Expression Pattern of Axin2 During Chicken Development

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Abstract

Canonical Wnt-signalling is well understood and has been extensively described in many developmental processes. The regulation of this signalling pathway is of outstanding relevance for proper development of the vertebrate and invertebrate embryo. Axin2 provides a negative-feedback-loop in the canonical Wnt-pathway, being a target gene and a negative regulator. Here we provide a detailed analysis of the expression pattern in the development of the chicken embryo. By performing in-situ hybridization on chicken embryos from stage HH 04+ to HH 32 we detected a temporally and spatially restricted dynamic expression of Axin2. In particular, data about the expression of Axin2 mRNA in early embryogenesis, somites, neural tube, limbs, kidney and eyes was obtained.

Introduction

Axin2 (also called Axil or Conductin) is a homologue of Axin protein. It shares approximately 45% of amino acids with Axin [1, 2]. The Axin protein houses four highly conserved functional domains throughout the species [3–5]. The N-terminal RGS-domain has been found to interact with the tumour suppressor gene adenomatous polyposis coli (APC) [1, 6]. Central in the Axin protein, binding sites for β-catenin and for the glycogen-synthase kinase 3 beta (GSK-3β) were described [4]. At the C-terminal region, the DIX-domain is located that resembles the sequence of a DIX—domain in dishevelled protein (Dvl) and promotes its interaction with Axin [7]. At its C-terminus, Axin also interacts with the protein phosphatase 2A (PP2A) [3, 8, 9]. Being responsible for the degradation of the downstream canonical Wnt-signalling pathway molecule β-catenin, Axin and Axin2 function as negative regulators of the canonical Wnt-signalling pathway [5, 10–12]. The Wnt-signalling pathway is one of the best elucidated signalling pathways. First, the canonical Wnt-pathway was described, followed by at least two non-canonical pathways. The pathway of planar cell polarity (PCP) and the Wnt/Ca⁺-pathway are referred to as non-canonical pathways [13–15]. These are described to establish orientation in epithelia (PCP) and to play a role in early embryonic ventral patterning (Wnt/Ca⁺-pathway) ([16] for review). Canonical and non-canonical Wnt-signalling are known to be enmeshed with each other, as their members partially contribute to more than one pathway [17, 18] and several Wnt ligands were described to activate both canonical and non-canonical pathways [19–22]. However, in this study, only the canonical pathway is of relevance, constituting a venue for the
Axin family [10]. Central to canonical Wnt-signalling stands the transcriptional activator β-catenin. When entering the nucleus, β-catenin can displace transcriptional repressors such as Groucho [23] from the TCF/LEF transcription factor, which now activates the transcription of target genes ([24] for review). In the unstimulated cell, a multi-protein complex mediates the degradation of β-catenin via the ubiquitin proteasome pathway [25, 26]. For this purpose, β-catenin is phosphorylated by one of the two kinases of the complex, GSK-3β or the casein kinase 1 (CK1). Axin acts as a central scaffold protein in the degradation complex by binding and thus bringing together all important components [3]. For this purpose, Axin and Axin2 contain highly conserved regions. GSK-3β phosphorylates β-catenin, which subsequently is ubiquitinated by the E3 ubiquitinase βTrCP and degraded by a proteasome [4, 27]. GSK-3β further phosphorylates Axin itself, leading to stabilization of its interaction with β-catenin [4]. APC and one of the relevant receptors in Wnt pathway, the low density lipoprotein related receptor LRP 5/6 are also known to be substrates of GSK-3β [28, 29]. Although GSK-3β is capable of phosphorylating β-catenin alone, Axin unites both proteins, facilitating and significantly accelerating this process [30]. Finally, this process represses β-catenin in the cytoplasm to a level that prohibits its access to the nucleus. In the presence of APC, the turnover of β-catenin increases [6, 31]. Previous studies suggest a role for APC in the recruitment of several β-catenin molecules to the environment of the destruction complex [5, 32, 33]. Upon arrival of Wnt-ligands, they bind to the seven-pass transmembrane receptor Frizzled and to its co-receptor, the low density lipoprotein related protein receptor (LRP) 5 or 6 [34–36]. This