Identification of New Regulators of Tendon Development Using the Zebrafish Model

Identification of New Regulators of Tendon Development Using the Zebrafish Model

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Abstract

Tendons transmit force from muscle to bone, enabling movement; ligaments connect bone to bone, maintaining stability. Despite their importance, tendon and ligament development is relatively uncharacterized. In this dissertation, the zebrafish is introduced as a model to study tendon development; tendon populations in zebrafish are homologous to their force-transmitting counterparts in higher vertebrates. The zebrafish craniofacial tendons and ligaments were identified based on expression analysis of genes enriched in mammalian tendons and ligaments (*scleraxis*, *collagen 1a2* and *tenomodulin*) or in zebrafish tendon-like myosepta (*xirp2a*). The craniofacial tendons and ligaments were fate-mapped to arise from cranial neural crest cells (CNCCs). Loss-of-function genetic approaches demonstrated that craniofacial and pectoral fin tendons require muscle for their maintenance and cartilage for their organization, with neither tissues required for their induction. Lastly, adult zebrafish and mammalian tendons and ligaments share similar ultrastructural properties.

The second part of this dissertation sought to understand the processes governing tendon cell induction. A zebrafish chemical screen identified Lovastatin and Simvastatin as compounds that affect craniofacial tendon development. Statins caused a dose-dependent expansion of the craniofacial and pectoral fin tendon programs. The expansion is specific to the tendon and ligament lineages, and is not observed in other musculoskeletal tissues. Chemical rescue and genetic loss-of-function experiments demonstrated that statin-mediated expansion of *scleraxisa*

is specific to the mevalonate pathway and Hmgcr inhibition. Furthermore, the expansion of tendon progenitors is mediated through inhibition of geranylgeranylation, and specifically geranylgeranyltransferase type I (GGTase I). We discovered that this phenotype is due to a change in cell fate specification, and not an increase in cell proliferation. Fate mapping experiments demonstrated that the expanded craniofacial *scleraxisa*-positive tendon progenitors arise entirely from CNCCs. As statin caused a reduction of CNCC-derived cartilage, statin is speculated to promote expansion of the craniofacial tendon program by directing CNCCs towards a tendon fate through regulation of GGTase I activity. Taken together, this dissertation advances our understanding of vertebrate tendon development. The first part establishes the zebrafish as a model to study tendon development; and the second part explores the regulation of the mevalonate pathway in tendon development.

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my parents, Debbie, and Cari

for their unconditional love.

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Chapter 1

Introduction

The human body has captured fascination since the Renaissance, as evident from drawings by Leonardo da Vinci (1452-1519) of the anatomy of the human body (Royal Collection, Windsor Castle, London, UK). A foundational component of the body are the tendons and ligaments - tendons transmit force generated by the contracting muscle to the bone, enabling movement; ligaments connect bone to bone, maintaining stability. Though tendons and ligaments differ in the types of tissues they connect, they have similar ultrastructure. (Jozsa and Kannus, 1997; Woo et al., 2000) Human tendons are capable of withstanding up to 9 kN of force, the equivalence of 12.5 times the body weight (Komi, 1990). The capacity of the tendon to tolerate tensile stress is unrivaled to that of other tissues. Tendon and ligament development is intimately connected with the development of the adjacent muscle, cartilage, and bone of the musculoskeletal system (Wortham, 1948). The initial fascination of the human body has lead to the morphological characterization of the tendons and ligaments, followed by subsequent identification of the molecular cues essential for their development and regeneration (Docheva et al., 2015; Huang et al., 2015; Nourissat et al., 2015; Subramanian and Schilling, 2015).

Studies of tendons have historically been embodied in the disciplines of anatomy, cellular physiology, and biomechanics, which resulted in a wealth of information addressing the fundamental architecture of newborn and adult mammalian tendons (Butler et al., 1978; Elliott, 1965; Kastelic et al., 1978). In stark contrast, the molecular signals involved in tendon development are largely unexplored due primarily to the absence of robust tendon markers. Accordingly, the discovery of *Scleraxis* as the earliest known marker of tendon and ligament progenitors that faithfully marks the lineage in all anatomical locations (Schweitzer et al., 2001) was a turning point in tendon biology as it paved the way for investigating the early formation of tendons and ligaments.

In the last decade, the use of *Scleraxis* in the mouse and chick systems has led to the discovery of several factors involved in tendon development (Schweitzer et al., 2010), and henceforth, provided fruitful insights into the molecular mechanisms governing tendon formation in the developing embryo. With the advent of high-throughput chemical genetic screens in zebrafish (Kaufman et al., 2009), we propose that the zebrafish model offers a vertebrate genetic system that can be utilized for discovering novel genetic pathways relevant to tendon biology. Such discoveries may exploit the preexisting literature of known bioactive compounds and clinical observational studies of tendon-associated conditions, and thereby, gain headway in elucidating the cellular and molecular mechanisms involved in tendon development.

Tendon/ Ligament Injuries

The ability of tendons and ligaments to transfer high tensile forces anchor them as the keystone components of locomotion for the entire lifetime of an organism. In the event of reduced or complete loss of function of the tendons/ligaments as a consequence of occupational-related injuries, sports-related injuries, or age-related degeneration, the effects can be incapacitating (Jozsa and Kannus, 1997). It is predicted that 10% of the population experience some form of Achilles tendinopathy before the age of 45 (Kujala et al., 2005). In the United States, an estimated $30 billion is spent annually on musculoskeletal injuries, with approximately 45% being connective tissue injuries (Praemer et al., 1999), which translates to an estimated 300,000 patients undergoing tendon repair (Pennisi, 2002). Due in part to their low cellular content (Jozsa and Kannus, 1997), low proliferation rates (Bi et al., 2007), and avascular environment (Petersen et al., 2000; Petersen et al., 2002), tendons and ligaments are highly inefficient at healing once damaged (Sharma and Maffulli, 2005). The high prevalence of

tendinopathies stands in contrast to the limited treatment options (Jozsa and Kannus, 1997), which are often plagued by potential complications that include re-rupture of the repair site (Sharma and Maffulli, 2005). While therapeutic interventions of connective tissue injuries warrant much promise (Moshiri and Oryan, 2013), successful pursuit in these endeavors requires a more intricate understanding of the molecular mechanisms governing the formation and development of tendons and ligaments.

Tendon structure

Biochemical composition

Tendons consist of tenoblasts (immature tendon cells) and tenocytes (mature tendon cells) embedded in an extracellular matrix. Tenoblasts and tenocytes comprise 90-95% of the cellular elements of tendons. (Jozsa and Kannus, 1997) The relative proportion of cells in the tissue decreases with age, speculated to be a consequence of increased extracellular matrix production, with a higher cell-to-matrix ratio in newborn tendons compared to that of aged tendons. The spindle-shaped tenoblasts are metabolically active in collagen and matrix protein biosynthesis, as exhibited by a high nucleus-to-cytoplasm ratio; whereas, the elongated tenocytes have a lower nucleus-to-cytoplasm ratio and reduced metabolic activity. (Ippolito et al., 1980) The remaining cellular elements comprise of a unique population of cells at the enthesis, synovial cells of the tendon sheath, and vascular cells of the arterioles (Jozsa and Kannus, 1997).

The non-cellular constituents of mammalian tendons are water (58% to 70% of the wet weight); collagen (~75% of dry weight), predominantly Collagen type I; elastin (2% of dry weight); proteoglycans (1% of dry weight); and inorganic substances (<0.2% of dry weight) (Elliott, 1965; Kjaer, 2004). Elastin is speculated to function in the recovery of the collagen

fibers back to the resting-state configuration following muscle contraction (Minns et al., 1973). The tendon matrix, comprised of proteoglycans, glycosaminoglycans (GAGs), structural glycoproteins, and a variety of small molecules, stabilizes the collagen skeleton and maintains ionic homeostasis (Jozsa and Kannus, 1997). Proteoglycans, glycosylated proteins covalently attached to a negatively-charged GAGs, function in the viscoelasticity of the tendon but do not contribute to the tensile strength (Puxkandl et al., 2002; Robinson et al., 2004). Inorganic components are essential for the formation and metabolism of collagen networks (Jozsa and Kannus, 1997). In adult mice with mutations in collagen or the tendon-enriched proteoglycans (i.e. Decorin, Fibromodulin, Lumican, Biglycan) (Hardingham and Fosang, 1992), the tendons have abnormal collagen fibrillogenesis, characterized by defects in structure, fibril diameter, and/or fibril quantity (Connizzo et al., 2013; Gaut and Duprez, 2016), further underscoring the importance of the matrix in tendon development.

Architecture

Tendons are a hierarchically organized tissue with the fundamental structural unit being the collagen fibril (Figure 1.1A) (Prockop and Kivirikko, 1995). They are characterized by a *D*-periodicity due to the axial arrangement of the collagen molecules (Kadler et al., 1996). Macroscopically, the collagen fibrils organize to form collagen fibers (primary bundle), fascicles (secondary bundles), tertiary bundles, and finally the tendon itself (Figure 1.1B) (Silver et al., 2003). The biomechanical properties of the tendon/ligament are dependent on the intra- and inter-molecular bonds of the collagen network (Butler et al., 1978). The tensile properties at each structural level may be visualized with a stress-strain curve (Figure 1.1C). The endotenon, a sheath of connective tissue, binds the collagen fibers together and provides a network accessible

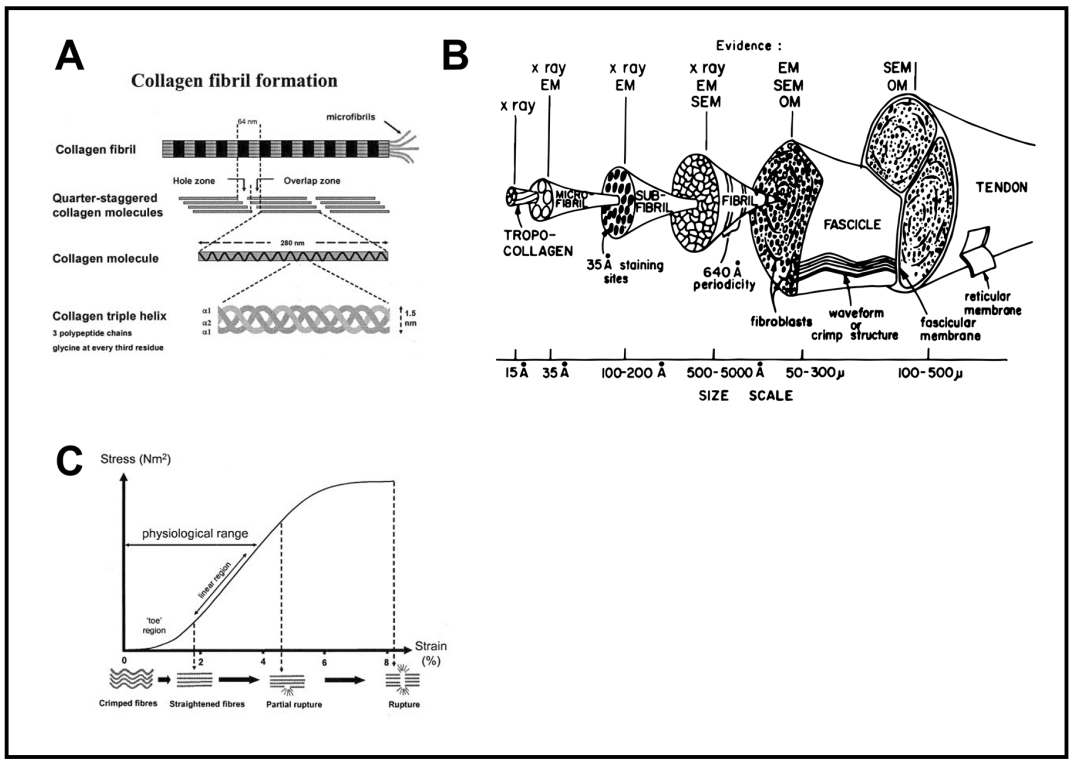

Figure 1.1. Architecture of tendons. (**A**) Microstructure of collagen fibrils. Collagen fibrils comprise of five collagen molecules arranged in a staggered configuration, packed and cross-linked with one another. Each collagen molecule consists of three coils of polypeptides arranged in a helical conformation. (**B**) Tendon hierarchy. (**C**) Tendon stress-strain curve. The curve is partitioned into three domains, each of which corresponds to deformation at different structural levels. In the resting state, collagen fibrils display a wavy configuration. In region I (>2% strain), the collagen fibrils lose their resting state macroscopic crimp and become increasingly straightened and paralleled. In region II (2-4% strain), the collagen fibrils permanently lose the wavy configuration due to intra-molecular lateral sliding of the collagen molecules and stretching of the of collagen triple-helices. In regions I and II, the resting state configuration is restored upon release of the tensile force. In region III (4-8% strain), microscopic rupture of the collagen fibers occurs due to failure of crosslinks as the collagen fibers slide pass one another. Above 8% strain, macroscopic failure occurs from intra-fibril damage as the stress has exceeded

the maximum load that the collagen fibers can withstand. (Butler et al., 1978; Fratzl et al., 1998; Jozsa and Kannus, 1997; Mosler et al., 1985) Reprinted from (Kastelic et al., 1978; Riley, 2004) with permission from Taylor & Francis Group and Oxford University Press, respectively.

Figure 1.1 (Continued).

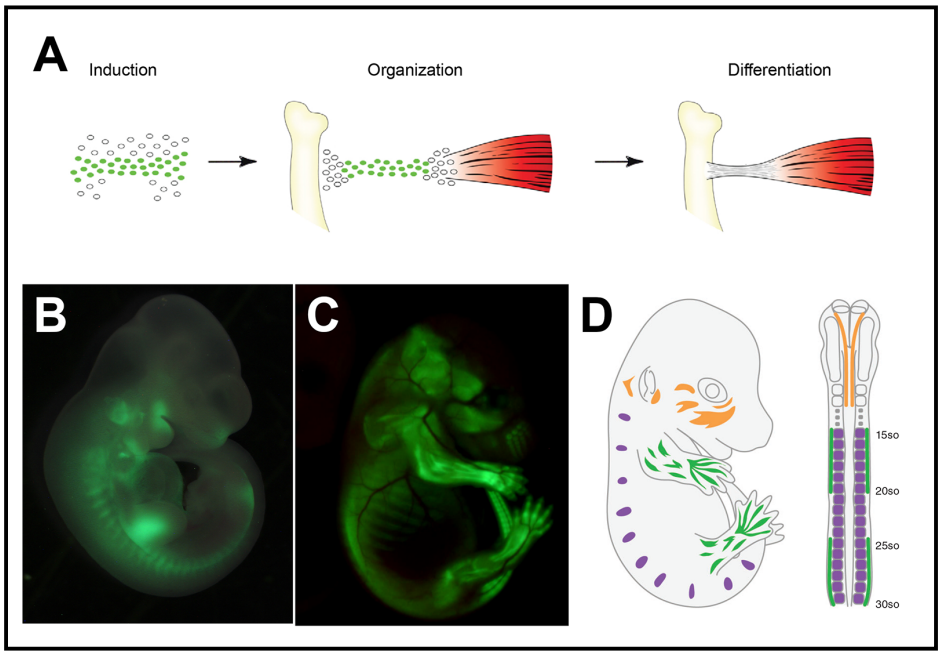

Figure 1.2. Vertebrate tendon development. (**A**) Stages of tendon developmental program. (**B, C**) mScxGFP mouse embryos at E10.5 (**B**) and E15.5 (**C**) reveal ScxGFP expression in tendon and ligament progentiors in the craniofacial, axial, and limb tissue. (**D**) Tendons of different anatomical location have distinct embryological origins. Cranial tendons are derived from the cranial neural crest (orange); axial tendons are derived from the somites (purple); and limb tendons are derived from the lateral plate mesoderm (green). (**B, C**) are from (Jenna Galloway and Richard Pearse, unpublished). (**A, D**) Reprinted with modifications from (Gaut and Duprez, 2016; Schweitzer et al., 2010) with permission from John Wiley & Sons and The Company of Biologists, Ltd, respectively.

to blood vessels, nerves, and lymphatics. The endotenon continues distally as the inner surface of the epitenon, which surrounds the entire tendon. (Elliott, 1965)

The hierarchical arrangement described persists for the entire length of the tendon, with alterations in the architecture at the insertion sites wherein the mechanisms for attachment of structurally and compositionally different materials must confer onto the tissues the ability to withstand tension, compression, and shear forces. At the myotendinous junction, the collagen fibrils are intercalated between the finger-like projections of the muscle cells (Tidball, 1984). The sarcolemma invaginations increase the junctional surface area by 13.2-fold (Tidball, 1984), and resultantly reduces the contraction-induced force applied per surface unit of membrane (Eisenberg and Milton, 1984). At the osteotendinous junction, the enthesis, there is a continuous avascular fibrocartilage gradient that gradually shifts from the uncalcified tendon to calcified bone (Benjamin et al., 1986).

Molecular regulation of tendon development

Tendon development in vertebrates comprise of three distinct stages – induction, organization, and differentiation (Figure 1.2A). Initially, a population of undifferentiated cells are specified towards a tendon fate through signaling from the adjacent mesenchymal cells. Afterwards, the tendon progenitors organize as loose primordia between the differentiating muscle and cartilage condensations. Lastly, the tendon progenitors condense and organize into structurally distinct tendons that connect with the muscle and cartilage/bone. (Schweitzer et al., 2010)

Scleraxis

Scleraxis is a basic helix-loop-helix (bHLH) transcription factor that was identified in a yeast two-hybrid screen of an embryonic mouse cDNA library for tissue-specific bHLH proteins that heterodimerize with the ubiquitously expressed bHLH protein E12 (Cserjesi et al., 1995). Members of the bHLH transcription factor family regulate cell specification and differentiation in a variety of developmental processes (e.g. neurogenesis, myogenesis, hematopoiesis, and pancreatic development) (Bain et al., 1994; Kadesch, 1993; Lee et al., 1995; Murre et al., 1989a; Porcher et al., 1996). The bHLH motif is characterized by a basic region preceding two amphipathic alpha-helices that are separated by a loop region. Dimerization of bHLH proteins, mediated through the HLH domain, results in formation of a bipartite DNA-binding domain that binds the E-box consensus CANNTG. (Murre et al., 1989a; Murre et al., 1989b) Scleraxis is known to regulate factors essential in tendon differentiation, and thus, postulated to function in extracellular matrix formation. Scleraxis has been demonstrated *in vitro* to activate the Collagen type I alpha 1 proximal promoter (Lejard et al., 2007), and to upregulate the expression levels of proteoglycans enriched in the tendon matrix, specifically, Decorin, Fibromodulin, and Lumican (Alberton et al., 2012). Moreover, Scleraxis can positively regulate the expression of the transmembrane glycoprotein Tenomodulin, a marker of differentiated tenocytes, *in vitro* and *in vivo* (Shukunami et al., 2006).

Scleraxis is robustly expressed in the tendon and ligament lineages from the progenitor to the differentiated state (Figure 1.2B, C) (Brent et al., 2003; Grenier et al., 2009; Schweitzer et al., 2001). To better understand the role of Scleraxis in tendon development, Scleraxis-deficient mice were generated by *Cre*-mediated excision of exon 1. Scleraxis null ($Scx^{-/-}$) mice are viable and mobile but have severely impaired mobility of their limbs and back. Further analysis of $Scx^{-/-}$

embryos determined that the force-transmitting and inter-muscular tendons are severely disrupted, whereas the muscle-anchoring tendons and ligaments are present. (Murchison et al., 2007) The presence of properly formed tendons and ligaments in *Scx*$^{-/-}$ embryos indicates that while Scleraxis is a faithful marker of the lineage, it is not required for the development of all tendons and ligaments.

Early growth response

The early growth response (Egr) proteins are a family of zinc-finger transcription factors (Joseph et al., 1988) that are the mammalian homologs to Stripe in the *Drosophila melanogaster* (Frommer et al., 1996). There are two known splice variants of the *stripe* transcript, *stripeA* and *stripeB* (Frommer et al., 1996). In the *Drosophila* thorax, StripeB is an essential regulator in the induction of tendon-like progenitors and StripeA is an essential regulator in the terminal differentiation of tendon-like cells (Becker et al., 1997; Fernandes et al., 1996; Volohonsky et al., 2007; Vorbruggen and Jackle, 1997). Conjointly, Egr1 and Egr2 are expressed in the developing tendons of the mouse and chick (Lejard et al., 2011). In the mouse limb, Egr1 and Egr2 are first detected by *in situ* hybridization at stages that correspond to differentiating tendon cells (Lejard et al., 2011), suggesting that Egr1 and Egr2 in vertebrates are not involved in specification of tendon cell fate. Nevertheless, it has been shown from *in vivo* studies that Egr1 and Egr2 are each sufficient to induce expression of *Scleraxis* and *Collagen type I alpha 1* (Lejard et al., 2011). To better understand the role of Egr1 and Egr2 in tendon development, mouse mutants have been examined for embryonic and adult tendon phenotypes. *Egr1*$^{-/-}$ and *Egr2*$^{-/-}$ embryos both displayed a slight reduction in collagen fibril number compared with controls (Lejard et al., 2011). The tails of adult *Egr1*$^{-/-}$ mice have a reduced quantity of individual tendons, greater

interfibrillar space, and reduced mechanical properties, compared with controls. Furthermore, the tendons of adult *Egr1*$^{-/-}$ mice display reduced expression of *Scleraxis*, *Tenomodulin*, *Biglycan*, and several tendon-associated collagens. (Guerquin et al., 2013) The Egr2 mouse knockout is perinatal lethal (Swiatek and Gridley, 1993). These findings support a later role for the Egr proteins in regulation of the tendon extracellular matrix.

Tenomodulin

Tenomodulin is a type II transmembrane protein that was identified based on its sequence homology to the cartilage-specific glycoprotein Chondromodulin-I (Yamana et al., 2001). Type II transmembrane proteins are characterized by a luminal C-terminus and cytoplasmic N-terminus (Chou and Elrod, 1999). *Tenomodulin* expression has been characterized in the axial and limb tendons and ligaments of chick, and observed to be temporally delayed in its onset of expression compared to *Scleraxis* (Shukunami et al., 2006), reflecting its induction at a more differentiated stage of the lineage. However, Tenomodulin is not tendon/ligament-specific, as it is also expressed in dense connective tissue such as the muscle sheath (epimysium), sclerocornea, and retinal pigment epithelium (Oshima et al., 2003; Shukunami et al., 2001; Yamana et al., 2001). To better understand the role of Tenomodulin in tendon development, Tenomodulin-deficient mice were generated by insertion of an IRES-LacZ neomycin cassette into the translation start site of exon 1. Tenomodulin null (*Tnmd*$^{-/-}$) mice display disruptions in the tendon, such as reduced proliferation in newborn tendons and reduced cell number in adult tendons. Moreover, ultrastructural analysis revealed greater variation of collagen fibril diameters with a shift towards a larger diameter in adult *Tnmd*$^{-/-}$ mice compared to wild-type littermates. (Docheva et al., 2005) These findings support a later role for Tenomodulin in tendon

development in collagen fibril maturation.

Fibroblast growth factor

The fibroblast growth factors (FGFs) are a family of structurally related proteins (Itoh, 2007) involved in a diverse array of developmental processes (Beenken and Mohammadi, 2009). FGFs are classified according to mechanism of action (i.e. intracrine, paracrine, or endocrine signaling) and structural homology (Itoh, 2007). Intracrine FGFs function as intracellular molecules in a FGF receptor (FGFR)-independent manner (Goldfarb et al., 2007; Schoorlemmer and Goldfarb, 2002). Paracrine and endocrine FGFs function by binding to and activating one of four FGFRs (FGFR1-4), single-pass transmembrane proteins with a cytoplasmic tyrosine kinase domain and three extracellular immunoglobulin-like domains (Mohammadi et al., 2005). Binding specificity of the ligand-receptor interaction is regulated through alternative splicing of FGFR transcripts (Ornitz and Itoh, 2015), distinct ligand binding affinities and signaling properties of FGFR variants (Ornitz and Itoh, 2015; Ornitz et al., 1996), and distinct temporal and spatial expression of the ligands and receptors (Beenken and Mohammadi, 2009).

FGF signaling has been demonstrated to be required for the induction of axial tendons progenitors. *FGF8*-expressing cells in the central myotome induce expression of *Scleraxis* in the sclerotome through interaction with the FGF receptor *FREK*-expressing cells at the myotomal border to subsequently give rise to the syndetome. FGF is sufficient for induction of *Scleraxis* and the *FREK* receptor is necessary for induction of *Scleraxis*, as demonstrated using the chick system by retroviral misexpression of FGF8 or a dominant-negative FREK receptor in the presomitic mesoderm, in addition to implantation of FGF8 or FGF4-soaked beads in the somite. (Brent et al., 2003) It was subsequently characterized that formation of the syndetome results

from transduction of myotomal FGF signals to the sclerotome through the intracellular Ets transcription factors Pea3 and Erm. Pea3 and Erm are both necessary and sufficient for FGF-dependent induction of *Scleraxis*. (Brent and Tabin, 2004) Moreover, FGF has been shown to be necessary and sufficient for *Scleraxis* in the limb mesenchyme (Brent et al., 2005; Edom-Vovard et al., 2002). However, this mechanism of a myotomal-derived signal is not employed in the induction of limb or branchiomeric tendons, which are muscle-independent processes (Brent et al., 2005; Grenier et al., 2009). Given the role of FGFs in patterning the limb bud (Martin, 1998), FGF is likely involved in tendon induction secondary to maintaining an undifferentiated, proliferative population of mesenchyme in the limb bud (Crossley et al., 1996; Mariani et al., 2008).

Transforming growth factor beta superfamily

The transforming growth factor beta (TGFβ) superfamily comprise of extracellular growth factors involved in a multitude of developmental processes (Dunker and Krieglstein, 2000), including the patterning of the axial and appendicular skeletons (Serra and Chang, 2003). The superfamily is classified into two subfamilies based on mechanism of action and sequence homology. The TGFβ/activin/nodal subfamily of ligands bind to type II receptors and have been characterized to activate intracellular signaling through the Smad2/3 pathway. The bone morphogenetic protein (BMP)/ growth differentiation factor (GDF) subfamily of ligands bind either to type I or type II receptors and have been characterized to activate intracellular signaling through the Smad1/5/8 pathway. Both type I and type II receptors are single-pass transmembrane proteins with a cysteine-rich extracellular domain and cytoplasmic serine/threonine kinase domain. (Chang et al., 2002)

Transforming growth factor beta subfamily

TGFβ signaling has been demonstrated to be required for the maintenance of tendon cell fate. Disruptions in TGFβ signaling, as studied in ligand-deficient (*Tgfb2*$^{-/-}$;*Tgfb3*$^{-/-}$, *Tgfbr2*^{Prx1Cre}) or tissue-specific receptor-deficient (*Tgfbr2*$^{fl/fl}$;*Wnt1-Cre*) embryos, result in normal initiation of *Scleraxis*-positive tendon progenitors and a subsequent near complete loss of the progenitors in all anatomical domains at tendon organization stages (Oka et al., 2008; Pryce et al., 2009). TGFβ2 has also been shown to be sufficient in inducing *Scleraxis* in organ culture (Oka et al., 2008; Pryce et al., 2009) and in cultured embryonic fibroblasts (Pryce et al., 2009). Furthermore, loss of GDF8, a muscle-specific TGFβ family member (McPherron et al., 1997) that signals through the Smad2/3 pathway (Rebbapragada et al., 2003), results in improper tendon formation in adult *GDF8*$^{-/-}$ mice (Mendias et al., 2008). Specifically, the limb tendons have decreased fibroblast density and are smaller in size (Mendias et al., 2008). Likewise, loss of Smad3 leads to adult *Smad3*$^{-/-}$ mice with limb tendons characterized by reduced tensile strength (Katzel et al., 2011), disruptions in the architecture of the collagen fibers, and reduced expression of tendon extracellular matrix proteins (Berthet et al., 2013). Taken together, TGFβ signals are important in the maintenance of tendon cell fate, though it is unclear if TGFβ directly or indirectly regulates *Scleraxis* as the disruptions to TGFβ signaling were not tissue-specific.

Bone morphogenetic protein / growth differentiation factor subfamily

Studies regarding BMP signaling are less conclusive. Initially, BMP signals have been shown to be necessary and sufficient to downregulate *Scleraxis,* as demonstrated by overexpression of BMP or the BMP antagonist Noggin in limb mesenchyme of chick (Schweitzer et al., 2001). In contrast, GDF5 (BMP14) and GDF7 (BMP12) have been shown to

regulate tendon matrix development. In adult *GDF7*$^{-/-}$ mice, the Achilles tendon have reduced concentration of glycosaminoglycan in the matrix and reduced diameter of collagen fibrils. Notwithstanding, these compositional and ultrastructural defects of the tendon did not translate to defects in its tensile properties (Mikic et al., 2006), perhaps due to functional redundancy of the GDFs. More pronounced, the Achilles tendon of adult *GDF5*$^{-/-}$ mice have reduced collagen content and reduced ability to tolerate tensile stress (Mikic et al., 2001). Taken together, a subset of BMP/GDF signals are involved in regulating tendon development. The seemingly contradictory findings may be accounted for by differences in the embryonic and post-natal regulation of tendon development, or perhaps attributable to species-specific differences. Additionally, it is unclear whether BMP indirectly regulates tendon development, with tendon disruptions occurring secondary to the skeletal defects (Salazar et al., 2016).

Formation and patterning of tendons

Tendons are categorized based on their anatomical location – craniofacial tendons, limb tendons, and axial tendons, each of which are derived from distinct embryological origins (Figure 1.2D). In the following section, I will present the embryonic development of the tendons/ligaments, the pertinent anatomy, and findings regarding the molecular crosstalk with the neighboring muscle and cartilage. I will additionally present notable differences in the anatomy and developmental programs between teleosts and amniotes, which diverged from a common ancestor about 450 million years ago (Figure 1.3) (Hedges, 2002), within an evolutionary context to address the structural and molecular differences in their body plans.

