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.

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 Collal (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 (Himits 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., 2013; 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
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-fertilization) 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. 1P, arrow), but is absent from the lateral regions (c and muscle (arrow) at 120 hpf. (E-H) Section in situ hybridization of scxa expression (arrowhead) between cartilage (c) and muscle (arrow) at 120 hpf. (E-S) Expression of scxa (E-H), tnm'd (I-L), xirp2a (M-P) and col1a2 (Q-S) at 72 hpf. All four genes are expressed at the attachment point of the sternohyoideus to the ceratohyal and basihyal cartilages (F,I,N,R, arrowhead; magnified in G,K,O,S). scxa, tnm'd 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 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: tnm'd and collagen 1a2 (col1a2). At 72 hpf, robust tnm'd 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, tnm'd 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...
Sca expression was found at muscle-to-cartilage or cartilage-to-cartilage attachments (Fig. 2A,B). In addition, sca, tnmd, col1a2, and xirp2a are co-expressed at these attachment sites. At 60-72 hpf, colocalization of xirp2a and sca 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 sca and tnmd transcripts were colocalized in the head and fin regions between 60 and 80 hpf (Fig. 2C,G-I). We also found that sca, xirp2a, and tnmd expression are temporally dynamic in the craniofacial region. Robust expression of sca 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, sca 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 sca, 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 sca at 48 hpf (Fig. 2K-M), and col1a2 and tnmd at 96 hpf (Fig. 2O). Together, the expression of sca, 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, sca 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-hyoideus 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.
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-fox3 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 (Ts650: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 ceratohalys. 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. S1LJ). 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 craniofacial tendon development, we examined scxa expression upon morpholino-mediated knockdown of both myod1 and myf5 or knockdown of myf5 in myod1myf5 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,
we observed a virtual loss of scxa expression in the head and fins of myod1-myf5-deficient embryos (Fig. 5LJ), 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 (Larbuissone et al., 2013; Walsh 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 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<sup>hs313</sup> or sox9a<sup>ahi1134</sup> 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

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 xip2a 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.
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).
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,II; 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 normal 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,1J,II, 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 myonemes 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
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 function for 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 tnmnd 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 tnmnd 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 tnmnd 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, 2014).
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
363 (Manfroid et al., 2012), sox9a
1134 (Yan et al., 2002) and myod1
261 (Hinits et al., 2011) were obtained from Zebrafish International Resource Center. Single embryos were genotyped for sox9p
363, sox9a
1134 and myod1
261 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'-ATTCCAGAGCCTGGTGGAAG-3' and 5'-GCCAGCATCTGCAGAAGG-3'); scxb (5'-TCATCAACCACACGCTCT-3' and 5'-TGGACAGTCCGTGATCGTA-3'); and β-actin (5'-TTCTGTTGATGAACTCCTGCGG-3' and 5'-TCGAGAAGTTTGAAGTTCGT-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 Expression Analysis System (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-dy 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: topa2a 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 scxa: scxa1 (5'-GATTTTCAACGCTTCCTCAGAGGCT-3'), scxa2 (5'-CCATCGCAAAAGACATC-ATCAACCT-3'), scxb1 (5'-ATCGCAAGAAGACTGCTCCTGACT-3') and scxb2 (5'-AGCTAGTTTCGATTTTGTCACCACT-3') were co-injected.

Cloning and expression analysis
scx transcripts were cloned into pCR2.1-TOPO (Invitrogen) and pBluescript through nested PCR using the following primers (5'-AGGATACGGTTCACGTTTG-3' and 5'-GCTGGTGATCGTACGGAAGAAG-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 AL921196), myog (GenBank accession number BC078421), sox9a (a gift from N. Trede, Huntsman Cancer Institute, Utah, USA), col1a2 (DY559926), col2a1 (Yan et al., 1993), irx2a (cb1049; GenBank accession number CP943681) and tnnm (GenBank accession numbers BC155615 and EV755477). Fluorescent in situ hybridization was performed as described previously (Talbot et al., 2010), with minor modifications. Digoxygenin 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-I6B3, 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 IgG2a-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 Zeno 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(sox10: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
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