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RESEARCH ARTICLE

STEM CELLS AND REGENERATION

The development of zebrafish tendon and ligament progenitors

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ABSTRACT

Despite the importance of tendons and ligaments for transmitting movement and providing stability to the musculoskeletal system, their development is considerably less well understood than that of the tissues they serve to connect. Zebrafish have been widely used to address questions in muscle and skeletal development, yet few studies describe their tendon and ligament tissues. We have analyzed in zebrafish the expression of several genes known to be enriched in mammalian tendons and ligaments, including *scleraxis* (*scx*), *collagen 1a2* (*col1a2*) and *tenomodulin* (*tnmd*), or in the tendon-like myosepta of the zebrafish (*xirp2a*). Co-expression studies with muscle and cartilage markers demonstrate the presence of *scxa*, *col1a2* and *tnmd* at sites between the developing muscle and cartilage, and *xirp2a* at the myotendinous junctions. We determined that the zebrafish craniofacial tendon and ligament progenitors are neural crest derived, as in mammals. Cranial and fin tendon progenitors can be induced in the absence of differentiated muscle or cartilage, although neighboring muscle and cartilage are required for tendon cell maintenance and organization, respectively. By contrast, myoseptal *scxa* expression requires muscle for its initiation. Together, these data suggest a conserved role for muscle in tendon development. Based on the similarities in gene expression, morphology, collagen ultrastructural arrangement and developmental regulation with that of mammalian tendons, we conclude that the zebrafish tendon populations are homologous to their force-transmitting counterparts in higher vertebrates. Within this context, the zebrafish model can be used to provide new avenues for studying tendon biology in a vertebrate genetic system.

KEY WORDS: Tendon, Craniofacial, Zebrafish

INTRODUCTION

Tendons transmit force between muscle and bone, using their biomechanical properties to store and release energy. Ligaments connect bone to bone and stabilize this movement. The discovery of scleraxis (*Scx*), a basic helix-loop-helix transcription factor, as the earliest marker of tendon and ligament progenitors provided the means to study the molecular mechanisms of tendon specification and maturation (Cserjesi et al., 1995; Schweitzer et al., 2001). Indeed, *Scx* is expressed in tendon and ligament cells from embryonic to adult stages, and in all anatomical locations where tendons and ligaments arise (Brent et al., 2003; Schweitzer et al., 2001). However, relative to the other musculoskeletal tissues, tendons and ligaments have received less attention, and many questions remain as to the molecular mechanisms underlying their development.

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The early *Scx*-expressing progenitors form and condense into tendon primordia that establish precise connections within the musculoskeletal system. Despite its expression in all tendon cells, *Scx* is not essential for their specification, as tendon progenitors are present in $Scx^{-/-}$ mutant mice (Murchison et al., 2007). Rather, *Scx* is necessary for the condensation and differentiation of specific tendon populations. Maturing tendon cells secrete a rich extracellular matrix, and *Scx* also promotes the expression of matrix genes, including *Col1a1* (Lejard et al., 2007) and tenomodulin (*Tnmd*) (Shukunami et al., 2006), which encodes a type II transmembrane glycoprotein that is important for tendon cell proliferation and collagen fibril maturation (Docheva et al., 2005).

Interactions between the musculoskeletal tissues are important for their development in specific anatomical contexts. In the limb and cranial regions, tendons form in the absence of muscle, yet require muscle for their maintenance, suggesting the development of these tissues is eventually mutually dependent (Edom-Vovard et al., 2002; Grenier et al., 2009). By contrast, the axial tendons require muscle for their induction through the action of FGF signaling (Brent et al., 2005, 2003; Brent and Tabin, 2004). Studies examining cartilage-tendon interactions have suggested that distinct regulatory programs exist in the formation of distal limb tendon and skeletal progenitors (Hurle et al., 1990; Kardon, 1998). However, the extent to which tendons require a properly formed cartilage template for their formation and differentiation remains unclear.

Zebrafish studies examining skeletogenesis (Medeiros and Crump, 2012) and myogenesis (Pownall et al., 2002) have demonstrated conserved developmental programs with those of mammals. The transcription factors *Sox9*, *Myod1* and *Myf5* all have zebrafish homologs that function analogously in the development of cartilage or muscle lineages (Hinits et al., 2011; Lin et al., 2006; Yan et al., 2002, 2005). In the zebrafish jaw, these cell types develop in close proximity and contribute to a functioning musculoskeletal apparatus before day 5 of development (Schilling and Kimmel, 1997). However, the tendon and ligament populations have never been characterized in this context. Head tendons and ligaments have been described in teleosts in terms of comparative morphology and feeding mechanics (Cubbage and Mabee, 1996; Diogo et al., 2008; Liem, 1967; Staab and Hernandez, 2010; Westneat, 1990). Most developmental studies have focused on the formation of the jaw joint (Miller et al., 2003; Nichols et al., 2013; Talbot et al., 2010) and have described the regions where muscles attach to cartilage as muscle insertion sites (Schilling and Kimmel, 1997). To date, there is no molecular or morphological comparison of the development of the tendon and ligament cell populations in the zebrafish. Here, through analysis of gene expression and morphology, we have identified the zebrafish tendon populations, establishing their location at the interface of muscle and cartilage tissues. In the craniofacial region, we have found that the tendons and ligaments derive from the neural crest. We demonstrate that the induction of the cranial and fin tendons do not require properly formed muscle or cartilage. However, interactions with these tissues are necessary for their maintenance and organization. By contrast, tendon gene

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expression in the myosepta is dependent upon muscle for its initiation. Together, our work demonstrates that zebrafish tendons and ligaments are homologous structures to higher vertebrate tissues, thus establishing the zebrafish as a model system to study vertebrate tendon development.

RESULTS

Cloning and expression of the zebrafish Scleraxis genes

To identify zebrafish tendon populations, we cloned *scleraxis*, a robust marker of developing mammalian tendons and ligaments. Zebrafish have two Scleraxis genes, *scleraxisa* (*scxa*; NM_001083069.1) and *scleraxisb* (*scxb*), that are located on chromosomes 19 and 16, respectively. The two proteins have 61% identity with each other, and zebrafish Scxa has 62% and Scxb has 56% identity with the mouse protein (EMBL-EBI ClustalW2 alignment). In the basic domain, which mediates DNA binding (Davis et al., 1990), zebrafish Scxa has 96% identity, and zebrafish Scxb has 90% identity with the corresponding region in mouse Scx.

To determine the expression of zebrafish *scxa* and *scxb*, *in situ* hybridization was performed at different developmental stages. The *scxa* transcripts are detectable by 40 hpf (hours post-fertlilization) in the pharyngeal arches, and between the myotomal boundaries along the anterior-posterior axis by 36 hpf (Fig. 1A-C; data not shown). The most robust expression of *scxa* is detected at 72 hpf, in two lateral stripes ventromedial to the palatoquadrate (Fig. 1E,F, arrow), centrally where the sternohyoideus (sh) meets the ceratohyal and basihyal cartilage elements (Fig. 1E,F, arrowhead; enlarged in 1G), and at the base of the cleithrum (Fig. 1H, arrow). Additional expression is observed in areas where the adductor mandibulae attaches to Meckel's cartilage, and at attachment points of the intermandibularis, interhyoideus and hyohyoideus muscles (Fig. 1F, asterisks). Although not evident by *in situ* hybridization during embryonic, juvenile or adult stages, *scxb* transcripts were detected by RT-PCR after 54 hpf and in juveniles and adults (data not shown). Based on this, all further analysis was performed with *scxa*.

