblast migration but mutant embryos are viable through the end of embryogenesis (Vogan et al., 1993). Using the *Scleraxis-GFP* (*ScxGFP*) tendon reporter (Pryce et al., 2007) to visualize tendons, we could not identify any tendons within the zeugopod of Sp^d limbs at E16.5, but found distinct tendons in the autopod, the most prominent of which were the major tendons that insert at the distal digits, the extensor digitorum communis (EDC) and flexor digitorum profundus (FDP) tendons (Fig. 1A-C).

To determine whether other tendons were also present in the autopod of Sp^d embryos, we analyzed transverse limb sections from E16.5 embryos. In sections through the wrist, the tendon pattern in the Sp^d limb was considerably different from that of wild type (WT) (Fig. 1D, E), but systematic analysis of serial sections through the autopod facilitated identification of individual tendons in the Sp^d mutant. In addition to the EDC and FDP, other extensor tendons were identified within the autopod, including the extensor carpi radialis longus /brevis (EL/B) and the extensor digiti quarti/quinti (EQ) (Fig. S1). However, the tendons that insert at or near the wrist, such as the flexor carpi ulnaris, were completely absent in Sp^d embryos. Surprisingly, a close inspection of the zeugopod in whole mount and transverse sections of Sp^d limbs also revealed thin dorsal and ventral trails of residual *ScxGFP* positive cells that extended from the wrist to the elbow (Fig. 1C). The pattern and organization of these cells was uncharacteristic of any tendon and likely represents residual progenitors that failed to differentiate in Sp^d limbs.

The distinctive appearance of autopod tendon segments in Sp^d embryos led us to examine whether these tendons persisted through embryogenesis, since in muscle-less chick limbs the distal tendons eventually degenerate (Kardon, 1998). We found however, that autopod tendon segments in Sp^d mutants not only persisted to the end of embryogenesis (E18.5), but generated an aligned collagen matrix indistinguishable at the ultrastructural level from that of WT embryos (Fig. 1N-O').

Muscle activity is required for individuation and robustness of limb tendons

While autopod tendon segments were formed in Sp^d embryos, tendon size was dramatically smaller than that of WT. Analysis of transverse sections through the autopod further demonstrated that several tendons were fused near the wrist, notably the EDC tendons, the EL/B tendons, and the EQ tendons (Fig. 1D, E, S1). To distinguish a direct requirement for

the presence of muscle from an indirect requirement for muscle forces in regulating tendon size and pattern, we examined tendon development in the absence of muscle activity. *Muscular dysgenesis (mdg)* is a spontaneous mutation in a voltage-gated calcium channel resulting in the loss of muscle excitation-contraction coupling and therefore muscle paralysis (Pai, 1965a; Pai, 1965b). Whole mount images and transverse sections through autopod levels revealed noticeably smaller tendons in *mdg* mutants compared to WT at E16.5 (Fig. 1 F-I), though not to the same extent as in *Sp^d* mutants, suggesting that muscle contraction plays a critical role in regulation of tendon growth, but the physical presence of muscle likely provides additional independent signals that regulate tendon size.

Unlike the *Sp^d* mutants, tendon formation in the zeugopod was not perturbed in *mdg* embryos (Fig. 1F-I). However, similar to *Sp^d* embryos, we identified tendon fusion in *mdg*, including fusion of the EDC tendons as well as the EL/B tendons, and the EQ tendons. Interestingly, these tendons were fused only near the wrist; in more proximal sections of *mdg* limbs, the fused tendons separated and tendon pattern was similar to WT (not shown). To determine the relationship between muscle activity and tendon fusion, we examined WT limbs at the onset of muscle activity, and found that the same tendons were fused at the wrist of E14.5 WT embryos (Fig. 1M, S2). These observations suggest that normal development of these extensor tendons (EDC, EL/B, and the EQ) consists of initial formation of a fused tendon anlage near the wrist, and that individuation of these anlagen into distinct tendons depends on muscle activity. Notably, TUNEL assays in WT embryos identified localized cell death at the EDC anlage at E13.5 and E14.5, underscoring the separation of the fused EDC anlage at the wrist into individuated EDC tendons by E15.5 (Fig. 1K, L), but cell death was not observed during the individuation of the other fused tendons. These results demonstrate that muscle contraction regulates some aspects of tendon size and patterning, but it is not essential for tendon formation or the continued maintenance and differentiation of tendon progenitors.

Tendon induction in the autopod is dependent on skeletal cues

The observations in muscle-less limbs suggest that development of the autopod and zeugopod segments of limb tendons are regulated by distinct and independent developmental programs. While we found that tendon segments within the autopod develop and persist in the absence of muscle, the signals that drive their development remain largely unknown. However, the induction of tendon progenitors along the forming cartilage condensations and a few published observations (Hurle et al., 1990; Yamamoto-Shiraishi and Kuroiwa, 2013) suggest a possible role for cartilage in autopod tendon induction. In the limb, cartilage specification requires the activity of the transcription factor *Sox9* (Akiyama et al., 2002), and subsequent differentiation of the cartilaginous anlagen depends on the activities of *Sox5* and *Sox6* (Smits et al., 2001). To examine the role of cartilage in limb tendon development, we therefore analyzed tendon development in mutants for these genes.

In *Sox5*^{-/-};*Sox6*^{-/-} double mutants in which cartilage condensations failed to differentiate (Smits et al., 2001), the majority of limb tendons appeared normal (Fig. S3). Conversely, tendon formation was severely affected in conditional *Sox9*^{ff};*Prx1*^{Cre} mutants, in which *Sox9* expression is eliminated in limb bud mesenchyme, resulting in early failure of mesenchymal condensations and the development of ‘skeletal-less’ limbs (Akiyama et al., 2002). *Scx* expression was completely absent from the autopod of *Sox9*^{ff};*Prx1*^{Cre} embryos at E13.5, but *Scx* expressing tendon structures could be detected in the zeugopod of these embryos (Fig. 2A, B).

Since apoptosis is very broad in the autopod mesenchyme of *Sox9*^{ff};*Prx1*^{Cre} mutant embryos beginning at E13.5 (Akiyama et al., 2002), we examined *Scx* expression at earlier stages. At E12.5, as the WT autopod was beginning to form distal digit protrusions, the *Sox9*^{ff};*Prx1*^{Cre}

mutant autopod appeared as a smooth paddle, indicating an early arrest of autopod development (Fig. 2C, D). While the pattern of *Scx* expression was normal in the stylopod and the zeugopod of mutant embryos, autopod expression was limited to a broad patch near the presumptive wrist (Fig. 2D). By E13.5, *Scx* expression was lost in the wrist and carpal levels of *Sox9^{ff};Prx1^{Cre}* mice, as the autopod failed to develop further. In contrast, the formation of zeugopod tendon segments was not disrupted in the same mutant limbs and the tendon pattern was similar to the tendons in WT limbs. Transverse sections taken through WT and mutant limbs in which the tendons were highlighted by *ScxGFP* and the muscles by MHC immunostaining showed a striking similarity between the individual tendon and muscle groups of WT and mutant embryos, suggesting that soft tissue differentiation and patterning at zeugopod levels were not dependent on skeletal signals (Fig. 2E, F).

To further test whether induction and differentiation of zeugopod tendons is indeed independent from tendon development in the autopod, we examined other mutants in which autopod development is selectively affected. It was previously shown that autopod development depends on the combined activities of the *Hoxa13* and *Hoxd13* genes (Fromental-Ramain et al., 1996). In a slightly milder allele combination of *Hoxa13^{+/-}; Hoxd13^{-/-}* mutant embryos, in which autopod development is also largely arrested, we found that the autopod segments of limb tendons again failed to form, while zeugopod segments of the same tendons developed normally (Fig. S4).

