
Early version, also known as pre-print

Link to published version (if available): 10.1096/fj.201802654RR

Link to publication record in Explore Bristol Research
PDF-document

This is the author accepted manuscript (AAM). The final published version (version of record) is available online via FASEB at https://www.fasebj.org/doi/10.1096/fj.201802654RR. Please refer to any applicable terms of use of the publisher.

University of Bristol - Explore Bristol Research
General rights
This document is made available in accordance with publisher policies. Please cite only the published version using the reference above. Full terms of use are available: http://www.bristol.ac.uk/pure/about/ebr-terms
Scleraxis genes are required for normal musculoskeletal development and for rib growth and mineralization in zebrafish

Erika Kague\textsuperscript{2}, Simon M. Hughes\textsuperscript{1}, Elizabeth Lawrence\textsuperscript{2}, Stephen Cross\textsuperscript{2}, Elizabeth Martin-Silverstone\textsuperscript{2}, Chrissy L. Hammond\textsuperscript{§,2}, and Yaniv Hinits\textsuperscript{§,1}

1 Randall Centre for Cell and Molecular Biophysics, School of Basic and Medical Biosciences, Faculty of Life Sciences and Medicine, King's College London, SE1 1UL, UK. 3rd floor North, New Hunt's House, Guy's Campus, King's College London, London SE1 1UL, UK. Tel.: +44 20 7848 6444; fax: +44 20 7848 6798

2 Department of Physiology, Pharmacology and Neuroscience, Medical Sciences, University of Bristol, University Walk, Clifton, BS8 1TD, UK.

Running title: zebrafish Scleraxis mutants

\textsuperscript{§}Corresponding authors: Yaniv Hinits; e-mail: yaniv.hinits@kcl.ac.uk, Chrissy L. Hammond; e-mail: chrissy.hammond@bristol.ac.uk.
Nonstandard Abbreviations

AR alizarin Red
AB alcian Blue
bHLH basic helix-loop-helix
BMD bone mineral density
BMI body mass index
DM dermomyotome
DGC dystrophin-associated glycoprotein complex
ECM extra-cellular matrix
H&E haematoxylin & eosin
HRP horseradish peroxidase
µCT micro-computed tomography
MTJ myotendinous junctions
MyHC myosin heavy chain
NMD nonsense-mediated decay
SHG second harmonic generation
Abstract

Tendons are essential part of the musculoskeletal system connecting muscle and skeletal elements to enable force generation. The transcription factor Scleraxis marks vertebrate tendons from early specification. Scleraxis-null mice are viable and have a range of tendon and bone defects in trunk and limbs but no described cranial phenotype. We report the expression of zebrafish Scleraxis orthologues: scxa and scxb in cranial and intramuscular tendons and in other skeletal elements. Single mutants for either scxa or scxb, generated by CRISPR/Cas9, are viable and fertile as adult fish. Whereas scxb mutants show no obvious phenotype, scxa mutant embryos have defects in cranial tendon maturation and muscle misalignment. Mutation of both scleraxis genes results in more severe defects in cranial tendon differentiation, muscle and cartilage dysmorphogenesis and paralysis, and lethality by 2-5 weeks indicating an essential function for Scleraxis for craniofacial development. At juvenile and adult stages, ribs in scxa mutants fail to mineralize and/or are small and heavily fractured. Scxa mutants also have smaller muscle volume, abnormal swim movement and defects in bone growth and composition. Scleraxis function is therefore essential for normal craniofacial form and function and vital for fish development.

Keywords

Zebrafish, scleraxis, tendon, ribs, development, musculoskeletal.

Introduction

The musculoskeletal system is formed by coordinated differentiation and morphogenesis of
skeletal muscle, tendon, ligament, cartilage, bone and associated joint cell types (reviewed in (1, 2)). Recent studies have shown that both signaling between component tissues, and physical forces deriving from muscular contraction or passive load can remodel various elements of the system to fit form to function (reviewed in (3)). In the integrated musculoskeletal system, resolving the primary cause of defects requires identification of the earliest failures – in muscle, bone or tendon – and determination of their secondary consequences during development of the entire system. With such detailed understanding, treatments for genetic or environmentally induced musculoskeletal pathologies may be more effective.

Tendons play an essential role in muscular control of the body by connecting muscle and skeletal elements, allowing force transmission. The earliest, and most persistent known marker for the tendon cell lineage is the basic helix-loop-helix (bHLH) transcription factor Scleraxis (4-6). Scleraxis (Scx) expression marks a somitic compartment called the syndetome in mammals and birds, from which the tendon precursors are derived (7). Scx is also expressed in pharyngeal arches and facial tendons of mouse embryos (8-10). Scx-null mice are viable but show a dramatic defect in tendon differentiation, resulting in a loss of intermuscular tendons and the tendons responsible for transmitting musculoskeletal force in the limbs, tail and trunk. However, short-range muscle-anchoring tendons such as the ones anchoring the intercostal muscles to the ribs and ligaments like the cruciate ligaments of the knee are unaffected (11). Scx is not required for tendon cell specification, as tendon progenitors are present in Scx\textsuperscript{-/-} mutant mice, but Scx is necessary for the condensation and differentiation of tendon cell populations (11). Experiments in mouse and chick have shown that Scx is required for secretion of structural extracellular matrix components including Col1a1, other tendon-associated collagens and tenomodulin (Tnmd) (12-14), for formation of
extended cytoplasmic extensions that support matrix organization, and in the crosstalk between tenocytes and endotenon cells (11).

Scx is also essential for developmental events beyond tendons themselves. Scleraxis is required for normal development of entheses that serve as insertion points for tendons onto bones (14-17). Most recently, Scleraxis was also implicated in regulation of fracture callus formation during bone healing (18). Thus, Scx promotes several aspects of musculoskeletal development in amniotes.

It has been suggested that Scx also functions in tendons of lower vertebrates. In frogs, Scx accumulates at the end of muscle fibers in the somites and the limbs, and is involved in inducing tendon matrix genes tenascin C and betaig-h3 (19, 20). In trout and zebrafish, the myosepta, sheets of connective tissue that separate the somite muscle blocks, are initially acellular but later contain cells expressing col1a1 and scleraxis (21, 22). Zebrafish have two Scx genes, scxa and scxb, but only scxa expression has been described (23, 24). Zebrafish scxa positive cranial tenocytes are located between muscles and the craniofacial skeleton, and co-express tendon markers such as tnmd, col1a2 and trsp4b (23-26). Interestingly, cranial and fin tendon progenitors can be induced in the absence of muscle or cartilage, whereas myosepal scxa expression requires muscle for its initiation (23) suggesting these tendon populations have different origin and regulation. As in mammals, zebrafish craniofacial tendon and ligaments are neural crest-derived (8, 23, 24, 26, 27). However, knockdown experiments in zebrafish with antisense morpholino oligonucleotides against both scxa and scxb were reported to have no effect on tnmd expression or create any craniofacial defects in the embryos (23). Whereas expression data implicate Scx in tendon development in various vertebrate groups, functional data derives mostly from mouse trunk and limbs. We set out to create a zebrafish loss-of-function model to increase understanding of Scx function across
vertebrates.

Here we describe the differential expression of scxa and scxb in embryonic, juvenile and adult zebrafish. Zebrafish single mutants for scxa or scxb are each viable allowing assessment of the adult musculoskeletal system. Mutations in scxa lead to embryonic defects in cranial tendon composition and shape and muscle misalignment. At juvenile and adult stages, Scxa is essential for growth and mineralization of the ribs and is required for normal swim movement, muscle volume and body composition. Lack of Scxa in the mutants also results in ectopic growth of bone in neural and haemal arches, while reducing jaw bone mineral density (BMD). Lack of Scxb alone has no obvious phenotype, but exacerbates the effect of lack of Scxa, indicating partially redundant function. Double mutants show severe cranial defects, but no obvious defect in somitic myotendinous junctions (MTJ). Double mutant embryos have reduced muscle growth and function and paralysis of the jaw leading to death at early juvenile stages.