Identification of zebrafish tendon and ligament progenitor cells

To confirm that *scxa* transcripts mark the developing tendons, we examined the expression of well-characterized mammalian tendon markers and components of the tendon matrix: *tnmd* and *collagen 1a2* (*col1a2*). At 72 hpf, robust *tnmd* expression is found in regions medial to the palatoquadrate, between the ceratohyals (Fig. 1I,J, arrow and arrowhead; enlarged in 1K) and surrounding the cleithrum (Fig. 1L, arrow), all similar areas to where *scxa* is expressed. We find *col1a2* expression at the attachment site to the sternohyoideus and in the lateral domains near the palatoquadrate (Fig. 1Q,R, arrow and arrowhead; enlarged in 1S). We also examined the expression of *xirp2a* (*Xin actin binding repeat-containing protein 2 alpha*), an actin-binding multi-adaptor protein found in myosepta (Otten et al., 2012). *xirp2a* is expressed at all sites of muscle-muscle and muscle-cartilage attachment in the head and fin, in addition to its previously characterized myoseptal expression (see Fig. 5E) (Otten et al., 2012). Interestingly, *xirp2a* is expressed near the sternohyoideus attachment to the lower jaw cartilage (Fig. 1M-N, arrowhead; enlarged in 1O) and at the base of the cleithrum (Fig. 1P, arrow), but is absent from the lateral regions near the palatoquadrate.

The expression of *scxa*, *tnmd* and *col1a2* in similar domains suggest that they are marking zebrafish tendons and ligaments. Section *in situ* hybridization for *scxa* confirmed its expression between muscle and cartilage (Fig. 1D, arrowhead). In triple-stained embryos, craniofacial

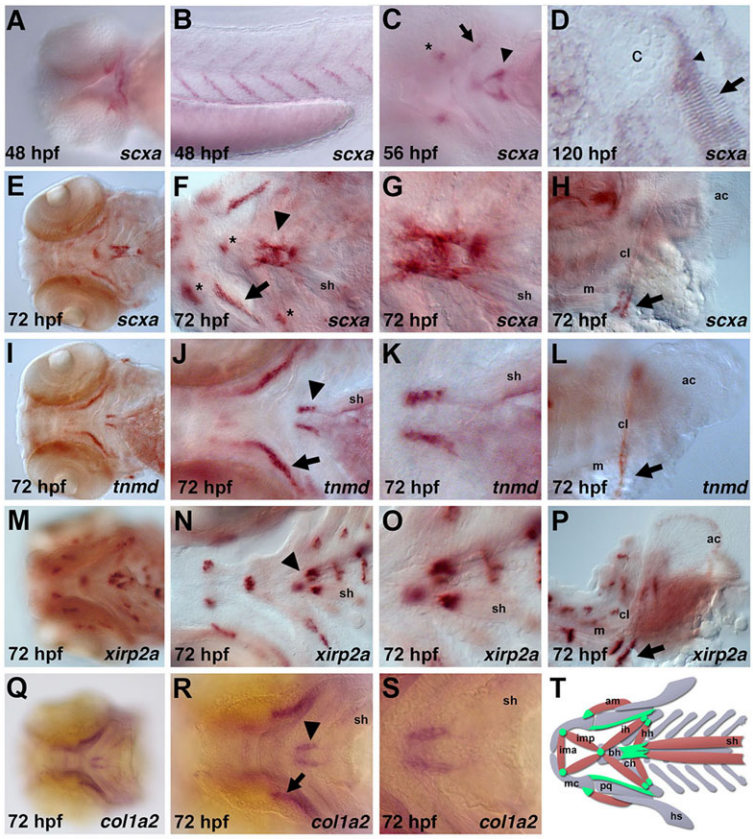

Fig. 1. Expression of tendon markers during zebrafish development. At 48 hpf, *scxa* is expressed in the (A) pharyngeal arches and (B) myosepta. (C) At 56 hpf, *scxa* is expressed in the craniofacial region (asterisk, arrow, arrowhead). (D) Section *in situ* hybridization of *scxa* expression (arrowhead) between cartilage (c) and muscle (arrow) at 120 hpf. (E-S) Expression of *scxa* (E-H), *tnmd* (I-L), *xirp2a* (M-P) and *col1a2* (Q-S) at 72 hpf. All four genes are expressed at the attachment point of the sternohyoideus muscles to the ceratohyal and basihyal cartilages (F,J,N,R, arrowhead; magnified in G,K,O,S). *scxa*, *tnmd* and *col1a2* are robustly expressed in two stripes ventromedial to the palatoquadrate (F,J,R, arrow). *scxa* and *xirp2a* are expressed at the adductor mandibulae, intermandibularis and hyohyoideus muscle attachment points (F, asterisks; N). *scxa*, *tnmd* and *xirp2a* are also expressed at the base of the cleithrum (H,L,P, arrow). (T) Schematic ventral view of zebrafish craniofacial muscle (red), cartilage (gray) and tendon/ligament (green) populations at 72 hpf. Only subsets of the muscle groups are depicted. All are ventral views of flat-mounted embryos except in B,H,L,P, which are lateral views, and in D, which is a coronal view. ac, actinotrichia; am, adductor mandibulae; bh, basihyal; c, cartilage; cl, cleithrum; ch, ceratohyal; hh, hyohyoideus; ih, interhyoideus; ima, intermandibularis anterior; imp, intermandibularis posterior; m, muscle; mc, Meckel's cartilage; pq, palatoquadrate; sh, sternohyoideus.

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scxa expression was found at muscle-to-cartilage or cartilage-to-cartilage attachments (Fig. 2A,B). In addition, *scxa*, *tnmd*, *col1a2* and *xirp2a* are co-expressed at these attachment sites. At 60-72 hpf, colocalization of *xirp2a* and *scxa* were observed in regions near the adductor mandibulae and where the interhyoideus intersects with the intermandibularis muscles (Fig. 2D-F, arrowhead), and their expression appeared distinct from myosin heavy chain staining in the muscle (Fig. 2E). The *scxa* and *tnmd* transcripts were colocalized in the head and fin regions between 60 and 80 hpf (Fig. 2C,G-I). We also found that *scxa*, *xirp2a* and *tnmd* expression are temporally dynamic in the craniofacial region. Robust expression of *scxa* at 60 hpf (Fig. 2F,G) is followed by weaker expression after 80 hpf (Fig. 2H,I), whereas *tnmd* expression is detected at 60 hpf but is stronger after 80 hpf (Fig. 2G-J,N). Based on this analysis, *scxa* expression appears to be downregulated as *tnmd* expression becomes upregulated, possibly reflecting cell differentiation events. *xirp2a* expression is robust at 96 hpf, and colocalizes with *tnmd* in regions proximal to the muscle (Fig. 2J, arrowhead). Domains that co-expressed *scxa*, *tnmd* and *col1a2*, but not *xirp2a* were medial to the palatoquadrate and in the sternohyoideus attachment proximal to the cartilage (Fig. 2D,I,J,N). In the myosepta, there is colocalization of *xirp2a* and *scxa* at 48 hpf (Fig. 2K-M), and *col1a2* and *tnmd* at 96 hpf (Fig. 2O). Together, the expression of *scxa*, *tnmd* and *col1a2* identifies two major regions of tendon and ligament populations in zebrafish craniofacial tissue (Fig. 1T; summarized in Fig. 7). One domain, medial to the palatoquadrate, is a ligament, connecting two cartilage elements of the jaw: the posterior-most region of Meckel's cartilage with the lateral-most region of the ceratohyal. The other region is a tendon located at the attachment site where the sternohyoideus muscles connect with the cartilage elements of the ventral jaw.