The failure of *Scx* induction in the autopod of skeletal-less limb buds suggested that prechondrogenic condensations may play a role in autopod tendon development; however, it was also possible that the effect on *Scx* expression was simply due to the severe disruption of autopod mesenchyme in the mutant embryos. We therefore examined the effect of a milder disruption of autopod chondrogenesis on tendon induction. Conditional targeting of *BMP2&4* in limb mesenchyme in *BMP2^{f/+};BMP4^{ff};Prx1^{Cre}* compound mutants results in loss of

posterior digits in the forelimb due to a posterior expansion of the limb bud and a failure of digit specification (Bandyopadhyay et al. 2006). Consistent with our previous observations, the loss of the posterior digits was accompanied by loss of their associated tendons (Fig. 2G), again highlighting the coupling between digit cartilage and tendon progenitor induction in the autopod.

To test whether cartilage was not only necessary but also sufficient for tendon induction, we next evaluated if formation of extra digits in mouse embryos would be accompanied by induction of an associated tendon, as was previously shown in chick embryonic experiments (Hurle et al., 1990). In the developing limb, *Gli3* functions as a key regulator of digit formation and loss of *Gli3* results in an array of extra digits (te Welscher et al., 2002; Vortkamp et al., 1992). Consistent with the results presented above, whole mount in situ hybridization for *Scx* expression revealed distinct, flexor and extensor tendons connected to each of the supernumerary digits in *Gli3^{Xt/Xt}* mutant limbs at E13.5 (Fig. 2H).

Collectively, these data indicate that cartilage condensations are both necessary and sufficient for induction of autopod tendons. Moreover, when combined with the results from muscle-less limbs, these results suggest a striking modularity in the formation of limb tendons. In skeletal-less limbs, tendons develop in the zeugopod despite the absence of their distal autopod segments. Conversely, in muscle-less limbs, the autopod segments of tendons were formed, but the zeugopod segments of these same tendons were missing and tendons that reside completely within the zeugopod and insert at the wrist were also completely missing (Fig. 1, S1). These results suggest a modular construction of limb tendons whereby independent developmental programs for the autopod and zeugopod tendon segments are combined to form contiguous functional entities.

Proliferation and lineage tracing reflect separate regulation of autopod and zeugopod tendon development

The distinct nature of the genetic programs that direct tendon formation in the autopod and zeugopod was also apparent when we analyzed proliferation and cell lineage in developing tendons. Using BrdU incorporation to label dividing cells in *ScxGFP* embryos, we evaluated proliferation in developing tendons between E12.5 and E15.5. While we observed extensive proliferation in all *ScxGFP* positive regions throughout the limb at E12.5, tendon progenitors in the autopod stopped proliferating by E13.5 and remained largely quiescent up to E15.5 (Fig. 3, S5). In contrast, tendon cells in the zeugopod showed high levels of proliferation through all stages of limb development (Fig. 3, S5). Notably, this difference in autopod and zeugopod tendon proliferation was consistent for all of the long tendons, so that within a single continuous tendon, such as the EDC and FDP, the autopod segments were non-proliferative at E14.5 while the zeugopod segments of the same tendons were highly proliferative.

Additional support for separate autopod and zeugopod tendon programs emerged from efforts to trace cell lineage in limb tendons, most distinctly when we evaluated the fate of progenitors that express the *Six2* homeodomain transcription factor. *Six2* was one of the first transcription factors shown to be expressed in developing tendons and it was subsequently implicated in limb muscle and tendon development (Laclef et al., 2003; Oliver et al., 1995; Yamamoto-Shiraishi and Kuroiwa, 2013). *Six2* expression is first detected in the developing autopod in a pattern that is very similar to that of *Scx*. However, in contrast with *Scx* expression, which highlights all tendon progenitors (Fig. 4A-E), in situ hybridization of transverse sections taken through the autopod showed *Six2* expression at E13.5 in distinct tendons and muscles, with strong expression in the FDP tendons, as well as the muscle progenitors comprising the flexor digitorum superficialis (FDS). While *Six2* expression was

also detected in the digit segments of the EDC tendons, *Six2* expression in EDC tendons was hardly detectable at the carpal level whereas it was still robust in the FDP tendons.

Because of the distinctive expression of *Six2* in FDP tendons, we used the inducible *Six2^{CreERT2}* deleter combined with the *Rosa-TdTomato* Cre reporter to trace the lineage of FDP tendon cells (Kobayashi et al., 2008; Madisen et al., 2010a). Pregnant *Six2^{CreERT2}* females were injected with tamoxifen at E12.5, labeling early *Six2* expressing cells, and the fate of *Six2* lineage cells was evaluated at E16.5. Cross sections through the forelimbs of these embryos showed a sharp demarcation in cell lineage. While the autopod segments of the FDP were strongly labeled and thus derived from the early *Six2* expressing cells, the zeugopod segments were mostly unlabeled (Fig. 4F-H). These results demonstrate that the autopod and zeugopod segments of the FDP are formed by different progenitor pools, again highlighting the distinctive processes that regulate tendon formation in these two limb compartments.

Integration of the autopod and zeugopod developmental programs of limb tendons

While we established that tendon development is modular and autopod or zeugopod tendon segments can develop in the absence of the other, normal tendon development does not involve independent development and fusion of two separate segments. To determine how the independent programs for tendon development in the autopod and zeugopod are integrated, we re-examined the initial events in limb tendon development. In WT embryos, *Scx* expression is first detected early in limb bud development at E10.5, but a recognizable pattern reflecting tendon organization emerges only at E12.5. By E13.5, the loosely organized progenitors condense to form well-delineated functional tendons (Schweitzer 2001, Murchison et al, Pryce et. al and Fig. 5A-F). Consistent with previous studies, we found that

Scx induction in the forming autopod and zeugopod of *Sp^d* muscle-less embryos was not significantly affected up to E12.5 (Bonnin et al., 2005; Schweitzer et al., 2001), however, differentiation and continued maintenance of the zeugopod tendon progenitors was impaired in the absence of muscles, leading to complete loss of tendons and tendon progenitors from the zeugopod by E13.5 (Fig. 5G, H and (Bonnin et al., 2005)).

These observations prompted us to examine the events surrounding the organization of the forming musculoskeletal system in these early stages. Interestingly, combined detection of tendon, muscle and cartilage progenitors (visualized by *Scx* in situ, and MHC and collagen type II immunostaining, respectively) revealed that at E12.5, loosely packed tendon progenitors were already positioned between the forming muscles and cartilage (Fig. 5E, F), similar to previous results from the chick limb ((Hurle et al., 1990; Kardon, 1998; Ros et al., 1995). The alignment and integration of the musculoskeletal system thus appears to begin at E12.5, under the influence of limb bud patterning cues even before the condensation of tendon progenitors to form load-bearing tendon structures at E13.5.

To determine the spatial relationships between the musculoskeletal tissues in these early stages, we next examined these tissues in whole mount preparations, using *Col2GFP* and *ScxGFP* limbs with muscles visualized by MHC staining or by Pax7Cre. Interestingly, we found that at E12.5, the muscles spanned the entire length of the short zeugopod skeleton and were attached to tendon progenitors induced in the autopod via short tendon segments near the forming wrist (Fig. 5I, arrows). Long tendons within the zeugopod were first apparent only at E13.5, and tendon elongation continued in later stages concurrent with zeugopod skeletal growth.

The tendon anlagen at the wrist therefore represent a distinct population of progenitors that plays a central role in the integration of the musculoskeletal system by connecting the autopod

tendon segments with their corresponding muscles in the zeugopod (Fig. 5). While the wrist progenitors appear contiguous with the autopod tendons already at E12.5, their unique nature is highlighted by the fact that unlike autopod progenitors, induction of these wrist tendon anlagen is independent of both skeleton and muscle since wrist progenitors can be observed at E12.5 in both ‘skeletal-less’ and ‘muscle-less’ mutants (Brackets in Figs. 5 and 2, respectively). These wrist elements are initially induced as blocks of tendon progenitors that connect groups of autopod tendons to muscles. For example, whole mount ScxGFP limbs at E12.5 showed a broad triangular patch of progenitors comprising the future EDC tendons (Fig 5I, S2), connecting directly to muscle. Similarly, fused wrist anlagen for the EL/B and the EQ tendons were also observed (Fig. 5I). These tendons elongate as individual tendons into the zeugopod but the fused elements at the wrist are subsequently separated only at E15.5 depending on muscle activity (Fig 1, S2).