heterodimeric complex binds Dvl at the cytoplasmatic tail of Fz [37] and initiates the recruitment of Axin and kinases (Gsk3b or Ck1) to the membrane, mediating the dissociation of the β-catenin-destruction complex [29, 38, 39]. Several studies have been performed in order to elucidate the exact molecular scenario after Wnt-binding. For instance, Wnt was described to induce Dvl, that is thought to recruit Axin-bound GSK-3β to the membrane, where the latter phosphorylates LRP 5/6 and as a consequence dissociates from Axin [29, 40]. The phosphorylation of LRP 5/6 can equally be achieved by CK1 [39, 41]. Axin priorly was dephosphorylated by another member of the β-catenin degradation complex, the protein phosphatase 1 (PP1) [42, 43]. Unphosphorylated Axin releases β-catenin [30, 40] and easily binds to LRP 5/6. Binding of Axin to Dvl that is connected to the cytoplasmatic tail of Frizzled, is proposed to facilitate this initial recruitment [27]. Previous studies have further proposed a model for the formation of so called signalosomes, built from multiple associated LRP/Axin complexes [44]. As a result of the dissociation from the β-catenin-destruction complex, β-catenin is not degraded any more, cytoplasmatic levels rapidly rise and it enters the nucleus [37]. A special function for Axin2 was found, when discovering its transcriptional dependence on TCF/LEF motive [45]. Axin2 expression therefore is initiated by canonical Wnt-signalling and provides a negative-feedback loop [27, 37, 45]. As this study aims to emphasize the relevance of Axin2 in regulating the Wnt-signalling pathway, it is important to mention the state of the art regarding the role of canonical Wnt-pathway in development and in disease. During development, canonical Wnt-signalling is described to be required for proper posterior axis formation and for the formation of the head [46–48]. Moreover, Wnt-signalling is known to be indispensable in the developing central and peripheral nervous system [49]. It is described to be involved to the segmentation clock during somitogenesis [50] and in the development of several other structures and organs, such as the limbs, the kidney, the gastrointestinal system, the sensory organs and the lungs ([37, 49] for review). In the adult, deregulation of the Wnt pathway cause several cancers and Wnt-signalling is required for stem cell self renewal [51, 52]. The regulation of Wnt-signalling via Axin and Axin2 impacts embryonic development and health in the adult, as described by many studies. Axin mutant mice failed to survive [11, 53] and display severe developmental defects. Mice with homozygous mutations in Axin2 developed a secondary caudal body axis...
and exhibited malformations of the skull due to premature fusion of cranial structures [54]. This malformation is an equivalent to the human disease craniosynostosis, that is described to develop on the basis of \textit{Axin2} mutations [55]. Another developmental defect associated with \textit{Axin2} abnormalities in mice and human is familial tooth agenesis and oligodontia [56, 56, 57]. Further, Axin is related to hepatocellular cancer [58, 59], ovarian cancers [60] and to medulloblastomas [61]. \textit{Axin2} mutations play a secondary role in familial adenomatous polyposis coli (FAP), when the causal mutation is not situated in APC and because proper function of APC requires Axin [62, 63]. Predisposition to colorectal cancer, when carrying mutations in \textit{Axin2} is described [58, 63]. Shedding similar functions than Axin, \textit{Axin2} was previously tested on its functional redundancy [54]. \textit{Axin2} was shown to be able to at least partially compensate for mutated Axin when expressed in the respective cells. Axin however, is expressed in small amounts in all embryonic tissues, while \textit{Axin2} expression was described to be restricted and dynamic during mouse development [11, 45]. Interestingly, Axin was described to be the limiting factor in \textit{Wnt} regulation, referring to its low cytoplasmatic levels [64]. \textit{Axin2} on the other hand, is highly expressed, suggesting an extensive role for \textit{Axin2} regulation in certain tissues. This observation, together with the fact that \textit{Axin2} is a target of \textit{Wnt}-signalling, indicates the importance of \textit{Axin2} mediated negative regulation in certain tissues. In this study, we demonstrate the dynamic expression pattern of \textit{Axin2} in the development of the chick.