Materials and Methods

Generation of mutant zebrafish lines and maintenance

CRISPR/Cas9 genome editing (28) was used to target scxa (zv11, Chr19: 3001919-3001938, GGGGGTGGCGACGGCTGAT) and scxb (zv11, Chr16: 31,396,781-31,396,801, GGCTATGGTTCCTTTAAGCT) and yielded two nonsense alleles for scxa (scxa^{kg141}, scxa^{kg170}) and one for scxb (scxb^{kg107}) all leading to premature stop codons upstream of the bHLH domain (Fig. S1). scxa^{kg141} carry a 4bp deletion leading to a frameshift after amino acid (aa) 64, adding a tail of two extra incorrect amino acids (aa) followed by a premature stop. scxa^{kg170} carry a 1 bp insertion also leading to a frameshift after aa 64, adding a 51 wrong aa
tail before a premature stop codon in exon 1. $scxb^{kg107}$ is a deletion of 12 bp combined with a 27 bp insertion, which creates an immediate UAG stop codon after 45 aa of the wild type protein (Fig. S1). The new mutant and transgenic lines: $Tg(actc1b:egfp)^{zf13}$ (29) and $TgBAC(col2a1a:mCherry)^{hu5910}$ (30) were maintained on AB wild type background. Staging and husbandry were as described (31).

mRNA in situ hybridisation and immunohistochemistry

In situ mRNA hybridization was performed as described previously (32) and adapted for juveniles, which were cut into three or four pieces, skinned, bleached and then treated with 50 µg/ml Proteinase K for 15 mins and re-fixed. Probes for $scxa$ and $scxb$ were made by amplifying from zebrafish cDNA (AB, 72hpf) a 1151 bp and 847 bp fragments, respectively, and cloning them into pGEM-Teasy vector (Promega). The following primers were used: 5’-CAGAAAGCCGGAGGAGTGTG-3’ and 5’-TGTGTATGCGCAGAAAGTGAC-3’ for $scxa$ and 5’-AGCAGGACTGGTTCTTCTTCTATTCA 3’ and 5’-CAGTGTTCGGTTCGTTTCGTA-3’ for $scxb$. For $tnmd$ and $xirp2a$ probes, the following primers (containing T3 polymerase site) were used: 5’-TCCACCCATCTCCTCTCAGA-3’ and 5’-GGATCCATTAAACCCTCACAAGGGAATGTGGGTAGTTGCAATGGAT-3’ for $tnmd$ and 5’-CTCAGCAGACCGTGGAAAC-3’ and 5’-GGATCCATTAAACCCTCACTAAAGGGAAGATGGGATGTGGGCTGTTCAACAT -3’ for $xirp2a$. Published probes include $Sox9a$ (33) and $tnnc$ (34). For immunohistochemistry, the following primary antibodies were used: sarcomeric myosin heavy chain (MyHC): A4.1025, 1:10, (35), MF20 (DSHB), 1:10, anti-GFP, 1:500 (rabbit, Torrey Pines or chicken, Abcam ab13970), anti-Tsp4 (Thbs4), 1:400 (Abcam ab211143, made against N-terminal recombinant fragment within zebrafish Thbs4), anti-alpha-Actinin, 1:500 (Sigma A7811). Secondary antibodies were
either HRP-conjugated (Vector) or Alexa dye-conjugated (Invitrogen). Samples for immunohistochemistry were fixed and stained as described (36). Wholemount pictures were taken on Olympus DP70 and dissected samples were flat mounted in glycerol and photographed on a Zeiss Axiophot with Axiocam using Openlab software.

**Alizarin red S and alcian blue staining**

Staining was performed as previously described (37).

**Micro-computed tomography (µCT)**

One-year old fish were fixed in 4% PFA for 72 h and dehydrated to 70% Ethanol. A total of 24 fish were scanned (7 scxa⁺, 3 scxb⁺, 14 siblings) using a Nikon XT H225ST CT scanner computed tomography (CT) scanner at a voxel size of 21 µm (scan settings 130 kV, 150 µA, 0.5 s exposure, 3141 projections), and selected regions rescanned at 5µm (130 kV, 53 µA, 0.7 s exposure, 3141 projections) without additional filters. Images were reconstructed using CT Pro 3D software (Nikon). Amira 6.0 was used to generate 3D volume renders. Soft tissues were discriminated by treating fixed fish with 2.5% phosphomolybdic acid for 14 days, as previously described (38) followed by µCT scanning. Muscle volume was calculated for a single transverse slice in two positions in the trunk: 1) at the level of the midpoint of the second last rib-bearing vertebra, and 2) at the midpoint of the 4th vertebra posterior to the previous position. Trunk musculature was segmented, and the volume calculated using the ‘Material Statistics’ module. Non-muscle volume in that position was calculated subtracting the muscle volume from the total volume.

Vertebral centrum volumes were calculated for the 7th and 8th thoracic vertebrae using the CT scans of unenhanced fish in Avizo 9.3 (FEI Visualization Sciences Group), by segmenting the
minimum volume possible around the neural canal, excluding all processes, trabeculae, spines and ribs in a transverse view. Volume was calculated using the 'Material Statistics’ module. Entire vertebral volume with ribs was measured on the 3rd thoracic vertebra, using the same method as above but segmenting the entire vertebra including all processes, trabeculae, spines and ribs. BMD was quantified as previously described (39).

**Second harmonic generation (SHG) imaging**

SHG images were acquired using 10x 0.3 NA water dipping lens, 880nm laser excitation and simultaneous forward and backward detection (440/20) in Leica SP8 AOPS confocal laser scanning microscope attached to a Leica DM6000 upright epifluorescence microscope with multiphoton lasers and confocal lasers allowing fluorescent and SHG acquisition of the same sample and z-stack. Microscope parameters for SHG acquisition were set as described previously (40). LASX (Leica) was used for image acquisition.

**Histology**

Following µCT, fish were rehydrated to PBST (1X PBS/ 0.01% Tween 20), decalcified in 1M EDTA-solution for 20 days, embedded in paraffin and sectioned at 8 µm. Sections were stained for Alcian blue and haematoxylin & eosin (H&E), as elsewhere (41) Pictures were taken using a GXML3200B with GXCAM camera (GX Microscopes).

**Body mass index and standard weight calculation of adult fish**

Adult fish from heterozygote incrosses, grown in tanks together, were anaesthetized with tricaine, blotted dry, and weighed nose-to-base of tail fin, length measured with a ruler and fin-clipped for genotyping. Body mass index (BMI) was calculated as ‘weight (g) x length\(^{-2}\)
(cm). Standard weight (K) was calculated using Fulton’s formula K = weight (g) x 100 x length^{-3} (cm)(reviewed in (42)).

**Fish tracking**

Two or three fish per movie were recorded in 8 litre tanks with a Nikon D3200 camera mounted above the tank at 1920x1080 resolution, 24.96 fps (see supplementary video Fig. S7). Fish motion was quantified using the Modular Image Analysis plugin (v0.5.17) (43) for Fiji (44, 45). Initially, the Fiji Color Deconvolution plugin (46) was used to convert the RGB-format video frames into greyscale while also enhancing the contrast of the fish from the background image of the tank. Next, the median time-projection image was subtracted from all frames to enhance the image. This image was subsequently binarized using the intermodes threshold (47) and median-filtered. Identified objects were size-filtered to remove noise spuriously detected as a fish. Individual fish were then tracked between frames using the Apache HBase implementation of the Munkres algorithm, with linking costs based on centroid separation (48). From these tracks, instantaneous (frame-to-frame) speeds were calculated. To remove false tracks, tracks lasting less than 50 frames were excluded from further analysis. To measure fish curvature, the binarized objects were skeletonized and a spline curve fit to this backbone using the Apache Math3 library (49); this permitted measurement of local curvature and backbone length.

The 100 frames in which the fish were most active were selected, and the instantaneous velocity and range of movement was compared between 6 homozygous and 6 heterozygous fish. Range of movement calculated by subtracting the minimum curvature from the maximum curvature for each fish. Each point on the graphs corresponds to the average value for each
fish and a two-way ANOVA was performed in GraphPad Prism version 7.04 to compare the mean value from each heterozygous fish to the mean value from each homozygous fish. The software is available and free to download (Supplementary file S8).

**Statistical Analysis**

Statistical analyses were performed using GraphPad Prism 7.00. The tests used, *n* numbers and sample sizes are indicated in the figure legends and significant *p*-values are shown on the figures. All tests met standard assumptions and the variation between each group is shown. Sample sizes were chosen based on previous, similar experimental outcomes and based on standard assumptions. No samples were excluded. Randomisation and blinding were not used except where the genotype of zebrafish was determined after experimentation.