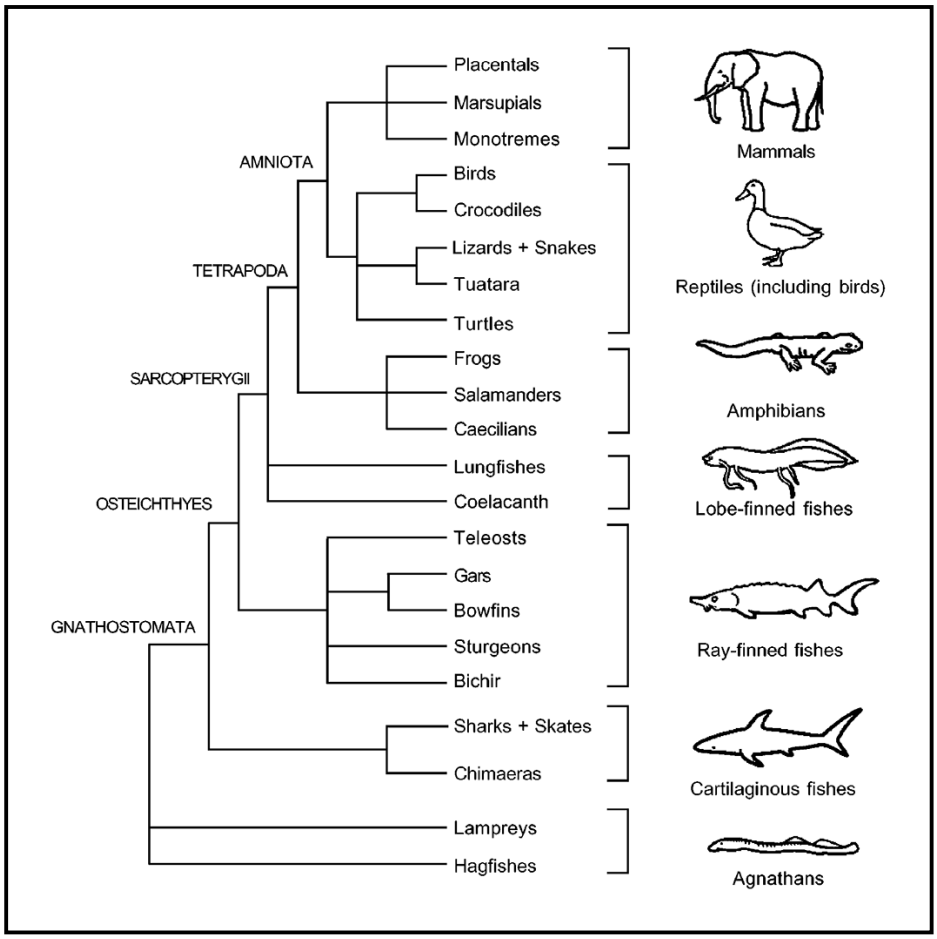

Figure 1.3. Phylogeny of gnathostomes based on morphological, paleontological, and molecular data. Disputable relationships are indicated as polytomies. Reprinted from (Meyer and Zardoya, 2003) with permission from Annual Reviews.

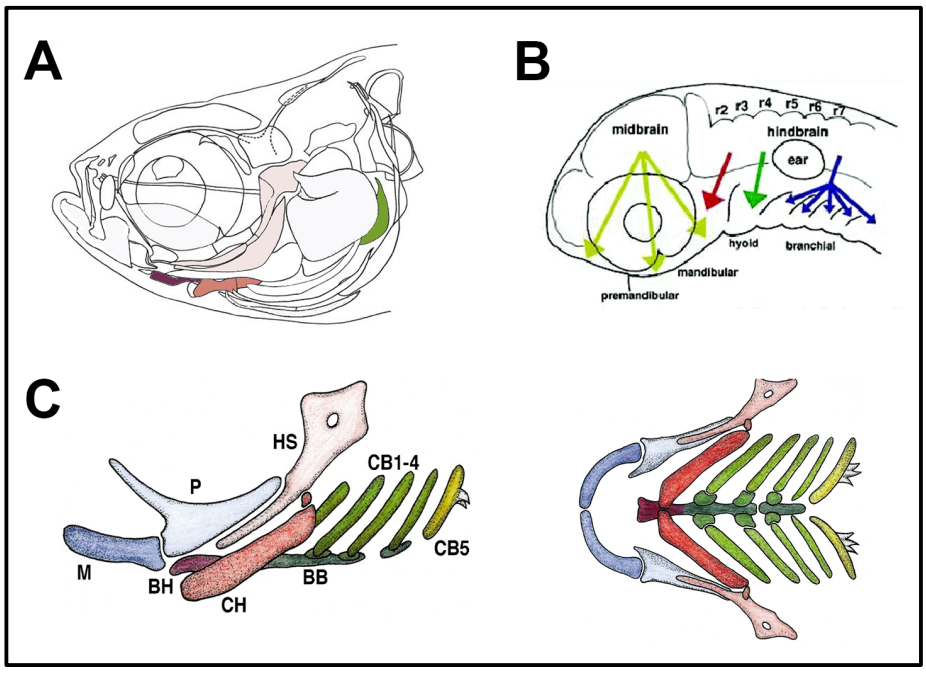

Figure 1.4. Skeletal development of the pharyngeal arches in teleost. (**A**) Diagrammatic view of craniofacial bones in the adult zebrafish, lateral view. (**B**) Streams of cranial neural crest cells migrate into pharyngeal arches at 24 hpf, lateral view. Stream 1 (red) forms the mandibular arch and gives rise to the trabeculae, Meckel's cartilage, and the palatoquadrate. Stream 2 (green) forms the hyoid arch and gives rise to the ceratohyal, hyosymplectic and basihyal. Stream 3 (blue) forms the branchial arches and gives rise to the pharyngobranchials, epibranchials, bilateral ceratobranchials, and the corresponding hypobranchials and basibranchials. (**C**) Cartilage elements of the lower jaw in the zebrafish larvae, lateral and ventral views. (**A, C**) Color scheme is as follows: mandibular arch (blue); hyoid arch (red); branchial arches (green). Reprinted with modification from (Knight and Schilling, 2006; Schilling et al., 1996; Verreijdt et al., 2006) with permission from Springer, Elsevier, and John Wiley & Sons, respectively.

Craniofacial tendons

Evolution of the jaw in gnathostomes (jawed vertebrates) provided a means for mastication (Figure 1.4A). Detailed fate mapping experiments have illustrated that the cartilage and connective tissue of the jaw originate from the cranial neural crest, a vertebrate-specific migratory and multipotent cell population (Le Douarin and Dupin, 2003). The cranial neural crest cells arise from the dorsal neural tube and delaminate to migrate as three streams (i.e. mandibular, hyoid, and postotic) into the pharyngeal arches (Lumsden et al., 1991; Trainor et al., 2002), along its periphery to envelope populations of myogenic progenitors (Le Douarin and Kalcheim, 1999) (Figure 1.4B). The cranial musculature originates from the paraxial mesoderm (Noden, 1983). Cells in the pharyngeal arches also give rise to the associated musculature and connective tissue of that arch-derived skeletal element. (Couly et al., 1993; Kontges and Lumsden, 1996; Le Lievre, 1978; Noden, 1978; Schilling and Kimmel, 1994). In vertebrates, the skeletal elements and muscle derived from the mandibular arch form the jaw, and those of the hyoid arch form the supporting structures of the jaw (Figure 1.4C).

The spatial arrangement of the embryonic tissue in the pharyngeal arches attributes to the developmental framework of the adult jaw in teleosts and amniotes. In the zebrafish, Meckel's cartilage articulates posteriorly with the palatoquadrates, which articulate anteriorly with the ethmoid plate to flank the roof of the oral cavity and posteriorly with the hyosympletics. The hyosympletics articulate dorsally with the auditory capsule to suspend the jaw. (Cubbage and Mabee, 1996; Schilling et al., 1996; Verreijdt et al., 2006). A chief mastication muscle is the masseter, which functions in elevation of the mandible during feeding, resulting in protrusion of the mandible. Another muscle group involved in mastication and deglutition is the stylohyoideus, a structure that provides attachment to the muscles located at the base of the oral cavity. (Diogo

et al., 2008; Liem et al., 2001) A similar characterization in the spatial arrangement of the cranial skeletal and muscle tissues is observed in amniotes (Diogo et al., 2008; Gray, 1918; Gross and Hanken, 2008; Helland, 1980; Liem et al., 2001; Sadler, 2010)

The jaw is one of the earliest musculoskeleton to develop in the zebrafish, with the cartilage and muscle progenitor populations present in the pharyngeal arches by 2 days post-fertilization (dpf) and feeding evident by 4 dpf (Schilling and Kimmel, 1997). The pharyngeal cartilage and muscle progenitor populations differentiate in synchrony and in close proximity to each other (Schilling and Kimmel, 1997), as is observed in musculoskeletal development of amniotes (Schilling, 1997). Furthermore, the processes governing skeletal development in the zebrafish are highly conserved to those in higher vertebrates. Several of the cranial bone develops via differentiation through a cartilage intermediate, followed by endochondral or perichondral ossification, while other directly ossify by intramembraneous ossification (Apschner et al., 2011). Skeletal development comprises of the following distinct phases – migration to target site, epithelial-mesenchymal interactions, condensation, and differentiation (Figure 1.5) (Hall and Miyake, 1995; Hall and Miyake, 2000). The condensation phase has been shown to be instrumental in patterning the size of the skeletal element, such that precocious differentiation results in smaller or the absence of skeletal elements, whereas prolonged condensation results in enlarged skeletal elements (Hall and Miyake, 1992). The cartilage matrix is comprised of proteoglycans and collagen fibrils, the most abundant are Aggrecan and Collagen type II, respectively. The matrix is synthesized during the condensation stage and continues until the chondrocytes terminally differentiate. Regulators of osteoblast differentiation include Runx2 and Osterix (Nakashima et al., 2002; Xiao et al., 2005; Yoshida et al., 2002). The different chondrogenic stages are associated with well-characterized unique and overlapping molecular

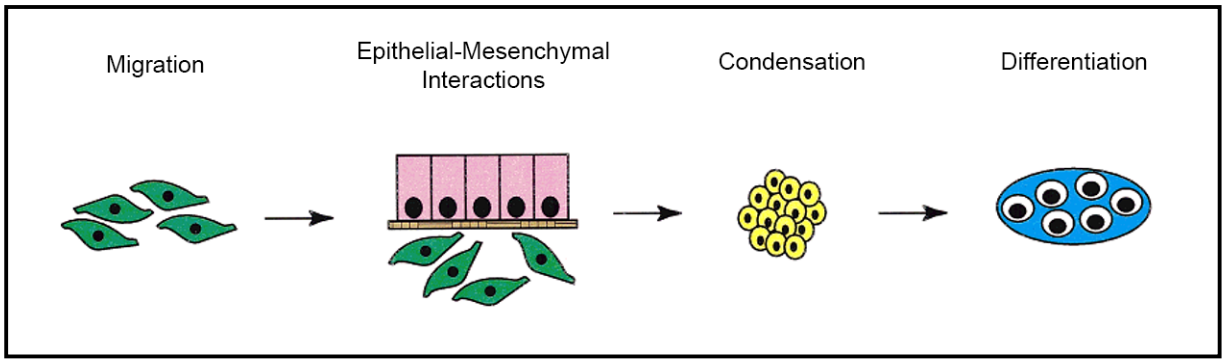

Figure 1.5. Stages of cartilage developmental program. Reprinted with modifications from (Hall and Miyake, 2000) with permission from John Wiley & Sons.

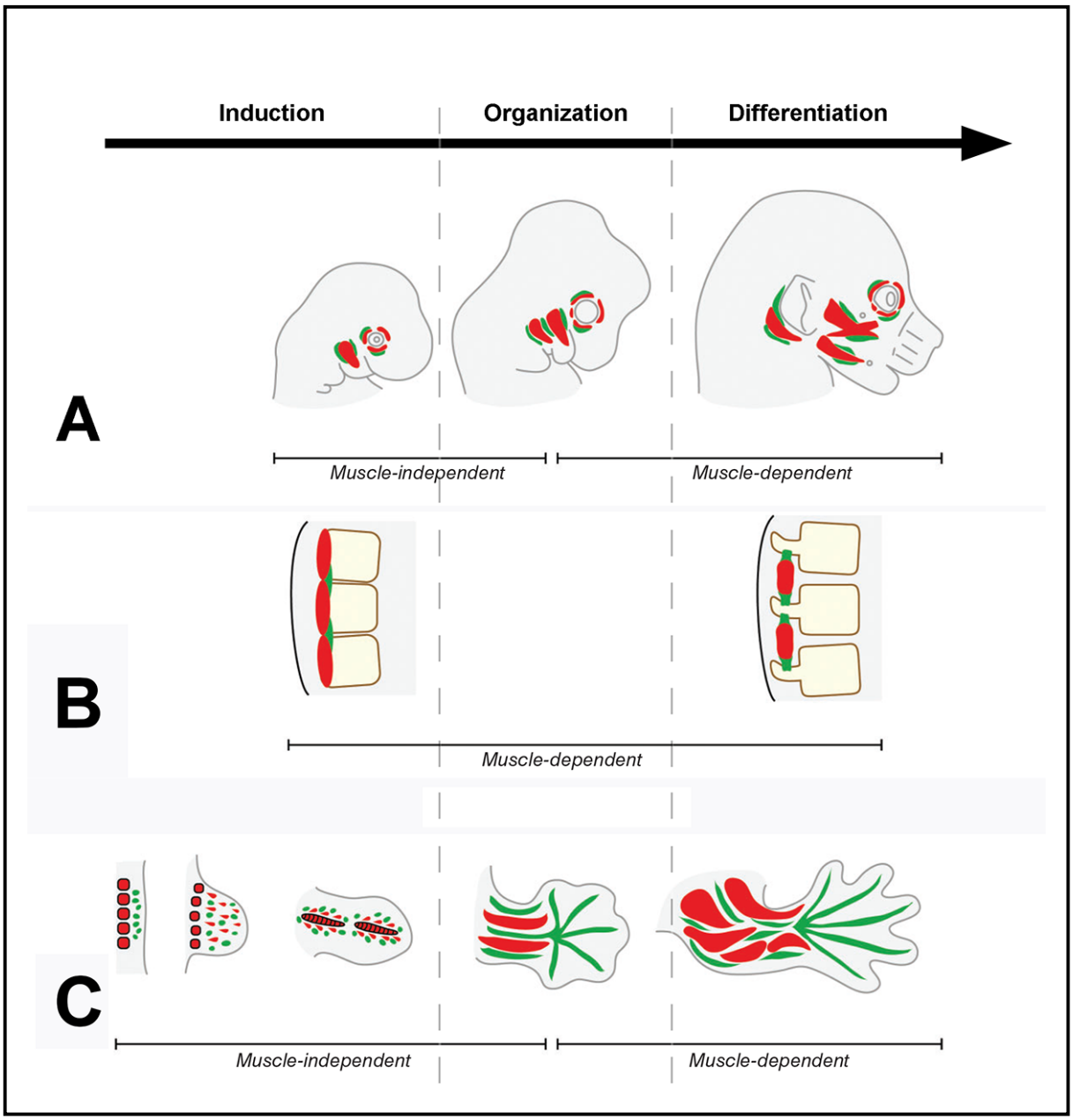

Figure 1.6. Influence of muscle in tendon development in vertebrates. (A) Cranial, (B) limb, and (C) axial tendon development. Reprinted with modifications from (Gaut and Duprez, 2016) with permission from John Wiley & Sons.

markers and signaling pathways. (Karsenty et al., 2009; Olsen et al., 2000; Verreijdt et al., 2006)

The spatial and temporal synchrony in the development of the cartilage, muscle, and tendons/ligaments (Hacker and Guthrie, 1998; Kontges and Lumsden, 1996; Trainor and Tam, 1995; Trainor et al., 1994), leading to their eventual intimate integration, asks about the extent to which their developmental programs are dependent on their respective interactions. The existence of a common embryonic origin for the skeletogenic and tenogenic lineages has made dissecting the dynamics of their interaction less straightforward. In contrast, the molecular crosstalk of the myogenic and tenogenic lineages is better characterized (Figure 1.6A). Examination of *Tbx1*$^{-/-}$ mutant mice, which lack differentiated branchiomeric muscles in the first arch (Dastjerdi et al., 2007; Kelly et al., 2004), established that while *Scleraxis*-positive tendon progenitors initiate independently of branchiomeric muscles, the muscle is required for the continued maintenance of tendon fate (Grenier et al., 2009). Likewise, while myogenic progenitors initiate independently of the cranial neural crest, the neural crest is critical for proper patterning and differentiation of myogenic fate (Rinon et al., 2007; Tzahor et al., 2003). Conclusions from the latter studies are based on analysis of the second pharyngeal arch of *Hoxa1*$^{-/-}$*;Hoxb1-3'RARE*$^{-/-}$ mutants (Gavalas et al., 2001) and constitutively active β-*catenin/Wnt1-Cre* mutants (Rinon et al., 2007). As differences exist in the the signaling factors that pattern the mandibular and hyoid arches, it is unclear whether such differences will translate to arch-specific differences in interactions with the neighboring myogenic progenitors. Signaling factors with spatial-temporal differences between the arches include the Hox transcription factors (Le Douarin and Kalcheim, 1999), and regulators of dorsal-ventral identity (e.g. Goosecoid and Barx1 (Swartz et al., 2012)). However, this caveat is unlikely given the evolutionary context – the Chordata-specific pharyngeal pouches (Graham and Smith, 2001) arose prior to the evolution

of the Craniata-specific neural crest-derived vertebrate head (Gans and Northcutt, 1983). As such, a framework applicable to the descendants of all pharyngeal arches in which initiation of the myogenic program is independent of the neural crest-derived tenogenic program is the most parsimonious.

Axial tendons

During craniate evolution, the axial skeleton became functionally and morphologically specialized; the predominant form of locomotion shifted from being axial-based to appendage-based (Janvier, 1996). In spite of these modifications, the axial musculoskeleton retained its plesiomorphic function in mobilization - the trunk of tetrapods functions in stabilization against gravitation and inertial forces and the body of the gnathostome fishes (chondrichthyes and osteichthyes) functions in lateral undulatory movement (Altringham and Ellerby, 1999; Schilling, 2011). In amniotes, the anterior-posterior axis is characterized by reiterations of vertebrae connected to one another by a series of ligaments, and to the musculature by a series of tendons (Gray, 1918; Head and Polly, 2015). In gnathostome fishes, the body axis is composed of longitudinally arranged W-shaped bundles of twitch fibers, the myomeres (Bone, 1989; Johnston et al., 1975). In actinopterygians, the myomeres are separated along the dorsal-ventral axis by the horizontal myoseptum, the predominant contributor of force transmission during undulatory movement (Gemballa et al., 2003b; Jayne and Lauder, 1995; Liem et al., 2001; Westneat et al., 1993). The horizontal myoseptum is a feature that is lost in the sarcopterygians (lobe-finned fish) (Gemballa and Ebmeyer, 2003; Gemballa et al., 2003a). In sarcopterygians and actinopterygians, the myomeres are separated along the anterior-posterior axis by highly organized collagenous chevron-shaped structures, the vertical myosepta (Gemballa et al., 2003a;

Nursall, 1956), which are postulated to function in force transmission (Gemballa and Vogel, 2002; Nursall, 1956; Westneat et al., 1993). Based on the similarities in architecture and function, the vertical myosepta and tendons are speculated to be functionally homologous structures (Bricard et al., 2014).

The axial tissues arise from serially homologous blocks of paraxial mesoderm, the somites, which consist of an epithelial wall enveloping a mesenchymal core of somitocoel cells (Christ and Ordahl, 1995). The somite undergoes epithelial-to-mesenchymal transition and differentiates into the dermamyotome, which gives rise to the dermis and muscle, and the sclerotome, which gives rise to the vertebrae and ribs (Borycki and Emerson, 2000; Brand-Saberi and Christ, 2000; Monsoro-Burq and Le Douarin, 2000). The somite of teleosts is composed predominantly of myotome with a relatively small ventral sclerotome, which migrates dorsally to surround the notochord (Devoto et al., 1996; Stickney et al., 2000). The formation of the axial cartilage and mytome is not synchronized – the cartilage template forms in the larvae (Bensimon-Brito et al., 2012; Javidan and Schilling, 2004), whereas differentiated myofibers are present in the embryo (Devoto et al., 1996). The vertebrae of teleosts are derived from two distinct lineages – the sclerotome gives rise to the centra and arches via direct ossification (Li et al., 2009; Renn et al., 2013; Spoorendonk et al., 2008), and the chordamesoderm-derived notochord gives rise to the chordacentra (Arratia et al., 2001; Grotmol et al., 2003; Inohaya et al., 2007; Nordvik et al., 2005). In contrast, the somite of amniotes is comprised predominantly of medially-positioned sclerotome, which migrates to surround the midline notochord and neural tube to give rise to the pericentrum and neural arches, respectively (Grotmol et al., 2003), via endochondral ossification (Peters et al., 1999; Zhao et al., 1997). The vertebral body of amniotes is exclusively derived from the sclerotome (Senthinathan et al., 2012). Given the differences in

the development of the vertebrae in teleosts and amniotes, there may exist species-specific differences in the interaction between the tenogenic and skeletogenic lineages.

The axial tendons of amniotes originate from the syndetome, a somitic compartment that is established by myotomal FGF signals to the adjacent sclerotome to induce formation of tendon progenitors (Figure 1.6B) (Brent et al., 2003). The specification of the sclerotome towards a tendon fate is mutually exclusive with a cartilage fate, with the default pathway towards tenogenesis (Brent et al., 2005; Brent et al., 2003). Although lineage tracing of the myosepta in teleosts is lacking, the sclerotome is postulated to give rise to the myosepta based on gene expression profile enriched in mammalian tendon matrix proteins (Bricard et al., 2014).

Limb tendons

In the shift from an aquatic to a terrestrial environment during the Devonian period, there was a correspondingly gradual evolution of the paired fins of sarcopterygians to the limbs of tetrapods (Cole and Currie, 2012). The limb skeletal and connective tissue are derived from the lateral plate mesoderm (Kieny and Chevallier, 1979; Onimaru et al., 2011; Saunders, 1948). In the limb bud of amniotes, there exists several signaling centers – the apical ectodermal ridge patterns the proximal-distal axis through FGF/Wnt-mediated signaling (Fernandez-Teran and Ros, 2008; Kengaku et al., 1998; Lu et al., 2008), the zone of polarizing activity patterns the anterior-posterior axis through Sonic hedgehog (Shh)-mediated signaling (Harfe et al., 2004; Zeller et al., 2009), mesenchyme-ectoderm interaction patterns the dorsal-ventral axis through Wnt/Engrailed-mediated signaling (Loomis et al., 1998), and the Hox code patterns digit identity (Tickle and Eichele, 1994; Zuniga, 2015). These signaling centers pattern the developing limb, which form bone by endochondral ossification, by regulating the induction and differentiation of

the progenitor population in the limb bud mesenchyme. A morphological divergence in the pectoral fin of teleosts is formation of the apical fold, the fin-ray envelope comprised of epithelial sheets that provide space for the formation of mesenchymal collagenous fibers, the actinotrichia (Wood and Thorogood, 1984). Due to similarities in gene expression profiles and regulatory program (e.g. Hox code) (Mercader, 2007; Yano and Tamura, 2013; Zuniga, 2015), the apical fold in zebrafish is postulated to be the equivalence of the apical ectodermal ridge in amniotes. Moreover, retinoic acid/Shh-mediated signals in the pectoral fin bud mediating anterior-posterior identity, indicating that the role of the zone of polarizing activity is conserved in zebrafish (Akimenko and Ekker, 1995). Consistent with this hypothesis, the skeletal program of the pectoral fin, which also arises from the lateral plate mesoderm (Mercader et al., 2006), and limbs of sarcopterygians is highly conserved in many aspects (Coates et al., 2002; Shubin et al., 1997). For example, the chondrogenic program of the pectoral fin of teleosts is similarly patterned by the *sox9* co-orthologs, *sox9a* and *sox9b*, which have distinct and overlapping roles (Yan et al., 2002; Yan et al., 2005). The limb muscles are derived from the somitic myotome , which delaminate into the lateral plate mesoderm (Chevallier et al., 1977; Christ et al., 1977; Ordahl and Le Douarin, 1992). Taken together, the patterning and differentiation of the lateral plate mesoderm-derived skeletal appendages in teleosts and amniotes are dependent on several common regulatory networks (Tickle, 2016; Yano and Tamura, 2013).

In contrast to the axial region but similar to the cranial tendons, specification of limb tendon progenitors is muscle-independent, but the maintenance of tendon fate is muscle-dependent (Figure 1.6C) (Bonnin et al., 2005; Brand et al., 1985; Brent et al., 2005; Edom-Vovard et al., 2002; Kardon, 1998; Kieny and Chevallier, 1979; Shellswell and Wolpert, 1977). Divergent from this archetype are the distal tendons of the autopod, which can initiate and

maintain its formation in muscleless limbs (Kardon, 1998; Kieny and Chevallier, 1979). Likewise, tendon progenitors are not required for the initiation of myogenic progenitors in the limb bud, but are required for proper organization of the myofibers (Kardon, 1998). Furthermore, while studies examining the crosstalk between cartilage and tendons are limited, those examining the enthesis and tuberosity of the limb suggest that the initiation of the Sox9-positive cartilage and Scleraxis-positive tendon populations may be independent of each other as well as the enthesis domain (Blitz et al., 2013; Sugimoto et al., 2013). This finding gains support from the formation of distal tendons following surgical removal of the terminal phalanx (Hurle et al., 1990).

Overview of the dissertation

Tendon development requires the coordination of many cellular processes. Cells must be specified towards a tendon fate and then organized, initially as loose cellular aggregates and then as structurally distinct tendons, at the anatomical interface of the differentiating cartilage and skeletal tissues. Here in the Introduction, I describe the structure of the tendon/ligament tissue, our current knowledge of the molecular pathways involved in tendon development, and the origins and developmental regulation of the craniofacial, axial, and limb tendons and skeleton. To date, the mechanism that coordinates the specification of tendon cells from a common skeletal progenitor during development remains to be deciphered.

Following this introduction, Chapter 2 presents the zebrafish as a genetic model for the study of vertebrate tendon development. The zebrafish offers the advantages of being amendable to high-throughput screening, in addition to genetic manipulations, transgenic and live-imaging approaches (Dahm and Geisler, 2006). Conserved expression of several genes in the zebrafish

with mouse and chick revealed the location of the craniofacial tendons and ligaments, which were subsequently demonstrated to be derived from the cranial neural crest. Our findings provide evidence that the tendons and ligaments in zebrafish morphologically, molecularly, and structurally resemble mammalian tendons and ligaments from embryonic to adult stages.

To better understand the processes that govern tendon cell induction, we performed a whole zebrafish embryo *in situ* hybridization screen using a known bioactive compound library. After screening over 1000 small molecules, two were discovered to cause an expansion of the craniofacial tendon progenitors. These positive-hits – Lovastatin and Simvastatin – were classified as statin compounds according to their mechanism of action. Statins are competitive inhibitors of 3-hydroxy-3-methylglutaryl-coA reductase (Hmgcr), the rate-limiting enzyme of mevalonate synthesis (Istvan and Deisenhofer, 2001). Chapter 3 explores the mechanism by which the mevalonate pathway regulates tendon development in the zebrafish and in higher vertebrates. We additionally demonstrate that the mevalonate pathway, specifically geranylgeranyltransferase type I (GGTase I) activity, is a critical regulator of *Scleraxis* expression and tendon fate specification. Taken together, our zebrafish studies characterize the mechanism and cellular processes that act in combination during cranial neural crest specification to establish an expanded population of craniofacial tendon progenitors.

Finally, Chapter 4 places these findings within an evolutionary context, examining the development of the craniofacial musculoskeleton and the mechanism of tendon specification in the cranial neural crest. This dissertation will hopefully serve as a foundation for future studies aimed at unraveling the factors responsible for tendon development.

Chapter 2

The development of zebrafish tendon and ligament progenitors

Attributions

I designed and performed the experiments examining the co-expression of *scleraxisa*, *tenomodulin*, *xirp2a*, *collagen 1a2*, and myosin heavy chain in the zebrafish. I cloned, sequenced, and analyzed the expression of *scleraxisb* in the zebrafish. I designed and characterized the *scleraxisa* and *scleraxisb* morpholinos. I characterized the role of *myf5* and *myod1*, in addition to that of the *sox9* co-orthologs, in tendon development. The following experiments were done in collaboration with Jenna Galloway – characterization of the regulation of *tfap2a* and *foxd3,* and fate mapping of Tg(*sox10:kaede*) embryos. Jenna Galloway characterized the expression of the genes *scleraxisa*, *tenomodulin*, *xirp2a*, and *collagen 1a2* in the zebrafish, and performed the chemical incubation experiments. Electron microscopy studies were performed with technical assistance from Mary McKee, Microscopy Core of the Program in Membrane Biology at Massachusetts General Hospital.

The development of zebrafish tendon and ligament progenitors

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<http://dev.biologists.org/content/141/10/2035.long>

Summary

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. In 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.

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 *Col1a1* (Lejard et al., 2007) and *tenomodulin* (*Tnmd*) (Shukunami et al., 2006), 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; Brent et al., 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; Yan et al., 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 does 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.

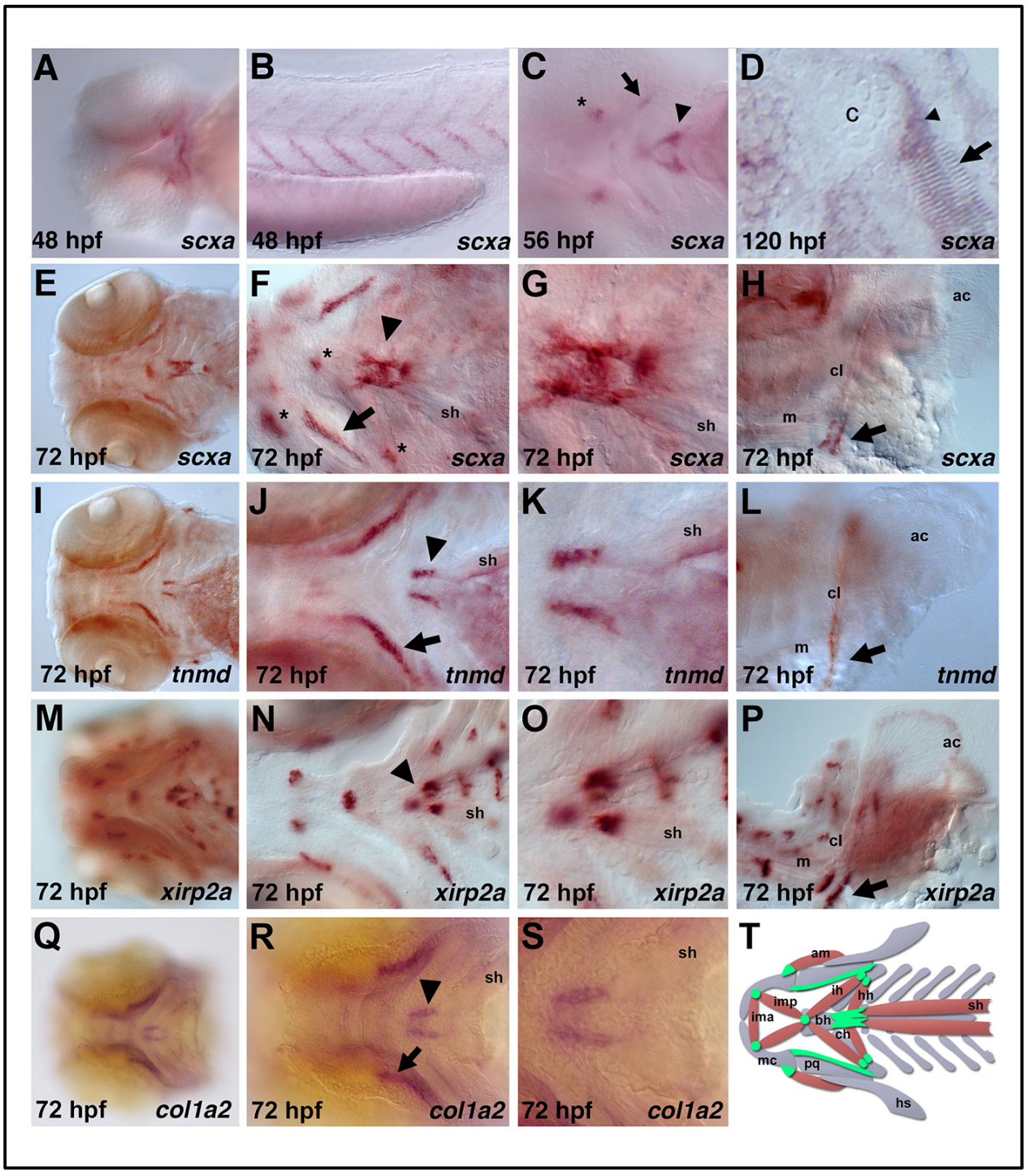
Results

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 Chromosome 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 (Figure 2.1A-C; data not shown). The most robust expression of *scxa* is detected at 72 hpf, in two lateral stripes ventromedial to the palatoquadrate (Figure 2.1E, F, arrow), centrally where the sternohyoideus (sh) meets the ceratohyal and basihyal cartilage elements (Figure 2.1E, F, arrowhead; enlarged in 2.1G), and at the base of the cleithrum (Figure 2.1H, arrow). Additional expression is observed in areas where the adductor mandibulae attaches

Figure 2.1. 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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to Meckel's cartilage, and at attachment points of the intermandibularis, interhyoideus, and hyohyoideus muscles (Figure 2.1F, 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*.