These observations suggest a new model for musculoskeletal integration that combines the distinct programs for autopod and zeugopod tendon development and suggests that zeugopod tendon development occurs in two stages: At E12.5, the muscles that span the zeugopod anchor to autopod induced tendons via tendon anlage whose induction is independent of muscle. Subsequently, these tendons elongate from the wrist into the zeugopod, in tandem with zeugopod skeletal growth. The long tendons are thus formed as continuous structures from two progenitor populations and the musculoskeletal system remains connected through this process.

Development of FDS tendons is also modular with a demarcation at the MCP joint

The developmental modularity we observed for limb tendons identified the wrist as a critical integration site for limb tendon development, with one exception - the FDS tendons. Like the FDP and EDC tendons, the FDS tendons extend from skeletal insertions at the interphalangeal joints to muscles in the zeugopod (Fig. 6A), but the FDS undergoes a unique

development program. FDS muscles differentiate in the autopod and starting at E14.5 translocate out of the autopod to achieve their final position in the zeugopod by E16.5 (Huang et al., 2013). While most limb tendons are already formed by E14.5, the FDS tendons at that stage consist only of a narrow flattened band attached to the distal end of FDS muscles and ‘cupping’ the FDP tendons near the metacarpophalangeal (MP) joint, (Fig. 6A) (Huang et al., 2013; Watson et al., 2009) Between E14.5 and E16.5, the FDS tendons grow both distally and proximally in tandem with muscle translocation, to form the digit and metacarpal/zeugopod segments of the tendon, respectively (Fig. 6A). Having identified modularity as a key feature in the development of limb tendons we next examined whether the FDS tendons also follow a similar developmental program.

The bidirectional growth of FDS tendons from the initial ‘cup’ anlage suggested a possible demarcation point for this tendon at the MP joint. Analyzing the cell lineage labeled by the *Sox9^{Cre}* deleter, which labels all cartilage cells as well as a subset of tendon cells (Blitz et al., 2013; Soeda et al., 2010; Sugimoto et al., 2013), we indeed identified a cell lineage boundary at the MP level of the FDS tendons. While the digit segments were largely composed of *Sox9* lineage cells, the metacarpal segments of FDS tendons were nearly devoid of these cells (Fig. 6B, C). The digit and metacarpal segments of the FDS tendons thus do not share a common cellular origin.

We previously showed that the metacarpal FDS segments are formed as the FDS muscles translocate from the paw (Huang et al., 2013). In cases where FDS muscle translocation was arrested (e.g. in paralyzed *mdg* mutant embryos), FDS tendon development was similarly arrested, suggesting a dependence on muscle. To investigate FDS tendon muscle dependency, we again examined the ‘muscle-less’ *Sp^d* mutant. Transverse sections at E16.5 revealed that while autopod FDP tendon segments were normal, the FDS tendons were completely absent in sections through the metacarpals (Fig. 7A-D). Notably, induction of

digit segments of the FDS tendons was not impaired in the absence of muscle, although these tendons were significantly smaller from those of WT embryos, suggesting that while induction of the digit segments of FDS tendons was not dependent on muscle, robustness of these tendons likely depends on connectivity and application of contractile forces. This observation was reinforced by analysis of FDS tendons in paralyzed *mdg* embryos and in *Scx*^{-/-} embryos, in which the FDS muscles are not connected to the digit tendon segments (Huang et al., 2013) and the FDS digit tendon segments were much reduced in size, though correctly patterned (Fig. 7)

The dependence of proximal but not distal segments of FDS tendons on muscle suggested an inherent similarity with the modular construction of other limb tendons. To test a possible requirement for cartilage, we examined FDS tendon development in the absence of critical genes that govern later aspects of skeletal development, since the severe autopod phenotype in *Sox9* mutants precludes analysis of the FDS tendons (Fig. 5) (Akiyama et al., 2002). In *Runx2*^{-/-} mutant embryos, in which bone formation is disrupted (Ducy et al., 1997; Komori et al., 1997; Otto et al., 1997), FDS tendons appeared normal (not shown). Conversely, *Sox5*^{-/-}/*Sox6*^{-/-} double null mutants, in which cartilage differentiation is disrupted (Smits et al., 2001) displayed modularity of the FDS tendons; in transverse sections of *Sox5*^{-/-}/*Sox6*^{-/-} E16.5 forelimbs, the digit segments of the FDS tendons were completely missing, but the metacarpal and zeugopod segments of the FDS tendons were normal. (Fig. S3). Surprisingly, Indian hedgehog null embryos (*Ihh*^{-/-}) in which endochondral ossification is impaired (St-Jacques et al., 1999; Vortkamp et al., 1996) harbored an identical FDS tendon phenotype, despite the very different skeletal phenotype (Fig. 7).

Taken together, these results demonstrate that development of the FDS tendons is also modular, with a developmental boundary at the MP joint instead of at the wrist. Induction of the distal (digit) tendon segment depends on a signal from the cartilage whereas development

of the proximal (metacarpal and zeugopod) segment depends on the interaction with muscle. We therefore conclude that while certain features of FDS tendon development are unique, the key aspects of modular development with distinct dependence on muscle and cartilage cues are universal for all limb tendons.

DISCUSSION

This study elucidates the principles that govern limb tendon development, establishing a novel conceptual framework for analysis of limb tendon phenotypes. We found that the development of the long autopod tendons is strikingly modular, in that independent programs regulate formation of the autopod and zeugopod segments of each tendon. We also identified a unique population of wrist tendon progenitors that mediate musculoskeletal integration by connecting the muscles in the zeugopod to the tendon progenitors induced at the autopod. While autopod tendon segments are formed by cartilage-dependent induction of tendon progenitors, the zeugopod segments are formed in two stages (Fig. 8): Integration occurs at E12.5 when differentiating muscles that span the length of the very short zeugopod connect via tendon progenitor anlagen at the wrist to newly induced tendon progenitors in the autopod. In the second stage of Elongation, muscle-dependent elongation of tendons occurs from the wrist anlagen, in tandem with zeugopod skeletal growth. While tendon elongation into the zeugopod occurs with an individual tendon for each muscle, the tendons are initially fused at wrist level, and complete individuation of the tendons is dependent on muscle activity and manifests only by E15.5.

Wrist tendon progenitor anlage coordinate musculoskeletal integration

Tendons function as contiguous structures that transmit the forces generated by muscle contraction from early embryonic stages. Integration of the system begins at E12.5, and structural continuity from zeugopod to autopod is enabled by the wrist tendon anlagen. Unlike autopod tendon progenitors, induction of the wrist anlagen is not dependent on cartilage or muscle; they function as short-range connectors between the autopod tendon elements and their respective muscles. However, nothing is known about the molecular and cellular mechanisms that mediate these events.

Autonomous induction of tendon progenitors that is not dependent on muscle was previously demonstrated in a number of studies (Bonnin et al., 2005; Edom-Vovard et al., 2002; Kardon, 1998; Schweitzer et al., 2001). Consistent with these studies, we showed that wrist tendon anlagen were induced in the absence of muscle but subsequently degenerated. The signals that regulate the initial induction of wrist tendon progenitors remain unknown, but it is likely that TGF β signaling may be involved in this process. We previously found that TGF β signaling is a potent inducer of tendon progenitors and loss of TGF β signaling resulted in complete loss of tendons (Pryce et al., 2009). Tendon loss is first manifested at E12.5 and wrist tendon anlagen are never detected in these mutants. It is therefore possible that induction of the wrist tendon anlagen is dependent on TGF β signaling. Interestingly, it was recently demonstrated that induction of a progenitor population for the cartilage and tendon components of the entheses depends on TGF β signaling (Blitz et al., 2013). The wrist tendon anlagen may therefore represent a unique version of such a progenitor population that, instead of mediating connection of muscle to cartilage mediates attachment of the muscles to the autopod tendon segments.

