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ORIGINAL PAPER

Development of ribs and intercostal muscles in the chicken embryo

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Abstract

In the thorax of higher vertebrates, ribs and intercostal muscles play a decisive role in stability and respiratory movements of the body wall. They are derivatives of the somites, the ribs originating in the sclerotome and the intercostal muscles originating in the myotome. During thorax development, ribs and intercostal muscles extend into the lateral plate mesoderm and eventually contact the sternum during ventral closure. Here, we give a detailed description of the morphogenesis of ribs and thoracic muscles in the chicken embryo (*Gallus gallus*). Using Alcian blue staining as well as Sox9 and Desmin whole-mount immunohistochemistry, we monitor synchronously the development of rib cartilage and intercostal muscle anlagen. We show that the muscle anlagen precede the rib anlagen during ventrolateral extension, which is in line with the inductive role of the myotome in rib differentiation. Our studies furthermore reveal the temporary formation of a previously unknown eighth rib in the chicken embryonic thorax.

KEYWORDS
chicken embryo, development, intercostal muscles, myotome, ribs, sclerotome, thorax

1 | INTRODUCTION

The thoracic body wall of a typical amniote vertebrate is characterized by the existence of ribs. Ribs are lateral extensions of the vertebrae that span the ventrolateral extent of the body wall and form the thoracic rib cage, which hosts the heart and the lungs. The ribs are interconnected by intercostal muscles, which enable movements of the ribs and the rib cage as a whole. The dynamic rib cage allows inspiration and expiration of the lungs and participates in locomotion.

The skeleton of the chicken thorax consists of seven rib-bearing thoracic vertebrae and the sternum (Schummer, 1973) (Figure 1). In the spine of the adult chicken, the first thoracic vertebra (Th1) is an individual vertebra, vertebrae Th2–5 are synostotically fused as *Notarium*, vertebra Th6 is again an individual vertebra and vertebra Th7 is fused with the *Synsacrum*. The seven pairs of ribs articulate with the vertebrae via the capitulum of the costal head and the tubercle of the costal neck: The capitulum forms a true joint with the vertebral body, and the tubercle with the transverse process of the same vertebra. A typical avian rib consists of two morphological elements: the vertebral and the sternal rib. The vertebral rib articulates with the vertebra and carries a prominent caudal extension, the uncinate process. The uncinate process forms a biomechanical connection to the caudally adjacent rib in order to stabilize the rib cage. The sternal rib articulates with the sternum and is denominated by some authors as separate *Os sternocostale*. Vertebral and sternal ribs project ventrocaudally and ventrocranially, respectively, and articulate

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by an intercostal joint in approximately right angle. The first rib as well as the last (seventh) rib in chicken deviate from the typical rib morphology as they do not have an uncinate process. The first and the second rib also deviate from the typical ribs as they lack a sternal part and are not connected to the sternum, but end freely in the thoracic musculature. In contrast to the true ribs which articulate with the sternum, these ribs are called floating ribs. The chicken sternum shows features typical for flying birds: a prominent ventral keel serving as the origin of the vast flight musculature and craniolateral processes serving as the origin for sternocostal and shoulder muscles (Ghetie, 1976; König, 2001; Schummer, 1973) (Figure 1).

The musculature of the chicken thorax consists of intercostal muscles and muscles connecting the ribs with surrounding skeletal elements. As already noted by Bellairs (Bellairs, 2005), the terminology of chicken muscles differs between authors and thorough literature on chicken myology is lacking. To clarify the anatomy of the chicken thorax muscles, we made schematic drawings of the individual muscle groups based on the available descriptions in the literature (Figure 2). Here, we used the terminology following Schummer, König and Liebich, and the anatomical atlas of Ghetie (Ghetie, 1976; König, 2001; Schummer, 1973). Like in mammals, the intercostal muscles are divided into an external and internal layer (*Mm. intercostales externi and interni*), which have intersecting muscle fibres and connect the vertebral part of two adjacent ribs, but not the sternal part. This is at variance with the situation in mammals, namely in human anatomy, where the internal intercostal muscles also connect the cartilaginous sternal part of the ribs. Another set of muscle fibres named costosternal or subcostal muscle extends between the craniolateral process of the sternum and the inner surface of the sternal ribs, thus bridging several segments. This muscle is comparable with the transversus thoracis muscle and the subcostalis muscles in humans. Dorsally, originating from the transverse process of the 13th cervical vertebra, the scalenus muscle is attached to the first two vertebral ribs, i.e. the floating ribs. Caudally adjacent, the levator costarum muscles extend between the transverse processes of the thoracic vertebrae and the dorsal surface of the caudally adjacent ribs. From the medial surface of the intercostal joints of the third to fifth ribs, the costoseptal muscle extends dorsomedially to the horizontal septum, which in birds, which do not have a muscular diaphragm, covers the ventral surface of the lungs (see Table 1 for a summary, and Figure 2 for an overview).

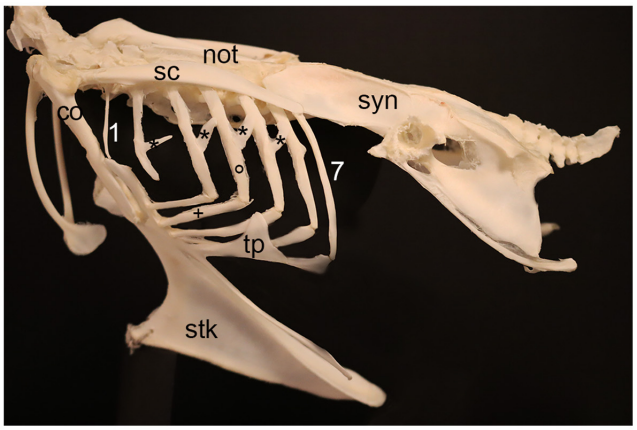


FIGURE 1 Skeletal preparation of the trunk of an adult chicken. The limbs as well as the ribs of the right thorax half are removed for reasons of clarity. Dorsal is to the top, cranial is to the left. First (1) and seventh (7) ribs are labelled by numbers. ° 4th vertebral rib; + 4th sternal rib; * uncinate processes. Abbreviations: Co, coracoid; not, notarium; sc, scapula; stk, sternal keel (*Carina sterni*); syn, synsacrum; tp, thoracic process of sternum.

The embryonic development of the ribs has been investigated by embryologists for almost 150 years (Scaal, 2021). Classical descriptive studies have identified the ribs as vertebral processes which originate from the sclerotomal compartment of the somites (Froriep, 1883; Schauinsland, 1906). This was later experimentally confirmed by lineage studies using carbon particles (Pinot, 1969; Seno, 1961),

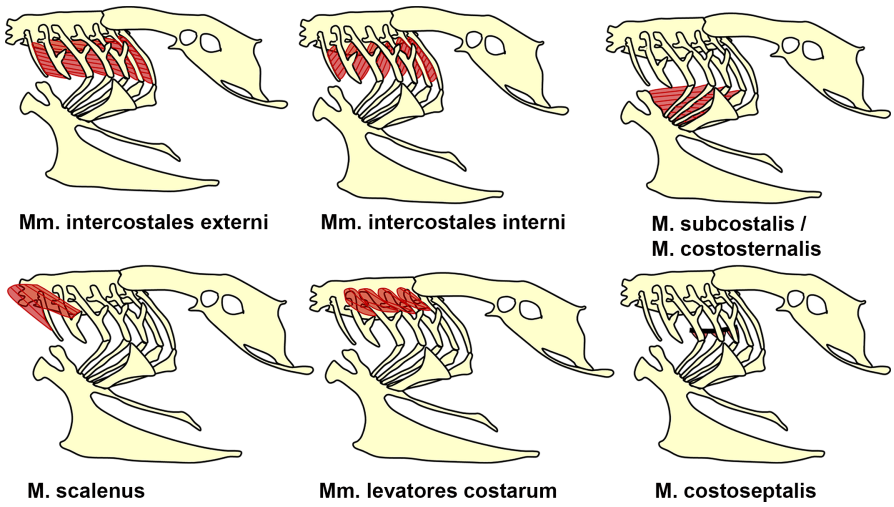


FIGURE 2 Schematic illustration of the thoracic muscles of the chicken. For a description of muscle origin and insertion, see Table 1. Muscles drawn according to non-illustrated data in the literature (Ghetie, 1976, König, 2001, Schummer, 1973).