**Genomic and protein comparison**

Clustal alignment and sequence pair distances were made using DNASTAR Lasergene Genomics Suite. Analysis of synteny was made using Ensembl zebrafish zv10 and the Genomicus synteny software version 93.01 (50)

**Results**

**Expression of scxa and scxb during zebrafish development**

Zebrafish have two orthologues of the mammalian Scx gene, *scxa* and *scxb* (23), with Scxa and Scxb showing 68.1% and 63.6 amino acid identity with mouse Scx and 68.3% and 63.2% with human SCX, respectively (Fig. S2A,B and (23)). Zebrafish *scxa* is syntenic to mammals, with the whole *scxa* gene positioned in the + strand inside intron 3 of *bop1* (Fig. S2C,
Ensembl GRCz11). In contrast, the scxb locus shows more rearrangements (Fig. S2C, Ensembl GRCz11). We sought to know where scleraxis genes are expressed in zebrafish and whether this relates to synteny. Expression of scxa, but not scxb, has been reported (23, 24, 26, 51). By 72 hpf, both scxa and scxb mRNAs were detected in tenocytes at the junctions between skeletal elements and head muscles (Fig. 1A,B). scxb mRNA overlapped scxa mRNA in the attachments of the Meckel’s adductor, the sternohyoideus and ocular muscles (Fig. 1A,B), similar to expression of tnmd and col1a2 (23). However, other tendons such as the mandibulohyoid junction and the intermandibularis tendon showed strong scxa and weak or no scxb expression (Fig. 1A,B). Strong expression of scxb and weak scxa was evident in the ocular muscle attachments (Fig. 1A,B). Both scxa and to a lesser extent scxb mRNAs are expressed at 72 hpf and beyond in somitic vertical myosepta (Fig. 1C,D and data not shown). Thus, scxa and scxb expression partially overlaps in cranial tendons and ligaments and intersomitic myotendinous junctions, although scxa mRNA appears more abundant in both during early larval stages.

During juvenile stages, scxa mRNA was detected in various skeletal elements such as the intermuscular tendons at the vertical myosepta, the fin radials, the joints in the fin bony rays (lepidotrichia) segments at SL 8.0-12.0 (Fig. 1E-G). Sections taken of stained juveniles show that scxa is not expressed at muscle ends at this developmental stage. Intriguingly, the chondrogenic marker sox9a mRNA is detected weakly in ribs, although it was observed strongly in fin endoskeletal elements (radials) and weakly in exoskeletal elements (fin rays) (Fig. 1H). scxb was only weakly detected in vertical myosepta (Fig. 1I,J). The head tendons and ligaments expressed strong scxa but little scxb at muscle attachments of the protractor hyoideus and intermandibularis anterior muscles that expressed the muscle marker tnnc (Fig. 1K-M). Thus, expression data shows that scxa is the predominant scleraxis gene expressed
at embryonic and juvenile stages.

**Lack of Scxa results in defective cranial tendons and ligaments and abnormal musculature**

In contrast to the tendon and bone phenotypes that have been reported in mice lacking Scx, morpholino knockdown of scxa and scxb in zebrafish yielded no obvious phenotype (11, 14, 18, 23). To analyze a complete loss of function of scxa and scxb in embryonic and adult fish, we generated stable mutant lines for scxa and scxb using CRISPR/Cas9 genome editing (Fig. S1). Scxa$^{kg141}$, scxa$^{kg170}$ and scxb$^{kg107}$ have premature stops before the bHLH domain, which is required for DNA binding and dimerization. As we have found no consistent difference between the two scxa mutant alleles, henceforth we have used the kg170 allele unless otherwise stated and refer to it as scxa$^{-/-}$. For simplicity, we refer to scxb$^{kg107}$ mutants as scxb$^{-/-}$. Analysis of the expression of scxa and scxb mRNA in scxa mutants shows reduced scxa signal in mutants compared with siblings, indicative of nonsense-mediated decay (NMD) (Fig. S2D). We observed no change in either the pattern or levels of expression of scxb in the scxa$^{-/-}$ mutants, suggesting that there is no compensatory upregulation of scxb (Fig. S2E). To investigate Scxa function, we imaged 7 dpf scxa mutants and siblings carrying a col2a1a:mCherry transgene by second harmonic generation (SHG) microscopy, which reveals myosin heads in muscle and collagen (mainly collagen I) in tendons and ligaments (40), combined with confocal imaging of the transgene that labels cartilage. No obvious changes to cartilage were observed (Fig. 2A-C). However, SHG revealed decreased signal in certain tendons and ligaments of scxa mutants such as the sternohyoideus tendon, suggestive of poor collagen organisation (compare Fig. 2A, A’, B, B’). To corroborate these results in scxa mutants, we performed
in situ hybridization for *tnmd* at 3 dpf in an incross of *scxa*<sup>+/-</sup>. Genotyped *scxa* mutants (9/9) showed reduced *tnmd* expression levels in cranial tendons, whereas cleithrum expression was largely preserved (Fig. 2D,D'). Using immunostaining for Tsp4b, a marker of tendons and ligaments (25), *scxa*<sup>-/-</sup> mutant embryos showed disorganization and changes to directionality and shape of cranial tendons and ligaments, which were variable between specific tendons and between individual mutants despite comparable levels of Tsp4b accumulation (8/8 of analysed mutants, Fig. 2E-G). Muscle fibres visualized with myosin heavy chain (MyHC) in *scxa* mutants showed a range of abnormalities, such as: fibers that extended beyond their normal boundaries, marked by Tsp4b, fibers that were misaligned or that crossed the midline and disorganized junctions (Fig. 2E-G, Fig. S3A and Table 1). Similar phenotypes were also seen in *scxa*<sup>kg141</sup> mutants (Fig. S3B-F and Table 1), and occasionally in *scxa*<sup>+/-</sup> embryos, though at lower penetrance (Fig. S3A and Table 1). We found no substantial *scxa* mutant phenotype in cartilage and bone by analysis of *Tg(col2a1:mCherry)* and Alizarin Red (AR)/Alcian Blue (AB) staining at 6 and 13 dpf (Fig. S4). Thus, lack of Scxa leads to disruption of tendon and ligament morphology and to defects in the attachment and orientation of cranial muscle fibers.

**Scxa adult fish are viable but show reduced body size and muscle volume, and abnormal swim behavior.**

Growth of zebrafish is dependent on feeding rate (52). To determine whether the cranial musculoskeletal defects in *scxa* mutants have consequences in later life, we examined growth of mutant fish and their siblings reared in the same tank. *scxa* homozygous mutants were viable and survived in relatively normal Mendelian ratios; 28/107 (26% mutants from the whole incross) adults from a heterozygote incross. Body measurements
of 15 months adult *scxa* mutant fish and their co-reared same-sex siblings showed that they weigh ~15% less (Fig. 3A). Overall body length was comparable between the different genotypes suggesting that under-feeding was not the main cause for lower weight (Fig. 3B). Moreover, mutants also show a significantly reduced body mass index (BMI) and standard weight (K, Fulton’s body condition factor), measures that would compensate for any differences in overall growth rate (Fig. 3C,D). We analyzed contrast-enhanced μCT to allow visualisation of soft tissue (Fig. 3I). Muscle volume is clearly smaller in mutants (compare Fig. 3I’’ and 3J’’). Quantification of muscle volume from μCT scans (Fig. 3K,L) shows that adult *scxa* mutant fish have significantly lower (~25% less) muscle volume than their co-habiting siblings (Fig. 3L). Non-muscle volume did not differ between the groups (data not shown). To test whether adult *scxa* mutant fish show altered swim behavior, we developed software to track videos of adult fish (Fig. 3N, see Materials and Methods). Both the range of movement (curvature) and instantaneous velocity of *scxa* mutants were significantly lower than those of co-reared heterozygotes (Fig. 3O, P). Thus, we conclude that fish lacking Scxa are thin, with reduced muscle and swimming ability compared with their siblings.

**Scxa adult fish lack rib mineralization and show bone growth and composition abnormalities.**

As juvenile zebrafish express *scxa* mRNA in ribs and other skeletal elements (Fig. 1), and our data from soft-tissue μCT showed missing ribs (Fig. 3J’,J’’) we sought to investigate in detail the zebrafish *scxa* mutant skeleton. We performed μCT on 15-month adult fixed fish (Fig. 4A-D), revealing severe lack of rib growth and mineralization (see below) and multiple bony outgrowths from neural and haemal arches in the mutants (Fig. 4B,D). AR staining
showed this phenotype in more detail (Figs. 4F-I and S5C-D’). We also used the µCT to test whether vertebral thickness differs between the groups; we extracted the centrum volume through image segmentation. However, no changes were observed, indicating that Scxa is not essential for vertebral bone thickness in zebrafish (Fig. S5A,C-D’). However, when bony structure at the same anteroposterior level is calculated to include rib and arches the mutants showed significant differences (Fig. S5A,C-D’). We calculated BMD in the lower jaw (dentary), the parietal skull and vertebral centrae. Jaw BMD is significantly lower in scxa than siblings, whereas skull and vertebral BMD were unchanged (Fig. 4E). Thus, scxa mutants have a range of skeletal and cartilaginous abnormalities in both trunk and skull.