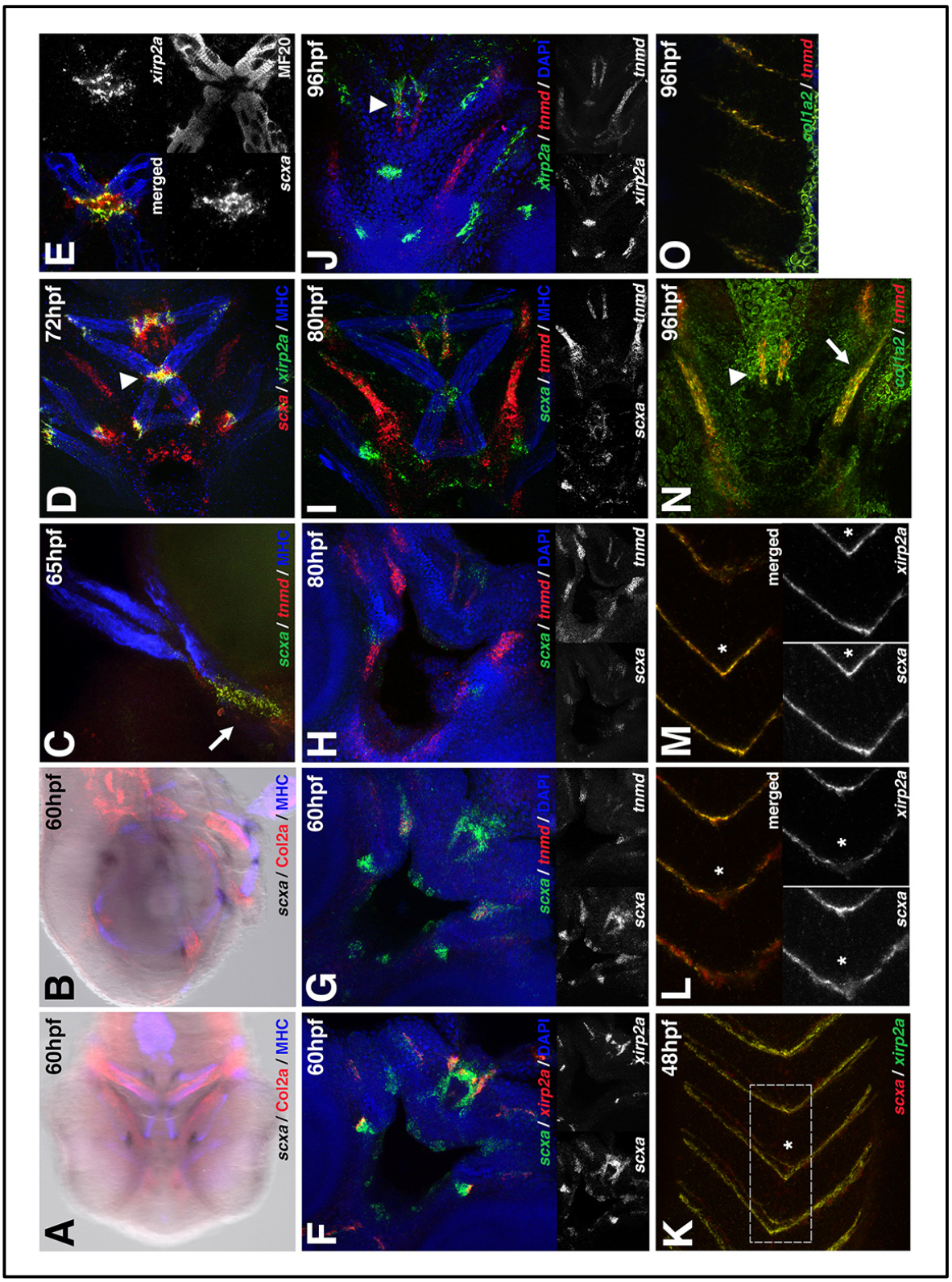
Identification of zebrafish tendon and ligament progenitor cells

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 (Figure 2.1I, J, arrow and arrowhead; enlarged in 2.1K) and surrounding the cleithrum (Figure 2.1L, 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 (Figure 2.1Q, R, arrow and arrowhead; enlarged in 2.1S). 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 (Figure 2.8E) (Otten et al., 2012). Interestingly, *xirp2a* is expressed near the sternohyoideus attachment to the lower jaw cartilage (Figure 2.1M, N, arrowhead; enlarged in 2.1O) and at the base of the cleithrum (Figure 2.1P, 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 (Figure 2.1D, arrowhead). In triple stained embryos, craniofacial *scxa* expression was found at muscle-to-cartilage or cartilage-to-cartilage attachments (Figure 2.2A, 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 (Figure 2.2D-F, arrowhead), and their expression appeared distinct from myosin heavy chain staining in the muscle (Figure 2.2E). The *scxa* and *tnmd* transcripts were colocalized in the head and fin regions between 60 and 80 hpf (Figure 2.2C, 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 (Figure 2.2F, G) is followed by weaker expression after 80 hpf (Figure 2.2H, I), whereas *tnmd* expression is detected at 60 hpf but is stronger after 80 hpf (Figure 2.2G-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 (Figure 2.2J, 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 (Figure 2.2D, I, J, N). In the myosepta, there is colocalization of *xirp2a* and *scxa* at 48 hpf (Figure 2.2K-M), and *col1a2* and *tnmd* at 96 hpf (Figure 2.2O). Together, the expression of *scxa*, *tnmd*, and *col1a2* identifies two major regions of tendon and ligament populations in zebrafish craniofacial tissue (Figure 2.1T; summarized in Figure 2.13). 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.

Figure 2.2. 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. Expressions of *scxa* and *tnmd* transcripts are 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 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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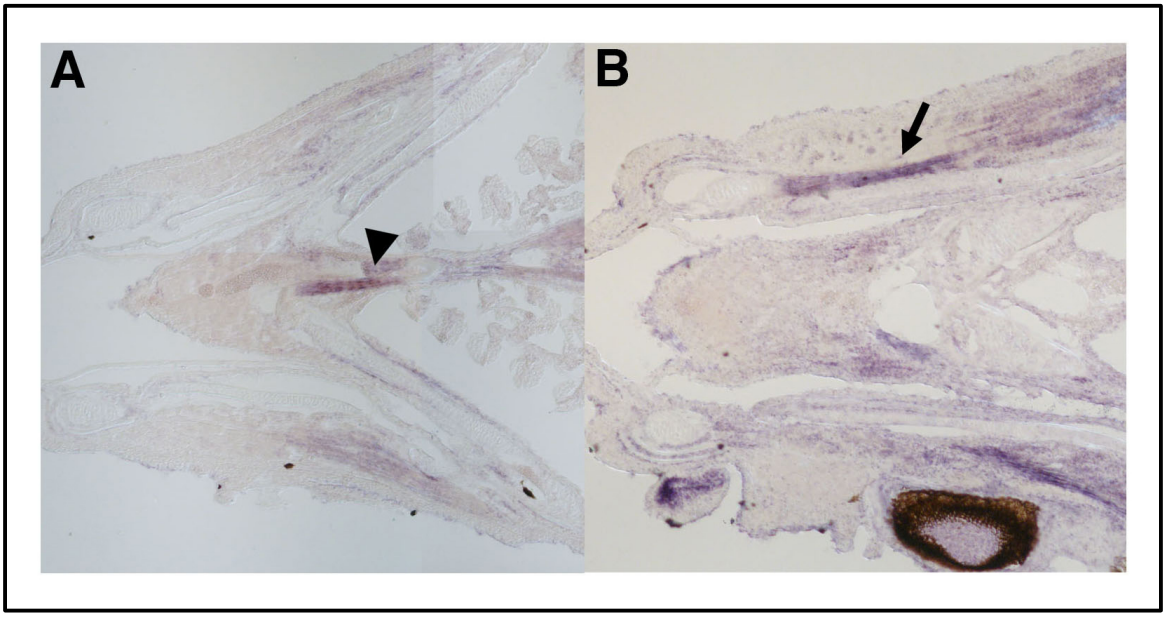


Figure 2.3. Craniofacial expression of *tnmd* in juvenile zebrafish. Coronal sections show *tnmd* expression near the sternohyoideus attachment (**A**, arrowhead) and in lateral regions attached to the mandible (**B**, arrow). *tnmd* is also detected in the perichondrium.

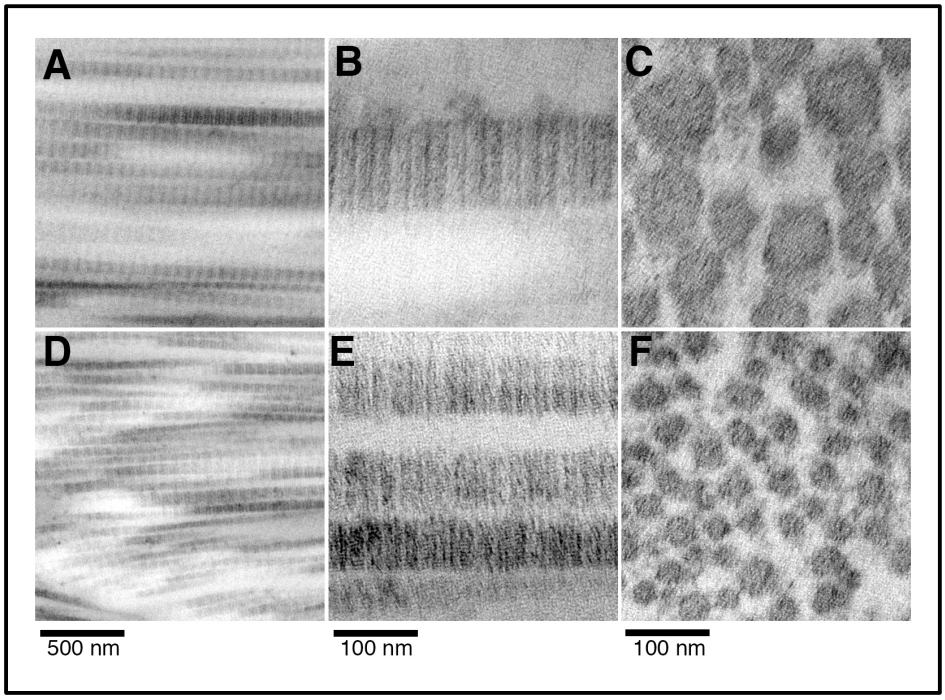


Figure 2.4. Ultrastructural analysis of adult zebrafish craniofacial tendons and ligaments. Transmission electron microscopy of ligament (**A, B**) and tendon (**D, E**) longitudinal sections reveal the parallel arrangement of collagen fibrils with characteristic periodicity. Ligament (**C**) and tendon (**F**) cross-sections reveal round collagen fibrils.

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 (Figure 2.3A, arrowhead) and in lateral tissue connecting to Meckel's cartilage (Figure 2.3B, 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 (Figure 2.4). 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 (Figure 2.5A, B, E, F; Figure 2.6A, B). Similarly, *scxa* is expressed, but in a disorganized pattern in *tfap2a* and *foxd3* single morphants compared with controls (Figure 2.5C, D, G, H; Figure 2.6C, 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 (Figure 2.5I–L; Figure 2.6A-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

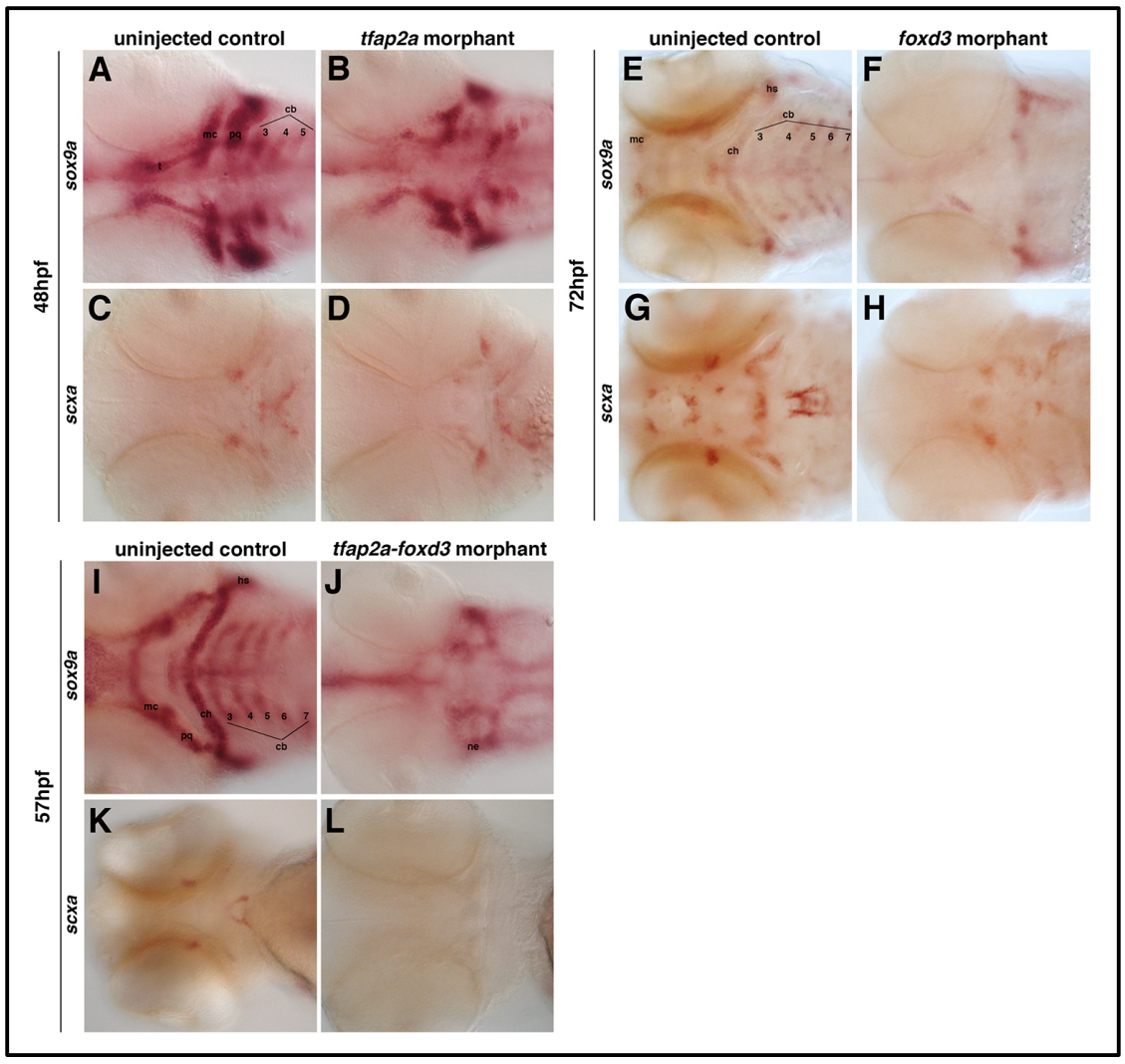


Figure 2.5. Proper neural crest development is required for craniofacial *scxa* expression. Morpholino-mediated knockdown of *tfap2a* results in (**A, B**) reduction of *sox9a*-positive pharyngeal cartilage and (**C, D**) an altered pattern of *scxa* expression at 48 hpf. Morpholino-mediated knockdown of *foxd3* results in (**E, F**) significant loss of *sox9a*-positive pharyngeal cartilage and (**G, H**) a significant loss of *scxa*-positive craniofacial tendon progenitors at 72 hpf. Morpholino-mediated knockdown of *tfap2a* and *foxd3* results in (**I, J**) complete loss of *sox9a*-positive pharyngeal cartilage (98%, n=58) and (**K, L**) *scxa*-positive craniofacial tendon progenitors (96%, n=46) at 57 hpf compared with controls. Ventral views of flat-mounted embryos.

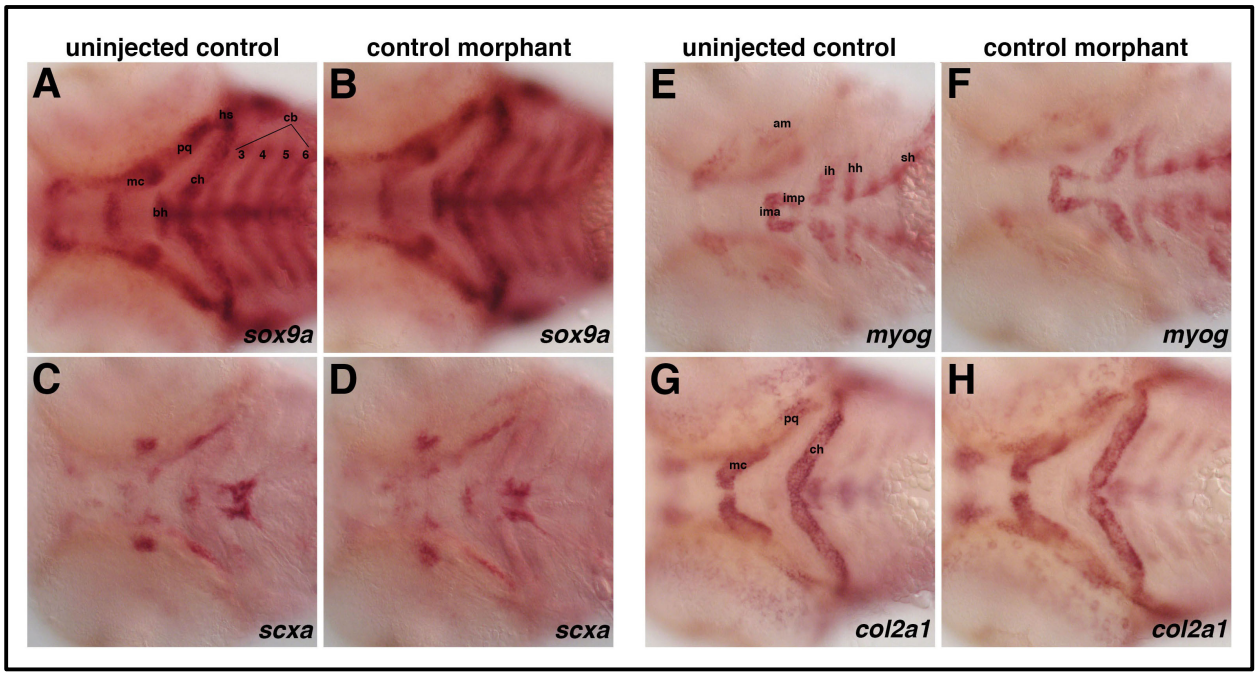
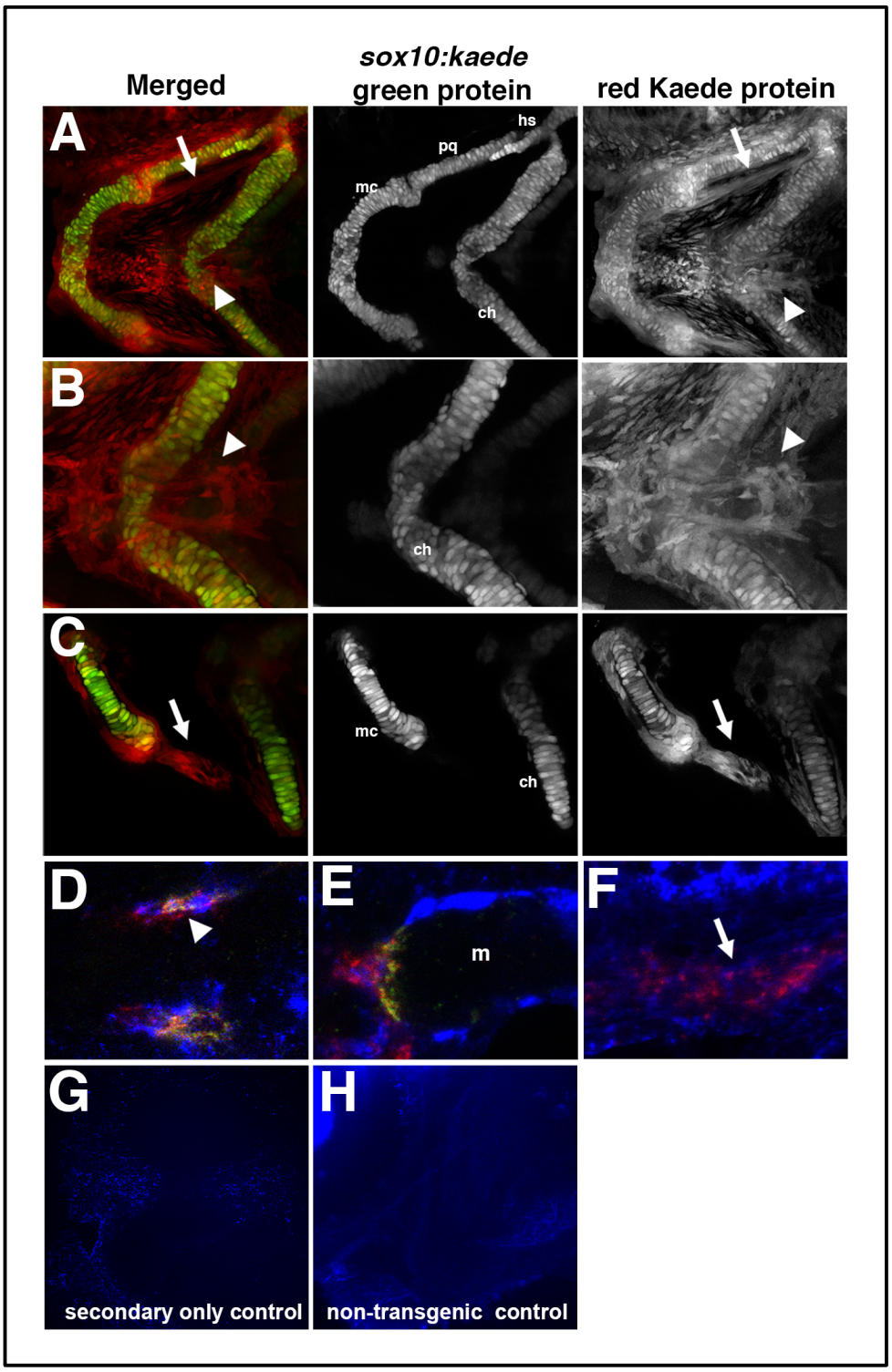


Figure 2.6. Control oligo morpholino does not affect the expression of musculoskeletal markers. Injection of control scrambled oligo at comparable concentrations to that of morpholinos used does not alter expression of *sox9a* (**A, B**)*, scxa* (**C, D**)*, myog* (**E, F**)*,* or *col2a1* (**G, H**) compared to controls at 60 hpf. am, adductor mandibulae; bh, basihyal; cb, ceratobranchial; ch, ceratohyal; hh, hyohyoideus; hs, hyosymplectic; ih, interhyoideus; ima, intermandibularis anterior; imp, intermandibularis posterior; mc, Meckel's cartilage; pq, palatoquadrate; sh, sternohyoideus.

Figure 2.7. Zebrafish craniofacial tendon populations are derived from the neural crest. (**A-C**) 72 hpf photoconverted *sox10:kaede* embryos express *sox10:kaede* green protein in the pharyngeal cartilage (**A**-**C** and middle panel), and the red Kaede protein from the 22 hpf photoconversion is found in the two major populations of craniofacial tendon progenitors (**A**, arrow and arrowhead; **B**, **C,** right panel). (**D-F**) Colocalization of *scxa* and Kaede protein in photoconverted *sox10:kaede* embryos at 72 hpf is observed in the sternohyoideus connection point (**D, E**; sternohyoideus muscle is labeled m) and in the ligament (**F**). A subset of the *scxa*-positive and Kaede-positive cells also co-expresses *xirp2a* transcripts. Arrows in (**A, C, F**) mark ligament medial to palatoquadrate; arrowheads in (**A, B, D**) mark tendon connecting the sternohyoideus to the ceratohyals. Controls for Kaede antibody staining for the secondary alone (**G**) or in non-transgenic embryos **(H**) did not show any staining when imaged at the same levels as in (**D-F**). cb, ceratobranchials; ch, ceratohyal; hs, hyosymplectic; m, muscle; mc, Meckel's cartilage; ne, neurocranium; pq, palatoquadrate.



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sox10:kaede CNCC descendants at 72 hpf. As a positive control for photoconversion, we observed CNCC-derived cartilage labeled with red Kaede protein (Figure 2.7A–C). 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 (Figure 2.7A, C; arrows) and in the tendon connecting the sternohyoideus to the ceratohyal (Figure2.7A, B, 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 (Figure 2.7D-F). The sternohyoideus tendon stained positive for *scxa*, *xirp2a* and Kaede (Figure 2.7D, F, arrowhead), and the ligament stained for *scxa* and Kaede (Figure 2.7F, arrow), while control antibody staining was negative (Figure 2.7G, H). Together, these findings establish that zebrafish craniofacial tendon and ligament cells are neural crest-derived.

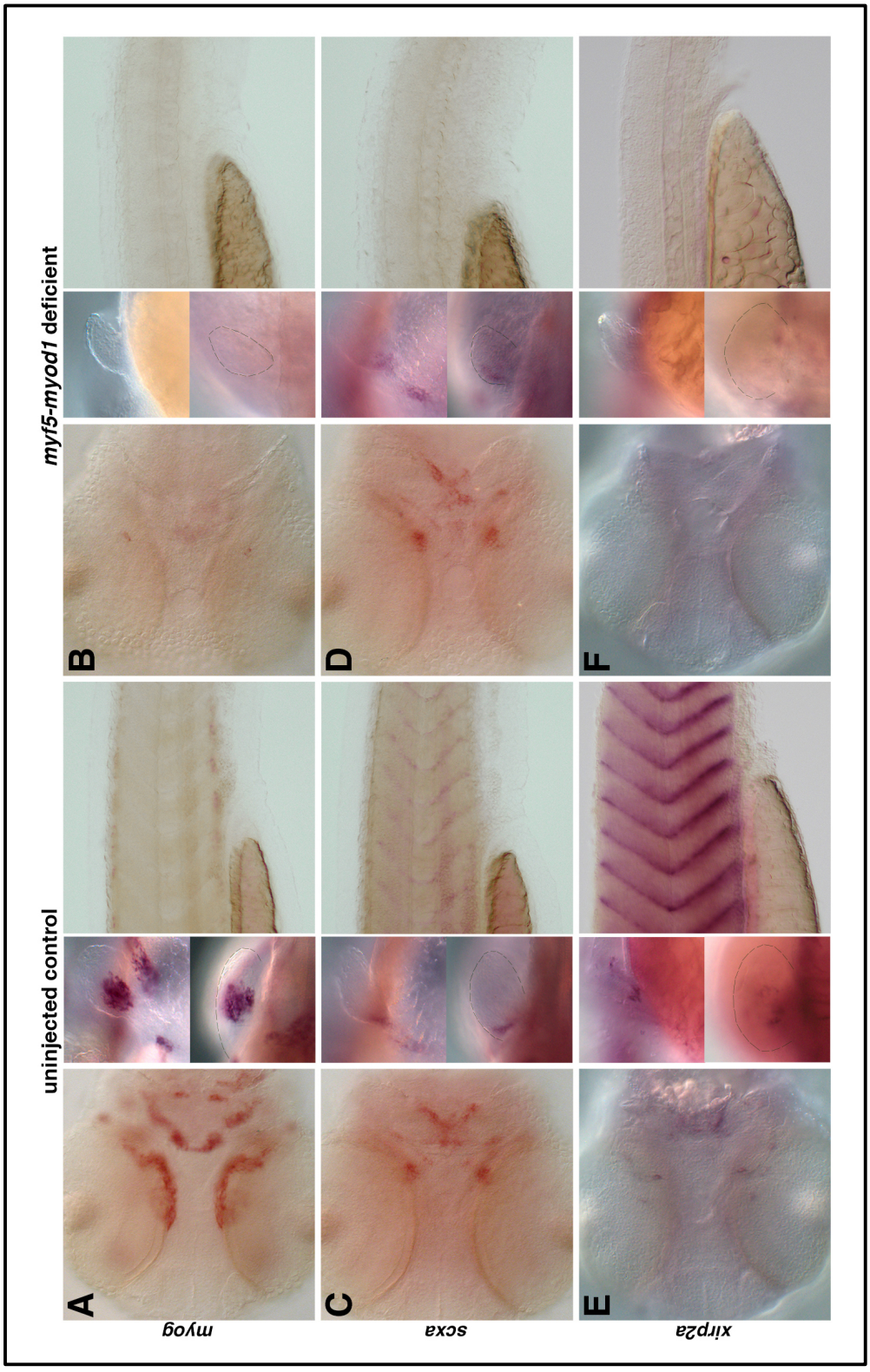
Role of muscle in tendon and ligament development

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 (Figure 2.6E, F; Figure 2.8A, B) and MHC expression is absent at 72 hpf (Figure 2.9A, B, E, F) compared with controls. By contrast, *scxa* expression is relatively normal at 53-58 hpf in the craniofacial and fin regions, but absent from the myosepta (Figure 2.8C, D). *xirp2a* expression was also lost in the myosepta (Figure 2.8E, 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 72hpf, we observed a virtual loss of *scxa* expression in the head and fins of *myod1-myf5*-deficient embryos (Figure 2.9C, D), indicating that muscles are required for the maintenance of *scxa* expression. *xirp2a* expression was lost in the head and fins at all stages examined (Figure 2.8E, F; Figure 2.9G, H), 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 in chemical inhibitors of FGF

Figure 2.8. The role of muscle in the specification of tendon populations. (**A, B**) Loss of *myod1* and *myf5* in 53-58 hpf 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).