Multiple roles for muscle and muscle activity in limb tendon development

Surprisingly, autopod tendon progenitors for the long tendons were not only maintained in *Sp^d* mutants, but differentiated and persisted through the end of embryogenesis, unlike the previous reports of tendon development in muscle-less limbs in chick (Kardon, 1998). The autopod tendons that developed in ‘muscle-less’ embryos were however dramatically smaller than those of WT, indicating that muscle forces may play a role in regulating tendon size during embryogenesis. Indeed, analysis of paralyzed embryos revealed thinner tendons at all levels of the limb. Muscles thus play a dual role in tendon development. On the one hand, muscle activity is required for lateral tendon growth, but on the other hand, the very presence of muscle is required for maintenance of the wrist tendon anlagen, and for tendon elongation into the zeugopod from wrist progenitors, since zeugopod tendons were formed in paralyzed mutants but missing in muscle-less mutants. The specific role of the muscles in these processes is yet to be determined; the muscles may simply function as anchoring elements that allow the connected tendon progenitors to experience the mechanical strain imparted by skeletal growth. However, the requirement for muscle may also be molecular or cellular in nature; elucidating the parameters that regulate tendon growth will therefore be the focus of future studies.

Cartilage is necessary and sufficient for induction of autopod tendon progenitors

In recent years, a number of studies have posited the existence of a chondro-tendo progenitor population (co-expressing *Scx* and *Sox9*) that gives rise to either chondrocytes or tenocytes during early development (Blitz et al., 2013; Sugimoto et al., 2013). The failure of cartilage differentiation in *Sox5^{-/-};Sox6^{-/-}* double mutants has also been associated with an expansion of axial tendon progenitors, suggesting a conversion of chondroprogenitors to a tenogenic cell

fate in the absence of these cartilage-specific transcription factors (Brent et al., 2005). It was therefore surprising that we did not find a comparable expansion of limb tendons or *Scx*-expressing tendon progenitors in response to the disruption of chondrocyte differentiation in *Sox9^{Prx1cre}*, *Sox5^{-/-};Sox6^{-/-}*, *BMP2^{f/+};BMP4^{ff};Prx1^{Cre}* or *Ihh^{-/-}* mutants. Conversely, we found that induction of autopod tendons was entirely dependent on cartilage, and tendon progenitors were not induced in the absence of cartilage in either *Sox9^{Prx1cre}* mutants or *BMP2^{f/+};BMP4^{ff};Prx1^{Cre}* double mutants, while tendons were induced in all of the supernumerary digits in *Gli3^{Xt/Xt}* mutant limbs.

These results suggest that in the autopod, cartilage may be the source of inductive factors for tendon progenitors. We previously showed that the condensing autopod cartilage expresses TGFβ2 ligand and that TGFβ signaling is a potent inducer of *Scx*, suggesting a model whereby TGFβs secreted from cartilage may induce tendon progenitors (Pryce et al., 2009). However, in the E12.5 autopod, early *Scx* expression is only detectable in subectodermal mesenchyme, and *Scx* is not expressed by mesenchymal cells closer to the cartilage condensation. An alternative model was recently suggested based on evidence that *Scx* expression can be induced by Wnt signaling in the autopod (Yamamoto-Shiraishi and Kuroiwa, 2013) and that BMP signaling is antagonistic to *Scx* expression (Schweitzer et al., 2001). In this context, *Scx* may be broadly induced by ectodermal Wnts and the restriction of *Scx* expression to the regions near the autopod cartilage condensations may be achieved by interdigital BMP expression (Knosp et al., 2004; Zou and Niswander, 1996). Since the cartilage condensations also express the BMP antagonist Noggin, the role of cartilage in this process may be maintenance of *Scx* expression by local repression of BMP signaling (Yamamoto-Shiraishi and Kuroiwa, 2013).

The unique development of FDS tendons highlights the general aspects of tendon modularity

The FDS tendon is a useful model for tendon modularity since its development is delayed relative to other tendons and there are genetic perturbations that only affect this specific tendon. Consistent with other limb tendons however, formation of the distal and proximal FDS segments is modular and each segment can differentiate in the absence of the other.

In a previous study, we found that proximal FDS tendon formation was tightly coupled to FDS muscle translocation. In mutants where FDS muscle translocation was partially arrested, proximal FDS tendon growth was like-wise arrested, such that the extent of muscle translocation dictated the extent of FDS tendon elongation (Huang et al., 2013). While active FDS muscle translocation is a unique process specific to this muscle, the mechanical signal imparted to the FDS tendon anlage is likely analogous to the mechanical signals imparted to the wrist tendon anlagen of the other limb tendons as the zeugopod skeleton elongates, further supporting our hypothesis that the main function of muscle in the process of tendon elongation is as a stable anchoring element.

Likewise, the requirement for skeletal signals in distal tendon induction was conserved in FDS tendon development. In *Sox5^{-/-};Sox6^{-/-}* and *Ihh^{-/-}* mutants, the digit segment of the FDS tendon was never induced. Since other tendons were largely unaffected while skeletal development was severely affected, these results suggest that *Ihh* and *Sox5/6* are not playing direct molecular roles in tendon differentiation, but that secondary signals from the skeleton may regulate FDS tendon development. Interestingly, while the skeletal phenotypes of these mutants are significantly different, a phenotypic feature common to both mutants is the loss of all phalangeal joints (Dy et al., 2010; St-Jacques et al., 1999). The possibility that induction of the FDS digit segment is dependent on cues from the forming joint is further supported by the fact that formation of the MP joint is concurrent with the initial stages of FDS digit tendon induction (Li et al., 2010). Moreover, the bilateral symmetry of the MP

joint also mirrors the bifurcated structure of the FDS digit tendons, suggesting that the cues governing joint symmetry may also regulate FDS digit tendon induction.

Tendon modularity: evolutionary perspective

The independent and modular development of autopod and zeugopod tendon segments is intriguing and likely reflects the evolutionary history of these tendons. In mammals, muscles are largely restricted to a single limb segment and do not cross the wrist or elbow (Diogo et al., 2009). In amphibians however, extrinsic muscles of the hand extend across the wrist and connect to tendons that are all contained within the autopod (Ashley-Ross, 1992; Diogo et al., 2009; Walthall and Ashley-Ross, 2006). The basic mechanisms that regulate autopod tendon formation are therefore likely conserved. For example, in regenerating newt limbs, tendons are formed in the autopod even when the regenerating limb does not include muscles (Holder, 1989), similar to the muscle-independent development of autopod tendon segments in the mouse. Since amphibians possess mostly autopod tendons, the mechanism of muscle-dependent tendon elongation which is characteristic of the zeugopod tendon segments, is either not significantly utilized in amphibians or may be a later evolutionary addition.

The absence of significant tendon elongation in amphibians is further emphasized by the differences in tail anatomy between mammals and amphibians. While the mouse tail contains a large number of long tendons that traverse large distances between muscles at the base of the tail and skeletal insertions in each of the tail vertebra (Shinohara, 1999) amphibian tails are largely muscular, connected to short local tendons. Notably, long tendons that extend across limb and tail segments can be found both in birds and lizards (Kardon, 1998; Proctor and Lynch, 1993; Zippel et al., 1999), suggesting that this simple mechanism, in which soft tissue growth is dictated by skeletal growth exists in most tetrapod clads.

The ability of tendons to accommodate skeletal growth by elongation provides an elegant solution for coordinated growth of musculoskeletal tissues. This simple rule of musculoskeletal growth implies that the dramatic changes in body size during growth of the individual or changes in length of individual bones through evolutionary mechanisms do not require coordinated modifications in the developmental programs of all musculoskeletal tissues. Instead, a change in skeletal growth would indirectly co-opt the soft tissues, and thus ensure coordinated growth. The implied simplicity of morphological change may be fundamental to the remarkable diversity of size and form in terrestrial vertebrates.