\section*{Materials and Methods}

\subsection*{Embryos}

Fertilized eggs of Gallus gallus domesticus were incubated at 37°C and 80\% relative humidity. Eggs were provided by a local breeder (Sörries-Trockels Vermehrungszucht). Staging was performed according to Hamburger and Hamilton [65].

The obtained chicken embryos were isolated, fixed in 4\% PFA for at least 24h. For description and analysis of the expression pattern of \textit{Axin2} during chicken development, chicken embryos in developmental stages HH 04 to HH 32 were proceeded in \textit{in-situ} hybridization.

\subsection*{Whole mount \textit{in-situ} hybridization}

Whole mount \textit{in-situ} hybridization was performed as previously described [66], using \textit{cAxin2} riboprobe for detection of \textit{Axin2} transcripts in all embryonic tissue. **Generation of a riboprobe for \textit{in-situ} hybridization.** The probe for \textit{cAxin2} \textit{in-situ} hybridization was generated from a pCMS-EGFP plasmid containing a full length \textit{Axin2} coding sequence. It was restricted using EcoRV and SmaI to obtain a 835bp fragment binding from bp926 to bp1788 on \textit{Axin2} mRNA (NCBI Reference Sequence: NC_006105.4). The purified fragment was blunted and cloned to pJET1.2/blunt Cloning Vector. From here, the fragment was excised using XbaI and XhoI and ligated to pBluescript II KS+ Vector. The obtained plasmid was suitable for generating a riboprobe in \textit{in vitro} transcription.

\subsection*{Sectioning}

**Vibratome-sections.** The embryos were embedded in 2, 5 – 4\% agarose gel and sectioned with Vibratome (Leica VT 1000 S) to 50–80\textmu m. Sections were collected and covered with cover slips and Aquatex (Merck).

**Cryo-sections.** \textit{In-situ} hybridized chicken embryos were embedded in Leica tissue freezing medium\textsuperscript{6} and frozen with liquid nitrogen. Obtained blocks were sectioned with Leica CM3050 S cryo-stat. Sections were collected on slides, dried and covered using Aquatex (Merck).
**Ethic statement.** According to German legislation, the use of embryonic vertebrates in an animal experiment needs approval only if the animal is in the last third of its embryonic development. In the case of chicken, this means that experiments done on animals before embryonic day 14 (E14) are not regarded as an animal experiment by the Tierschutzgesetz, and therefore, do not need approval or governmental permission.

The chicken embryos sacrificed for this work were between developmental stages HH+04 (E1) and HH32 (E7.5). All embryos were sacrificed at the end of the study by opening the shell and tearing the allantois and amnion with forceps. Thereafter, the embryos were immersed in 4% PFA/PBS solution for fixation. No permits were required for the described study, which complied with all relevant regulations.