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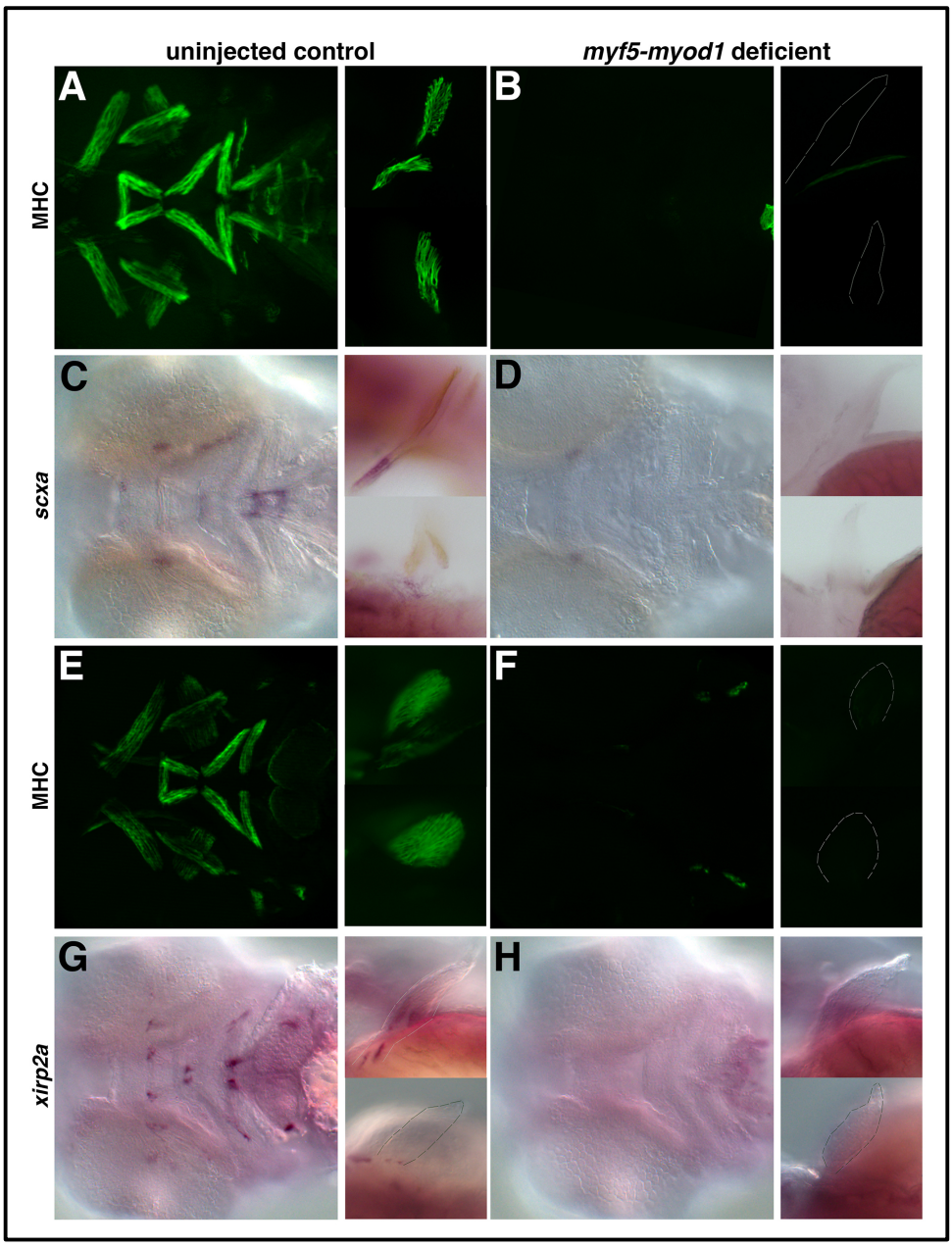

Figure 2.9. Differentiated muscle is required for the maintenance of tendon populations. *myod1*$^{-/-}$ and *myf5*-deficient embryos at 72 hpf have (**A, B, E, F**) complete loss of myosin heavy chain (MHC) expression in the craniofacial and pectoral fin tissue and (**C, D**) a virtual loss of *scxa* expression in the head and pectoral fin tissue. (**G, H**) Expression of *xirp2a* is also missing (100%, n=19). Fluorescent images of MHC-stained flat-mounted embryos in (**A, B, E, F**) correspond to the same embryos in brightfield (**C, D, G, H**).

(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 (Figure 2.10B), and in embryos treated with the TGFβ pathway inhibitor, *scxa* expression was reduced (Figure 2.10C). 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; Yan et al., 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 (Figure 2.6G, H; Figure 2.11A, B). *scxa* is expressed in *sox9a*-deficient embryos at 56 and 72 hpf (Figure 2.11E, H), indicating that *scxa*-positive tendon progenitors are specified in the

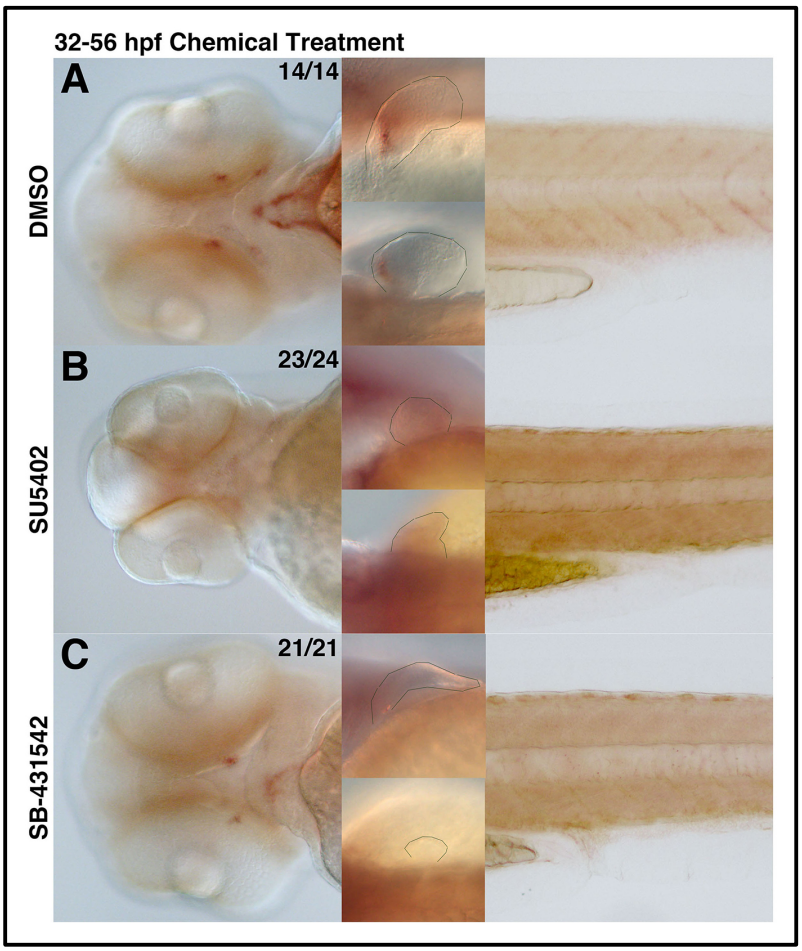

Figure 2.10. *scxa*-positive tendon populations are affected upon loss of FGF and TGFβ signaling. *scxa* expression in embryos upon incubation in chemical from 32-56 hpf. (**A**) Treatment with DMSO does not alter *scxa* expression and is used as a positive control. (**B**) Treatment with FGFR inhibitor (SU5402) results in complete loss of *scxa*-positive population in the craniofacial, pectoral fin and myoseptal tissue. (**C**) Inhibition of TGFβ signaling (SB-431542) results in a significant reduction of *scxa*-positive population in the craniofacial, pectoral fin and myoseptal tissue.

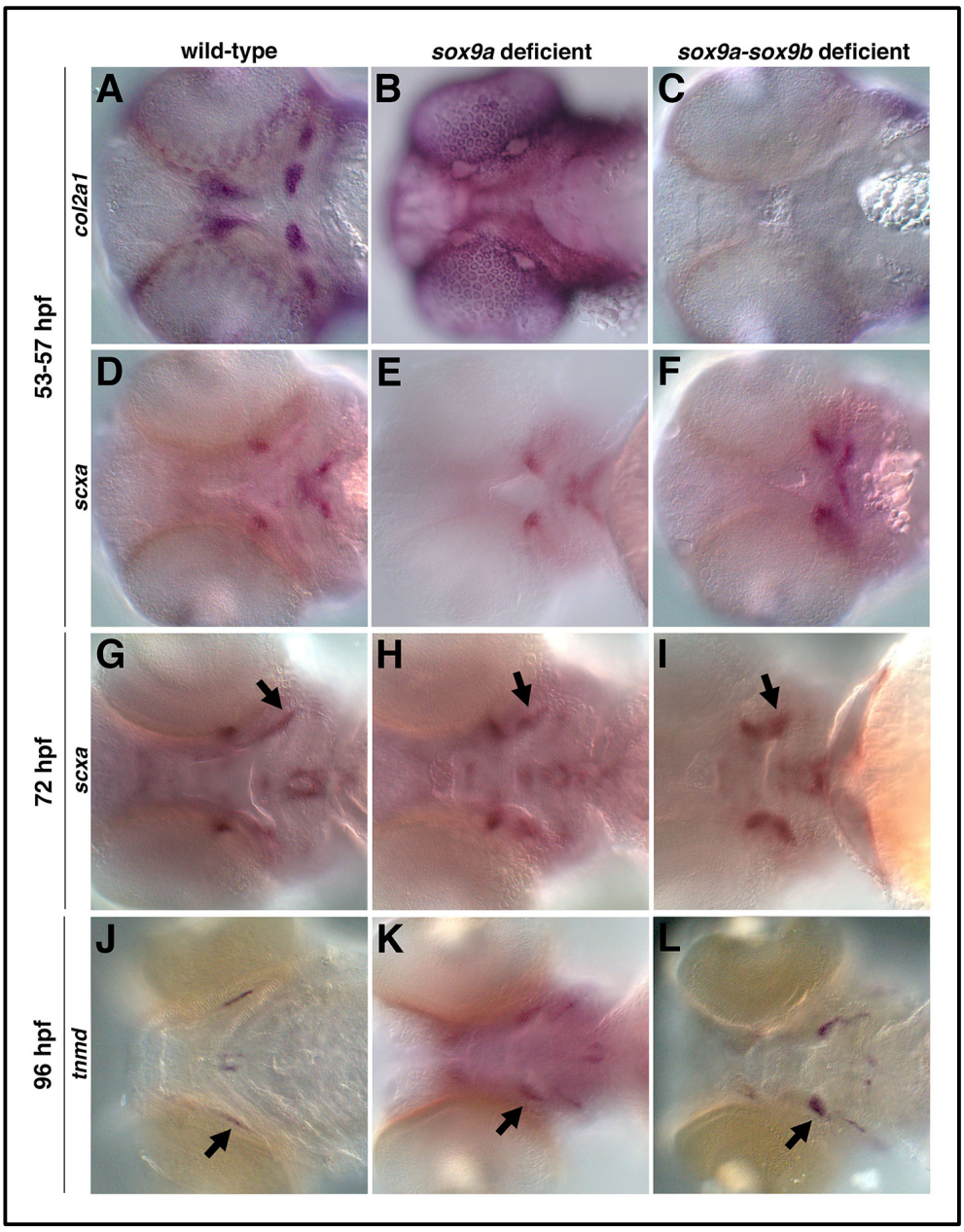

Figure 2.11. The role of cartilage in the specification and organization of tendon and ligaments. *col2a1*-positive pharyngeal cartilage was lost in *sox9a*- (**B**) and *sox9a-sox9b*-deficient embryos (88%, n=56) (**C**) compared with controls (**A**). (**D-F**) *sox9a*- and *sox9a-sox9b*-deficient 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.

Figure 2.11 (Continued).

Figure 2.12. Pectoral fin tendon populations are specified and maintained in the absence of cartilage. (**A, B, D, E**) *sox9a* morphants have loss of *col2a1*-positive pharyngeal cartilage and altered pattern of *scxa* expression at 53-57 hpf. (**C, F**) *sox9a-sox9b*-deficient embryos have lost *col2a1*-positive pharyngeal cartilage, yet retain *scxa* expression at 53-57 hpf. (**G-I**) Embryos deficient in *sox9a* and/or *sox9b* transcripts express *scxa* at 72 hpf. (**J-L**) Embryos deficient in *sox9a* and/or *sox9b* transcripts express *tnmd* at 96 hpf. Representative lateral (left) and dorsal (right) views of pectoral fin tissue are indicated for each category of embryos.

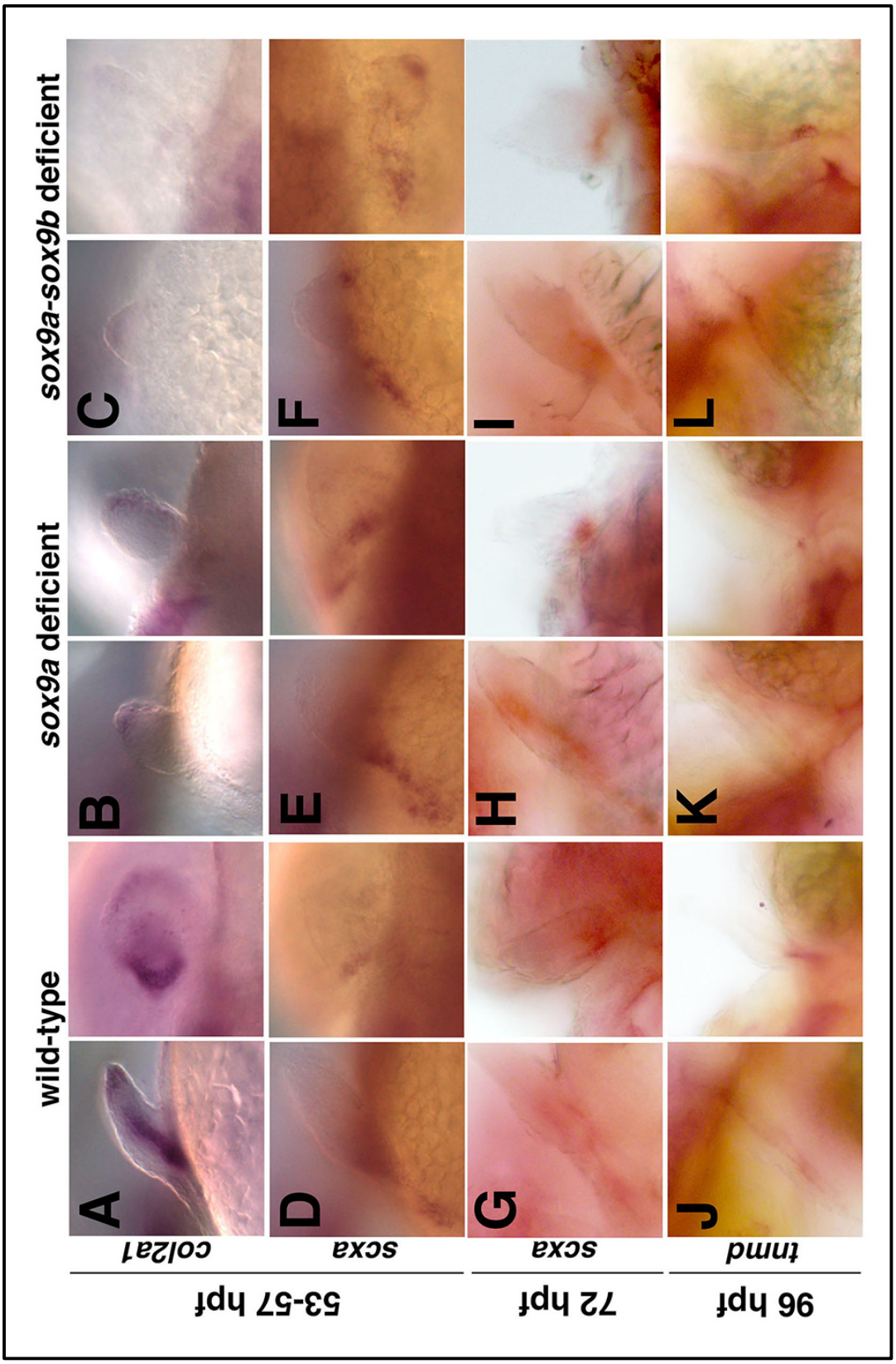

Figure 2.12 (Continued).

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 observed these changes in the *sox9a-sox9b*-deficient embryos at 53-57 hpf upon examination of *col2a1* expression (Figure 2.11C; 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 (Figure 2.11F, I, L; Figure 2.12; 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 (Figure 2.11G, 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 (Figure 2.13). 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

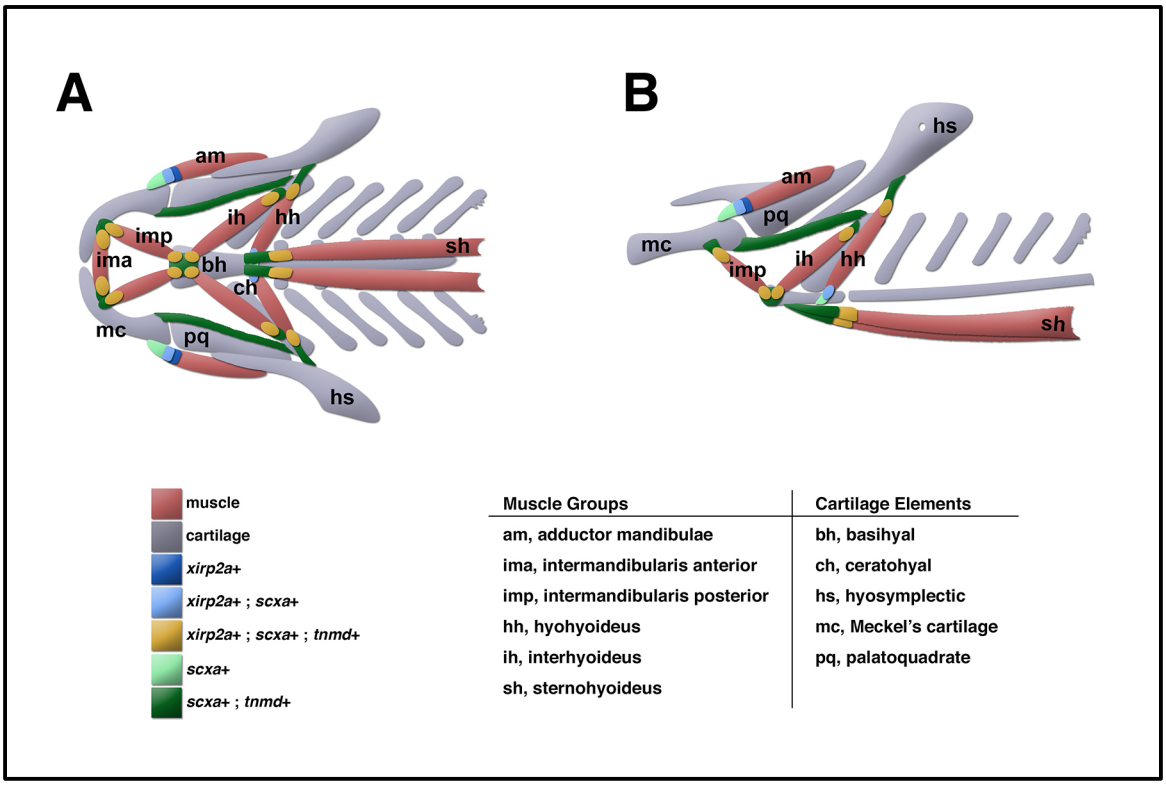

Figure 2.13. *scxa*, *tnmd*, and *xirp2a* expression in zebrafish craniofacial musculoskeletal tissue. (**A**) Ventral and (**B**) lateral views of craniofacial muscle (red), cartilage (gray), and tendons/ligaments. Populations of tendons and ligaments are colored according to their expression of *scxa*, *tnmd*, and/or *xirp2a*. Only the major muscle groups attached to the tendons are depicted.

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 (Figure 2.1B; 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 *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 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 due 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 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 since they are amenable to high-throughput screening approaches and live-image analysis of patterning events (Dahm and Geisler, 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.

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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 (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'-TCATCACCACCACAACGTCT-3' and 5'-TGTGCAGTTCGTTCAGTTCA-3'); and *ß-actin* (5'-TTCCTGGGTATGGAATCTTGCGGT-3' and 5'-TCGAGAGTTTAGGTTGGTCGTTCGT-3'). Embryos at 72 hpf were pooled, and *sox9a-sox9b*-deficient embryos were identified 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 are: *scxa* (Dr03104896) and *β-actin* (Dr03432610).

Morpholinos and injection

Morpholinos (Gene Tools) were injected at the 1-cell stage as described: *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'–GATTTCTAACGCTCTCCACAAGGCT–3'), *scxa2* (5'–CCATCGCAAAAGACATCATCAACTT–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'–GCTGGGTGTACGCAAGAAGAG–3'). Colorimetric *in situ* hybridization was performed in whole mount or on 10μM 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* (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 (Perkin-Elmer). Antibody staining was performed (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 (Perkin Elmer). 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. Figure 2.2A, 2.2B were generated with the Zeiss Zen software by overlaying the red, green and brightfield channels of each image, and the opacity and levels were adjusted in Photoshop to optimize the visualization of all three stains. Figure 2.3A, 2.3B were tiled together in Photoshop from several images taken of the same section. Figure 2.7A, 2.7B were also tiled together in Photoshop and using the tiling function on the Zen software during image capture. Some images for Figure 2.2 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.

Chapter 3

The mevalonate pathway through GGTase I regulates tendon cell induction during development

Attributions

I designed, performed, and analyzed the proliferation assays and the fate mapping of Tg(*sox10:kaede*) embryos. I characterized the role of hmgcrb, fntβ, and pggt1β, in addition to that of the Rho family of small G-proteins, in tendon development. I performed the qPCR assays and cell quantification upon chemical treatment. The following experiments were done in collaboration with Jenna Galloway – screening of the NINDS CC2 library and subsequent validation of the positive hits, in addition to expression analysis of musculoskeletal markers. Jenna Galloway generated the Tg(*scxa:mCherry*) line in the zebrafish. Matthew King, a former technician, performed the chemical rescue experiments and helped to establish the Tg(*scxa:mCherry*) line.

The mevalonate pathway through GGTase I regulates tendon cell induction during development

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Summary

Tendons and ligaments are necessary for force transmission and stability in the musculoskeleton. Surprisingly, the pathways regulating their specification are poorly understood. We sought to understand the processes that govern tendon cell induction using a zebrafish *in situ* hybridization small molecule screen. In this screen, Lovastatin and Simvastatin were discovered to promote a dose-dependent expansion of the craniofacial and pectoral fin tendon program. Chemical rescue experiments indicate that the statin-mediated expansion of *scleraxisa* is specific to the mevalonate pathway, and mediated by inhibition of geranylgeranyltransferase type I (GGTase I). Hmgcr inhibition specifically expands the tendon lineage of the musculoskeleton, and negatively impact cartilage and bone formation. Surprisingly, the expansion is not a consequence of increased cell proliferation. Instead, the expanded populations of craniofacial *scleraxisa*-positive cells are descendants of the cranial neural crest that we believe have been recruited from a chondrogenic and osteogenic fate. Taken together, we demonstrated that the GGTase I is a critical regulator of vertebrate tendon fate specification.

Introduction

The ability of tendons and ligaments to transmit and stabilize high tensile forces anchor them as the keystone components of the musculoskeletal system. Adult tendons have a hierarchically organized collagen network, which contributes to the mechanical integrity of the tissue. Their functional role, however, leads to a high incidence of occupational-related injuries, sports-related injuries, and age-related degeneration. (Jozsa and Kannus, 1997). Unfortunately, tendons are highly inefficient at healing once damaged (Sharma and Maffulli, 2005) and therapeutic intervention is currently unable to attain long-term functional repair of the injured

tissue (Rodrigues et al., 2013). A comprehensive understanding of the pathways regulating tendon tissue formation would have significant impacts in the clinical realm.

The tendon progenitor cells are specified in the craniofacial, axial, and limb tissues of the developing embryo in close association with the skeletal progenitors. The cranial skeletal and tendon progenitors are derived from the cranial neural crest (Chen and Galloway, 2014; Couly et al., 1993; Kontges and Lumsden, 1996; Le Douarin and Kalcheim, 1999; Le Lievre, 1978; Lumsden et al., 1991; Noden, 1978; Schilling and Kimmel, 1994). The axial skeletal and tendon progenitors are derived from a common somitic compartment, the sclerotome (Brand-Saberi and Christ, 2000; Brent et al., 2003; Monsoro-Burq and Le Douarin, 2000). The mesenchymal cells of the sclerotome lying immediately adjacent to the myotome in the developing somite are specified towards a tendon fate, giving rise to the syndetome, in response to myotomal FGF signaling (Brent et al., 2003). The limb cartilage, bone, and tendon progenitors are derived from the lateral plate mesoderm (Hurle et al., 1989; Hurle et al., 1990; Kieny and Chevallier, 1979; Ros et al., 1995; Saunders, 1948), and specifically, from a *Sox9*-expressing cell population (Akiyama et al., 2005). It remains to be elucidated the mechanism by which cells of the cranial neural crest and lateral plate mesoderm are specified towards a chondrogenic or tenogenic lineage. The molecular signals involved in specifying the tendon progenitors are largely unknown, and this is primarily due to the absence of robust early markers prior to the discovery of Scleraxis. Scleraxis is a basic helix-loop-helix transcription factor (Cserjesi et al., 1995) that faithfully marks the tendon and ligament lineages in all anatomical locations (Brent et al., 2003; Chen and Galloway, 2014; Grenier et al., 2009; Schweitzer et al., 2001). Scleraxis has been shown to regulate extracellular matrix proteins – Scleraxis activates the Collagen type I alpha 1 proximal promoter (Lejard et al., 2007), and positively regulates the expression of the

transmembrane glycoprotein Tenomodulin, a marker of differentiated tenocytes (Shukunami et al., 2006). Although *Scleraxis* is a faithful marker of the lineage, it is not required for the development of all tendons and ligaments, as the muscle-anchoring tendons and ligaments properly form in *Scleraxis* null mutants (Murchison et al., 2007). Studies in mouse and chick have implicated fibroblast growth factor (FGF) (Brent et al., 2003; Brent and Tabin, 2004) and transforming growth factor beta (TGFβ) signals (Oka et al., 2008; Pryce et al., 2009) in the regulation of tendon development. However, neither of these signals is required for the induction of tendon progenitors in all anatomical locations. Overall, the basic molecular landscape of tendon biology is extensively uncharted.

To discover new pathways that govern tendon cell induction, we utilized the zebrafish model as it is a powerful vertebrate genetic system amendable to high-throughput screening owing to its small size, high fecundity, and permeability to small molecules. An *in vivo* system whereby zebrafish are chemically treated in a physiologically-relevant state offers the benefit of providing spatial information regarding tissue specificity and toxicity. (Mathias et al., 2012) Chemical screens in zebrafish have contributed to a better understanding of biological processes (Kaufman et al., 2009; Peterson et al., 2000), such as development of the heart (Choi et al., 2013), liver (North et al., 2007), and kidney cysts (Cao et al., 2009). From an *in vivo* zebrafish screen for regulators of hematopoietic stem cells, the identification of Prostaglandin E2 have led to clinical trials for its usage in hematopoietic transplantation therapy (Goessling and North, 2014), underscoring the conservation in the developmental programs of zebrafish and humans. We have previously established the zebrafish as a model for the study of vertebrate tendon development, demonstrating that the tendons and ligaments of zebrafish have similarities in their gene expression, morphology, collagen ultrastructural arrangement and developmental regulation

to that of mammalian tendons (Chen and Galloway, 2014). With the precedence for chemical screens of biologically annotated bioactives in zebrafish, we speculated that unknown pathways involved in vertebrate tendon development may be identified through similar measures.

In this study, statins were identified in a screen-based approach in zebrafish for regulators of tendon development. Statins are a class of HMG-CoA reductase (Hmgcr) inhibitors (Istvan and Deisenhofer, 2001) that are clinically prescribed as cholesterol-lowering agents (Vaughan and Gotto, 2004). In this study, we demonstrate that hmgcr inhibition causes an expansion of the craniofacial and pectoral fin tendon programs in the zebrafish embryo. This phenomenon is shown to be specific to the tendon progenitors of the musculoskeleton and does not expand other musculoskeletal lineages such as the muscle, cartilage and bone. This expansion is not a consequence of increased proliferation of a *scleraxisa* (*scxa*)-positive cell population, but instead appears to be caused by a recruitment of neural crest progenitors towards the tendon lineage. The expanded domain of craniofacial *scxa*-positive tendon progenitors was fate mapped to be descendants of the cranial neural crest, whose differentiation towards a cartilage fate is hampered upon statin exposure. We further demonstrated that the interaction is mediated by inhibition of the geranylgeranylation branch of the mevalonate pathway, and specifically geranylgeranyltransferase I (GGTase I). Taken together, we have established the GGTase I activity is a critical regulator of tendon fate specification.

Results

Statin promotes expansion of zebrafish craniofacial and pectoral fin tendon programs

A chemical screen of a known bioactive compound library, the National Institute of Neurological Disorders and Stroke Custom Collection 2 (NINDS CC2) library (Figure 3.1A),

Figure 3.1. Chemical screen in zebrafish identify statin as regulator of craniofacial *scxa*-positive tendon progenitors. (**A**) Schematic of small molecule screen design. The NINDS Custom Collection 2 library, which contains 1040 known bioactive compounds, was screened in duplicate. Wild-type zebrafish embryos were incubated with the individual compounds from 32-56 hpf and alterations in craniofacial *scxa* were assessed by whole-mount *in situ* hybridization at 56 hpf. (**B-E**) Expression of *scxa* at 56 hpf upon chemical incubation from 32-56 hpf. (**B**) *scxa* is expressed in the pharyngeal arch (asterisk, arrow, arrowhead) of DMSO-treated embryos, which served as a positive control. (**C**) *scxa* is lost in the pharyngeal arches of SU5402-treated embryos, which served as a negative control. Treatment with Lovastatin (**D**) and Simvastatin (**E**), positive hits identified from the screen, caused expansion of *scxa* in the pharyngeal arches (arrow) compared with controls. Ventral views of flat-mounted embryos.