TABLE 1 List of intrinsic muscles of the thoracic ventrolateral body wall (Ghetie, 1976; König, 2001; Schummer, 1973)

Muscle	Origin	Insertion	Innervation
Mm. intercostales externi	Caudal margin of the vertebral ribs and the uncinate processes	Cranial margin of caudally adjacent ribs	Nn. intercostales
Mm. intercostales interni	Cranial margin of the vertebral ribs	Caudal margin of cranially adjacent ribs	Nn. intercostales
Mm. subcostales (costosternales)	Proc. craniolateralis sterni	Inner surface of sternal ribs Th3–6	Nn. intercostales
M. scalenus	Proc. transversus of C13 (partially also of Th1)	Cranial margin of floating ribs Th1 and 2	Ventral rami of Nn. cervicales
Mm. levatores costarum	Transverse processes of Th2–5	Cranial margin of caudally adjacent proximal ribs	Dorsal rami of Nn. thoracales
M. costoseptalis	Medial surface of intercostal joints of Th3–5	Septum horizontale	Nn. intercostales

the quail–chick chimerization technique (Chevallier, 1975; Christ et al., 1974; Olivera-Martinez et al., 2000) and LacZ retroviral transfection (Evans, 2003). These studies furthermore revealed that the ribs originate from two different sclerotomal subdomains: The head and neck region of the vertebral rib, which forms the costovertebral joints, originates from the central sclerotome. The vertebral and sternal rib shaft originates from the lateral sclerotome. Moreover, the head and neck region of the ribs is formed by sclerotomal cells of a single somite, whereas the rib shaft is formed by sclerotomal cells of two adjacent sclerotomes, thus undergoing resegmentation (Bagnall et al., 1988; Evans, 2003; Huang et al., 2000). Integrating morphological and embryological data, Aoyama et al (Aoyama et al., 2005) proposed to discriminate three parts of the developing avian rib: the proximal part consisting of costal head and neck, the vertebro-distal part consisting of the shaft of the vertebral rib including the uncinate process, and the sterno-distal part consisting of the sternal rib (reviewed in Scaal (2016) and Scaal (2021)).

Research on the molecular regulation of rib development has seen a lot of progress in recent years, which has mainly been gained in chicken and mouse embryos. It became clear that the regulation of proximal rib development is fundamentally different from that of vertebro-distal and sterno-distal rib components. Whereas development of the proximal ribs is closely linked to signalling pathways regulating the development of the vertebral bodies, development of the vertebral and sternal rib shaft is strongly coupled to intercostal muscle development (Scaal, 2021).

The development of the thoracic musculature is not well understood. According to the literature, intercostal muscle in chicken arises from the myotomes of somites 19–26 (Chevallier, 1975; Evans, 2003). The thoracic muscles are derivatives of the myotomal compartment of the somites, which is formed by cell recruitment from the epithelial dermomyotome. As opposed to the back musculature, which derives from the epaxial myotomes of the somites, the thoracic and the abdominal muscles originate from the hypaxial myotomes (Scaal & Marcelle, 2018). Initially, cells from the ventrolateral lip of the dermomyotome translocate to form the ventrally adjacent hypaxial myotome, which is subsequently supplemented by secondary myotomal cells from the entire hypaxial dermomyotomal sheet (Cinnamon et al., 1999; Gros et al., 2004; Gros et al., 2005). During subsequent growth of the myotome in ventrolateral direction, the dermomyotomal ventrolateral lip remains associated with the ventrolateral myotomal tip to continue cell supply until myotomal expansion is completed. In mouse embryos, for instance, this myotomal growth requires Pax3 and Six1/4 activity in the dermomyotomal/myotomal cells (Grifone et al., 2005; Laclef et al., 2003; Tremblay et al., 1998) and Pitx2 activity in the resident somatopleural cells (Eng et al., 2012).

From this short overview of thoracic wall development, it becomes evident that ribs and intercostal muscles develop in close spatial and functional association. The muscle anlage has been shown to be an important signalling centre for rib development, whereas reciprocal signalling from the rib anlage to the myotome has not yet been reported (Scaal, 2021). In spite of the growing knowledge of the molecular networks regulating thorax development, the morphological basis of rib and intercostal muscle formation has not yet been sufficiently described. Notably, a thorough description of the progressive stages of rib and intercostal muscle growth from their origin in the somites to the definitive bauplan is lacking.

In this work, we characterize rib and intercostal muscle development in the chicken embryo with a focus on the temporal and spatial progress during development (Figure 3). We describe the progressive formation of ribs in whole-mount embryos using Sox9 as an early cartilage marker including the precartilaginous condensations (Akiyama et al., 2002; Lefebvre et al., 2019) and Alcian blue staining, which marks cartilage glycosaminoglycans (Steedman, 1950), to revisit the development of the embryonic thoracic skeleton. We show the formation of thoracic muscles by using Desmin as a marker of the muscle lineage from somitic muscle precursor cells (Kaehn et al., 1988) to the fully developed musculature (Lazarides and Balzer, 1978). We furthermore use vital dye labelling of somite cells with the vital dye DiD to identify somite cells irrespective of their differentiation state. By combining these approaches in the same embryo, we analyse the spatial relationship between rib and muscle anlagen in the course of thorax development. Our data show that intercostal muscle growth slightly precedes rib elongation, thus underlining the role of intercostal muscle anlagen as a signalling centre

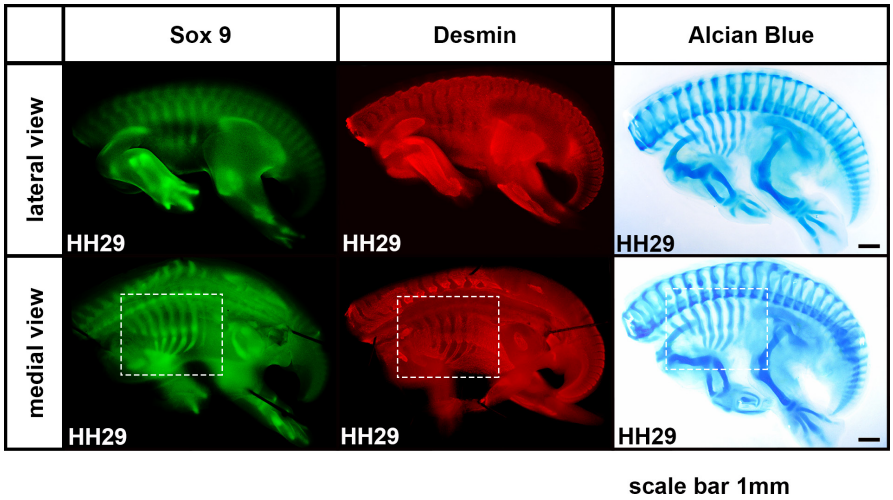


FIGURE 3 Overview of the embryo preparations and stainings used to monitor thorax development. The upper series shows a HH-stage 29 chicken embryo after removal of the head and neck and cut in half in the paramedian plane in lateral view, which is the most commonly used perspective in vertebrate embryology. The lower series shows a likewise bisected HH-stage 29 chicken embryo in medial view after removal of the viscera, which is less familiar but better shows the ribs as well as the intercostal muscles as they are not concealed by the limbs and limb-associated musculature. This medial view is used in the following figures in this work. Dorsal is to the top and cranial is to the left. Sox9 (green) stains the cartilage lineage, Desmin (red) stains the muscle lineage and Alcian blue (blue) stains the differentiating cartilage. The frame in D–F shows the area of the thorax shown in Figures 4–6. Scale bar 1 mm.

regulating rib development. Unexpectedly, we discovered a temporary eighth rib in almost half of the embryos examined, which has so far not been described and does not persist to adulthood.