METHODS

Mice

Existing mouse lines used in these studies were previously described: *ScxGFP* tendon reporter (Pryce et al., 2007), *Col2GFP* cartilage reporter (Grant et al., 2000), *Six2^{CreERT2}* (Kobayashi et al., 2008), *Sp^d* (Vogan et al., 1993), *mdg* (Pai, 1965a; Pai, 1965b), *Sox9^{ff}* (Akiyama et al., 2002), *Prx1^{Cre}* (Logan et al., 2002), *Gli3^{Xt/Xt}* (Vortkamp et al., 1992), *Sox9^{Cre}* (Akiyama et al., 2005), *Ihh^{-/-}* (St-Jacques et al., 1999), *Sox5^{-/-}*; *Sox6^{-/-}* (Lefebvre et al., 2001), *Hoxa13^{+/-}*; *Hoxd13^{-/-}* (Davis and Capecchi, 1996; Stadler et al., 2001), *BMP2^{ff/+}*; *BMP4^{ff}* (Bandyopadhyay et al., 2006; Selever et al., 2004), *Scx^{-/-}* (Murchison et al., 2007), *Ai14 Rosa26-TdTomato* reporter (*RosaT*) (Madisen et al., 2010b).

Histology

Standard protocols for whole mount and section in situ hybridization, immunostaining, BrdU and TUNEL labeling were performed as previously described (Murchison et al., 2007).

Patterning of limb tendons and muscles was acquired in serial transverse sections (Watson et al., 2009). A monoclonal antibody for My32 (Sigma), was used to detect muscle-specific type II myosin heavy chain (MHC).

Transmission Electron Microscopy

E18.5 mouse limbs were fixed in 1.5% glutaraldehyde/1.5% paraformaldehyde (Tousimis Research Corporation, Rockville, MD) in Dulbecco's serum-free media (SFM) containing 0.05% tannic acid, followed by an extensive rinse in SFM, then post-fixation in 1% OsO₄. Samples were washed in SFM then dehydrated in a graded series of ethanol to 100%, rinsed in propylene oxide, and infiltrated in Spurr's epoxy. Samples were polymerized at 70°C over 18 hours.

Whole Mount Confocal Microscopy

Mouse forelimbs from E12.5-E14.5 were fixed in 4% paraformaldehyde overnight at 4°C. Following fixation, whole mount immunostaining for MHC was carried out for E12.5 and E13.5 limbs as previously described (DeLaurier et al., 2006). Limbs were then incubated in Sca/e2 solution at 4°C until cleared (Hama et al., 2011). The Zeiss LSM780 laser scanning confocal microscope was used to acquire 10x tiled z-stack images. Image processing was carried out using Zeiss Zen software to stitch tiles and obtain maximum intensity projection images.

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AUTHOR CONTRIBUTIONS

AHH, TJR, BP, JW, SW contributed to experiments and data collection. AHH and RS contributed to conception, data analysis and interpretation. ST and DK contributed to TEM data collection and analysis. FL, VL, BH, HSS, HA contributed mutants embryos generated by their labs for the project. AHH and RS prepared and edited the manuscript prior to submission.

FIGURES

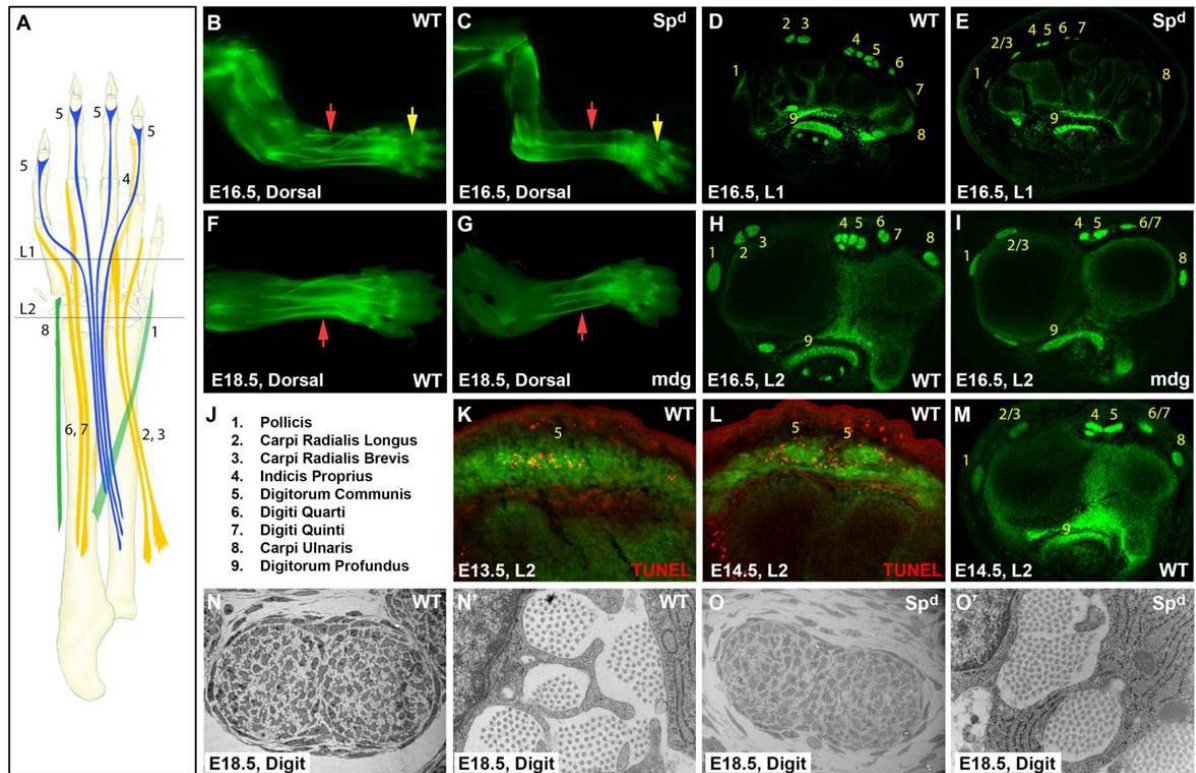


Figure 1: Developmental modularity of limb tendons is revealed by muscle-independent autopod tendon development and muscle-dependent zeugopod tendon development. (A) Schematic of long extensor tendons from their muscle origins to skeletal insertions. (B, C) Whole mount and (D, E) transverse section images of *ScxGFP* WT and *Sp^d* limbs at E16.5. (F, G) Whole mount and (H, I) transverse sections of *mdg* limbs at E16.5. (J) Table of numerical tendon assignments. (K, L) TUNEL staining of WT EDC tendon near the carpals at E13.5 and E14.5. (M) *ScxGFP* WT limb at E14.5. (N-O') TEM of WT and *Sp^d* FDP tendon at digit level. Yellow and red arrows highlight autopod and zeugopod tendons, respectively.