We next investigated tendon and ligament tissues of juvenile and adult zebrafish. In juvenile stage zebrafish, we detected strong expression of *tnmd*, a robust marker of differentiating tendons (Docheva et al., 2005), near the sternohyoideus connection (Fig. 3A, arrowhead) and in lateral tissue connecting to Meckel's cartilage (Fig. 3B, arrow). At these later stages, *scxa* is weakly expressed by section *in situ* hybridization and detected by RT-PCR in isolated adult tendon and ligament tissue (data not shown). To determine the ultrastructural characteristics of adult tendons and ligaments, electron microscopy analysis was performed on the tendon connecting one of the subdivisions of the adductor mandibulae and the mandibulo-hyoid ligament. Similar to the ultrastructure of mammalian tendons and ligaments (Ezura et al., 2000), those of zebrafish show a circular collagen fibril arrangement in cross-section, and parallel collagen fibrils with a characteristic periodicity in longitudinal section (Fig. 3C-H). Together, these data demonstrate that zebrafish craniofacial tendons and ligaments molecularly, morphologically and structurally resemble mammalian tendons and ligaments from embryonic to adult stages.

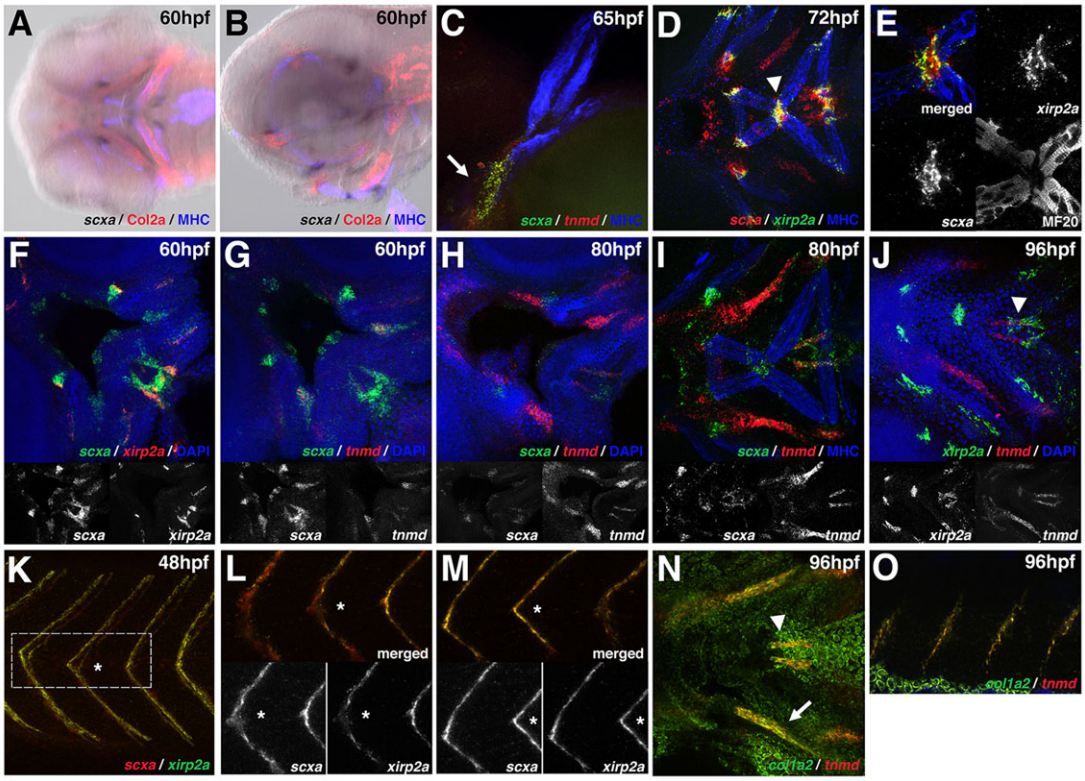

Fig. 2. Tendon genes mark discrete domains joining muscle and cartilage. (A,B) *scxa* is expressed at points of attachment of muscle-to-cartilage and cartilage-to-cartilage in the craniofacial tissue at 60 hpf. Images were generated by overlaying the bright-field and fluorescent channels. (C) *scxa* and *tnmd* are co-expressed at the base of the cleithrum (arrow). (D-F) *scxa* and *xirp2a* are co-expressed in muscle attachment points, e.g. where the interhyoideus and intermandibularis muscles intersect (D, arrowhead; enlarged in E). Single channels of confocal image are shown in E. (G-I) Colocalization of *scxa* and *tnmd* is detected between 60 and 80 hpf. Expression of *scxa* and *tnmd* transcripts is temporally dynamic: there is robust expression of *scxa* at 60 hpf followed by weaker expression after 80 hpf; *tnmd* is weakly expressed at 60 hpf and increases in expression after 80 hpf. (J) At 96 hpf, *xirp2a* and *tnmd* are co-expressed in regions proximal to the muscle (arrowhead). (K-M) *scxa* and *xirp2a* are co-expressed in the tail myosepta in regions medial (L) and lateral (M) to the notochord. Single channels of confocal images are shown in L,M and an asterisk marks the corresponding myoseptum in the same embryo. (N,O) At 96 hpf, *tnmd* and *col1a2* are co-expressed in regions medial to the palatoquadrate (N, arrow), at the sternohyoideus connection point (N, arrowhead), and in the myosepta (O). Ventral (A,D-J,N) and lateral (B,C,K-M,O) views of flat-mounted embryos.

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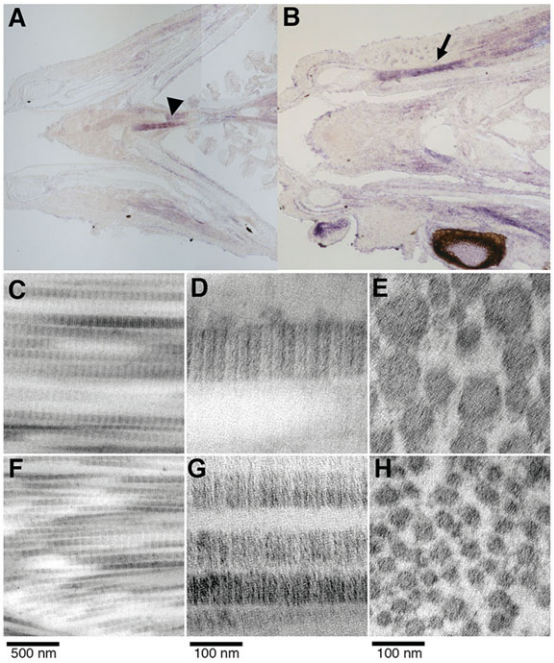

Fig. 3. Expression and ultrastructural analysis of adult zebrafish craniofacial tendons and ligaments. (A,B) Coronal sections of juvenile zebrafish show *tnmd* expression near the sternohyoideus attachment (arrowhead) and in lateral regions attached to the mandible (arrow). *tnmd* is also detected in the perichondrium. Transmission electron microscopy of ligament (C,D) and tendon (F,G) longitudinal sections reveal the parallel arrangement of collagen fibrils with characteristic periodicity. Ligament (E) and tendon (H) cross-sections reveal round collagen fibrils.

Zebrafish craniofacial tendons and ligaments are derived from the neural crest

In higher vertebrates, head tendons along with other cranial skeletal tissues are derived from the neural crest, while head musculature originates from the mesoderm (Le Douarin, 1982). The neural crest origin of zebrafish head skeletal structures has been established (Schilling and Kimmel, 1994), but it is not understood whether cranial tendons and ligaments are also neural crest derived. To determine this, we first tested whether cranial tendon formation requires proper neural crest development, using morpholino-mediated knockdown of the transcription factors *foxd3* and *tfap2a*, which are essential neural crest regulators (Arduini et al., 2009; Wang et al., 2011). In *tfap2a* and *foxd3* single morphants, neural crest development is disrupted but not altogether missing (Arduini et al., 2009; Barrallo-Gimeno et al., 2004; Montero-Balaguer et al., 2006; O'Brien et al., 2004; Wang et al., 2011). As a control to discern the knockdown efficiency, we assessed expression of *sox9a*, a marker of neural crest and cartilage cells. Consistent with reported results, *sox9a* transcripts are present, but the pattern of expression is abnormal in *tfap2a* and *foxd3* single morphants compared with controls (supplementary material Fig. S1A-D and Fig. S2A,B). Similarly, *scxa* is expressed, but in a disorganized pattern in *tfap2a* and *foxd3* single morphants compared with controls (supplementary material Fig. S1E-H and Fig. S2C,D). As loss of both *tfap2a* and *foxd3* causes a complete absence of all neural crest derivatives (Arduini et al., 2009; Wang et al., 2011), we next examined *scxa* expression. In *tfap2a-foxd3* double morphants, we observed a loss of *scxa* and *sox9a* expression in the pharyngeal arch regions compared with controls (Fig. 4A-D; supplementary material Fig. S2A-D), indicating that proper neural crest development is required for *scxa* expression in craniofacial regions.