2 | METHODS

2.1 | Embryos

For all experiments, fertilized chicken eggs (*Gallus gallus* domesticus, White Leghorn) provided by academic and commercial breeders (Lehr- und Forschungsstation Gut Frankenforst, Agricultural Faculty, University of Bonn; LSL, Dieburg) were incubated at 37.5°C and at 55% humidity until they reach the required age according to Hamburger and Hamilton (HH-stages) (Hamburger & Hamilton, 1951). Embryos were isolated, decapitated and cut in half in para-sagittal plane. To enable a medial view of the ribs, all internal organs were carefully removed and only the body wall was preserved.

2.2 | Alcian blue staining

Embryos of HH-stages 25–37 were isolated, prepared as described above, fixed in 70% ethanol in 1xPBS buffer overnight at 4°C, dehydrated in a rising alcohol series and incubated in 99.9% acetone overnight at 4°C. Depending on the HH-stages, the specimens were stained with Alcian blue solution (0.02% Alcian Blue 8GX [Sigma], 70% ethanol, and 30% acetic acid) for 3–6 h. Embryos were rehydrated in a descending ethanol series and cleared with 1% potassium hydroxide in H_2O for 1.5 to 5 h depending on the stages examined. We used glycerol (100%) as a fixation medium. Specimens were examined and photographed using a Leica MZ16F stereomicroscope.

2.3 | Double immunohistochemistry

Embryos of HH-stages 24–30 were isolated, prepared as described above, fixed in 4% paraformaldehyde at 4°C overnight, washed twice in 1xPBS, dehydrated in methanol and stored at −20°C. After rehydration with 1xPBS, the embryos were permeabilized with 5 mg/ml hyaluronidase (Sigma) for 2 h at 37°C, blocked for 4 h with blocking solution (10% donkey serum and 10% goat serum in 0,1% PBST) at 37°C and incubated for 2 days with primary antibody solution at 4°C (mouse anti-Desmin, Invitrogen MA5-13259, dilution 1:250; rabbit anti-Sox9, Sigma HPA001758, dilution 1:150).

After washing in 1xPBS, the embryos were incubated with secondary antibodies (donkey anti-mouse AF568 IgG (H + L), Invitrogen A10037, dilution 1:500, or donkey anti-rabbit AF568 IgG (H + L), Life technologies A10042, dilution 1:500; combined with goat anti-rabbit AF488 IgG (H + L) Invitrogen A1108, dilution 1:500, or goat anti-mouse IgG DyLight™ 488, Invitrogen, SA241239, dilution1:500, in 1% BSA in 0,1% PBST) for 2 days and washed again in 1xPBS. Finally, the embryos were again fixed with 4% paraformaldehyde in 1xPBS buffer solution and stored at 4°C. Specimens were examined and photographed using a Leica MZ16F stereomicroscope.

2.4 | Injection of vital dyes in ovo

Eggs containing embryos at HH-stages 17–18 were windowed and injected with India ink (Rotring, diluted 1:10 with 1xPBS buffer) into

the yolk underneath the embryo for visualization. Depending on the stage of the embryo, a series of 5–8 somites were injected in the somitocoel or underneath the dermomyotome with a cell-labelling solution (DID´ in DMF [2 mM], Invitrogen D7757) using injection needles made from borosilicate glass capillaries (O.D. 1.5 mm; I.D. 1.10 mm; Science Products GmbH) drawn on a Sutter P-97 Puller. Thus, both sclerotome and dermomyotome/myotome cells were labelled.

The injected embryos were covered with 1–1,5 ml 1xPBS, the egg window closed with adhesive tape and reincubated until they had reached the desired stage. Embryos at HH-stages 24–30 were isolated, prepared as described above and fixed with 4% paraformaldehyde. Specimens were examined and photographed using a Leica Thunder stereomicroscope. Where applicable, after the cell labelling procedure embryos were additionally subject to immunohistochemistry as described above.

3 | RESULTS

3.1 | Morphogenesis of the ribs

To revisit the morphogenesis of the rib cage in the chicken embryo, we performed Alcian blue cartilage stainings of embryos from HH-stage 25 (E.5) to HH-stage 37 (E.11) (Figure 4). Alcian blue stains glycosaminoglycans like hyaluronan and chondroitin sulphate in the developing cartilage (Steedman, 1950).

At HH-stage 25, the vertebral bodies are visible without clear segmental separation by intervertebral discs. The vertebral arches and the costal processes have formed as dorsal and ventral extensions of the vertebrae, respectively. Costal processes are visible in every segment. In the thorax, the costal processes represent the vertebral part of the ribs.

At HH-stage 26, Alcian blue-negative intervertebral disc anlagen separate the series of vertebral bodies. The costal processes of the last cervical vertebrae and all seven thoracic vertebrae considerably increased in length and thickness. Also caudal to the seventh rib, in the first lumbal segment, a costal process is faintly visible. The prospective sternal parts of the ribs in Th3–7 have still not yet formed but are indicated by thin stripes of staining. The anlage of the scapula is visible anterior to the thorax and extends caudally unto the first rib.

At HH-stage 27, the sternal ribs of Th3 to Th6 appear as thick, bud-like condensations. They are incompletely separated from the vertebral ribs by an apparent constriction of the rib anlage. The stripe-like staining presaging the sternal ribs in HH26 has disappeared. The sternal rib of the seventh rib is not yet visible. The costal process of the first lumbal segment is still discernible. At this stage, the sternum starts to feebly appear in the staining, with a gap to the distal tip of the sternal rib buds.

At HH-stage 28, the sternal rib anlagen have stretched from round buds to longitudinal, yet still short pieces of cartilage. The connection to the vertebral ribs shows only weak staining, indicating the conversion of cartilage to connective tissue at the prospective intercostal joints. The scapula does not extend further caudally and still reaches only to the first rib. The costal process of the first lumbal segment has disappeared in most cases, but as detailed below, persists in some cases to form a temporary eighth rib.

At HH-stage 30, the vertebral ribs have elongated markedly. The sternal ribs of Th3–Th6 have also lengthened considerably and developed to a similar thickness as the vertebral ribs. Both rib parts appear slightly curved convexly in caudal direction. The prospective intercostal joint is still visible but only weakly stained. In spite of the robust development of the sternal ribs of Th3–6, the sternal rib of Th7 is still not detectable. There is a clear gap between the distal tips of the sternal ribs and the sternal cartilage. The scapula now extends into the thoracic region up to the second or third rib.

At HH-stage 31 and 32, vertebral and sternal ribs form a marked angle of about 90° to 100° pointing caudally, and the formation of the sternal part of the seventh rib commences. The intercostal joints are now Alcian blue-negative, indicating their consistence of connecting tissue. The scapula extends caudally up to the fourth rib.

At HH-stage 33, the proximal ribs show the bifurcation of the proximal rib part in capitulum of the head and tubercle of the costal neck. In the late stage 33, the uncinate processes of the second to fifth ribs become visible as individual cartilaginous condensations which are separated from the vertebral ribs by a clear gap without staining. In contrast to the sternal ribs of Th3–6, the sternal rib of Th7 is still short and distant from the sternum. The sternum is now a well-defined cartilaginous structure with distinct craniolateral and caudolateral processes, and the *Facies articularis* at the position of the prospective costosternal joints. The prominent thoracic process of the sternum, which extends lateral to the sternal ribs in the adult, has not yet formed. The scapula extends caudally up to the fourth or fifth rib.

At HH-stages 34 and 35, the costal capitulum and tubercle have further taken shape and form a sharply delineated articular cleft with the vertebrae. The sternal part of the seventh rib has still not reached the sternum. The scapula extends up to the fifth rib.

At HH-stage 37, the sternal rib of Th7 has reached the sternum. In the central part of the vertebral ribs, the Alcian blue staining fades due to the onset of ossification. Apart from the uncinate processes, which still do not connect to the vertebral ribs, the adult morphology of the ribs and the costovertebral, intercostal and costosternal joints is largely reached. A rudimentary uncinate process may form at the sixth rib. The morphogenesis of the sternum is also largely completed, with the exception of the thoracic process. The scapula reaches its final craniocaudal extent up to the sixth rib.