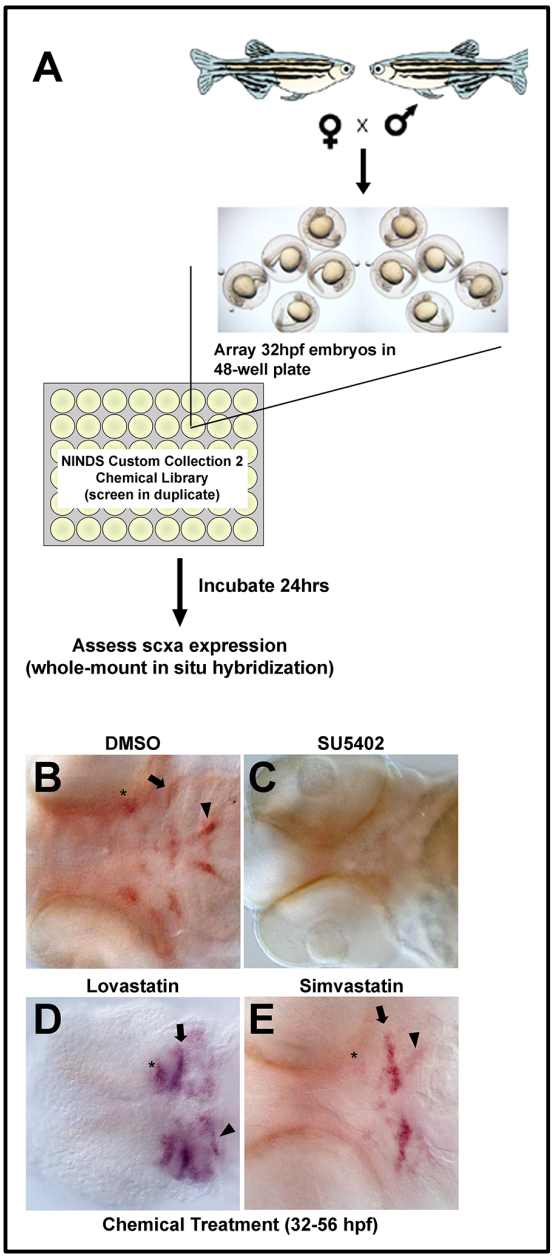
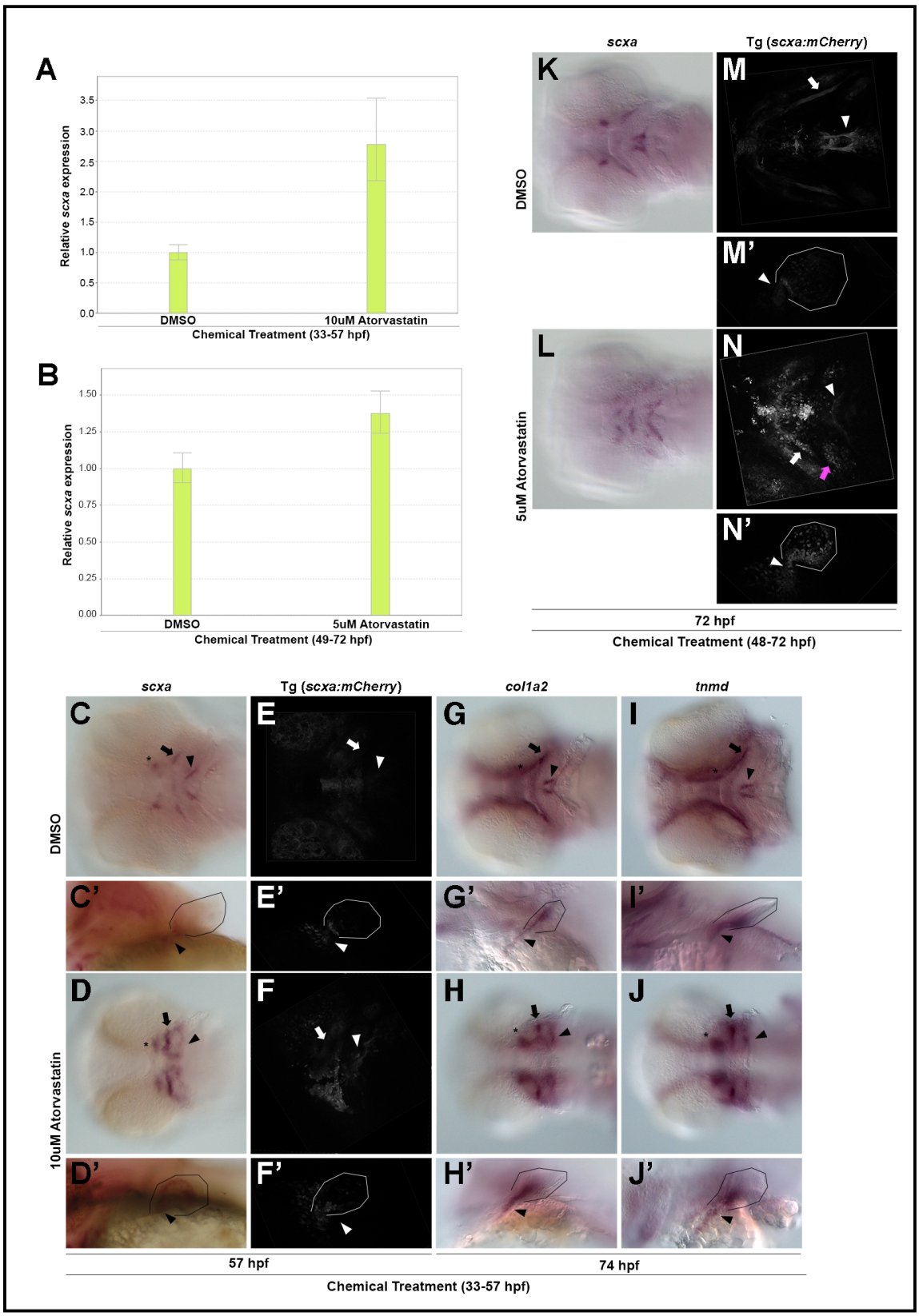


Figure 3.1 (Continued).

was conducted to identify new pathways regulating tendon development in zebrafish. Wild-type embryos were incubated with the individual chemicals from 32-56 hours-post-fertilization (hpf) and subsequently examined at 56 hpf for altered craniofacial *scxa* expression compared with controls (Figure 3.1B, 3.1C), as assessed by *in situ* hybridization. *scxa* is first detected in the pharyngeal arches of the zebrafish by 40 hpf (Chen and Galloway, 2014). A large proportion of the compounds (97.69%; 1016/1040) failed to alter *scxa* expression, whereas 2 (0.19%) were toxic and resulted in death, 10 (0.96%) resulted in developmental delay and unaltered *scxa* expression, 3 (0.29%) resulted in general and non-specific morphological abnormalities, 2 (0.29%) increased *scxa* expression, and 1 (0.10%) decreased *scxa* expression. Of the 24 compounds initially identified to affect *scxa* expression, 6 of the compounds are commercially unavailable and therefore could not be validated. The compounds that were identified to increase craniofacial *scxa* expression were Lovastatin and Simvastatin (Figure 3.1D, E). To determine if statins as a class of compounds cause expansion of *scxa*, we tested additional commercially available statin compounds that differ in pharmacokinetic properties, Atorvastatin and Fluvastatin (Igel et al., 2001). All four statin compounds caused a dose-dependent expansion of craniofacial *scxa* expression in wild-type embryos at 56 hpf following incubation from 32-56 hpf. Atorvastatin treatment resulted in the most robust phenotype (data not shown), thus, all further analysis was performed with this compound. In performing a time series of statin treatment, we found that Atorvastatin treatment from 48-72 hpf resulted in a similar expansion of *scxa* by *in situ* hybridization (Figure 3.2K, L). The statin-mediated upregulation of *scxa* was also confirmed by qPCR at both stages (Figure 3.2A, B).

To determine the effect of statin on the tendon program and in the different anatomical regions where tendons arise, we examined the expression of tendon markers and components of

Figure 3.2. Statin promotes expansion of craniofacial and pectoral fin tendon programs. (**A, B**) qPCR quantification of the relative expression levels of *scxa*. Atorvastatin increased *scxa* expression at 57 hpf (**A**) and 72 hpf (**B**) upon incubation from 33-57 hpf and 49-72 hpf, respectively, compared with controls. The ratio of *scxa* to *β-actin* reference was quantified, and normalized to the control sample; 125 embryos were pooled per condition; error bars represent analytical replicates (n=4). Significance of $p < 0.0001$ (1-tailed 2-sample t-test) was verified in 3 independent experiments for both treatment windows. (**C-J'**) Craniofacial and pectoral fin expression of *scxa* (**C-D'**) and the Tg(*scxa:mCherry*) (**E-F'**) at 57 hpf, and expression of *col1a2* (**G-H'**) and *tnmd* (**I-J'**) at 74 hpf upon chemical incubation from 33-57 hpf. (**K-N'**) Craniofacial and pectoral fin expression of *scxa* (**K, L**) and the Tg(*scxa:mCherry*) (**M-N'**) at 72 hpf upon chemical incubation from 48-72 hpf. Atorvastatin caused an expansion in the expression of all three genes (*scxa, col1a2*, *tnmd*) and the Tg(*scxa:mCherry*) in the pharyngeal arches (arrow); and at the base of the cleithrum (arrow), extending distally to the actinotrichia, compared with controls. Ventral views of the craniofacial region and lateral views of the pectoral fin in flat-mounted embryos.



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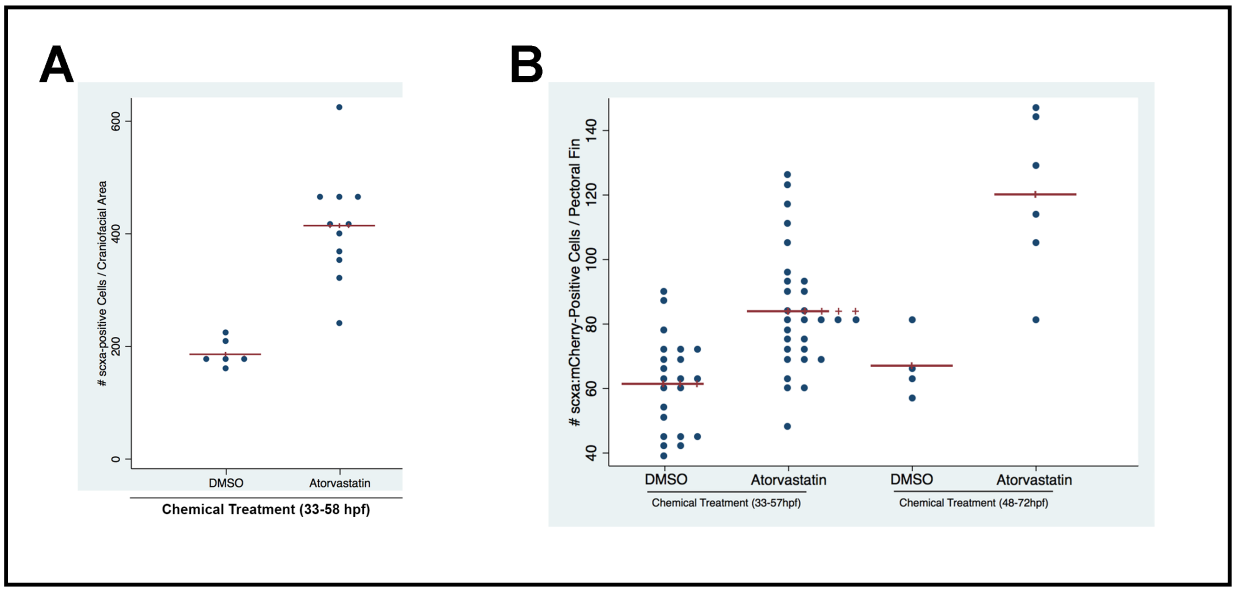


Figure 3.3. Statin increases the quantity of craniofacial and pectoral fin *scxa*-positive tendon progenitors. (**A**) Quantification of *scxa*-positive cells in the craniofacial region as assessed from confocal images of embryos processed by fluorescent *in situ* hybridization and counterstained with Hoechst 33342. Atorvastatin increased the quantity of *scxa*-positive cells ($p < 0.0001$, 1-tailed 2-sample t-test) at 58 hpf upon chemical incubation from 33-58 hpf, compared with controls. (**B**) Quantification of *scxa*-positive cells in the pectoral fin as assessed from confocal images of Tg(*scxa:mCherry*) embryos. Atorvastatin increased the quantity of *scxa*-positive cells at 57 hpf ($p < 0.0001$, 1-tailed 2-sample t-test) and at 72 hpf ($p = 0.0013$, 1-tailed 2-sample t-test) upon chemical incubation from 33-58 hpf and 48-72 hpf, respectively, compared with controls.

the tendon matrix, *scxa*, *tenomodulin* (*tnmd*), and *collagen type I alpha 2* (*col1a2*), in the craniofacial region, pectoral fin, and myosepta (Brent et al., 2003; Chen and Galloway, 2014; Lejard et al., 2007; Schweitzer et al., 2001; Shukunami et al., 2006). We found that embryos incubated with Atorvastatin from 33-57 hpf and 48-72 hpf have an expanded domain of *scxa* in the craniofacial and pectoral fin regions at 57 hpf (Figure 3.2C-F') and 72 hpf (Figure 3.2K-N'), respectively, compared with controls. Using confocal imaging of Atorvastatin-treated and control embryos, we were able to determine that the expansion in *scxa* expression reflects an over 2-fold increase in the quantity of *scxa*-positive cells in the craniofacial (Figure 3.3A) and pectoral fin (Figure 3.3B) regions. In addition, incubation with Atorvastatin from 33-57 hpf caused expansion of *col1a2* (Figure 3.2G-H') and *tnmd* (Figure 3.I-J') at 74 hpf in the craniofacial and fin regions compared with controls. In contrast, Atorvastatin affects neither the expression of *scxa* nor the quantity of *scxa*-positive cells in the myosepta (data not shown). Incubation with Atorvastatin from 33-57 hpf caused a reduction in *col1a2* and *tnmd* at 74 hpf in the myosepta when compared with controls (data not shown). As statin induces significant myopathy of myomeres (Hanai et al., 2007) and muscle is required for proper *scxa* expression in the axial region (Brent et al., 2003; Chen and Galloway, 2014), the myoseptal expression of *col1a2* and *tnmd* may be more sensitive than craniofacial and limb regions to muscle defects. Taken together, these data demonstrate that a screen-based approach in zebrafish has identified statin as a regulator of tendon development, wherein statin promotes an expansion of the craniofacial and pectoral fin tendon programs by increasing the quantity of tendon progenitors.

Inhibition of Hmgcr and mevalonate production expands the tendon program

As many drugs can affect multiple pathways in addition to their known characterized

target, we first sought to confirm that our phenotype resulted from statin inhibition of 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGCR), the rate-limiting enzyme of the mevalonate pathway (Istvan and Deisenhofer, 2001). Zebrafish have two HMGCR orthologs, *hmgcra* and *hmgcrb*, which are located on Chromosome 5 and 21, respectively. *hmgcra* is expressed in the larval liver and intestine; *hmgcrb* is maternally transcribed and ubiquitously expressed during early development. (Thorpe et al., 2004) As *hmgcrb* is expressed during the spatial-temporal domain of interest, we focused on *hmgcrb* for further analysis. To test whether the statin-mediated expansion in craniofacial *scxa* is due to inhibition of Hmgcrb, we examined *scxa* expression in *hmgcrb*-deficient embryos by *in situ* hybridization. In a subset of the *hmgcr1b*^{s617} mutants (D'Amico et al., 2007; Mapp et al., 2011), identified by their severe pericardial edema, *scxa* is moderately expanded at 60 hpf when compared with controls (Figure 3.4A, B). Furthermore, the complete loss of the maternal and zygotic *hmgcrb* in zebrafish, achieved by morpholino-mediated knockdown, resulted in severe morphological defects. This finding is consistent with the observation that the *Hmgcr* knockout in mouse is embryonic lethal (Ohashi et al., 2003), suggestive of an essential role for HMGCR in early mouse and zebrafish development. For this reason, a synthetic lethal approach was employed to induce acute loss of Hmgcrb within a specified developmental time window and to circumvent its earlier embryonic requirement. Embryos exposed to lower concentrations of the *hmgcrb* morpholino and Atorvastatin in combination displayed an expansion of *scxa* at 57 hpf compared with controls (Figure 3.4C-F), suggesting an essential role for Hmgcrb inhibition in the statin-mediated expansion of craniofacial *scxa*. To provide unequivocal evidence that inhibition of the mevalonate pathway is responsible for the phenotype observed, we rescued the expansion in craniofacial *scxa* expression to wild-type levels with metabolites of the mevalonate pathway.

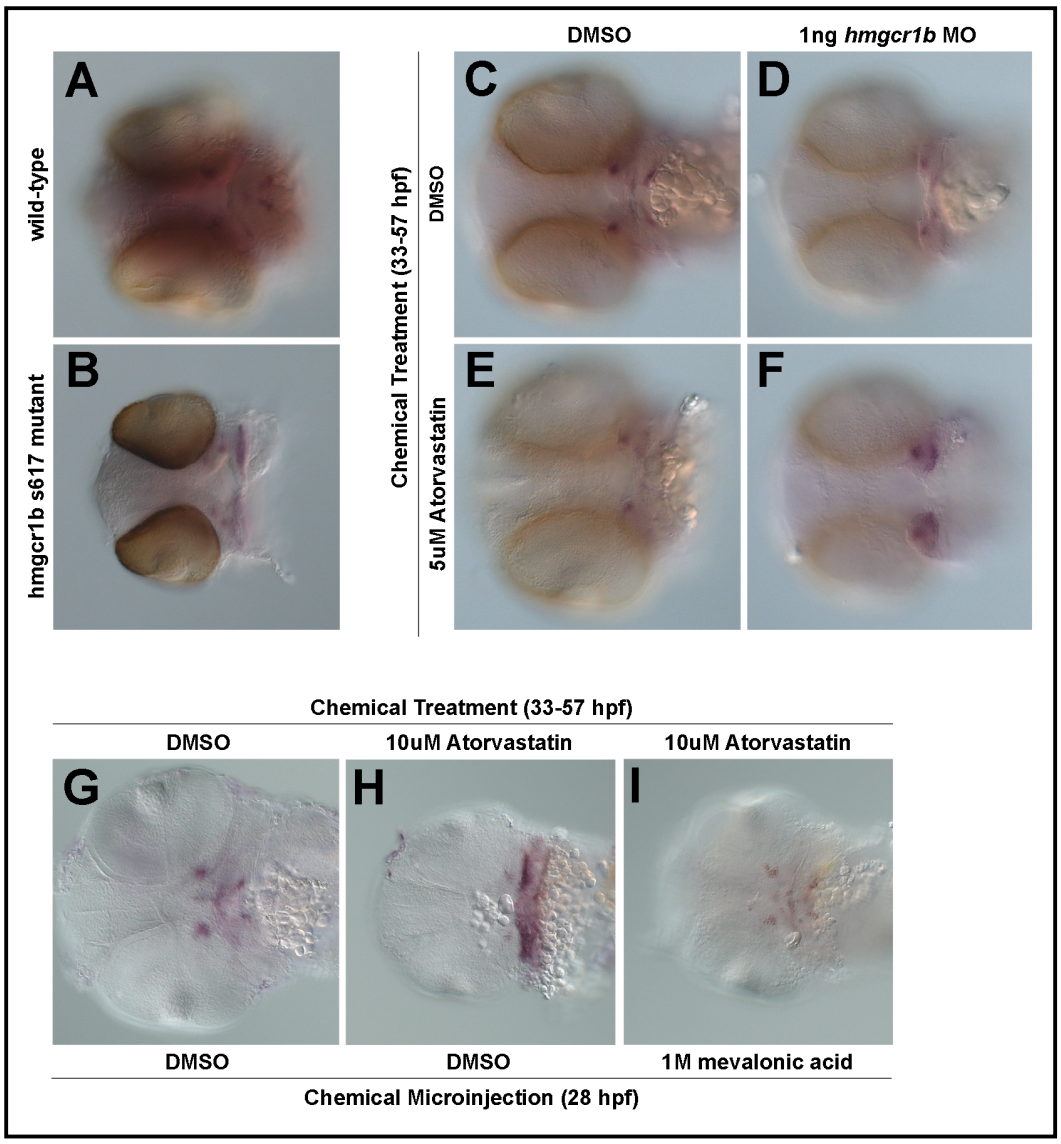


Figure 3.4. Role of Hmgcrb inhibition in statin-mediated expansion of *scxa*-positive tendon progenitors. (**A, B**) Expression of *scxa* at 60 hpf is moderately expanded in a subset of the *hmgcr1b*^{s617} mutants, identified by their severe pericardial edema (D'Amico et al., 2007; Mapp et al., 2011). (**C-F**) Expression of *scxa* is expanded at 57 hpf in embryos exposed to synthetic lethal interaction of *hmgcr1b* morpholino-mediated knockdown and chemical treatment from 33-57 hpf. (**G-I**) Expression of *scxa* at 57 hpf is rescued to wild-type levels (**G**, 100%; n=11) in embryos incubated with Atorvastatin from 33-57 hpf (**H**, 100%; n=11) and injected with mevalonic acid at 28 hpf (**I**, 94%; n=18). Ventral views of flat-mounted embryos.

Microinjection of mevalonic acid at 28 hpf, the immediate metabolite targeted by statin, rescued *scxa* expression at 57 hpf following incubation with Atorvastatin from 33-57 hpf (Figure 3.4G-I). Together, these data establish the inhibition of Hmgcrb as the mechanism by which statin promotes expansion of craniofacial *scxa*-positive tendon progenitors in the zebrafish.

Cranial neural crest patterning and tendon developmental regulators

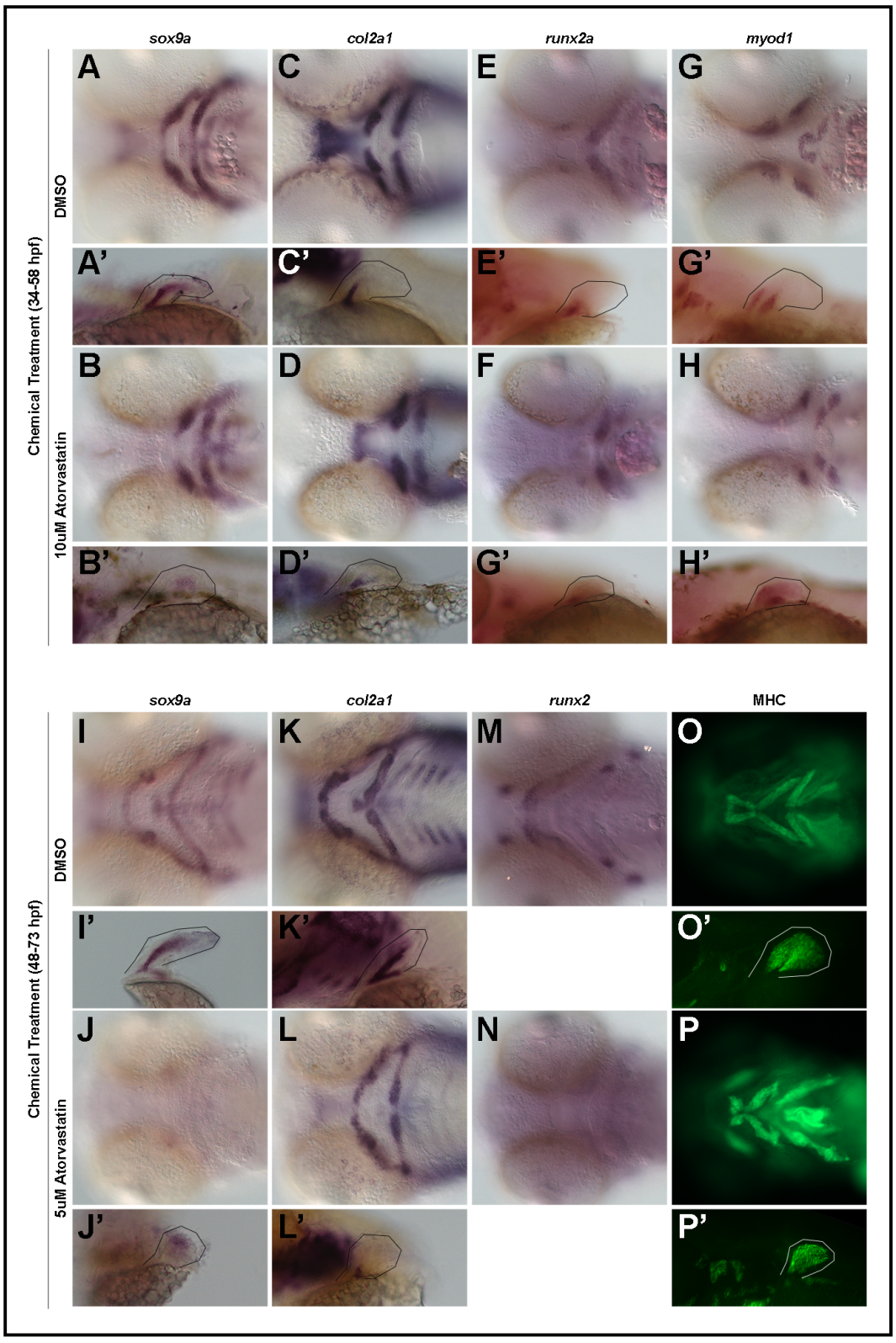
To understand if Hmgcr inhibition causes major changes in craniofacial patterning and tendon pathway regulators, we examined markers of pharyngeal arch patterning and of developmental pathways known to be involved in tendon development within the temporal window of interest. To investigate anterior-posterior and dorsal-ventral arch polarity, we examined the expression patterns of *hand2*, *bapx1*, *hoxa2b*, *hoxb2a* by *in situ* hybridization at 48 hpf upon chemical treatment from 33-48 hpf (Hunter and Prince, 2002; Miller et al., 2003). We found the expression of these genes were preserved in Atorvastatin-treated embryos compared with controls (data not shown), indicating that dorsal-ventral and anterior-posterior arch polarity were not affected. Analysis of TGFβ (*tgfbi*), FGF (*pea3*), and Wnt (*wnt4a*, *wnt9a*, *wnt5b, frizzled7a*, *frizzled7b*), which are components of pathways capable of promoting tendon fates (Brent and Tabin, 2004; Pryce et al., 2009; ten Berge et al., 2008; Yamamoto-Shiraishi and Kuroiwa, 2013), does not show upregulation in Atorvastatin-treated embryos compared with controls (data not shown). Moreover, analysis of Tg(*6xTcf/LefBS-miniP:d2eGFP*) (Shimizu et al., 2012), a marker of Wnt/β-catenin mediated Tcf transcriptional activity, at 60 hpf and 74 hpf upon chemical treatment from 33-57 hpf and 48-72 hpf, respectively, does not show upregulation of the transgene in Atorvastatin-treated embryos compared with controls (data not shown). Together, this suggests that statin-mediated expansion of *scxa* is not mediated through expanded

TGFβ, Wnt or FGF activity.

Statin inhibits chondrogenesis in craniofacial and pectoral fin regions

To test the specificity of the statin-mediated expansion for the tendon lineage, we examined the other tissue types in the craniofacial and pectoral fin musculoskeleton by *in situ* hybridization. Incubation with Atorvastatin from 34-58 hpf caused a slight reduction at 58 hpf in the craniofacial expression of *sox9a* ((*sex determining region-Y)-box 9a*) (Figure 3.5A, B), a marker of chondroprogenitors (Yan et al., 2002), *collagen type II alpha 1* (*col2a1*) (Figure 3.5C, D), a component of the cartilage matrix (Yan et al., 2002), and *runx2a* (*runt-related transcription factor 2a*) (Figure 3.5E, F), a marker of differentiating osteoblasts (Flores et al., 2006). Incubation with Atorvastatin from 48-73 hpf resulted in a significant reduction in *col2α1* (Figure 3.5K, L) and complete loss of *sox9a* (Figure 3.5I, J) and *runx2a* (Figure 3.5M, N) at 73 hpf in the craniofacial region. In the fin, the expression of *sox9a* (Figure 3.5A', B'), *col2a1* (Figure 3.5C', D'), and *runx2a* (Figure 3.5E', F'), were extensively reduced at 58 hpf upon Atorvastatin treatment from 34-58 hpf. Following Atorvastatin treatment from 48-73 hpf, the fin expression of *sox9a* is lost in the scapulocoracoid and extensively reduced in the endochondral disc of the fin bud (Figure 3.5I', J'); expression of *col2a1* is lost in the endochondral disc and extensively reduced in the scapulocoracoid (Figure 3.5K', L'). *runx2* expression is not detected in the pectoral fin at 73 hpf in DMSO- or Atorvastatin-treated embryos (data not shown). Examination of the muscle revealed that craniofacial expression of *myogenic differentiation 1* (*myod1*) (Figure 3.5G, H), a marker of myoblast progenitors (Lin et al., 2006), at 58 hpf and myosin heavy chain (MHC) (Figure 3.5O, P) (Blagden et al., 1997) at 73 hpf were spatially disrupted upon incubation with Atorvastatin from 34-58 hpf and 48-73 hpf, respectively. The disrupted

Figure 3.5. Effect of statin on craniofacial and pectoral fin musculoskeleton. (**A-H'**) Craniofacial and pectoral fin expression of *sox9a*, *col2a1*, *runx2a*, and *myod1* at 58 hpf upon chemical incubation from 34-58 hpf. (**I-P'**) Craniofacial and pectoral fin expression of *sox9a*, *col2a1*, *runx2a*, and myosin heavy chain (MHC) at 73 hpf upon chemical incubation from 48-73 hpf. Atorvastatin caused reduction in the craniofacial expression of *sox9a* (**A, B**), *col2a1* (**C, D**), and *runx2a* (**E, F**) at 58 hpf compared with controls. At 73 hpf in the craniofacial region, atorvastatin caused a drastic reduction in *col2a1* (**K, L**) and a complete loss of *sox9a* (**I, J**) and *runx2a* (**M, N**) compared with controls. At 58 hpf and/or 73 hpf in the pectoral fin, Atorvastatin caused extensive reduction in *sox9a* (**A', B', I', J'**), *col2a1* (**C', D', K', L'**), and *runx2a* (**E', F'**) compared with controls. Atorvastatin caused spatial disruptions in the craniofacial expression of *myod1* at 58 hpf (**G, H**) and MHC at 73 hpf (**O, P**) compared with controls. Atorvastatin does not alter pectoral fin expression of *myod1* at 58 hpf (**G', H'**) and MHC at 73 hpf (**O', P'**) compared with controls. Ventral views of craniofacial region and lateral views of pectoral fin in flat-mounted embryos.