The µCT also showed no mineralization in the thoracic ribs in 7/7 scxa+/– homozygous mutants, while 7/7 scxa+/- and 4/4 scxa+/- siblings had normal ribs (Fig. 4A-D). Alizarin red (AR) staining on similar age adults also revealed a lack of mineralization in ribs in 6/6 scxa mutants, whereas 7/7 scxa+/- showed normal ribs (Fig 4F-I). Most juvenile mutants stained for AR also lack mineralization (7/9 analyzed mutants), whereas the remaining 2/9 showed a milder phenotype with uneven mineralization: some ribs missing and others displaying residual mineralization (see Fig. 5). No siblings lacked mineralization (0/22 analyzed siblings). However, close examination of mutants lacking mineralization revealed that some unstained, glossy, rib tissue was present (Fig. 4J), suggesting that Scxa is required for normal mineralization or growth, rather than formation of ribs per se. Indeed, Haematoxylin and Eosin (H&E) combined with alcian blue (AB) staining on adult sections unveiled rib-like structures between the myotomes (Fig, 4K,K’,L,L’). This histological analysis also revealed an accumulation of fibrous tissue in the intervertebral disk (IVD), suggesting abnormality with the notochord sheath cell layer (Fig. 4M, M’).
Scxa mutants have abnormal rib mineralization from patterning stage

To understand better the nature of the rib growth and mineralization defect, we stained juveniles with AR+AB at various stages around the time when ribs are formed. Ribs develop anteriorly to posteriorly beginning at 5.8 mm SL, as seen by AR or Calcein staining (53, 54). At juveniles of SL 5.8 mm, although no mineralized ribs were detected in any genotype, the arrangement of the vertical myoseptum and the rib region appeared abnormal in the mutants (Fig. 5A,A'; 2/2 scxa mutants analyzed). Juveniles reaching SL 6.5 mm had initiated mineralization in the anterior ribs (ribs 5 and 6), but scxa mutants at that stage lack mineralized ribs. However, the Weberian ossicles located anterior to the ribs, developed normally (Fig. 5B,B'; 4/4 larvae examined). In SL 10.5 mm juveniles, ribs had formed in siblings (Fig. 5C), but scxa mutants either lacked mineralized ribs (Fig. 5C'; 3/5 mutants analyzed) or showed a milder phenotype with some ribs missing and others small, bent and twisted (Fig. 5D,D'; 2/5 mutants analyzed) as occasionally found in adult (Fig. S5E,E'). These defective ribs are reminiscent of ribs in mutants where altered collagen composition causes weak and bent ribs that may reflect a history of repeated fracture repair (55). These observations show that scxa mutants have defects in the tendon-like regions between the myotomes where ribs form already at the patterning stage, rather than it being a remodelling in response to later events.

Scxa mutants maintain normal myotendinous junctions in the somites

During development, muscle fibres from each side of the vertical myoseptal somite border align, connect, and secrete ECM proteins that create a distinctive extracellular myotendinous junctions (MTJ) structure. Anchorage to this MTJ allows fibres to transmit and withstand contraction forces (reviewed in (56)). Many tendon markers are expressed at the MTJ either
by the neighboring muscle cells or by fibroblast-like cells proposed to be tenocytes (22). Indeed, by 4 dpf we observed fibroblast-like cells with the matrix protein Tsp4b surrounding their nuclei and with long processes extended into the myosepta (Fig. S6A, A’). Given that scxa is expressed early in the MTJ (Fig. 1A and (23)), we tested whether defective early MTJ patterning might underlie the rib and muscle defects. In situ hybridisation for xirp2a mRNA, a marker for somitic MTJ, for embryos from a scxa<sup>+/−</sup> incross at 52 hpf showed reduced expression in 9/9 genotyped scxa mutants (Fig. 6A) but not in siblings (17/17). At 72 hpf, tnmd mRNA, a marker for maturing tenocytes, was also decreased in scxa mutant larvae (9/9 genotyped mutants) but not in siblings from a similar cross (Fig. 6B). In contrast, immunohistochemistry for Tsp4b protein, which controls matrix assembly in the MTJ (25), yielded no detectable change in level or distribution in scxa mutants (Fig. 6C; Fig. S6B 5/5 analyzed mutants). We conclude that some but not all MTJ markers are reduced in scxa mutants.

To determine whether MTJ defects were secondary to muscle defects at these early stages, scxa mutants were bred into the transgenic Tg(<i>actc1b:egfp</i>)<sup>zfl3</sup>. Somite muscle architecture appeared normal as seen by myosin heavy chain (MyHC), alpha-actinin and GFP distribution (Fig. 6C and Fig. S6D). The ECM protein Laminin and the fibre-end associated cytoskeletal link protein Dystrophin also did not distinguish mutants from their siblings (Fig. S6D and data not shown). Thus, early myogenesis appears normal in scxa mutants.

**Scxb mutants are viable and show no obvious defects**

We also examined scxb homozygous mutants at embryonic, juvenile and adult stages. During embryonic stages, scxb mutants show neither tendon or muscle craniofacial abnormalities (Figs. 2C,C’, S3G and Table 1), nor difference in AR/AB staining at 6 dpf (data
not shown). Somitic MTJ of scxb mutant embryos also developed normally as seen by normal Tsp4b distribution in the myosepta (5/5 mutants and 13/13 genotyped sibling larvae), and somitic muscle volume and structure was unchanged (MyHC, Figs. 3M, S6C). Adult scxb mutants are viable and survive in normal numbers 9/39 (23% mutants from a heterozygote incross). Measurement for 4 and 12 months old scxb mutants showed no significant differences between scxb mutants and siblings in weight, length, BMI and standard weight (Fig. 4D-F and data not shown). The skeleton was evaluated by μCT (3/3) and AR (4/4, Fig. S5F,F’), and the mutants showed to be normal and indistinguishable from siblings (Fig. S5F,F’,G,G’ and data not shown). Thus, the lack of Scxb has no detectable effect on the development of zebrafish musculoskeletal system.

**Lack of Scxa and Scxb leads to lethal jaw defects**

We generated double mutants from incrosses of $scxa^{+/kg170};scxb^{+/-}$. Double mutants were indistinguishable from their siblings before 4 dpf and were viable in normal Mendelian numbers up to early juvenile stages (7/138 from total number of genotyped embryos at 6-13 dpf, $\chi^2$ test p=0.567). Immunostaining for Tsp4b and MyHC showed no phenotypic abnormality in the MTJ (Fig. 7D, compare with Fig. 6C) showing that scxa and scxb combined function is not essential for early somite MTJ development.

In embryos from incrosses of $scxa^{+/kg170};scxb^{+/-}$, a subset had abnormal lower jaw, hanging open from 4 dpf, (Fig. 7A,B and A’,B’). This phenotype showed in 22/533 embryos (4.1% of all embryos) of which $scxa^{-/-};scxb^{+/-}$ (16/22) and $scxa^{-/-};scxb^{+/-}$ (6/22, ~10% penetrance). No normal embryos (25 genotyped) were double mutant but one embryo was $scxa^{-/-};scxb^{+/-}$. The jaw morphology defect caused reduced jaw movement, although they were motile and able to swim (data not shown). At 13 dpf, $scxa^{-/-};scxb^{-/-}$ larvae had a similar jaw phenotype and
severe growth retardation (3/45, 6.6% from all embryos; Fig. 7C,C'), and by 34 dpf, no surviving \textit{scxa}^{-/-};\textit{scxb}^{-/-} fish were obtained (0/41, 0% from all embryos). This was confirmed by an incross of \textit{scxa}^{+/kg141};\textit{scxb}^{+/} that yielded no double mutants (0/63, 0% from whole surviving incross, tested at 12 months of age). Overall, lack of \textit{scxa}^{-/-};\textit{scxb}^{-/-} fish is significant ($\chi^2 =0.008$), indicating that they die in the period between 2 and 5 weeks of age. \textit{scxa}^{-/-};\textit{scxb}^{-/-} fish were found in expected Mendelian numbers for both incrosses.