3.2 | Temporal formation of an eighth rib

As mentioned above, we unexpectedly identified an eighth rib anlage caudal to the seventh rib, i.e. at the level of the first lumbal

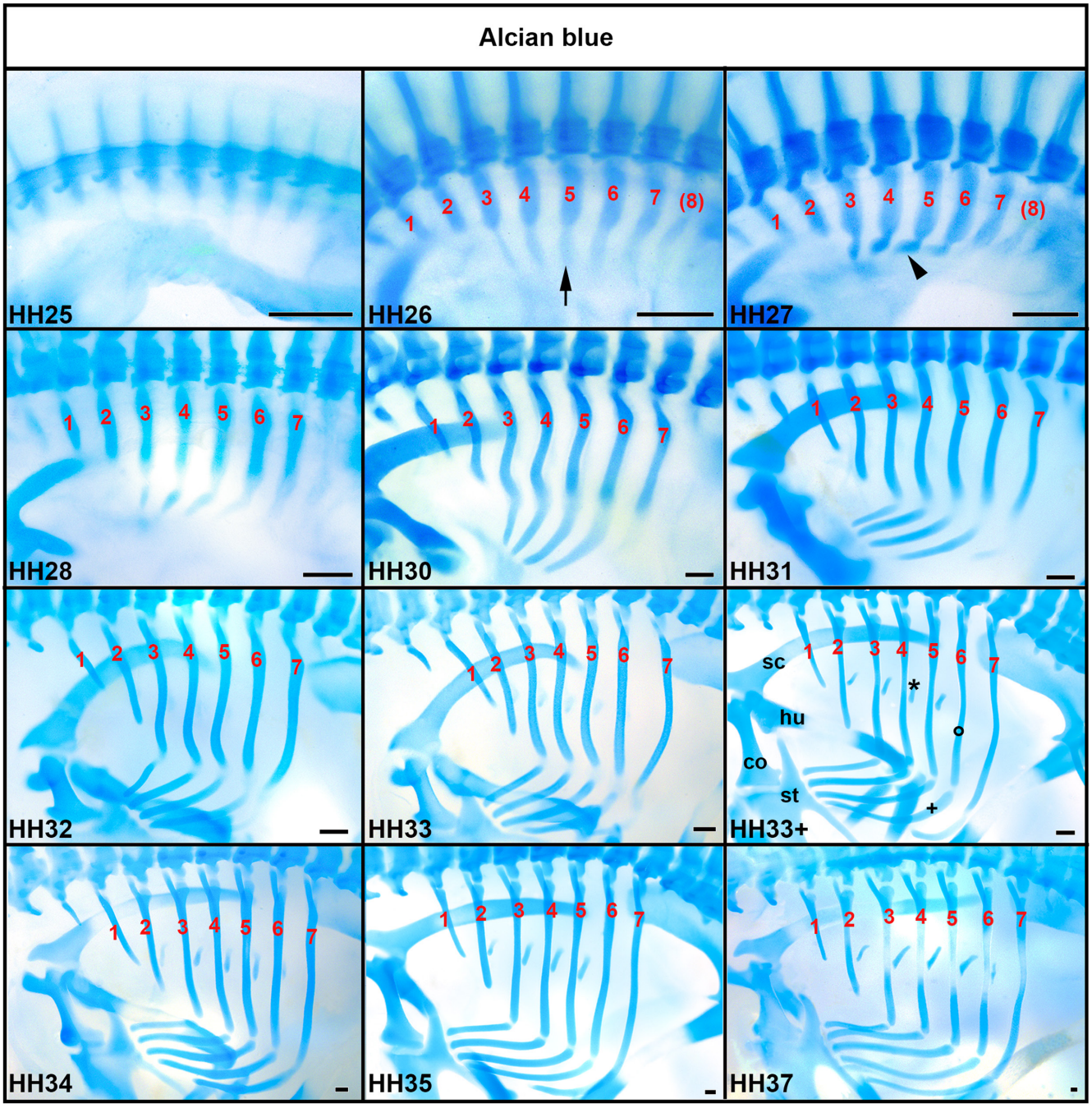


FIGURE 4 Alcian blue staining of the chicken thorax at HH-stages 25–37. Embryos have been cut in half and photographed in medial view. Dorsal is to the top and cranial is to the left. The ribs are numbered according to the thoracic segments in cranial-to-caudal sequence. HH26, arrow indicates an Alcian blue-positive, narrow stripe preceding sternal rib formation. HH27, arrowhead indicates an early, bud-like sternal rib anlage. HH33+, elements of the forming cartilaginous thorax are exemplarily labelled: Sc, scapula; hu, humerus; co, coracoid; st, sternum; ° vertebral rib; +, sternal rib; * uncinate process. Scale bar 500 μm.

segment, in almost half of the embryos examined (Figure 5, see also Figure S1 for variants of eighth ribs occurring from HH-stages 30–37). It is detectable as elongated costal process already at HH-stage 26, and appears around HH-stage 31 as a fine cartilaginous anlage much thinner than the regular ribs, lacking a sternal part in most cases. In one of the cases examined (n = 42), the eighth rib had a close-to-normal morphology including a sternal rib (see Figure 5 at HH34), and in some cases, the eighth rib was very thin and split in two fragments (see Figure S1, HH35+), which likely illustrates the process of degeneration of the eighth rib at later stages. We did not examine embryos beyond HH-stage 37. Nevertheless, we strongly suggest that the eighth rib degenerates at older stages. To our knowledge the formation of an eighth rib in adult chicken anatomy, even as a variant, has not yet been described in the literature. When quantifying the occurrence of an eighth rib at stages in which the eighth rib is best visible (HH-stages 31 to 35), we found an eighth rib

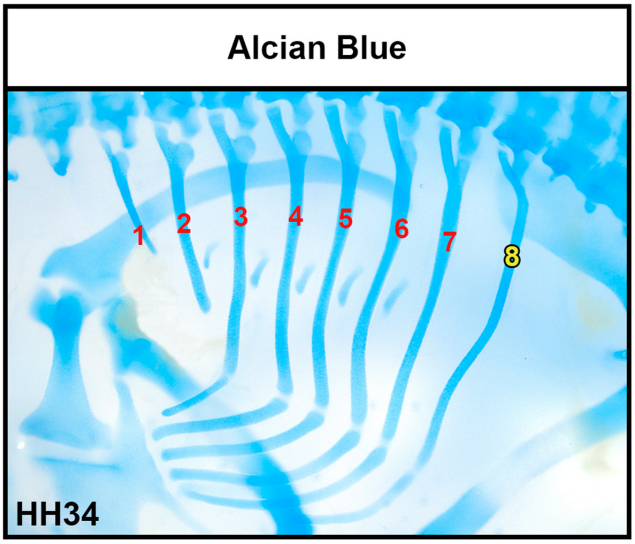


FIGURE 5 Alcian blue staining of the chicken thorax at HH-stage 34 showing a specimen with a well-formed eighth rib. Embryo has been cut in half and photographed in medial view. Dorsal is to the top, cranial is to the left. The ribs are numbered according to the thoracic segments in cranial-to-caudal sequence. The specimen here shows an eighth rib including both the vertebral and sternal rib parts. For an overview of specimens with eighth ribs with variable morphology, see Figure S1.

in 18 of a total of 42 specimens examined by Alcian blue staining, i.e. in 40% of the cases.

3.3 | Rib and intercostal muscle development

Both ribs and intercostal muscles originate from the somites. They form by ventrolateral extension of the lateral sclerotomes and the hypaxial myotomes, respectively, and grow into the somatic lateral plate mesoderm, the somatopleure.