These results could be explained either by the tendons themselves being derived from neural crest or, in principle, by their being distinct in origin but requiring neural crest input for their formation. To distinguish between these possibilities, we performed a fate-mapping experiment using a photoconvertible Kaede protein, the expression of which is restricted to the neural crest lineage in the *sox10:kaede* transgenic line (*Tg(sox10:kaede)*) (Dougherty et al., 2012). Upon exposure to ultraviolet light, Kaede protein is irreversibly photoconverted from green to red, allowing cell fate to be followed several days post-photoconversion (Ando et al., 2002). Using this photoconversion lineage-tracing strategy with the *Tg(sox10:kaede)* line, we tested whether the cranial neural crest cells (CNCCs) give rise to tendons and ligaments in the head. We photoconverted *Tg(sox10:kaede)* CNCCs at 22 hpf, and examined the location of the *sox10:kaede* CNCC descendants at 72 hpf. As a positive control for photoconversion, we observed CNCC-derived cartilage labeled with red Kaede protein (Fig. 4E-G). The cartilage at these stages also expresses the *sox10:kaede* green protein, consistent with previous reports for this transgene (Dougherty et al., 2012; Dutton et al., 2008). Other cells exclusively expressed the red Kaede protein, identifying them as descendants of the *sox10:kaede* CNCCs. Subsets of these red Kaede populations were located in regions identified to be tendons and ligaments, specifically in the ligaments medial to the palatoquadrate (Fig. 4E,G, arrows) and in the tendon connecting the sternohyoideus to the ceratohyal (Fig. 4E,F, arrowheads). The ligament near the palatoquadrate appears to be physically anchored to the retroarticular process of Meckel's cartilage and the ceratohyal, whereas the tendon attaching to the sternohyoideus muscles are connected to the center of the ceratohyals. Double staining for *scxa* and *xirp2a* transcripts and Kaede protein confirmed that the tendons and ligaments originate from the neural crest (Fig. 4H-J). The sternohyoideus tendon stained positive for *scxa*, *xirp2a* and Kaede (Fig. 4H,I, arrowhead), and the ligament stained for *scxa* and Kaede (Fig. 4J, arrow), while control antibody staining was negative (supplementary material Fig. S1I,J). Together, these findings establish that zebrafish craniofacial tendon and ligament cells are neural crest derived.

Role of muscle in tendon and ligament development

To test the function of muscle in zebrafish tendon development, we examined tendon gene expression in embryos lacking essential regulators of myogenesis: *myod1* and *myf5*. Loss of either gene alone alters the formation of specific cranial muscles but does not disrupt the development of all head musculature (Hinits et al., 2011; Lin et al., 2006). However, loss of both *myod1* and *myf5* causes a complete absence of all differentiated craniofacial muscles (Hinits et al., 2011; Lin et al., 2006). To determine the effect of muscle loss on zebrafish tendon development, we examined *scxa* expression upon morpholino-mediated knockdown of both *myod1* and *myf5* or knockdown of *myf5* in *myod1*^{fh261} mutants. To control for the extent to which myogenesis was inhibited, we examined expression of either *myogenin* (*myog*), a marker of differentiating muscle cells, or myosin heavy chain (MHC). In *myod1-myf5*-deficient embryos, *myog* expression is completely missing at 53-58 hpf and MHC expression is absent at 72 hpf compared with controls (Fig. 5G,H,K,L; supplementary material Fig. S2E,F). By contrast, *scxa* expression is relatively normal at 53-58 hpf in the craniofacial and fin regions, but absent from the myosepta (Fig. 5C,D). *xirp2a* expression was also lost in the myosepta (Fig. 5E,F). These findings indicate that interactions with the muscle are necessary for proper *scxa* and *xirp2a* expression in the axial regions, but not required for induction of *scxa*-positive craniofacial and fin tendon progenitors. At 72 hpf,

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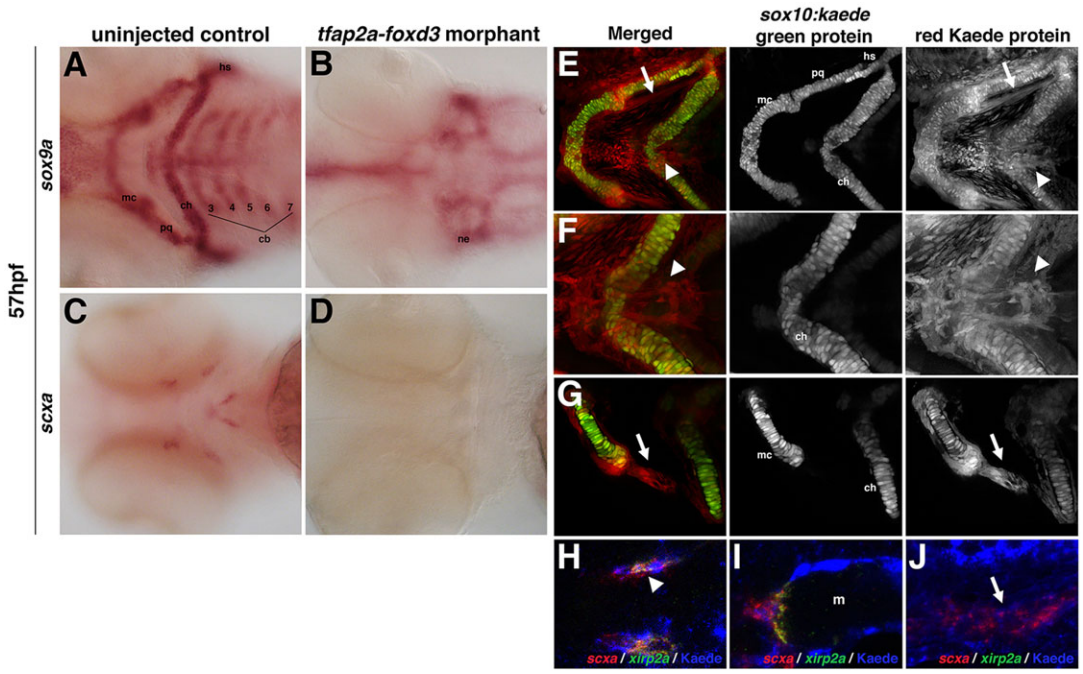