To investigate further the ‘hanging jaw’ phenotype, we stained 6 dpf larvae from an incross of \textit{scxa}^{+/kg170};\textit{scxb}^{+/-} for AR/AB (Fig. 7E-G and E’-G’). \textit{scxa}^{-/-};\textit{scxb}^{-/-} fish show a clear change of position of the Meckel's cartilage (Fig. 7B',E'). The joint at the anterior tip of the Meckel's cartilage contains many rounded cells that are undifferentiated as opposed to elongated mature cartilage in siblings (compare Fig. 7F',G' and F,G). All double mutants (6/6) show a severe reduction in Tsp4b and disorganisation in cranial tendons and ligaments, most severely in the mandibulohyoid junction between 4 and 13 dpf (Fig. 7I,I'). Functional muscle has been shown to be required for normal jaw morphology (57, 58). We found that \textit{scxa}^{-/-};\textit{scxb}^{-/-} larvae had more severe defects in jaw muscles, particularly in the intermandibularis posterior and interhyoideus muscles. A large proportion of the intermandibularis posterior muscle fibres from either side of the midline extended beyond the mandibulohyoid junction, where they normally end, until their displaced meeting, while other appeared to change angle and also be part of the interhyoideus muscle (compare Fig. 7H',I' and H,I and Table 1). Thus, lack of both scleraxis genes in zebrafish cause substantial tendon, muscle and cartilage defects resulting in paralysis of the jaw with lethal consequences.
Discussion

The findings described here provide genetic demonstration of three major points. Firstly, that scxa is required for correct skeletal development, including rib growth and mineralization, morphology of vertebral arches, normal swimming behavior and trunk muscle composition. Secondly, scxa mutation leads to embryonic defects in cranial tendon formation and muscle misalignment. Thirdly, whereas loss of scxb alone does not lead to severe phenotypes, scxb is required in the absence of scxa, as loss of both leads to lethal jaw paralysis. From this we conclude that scxa and scxb have overlapping functions in tendon formation.

**scxa and scxb expression and function during embryonic stages**

In addition to confirming the expression of scxa in the craniofacial tendon precursors and the MTJ (23, 24), we demonstrate that scxa mRNA is expressed in the full extent of the vertical myosepta at juvenile stages. Loss of Scxa affects the structure and shape of various tendons, most notably ones connecting the jaw muscles to the jaw. This leads to abnormal connections, ectopic growth and misalignment of fibres of the craniofacial musculature. scxa mutants are viable however, surviving to adulthood despite a range of phenotypes. Although craniofacial cartilage and bone appear normal in embryos, our μCT data show significantly lower BMD in jaw of adult scxa mutants. The jaw bones mineralize much later than our larval analyses. This may reflect changes to muscle activity due to the above tendon and muscle phenotypes leading to changes in the mechanical load of certain areas. Mechanical loading has been previously shown in humans and fish to affect bone properties, specifically BMD (59, 60). We show that scxb is expressed in a subset of cranial tendons, and weakly in the intersomite MTJs. Loss of Scxb alone led to no obvious phenotype, in the presence of wild type Scxa. However, mutation of scxb in scxa mutants exacerbates the phenotype, such that
muscles are misaligned, extend into different muscles, and lack attachment to the skeleton. This leads to jaw paralysis, resembling the flaccid paralysis observed upon treatment with MS222 (58). We observe growth retardation and death in the early juvenile stages, likely from starvation. We, and others, have previously shown that periods of larval immobility affect the formation of jaw skeletal elements and joints (57, 58, 61, 62). Similarly, we show that tendon malformation can affect the muscle and the morphogenesis of the jaw, indicating synergy in development of the cranial musculoskeletal system. Some of the defects are reminiscent of the zebrafish cyp26b1 mutant tendon phenotypes (24). In cyp26b1 mutants, tenoblasts fail to condense into nascent scxa-expressing tendons, affecting muscle projection and misdirecting it. Our data shows that Scleraxis function in tenoblasts contributes to the maturation of cranial tendons. None of the described mouse loss-of-function models reported head phenotypes (11, 14). However, Scx is expressed in pharyngeal arches and facial tendons of mouse embryos (8-10), and other mutations affecting mouse tongue muscle tendons resulted in tongue muscle abnormalities and dysmorphogenesis (63, 64). Facial tendons in mouse, chick and zebrafish have a common origin from cranial neural crest cells (8, 23, 27). They are also similar in function although different fish, birds and mammals have varied feeding strategies and mandibular morphology (65, 66) such that cranial phenotypes stemming from tendon defects may differ between species.

The role Scxa and Scxb in the somitic MTJs seems to be limited at embryonic stages. We detected mild downregulation of tendon markers and downstream targets such as tnmd and collagens, comparable with mammals and birds (11-14). However, we found no evidence for downregulation of key components of the ECM such as Thrombospondin 4b and Laminin and functional embryonic phenotype, nor did we detect damaged muscle, loss of sarcomeric structure or somitic boundary compromise in either the single or double mutants at larval
stages. This contrasts with the phenotype observed upon loss of the dystrophin-associated glycoprotein complex (DGC) components (reviewed in (67) and (56)). This may reflect low levels of expression of scxa and scxb at embryonic stages, but other genes, likely in the fibres themselves could control these ECM components at the somitic MTJs. It suggests that the muscle-dependent DGC and integrin complexes are independent from tendon development and are sufficient to connect the somitic muscle blocks even when the tendons are impaired, preventing damage, at least during embryonic stages.

**Scleraxis function in rib mineralization**

Our data show that Scxa is strongly expressed in the intramuscular tendons adjacent to the developing ribs and is essential for rib growth and mineralization. In scxa mutants we see severe defects in rib structure, such that mutants lack mineralized ribs. The tissue appears to become fibrous, rather than bony. scxa mutants display changes to the structure of the vertebral arches, which are wide and irregular, despite the normal myotome patterning seen at larval stages.

Many studies have indicated that all parts of the ribs are derived from the sclerotome compartment of the somites (68-71), whereas other studies have suggested that the ribs can be divided into three regions and that rib development also depends on the dermomyotome (DM) (72, 73). In addition, manipulations in chick, that led to loss of Scx expression, such as separating the ectoderm physically from somites or changes to MKP3 levels affecting ERK signaling strength resulted in defective distal rib development (74, 75). Although in mice Scx is expressed in rib primordia (8, 11, 13), the ribs and the tendons that connect them to the intercostal muscles were unaffected in one Scx^{−/−} allele (11), and the rib cage was decreased in size for Scx^{cre/cre} allele (14). Conditional inactivation of Sox9 in Scx^{+}Sox9^{+} cells in
$\text{ScxCre;Sox9}^{\text{flox/flox}}$ mice, caused a loss of all but the proximal rib cage (76). Mammalian tendon-bone attachments including the patella, deltoid tuberosity, olecranon and other eminences express both Sox9 and Scx (16, 76, 77). It is unclear if scxa is expressed in rib precursors in fish and whether it has similar role in mineralization of the ribs as in mineralisation of these mammalian eminences (11, 14, 16, 17, 76, 77). Function may also be maintained in the distal parts of the ribs in some amniotes. Ribs protect internal organs in fish and amniotes. The development of lungs and the requirement to protect the respiratory system with a strong bony rib cage may have shifted rib development to be more dependent on Sox9 in land living animals, and so affecting rib composition and strength.

Interestingly, we found that zebrafish lacking rib bone are viable, but their swimming performance is altered. This may indicate that the ribs play a mechanical role in swimming. Indeed, some studies have highlighted correlations in intramuscular ossification in fish that differ in their swimming modes (78, 79). The reduced volume of trunk musculature and body weight in scxa adult mutants could be linked to the altered swim behavior seen in the mutants. This in turn could be due to musculoskeletal attachments to the ribs and other intramuscular bones between the myomeres that are not capable of transmitting the full force from muscle contractions. Alternatively altered intramuscular attachments in the trunk or abnormal fin attachments could alter both swim performance and preclude rib development.

Whereas scxa;scxb double mutants are smaller in length and have paralyzed jaw, both linked to reduced feeding, scxa mutants have normal standard length, and their jaw movement seem normal. However, we cannot rule out the possibility that reduced feeding is affecting the lower weight of scxa mutants, which in turn may be due to the mutants being outcompeted by their siblings.