In order to monitor the development of the ribs and the intercostal muscles synchronously in the same embryo, we double-stained embryos from HH-stage 24 to HH-stage 30 with antibodies against Sox9 and Desmin (Figure 6). Sox9 is a marker of the cartilage lineage already in the sclerotome and up to cartilage differentiation (Akiyama et al., 2002). Desmin is a marker of the muscle lineage and is found in early somitic muscle precursor cells as well as in differentiated muscle fibres (Kaehn et al., 1988, Lazarides and Balzer, 1978). We isolated the thoracic body wall of embryos cut in half in the median plane, and monitored both markers in medial view so that the limb-derived musculature did not conceal the rib cage. We were especially interested to investigate whether both cell populations extend simultaneously, or whether one of the two somite-derived cell populations grows in advance of the other.

At HH-stage 24, the Sox9-positive lateral sclerotomes have formed short ventrolateral extensions, the costal processes. The costal processes at thoracic levels are still of similar length as those of the adjacent cervical and lumbar regions. In between the rib anlagen, the hypaxial myotomes appear as Desmin positive, strictly segmental muscular stripes that extend from the somites in ventrolateral direction. The muscle anlagen extended farther ventrally than the rib anlagen, preceding the costal processes clearly in growth.

At HH-stage 25, the same pattern persists, but both rib and intercostal muscle anlagen have visibly elongated.

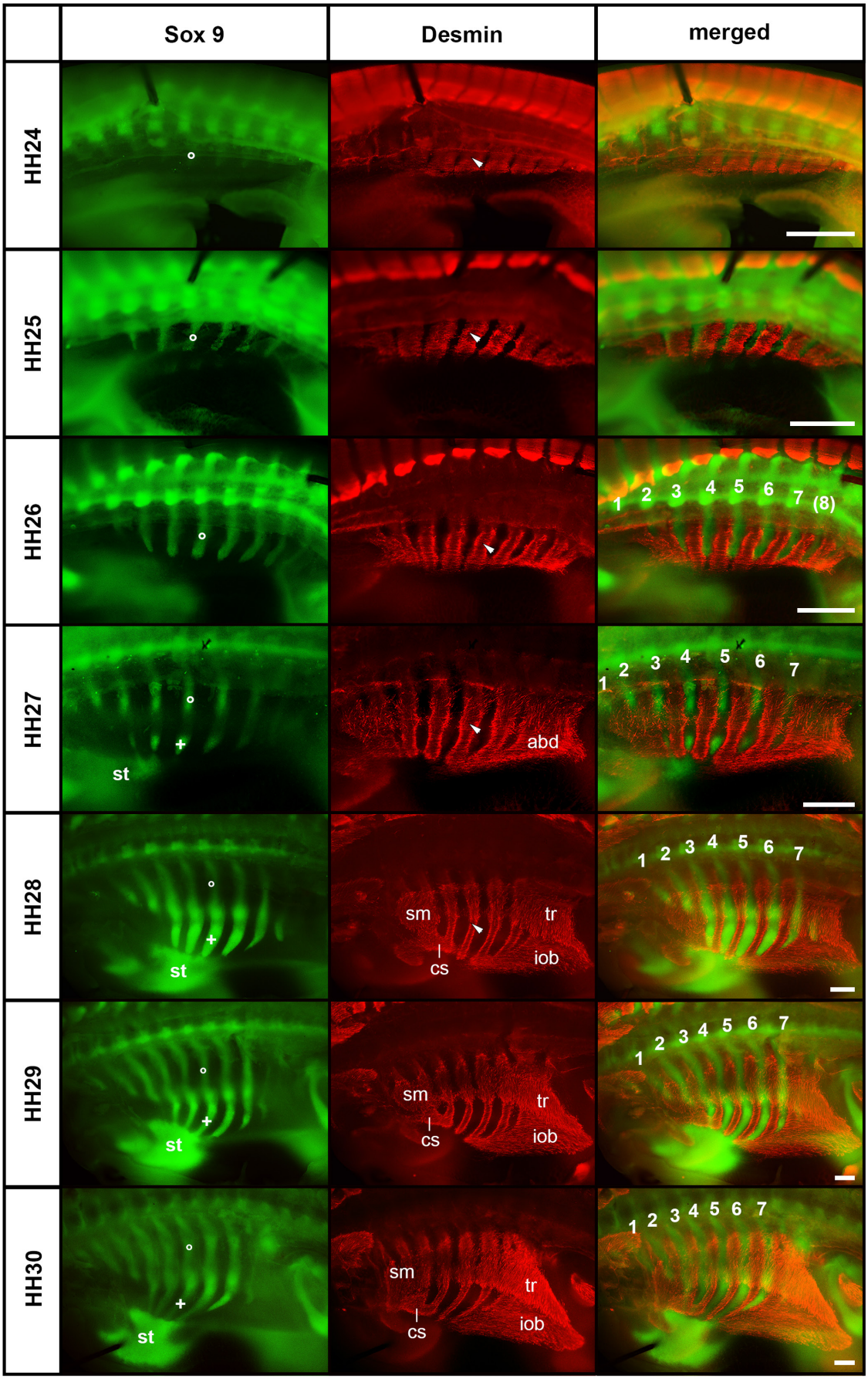
At HH-stage 26, the muscle anlagen are still extending further ventrally than the adjacent rib anlagen. Distal to the tips of the costal processes, some myotomal cells have started to extend longitudinally to the neighbouring segments, thus bridging the prospective rib area. At this stage, the Sox9-positive rib anlagen represent the vertebral rib parts; the sternal rib parts are not yet visible.

At HH-stage 27, the rib anlagen still lag behind the growing intercostal muscles. In the caudal part of the thorax, segment-overspanning muscle fibres are visible at the distal tips of the intercostal spaces, which are cranial extensions of the abdominal musculature. The sternal rib anlagen are now visible as short, oval structures at a clear distance to the Sox9-positive sternal cartilage. Between vertebral and sternal ribs, a zone of minor Sox9 staining represents the future intercostal joint.

At HH-stage 28, the caudally pointing angle between both rib parts starts to form. The gap between the sternal ribs and the sternum is narrow but still distinct. Concomitant with the arrival of the intercostal muscles at the sternum, the ribs catch up and reach the same length. Growing in from caudal and dorsal, the growing abdominal muscle sheets of the internal oblique and transversal muscles increasingly cover the ribs in the caudal part of the thorax. A muscle blastema with rather irregular, largely dorsoventral fibre orientation appears at the level of Th1 and Th2. We speculate that this muscle blastema will give rise to the scalenus muscle, which is not yet distinguishable at the stages examined. The intercostal muscles between the sternal ribs start crossing the ribs and attaching to the sternum, thus forming the costosternal muscle anlage.

At HH-stage 29, the sternal ribs Th3–5 have approached the sternum very closely, without visible articular clefts, while Th6 and Th7 still end freely in the body wall. Caudal to Th7, in about 40% of the embryos, the rudiment of an eighth rib is discernible as a very faint stripe of Sox9 expression. From caudal, the internal oblique abdominal muscle is inserted at Th7 and the distal parts of Th5 and Th6. From dorsal, the transverse abdominal muscle is growing ventrally along the vertebral part of Th5–Th7.

At HH-stage 30, neither the sternal rib of Th7 nor the rudimentary eighth rib have reached the sternum. With respect to the musculature, the situation is similar to HH-stage 29, with the intercostal muscles reaching the sternum, the abdominal muscles contacting and partially covering the ribs from dorsal and caudal, and the putative anlage of the scalenus, levator costarum and costosternal muscles covering the first two ribs in the anterior part of the thorax. The anlage of the costosternal muscle now extends further caudally and reaches the sternal part of the third rib.


Sox 9
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1 2 3 4 5 6 7 (8)
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FIGURE 6 Double whole-mount immunohistochemical stainings of the chicken thorax of HH-stage 24–30 using antibodies against Sox9 (green, left column) and Desmin (red, middle column) and overlays of both markers (right column). Dorsal is to the top and cranial is to the left, medial view. Sox9 is a marker of the chondrogenic lineage labelling the ribs, and Desmin is a marker of the myogenic lineage labelling musculature. Rib anlagen are numbered from Th1 to Th7. Arrowhead, intercostal muscle anlage. ° costal process and vertebral rib. + sternal rib. Abd, abdominal musculature before separation in anatomical muscle anlagen. Cs, costosternal muscle anlage; Iob, internal oblique abdominal muscle; Sm, putative anlage of the scalenus muscle; St, sternum; Tr, transverse abdominal muscle. Scale bar 500 μm.