Fig. 4. Zebrafish craniofacial tendon populations are derived from the neural crest. Morpholino-mediated knockdown of *tfap2a* and *foxd3* results in (A,B) complete loss of *sox9a*-positive pharyngeal cartilage (98%, *n*=58) and (C,D) *scxa*-positive craniofacial tendon progenitors (96%, *n*=46) at 57 hpf compared with controls. (E-G) 72 hpf photoconverted *sox10:kaede* embryos express *sox10:kaede* green protein in the pharyngeal cartilage (E-G and middle panel), and the red Kaede protein from the 22 hpf photoconversion is found in the two major populations of craniofacial tendon progenitors (E, arrow and arrowhead; F,G, right panel). (H-J) Colocalization of *scxa* and Kaede protein in photoconverted *sox10:kaede* embryos at 72 hpf is observed in the sternohyoideus connection point (H,I; sternohyoideus muscle is labeled m) and in the ligament (J). A subset of the *scxa*-positive and Kaede-positive cells also co-expresses *xirp2a* transcripts. Arrows in E,G,J mark ligament medial to palatoquadrate; arrowheads in E,F,H mark tendon connecting the sternohyoideus to the ceratohyals. cb: ceratobranchials; ch, ceratohyal; hs, hyosymplectic; m, muscle; mc, Meckel's cartilage; ne, neurocranium; pq, palatoquadrate.

we observed a virtual loss of *scxa* expression in the head and fins of *myod1-myf5*-deficient embryos (Fig. 5I,J), indicating that muscles are required for the maintenance of *scxa* expression. *xirp2a* expression was lost in the head and fins at all stages examined (Fig. 5E,F,M,N), suggesting that muscle is required for *xirp2a* expression. After 80 hpf, the effectiveness of the morpholino knockdown was reduced as MHC staining returned in *myod1-myf5*-deficient embryos. Nevertheless, our findings demonstrate that muscle is required for *scxa* and *xirp2a* expression in the myosepta, and for maintaining *scxa* expression in the craniofacial and fin regions.

Studies in mouse and chick have established that FGF signals from muscle are important for the induction of *Scx* progenitors, and that TGFβ signaling is involved in tendon cell maintenance (Brent et al., 2003; Brent and Tabin, 2004). To test the requirement for these signals in zebrafish tendon cell development, we incubated embryos with chemical inhibitors of FGF (SU5402) and TGFβ (SB-431542) pathways at 32 hpf and examined the effect on *scxa* expression at 56 hpf. We found that *scxa* expression was lost in all anatomic locations in SU5402-treated embryos (supplementary material Fig. S3B), and in embryos treated with the TGFβ pathway inhibitor, *scxa* expression was reduced (supplementary material Fig. S3C). As both molecules have important roles in other contexts, especially in neural crest development (Larbuisson et al., 2013; Walshe and Mason, 2003), and SU5402 can affect other receptor tyrosine kinase pathways (Mohammadi et al., 1997; Sun et al., 1999), it cannot be concluded whether these pathways act directly or indirectly on *scxa* expression. Nevertheless, our results are consistent with previously established roles for these pathways in other systems.

Role of cartilage in tendon and ligament development

Having demonstrated a crucial role for muscle in the induction of *scxa* expression in axial tendon cells and maintenance of *scxa* expression in cranial and fin tendon and ligament populations, we next tested whether interactions with cartilage are important for tendon and ligament development. We examined the effect of loss of the *Sox9* co-orthologs *sox9a* and *sox9b*, which have redundant and gene-specific functions in neural crest and pharyngeal cartilage development (Yan et al., 2005). *sox9a* is essential in the formation of the Alcian Blue-positive cartilage structures in the pharyngeal arches and pectoral fins, and *sox9b* is important for proper neural crest development (Yan et al., 2002, 2005). As a control of *sox9a* knockdown efficiency, we examined expression of the differentiated cartilage marker *col2a1*, and found a consistent loss of *col2a1*-positive cartilage elements in *sox9a* morphants compared with controls (Fig. 6A,B; supplementary material Fig. S2G,H). *scxa* is expressed in *sox9a*-deficient embryos at 56 and 72 hpf (Fig. 6E,H), indicating that *scxa*-positive tendon progenitors are specified in the absence of differentiated cartilage. In all *sox9b* morphants and embryos resulting from *sox9b*^{fh313} heterozygous mutant crosses, *scxa* expression was present, although there was a reduction in the size of the *scxa* expression domains and in the *col2a1*-expressing cartilage elements at 48 and 57 hpf (data not shown). These results are likely a consequence of the requirement for *sox9b* in proper neural crest development (Yan et al., 2005). To dissect the functional role of both *sox9* genes in tendon development, we injected morpholinos targeting either *sox9a* or *sox9b* into *sox9b*^{fh313} or *sox9a*^{hi1134} mutant embryos, respectively, and examined tendon gene expression. Loss of both *sox9a* and *sox9b* causes loss of all pharyngeal arch cartilage and a disruption in otic vesicle formation (Yan et al., 2005). We

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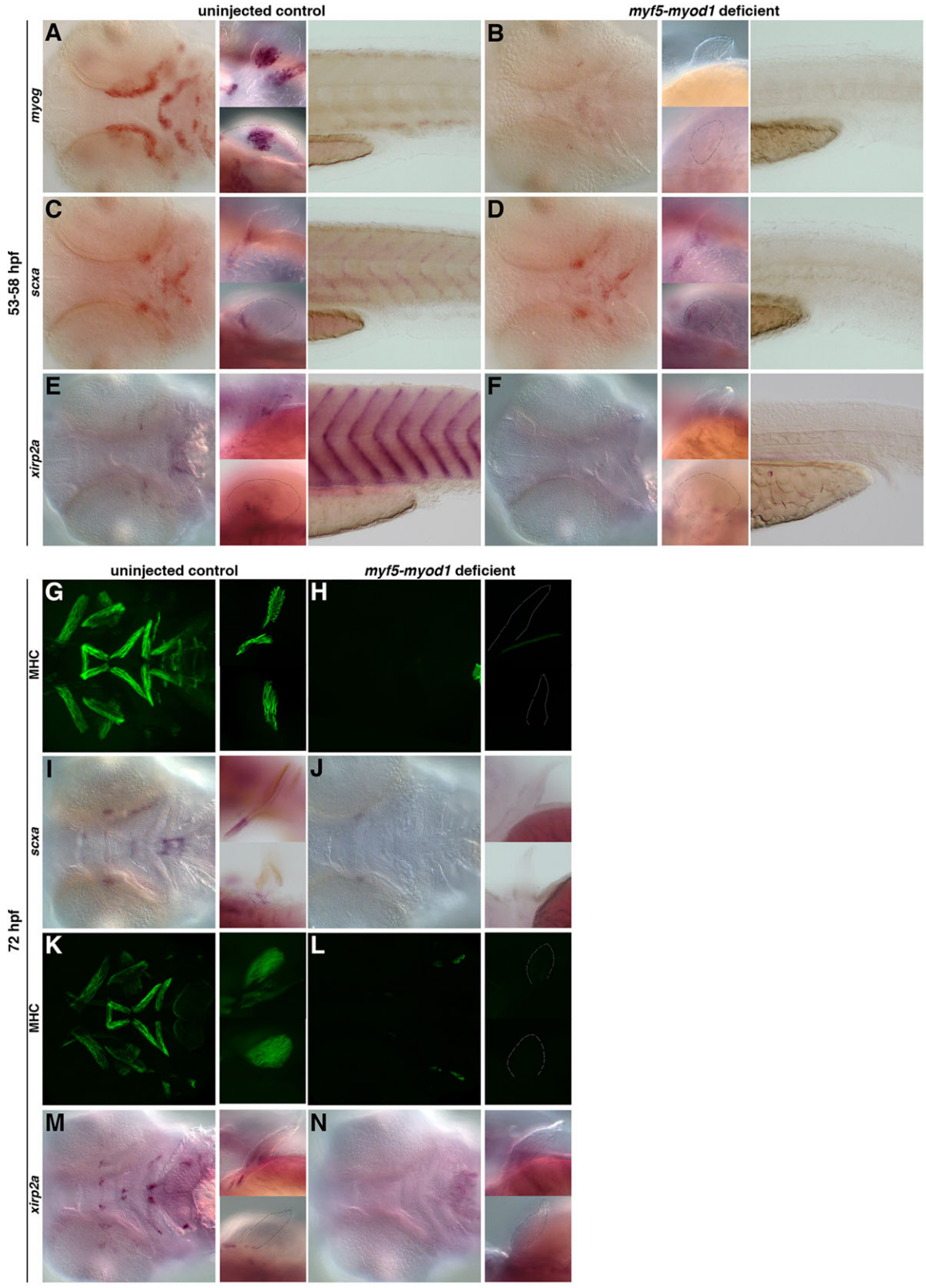