**Results and discussion**

0.1 Expression pattern of cAxin2 during early chicken embryogenesis

After whole mount *in-situ* hybridization, a dynamic expression pattern of Axin2 was found from stages HH 04 to 32. In early embryogenesis, Axin2 expression was observed in the primitive streak (ps)\(^{(1)}\) (A black arrow, B, C, D) and in the Hensen's node (hn)\(^{(1)}\) (B red arrow, C red arrow, D, E). Additionally, the head fold (hf) heavily expresses Axin2 from stage HH 07 + onwards \(^{(1)}\) (B, C black arrows). During further development, in stage HH 10, Axin2 transcripts were detectable in the Hensen's node (hn), posterior presomitic mesoderm (psm) \(^{(1)}\) (E) and medially in the freshly segmented paraxial mesoderm (dml-dorso-medial lip) \(^{(1)}\) (E, E1 red arrow). Transversal sections were performed to analyse the expression of Axin2 during early embryogenesis in detail. They present gastrulation and neurulation processes, where the maturation can be observed in a cranial to caudal axis. The green bars in the whole mount specimens indicate the sectioning level. Sections of HH stage 08 \(^{(1)}\) (Fig 1, C.1, C.2, C.3) show the caudally regressing primitive streak (ps) with the primitive groove (pg). The primitive folds (pf) of the ectoderm and the developing mesoderm underlying the primitive groove (pg) express Axin2 \(^{(1)}\) (Fig 1, C.1, C.2, C.3). Further, the transversal section of the head fold (hf) in HH stage 08 (C.4) shows intense expression of Axin2 in medial parts, facing towards the lumen of the anterior neuropore. In HH 09, during the primary neurulation process, cranially to Hensen's node (hn) \(^{(1)}\) (Fig 1, D, D.1, D.2), only little Axin2 is expressed in the neural groove (ng) \(^{(1)}\) (Fig 1, D.1, D.2) and in the elongating notochord (nc) \(^{(1)}\) (Fig 1, D.1 and D.2). At this stage the head folds (hf) at mid-brain (mb) level have converged \(^{(1)}\) (Fig 1, D.3) and Axin2 expression is increased in the medial neural folds. In Fig 1, E.2, E.3, E.4 and E.5 (HH 10), the segmental plate mesoderm (spm) is formed, as the neural folds (nf) extend distally to form the neural tube (nt). Axin2 is expressed in the neural groove (ng) and in the notochord (nc) \(^{(1)}\) (Fig 1, E.4, E.5). At HH stage 10 more cranially, first somites (so) are shaped in the segmental plate mesoderm \(^{(1)}\) (Fig 1, E.2, E.3), as the neural folds (nf) fuse to form the neural tube (nt). In sections E.6 to E.8 \(^{(1)}\) (Fig 1), the development of the caudally shifted Hensen's node (hn) is depicted. Axin2 expression is restricted to the central Hensen's node \(^{(1)}\) (Fig 1, E.6, E.7, E.8) expanding towards the ventral axial mesoderm (am). In picture E.6 \(^{(1)}\) (Fig 1), the prechordal mesoderm (pcm) is heavily stained for Axin2.

By stage HH 11, the expression of Axin2 in the dorso-medial lip (dml) appears \(^{(1)}\) (A, A.1 black arrows). This expression intensifies as the somites mature \(^{(2)}\) (HH 14: B, black arrow and HH 15: C, C.1 black arrows). Additionally, the posterior neuropore (pnp) is intensively stained for Axin2 \(^{(2)}\) (A, B, C). Regarding the head of the depicted embryos in Fig 2, Axin2 expression is visible predominantly in the mid-brain (mb) \(^{(2)}\) (A, B.1, C white arrow). During secondary neurulation, which describes the elongation of the neural tube (nt) into the tail bud, Axin2 is expressed centrally in the tail bud mesoderm (tbm) \(^{(2)}\) (C.2, C.3, C.4) in...
Fig 1. Expression of cAxin2 in stages HH 04+ to HH 10. Overviews and transversal sections of chicken embryos. Green bars in overviews (C, D, E) indicate sectioning level. (A) HH 04+: Axin2 transcripts in ps (black arrow). (B) HH 07+: expression intensified in ps, hn(red arrow) and hf (black arrow). (C) HH 08: expression in hf (black arrow), hn (red arrow) and ps. (C.1) Axin2 expression in pf and pg. (C.2) intense staining in the pf. (C.3) expression thickened ectoderm as a first step of neurulation. (C.4) transcripts in the most medial inner epithelium of the hf. (D) HH 09: expression in hn, faintly in the psm and in the mb. (D.1, D.2) expression in the ng and in the nc. (D.3) strong expression in the medial layer of the hf. (E) HH 10: strong Axin2 expression in hn and psm, as in the mb. (E.1) higher magnification of the so and nt shows expression in the medial so, the dml. (E.2) faint staining in medio-dorsal epithelium of the early so and in the developing nt. (E.3) transcripts rarely detectable in the nf prior to closing. (E.4) expression of Axin2 in the nc and in the annealing nf. (E.5) expression in the centre of the nf and in the nc. (E.6, E.7, E.8) upheaval of the nf in the distal-most hn. (E.6) expression in the centre of the future nt and in the pcm. (E.7, E.8) transcripts in the folding neuroectoderm expanding towards the am. pe-primitive streak, hn-Hensen’s node, hf-head fold, pf-primitive folds, pg-primitive groove, psm-presomitic mesoderm, mb-mid-brain, ng-neural groove, nc-notochord, so-somites, nt-neural tube, nf-neural fold, spm-segmental plate mesoderm, pcm-prechordal mesoderm, am-axial mesoderm.