Both our expression and functional data points to a greater role for scxa than for scxb in both
head and trunk during development, especially at juvenile stages. This is likely reflecting the high synteny of scxa, but not scxb, to the mammalian orthologues, likely keeping ancestral regulatory elements intact. However, Scxb protein is very similar to Scxa protein; 70.8% identity between the two proteins, almost identical bHLH domain (Fig. S2A,B), overlapping expression and the ability to replace the function of the other to some extent.

In summary, we have shown that Scleraxis has an essential role in the normal development of the musculoskeletal system in fish. Its essential function in differentiation and maturation of tendons, and in ossification of skeletal elements that express Sox9 and Scx, are conserved with other vertebrates. Zebrafish are thus a useful model to study the close relationship of muscle, tendon and bone in development and disease of joints.

Acknowledgments

EK, EMS and CLH are funded by Arthritis Research UK Grants 19476, 21937 and 21121, SMH is a member of MRC scientific staff with Programme Grant G1001029 and MR/N021231/1 support, EL is funded by the Wellcome Trust Dynamic Molecular Cell Biology PhD programme, SC is partially funded by the Elizabeth Blackwell Institute, through its Wellcome Trust ISSF Award, YH was funded by BHF grant PG/14/12/30664.

Author Contributions Statement: YH and SMH conceived the study, YH and CLH managed the workplan, EK, EMS, EL, SC, CLH and YH performed and analyzed experiments and wrote the manuscript.

Competing Interests
None of the authors have any competing financial interests.
References

Kague et al.  Page 29


42. Cross, S. (2018) Modular Image Analysis V0.5.17. . In Zenodo


Ligament versus bone cell identity in the zebrafish hyoid skeleton is regulated by mef2ca. *Development* **143**, 4430-4440


### Table 1

<table>
<thead>
<tr>
<th>Phenotype*</th>
<th>+/+</th>
<th>scxa&lt;sup&gt;+&lt;/sup&gt;</th>
<th>scxa&lt;sup&gt;−/−&lt;/sup&gt;</th>
<th>scxb&lt;sup&gt;−/−&lt;/sup&gt;</th>
<th>scxa&lt;sup&gt;−/−&lt;/sup&gt;;scxb&lt;sup&gt;−/−&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>intermandibularis anterior:</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>overextension</td>
<td>0/10</td>
<td>0/14</td>
<td>1/8</td>
<td>0/5</td>
<td>1/4</td>
</tr>
<tr>
<td>ectopic fibers</td>
<td>0/10</td>
<td>0/14</td>
<td>1/8</td>
<td>0/5</td>
<td>0/4</td>
</tr>
<tr>
<td>muscle fibers misaligned</td>
<td>0/10</td>
<td>0/14</td>
<td>2/8</td>
<td>0/5</td>
<td>2/4</td>
</tr>
<tr>
<td><strong>intermandibularis posterior:</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>overextension</td>
<td>2/10</td>
<td>5/14</td>
<td>8/8</td>
<td>2/5</td>
<td>4/4</td>
</tr>
<tr>
<td>ectopic fibers</td>
<td>0/10</td>
<td>1/14</td>
<td>1/8</td>
<td>0/5</td>
<td>4/4</td>
</tr>
<tr>
<td>muscle fibers crossing midline</td>
<td>0/10</td>
<td>1/14</td>
<td>6/8</td>
<td>1/5</td>
<td>4/4</td>
</tr>
<tr>
<td><strong>hyohyoideus:</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>overextension</td>
<td>1/10</td>
<td>5/14</td>
<td>5/8</td>
<td>0/5</td>
<td>4/4</td>
</tr>
<tr>
<td>ectopic fibers</td>
<td>0/10</td>
<td>1/14</td>
<td>2/8</td>
<td>0/5</td>
<td>4/4</td>
</tr>
<tr>
<td>muscle fibers crossing midline</td>
<td>0/10</td>
<td>2/14</td>
<td>5/8</td>
<td>0/5</td>
<td>3/4</td>
</tr>
<tr>
<td><strong>sternohyoideus:</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>overextension</td>
<td>0/10</td>
<td>2/14</td>
<td>6/8</td>
<td>0/5</td>
<td>4/4</td>
</tr>
<tr>
<td>ectopic fibers</td>
<td>0/10</td>
<td>2/14</td>
<td>2/8</td>
<td>0/5</td>
<td>4/4</td>
</tr>
<tr>
<td>muscle fibers crossing midline</td>
<td>0/10</td>
<td>5/14</td>
<td>6/8</td>
<td>2/5</td>
<td>4/4</td>
</tr>
<tr>
<td><strong>adductor mandibularis:</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>overextension</td>
<td>0/10</td>
<td>1/14</td>
<td>4/8</td>
<td>0/5</td>
<td>0/4</td>
</tr>
<tr>
<td>ectopic fibers</td>
<td>0/10</td>
<td>2/14</td>
<td>3/8</td>
<td>1/5</td>
<td>0/4</td>
</tr>
</tbody>
</table>

*Analyzed at 4 dpf in embryos from scxa<sup>−/−</sup>;scxb<sup>−/−</sup> or scxa<sup>−/−</sup>;scxb<sup>−/−</sup> incrosses from IHC of Tsp4b and MyHC (A4.1025).
Figure Legends

**Figure 1. Comparison of scxa and scxb expression during embryonic and juvenile stages.** In situ hybridisation for indicated genes shown in lateral (C-J) or ventral view (A,B, K-M). **A-B.** At 72hpf, *scxa* is detected in Meckel’s adductor tendon (mat), the intermandibularis tendon (imt), the mandibulohyoid junction (mhj), the sternohyoideus tendon (sht) and hyohyoideus junction (hhj). *scxb* is expressed at the sternohyoideus tendon and hyohyoideus junction, the Meckel’s adductor tendon and the ocular muscle tendons (ot). **C,D.** At 72 hpf, *scxa* and *scxb* are expressed weakly at the myosepta (blue arrowheads) and the caudal fin (black arrows). **E-J.** In situ hybridisation for *scxa* (E-G), *sox9a* (H) and *scxb* (I,J) at juvenile stages as indicated showing expression in the trunk in the posterior vertical myosepta (arrowheads), and the more anterior myosepta near the thoracic ribs (arrows), intermuscular bones (white arrowheads), fin radials (blue arrowheads), in gills (blue asterisk) and between fin bony ray segments (purple arrowheads). Sagittal sections in rib area (E’) and myosepta in anal fin area (E”) show *scxa* staining in intramuscular tendons separated from muscle fiber (mf) ends (yellow arrowheads) by an unstained tendon-sheath or extra cellular matrix (brackets). **K-M.** *scxa* expression at juvenile stages in head tendons including: protractor hyoideus tendons (orange arrow) and intermandibularis anterior tendon (green arrows) at the attachments of the cranial muscles (labelled with *tnnc*) L: Protractor hyoideus-dorsal and ventral (pr-h) and the intermandibularis anterior (ima). No *scxb* expression is detected in cranial tendons at juvenile stages. Scale bars, 100 µm (A-D, E’, E”), 0.5 mm (E-M).

**Figure 2. Cranial tendons, ligaments and muscles of scxa<sup>−/−</sup> mutants are abnormal and disorganized.**

**A-C.** Second harmonic generation (SHG) imaging shows both collagen arrangement in
tendons and ligaments and myosin heads in muscle (light blue) combined with confocal imaging of the transgene TgBAC(col2a1a:mCherry)hu5910 (cartilage, purple) of 7 dpf scxa<sup>+/−</sup>, scxb<sup>+/−</sup>, and wt (+/+) larvae. Shown are representative maximum projections images of the stacks (A-C) and single scans at comparable Z positions (A’-C’). Decreased SHG signal was observed in the ligaments connecting the Meckel’s and palatoquadrate cartilages of scxa and scxb mutants (white arrows), and in the sternohyoideus tendon (yellow arrows) connecting the sternohyoideus muscle and the basihyal cartilage (asterisk) of scxa mutants. D. In situ hybridisation for tnmd for 3 dpf scxa<sup>+/−</sup> and their siblings (+/+) showing reduced tnmd mRNA levels in scxa mutant, such as the sternohyoideus tendon (black arrowhead), and the ligaments connecting the Meckel’s and palatoquadrate cartilages (red arrowhead). Expression at the base of the cleithrum is maintained (asterisk). E-G. Confocal stacks of 4 dpf embryos from a scxa<sup>+/−</sup> incross, immunostained for MyHC (A4.1025, green) and Tsp4b (red). Specific defects in tendon structure and directionality in scxa mutants (white rectangles, E) are shown in higher magnification panels for mandibulohyoid junction (E’,F’) and ceratohyal tendon (E”, F”). Scxa mutants show varied array of muscle defects such as abnormal overextension or crossing the midline of muscle fibres (F,G compared with E) and detached fibres in interhyoides and hyohyoides (arrowheads, F). The magnified area (G’-G’’) shows ectopic fibre in the adductor mandibularis (white arrow, G’), ectopic misguided fibre from the interhyoides is crossing the midline and growing towards the interhyoides across the midline (yellow arrow, G’”) and ectopic extention of sternohyoides tendon (blue arrowhead) is attracting overextended fibres (green arrowhead). mc, Meckel’s cartilage, pq, palatoquadrate cartilage, cht, ceratohyal tendon, mat, Meckel’s adductor tendon, mhj, mandibulohyoid junction, pqat, palatoquadrate adductor tendon, sht, sternohyoides tendon, am, adductor mandibularis hh, hyohyoides, ima, intermandibularis anterior, imp, intermandibularis posterior,
ih, interhyoides, sh, sternohyoideus. All images in ventral view, anterior to left. Scale bars 100 µm, except F'-F'' and G'-G''', 20 µm.