Fig. 5. The role of muscle in the specification and maintenance of tendon populations. (A,B) Loss of *myod1* and *myf5* results in the complete absence of *myog*-positive differentiated muscles in the head (left), fin (middle) and tail (right) (93%, *n*=67). (C-F) In *myod1-myf5*-deficient embryos at 53-58 hpf, *scxa* expression is lost in the myosepta (C,D), and *xirp2a* expression is completely absent in the craniofacial, pectoral fin and myoseptal tissue (E,F) compared with controls. However, loss of differentiated muscle (C,D) does not alter expression of *scxa*-positive tendon progenitors in the craniofacial or pectoral fin tissue (97%, *n*=32). (G-N) At 72 hpf, *myod1*$^{-/-}$ and *myf5*-deficient embryos have (G,H,K,L) complete loss of myosin heavy chain (MHC) expression in the craniofacial and pectoral fin tissue and (I,J) a virtual loss of *scxa* expression in the head and pectoral fin tissue. (M,N) Expression of *xirp2a* is also missing (100%, *n*=19). Fluorescent images of MHC-stained flat-mounted embryos in G,H,K,L correspond to the same embryos in brightfield (I,J,M,N).

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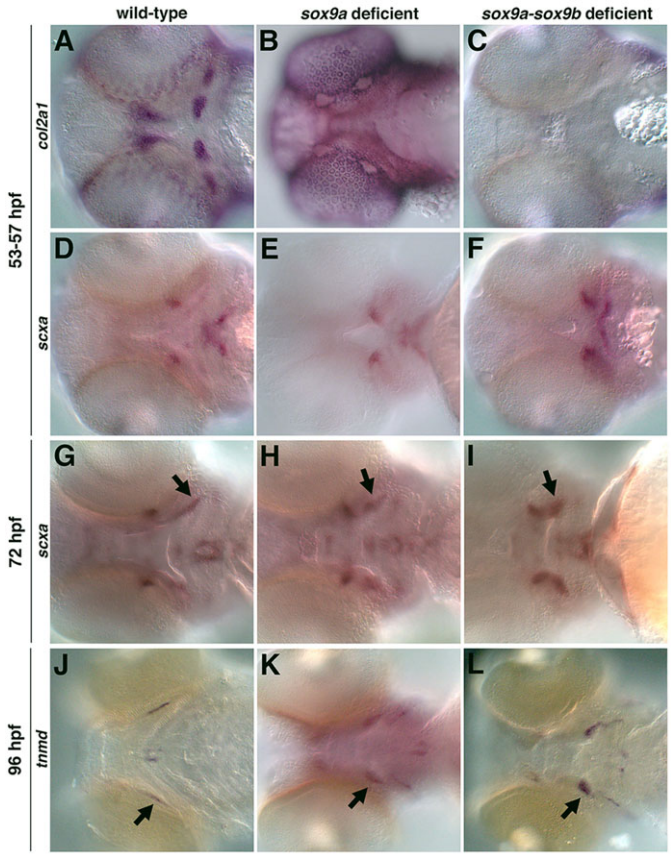

Fig. 6. The role of cartilage in the specification and organization of tendon and ligaments. *col2a1*-positive pharyngeal cartilage was lost in *sox9a*- (B) and *sox9a-sox9b*-deficient embryos (88%, *n*=56) (C) compared with controls (A). (D-F) *sox9a*- and *sox9a-sox9b*-deficient embryos (93%, *n*=42) express *scxa* but in an altered pattern compared with controls. *sox9a-sox9b*-deficient embryos were identified by the loss of their otic vesicle, which only occurs in the absence of both genes. Similar expression was observed for morpholino-mediated knockdown of *sox9a* and *sox9b* together, and with *sox9a* or *sox9b* morpholino injection into crosses of *sox9a*^{hi1134} or *sox9b*^{fh313} mutants, respectively. (G,H,J,K) At 72 hpf and 96 hpf, *sox9a*- and *sox9a-sox9b*-deficient embryos (82%, *n*=57) express *scxa* and *tnmd*, but in an abnormal pattern compared with controls. The *scxa*- and *tnmd*-expressing ligaments (H,I,K,L, arrows) appear shorter and wider than controls. Expression posterior and lateral to the ligaments in K,L marks the branchiostegal rays.

observed these changes in the *sox9a-sox9b*-deficient embryos at 53-57 hpf upon examination of *col2a1* expression (Fig. 6C; data not shown). At all stages examined, none of the *sox9a-sox9b*-deficient embryos lost *scxa* or *tnmd* expression in the craniofacial, fin or myoseptal regions (Fig. 6F,I,L; supplementary material Fig. S4; data not shown), demonstrating that the *Sox9* co-orthologs and properly formed cartilage elements are not required for induction of tendon cell fate in zebrafish. However, cartilage is necessary for the organization of the tendon progenitors, as the expression of *scxa* and *tnmd* at 72 hpf and 96 hpf, respectively, appeared abnormal in *sox9a* and *sox9a-sox9b*-deficient embryos. The ligaments, in particular, were affected, appearing shorter and not as elongated as in control embryos (Fig. 6G,I,J,L, arrows). Together, these results suggest that interactions with the cartilage are necessary for the tendon progenitors to organize properly within the musculoskeletal system.

DISCUSSION

We have identified the cranial tendon and ligament progenitor populations in the zebrafish and have shown that they form at the intersection between developing muscle and cartilage or between cartilage segments (Fig. 7). The zebrafish tendons express the same markers as mammalian and avian tendons, including *scxa*, *tnmd* and *col1a2*, and likewise display similar adult collagen fibril arrangement. Zebrafish craniofacial tendons are derived from neural crest tissues, and their initial specification is independent of interactions with the neighboring muscle and cartilage. However, in zebrafish lacking properly formed muscle, *scxa* expression is not maintained in the craniofacial and fin regions. These findings mirror those in the mouse jaw and avian limb, where muscle loss results in normal initiation of *Scx* expression, but in a loss of its maintenance (Edom-Vovard et al., 2002; Grenier et al., 2009). Interestingly, *scxa* expression in the ligaments connecting Meckel's to the ceratohyal cartilages is also not maintained in zebrafish that lack properly formed muscle, possibly indicating a requirement for long-range signals arising from the muscle or for the movement the muscle produces. Muscle contraction is important in the development of many tissues, including the joints, cartilage and tendon-bone insertions (Kahn et al., 2009; Shwartz et al., 2012). *Scx* expression, in particular, is sensitive to changes in mechanical stimuli in adult tendons (Maeda et al., 2011). Furthermore, muscleless and aneural chick wings lose *Scx* expression in all regions of the proximal limb, and although no direct analysis of ligament fates was performed, *Scx* expression was absent in areas near cartilage elements (Edom-Vovard et al., 2002; Kardon, 1998).