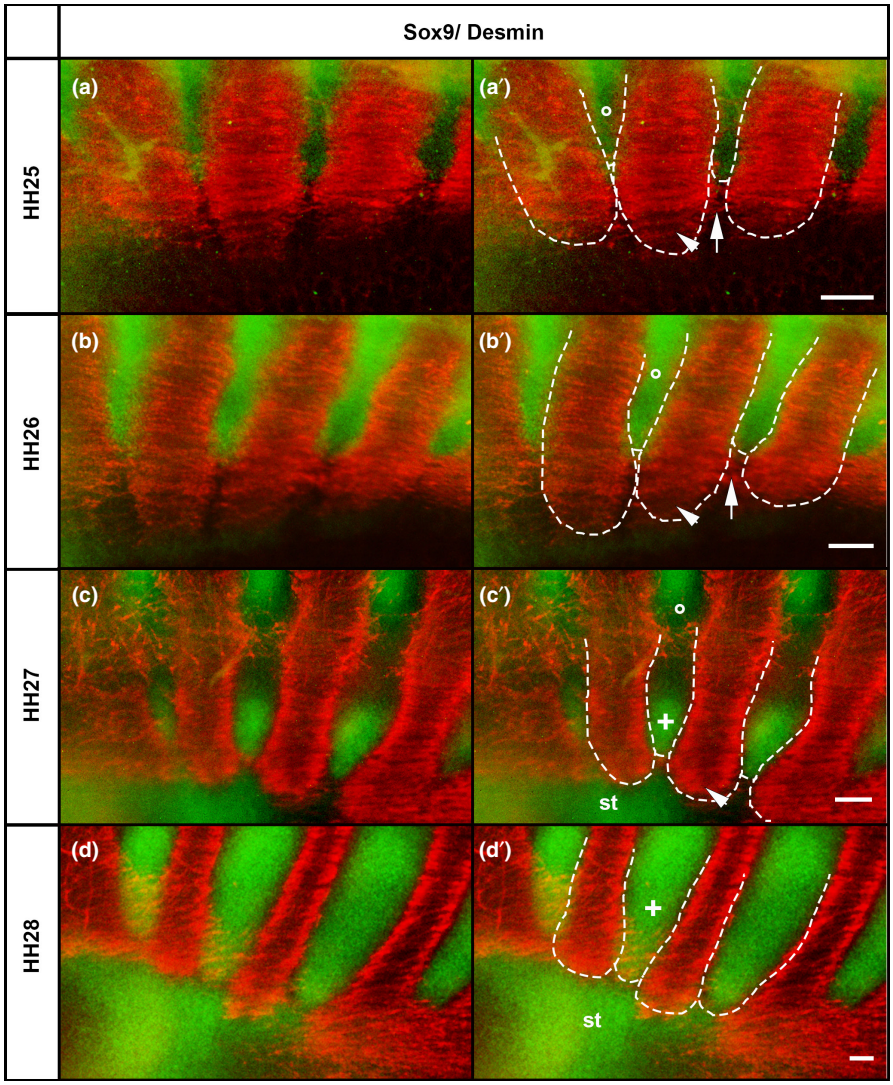

FIGURE 7 Close-ups showing the differentiation front of ribs and intercostal muscles in the ventrolateral body wall. Double whole-mount immunohistochemical stainings of the chicken thorax of HH-stages 25–28 using antibodies against Sox9 (green) and Desmin (red) in an overlay of both markers in the same embryo. Dorsal is to the top and cranial is to the left, medial view. The left column (a–d) shows the original stainings, and the right column (a' to d′) shows the identical specimens, with the outline of the rib and intercostal muscle anlagen drawn in for clarity. Note that the muscle anlagen extend more ventrally than the rib anlagen (a–c), and reach the same ventral level only at HH-stage 28 when both anlagen have reached the sternum (d). Arrowhead, distal tip of intercostal muscle anlagen showing loose and variable muscle fibres. Arrow, muscle fibres extending into the domain of the prospective rib anlage. °, costal process and vertebral rib anlage. +, sternal rib anlage. St, sternal anlage. Scale bar 100 μm.

3.4 | Dynamics of the ventrolateral differentiation front of ribs and intercostal muscles

In the description of rib and intercostal muscle development above, it became clear that during the extension of both anlagen into the lateral plate mesoderm, the muscle anlagen grow in advance of the rib anlagen. To investigate this in more detail, we observed the growth front of both myogenic and chondrogenic somite derivatives at a higher resolution (Figure 7).

At HH-stage 25, the hypaxial myotomes are strictly segmental, regular strands of parallel muscle fibres. At their distal tips, they show fibres of shorter length which are less densely packed

compared to more proximal levels. The Sox9-positive rib anlagen are thin and positioned in between the myotomes, ending with their distal tips clearly proximal to the tips of the muscle anlagen. The contact zone between myotomes and costal processes does not show a clear border between both cell populations, indicating their early differentiation state. At the distal region of the myotomes, which is not in contact with the rib anlagen, individual muscle fibres grow beyond the previous myotomal segment borders and extend longitudinally into the prospective rib region.

At HH-stage 26, more muscle fibres of the myotomal tips extend into the prospective rib region, so that the myotomal tips appear broader in width than the more proximal parts. In the distalmost part, the fibre arrangement is even less regular than at earlier stages, including short fibres which have obviously not yet extended to their definitive segment spanning length.

At HH-stage 27, the myotomal tips extend slightly beyond the sternal rib anlagen, and show an increased width distal to the latter. Distal to the cranialmost ribs, the sternal anlage appears as diffuse, Sox9-positive zone.

At HH-stage 28, the distal tips of both muscle and rib anlagen are at approximately the same level, as they both have reached the sternal anlage. The contact zone between muscles and rib anlagen is sharply delineated, indicating the advanced differentiation state of muscle and cartilage zones and the prospective muscle attachment to the ribs.

In addition to the Desmin–Sox9 double stainings, we performed DiD vital dye labelling experiments in which all somite cells including both, muscle and cartilage precursor cells, were labelled to be identified in the somatopleural environment (Figure 8). In HH-stage 26 embryos, we observed stripes with no or very few somite-derived cells in between the distal tips of the myotomes and distal to the Sox9-positive rib anlagen. These stripes co-localize with the Alcian blue-positive stripes preceding the sternal ribs mentioned above (Figure 4, HH26).

4 | DISCUSSION

The developmental mechanisms leading to the formation of the rib cage and its associate musculature are of clinical interest with respect to thoracic malformations (Al-Qadi, 2018; Canavese & Dimeglio, 2013), and are a model system for basic research on the molecular interactions of skeletal and muscular tissues during embryogenesis (Galea et al., 2021). Nevertheless, a comprehensive description of thorax development has been lacking in the literature. Here, we describe the concurrent development of ribs and intercostal muscles in the chicken embryo from HH-stage 24 (embryonic day 4) up to HH-stage 37 (embryonic day 11). Our data show that the intercostal muscles develop in advance of the adjacent rib anlagen during ventrolateral extension. We furthermore found the variable and temporal presence of an as-yet undiscovered eighth rib during thorax development.

4.1 | Rib development

In order to monitor the morphogenesis of the rib cage, we performed Alcian blue stainings of chicken embryos at consecutive stages from the formation of costal processes to the completion of the definitive rib morphology (Figure 4). Alcian blue is a well-known staining method for cartilage, staining glycosaminoglycans of the cartilage extracellular matrix and thus marking the chondroprogenitor stage of chondrogenesis as well as the differentiated cartilage (Scott, 1970; Scott & Mowry, 1970; Steedman, 1950).