**Figure 3. Lack of Scxa in adult affects body measurements and swim behavior.**

A-H. Weight, length, BMI and standard weight comparison between mutants and sibling adult fish from a scxa<sup>+/−</sup> incross (13 siblings and 7 scxa<sup>+/−</sup> males, 18 siblings and 6 scxa<sup>+/−</sup> females, A-D) and a scxb<sup>+/−</sup> incross (21 siblings and 5 scxb<sup>+/−</sup> males, 6 siblings and 2 scxb<sup>+/−</sup> females, E-H). Males and females are presented separately as were found significantly different. I-J. Contrast enhanced µCTs to show soft tissue of scxa<sup>+/−</sup> mutant (J-J'') and a +/+ sibling (I-I''). Magnified scans show lack of ribs (black arrows) in the mutant. I'' and J'' show transverse optical sections at indicated positions in I and J, dashed arrows indicate the vertebrae. K. Myotome volume from co-reared similar length fish are calculated from scans, by creating a virtual ‘steak’ between two ribs (red area, see materials and methods). L,M Quantification of muscle volume in scxa<sup>+/−</sup> adult mutants (L) and scxb<sup>+/−</sup> (M) and their respective siblings. N-P. Swimming performance was calculated from videos taken from above the tank (schematic drawing in N, see also supplementary video, Fig. S7) and the range of movement (curvature, M) and instantaneous velocity (N) were calculated as detailed in materials and methods. Two-way ANOVA statistics with Sidak’s post-hoc tests performed in A-H. Unpaired t-test with Welch’s correction performed in L,M, and two-way ANOVA performed in O,P, significant p-values indicated on graphs.

**Figure 4. Adult scxa homozygous mutants have skeletal abnormalities.**

A-E. 3D volumetric isorenders from µCt data of scxa<sup>+/−</sup> and wt sibling showed absence of mineralized ribs (green dashed line above the rib region) (C, compare to A), and protruding
jaws (green arrows) in mutants. Thoracic ribs region was magnified to show details of the vertebrae (B,D). Small bony structures were observed branching from the haemal arches (green arrowheads) and vertebrae misalignments were often present (red dashed arrow) in scxa mutants. E. Bone mineral density (BMD) values were calculated from three distinctive bones: jaw (green arrows in A,C), vertebrae and the parietal bone (yellow arrowhead in A,C). One-way ANOVA statistics with Tukey’s post-hoc test performed, p-values indicated. F-J. Alizarin Red stained adult scxa<sup>−/−</sup> mutants show no signal in ribs (H, under green line, compared with F), and neural (black arrowheads) and haemal arches (yellow arrowheads) have extensive bony growth (vertebrae 15-19 magnified in G,I). Fibrous, almost transparent ribs are seen in high magnification (black arrows, J), with the odd mineralized rib tissue (red arrowhead in H,J). The area rostrally to rib 5 is formed normally. ps/r4- parapophysis and rib 4. K-M. Adult wt and scxa mutant zebrafish sagittal (K,K’,L,L’) and transversal (M,M’) paraffin sections stained with Hematoxylin and Eosin and Alcian blue. Existing rib structure in scxa mutant is short, thin and wavy. The intervertebral disk (ivd) is shown in M,M’. The notochord string (ns) connecting the dorsal and ventral of the V shape is normal. However the IVD is enriched with fibrous tissue (more purple, green arrowheads). m, muscle, n, notochordal cells. Scale bars: A-D,F-J, 1mm and K-M, 100 µm.

Figure 5. *Scxa is is required for rib mineralization and its patterning.*

Alizarin red staining for fish from a scxa<sup>+/−</sup> incross at the indicated juvenile stages At SL 5.8 mm, increased DIC shows that the junction region between muscle and rib (yellow arrows) is already abnormal (A,A’). At SL 6.5 mm, the first anterior ribs (r5 and r6) are lacking (asterisks in B’, compared with B). A severe lack of mineralized ribs (black asterisks) and mineralized rib fragments (white arrowhead) are seen at SL 10.5 mm (C’, compare to C). In another mutant
(D), some ribs are missing (asterisks in D), while other are highly fractured and healed (ribs 5-9 magnified and marked by red arrowheads in D’). Note that rib 4 (r4) is formed normally (arrows B-D). All scale bars, 100µm.

**Figure 6. Scxa mutants have reduced levels of tendon markers but somitic myotendinous junctions appear normal.**

A,B. In situ hybridisation for xirp2a at 52 hpf (A) and tnmd at 3 dpf (B) for scxa−/− and their siblings (+/+), lateral view, anterior to left. In B, main image shows the anterior somites, inset-whole embryo. Both xirp2a and tnmd mRNA levels are reduced at the MTJs at the somitic borders (arrowheads). C. Confocal stacks of immunodetection of MyHC (A4.1025) and Tsp4b in somites 11-14 of 4 dpf embryos of scxa−/− and siblings showing normal distribution of Tsp4b and normal muscle structure. All scale bars, 100µm.

**Figure 7. Scxa;scxb double mutants have a jaw phenotype and severe musculoskeletal defects.**

A-C’. Live transmitted light and red fluorescent images in lateral view, anterior to left of genotyped scxa−/−;scxb−/− double mutants (A’-C’) compared with siblings (A-C, shown is scxa+/− scxb+/−). Mutants show a ‘hanging open’ jaw. The col2a1:mCherry transgene (B) shows the dropping Meckel’s cartilage (mc). At 13 dpf, fish are much smaller than siblings (whole fish insets in C). D. Confocal stacks of immunodetection of MyHC (A4.1025) and Tsp4b in 4 dpf embryos of scxa−/− scxb−/− showing normal distribution of Tsp4b and normal muscle structure (compare with Fig. 6C). E-G’. Alcian blue/Alizarin red staining for cartilage and bone for scxa−/− scxb−/− (E’-G’) and sibling from the same cross (E-G), shown in lateral (E) and ventral (F,G) views showing the ‘hanging’ jaw phenotype. The magnified area from Meckel’s cartilage
shows many less differentiated rounded cells at its most anterior tip, near the joint (arrow, G'). Compared with elongated mature cells in sibling (arrow, G). H-I'. Confocal stacks showing immunodetection of cranial muscles (MyHC, A4.1025) and tendons (Tsp4b) of 4 dpf embryos of scxa+/−;scxb+/− incross in ventral view. Tsp4b is highly reduced in tendons and ligaments of double mutants as shown for mandibulohyoid junction (mhj) (magnified area, I,I'). Many muscle fibres in scxa−/−;scxb−/− extend the length of the intermandibularis posterior (imp) and the interhyoideus (ih) (yellow arrowheads, I'), while others extend far beyond their normal end at the mandibulohyoid junction until their meeting point (white arrowheads, I'). All scale bars, 100µm. hhj, hyohyoideus junction, ima, intermandibularis anterior, imt, intermandibularis tendon, mc, Meckel's cartilage, sh, sternohyoideus, sht, sternohyoideus tendon.
Fig. 2
Fig. 3

A. Comparison of weight distributions between males and females with significance levels p=0.047 and p=0.014.

B. Comparison of length distributions between males and females.

C. Comparison of BMI distributions between males and females with significance levels p=0.004 and p=0.012.

D. Comparison of standard weight distributions between males and females.

E. Comparison of weight distributions between males and females.

F. Comparison of length distributions between males and females.

G. Comparison of BMI distributions between males and females.

H. Comparison of standard weight distributions between males and females.

I. Comparison of images of +/+ and scxa-/- genotypes.

J. Comparison of images of scxa-/- and scxb-/- genotypes.

K. Comparison of muscle volume distributions between +/+ and scxa-/- genotypes.

L. Comparison of muscle volume distributions between scxb+/- and scxb-/- genotypes with significance level p=0.006.

M. Comparison of range of movement (curvature) distributions between scxa+/- and scxa-/- genotypes with significance level p<0.001.