In the amniote axial skeleton, in contrast to the limb and cranial regions, signals from the muscle are required for the formation of the syndetome: the somitic compartment of tendon progenitors (Brent et al., 2003). We observed loss of *scxa* expression in the myosepta in embryos lacking muscle, suggesting a similar mode of regulation in zebrafish. The syndetome in amniotes arises from distinct regions of the sclerotome, whereas the somitic origins of the myosepta in fish have not been well defined. At embryonic and larval stages, the zebrafish myosepta connect adjacent myomeres and function in undulatory locomotion. Although a sclerotome is present at this time, cartilage does not appear until mid-larval stages in the axial region (Bird and Mabee, 2003). It has been demonstrated that the horizontal myosepta, which separate the epaxial and hypaxial musculature, are derived from the myotomal muscle pioneer cells (Devoto et al., 1996; Felsenfeld et al., 1991; Hatta et al., 1991; Schweitzer et al., 2005). By contrast, the vertical myosepta are believed to be of sclerotomal origin, making them analogous to mammalian axial tendon tissue (Bricard et al., 2014; Charvet et al., 2011). In the developing somite of zebrafish, which comprises predominantly myotomal cells, the sclerotomal cells form in the ventralmost domain and migrate dorsally to eventually surround the notochord and neural tube (Morin-Kensicki and Eisen, 1997; Stickney et al., 2000). Interestingly, we observed strong *scxa* expression in ventral myoseptal regions between 36 and 48 hpf (Fig. 1B; data not shown). These regions may represent the early sclerotomal cells thought to form the myosepta in trout (Bricard et al., 2014). In addition, we detect *scxa* in only the vertical and not the horizontal myosepta. Together, these results suggest that *scxa* is marking an early syndetome equivalent in zebrafish, but lineage-tracing experiments are necessary to confirm the somitic origins of the *scxa*-expressing myoseptal cells.

Lineage studies show that axial tendons and proximal limb tendons and ligaments arise from an early common Sox9-positive progenitor (Akiyama et al., 2005; Soeda et al., 2010; Sugimoto et al., 2013), yet a requirement for *Sox9* in the cranial tendon and ligament lineages has not been established. In the mouse, deletion of the transcription factors *Sox5* and *Sox6*, which are downstream of *Sox9* and important for cartilage differentiation, results in the expression

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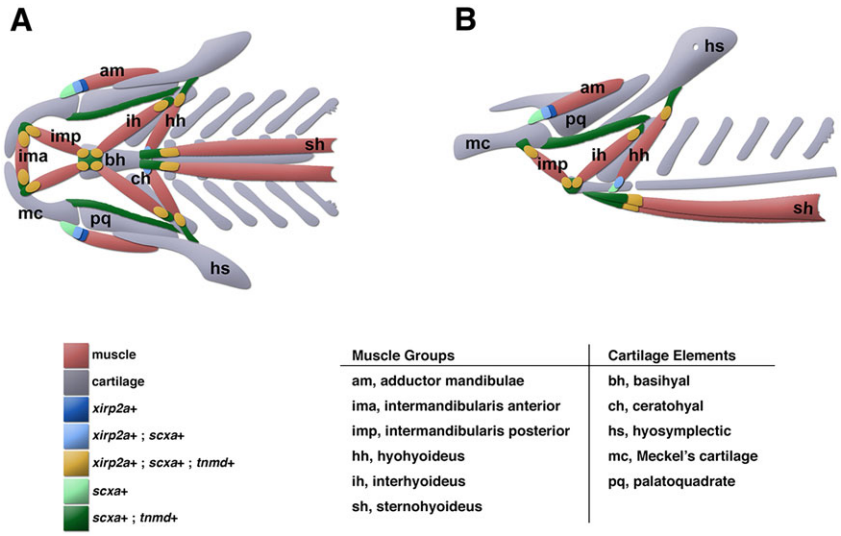

Fig. 7. *scxa*, *tnmd* and *xirp2a* expression in zebrafish craniofacial musculoskeletal tissue. (A) Ventral and (B) lateral views of craniofacial muscle (red), cartilage (gray) and tendons/ligaments. Populations of tendons and ligaments are colored according to their expression of *scxa*, *tnmd* and/or *xirp2a* based on expression data of 60-96 hpf zebrafish embryos. Only the major muscle groups attached to the tendons are depicted.

of tendon markers in regions that would form rib cartilages, indicating a dual role for *Sox5* and *Sox6* in promoting cartilage differentiation and suppressing tendon fates (Brent et al., 2005). However, this re-specification of cartilage towards a tendon cell fate in the mouse *Sox5-Sox6* double mutants was not observed in the limbs and cranial regions. Although we did observe an alteration in the pattern of the cranial tendon progenitors in the absence of *sox9*, we did not detect an increase in *scxa* expression by qPCR (data not shown). Our data demonstrate that tendon cells can form in the absence of a proper cartilage template, and even in the absence of both *sox9* genes, suggesting that tendon and ligament lineages form independently of the cartilage program. These findings gain support from studies in chick that report formation of tendon fibers in distinct locations upon surgical removal of the terminal phalanx (Hurle et al., 1990; Kardon, 1998). In addition, recent studies examining the formation of bone eminences have shown that loss of *Sox9* in *Scx*-expressing cells has no effect on limb tendon development (Blitz et al., 2013). Our studies demonstrate that a properly formed cartilage template is necessary for the organization of the ligament cells, further supporting the notion that later interactions with neighboring musculoskeletal tissues are important for their coordinated development. Throughout these and other studies, there are apparent similarities between the limb and cranial tendon development programs (Edom-Vovard et al., 2002; Grenier et al., 2009). However, it remains unclear, given the differences in the regulation of the cranial and axial musculature (Harel et al., 2009; Sambasivan et al., 2009), whether distinct modes of regulation in tendon development exist in different anatomical regions.

Since the discovery of *Scx* as the first marker of tendon progenitors in vertebrates over a decade ago, the mechanism by which Scx regulates tendon development is still not completely understood. Data from the $Scx^{-/-}$ mutant mice indicates that loss of Scx results in a loss of tendons: specifically, the force-transmitting and inter-muscular tendons. However, other categories of tendons, such as the muscle-anchoring tendons and ligaments, do form, and the null mice survive to adulthood (Murchison et al., 2007). We tested morpholinos targeted to *scxa* and *scxb*, but even at the highest concentrations, we did not detect any changes in *tnmd* expression or in jaw morphology (data not shown). The inability to detect an early function for *scx* is not surprising given that the mouse knockouts are viable and do not have any reported defects in their cranial morphology (Murchison et al., 2007). However, a role for *scxa* or *scxb* in later tendon developmental events may exist in the fish, but cannot be evaluated owing to the limitations of morpholino-based loss-of-function approaches.

The jaw morphology of the cypriniforme fishes is very diverse (Hernandez et al., 2008; Staab et al., 2012) due to pressures for prey capture and food consumption. Feeding advantages are important for ensuring survival, especially early in life as mortality is high at larval stages (Houde and Schekter, 1980). For zebrafish, which begin feeding by 5 dpf (days post-fertilization), it is essential to have a functioning cranial musculoskeletal apparatus. Studies of feeding mechanics of larval zebrafish identify three main phases of the food strike, which primarily involve the depression of the hyoid arch through the contraction of the sternohyoideus. Additionally, it is thought that the muscles attaching to the cleithrum help to prevent its anterior displacement, which allows efficient opening of the buccal cavity (Hernandez et al., 2002). Interestingly, the major regions of overlapping *scxa* and *tnmd* expression are found exactly at these attachment points, where the sternohyoideus muscles meet the ceratohyal cartilages and where the sternohyoideus attaches to the cleithrum. The other main location of *scxa* and *tnmd* co-expression is the ligament connecting the posterior end of Meckel's cartilage to the hyoid arch. In other fish species, the mandibulo-hyoid ligament acts along with the hyoid bone like a pulley to open the mandible through the transduction of force originating from the sternohyoideus (Pitcher, 1986; Van Wassenbergh et al., 2013). Consequently, the mandibulo-hyoid ligament functions primarily in enabling movement, rather than maintaining stability, as is a defining characteristic of ligaments. These ligaments are likely serving a similar purpose in the zebrafish larvae, although functional tests would be necessary to demonstrate this hypothesis. Ultimately, we determined that the main locations of *scxa* and *tnmd* co-expression coincide with regions that have been associated with force production for feeding. The location of the early craniofacial tendon progenitors is significant for future studies aimed at understanding the evolution of functional morphology in feeding behaviors.