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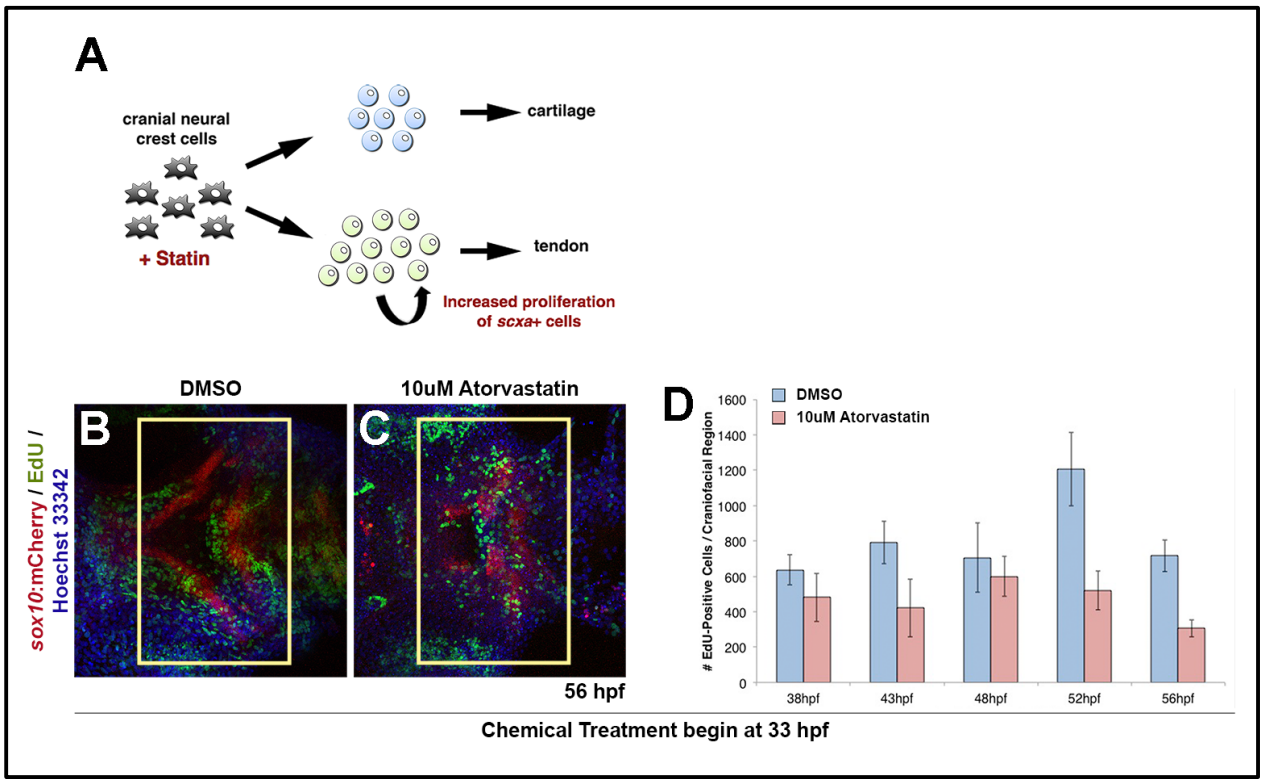

Figure 3.6. Statin-mediated expansion of tendon progenitors is not due to proliferation of craniofacial region. (**A**) Schematic of a proliferation mechanism. Statin-mediated changes are indicated in red. (**B, C**) Tg(*sox10:mcherry*) embryos at 56 hpf upon chemical treatment from 33-56 hpf that have been fluorescently labeled for EdU-positive cells. (**D**) Quantification of EdU-positive cells in the craniofacial region at 5-hour intervals upon chemical incubation from 33 hpf, as assessed from confocal images of embryos processed by fluorescent immunohistochemistry. Atorvastatin does not increase the quantity of EdU-positive cells in the craniofacial region at 38 hpf, 43 hpf, 48 hpf, 52 hpf, or 56 hpf, compared with controls. (n=3 embryos) were examined per condition. The yellow-colored boxes in (**B, C**) mark the craniofacial domain for which the counts were made. Ventral views of craniofacial region in flat-mounted embryos.

morphology of the craniofacial muscle is consistent with a deficiency in *sox9a* (Yan et al., 2002), as differentiating cartilage elements are essential for the patterning of pharyngeal muscles. The fin expression of *myod1* (Figure 3.5G', H') and MHC (Figure 3.5O', P') appeared to be relatively unaltered. Overall, this expression analysis demonstrates that statin causes expansion of tendon progenitors and a loss of cartilage and bone progenitors in the craniofacial and pectoral fin regions.

Role of proliferation in statin-mediated expansion of tendon progenitors

The increased numbers of tendon progenitor cells could result from increased proliferation of the *scxa*-expressing cells or from conversion of alternative cell types to the tendon lineage. To determine if increased proliferation causes the statin-mediated expansion of tendon progenitors (Figure 3.6A), we performed several assays including EdU (5-ethynyl-2'-deoxyuridine) and Phospho-Histone H3 (PH3) staining to assess the number of proliferating cells at multiple stages of statin treatment. For the craniofacial tendon progenitors, we quantified the number of proliferative cells based on incorporation of the thymidine analogue EdU into DNA during replication (Salic and Mitchison, 2008). EdU pulse-labeling was performed on embryos in 5-hour intervals following chemical treatment from 33 hpf until 56 hpf. EdU staining was performed on Tg(*sox10:mcherry)* transgenic embryos and the number of EdU-positive cells were quantified for a defined craniofacial region based on *sox10:mcherry* expression. Quantification of EdU-positive cells at multiple stages illustrated that Atorvastatin-treated embryos do not have more proliferative cells compared with controls (Figure 3.6B-D). However, subtle changes in the craniofacial *scxa*-positive domain may not be detected by this analysis. Accordingly, we tested the effect of the pharmacological inhibition of cellular proliferation with Aphidicolin, a DNA

polymerase inhibitor (Spadari et al., 1984), on craniofacial *scxa* expression by *in situ* hybridization and immunohistochemistry. We used a concentration of Aphidicolin that effectively reduces the quantity of cells expressing Phospho-Histone H3 (Figure 3.7C, F), a marker of mitotic cells (Ladstein et al., 2010). Embryos treated from 34-58 hpf with Atorvastatin and Aphidicolin in combination maintained an expansion in *scxa* expression at 58 hpf (Figure 3.7A-D). Quantification of the number of PH3-positive cells indicated that Atorvastatin treatment does not promote proliferation at 58 hpf in either the craniofacial region (Figure 3.7F) or specifically the *scxa*-positive domain of the craniofacial region (Figure 3.7G). In contrast, proliferation does contribute to the growth of the population of *scxa*-positive cells during normal development (Figure 3.7E). Similar findings were concluded for the fin (Figure 3.7A', B', H, I). Taken together, the data demonstrates that there is no increase in proliferation in the musculoskeletal tissue or specifically in the *scxa*-positive region, and therefore, does not contribute to the statin-mediated expansion of craniofacial and fin *scxa*-positive tendon progenitors in zebrafish.

Expanded domain of *scxa*-positive tendon progenitors are derived from the neural crest

The craniofacial cartilage and tendon lineages, two tissue types that are oppositely affected by statin, arise from a common progenitor, the cranial neural crest (Chen and Galloway, 2014; Schilling and Kimmel, 1994). To determine the embryonic origin of the expanded *scxa*-positive domain, we performed a fate-mapping experiment using the restricted expression of the Kaede protein to the neural crest lineage in the Tg(*sox10:kaede*) (Dougherty et al., 2012). The sensitivity of the anti-kaede antibody used allows detection of the descendants of the *sox10:kaede*-positive lineage, including cells that have turned off expression of the transgene.

Figure 3.7. Statin-mediated expansion of tendon progenitors is not due to increased proliferation of *scxa*-positive cells. (**A-D**) Craniofacial and pectoral fin expression of *scxa* and Phospho-Histone H3 (PH3) at 58 hpf upon chemical incubation from 33-58 hpf, as assessed by fluorescent *in situ* hybridization. Similar expression changes in *scxa* were observed when assessed by colorimetric *in situ* hybridization (data not shown). Expression of *scxa* in the pharyngeal arches is reduced in embryos treated with Aphidicolin (**C**), and expanded in embryos treated with Atorvastatin alone (**F**) and in combination with Aphidicolin (**D**), compared with controls (**A**). (**E-I**) Quantification of cells in the craniofacial and pectoral fin regions at 57/58 hpf upon chemical incubation from 33-57/58 hpf, as assessed from confocal images of embryos processed by fluorescent *in situ* hybridization and immunohistochemistry. (**E**) Quantification of craniofacial *scxa*-positive cells. Proliferation contributes to the growth of the *scxa*-positive domain ($p < 0.0001$, 1-tailed 2-sample t-test). A decrease in cellular proliferation does not hamper the Atorvastatin-mediated expansion of *scxa* ($p < 0.0001$, 1-tailed 2-sample t-test). (**F**) Quantification of craniofacial PH3-positive cells. Atorvastatin does not increase the quantity of PH3-positive cells in the craniofacial region compared with controls ($p = 0.8515$, 1-tailed 2-sample t-test). The concentration of Aphidicolin used is effective at reducing proliferation in the craniofacial region ($p = 0.0001$, 1-tailed 2-sample t-test). (**G**) Quantification of cells that co-express *scxa* and PH3 in the craniofacial region indicated no increase in Atorvastatin-treated embryos compared with controls ($p = 0.2904$, 1-tailed 2-sample t-test). (**H**) Quantification of PH3-positive cells in the pectoral fin indicated no increase in Atorvastatin-treated embryos compared with controls ($p = 0.4728$, 1-tailed 2-sample t-test). (**I**) Quantification of cells that co-express the *scxa* transcript and PH3 protein in the pectoral fin indicated no increase in Atorvastatin-treated compared with controls ($p = 0.4437$, 1-tailed 2-sample t-test). The yellow-

colored boxes in (**A, B, C, D**) mark the craniofacial domain for which the counts were made. Ventral views of craniofacial region and lateral views of pectoral fin in flat-mounted embryos. Red bar in graph indicates mean value. Astatin ADC, Atorvastatin-Aphidicolin co-treatment.

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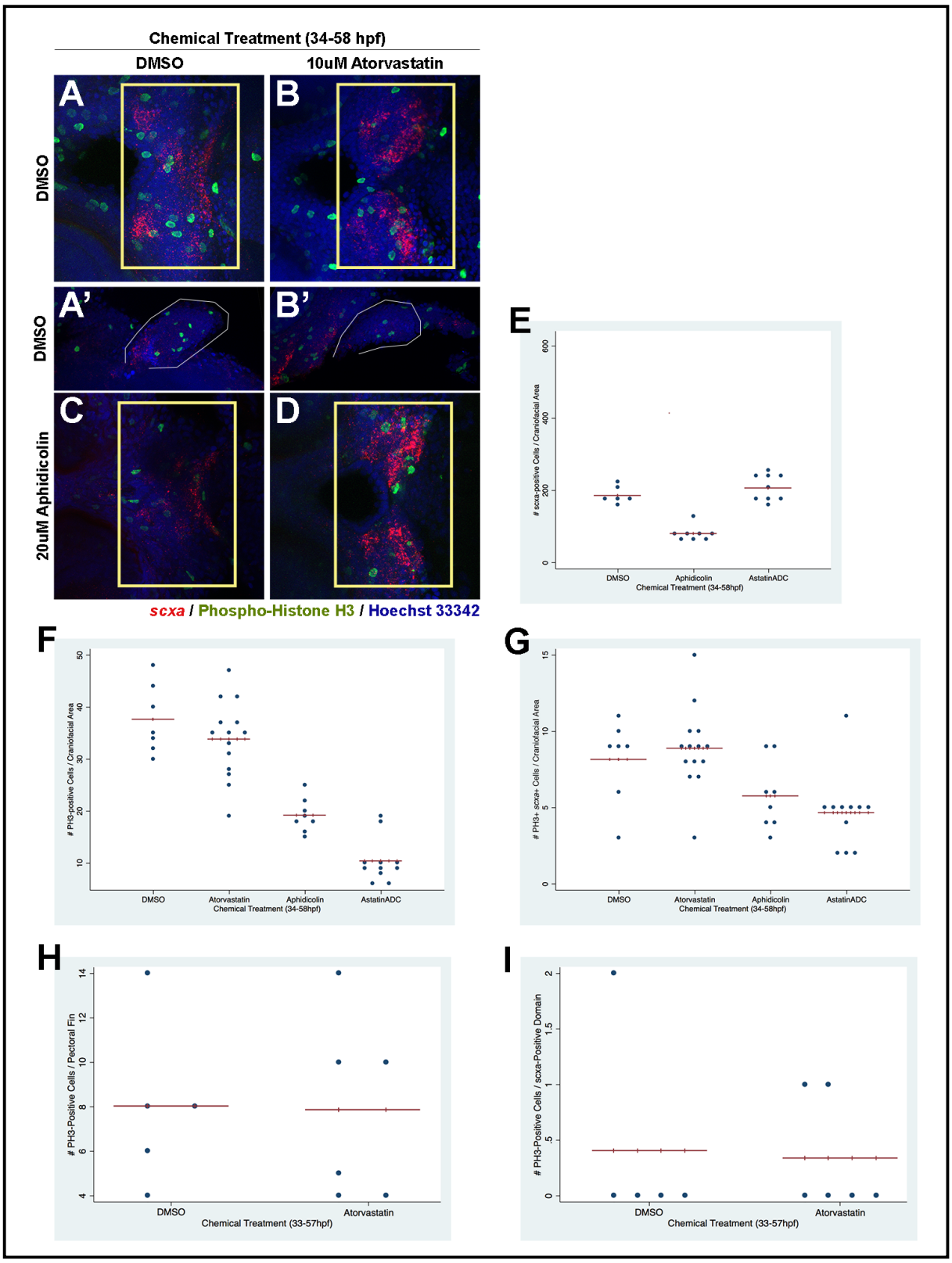

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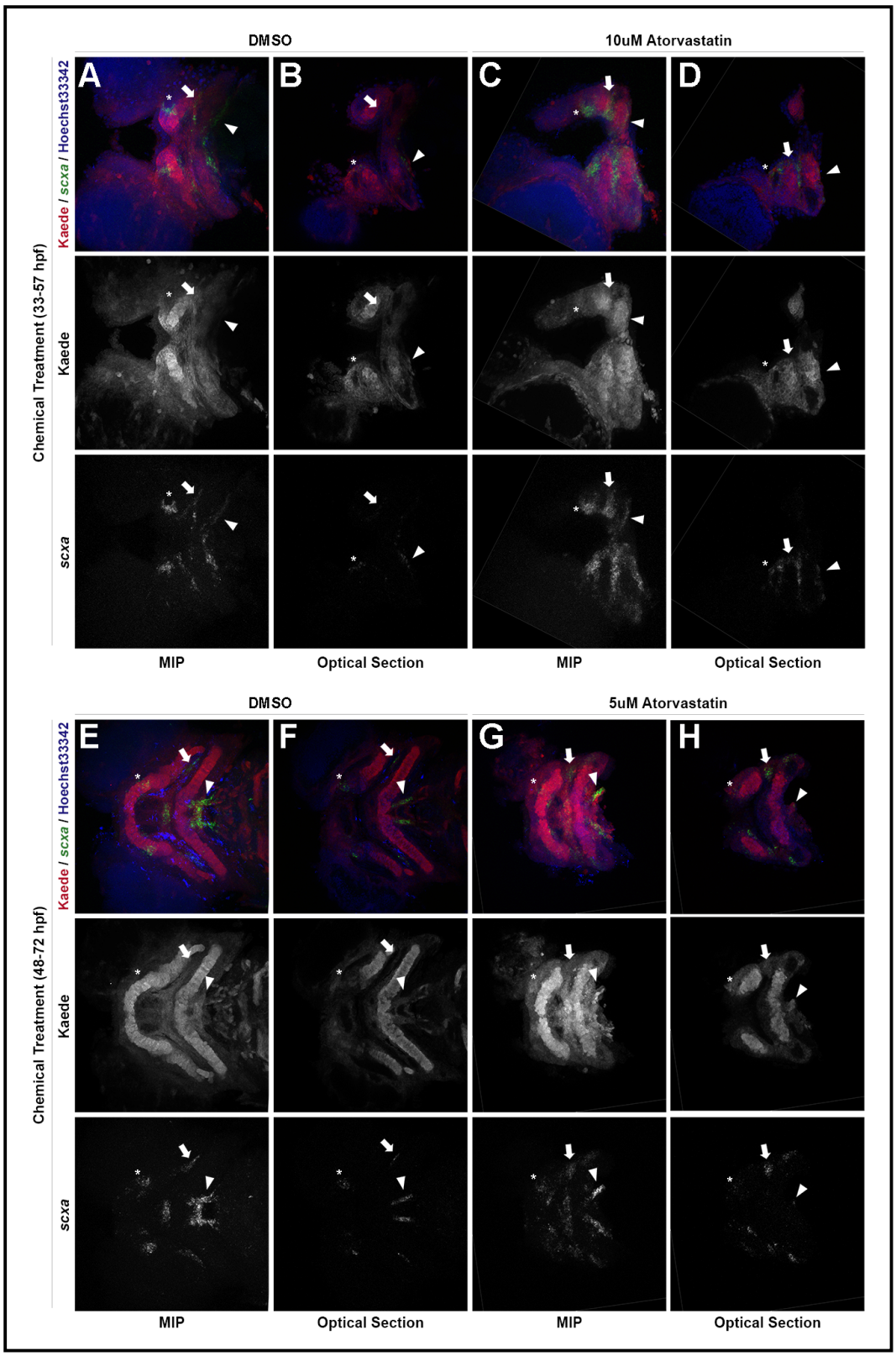
Confocal images of the craniofacial region of DMSO-treated (n=4 per timepoint) and Atorvastatin-treated (n=8 per timepoint) embryos that had been fluorescently stained for the presence of the Kaede protein and the *scxa* transcript, as well as counterstained with Hoechst 33342, were analyzed for Kaede and *scxa* colocalization. For each embryo examined, optical sections spanning the entire *scxa*-positive expression domain were analyzed to assess whether every *scxa*-positive cell co-expressed the Kaede protein. We found that the *scxa*-positive population in the Tg(*sox10:kaede*) embryos co-expressed the Kaede protein in all embryos examined following DMSO and Atorvastatin treatment from 33-57 hpf (Figure 3.8A-D; Figure 3.9A, B; Figure 3.10A) and 48-72 hpf (Figure 3.8E-H; Figure 3.9C, D; Figure 3.10B). Taken together, our data suggests that the statin-mediated expanded *scxa*-positive domain is exclusively derived from the cranial neural crest.

Statins increase the number of *scxa*-positive tendon progenitors, which is not accounted for by an increase in proliferation, and the *scxa*-positive cells are descendants of the cranial neural crest. As the population of cartilage and bone progenitors is reduced following statin treatment, and the cranial neural crest gives rise to the cranial cartilage and bone lineage, we speculate that the population of cells in the expanded *scxa*-positive domain are recruited from the cartilage lineage and is subsequently directed towards a tendon fate. To test the hypothesis of a statin-mediated recruitment of cranial neural crest cells towards the tendon lineage and to determine which stage of skeletal development this occurs, we examined a transgenic line that marks differentiated chondrocytes, Tg(*col2a1:eGFP*) (Dale and Topczewski, 2011). If statin treatment converts cells that have already progressed down the chondrocyte lineage, we would expect co-expression of *col2a* and *scxa* in subsets of cells in the statin-treated embryos compared with controls. Analysis of Tg(*col2a:eGFP;scxa:mCherry*) embryos illustrated that at 72 hpf in the

Figure 3.8. Statin-mediated expanded craniofacial *scxa*-positive domain is derived from the cranial neural crest. (**A-H**) Expression of *scxa* and Kaede in Tg(*sox10:kaede*) embryos at 57 hpf (**A-D**) and 72 hpf (**E-H**) upon chemical incubation from 33-57 hpf and 48-72 hpf, respectively. Co-localization of *scxa* and Kaede in Tg(*sox10:kaede*) embryos at 57 hpf and at 72 hpf is observed in all *scxa*-positive domains of DMSO- and Atorvastatin-treated embryos. The sensitivity of the anti-kaede antibody used allows detection of descendants of sox10:kaede-positive lineage that has turned off expression of Tg(*sox10:kaede*). Asterisks mark anterior-most domain of *scxa*, arrows mark middle domain of *scxa*, and arrowheads mark posterior-most domain of *scxa* in the pharyngeal arch. Ventral views of flat-mounted embryos.



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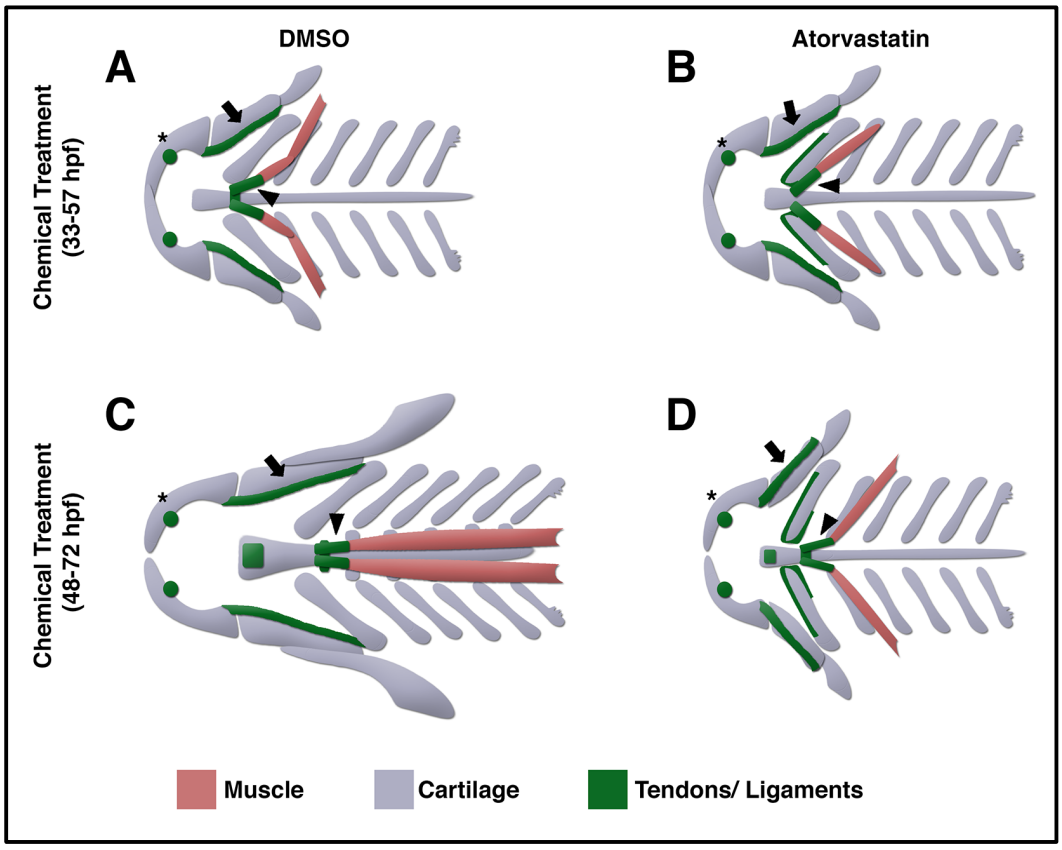

Figure 3.9. Schematic of statin-mediated expanded craniofacial scxa-positive domain in zebrafish. Expression of scxa at 57 hpf (**A, B**) and 72 hpf (**C, D**) upon chemical treatment from 33-57 hpf and 48-72 hpf, respectively. Asterisks mark anterior-most domain of scxa, arrows mark middle domain of scxa, and arrowheads mark posterior-most domain of scxa in the pharyngeal arch. Ventral views of craniofacial muscle (red), cartilage (gray), and tendons/ligaments (green) based on maximum intensity projection images from (**Figure 3.8A-H**). Only sternohyoideus muscle group attached to tendons is depicted.

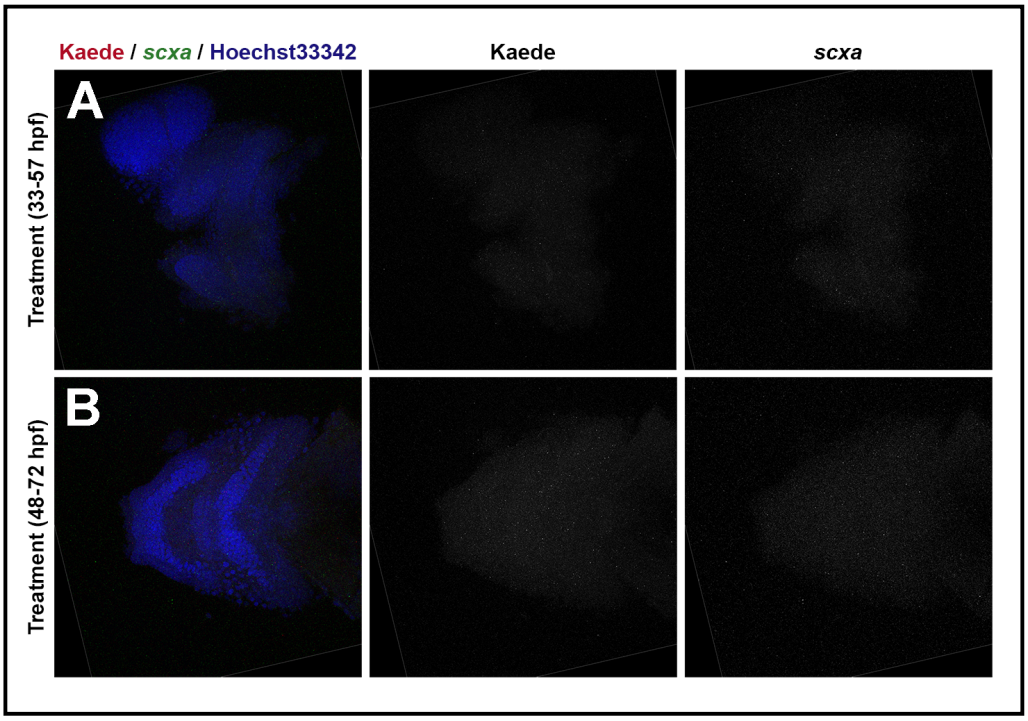

Figure 3.10. Secondary antibody only controls for Kaede antibody staining. Ventral views of flat-mounted embryos imaged at the same levels as in (**Figure 3.8A-H**).

Figure 3.11. Statin-mediated expanded craniofacial *scxa:mCherry*-expressing cells do not colocalize with *col2a:eGFP*-expressing cells. Craniofacial region of Tg(*col2:eGFP; scxa:mCherry*) embryos at 72 hpf upon chemical incubation from 48-72 hpf. (**A-C**) In the DMSO controls, the *col2:eGFP* and *scxa:mCherry* reporters are expressed in spatially distinct domains, with the exception of the junction points at which the cartilage and tendon progenitors meet (**A, B**, yellow-colored arrow). (**D-F**) In the Atorvastatin-treated embryos, the *scxa:mCherry* reporter (**D, E**, blue-colored arrow) is spatially expressed in putative cartilage elements (**D, E**, orange-colored arrow) though there is an absence of *scxa:mCherry*-positive cells co-expressing *col2a:eGFP* in these regions. All are ventral views of flat-mounted embryos. White-colored arrow marks mandibulo-hyoid ligament medial to the palatoquadrate; white-colored arrowhead marks tendon located at the sternohyoideus attachment site.

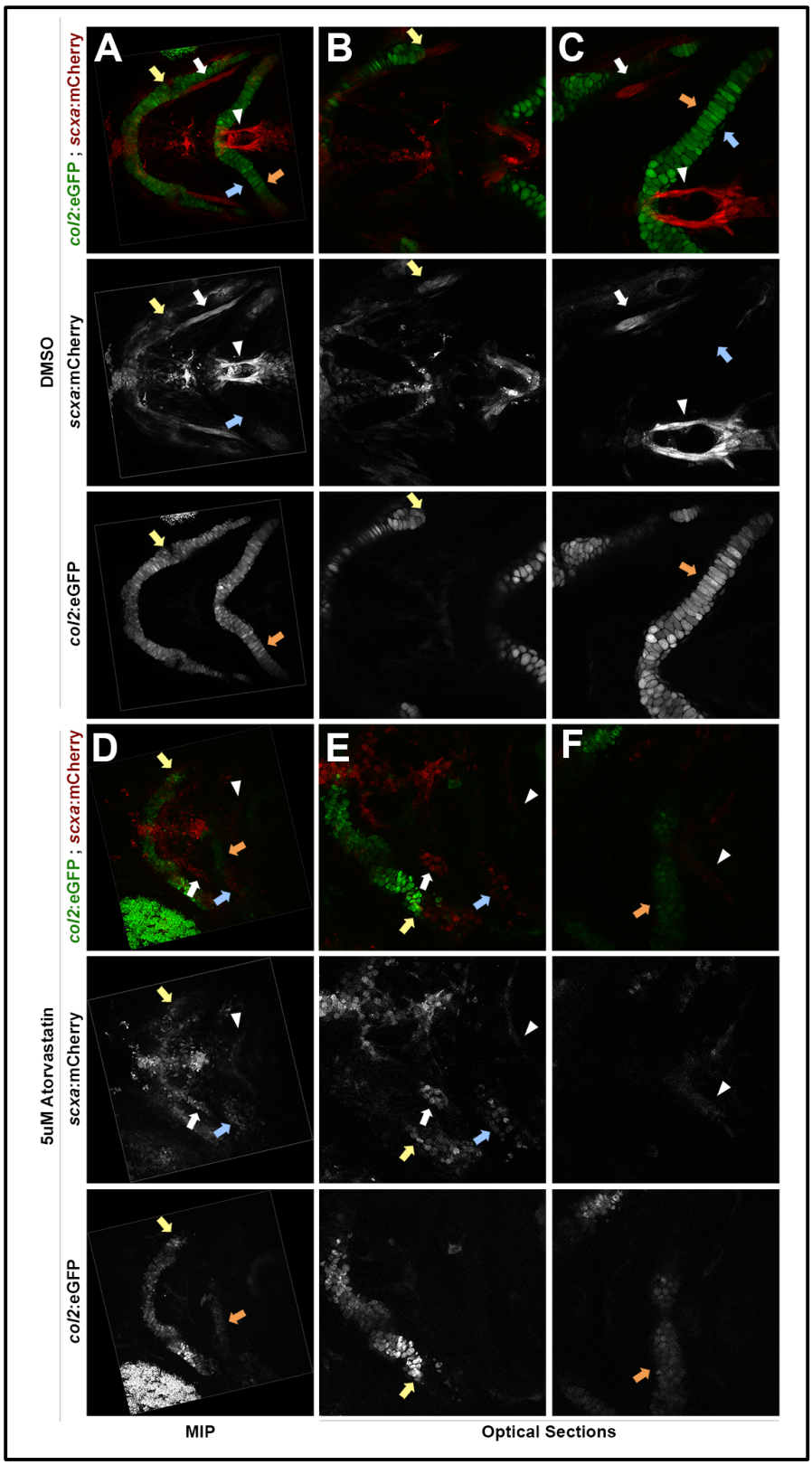

Figure 3.11 (Continued).

craniofacial region (Figure 3.11A-C), the *col2a:eGFP*-positive cartilage progenitors and *scxa:mCherry*-positive tendon progenitors are present in spatially distinct domains, with the exception of junction points at which the two tissues meet (Figure 3.11A, B, D, E, yellow-colored arrow). Atorvastatin treatment (Figure 3.11D-F) promotes expression of *scxa:mCherry*-positive tendon progenitors (Figure 3.11D, E, blue-colored arrow) in the putative cartilage elements (Figure 3.11D, F, orange-colored arrow). However, these *scxa:mCherry*-positive cells do not co-express *col2a:eGFP*, suggesting that the *scxa*-positive cells of the expanded domain have already been recruited prior to the onset of *col2a* expression. Our next step is to assess whether the statin-mediated conversion occurs by recruitment of *sox9a*-expressing skeletal progenitors, or alternatively, at the earlier stage of *sox10*-expressing cranial neural crest cells. A statin-mediated recruitment of *sox9a*-expressing or *sox10*-expressing progenitors would distinguish between a role for the mevalonate pathway in enforcing tendon specification in a common skeletal progenitor or in a multipotent neural crest cell population, respectively. By examining markers of other neural crest-derivatives (e.g. glial cells, melanocytes, neurons (Le Douarin and Kalcheim, 1999)), we may begin to delineate between these two possibilities.