4.1.1 | Morphogenesis of the ribs

According to Romanoff (Romanoff, 1960), who is referring to classical earlier work (Fell, 1939; Gladstone & Wakeley, 1932; Knopfli, 1919), the ribs, as well as the costal processes at thoracic and cervical level, become morphologically visible during E6 (HH-stage 28–29), when they consist of “early precartilage”. This is considerably later than our earliest detection of Alcian blue-stained costal processes (HH-stage 25) and Sox9-stained costal processes (HH-stage 24). This discrepancy might be due to the different cartilage detection approaches, as Knopfli (Knopfli, 1917; Knopfli, 1919) was using histological analysis or methyl green staining to identify

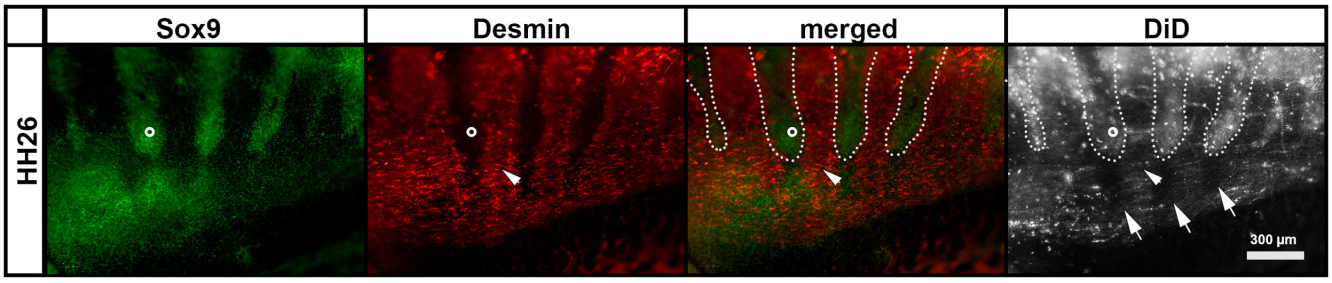

FIGURE 8 Triple stainings of the differentiation front of ribs and intercostal muscles in the ventrolateral body wall of a HH-stage 26 chicken embryo using antibodies against Sox9 (green) and Desmin (red), as well as DiD vital dye labelling of all somite cells. Dorsal is to the top and cranial is to the left, medial view. Note the stripe-like areas distal to the rib anlagen and in between the muscle anlagen, which contain no or few somite cells (arrows), indicating growth corridors of the rib anlagen in a somatopleural environment. Arrowhead, Desmin-positive intercostal muscle anlage. °, Sox9-positive rib anlage.

the ribs. Moreover, these data are difficult to correlate as the notion of "early precartilage" used by Romanoff (Romanoff, 1960) is not specified in molecular terms. In the thorax, at HH-stages 25 and 26, the costal processes are continuous with the vertebrae and correspond to the anlagen of the vertebral part of the ribs. This is in line with Knopfli (Knopfli, 1917), who pointed out the continuity of developing vertebrae and ribs and thus justly argued for a sclerotomal origin of the ribs. Distal to the vertebral ribs, presaging the location of the prospective sternal ribs, thin stripes of Alcian blue stainings are discernible. As these stripes do not appear in Sox9 stainings, we interpret them as migratory corridors for the sternal rib precursor cells containing extracellular matrix GAGs like hyaluronan, which is stained by Alcian blue, rather than proper sternal rib anlagen. Our vital dye stainings of somites (Figure 8) reveal that these putative migratory corridors are largely devoid of somite cells, thus confirming this hypothesis, notwithstanding the presence of somatopleural cells in the corridors. At HH-stage 27, in agreement with Romanoff (Romanoff, 1960), the sternal ribs first appear as oval structures which are separated from the vertebral part of the rib by a constriction of the rib anlage. They are separated from the ventrolaterally adjacent sternal anlage by a thin cleft. In the following stages, the sternal rib anlage stretches ventrally to acquire the longitudinal form of the definitive sternal rib. According to Romanoff (Romanoff, 1960), and in agreement with our results, proper chondrification starts at E7 (HH-stage 30–31) in the vertebral rib part and slightly later in the sternal rib part. At that stage, both parts of the rib start to extend caudally at their junction to form an obtuse angle opening in cranial direction.

The articulations between both rib parts (intercostal joint) and between rib and sternum (sternocostal joint) form at approximately the same time as the joints between vertebrae and ribs (costovertebral joints). The intercostal joint has been inconsistently characterized in the literature. Whereas some textbooks call it *Synchondrosis intercostalis* (König, 2001), thus inferring a cartilaginous synarthrosis, other authors assume a syndesmotic joint composed of connective tissue (Romanoff, 1960). As the embryos used in our study did not reach sufficient age to decide on this question, we need to leave it open here. In addition, the mode of intracostal joint formation is controversial in classical literature. Whereas Lutz (Lutz, 1942) held that the connective tissue of the joints arises directly from mesenchyme without passing a cartilaginous stage, Knopfli (Knopfli, 1917) stated that both rib parts first form a solid cartilaginous structure, and intercostal joints form only secondarily. Our Alcian blue data argue for the latter model, as we observe Alcian blue staining throughout both rib parts up to HH-stage 29 even though the prospective joint region is thinner and less intensely stained than the skeletal elements. Sox9 as a marker for sclerotomal derivatives, which is also expressed in precartilage tissue, is less clear in this respect, as there is a marked downregulation of Sox9 staining in the region of the prospective intercostal joints at HH-stage 27, whereas at HH-stage 28 both rib parts form a continuous Sox9 domain again (Figure 6).

The uncinate processes, according to Romanoff (Romanoff, 1960), form as separate cellular condensations slightly more dorsal than their definitive location. They chondrify and fuse to the vertebral ribs at E8 or E9, thereby reaching their definitive location by ventrolateral displacement. In line with this, we identify individual Alcian blue-positive uncinate process anlagen from HH-stage 33, i.e. E7-8, onwards, but our data argue for later fusion to the vertebral ribs as at HH-stage 37, i.e. E.11, the uncinate processes are still clearly separate (Figure 4).

Ossification of the ribs is not studied here, but has been reported to occur separately in the vertebral and sternal parts starting between E10 (i.e. HH-stage 36) and E12 (i.e. HH-stage 38). Ossification starts in the vertebral rib shaft, continues in the costal neck region and, finally, around E16 (i.e. HH-stage 42), reaches the sternal rib. This is in agreement with our observation that at HH-stage 37, Alcian blue-reduced zones appear in the centre of both rib anlagen, which correspond to the forming osseous foci in the ribs. At hatching, the largest part of the proper ribs is ossified, whereas ossification of the uncinate processes occurs postnatally (Romanoff, 1960).

4.1.2 | Temporal formation of an eighth rib

An unexpected yet very interesting observation in our experiments is the variable occurrence of an eighth rib (Figures 5 and Figure S1). The existence of this rib has not been reported in the literature so far. It is a temporary structure and is found in ca. 40% of the chicken embryos. It becomes first visible at HH-stage 26 as a long costal process, is best developed around HH-stage 34 and is visible in some cases at least up to HH-stage 37. In the vast majority of the embryos examined, the eighth rib is a very delicate and thin floating rib consisting only of the vertebral rib part, whereas we found only one specimen which also developed a sternal rib. However, although rare, this means that the first lumbal segment has the potential to form a complete rib. The ephemeral and delicate nature of the eighth rib is obviously the reason why it has not yet been identified so far. It is likely that this eighth rib is a rudiment of ancestral galliform birds which might have had more ribs than the extant chicken. The number of ribs in birds is quite variable, with true ribs ranging from three in some pigeon species to nine in swans, and floating ribs in variable numbers (Stresemann, 1934). Lutz (1942) reports that in embryos of the Emu, which have three presternal floating ribs, four true ribs and three poststernal floating ribs, there are additional mesenchymal rib anlagen visible cranial and caudal to these which do not reach the cartilaginous stage, and which disappear during later development. This illustrates the evolutionary and developmental flexibility of the thoracic bauplan in birds, which may account for the eighth rib rudiment in chicken.