With the discovery that the craniofacial tendons in zebrafish are molecularly and functionally similar to those of higher vertebrates, it is now feasible to use the zebrafish as a new model to study the regulation of tendon formation and differentiation. The zebrafish offers many advantages as a developmental and genetic system because they are amenable to high-throughput screening approaches and live-image analysis of patterning events (Dahm and Geisler,

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2006). Therefore, the zebrafish provides a powerful opportunity to gain new insights into the regulation of tendon specification events and expand our understanding of cellular behaviors governing tendon patterning.

MATERIALS AND METHODS

Fish maintenance, genotyping and chemical treatments

Zebrafish were staged and maintained as described (Kimmel et al., 1995; Westerfield, 1995). *sox10:kaede* (Dougherty et al., 2012) were obtained from Dr Eric Liao (Massachusetts General Hospital, Boston, MA, USA), and *sox9b*^{fh313} (Manfroid et al., 2012), *sox9a*^{hi1134} (Yan et al., 2002) and *myod1*^{fh261} (Hinits et al., 2011) were obtained from Zebrafish International Resource Center. Single embryos were genotyped for *sox9b*^{fh313}, *sox9a*^{hi1134} and *myod1*^{fh261} as described previously (Hinits et al., 2011; Manfroid et al., 2012). For chemical treatments, embryos were incubated in 0.1% DMSO, 5 µM SU5402 and 50 µM SB-431542 (Tocris) from 32-56 hpf. All animal work was performed with IACUC approval.

RT-PCR and qPCR

Whole zebrafish or adult zebrafish tendons, ligaments and liver (negative control) were used for RNA extraction and cDNA synthesis (Invitrogen #18373-019; ThermoScientific #K1621). No-reverse transcriptase controls and no-template controls for each primer set did not amplify products. Primer sequences are: *scxa* (5′-ATTCGAGAGCCTTGTGGAGA-3′ and 5′-GCAGCATCTGCAGTCAAGAG-3′); *scxb* (5′-TCATCACCACCACA-ACGTCT-3′ and 5′-TGTGCAGTTCGTTCAGTTCA-3′); and *β-actin* (5′-TTCCTGGGTATGGAATCTTGCGGT-3′ and 5′-TCGAGAGTTTA-GGTTGGTCGTTCGT-3′). Embryos at 72 hpf were pooled, and *sox9a-sox9b*-deficient embryos were identified by otic vesicle loss. RNA extraction (Qiagen #74104), cDNA synthesis (Roche #04379012001) and TaqMan Fast Universal gene expression assays with a StepOnePlus Real-Time PCR Machine (Applied Biosystems) were performed. There were no-reverse transcriptase controls for each sample, and reactions were performed in quadruplicate. Sample expression was normalized to *β-actin* and the FAM-dye probes used were: *scxa* (Dr03104896) and *β-actin* (Dr03432610).

Morpholinos and injection

Morpholinos (Gene Tools) were injected at the one-cell stage as described previously: *tfap2a E212* (O'Brien et al., 2004), *foxd3* (Montero-Balaguer et al., 2006), *myod1* (Lin et al., 2006), *myf5* (Lin et al., 2006), *sox9a* (Yan et al., 2002) and *sox9b* (Yan et al., 2005). Standard control oligo (5′-CCTCTTACCTCAGTTACAATTTATA-3′) was injected at equivalent concentrations. Morpholinos designed against *scx*: *scxa1* (5′-GATTTCTA-ACGCTCTCCACAAGGCT-3′), *scxa2* (5′-CCATCGCAAAAGACATC-ATCAACTT-3′), *scxb1* (5′-ATCGCAAAAGACATGCCTCAGTACT-3′), and *scxb2* (5′-AGCTTAGTTTCGATTTTACACCCAA-3′) were co-injected.

Cloning and expression analysis

scxb transcripts were cloned into pCR2.1-TOPO (Invitrogen) and pBluescript through nested PCR using the following primers (5′-AGGGATACGGTTCACGTTTG-3′ and 5′-GCTGGGTGTACGCA-AGAAGAG-3′). Colorimetric *in situ* hybridization was performed in whole mount or on 10 µM paraffin sections as described (Brent et al., 2003), with minor modifications. Probes include: *scxa* (accession numbers AL923903 and AL921296), *myog* (GenBank accession number BC078421), *sox9a* (a gift from N. Trede, Huntsman Cancer Institute, Utah, USA), *col1a2* (DY559926), *col2a1* (Yan et al., 1995), *xirp2a* (cb1045; GenBank accession number CF943681) and *tnmd* (GenBank accession numbers BC155615 and EV754577). Fluorescent *in situ* hybridization was performed as described previously (Talbot et al., 2010), with minor modifications. Digoxigenin and fluorescein-labeled probes were revealed using TSA-fluorescein/Cy3 substrates (PerkinElmer). Antibody staining was performed as described previously (Clement et al., 2008), with minor modifications. Primary antibodies (1:500) were anti-collagen type II [II-II6B3, Developmental Studies Hybridoma Bank (DSHB)], anti-sarcomere (MF20, DSHB), anti-myosin heavy chain (A4.1025, DSHB) and anti-kaede (MBL #PM102). Secondary antibodies (1:500) from Southern Biotech were goat anti-mouse IgG1-HRP, goat anti-mouse IgG2b-HRP and rat anti-mouse IgG2a-HRP. Secondary antibodies from Life Technologies were goat anti-mouse Alexa Fluor 647 (1:400), Alexa Fluor 488 goat anti-mouse-IgG (1:450) and goat anti-rabbit-HRP (1:500). Detection was performed using TSA-Cy3/Fluorescein/Cy5 substrates (PerkinElmer). DAPI staining was performed where indicated.

Image analysis

Embryos were imaged using a Zeiss upright compound microscope, Zeiss AxioZoom V16 with ApoTome2 or Nikon Eclipse 80i, and images were acquired using Nikon ACT-1, Zeiss Zen or NIS Elements. Confocal images were taken using a Zeiss LSM 710 NLO microscope and images were acquired and processed with Zeiss Zen, Imaris (Bitplane) or ImageJ software using the maximum-intensity projection feature applied to *z*-stacks and/or tile-stitching. Fig. 2A,B were generated with the Zeiss Zen software by overlaying the red, green and bright-field channels of each image, and the opacity and levels were adjusted in Photoshop to optimize the visualization of all three stains. Fig. 3A,B were tiled together in Photoshop from several images taken of the same section. Fig. 4E,F were also tiled together in Photoshop and using the tiling function on the Zen software during image capture. Some images for Fig. 2 had their channels switched to keep the consistency of gene color scheme.

Fate mapping

Tg(s*ox10:kaede*) embryos were mounted in low-melting point agarose with tricaine at 22 hpf. Craniofacial regions of embryos were photoconverted using the DAPI channel, with regional selectivity accomplished by varying the size of the pinhole. At 72 hpf, representative photoconverted Tg(*sox10:kaede*) embryos were mounted and imaged or were processed for expression analysis.

Ultrastructural analysis

Craniofacial tendon and ligament tissues of adult zebrafish were processed and analyzed with a JEOL 1011 electron microscope at the Microscopy Core of the Program in Membrane Biology (PMB) at Massachusetts General Hospital.

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Competing interests

The authors declare no competing financial interests.

Author contributions

J.W.C. and J.L.G. designed and performed the experiments, analyzed the data generated and wrote the paper.

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Supplementary material

Supplementary material available online at
http://dev.biologists.org/lookup/suppl/doi:10.1242/dev.104067/-/DC1

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