Statin promotes the tendon program through inhibition of GGTase I

To dissect the molecular pathways and identify the components responsible for expanding tendon progenitors following statin treatment, we used a combination of genetic and chemical loss and gain of function approaches. The mevalonate pathway metabolizes the biosynthesis of cholesterol and isoprenoids (Figure 3.12A) (Goldstein and Brown, 1990; McTaggart, 2006). Isoprenoids are covalently attached to proteins by the post-translational modification processes of farnesylation or geranylgeranylation. Farnesylation is the addition of

the consensus sequence CaaX by farnesyl transferase (FTase); geranylgeranylation is the addition of the consensus sequence CAAL by geranylgeranyl transferase I (GGTase I) or GGTase II (Rab GGTase) (McTaggart, 2006). We examined the requirement of mevalonate-derived metabolites in the statin-mediated expansion of craniofacial *scxa*-positive tendon progenitors by *in situ* hybridization. To test the requirement of the cholesterol and prenylation branches in the expansion of *scxa*, we incubated embryos from 33-57 hpf with pharmacological inhibitors of GGTase I (GGTI-286) (Lerner et al., 1995), FTase (FTI-277) (Lerner et al., 1995), and 2,3-oxidosqualene:lanosterol cyclase (RO48-8071) (Morand et al., 1997). Inhibition of these branches in combination caused expansion of *scxa* at 57 hpf compared with controls (Figure 3.12B, C), which phenocopies that of embryos treated with Atorvastatin alone from 33-57 hpf (Figure 3.12D). In addition, microinjection at 28 hpf of farnesyl pyrophosphate, a branch-point intermediate for the cholesterol and prenylation branches (McTaggart, 2006), rescued *scxa* expression at 57 hpf following incubation with Atorvastatin from 33-57 hpf (n=7, 57% rescued), compared with DMSO-microinjected controls (n=43, 0% rescued). To test the requirement of the prenylation branch in the expansion of *scxa*, we used morpholinos targeted to *FTase beta* (*fntβ*) and *protein GGTase I beta* (*pggt1β*) (Mapp et al., 2011), and found that upon loss of both *FTase beta* (*fntβ*) and *protein GGTase I beta* (*pggt1β*) there was an expansion of *scxa* in the craniofacial region compared with controls at 60 hpf (Figure 3.12E, F) , suggesting the tendon cell expansion results from inhibition of prenylation rather than cholesterol synthesis. This expansion of *scxa* is maintained in *pggt1β* morphants alone (Figure 3.12G), whereas *scxa* is reduced in *fntβ* morphants (Figure 3.12H), indicating geranylgeranylation as the branch responsible for tendon cell expansion. In the pectoral fin, there was a corresponding expansion of *scxa* in *pggt1β* morphants alone, and a reduction in scxa in the *fntβ* morphants, compared with

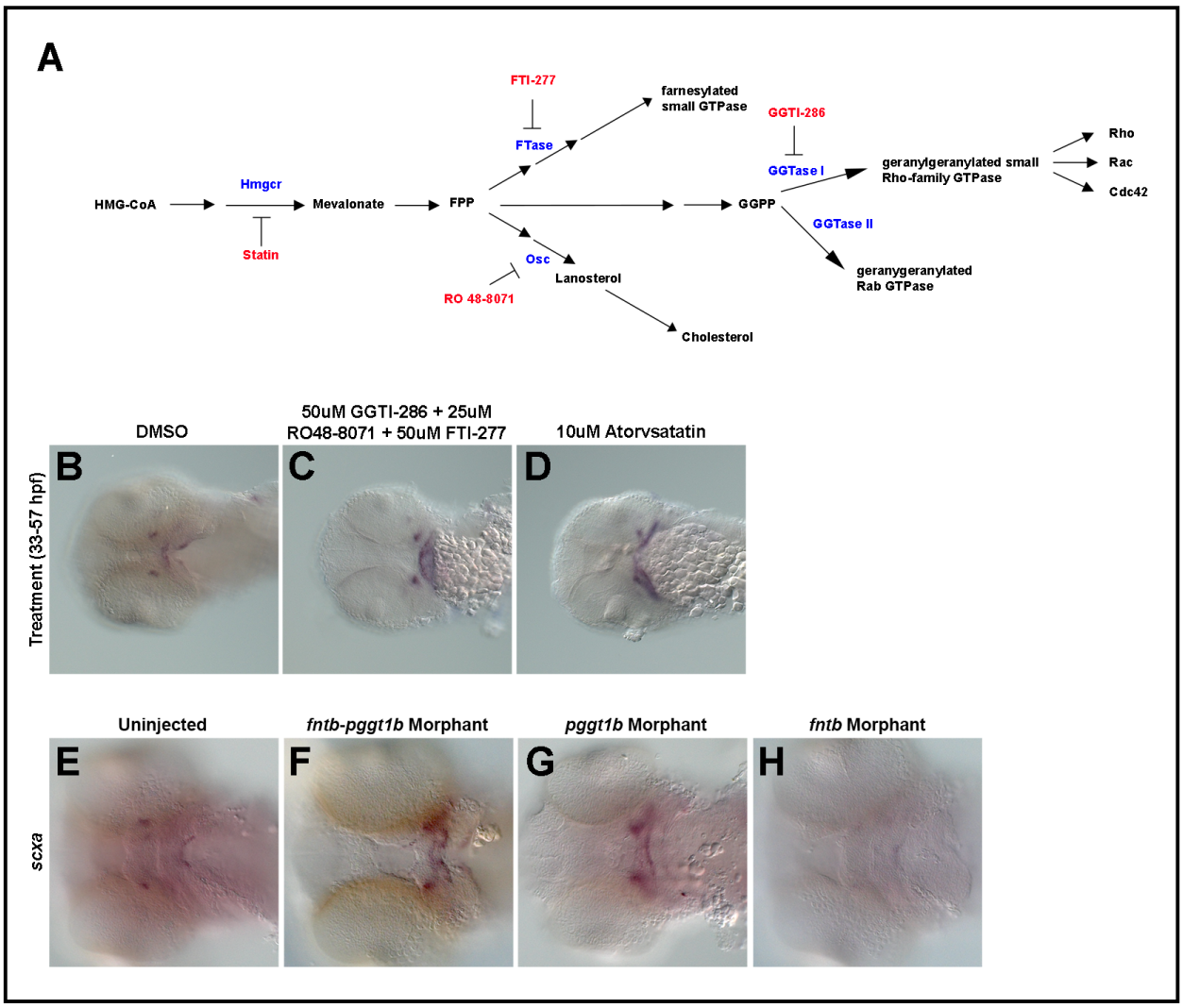

Figure 3.12. Role of the mevalonate pathway in statin-mediated expansion of *scxa*-positive tendon progenitors. (**A**) Simplified schematic of the Hmgcr pathway. Enzymes involved in the pathway are indicated in blue; chemical inhibitors are indicated in red. (**B-D**) Expression of *scxa* at 57 hpf, compared with controls (100%, n=6) (**B**), is expanded upon co-treatment with GGTI-286, RO48-8071, and FTI-277 following chemical incubation from 33-57 hpf (30%; n=10) (**C**). This expansion in *scxa* phenocopies that of Atorvastatin-treated embryos (**D**). (**E-H**) Expression of craniofacial *scxa* at 60 hpf, compared with controls (100%, n=41) (**E**), is expanded in fntβ-pggt1β (73%, n=103) (**F**) and pggt1β morphants (60%, n=89) (**G**), and reduced in fntβ morphants (29%, n=17) (**H**). Ventral views of the craniofacial region in flat-mounted embryos.

HMGCoA, 3-hydroxy-3-methylglutaryl-coenzyme-A; Hmgcr, HMG-CoA reductase; FPP, farnesyl pyrophosphate; FTase, farnesyl transferase; Osc, 2,3-oxidosqualene:lanosterol; GGPP, geranylgeranyl pyrophosphate; GGTase, geranylgeranyl transferase; fntβ, farnesyl transferase beta subunit; pggt1β, protein geranylgeranyl transferase 1 beta subunit.

Figure 3.12 (Continued).

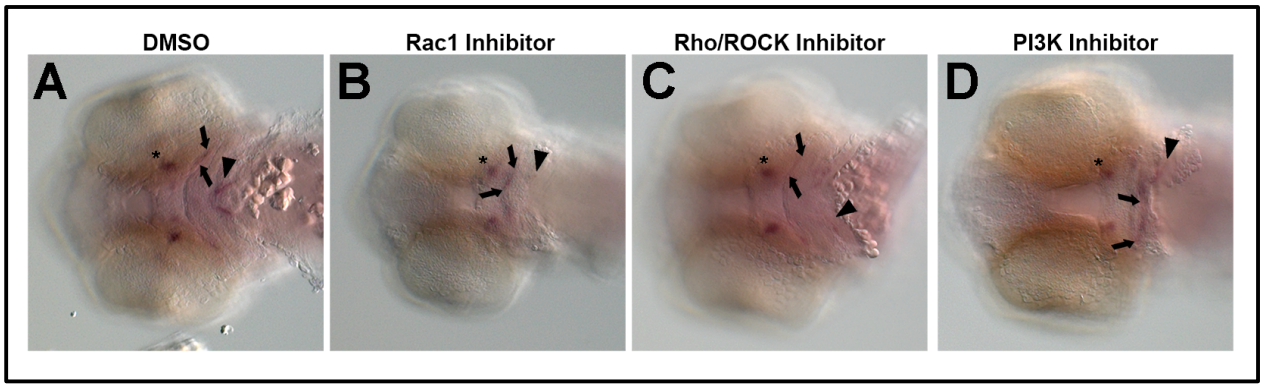

Figure 3.13. Molecular dissection of the statin-mediated expansion of *scxa*-positive tendon progenitors. (**A-C**) Expression of *scxa* upon chemical incubation with inhibitors of the small Rho GTPases from 33-57 hpf and *in situ* hybridization at 57 hpf. Expression of *scxa*, compared with controls (100%, n=31) (**A**), is expanded upon inhibition of Rac1 (10 μM EHT1864; 94%, n=17) (**B**), and reduced upon inhibition of Rho/ROCK signaling (75 μM Y27632; 100%, n=11) (**C**). (**D**) Expression of *scxa* is expanded, compared with controls, upon inhibition of PI3K (10 μM LY294002; 100%, n=9) from 33-57 hpf and *in situ* hybridization at 57 hpf. Asterisks mark anterior-most domain of *scxa*, arrows mark middle domain of *scxa*, and arrowheads mark posterior domain of *scxa* in the pharyngeal arch. Ventral views of the craniofacial region and lateral views of the pectoral fin in flat-mounted embryos.

controls (data not shown). In further support of a requirement for the geranylgeranylation branch in the expansion of *scxa*, microinjection at 28 hpf of geranylgeranyl pyrophosphate, an intermediate unique to the geranylgeranylation branch (McTaggart, 2006), rescued *scxa* expression at 57 hpf following incubation with Atorvastatin from 33-57 hpf (n=19, 47% rescued), compared with DMSO-microinjected controls (n=43, 0% rescued) . Together, these data indicate that inhibition of geranylgeranylation alone and GGTase I specifically is required for the statin-mediated expansion of craniofacial *scxa*-positive tendon progenitors in zebrafish.

GGTase I catalyzes the geranylgeranylation of the Rho family of GTPases, well-characterized regulators of extracellular signaling that control actin cytoskeleton organization, transcriptional activation, and mitogenesis (McTaggart, 2006; Van Aelst and D'Souza-Schorey, 1997). We examined the role of the Rho family members, Ras homolog (Rho) and Ras-related C3 botulinum toxin substrate (Rac), in craniofacial *scxa*-positive tendon progenitors by performing chemical incubation with specific inhibitors from 33-57 hpf and *in situ* hybridization for *scxa* at 57 hpf. There are three characterized Rac GTPases in vertebrates, the ubiquitously expressed Rac1 GTPase, the hematopoietic-specific Rac2 GTPase, and the neural-specific Rac3 GTPase (Bolis et al., 2003; Didsbury et al., 1989; Malosio et al., 1997; Shirsat et al., 1990). There are three characterized Rho GTPases in vertebrates, RhoA, RhoB, and RhoC, which are all ubiquitously expressed and involved in regulation of the cytoskeleton, with differences in their intracellular functions (Wheeler and Ridley, 2004). We observed that inhibition of Rac1 GTPase (EHT1864) (Onesto et al., 2008; Shutes et al., 2007) caused expansion of *scxa*, whereas inhibition of ROCK (Rho-associated protein kinase) signaling (Y27632) (Uehata et al., 1997) caused a reduction in *scxa*, compared with controls (Figure 3.13A-C). Together, these data demonstrate that inhibition of Rac1 GTPase causes a similar expansion of craniofacial *scxa*-

positive tendon progenitors in zebrafish to that of statin.

Activation of several Rho GTPases have been shown to be dependent on G-protein coupled receptors (GPCR)-mediated signaling (Bhattacharya et al., 2004). Heterotrimeric GPCRs, seven transmembrane domain receptor proteins, are the largest and most diverse family of membrane receptors (Oldham and Hamm, 2008). PI3K has been shown *in vitro* to be activated by the G-protein beta-gamma subunits (Stephens et al., 1994), an interaction that is Rac1-dependent (Ma et al., 1998). We observed that inhibition of PI3K (LY294002) (Vlahos et al., 1994) from 33-57 hpf and *in situ* hybridization for *scxa* at 57 hpf caused an expansion of *scxa,* compared with controls (Figure 3.13A, D). We speculate that statin disruption of beta-gamma subunit-dependent GPCR signaling alters PI3K/Rac1 signaling, and ultimately causes the statin-mediated expansion of *scxa*.

Discussion

We have delineated the mechanism by which statin regulates tendon development using the zebrafish model. Rather than promoting proliferation of the tendon progenitor cells, we have found that Hmgcr inhibition recruits additional cells to the tendon program from the neural crest lineage. We believe that fate conversion occurs at the expense of the cranial neural crest-derived skeletal subtypes, cartilage and bone. Furthermore, we have found that the tendon cell expansion occurs through inhibition of the geranylgeranylation branch of the mevalonate pathway. We believe this specifically involves the Rac1 GTPases, in a signaling cascade containing the G-protein beta-gamma subunits and PI3K.

Zebrafish chemical genetic screens

Taken together, this study provides further evidence that zebrafish chemical screens may identify novel molecular pathways involved in modulation of biological processes, ranging from those associated with early development to organogenesis (Rennekamp and Peterson, 2015). Chemical screens are advantageous over the large-scale ENU mutagenesis screens in zebrafish (Mullins et al., 1994; Solnica-Krezel et al., 1994) in that there is minimal space requirement as the screening is performed during embryonic development of the F1 generation. Moreover, the external development of embryos allows temporal modification of the chemical exposure window, and thereby, permits the characterization of later roles of pathways essential for early development. In addition, the permeability of zebrafish to small molecules (Peterson et al., 2000) bypasses the issue of low bioavailability, often the result of drug design focused towards applications in human therapies, and offers a system to investigate the mechanisms of pleiotropic effects.

Tendon-cartilage lineage induction

Our study demonstrates that statin negatively affects the differentiation of pre-chondrogenic progenitors. During development, FGF is necessary for proper formation of skeletal elements, specifically in patterning the proximal-distal identity (Lewandoski et al., 2000; Lu et al., 2006; Mariani et al., 2008; Minowada et al., 1999), with downstream intracellular signaling cascades mediated in part by the RAS (retrovirus-associated DNA sequences) pathway (Ornitz and Itoh, 2015). It is possible that statin, through farnesylation-mediated Ras inhibition (Bollag and McCormick, 1991), negatively affects chondrogenesis (Walshe and Mason, 2003). However, other studies have indicated that statin promotes cartilage development (Yamashita et

al., 2014). FGFR gain-of-function activating mutations are associated with skeletal dysplasia, such as achondroplasia (Cohen, 1998; Matsui et al., 1998), and statin treatment was able to rescue this skeletal defect to some effect. This is consistent with the finding that attenuation of activated FGFR signaling by C-type natriuretic peptide can partially rescue achondrodysplasia–related bone defects (Lorget et al., 2012). Furthermore, statins function by inhibiting the mevalonate pathway, and by extension, cholesterol biosynthesis and protein prenylation, which are known to be involved in a multitude of signal transduction cascades related to cell growth and differentiation, cytoskeleton organization, and metabolism (Goldstein and Brown, 1990; Marcuzzi et al., 2016; McTaggart, 2006; Thurnher et al., 2013). Approximately 300 prenylated proteins are predicted to exist in the human proteome (Sebti, 2005), underscoring the impact of the mevalonate pathway in regulation of a diverse array of biological processes. We speculate that these contrasting roles for statin in chondrogenesis may reflect mevalonate biosynthesis being an upstream regulator of specific pathways that act in different ways at distinct developmental stages.

Cranial neural crest lineage specification

We have thus far established that *scxa*-positive cells of the expanded domain following statin treatment have already been recruited from the cranial neural crest lineage prior to chondrocyte differentiation. Therefore, we speculate that statin-mediated conversion occurs by recruitment of an earlier developmental stage, specifically, of the *sox9a*-expressing skeletal progenitors (Figure 3.14A) or *sox10*-expressing cranial neural crest cells (Figure 3.14B). The difference in these two populations lies in their degree of cell potency and the microenvironment in which they reside.

Neural crest cells are a multipotent population that is able to differentiate towards a variety of lineages (Le Douarin and Kalcheim, 1999), though it is not well-characterized if and when the potency of these cells become restricted. Subpopulations of the cranial neural crest are postulated to become restricted in potentiality during ventral migration (Le Douarin and Smith, 1988), a hypothesis supported by the observation that migrating cranial neural crest cells contain both pluripotent and lineage-restricted populations when cultured *in vitro* (Baroffio et al., 1991). Moreover, the cranial neural crest gives rise to the all the membranous bones of the skull through a common osteo-chondrogenic progenitor population, as demonstrated by the loss of all membranous bones upon inactivation of Osterix (Osx), a marker of differentiated osteoblasts (Nakashima et al., 2002), in *Sox9*-expressing cells ($Osx^{flox/LacZ}$;*Sox9-Cre*) (Akiyama et al., 2005). Prior to ventral migration, this population of osteo-chondrogenic progenitors is segregated into two distinct lineages, giving rise to either the bones that form by endochondral ossification or those that form by intramembranous ossification. Inactivation of Sox9 in migrating neural crest cells ($Sox9^{flox/flox}$;*Wnt1-Cre*) results in lost of endochondral bone but not intramembranous bones (Mori-Akiyama et al., 2003). Such cell fate specification prior to ventral migration is not unique to the skeletal lineage, and has similarly been observed in specification of melanoblasts (Reedy et al., 1998). Given that our studies examined the role of GGTase I activity after ventral migration was completed and cranial *sox9a* expression was uniformly lost upon statin treatment, we speculate that the statin-mediated conversion occurs by recruitment of a progenitor population whose potency is at least restricted towards a skeletal lineage.

Extracellular cues influence the permissiveness of a microenvironment to direct cell fate maintenance stage, and does not affect the axial tendon program (Schweitzer et al., 2001). Such anatomical and temporal differences in response to extracellular signaling underscores the

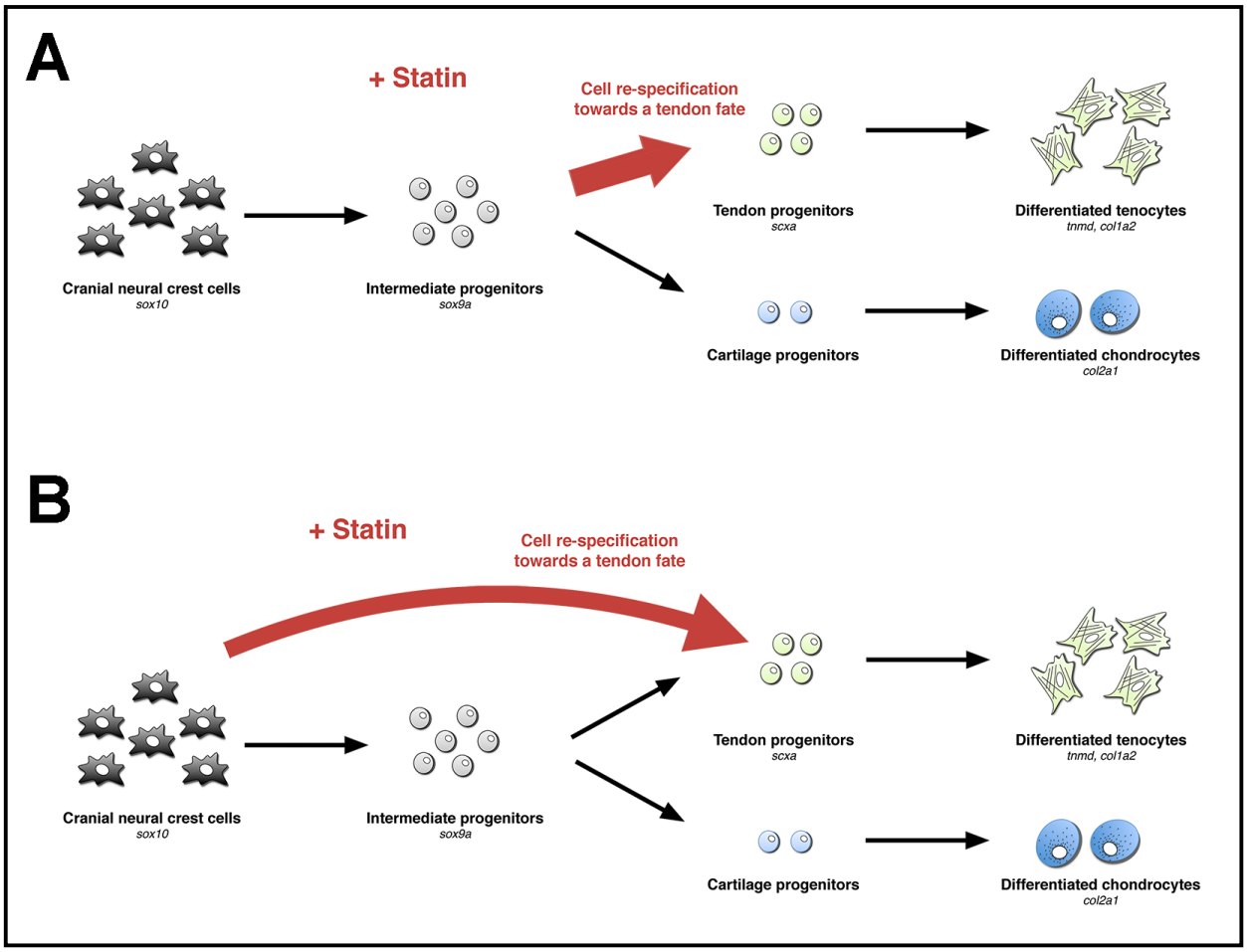

Figure 3.14. Models for mechanism of statin-mediated expansion of tendon program. (A) Cell fate conversion occurs by recruitment of *sox9a*-expressing skeletal progenitors. (B) Cell fate conversion occurs by recruitment of *sox10*-expressing cranial neural crest cells.

dynamic complexity of what constitutes a permissive domain for induction of tendon fates. In our studies, expression analysis of tendon markers revealed that the statin-mediated expansion of the craniofacial tendon program is localized to a defined domain of the pharyngeal arch. The specification. It has been shown from *in vivo* chick studies that signals from the adjacent ectodermal are necessary for the induction but not maintenance of limb tendon fates, and BMP signals are sufficient in repressing axial and limb tendon fates. Inhibition of BMP is sufficient in promoting tendon fates in the limb bud mesenchyme during the induction stage but not the restriction in the expansion of the tendon program indicates that not all populations of cranial neural crest-derived mesenchyme are competent to activate transcription of *scxa* upon inhibition of the mevalonate pathway. Moreover, the expanded domain of *scxa*-positive cells is bilaterally aggregated in a single domain, suggesting that they arise from a single pharyngeal arch primordium. Consequently, we speculate from the localized domain of expansion in *scxa*-positive tendon progenitors, and an unaffected dorsal-ventral and anterior-posterior arch polarity, that a permissive microenvironment for statin-mediated transcriptional activation of *scxa* may be established by arch-specific signaling.

Interaction between lateral plate mesoderm lineages in formation of pectoral fin

Our analysis of the pectoral fin revealed that statin promotes expansion of the tendon program. This expansion is associated with a loss of *sox9a* in the scapulocoracoid and a significant reduction of *sox9a* in the endochondral disc of the pectoral fin. Differences in the degree of reduction of *sox9a*, a marker of cartilage cells, in the different domains of the pectoral fin may be accounted for by differences in the cartilage developmental program. Endochondral ossification of the pectoral fin is mediated by the *sox9* co-orthologs, *sox9a* and *sox9b*, which

have overlapping expression domains in the fin bud and are functional redundant with each other (Yan et al., 2005). Specifically, *sox9a* is expressed at low levels in the scapulocoracoid and endochondral disc; *sox9b* is robustly expressed in the endochondral disc (Plavicki et al., 2014; Yan et al., 2005). Therefore, the presence of *sox9b* in the endochondral disc may be a contributing factor in the maintenance of a low level of *sox9a* upon statin treatment, as differences may exist in the effect of mevalonate inhibition on *sox9a*-positive cells depending on whether there is co-expression of *sox9b*.

In the zebrafish, it is speculated that the cartilage (Ahn et al., 2002; Kague et al., 2012) and tendon lineages of the pectoral fin are derived from a common progenitor, the lateral plate mesoderm. It has been demonstrated in the limb bud that the tendons are derived from the lateral plate mesoderm in chick (Wortham, 1948), and the cartilage and tendon lineages are derived from a common progenitor population in mouse (Pearse et al., 2007). However, the absence of specific lineage tracing lines for the lateral plate fin bud mesoderm prevents a similar fate-mapping of the statin-mediated expanded *scxa*-positive domain in the pectoral fin bud as was performed for the craniofacial region.

Scleraxis, Rac1 GTPases, and the cytoskeleton

Cell morphology influences the differentiation of mesenchyme towards chondrogenic, osteogenic, and adipogenic lineage (McBeath et al., 2004; Solursh et al., 1982). The cytoskeletal network of actin filaments regulates cell morphology (Zanetti and Solursh, 1984) through nonmuscle myosin II (NMM II)-mediated focal adhesion dynamics (Cai et al., 2006), and this has been shown to be critical in the differentiation of naïve mesenchyme stem cells towards different lineages *in vitro* (Engler et al., 2006). Focal adhesions relay external mechanical cues

between the extracellular matrix and the cell (Bershadsky et al., 2003), and modify the assembly of the cytoskeleton in response to matrix stiffness (Beningo et al., 2001; Discher et al., 2005). In tenocytes, cell morphology influences gene expression, such as that of Collagen type I (Li et al., 2008; von der Mark et al., 1977), and the actin cytoskeleton functions in proper organization of the collagenous extracellular matrix (Canty et al., 2006). As the Rho family of GTPases are known to pattern the cytoskeleton (Takai et al., 2001), interaction between the Rho GTPases may regulate the cell fate decision of cranial neural crest mesenchyme in the differentiation towards a chondrogenic versus tenogenic lineage. This model, however, is dependent upon the effect of the Rho family of GTPases on *scxa* expression being cell autonomous. This possibility may be addressed through gain- and loss-of-function studies whereby signaling by the small Rho GTPases Rac1 and RhoA are altered in a Gal4/UAS-mediated tissue-specific manner. Rac1 and RhoA activity may be upregulated by expressing constitutively active forms of the proteins, $Rac1^{V12}$ and $RhoA^{V14}$, respectively; Rac1 and RhoA activity may be attenuated by expressing the Cdc42/Rac interactive binding (CRIB) domain of PAK1 and the Rho-binding domain of protein kinase N, respectively, targeted to the membrane (Kardash et al., 2010).

Nevertheless, it is interesting to speculate that cytoskeletal changes caused by alteration in Rac1 could underlie the alteration in cell fates observed upon statin treatments. We observed that Y27632-mediated inhibition of Rho/ROCK signaling reduced, and EHT1864-mediated inhibition of Rac1 expanded, expression of *scxa*-positive tendon progenitors, suggesting that Rho/ROCK signaling is required for and Rac1 GTPases suppresses tenogenic fate. The former finding is consistent with a role for Rho/ROCK signaling in mediating gene expression of tendon markers (i.e. *Scx* and *Tnmd*) of mesenchymal stem cells *in vitro* (Maharam et al., 2015). Furthermore, Rac1 is necessary and sufficient in promoting early chondrogenesis and

hypertrophic differentiation, whereas Rho is necessary and sufficient in repressing chondrocyte maturation (Jungmann et al., 2012; Wang and Beier, 2005; Wang et al., 2004; Woods et al., 2005; Woods et al., 2007). Rho/ROCK signaling regulates the actin cytoskeleton, with a role in suppressing cortical actin organization, a characteristic of differentiated chondrocytes (Woods et al., 2005). Our data parallels this finding as we observe loss of *sox9a*-positive cartilage progenitors upon statin treatment. *Sox9* levels is dependent on actin and microtubule dynamics (Woods et al., 2005), in part through signaling that is positively regulated by the Rac1 pathway (Woods et al., 2007) and negatively regulated by the Rho/ROCK pathway (Woods et al., 2005). The necessity of Rac1 and/or Rho GTPases in the regulation of *sox9a* expression may be tested in the zebrafish to assess whether this crosstalk is conserved in the osteo-chondrogenic program of teleosts. Although additional experiments are necessary to support our model, it is tempting to speculate that statin promotes expansion of the craniofacial and pectoral fin tendon program by influencing the actin cytoskeleton, and consequently, the morphology, of cranial neural crest cells via a Rac1-mediated mechanism to promote differentiation towards a tenogenic fate and repress differentiation towards a chondrogenic fate.

Conservation of the mevalonate pathway in mammalian tendon development

The craniofacial cartilage program between teleosts and mammals is highly conserved (Medeiros and Crump, 2012; Schilling and Kimmel, 1997), and as such, we speculate that the role of mevalonate biosynthesis in tendon development would likewise be conserved in higher vertebrates. HMGCR is essential for early mouse development, as evident by the embryonic lethality of the *Hmgcr* knockout (embryos die prior to embryonic day 8.5) (Ohashi et al., 2003). The role of the mevalonate pathway after gastrulation and prior to the onset of *Scx* expression

may be studied using mice homozygous for a floxed Hmgcr or a Rac1 allele (Glogauer et al., 2003; Nagashima et al., 2012; Srinivasan et al., 2009; Zhou et al., 2013). The mouse would offer clear advantages in the ability to perform tissue- and stage-specific loss-of-function analyses. Such Cre drivers as Wnt1, Prx1, or Sox9 would aid in determining the effects of *Hmgcr* deletion specifically in the migrating neural crest cells (Chai et al., 2000; Danielian et al., 1998) limb mesenchyme (Logan et al., 2002), or skeletal progenitors (Akiyama et al., 2005), respectively.

In addition, micromass cultures, in which cells aggregate and mimic the process of chondrogenesis, provide a means to dissect pathway function directly on the limb bud progenitors *in vitro*. These types of analyses in mammals would further reveal the mechanism by which the mevalonate pathway regulates tendon development.

Zebrafish chemical genetic screens

Taken together, this study provides further evidence that zebrafish chemical screens may identify novel molecular pathways involved in modulation of biological processes, ranging from those associated with early development to organogenesis (Rennekamp and Peterson, 2015). Chemical screens are advantageous over the large-scale ENU mutagenesis screens in zebrafish (Mullins et al., 1994; Solnica-Krezel et al., 1994) in that there is minimal space requirement as the screening is performed during embryonic development of the F1 generation. Moreover, the external development of embryos allows temporal modification of the chemical exposure window, and thereby, permits the characterization of later roles of pathways essential for early development. In addition, the permeability of zebrafish to small molecules (Peterson et al., 2000) bypasses the issue of low bioavailability, often the result of drug design focused towards applications in human therapies, and offers a system to investigate the mechanisms of pleiotropic

effects.