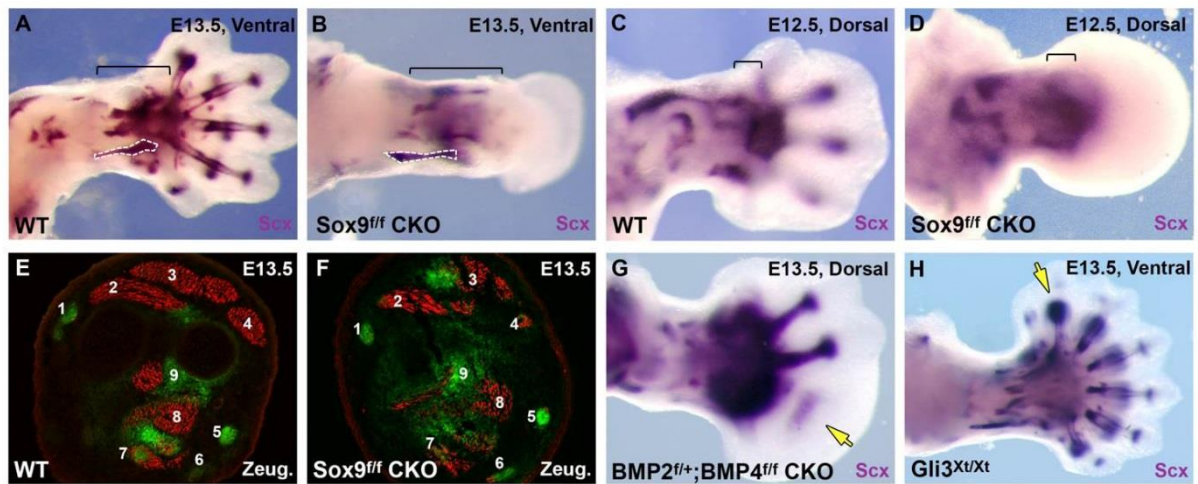


Figure 2: Autopod tendon development depends on cartilage. (A-D) Whole mount in situ hybridization for *Scx* expression of WT and *Sox9^{Prx1Cre}* limbs at E12.5 and E13.5. Analogous zeugopod tendons are outlined in A, B. Brackets delineate wrist tendon progenitors at E12.5 and zeugopod tendons derived from those progenitors at E13.5. (E, F) Transverse sections of *ScxGFP* WT and *Sox9^{Prx1Cre}* zeugopod stained for MHC at E13.5. (G, H) Whole mount in situ hybridization for *Scx* expression of conditional *BMP2^{ff/+};BMP4^{ff};Prx1^{Cre}* limbs and *Gli3^{Xt/Xt}* limb at E13.5. Yellow arrows highlight missing or extra tendons in G, H.

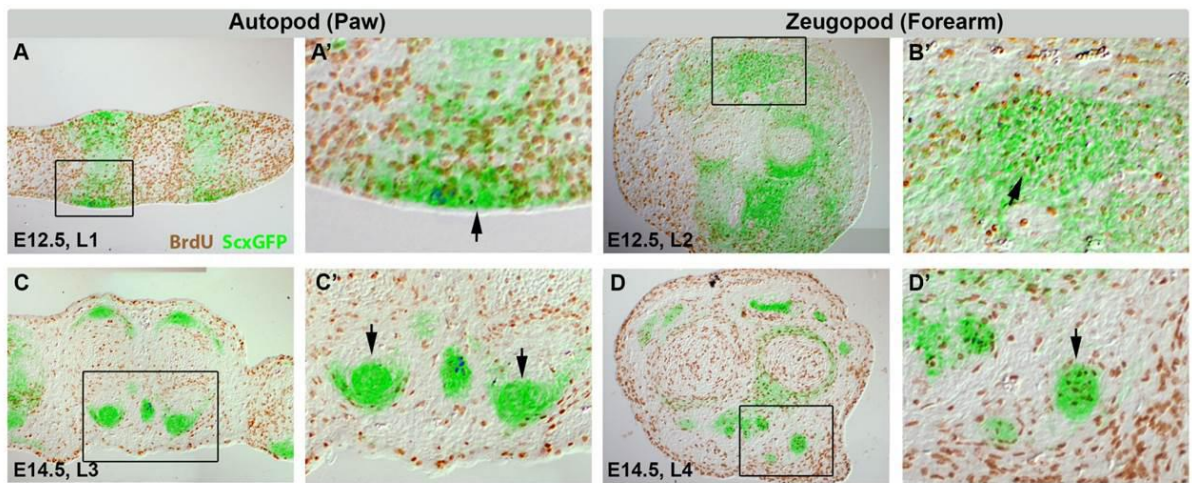


Figure 3: Distinct regulatory patterns of tenocyte proliferation in autopod and zeugopod tendon segments. BrdU was detected using DAB staining and the images were overlaid with *ScxGFP* signal from an immediate alternate section to highlight cell proliferation in tendons. (A) Autopod and (B) zeugopod tendons at E12.5. (C) Autopod and (D) zeugopod tendons at E14.5. Enlarged views of boxed areas in A-D shown in A'-D', respectively.

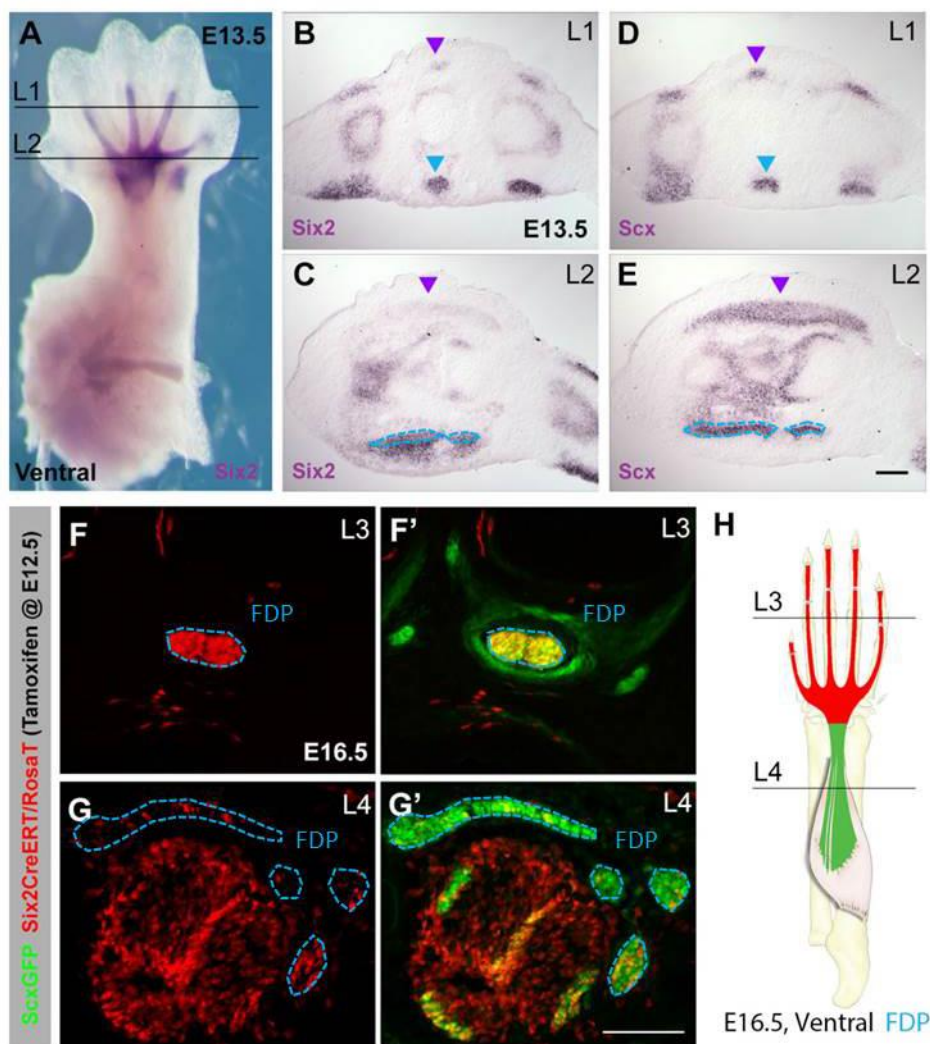


Figure 4: The autopod and zeugopod segments of the FDP tendons are derived from separate progenitor populations. (A) Whole mount in situ hybridization for *Six2* at E13.5. (B, C) *Six2* and (D, E) *Scx* in situ hybridization of transverse sections through levels indicated in A. (F, G) Transverse sections through limb of *Six2^{CreERT2};RosaT;ScxGFP* embryos at E16.5; tamoxifen was given at E12.5. (H) Schematic showing lineage distribution in FDP tendons. Blue and purple triangles and outlines indicate FDP and EDC tendons, respectively. FDS muscles and tendons are also derived from *Six2*-lineage cells (non-outlined red tissues in G, G').