N. Comparison of instantaneous velocity distributions between scxa+/- and scxa-/- genotypes with significance level p<0.001.
Fig. 6

A  52 hpf

B  72 hpf

+/+

scxa -/

xirp2a

tnmd

Merge  Tsp4b  MyHC

C  4 dpf

+/+

scxa -/


Fig. 7
Figure S1. Generating mutant alleles for *scxa* and *scxb*. A. Schematic representation of *scxa* and *scxb* gene and protein and the new mutant alleles for *scxa* (*kg141* and *kg170*), and *scxb* (*kg107*). Each gene has two exons (Coding sequence shown by filled boxes, UTRs-white boxes, introns are marked with a black line). All mutations produce truncated proteins devoid of the basic and helix–loop–helix domains (bHLH, light blue). Beneath, DNA and protein sequence of wild type (wt) and mutant alleles are shown. CRISPR target sequence is underlined in the wt DNA sequence. Deleted nucleotides are shown by (-) in mutants or highlighted in the wt sequence and inserted nucleotides are shown with thick underscore. Presumed stop codons are shown in red text. Amino acid (aa) tails after frameshifts are underlined (only shown partially for *kg170*).
Figure S2. Scxa is the more conserved homologue of the mammalian Scx.

A. Clustal alignment of the translation product of the zebrafish scxa and scxb genes with representatives of other major vertebrate groups (human, mouse, chicken, Xenopus laevis and Fugu rubripes).

B. Sequence pair distances of representative the above proteins using the Clustal method with PAM 250 residue weight table.

C. Synteny diagram based on Genomicus software and Ensembl GRCz11 showing similar position of scxa gene inside intron 3 of the Bop1 gene on the other strand, as described for mouse Scx (11), whereas scxb locus show changes and rearrangements compared with mouse and human genomes.

D-E. In situ hybridisation for scxa (D) and scxb (E) for 3 dpf embryos from a scxa+/- incross. Cranial expression is shown in ventral view. scxa mRNA levels in scxa mutant (11/50 embryos from the incross) are reduced compared with heterozygote (29/50) and +/- embryos (10/50), genotypes confirmed (D). scxb mRNA levels and spatial pattern do not differ between genotypes, genotypes confirmed (E). scale bars-100µM.
Figure S3. Cranial tendons, ligaments and muscles of $\text{scxa}^{\text{kg141}}$ mutants are abnormal and disorganized. Confocal stacks of cranial muscles using immunofluorescence for MyHC (A4.1025, red in A), Tsp4b (red) and MyHC (MF20, green) in B-D, GFP (green) and alpha-actinin (blue) in E,F and Tsp4b (red) and MyHC (A4.1025, green) in G. All in ventral view, anterior to left. A. Embryos from a $\text{scxa}^{\text{kg170/+}}$ incross at 4 dpf; $\text{scxa}^{-/-}$ mutants had misaligned fibres and tri- and four-way abnormal junctions (yellow arrowhead). Some $\text{scxa}^{-/-}$ embryos had some milder defects (white arrows). B-D. 6 dpf embryos from a $\text{scxa}^{\text{kg141/+}}$ incross; the matrix protein, Tsp4b was downregulated in mutants, and some tendons such as the mandibulohyoid junction and intermandibular tendon (C), the sternohyoides and hyohyoides tendons (D) were misshapen and showed decreased matrix condensation. Muscle fibre defects contained fibres connecting to wrong muscles and disorganized junctions. E,F. 5 dpf embryos from a $\text{scxa}^{\text{kg141/+}};Tg(\text{actc1b:egfp})^{\text{zf13}}$ incross. F shows magnified boxed region in E. Mutant embryos had fibres from the interhyoides muscle growing in the wrong direction towards the hyohyoides junction (white arrow). G. Both tendons and muscles of 4 dpf embryos from a $\text{scxb}^{-/-}$ incross looked normal. mhj, mandibulohyoid junction, ima, intermandibularis anterior, imp, intermandibularis posterior, imt, intermandibular tendon, sht, sternohyoides tendon, hhj, hyohyoides junction, ih, interhyoides, hh, hyohyal, sh, sternohyoides. All scales 100µm except A, 50µm.
Figure S4. Cranial bone and cartilage show no obvious defects at embryonic and juvenile stages. A. Alcian blue and Alizarin red double staining for cartilage and bone for 6 dpf scxa+/kg170 incross embryos, showing as flatmounts of the pharyngeal skeleton (ventral view, left) or neurocranium (dorsal view, right). B-C. 13 dpf scxa+/kg170;Tg(col2a1:mCherry) incross juveniles shown for live mCherry expression (ventral view, B) and Alcian blue and Alizarin red double staining (pharyngeal skeleton, ventral view, C, left and neurocranium, dorsal view, C, right). No dramatic differences are detected beyond normal variation.
Figure S5. Adult scxa mutants show skeletal defects in trunk but not in skull.
A. Vertebral centrum volumes calculated from μCT scans of genotyped adults from a scxa+/− incross (n=3 per genotype) using the minimum volume possible around the neural canal, excluding (for 7th and 8th thoracic vertebrae) or including (for the 3rd thoracic vertebra) all processes, trabeculae, spines and ribs in a transverse view. Vertebrate centrum alone is similar between all genotypes, but when the above skeletal elements were added, volume in scxa−/− fish is significantly smaller than siblings. One-way ANOVA statistics with Tukey’s post-hoc test performed, p-values indicated. B-D’, F-G’.
B. Alizarin Red staining for adult scxa−/− (B’-D’) and siblings (B-D) or scxb−/− mutants (F’,G’) and their siblings (F,G). Skull of scxa mutants appeared normal whereas staining in ribs was missing. Skull (arrowhead), jaw (yellow arrowhead) and ribs (arrows) looked normal in scxb mutants. Dissected thoracic 12th vertebrae from scxa−/− mutant and sibling are shown in lateral and frontal views for details of bony growth in arches (C’,D’ compared with C,D).
E, E’. Adult scxa mutant zebrafish sagittal paraffin section stained with Hematoxylin and Eosin and Alcian blue showing fractured and healed rib fragment (magnified in E’). Scale bars, 100µm in E,E’, 1mm in B,B’,F,F’, 0.5mm in C-D’.
Figure S6. Myotendinous junctions and muscle of scxa<sup>kg141</sup> and scxb mutants appear normal.

A-A'. Small confocal stacks of immunofluorescence for MyHC (A4.1025, green), Tsp4b (red) and nuclear stain (Hoechst) of 4 dpf wt embryos somites 11,12, in lateral view, showing cells (magnified in A') at the edge of myosepta (white arrowheads) surrounded by the somites and the neural tube (nt), in which single nuclei (yellow arrowheads) are surrounded by Tsp4b matrix stain and extend processes of Tsp4b along the myosepta (arrows). m, muscle fibres. B,C. Lateral view of somites 11-14 of confocal stacks for immunofluorescence of MyHC (A4.1025, green), Tsp4b (red) of 6 dpf embryos from a scxa<sup>kg141/+</sup> incross (B) or 4 dpf of a scxb<sup>1</sup> incross (C). No major differences in either MyHC or Tsp4b are detected. D. Lateral view of confocal stacks for GFP, Laminin and sarcomeric alpha-actinin of somites 11-14 from 2 dpf scxa<sup>kg141/+</sup>;Tg(actc1b:egfp)<sup>zfr13</sup> incross embryos. No major differences between genotypes are detected. All scale bars 50µm, except A-A', 20µm.
Figure S7. Video: Swim behavior differences between mutant and sibling adult fish. Split screen showing two video fragments shot from above, taken from the original videos of scxa<sup>+/kg170</sup> and scxa<sup>+kg170/kg170</sup> adult fish swimming in a tank.

Figure S8. Parameter file for downloading for use in the “Modular image analysis” software.