Conclusion

The identification of a role for the mevalonate pathway in directing tendon fate specification of the cranial neural crest provides insight into the processes that govern tendon cell induction. The involvement of GGTase I activity in tendon development suggests the contribution of cytoskeletal dynamics in directing tendon cell fates. Lastly, our studies highlight the advantages of the zebrafish system and chemical screening for discovering novel genetic pathways involved in highly conserved biological processes.

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Materials and Methods

Fish maintenance and genotyping

Zebrafish were staged and maintained as described (Kimmel et al., 1995; Westerfield, 2000). *sox10:kaede* (Dougherty et al., 2012), *sox10:mCherry* (Kong et al., 2014), and *sox10:eGFP* (Curtin et al., 2011) were obtained from Dr. Eric Liao (Massachusetts General Hospital, Boston, MA, USA), *hmgcr1b*^{s617} were obtained from Dr. Masazumi Tada (University College London, London, UK), col2*:eGFP* (Dale and Topczewski, 2011) were obtained from Dr. Jacek Topczewski (Northwestern University, Chicago, IL, USA). Heterozygous adults were genotyped for *hmgcr1b*^{s617} (D'Amico et al., 2007) as described previously. The *scxa:mcherry* transgenic was generated using a bacterial artificial chromosome (BAC) containing the entire *scxa* genomic locus (CHORI BACPAC Resources). Using constructs and protocols provided by Stefan Schulte-Merker (Bussmann and Schulte-Merker, 2011), *mCherry* was recombineered into the first exon of the *scxa* BAC (CH211 251g8) and tol2 sites were introduced into the vector backbone. Properly targeted BACs were confirmed by PCR, prepared and injected into 1-cell stage embryos. Injected embryos were raised to adults and *mCherry*-expressing offspring were identified and raised to establish the line. Expression analysis by whole-mount *in situ* hybridization demonstrated faithful expression of *mCherry* with *scxa* at all developmental stages examined. All animal work was performed with IACUC approval.

Chemical screen design and validation testing

Wild-type stage-matched embryos were arrayed into 48-well plates (~10 embryos per well) of individual test compounds and exposed from 32-56 hpf. The compound library used, the NINDS CC2 (1040 known bioactives), was obtained from the Institute of Chemistry and Chemical

Biology, Longwood Screening Facility. The compounds were dissolved in DMSO and subsequently diluted in E3 buffer to a final concentration of ~13μM. Each compound was screened in replicate; DMSO and SU5402 served as controls. *In situ* hybridization for *scxa* expression was performed to assess craniofacial tendon progenitors. Qualitative scoring (number of embryos with altered *scxa* expression per number scored) was conducted using the following criteria: normal/unchanged, decreased/absent, and increased. Annotations were made regarding any changes in the spatial domain of *scxa*.

qPCR

Zebrafish embryos were treated with Atorvastatin from 33-57 hpf (+/- 1 hpf) and 48-72 hpf (+/- 1 hpf). Embryos, collected at 57 hpf and 72 hpf (+/- 1 hpf), were pooled and used for RNA extraction (Qiagen #74104) and cDNA synthesis (Roche #04379012001). TaqMan Fast Universal gene expression assays with a StepOnePlus Real-Time PCR Machine (Applied Biosystems) were performed. No-reverse transcriptase controls were used 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).

Expression analysis

Colorimetric *in situ* hybridization was performed in whole-mount as described (Brent et al., 2003) with minor modifications. Probes include: *scxa* (accession numbers AL923903 and AL921296), *col1a2* (GenBank accession number DY559926), *tnmd* (GenBank accession numbers BC155615 and EV754577), *myod1* (GenBank accession number BC078421), *sox9a* (a gift from N. Trede, Huntsman Cancer Institute, Utah, USA), *col2a1* (Yan et al., 1995), and

runx2a (Burns et al., 2002). Fluorescent *in situ* hybridization was performed as described previously (Talbot et al., 2010), with minor modifications. Digoxigenin-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 were (1:500) anti-kaede (MBL #PM102), (1:500) anti-myosin heavy chain (A4.1025, Developmental Studies Hybridoma Bank), (1:200) anti-phospho-Histone H3 (Millipore #06-570). Secondary antibodies from Southern Biotech was (1:500) goat anti-mouse IgG2b-HRP. Secondary antibody from Jackson ImmunoResearch Laboratories were (1:200) Alexa Fluor 488 donkey anti-rabbit IgG and (1:500) donkey anti-rabbit IgG-HRP. Detection was performed using TSA-Fluorescein substrates (PerkinElmer). Hoechst 33342 (Invitrogen) staining was performed where indicated.

Image analysis

Embryos were imaged using a Zeiss AxioZoom V16 with ApoTome2 or Nikon Eclipse 80i, and images were acquired using 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 or ImageJ software using the maximum-intensity projection feature applied to z-stacks. The opacity and levels were uniformly increased with Photoshop software to optimize the visualization of (Figure 3.8 and Figure 3.10).

Morpholino design, pharmacological treatment, and microinjection

Morpholinos (Gene Tools) were injected at the one-cell stage as described previously: *fntb ATG* (Mapp et al., 2011), *hmgcr1b ATG* (D'Amico et al., 2007), *pggt1b ATG* (Mapp et al., 2011).

Morpholinos were injected at the following concentrations: *fntb* (1.4 ng alone and 2.1 ng in *fntb-pggt1b* morphants); *pggt1b* (5.5 ng alone and 2.8 ng in *fntb-pggt1b* morphants); 1.0 ng *hmgcr1b*. Embryos were incubated in compounds from Tocris Bioscience (Atorvastatin, Lovastatin, Fluvastatin, FTI-277, SU5402), Sigma-Aldrich (Simvastatin, Aphidicolin), Calbiochem (GGTI-286), and Cayman Chemical (RO48-8071, EHT1864, LY294002, Y27632), at the concentrations and developmental stages indicated. The pharyngeal cavity of embryos was injected at 28 hpf. All compounds were from Sigma-Aldrich, resuspended in DMSO, and injected at following concentrations: 2.4 mM geranylgeranyl pyrophosphate, 2.5 mM farnesyl pyrophosphate, 1.0 M mevalonolactone. The compounds were resuspended in DMSO, except FTI-277 (distilled water) and GGTI-286 (100% ethanol). All compounds were diluted in E3 buffer to a final concentration.

5-ethynyl-2'-deoxyuridine (EdU) pulse labeling

Tg(*sox10:mCherry*) embryos were chemically treated at 33 hpf, pulsed with EdU for 20-minutes in 5-hour intervals, and subsequently chased with E3 Buffer for 30-minutes prior to fixation. Detection was performed with Click-iT EdU Alexa Fluor 488 (ThermoFisher Scientific #C10337). Hoechst 33342 (Invitrogen) staining was performed where indicated.

Chapter 4

Discussion

Zebrafish tendon development

This dissertation describes our work on the developmental regulation of tendon progenitors using the zebrafish model. Through expression analysis of genes known to be enriched in mammalian tendons and ligaments (*Scleraxis*, *Collagen type I alpha 2* and *Tenomodulin*) or in the zebrafish tendon-like myosepta (*xirp2a*), we identified the locations of the craniofacial tendons and ligaments in the zebrafish. Moreover, we fate-mapped the craniofacial tendons and ligaments to be descendants of the cranial neural crest, which also gives rise to the cranial skeletal elements. Our studies focused predominantly on the craniofacial tendons in the zebrafish due in large part to their resemblance to tendons in higher vertebrates. The developmental programs of the myosepta and pectoral fin tendon progenitors in the zebrafish also were found to be similar to those of mammals. Finally, we demonstrated that mevalonate biosynthesis is an essential regulator in the specification of the cranial neural crest cells towards a tenogenic cell fate, specifically, through mediating GGTase I activity. Our analyses of the cellular processes governing tendon cell induction provide insight for understanding the contribution of geranylgeranylation in the emergence of alternate lineages from a common progenitor population.

In this chapter, the implications of our experimental conclusions are placed within the larger framework of the development of the musculoskeletal system and clinical relevance. Accordingly, I first discuss the future of studying tendon biology in the zebrafish. Next, I examine the coordinated development of the musculoskeletal tissues and the factors regulating specification of the skeletal and connective tissue lineages. Finally, I speculate on the possible clinical implications considering the widespread use of statins in the population.

Zebrafish as a model to study tendon biology

Zebrafish genetics informs vertebrate biology

The zebrafish model is a powerful vertebrate genetic system with a genome and body plan similar to that of higher vertebrates (Patton and Zon, 2001). In addition to the advantageous husbandry and life-cycle considerations (Patton and Zon, 2001), the zebrafish system offers a diverse palette of genetic tools. The mechanisms governing developmental processes have in large part been elucidated through forward genetic screening (Lawson and Wolfe, 2011). The first large-scale screens in zebrafish, in which mutagenesis was mediated by N-ethyl-N-nitrosourea (ENU), discovered an exponential cohort of novel genes and signaling pathways essential for vertebrate development (Driever et al., 1996; Haffter et al., 1996). Recently, the development of high-throughput platforms has promoted the use of small molecule chemical screening in zebrafish to identify novel regulators of developmental processes with the potential of therapeutic benefits (Rennekamp and Peterson, 2015). The role of the genes and signaling pathways identified from these forward genetic screens have then been characterized using chemical and reverse genetic approaches, such as pharmacological agonists and inhibitors, morpholinos, and clustered regularly interspaced short palindromic repeats (CRISPR)/ CRISPR-associated protein 9 (Cas9)-targeted mutagenesis, and in loss-of-function and phenotype rescue experiments (Kok et al., 2015; Lawson and Wolfe, 2011; Varshney et al., 2015).

Our studies of statin-mediated expansion of the craniofacial and pectoral fin tendon programs determined a role for the mevalonate pathway in transcriptional regulation of *scxa* in CNC-derived progenitors. The intricacies of the transcriptional networks involved in differentiation of the tendon lineage may be unraveled upon transcriptomic profiling of pan-neural crest cells, as well as that of the skeletal lineages, upon chemical treatment with statin and

the more proximal targeted compounds found to modulate *scxa* activity (e.g. GGTase I and Rac1 inhibitors). The resultant global and tissue-specific perspective of tendon induction would likely to be fruitful in characterizing the basic landscape of tendon biology. The reverse genetic tools available in the zebrafish, in combination with transgenic approaches, presents the zebrafish as a system that is amenable for rapid analysis of candidates identified from such an approach.

Conservation of tendon program between teleosts and amniotes

Our studies have illustrated that a high level of interspecies conservation exists in the tendon program (Chen and Galloway, 2014). We showed that the cranial neural crest-derived craniofacial tendons and ligaments of teleosts and amniotes share similar molecular, morphological, and structural characteristics from embryonic to adult stages. The tendon and ligament progenitor populations initiate at the junctional interface between developing muscle and cartilage or between cartilage segments, respectively. The tissue is enriched in expression of *Scleraxis*, *Tenomodulin*, and *Collagen type I alpha 2*, and in the adult, the tissues are characterized by a *D*-periodicity resulting from a highly organized network of collagen fibrils. (Brent et al., 2003; Docheva et al., 2005; Schweitzer et al., 2001) In addition to displaying similar structure and gene expression profiles, the molecular regulation of the developing tendons and ligaments of teleosts and amniotes are conserved. We demonstrated that the initiation of cranial and pectoral fin tendons is a muscle-independent process, though its continued maintenance is a muscle-dependent process. In contrast, we found that the initiation of the axial tendons is a muscle-dependent process. These findings are similar to studies investigating the role of the muscle in tendon development in the mouse jaw and avian limb (Edom-Vovard et al., 2002; Grenier et al., 2009).

The interspecies conservation in tendon development described in our studies supports the feasibility of using the zebrafish model for studies investigating tendon development in the embryo and adult. As each model system has its advantages and limitations, studies of highly conserved developmental processes provides the benefit being able to utilize a repertoire of genetic tools in different species to address the inquiries of interest. Overall, we speculate that the transcriptional networks involved in the induction and maintenance of the tendons and ligaments are similar across vertebrate species, with differences in the developmental program between the craniofacial, axial, and limb tendons, which arise from distinct embryonic origins.

Developmental and regeneration studies in the zebrafish

Cell ablation studies are utilized to understand mechanisms of tissue regeneration and intercellular interactions in lineage specification and developmental processes. An ablation method available in zebrafish is nitroreductase (NTR)-mediated cell ablation upon exposure to metrodinazole (Mtz) (Curado et al., 2007). The NTR enzyme, derived from *Escherichia coli*, converts nitroimidazole substrates, such as the non-toxic prodrug Mtz, into a cytotoxic DNA cross-linking agent (Anlezark et al., 1992; Edwards, 1993; Lindmark and Muller, 1976). The NTR/Mtz system attains spatial control from expression of NTR mediated by a tissue-specific promoter; and attains temporal control from modulating the developmental stage of Mtz exposure (Curado et al., 2007). Taken together, the NTR/Mtz system provides a means to ablate cells in a controlled and reproducible manner. Using the NTR/Mtz technology, we may examine the regenerative potential of the tendon populations in the zebrafish at different developmental stages upon ablation of *scxa*-expressing tendon progenitors. Moreover, in having demonstrated that the cranial neural crest cells give rise to the *scxa*-expressing cells in the statin-mediated

expanded domain, we may assess whether a particular cell lineage (i.e. *sox9a*-expressing skeletal progenitors and/or *sox10*-expressing cranial neural crest cells) contributes to this expanded domain by examining the effect caused by their ablation. Additionally, ablation of *scxa*-expressing tendon progenitors would test whether they are critical for establishing a permissive environment for the statin-mediated expansion of the tendon program despite not contributing to the expanded population of *scxa*-positive cells.

in vivo *imaging modalities in the zebrafish*

The availability of transgenic reporter lines and their external development provides tools to perform tissue-specific labeling and lineage tracing studies in live zebrafish. We have generated a *mCherry* transgenic line in zebrafish, expressed using regulatory elements derived from the endogenous *scxa* locus. The expression pattern of the *scxa:mCherry* transgene recapitulates the endogenous *scxa* expression, robustly marking the developing tendons and ligaments of the craniofacial, pectoral fin, and axial tissue in zebrafish. This reporter line facilitates our investigation in examining the dynamics of musculoskeletal development. Using time-lapse confocal microscopy, the craniofacial *scxa:mCherry*-positive tendon progenitors are visually traced as they initiate and subsequently differentiate into structurally distinct structures that connect the developing cartilage and muscle progenitors. The use of confocal microscopy of transgenic zebrafish expressing reporters in different cell lineages can assess the existence of multipotent progenitor populations that gives rise to the tenogenic, chondrogenic, and/or osteogenic lineages; and descendants of the *sox10*-expressing cranial neural crest cells may be fate-mapped using the Tg(*sox10:kaede*) (Dougherty et al., 2012). Furthermore, confocal-based imaging of the *ubi:Zebrabow* and *UAS:Zebrabow* transgenic lines allows for unbiased clonal fate

mapping (Pan et al., 2013).

Tendons and ligaments are a hierarchically organized collagenous tissues (Elliott, 1965; Prockop and Kivirikko, 1995), comprised predominantly of Collagen type I, an architecture that enables the tissue to be imaged using second harmonic generation microscopy (Cox et al., 2003). Second harmonic generation is a process that permits visualization of the organization and density of the collagen fibrils (Campagnola, 2011). Our studies have demonstrated that the ultrastucture of the craniofacial tendons and ligaments in adult zebrafish is similar to that of tendons and ligaments in mammals, and therefore, second harmonic generation imaging of the tendons and ligaments in zebrafish in varying degrees of pathological conditions may provide insight into the dynamics of tendon pathology, healing, and regeneration.

Skeletal-tendon fate decisions of cranial neural crest and limb/fin bud progenitors

Plasticity of skeletal-tendon fates

The axial skeletal tissues of amniotes arise from the sclerotome compartment of the somite (Brand-Saberi and Christ, 2000; Christ and Ordahl, 1995; Monsoro-Burq and Le Douarin, 2000). In the sclerotome, *Scx* and *Pax1* are expressed in mutually exclusive domains (Brent et al., 2003). The activity of Pax1, a marker of early sclerotome cells (Wallin et al., 1994), is sufficient to attenuate *Scx* expression (Brent et al., 2003). The spatial exclusivity of these transcripts in the axial tissue is a consequence of myotomal FGF-mediated downregulation of *Pax1* in the adjacent sclerotome during syndetome formation. Therefore, the axial tendon and skeletal progenitors are both descendants of the *Pax1*-expressing cells of the sclerotome. The differentiation of these *Pax1*-expressing cells towards a cartilage lineage is mediated by active repression of tendon induction in the dorsolateral sclerotome. It has been demonstrated that upon

formation of mesenchymal condensations, which do not undergo chondrocyte differentiation (*Sox5*$^{-/-}$*; Sox6*$^{-/-}$) (Smits and Lefebvre, 2003), the *Sox9*-expressing mesenchymal condensations activate *Scx* expression, resulting in an expanded domain of *Scx*-positive tendon progenitors (Brent et al., 2005). Sox5 and Sox6, markers of chondroprogenitors, are known downstream targets of Sox9 in chondrocyte differentiation and this work would suggest that these factors also are required to repress tendon fates (Akiyama et al., 2002; Lefebvre et al., 2001; Lefebvre et al., 1998). Moreover, the expression of *Scx* in the undifferentiated *Sox5*$^{-/-}$*; Sox6*$^{-/-}$ skeletal elements is not a consequence of increased FGF signaling, as is the case of the Scx-expressing cells in the syndetome, and is restricted to the dorsolateral domain of the sclerotome despite expression of *Sox9* in other domains in the sclerotome (Brent et al., 2005). These findings suggest that differences exist in the regulatory network within subdomains of the sclerotome, which leads to variation in the permissiveness of a sclerotomal domain to induce the tendon program. Taken together, the *Pax1*-expressing skeletal progenitor population is plastic in its specification such that modulations in its developmental program will promote tendon cell fate.

In our studies, we have demonstrated that in the zebrafish, the craniofacial skeletal and tendon progenitors are descendants of common progenitors in the cranial neural crest. Upon loss of a cartilage template (*sox9a*^{hi1134}) (Yan et al., 2002; Yan et al., 2005), the pattern of the *scxa*-positive tendon progenitors is altered but an increase in *scxa* expression was not observed when assessed by qPCR. This finding indicates that the Sox9-mediated repression of *Scx* in the specification of the axial skeletal progenitors is not observed in the specification of the craniofacial skeletal progenitors. Rather, based on our investigation of the statin-mediated expansion of the craniofacial tendon program, the mevalonate pathway is involved in mediating the specification of the craniofacial skeletal progenitors. These findings underscore the variation

in the specification mechanism of skeletal progenitors across anatomical domains. Alternatively, these differences may be accounted for by interspecies variation.

Specification of cranial skeletal lineages in teleost and amniotes

The cranial skeletal elements of teleosts and amniotes arise from the cranial neural crest cells, which delaminate from the dorsal neural tube and migrate into the pharyngeal arches (Le Douarin and Dupin, 2003; Lumsden et al., 1991; Schilling and Kimmel, 1994). The anterior-posterior positional identity of the cranial neural crest cells is conferred by the Hox transcriptional factors (Knight and Schilling, 2006; Le Douarin and Kalcheim, 1999; Trainor, 2005). Loss-of-function studies in mouse, chick, and zebrafish have illustrated that alterations in the Hox code result in homeotic transformations of the cranial neural crest-derived skeletal elements in the pharyngeal arches. Loss of Hoxa2 in mouse causes transformation of the second arch derivatives into structures characteristic of the first arch (Gendron-Maguire et al., 1993; Rijli et al., 1993). Similar homeotic transformations were observed upon disruption of *hoxa2* and *hoxb2* expression in zebrafish (Hunter and Prince, 2002; Miller et al., 2004). Conversely, overexpression of Hoxa2 in the first branchial arch transforms its identity to that of the second branchial arch in chick (Grammatopoulos et al., 2000). Furthermore, transcriptional regulators of dorsal-ventral arch, such as Endothelin (Clouthier and Schilling, 2004; Kimmel et al., 2003), are similarly involved in patterning the identity of the skeletal elements. Taken together, the signaling pathways characterized in patterning the identity of the cranial skeletal elements affect the cranial neural crest cells in the mesenchymal condensation stage, prior to overt chondrocyte differentiation.

Our studies have demonstrated that the phenotype of a statin-mediated expansion of *scxa-*

positive cells and loss of *sox9a*-positive cells is not a consequence of either anterior-posterior or dorsal-ventral arch polarity, as the expression patterns of *hand2*, *bapx1*, *hoxa2b*, *hoxb2a* were preserved following statin treatment compared with controls. Additionally, we demonstrated that statin-mediated recruitment of neural crest progenitors towards the tendon lineage occurs prior to chondrocyte differentiation, as indicated by *col2a1* expression, a finding that is consistent with regulation in cell fate specification during the mesenchymal condensation stage. Furthermore, the statin-mediated expansion of the craniofacial tendon program is restricted to a subdomain of the pharyngeal arch, whereas the *sox9a*-expressing cells, which we speculate are the source of the expanded domain of *scxa*-positive cells, are lost throughout the entire pharyngeal arch. The spatial contrast in the effect of statin on the expression of *sox9a* compared to *scxa* suggests that the plasticity of skeletal progenitors in the pharyngeal arch to differentiate towards a tendon lineage is established by arch-specific signaling. By extension, it highlights that the cranial skeletal progenitors retain their multipotent potential long after ventral migration of the cranial neural crest cells.

Specification of limb bud/ pectoral fin skeletal lineages in teleost and amniotes

The lateral plate mesoderm is known to give rise to the limb skeletal elements of amniotes (Kieny and Chevallier, 1979; Saunders, 1948), and the pectoral fin skeletal elements of teleosts (Mercader et al., 2006). The limb progenitors of amniotes are specified by several signaling centers in the developing limb through modulation of the chondrogenic program. The major signaling centers are the apical ectodermal ridge (FGF/Wnt signaling), the zone of polarizing activity (Shh signaling), mesenchyme-ectoderm interaction (Wnt/Engrailed signaling), and the Hox code, which patterns the proximal-distal axis, the anterior-posterior axis, the dorsal-

ventral axis, and digit identity, respectively (Fernandez-Teran and Ros, 2008; Harfe et al., 2004; Kengaku et al., 1998; Loomis et al., 1998; Lu et al., 2008; Tickle and Eichele, 1994; Zeller et al., 2009; Zuniga, 2015). The progenitors of the pectoral fin bud, while morphologically different from the limb bud, are specified by a similar cohort of regulatory factors (Tickle, 2016; Yano and Tamura, 2013). The major signaling centers in the zebrafish are the apical ectodermal fold, Shh signaling, and the Hox code, which patterns the proximal-distal axis, the anterior-posterior axis, and segment identity, respectively (Akimenko and Ekker, 1995; Yano and Tamura, 2013; Zuniga, 2015). Additionally, the limb bud and pectoral fin bud both develops via a sox9-mediated chondrogenic program (Akiyama et al., 2002; Yan et al., 2002; Yan et al., 2005). Taken together, these conserved signaling centers in amniotes and teleosts pattern the developing limb bud and pectoral fin bud, respectively, by regulating the Sox9-mediated chondrogenic program of the lateral plate mesoderm-derived mesenchymal condensations.

Our studies demonstrated that statin causes an expansion of the tendon program and a lost of *sox9a*-expressing cells in craniofacial and pectoral fin tissue, but not in the axial tissue. Similar to the craniofacial region, the statin-mediated expansion of the pectoral fin tendon program is restricted to subdomains, specifically, the ventral fin region closest to the scapulocoracoid displayed the most robust expansion of *scxa*-positive tendon progenitors. Additionally, the statin causes a lost of sox9a-expressing cells throughout the fin, except for the dorsal-most region of the endochondral disc, where there is a significant reduction in *sox9a* expression. We speculate that the spatially confined domain of statin-mediated expansion of pectoral fin *scxa*-positive tendon progenitors is a consequence of differences in the permissiveness of the developmental microenvironments of the subdomains to activate transcription of *scxa* upon inhibition of the mevalonate pathway. We hypothesize that the

permissiveness of the microenvironment is established in part by a signaling center involved in establishing the dorsal-ventral axis in the pectoral fin. Taken together, we speculate that the modulations in the developmental program of the skeletal progenitors that allows for transcriptional activation of *scxa* in the craniofacial tissue is similar to that in the pectoral fin tissue.

Cytoskeletal dynamics and skeletal differentiation

The cytoskeletal network of actin filaments is a critical regulator of cell fate specification *in vitro* (Engler et al., 2006). A class of cytoskeletal regulators are the Rho family of small GTPases (Chardin et al., 1989; Paterson et al., 1990), of which the major family members are Rho, Rac, and Cdc42. This family of small GTPases are involved in cytoskeletal organization, with members regulating different aspects of the cellular architecture. The Rac proteins regulate formation of lamellipodia, thin actin sheets that accumulate at the distal-most region of the cell. The Cdc42 proteins regulate filopodia, bundles of long actin filaments that are arranged in the direction of protrusion. The Rho proteins regulate formation of stress fibers, which transverse the cell and interact with the extracellular matrix through focal adhesions. (Takai et al., 2001) It has been demonstrated *in vitro* that focal adhesion-mediated modulation of the cytoskeleton directs specification of naïve mesenchyme stem cells towards different lineages according to the extracellular microenvironment, specifically, matrix stiffness (Engler et al., 2006).

Our studies determined that the statin-mediated expansion of *scxa*-positive tendon progenitors is mediated by inhibition of GGTase I, which catalyzes the geranylgeranylation of the Rho family of small GTPases (McTaggart, 2006). We speculate that these small GTPases may regulate the differentiation of cranial neural crest mesenchyme towards a tenogenic lineage.

To test the requirement of the Rho family of small GTPases in mediating transcriptional activation of *scxa* in the cranial neural crest cells, a Gal4/UAS-mediated tissue-specific modulation of the RhoA and Rac1 activity may be utilized. Specifically, Rac1 and RhoA activity may be upregulated with constitutively active forms of the proteins, $Rac1^{V12}$ and $RhoA^{V14}$, respectively, and attenuated with the Cdc42/Rac interactive binding (CRIB) domain of PAK1 and the Rho-binding domain of protein kinase N, respectively (Kardash et al., 2010). Findings from these studies will indicate whether the statin-mediated expansion of the tendon program that we have determined to be mediated from GGTase I activity is a cell autonomous event. Additionally, these experiments will indicate which subset of the small GTPases is involved in mediating the statin-mediated phenotype.

Conservation of Hmgcr regulation of tendon fate in higher vertebrates

The developmental programs of the craniofacial cartilage and tendon between teleosts and mammals are highly conserved (Brent et al., 2003; Chen and Galloway, 2014; Edom-Vovard et al., 2002; Grenier et al., 2009; Medeiros and Crump, 2012; Schilling and Kimmel, 1997). We speculate that the role of the mevalonate pathway in regulating tendon development is conserved in the mammalian lineage. The mevalonate pathway, specifically Hmgcr inhibition, is essential for early development of teleosts and mammals, as demonstrated from loss-of-function studies in the zebrafish (Chapter 3) and mouse (Ohashi et al., 2003). To test the role of the mevalonate pathway in mammalian tendon development, the mouse model offers the ability to genetically knockout Hmgcr or its downstream targets with spatial and temporal specificity. Temporal specificity of loss- and/or gain-of-function in Hmgcr or Rac1 activity may be attained using mice homozygous for the respective floxed alleles (Glogauer et al., 2003; Nagashima et al., 2012;

Srinivasan et al., 2009; Zhou et al., 2013). Spatial control may be attained using Cre drivers for Wnt1 or Prx1, which would induce deletion of these alleles specifically in the migrating neural crest cells (Chai et al., 2000; Danielian et al., 1998) or limb mesenchyme (Logan et al., 2002), respectively. A Cre driver specific for the somitic sclerotome to examine the axial tissue does not currently exist. Alterations in the expression level of Scx may be visualized in these mutant lines when crossed onto a *ScxGFP* reporter (Pryce et al., 2007) background. Furthermore, the role of Hmgcr and Rac1 activity in mediating a cell fate conversion by recruitment of skeletal progenitors may be assessed using a Cre driver for Sox9 or Bapx1, critical regulators of chondrocyte development (Akiyama et al., 2005; Sivakamasundari et al., 2012). Sox9 is a master regulator of chondrogenesis that is required at the multiple stages, including the earliest steps of mesenchymal condensation formation and chondrocyte differentiation (Akiyama et al., 2002; Bi et al., 1999; Bi et al., 2001; Healy et al., 1999; Zhao et al., 1997). Therefore, an advantage in using a Cre driver for Bapx1, a downstream target of Sox9 (Yamashita et al., 2009) and negative regulator of chondrocyte hypertrophy (Provot et al., 2006), is that analysis of the role of Hmgcr and Rac1 activity is developmental confined to the *Sox9*-expressing prehypertrophic chondrocytes.

Therapeutic relevance

The discovery of statins as a regulator in the induction of tendon fates has implications for its use in a clinical setting. Statins, prescribed for treatment of hypercholesterolemia, cause significant reductions in plasma cholesterol levels (Brown and Goldstein, 1991; Havel and Rapaport, 1995). Clinical studies have demonstrated beneficial pleiotropic effects associated with statins, such as amelioration of cardiovascular-related morbidity and mortality in patients

with and without coronary heart disease symptoms (Downs et al., 1998; Lewis et al., 1998; Ridker et al., 2008; Riegger et al., 1999; Sacks et al., 1996). Based on our studies suggesting a role for the mevalonate pathway in promoting a tenogenic fate through recruitment of a common skeletal progenitor population, we speculate that statins may contribute to therapeutic management of degenerating tendon tissues.

The pathological development of fibrocartilage is found in clinical presentations of degenerative conditions that oppositely affect expression of chondrogenic and tenogenic markers. In the degeneration of cruciate ligaments, which contributes to progression of osteoarthritis, the tissue displays an expression profile associated with increased chondrogenic potential, as indicated by Sox9 and Collagen type II expression, and reduced tenogenic potential, as indicated by Scx and Collagen type I expression, compared with controls (Kumagai et al., 2012; Nakahara et al., 2013). Moreover, in rotator cuff tendinitis, the collagen matrix of the tissue is found to be remodeled with an increase in Collagen type III content and a decrease in Collagen type I content compared with controls (Riley et al., 1994). Similar findings were observed in human tissue diagnosed with patellar tendinitis (Samiric et al., 2009) and in rat patellar tendon upon collagenase-induced degeneration (Lui et al., 2009). A potential treatment in mitigating such pathological progression of such degenerative tissue is to suppress the chondrogenic differentiation of the tissue. If statins are found to regulate the developmental program of skeletal and tendon fates in a cell autonomous manner, statin therapy may be an effective therapeutic intervention in promoting tendon fates and repressing cartilage fates. More precisely, treatment with more proximal targeted compounds that modulate *Scx* activity (e.g. GGTase I and Rac1 inhibitors) may offer a more targeted effect with less pleiotropic events.

Conclusion

This dissertation establishes the zebrafish as a model for the study of tendon development and then using the zebrafish as a model, examines the processes that guide cell fate specification of the cranial neural crest lineage. We are optimistic that our discovery of the role of the mevalonate pathway will be relevant to mammalian tendon development and may advance the development of clinical therapeutics targeted towards tendinopathies. Follow-up studies aimed at identifying additional regulators of tendon development will benefit from the genetics offered by the zebrafish and mouse models.

Chapter 5

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