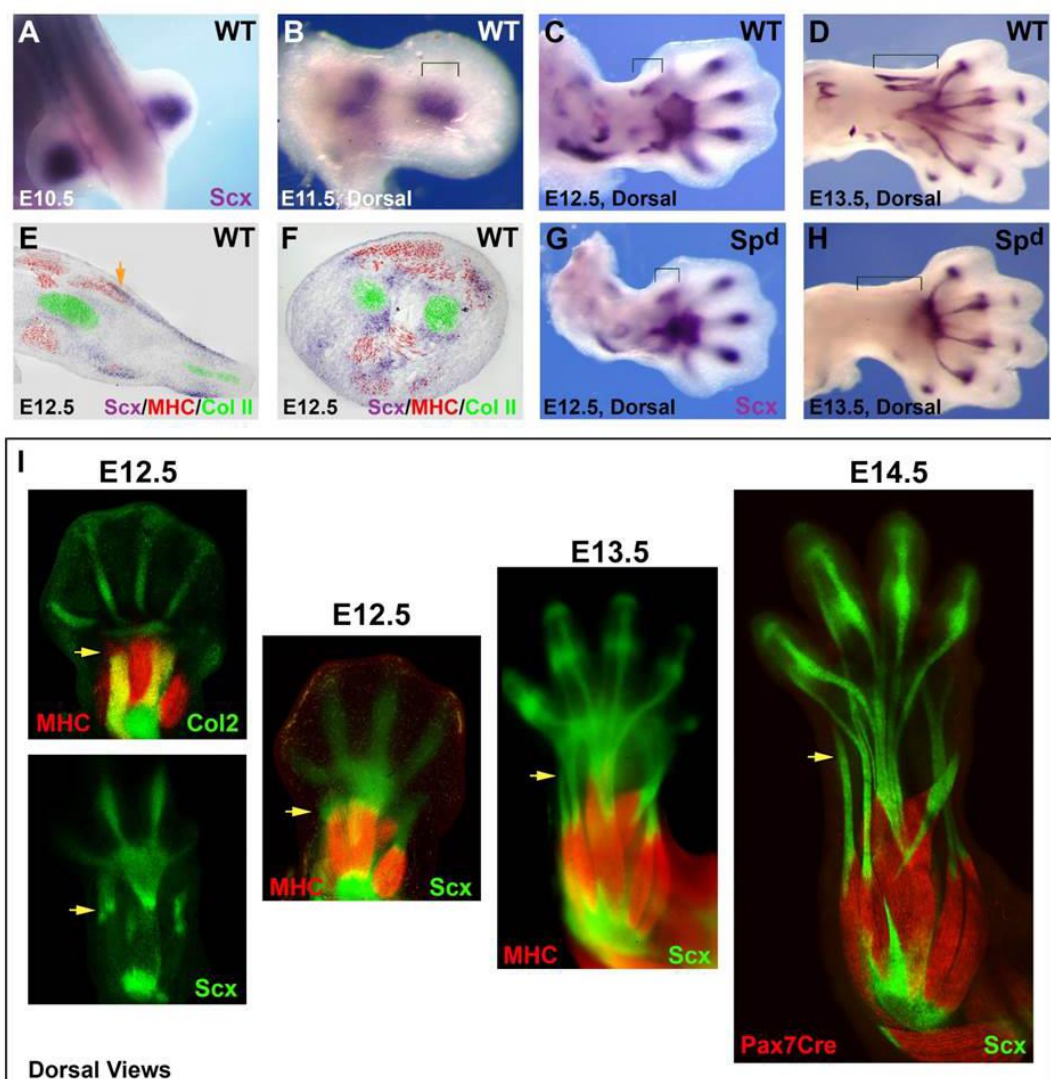


Figure 5: Zeugopod tendons develop via muscle anchoring, followed by elongation in parallel with skeletal growth. (A-D) Whole mount in situ hybridization of WT limbs for *Scx* from E10.5 to E13.5. (E) Sagittal and (F) transverse sections of E12.5 limbs stained for MHC and collagen type II for muscle and cartilage, respectively. (G, H) *Scx* expression in whole mount *Sp^d* limbs compared to (C, D) WT at E12.5 and E13.5. Brackets delineate wrist tendon progenitors at E12.5 and zeugopod tendons derived from those progenitors at E13.5. (I) Whole mount MHC-stained *Col2GFP* limb at E12.5 and whole mount *ScxGFP* limb at E12.5 (left). Whole mount *ScxGFP* limbs with muscle labeled by MHC staining at E12.5 and E13.5 (center) or Pax7Cre at E14.5 (right). Yellow arrows highlight wrist. Individual tendons in I can be identified using the schematic and table in **Figure 1**.

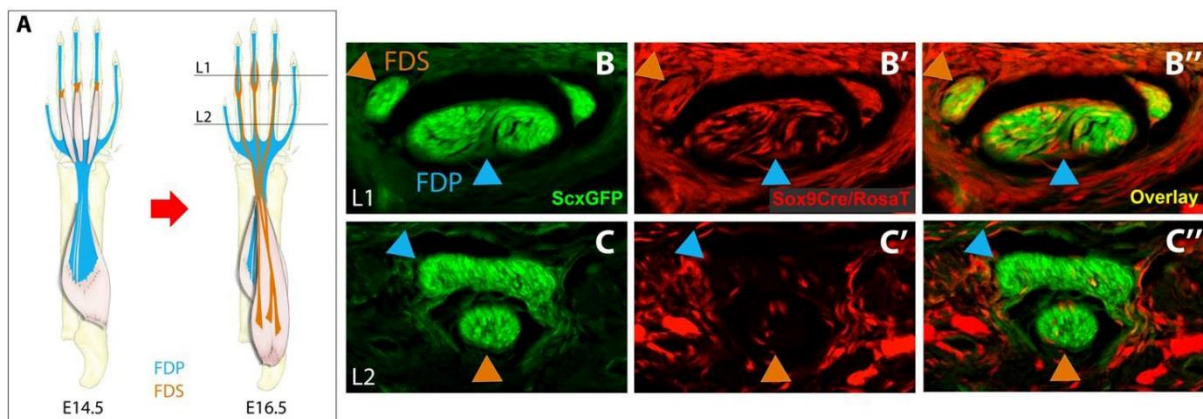


Figure 6: FDS tendons are formed via distinct cell populations. (A) Schematic of FDS tendon and muscle development. Adapted from (Huang et al., 2013). (B, C) Lineage tracing with *Sox9^{Cre}* and *Rosa26-TdTomato* at E16.5. B', B'' and C', C'' show *TdTomato* signal and *ScxGFP* and *TdTomato* overlays, respectively. Orange and blue triangles highlight FDS and FDP tendons, respectively.

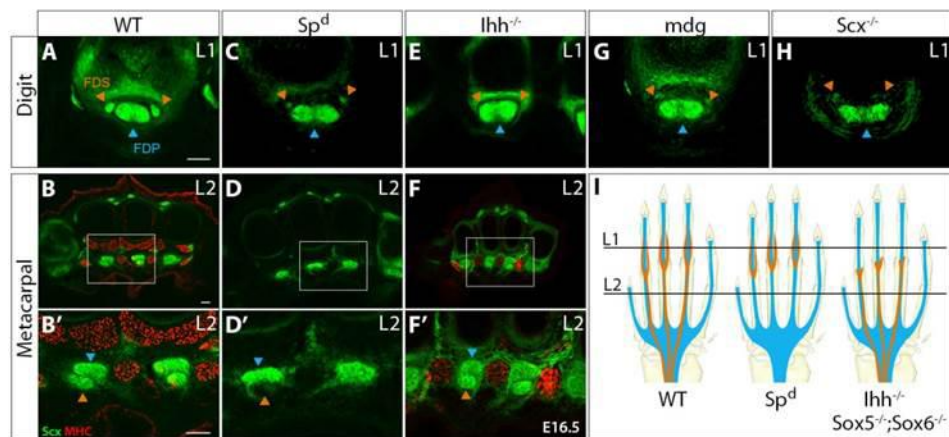


Figure 7: FDS tendon development is also modular and depends on muscle and cartilage. Transverse sections stained with MHC from (A, B) WT, (C, D) *Sp^d*, and (E, F) *Ihh^{-/-}* embryos at E16.5. Enlarged views of boxed areas in B, D, F shown in B', D', F', respectively. Digit FDS tendons in (G) paralyzed *mdg* mutants and (H) *Scx^{-/-}* mutants. (I) Schematic indicates digit and metacarpal levels and depict modular development of FDS tendons. Orange and blue triangles highlight FDS and FDP tendons, respectively.