As the corresponding vertebra of the eighth rib in chicken is the first lumbar vertebra, this indicates a morphological shift from thoracic to lumbar identity of this segment both in ontogeny and phylogeny. Interestingly, the seventh thoracic vertebra, as all lumbar and

sacral vertebrae and some caudal vertebrae, is fused to the synsacrum, which consists of a slightly variable total number of fused vertebrae ranging from 15– to 16 segments. Thus, the morphological attribution of synsacral vertebrae to thoracic, lumbar, sacral and caudal vertebrae is anatomically difficult, which opens the possibility that the first lumbar vertebra has emerged from an ancestral eighth thoracic vertebra during evolution. The molecular basis for the development of an eighth rib is difficult to explain. The segmental identity of thoracic and lumbar vertebrae is determined by the expression of Hox genes. In both chicken and mouse embryos, the anterior border of *Hoxc6* expression marks the cervico-thoracic transition, and the anterior border of the expression of *Hox9 a,b,c* paralogues marks the thoraco-lumbar transition. The thoraco-lumbar transition in chicken has been identified at the level of somite 26 (Burke et al., 1995). Genetic experiments in the mouse have shown that rib formation in the thorax is promoted by *Hox6* genes, and is blocked by *Hox10* genes at lumbar levels. Additional ribs in lumbar segments have been observed in mice overexpressing Hoxc6 (Vinagre et al., 2010), in quadruple mutants of Hox 9 (McIntyre et al., 2007) and in triple mutants of Hox10 (Wellik & Capecchi, 2003), the latter developing lumbar ribs of a relatively normal phenotype. In animals that secondarily develop ribs at lumbosacral levels, like snakes, the rib-inhibiting activity of Hox10 is suppressed by a modified Myf5 enhancer which does not allow binding of Hox10, but still allows binding of rib-promoting Hox6 and Pax3 (Guerreiro et al., 2013; Woltering et al., 2009). To date, no such mechanism has been described in the chicken embryo, and the ephemerality of the eighth rib remains obscure. An explanation of the temporary formation of the eighth rib in chicken, therefore, awaits respective molecular investigations.

4.2 | Intercostal muscle growth precedes rib growth

Our double-staining experiments, which were done to visualize the concurrent differentiation of ribs and intercostal muscles in the same embryo, clearly show that in the early phase of thorax development, up to HH-stage 27, the muscle anlagen grow in advance of the rib anlagen (Figures 6, 7). Both anlagen, rib and musculature, were stained with early markers, the expression of which starts as early as in the sclerotomal and myotomal compartment of the somites, respectively, and persists well up to cartilage and muscle differentiation. Due to the early onset of expression of both markers, it is valid to conclude that they duly illustrate the respective lineages in their position relative to each other. In confirmation of this, we found the intermyotomal spaces distal to the rib anlagen largely free of somite cells (Figure 8), indicating that they are corridors for the elongation of the Sox9-positive rib anlagen. Recent cell labelling studies have shown that the growing sternal rib anlagen are accompanied by dynamic populations of somatopleural cells (Sakamoto et al., 2022), indicating that these corridors are not cell-free regions, but that the invading somite cells rather supplant the somatopleural cells during extension.

4.2.1 | Muscular signals are a prerequisite for rib growth

Our findings are in agreement with molecular data showing that signals from the myotome are required for rib development, but not vice versa (Scaal, 2021). Proximal rib formation depends on Shh signalling from the notochord–floorplate complex (Aoyama et al., 2005; Chiang et al., 1996; Teillet et al., 1998) and requires expression of the paired box transcription factor Pax1 in the central sclerotome (Dietrich & Gruss, 1995; Wallin et al., 1994), thus being closely linked to vertebral development but independent of muscle development. In contrast, formation of the rib shaft of the vertebral and the sternal rib (i.e. the vertebro-distal and sterno-distal rib components according to Aoyama et al (Aoyama et al., 2005)) is independent of Pax1, but requires FGF8 and PDGF signalling from the myotomal muscle anlagen (Huang et al., 2003; Soriano, 1997; Tallquist et al., 2000). Consequently, mouse mutants showing hypaxial muscle defects like *Splotch* (*Pax3*) mutants (Dickman et al., 1999; Henderson et al., 1999), *Myf5* mutants (Braun et al., 1992) and *Six* mutants (Grifone et al., 2005; Laclef et al., 2003) also have severe defects in distal rib development. Hoxc6, which is determining thoracic identity along the body axis and thus rib development in the vertebrae, has been shown to act on a *Myf5/Myf6* enhancer (Vinagre et al., 2010), thus underlining the prime importance of muscle-derived signalling for rib development. Interestingly, proper rib formation does not only require early signalling from the myotome to the sclerotome, but also ongoing signalling from intercostal musculature to the ribs during later stages (Wood et al., 2020). Unlike the vertebral ribs, formation of the sternal ribs additionally requires BMP signalling from the lateral plate mesoderm (Sudo et al., 2001). Our data show that Desmin-positive myotomal muscle anlagen develop in advance of the Sox9-positive rib anlagen, thus, as it were, paving the way for the following sclerotomal cells which will differentiate to cartilage. We hypothesize that this temporal delay allows the myotomal cells to operate the signalling cascades leading to FGF secretion, thus creating the required molecular environment necessary for rib cartilage formation.

4.2.2 | Evolution of rib morphology by muscular cues?

This piloting role of the myotome in rib development is interesting with respect to the still largely unknown mechanisms of rib cage morphogenesis. Ribs show many variations not only in their three-dimensional anatomical form between taxa but also within the same organism at different segmental levels. If rib development follows intercostal muscle development, with the ribs growing in the space between the hypaxial myotomal muscle masses, signals shaping the thorax at early stages likely act on the musculature, rather than directly on the rib anlagen. This concept is in line with the findings of Vinagre et al. (2010) on the rib-inducing activity of Hox genes acting via muscle regulatory elements. It is therefore tempting to

speculate that evolutionary changes in rib cage morphology are driven by modifications in the muscular rather than the skeletal thorax components.

Our data show that the space between the rib anlagen is filled with segmental intercostal muscles, both in vertebral and sternal rib parts. In adult chicken, however, the musculature at the level of the sternal ribs is represented by the segment-crossing costosternal muscles (Figures 2 and 6), whereas the sensu stricto intercostal musculature is limited to the vertebral rib part. The cellular and molecular basis of this differential muscle behaviour is unknown.

4.3 | Summary and perspectives

We provide an illustrated overview of the development of the thorax in the chicken embryo, with emphasis on the development of the ribs and the intercostal muscles. Our studies reveal the existence of a previously unrecognized eighth rib forming variably and temporarily in the first lumbar segment. We show that the myotome-derived intercostal muscles invade the ventrolateral body wall in advance of the sclerotome-derived ribs, thus supporting the concept of the regulation of rib development by intercostal muscles. Based on our data, new questions arise which await further studies. It will be interesting to decipher the molecular network responsible for the regulation of the coordinate yet offset growth of intercostal muscle and ribs, and to identify the molecular variables within this network leading to morphological differences between ribs at different segmental levels and between species. Another open question is how the segmental muscle anlagen between the sternal ribs are transformed, or replaced, to form the segment-crossing costosternal muscles. It will further be interesting to examine the genetic basis of the temporary formation of the eighth rib from the first lumbar vertebra with respect to Hox gene expression in the chicken. From an evolutionary point of view, it will be rewarding to examine whether the principle of muscle growth preceding rib growth is only the case in the dynamic rib cage of amniotes, or also in anamniote taxa which do not need rib excursions for breathing. To conclude, using the paradigm of rib and intercostal muscle development as a model system, further studies on the molecular interaction of muscle and skeleton may shed more light on the principles of morphogenesis both in embryonic development and in evolution.

AUTHOR CONTRIBUTIONS

Julia Khabyuk: acquisition of data, data analysis/interpretation, critical revision of the manuscript and approval of the article. Felicitas Pröls: data analysis/interpretation, critical revision of the manuscript and approval of the article. Margarethe Draga: acquisition of data, data analysis/interpretation, critical revision of the manuscript and approval of the article. Martin Scaal: concept/design, data analysis/interpretation, drafting of the manuscript, critical revision of the manuscript and approval of the article.

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DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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