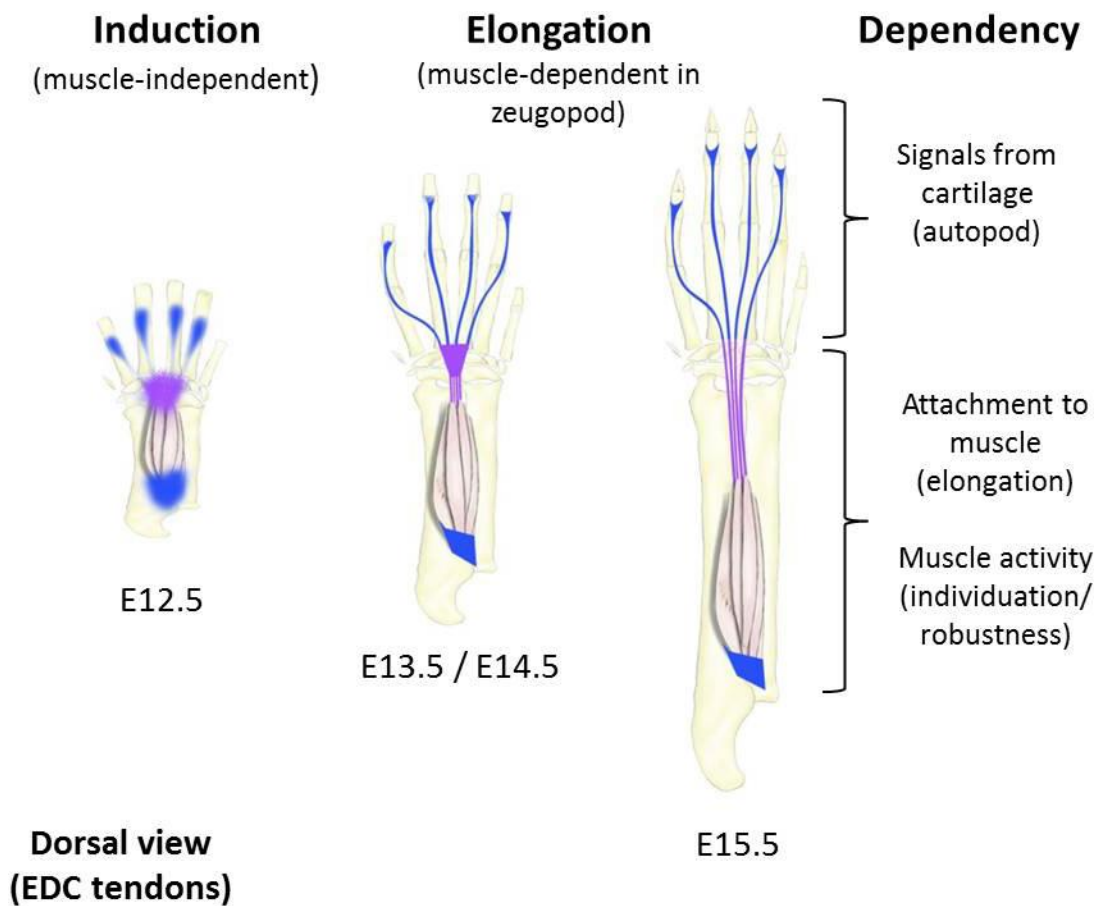


Figure 8: Model of zeugopod tendon formation as a distinctly regulated developmental process. Muscle-independent tendon induction of wrist progenitors occurs at E12.5, thus integrating the musculoskeletal tissues (muscle-tendon-cartilage). While autopod tendon development requires signals from cartilage, zeugopod tendons undergo a muscle-dependent elongation phase, in parallel with skeletal growth, such that the extent of skeletal growth dictates the extent of tendon elongation. The requirement for muscle for elongation lies in early attachment to the wrist tendon anlagen, and subsequent individuation and robustness of tendon depends on muscle forces. Blue highlights autopod tendon progenitors and tendons, while purple highlights the wrist anlagen and the wrist-derived zeugopod tendons. While the EDC tendons are shown here, this model of tendon formation applies to all of the autopod tendons, with the exception of the FDS tendons which are highlighted separately in **Figure 9**.

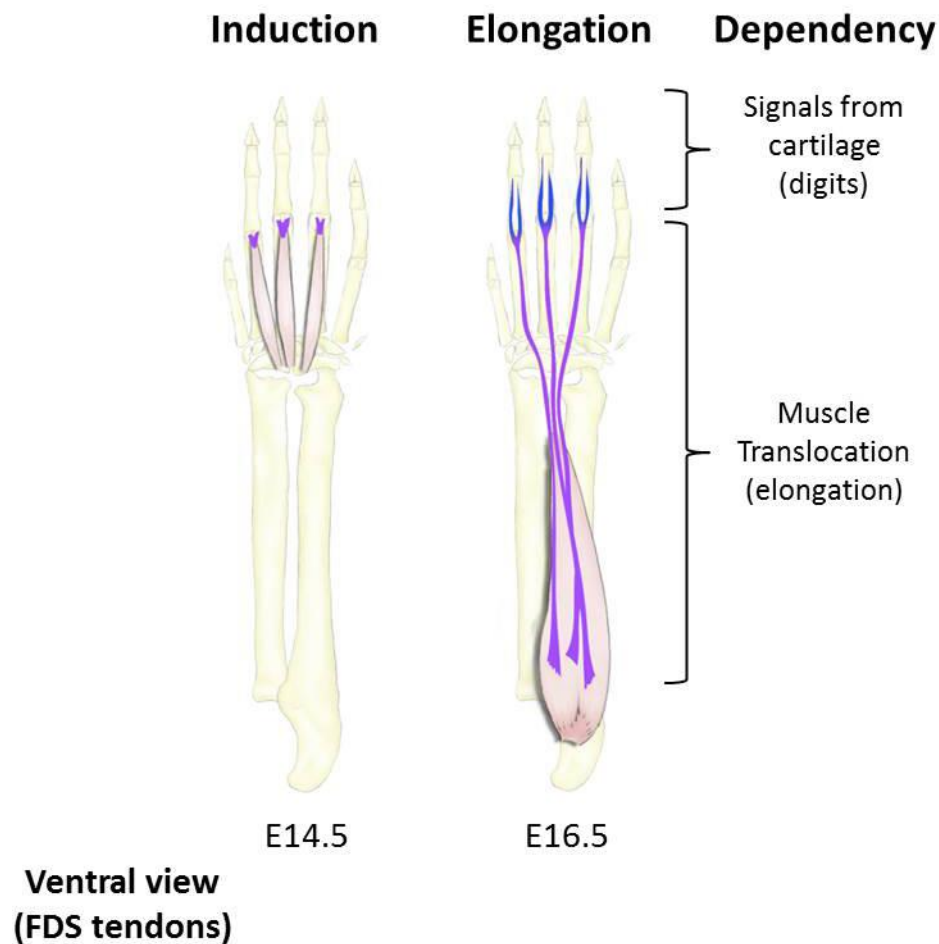


Figure 9: Model of FDS tendon formation and its unique features. Like the other autopod tendons, FDS tendons form first by induction of a short-range anlagen attached to muscle, followed by modular formation of independent distal and proximal tendon segments that depend on signals from cartilage and muscle, respectively. Unlike the other tendons, FDS tendon development is delayed relative to all other tendons, the developmental boundary for modularity is the MP joint instead of the wrist, and proximal tendon elongation is regulated by a unique process of active muscle translocation from the paw into the arm (described in (Huang et al., 2013)). Overall, the development of FDS tendons is consistent with our general conceptual framework for limb tendon development, yet its unique features highlight these tendons as a useful model tendon to test modularity and tendon elongation.

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