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HH 15 and ventrally in the recently formed secondary neural tube (snt) and secondary notochord (snc) (Fig 2, C.2).

In stage HH 14 at mid-brain level (Fig 2, B.1), the anterior neuropore has closed and Axin2 expression has shifted to a patch in the ventral mesoderm, flanking the mid-brain (mb) (Fig 2, B.1 black arrow). In HH stage 15, Axin2 expression is detectable in the developing sensory organs, eye and ear, for the first time (Fig 2, C.5). Axin2 mRNA was detected in the otic cup (oc) (Fig 2, C.5) and adjacent hind-brain (hb), as well as in the out-pocketing optic vesicle (ov) (Fig 2, C.5). The optic vesicle (ov) forms laterally from the prosencephalon, where Axin2 is transcribed in the medial wall.
Previous studies have investigated the role of Wnt-signalling during gastrulation, neurulation, axis- and head formation. In the early patterning events of the vertebrate body, canonical Wnt-signalling is believed to first act as dorsalizing and later as posteriorizing signal [67, 68]. In concordance to that, several Wnt-mutant mice exhibit truncated posterior axis, lost tail formation and disturbed somitogenesis [69, 70]. Experiments in chicken and Xenopus resulted in axis duplication and disturbed head formation after Wnt overexpression [71]. Proper formation of the head requires Wnt inhibition in the anterior embryonic tissue [72–74]. Ectopic expression of Wnt inhibitors was found to induce notochord formation [75]. The examination of Axin knockouts revealed its function in ventralizing the respective tissue and in inhibiting posterior axis formation [11]. Furthermore, Axin loss of function in Xenopus resulted in disturbed closure of neural folds, head folds and the duplication of the allantois [76]. These findings together with the our new observed expression of Axin2 during chicken embryogenesis support the idea that appropriate regulation Wnt-signalling via Axin2 influences body patterning, axis elongation and head formation. The expression of several Wnts in the chicken primitive streak and Hensen’s node reinforce this hypothesis [77].

0.2 Expression pattern of cAxin2 in stages HH 17 to 32

At HH stage 17, the chicken limb buds (lb) are distinguishable, expressing Axin2 mRNA from their onset (Fig 3, A red arrows). During the rapid outgrowth of the limb buds (lb) the Axin2 expression increases (Fig 3, HH 19: C, HH 20: D.1, HH 21: E.3, HH 22: F.2). The apical ectodermal ridge (aer) is notably stained (Fig 3, D.1 white arrow, E.3 black arrowhead, F.2 black arrow). In somites (so), Axin2 expression shifts from the medial somite to the intersomitic furrow (isf) (Fig 3, HH 21: E.1 white arrow; HH 22: F, F.2 white arrows). After whole mount in-situ hybridization, the neural tube (nt) is stained for Axin2 in two longitudinal stripes, at first weakly (Fig 3, HH 19: C.1, HH 20: D.2 black arrowhead), then stronger (Fig 3, HH 21: E.1 black arrowhead, HH 22: F.1 black arrowhead). Moreover, the mesenchyme of the sprouting tail bud (tb) expresses Axin2 (Fig 3, HH 17: A, HH 19: C.3 black arrowhead, HH 22: F.4 red arrow). As well as at other expression sites, Axin2 transcription relatively increases during maturation of the respective tissue or organ. At the head region, Axin2 is expressed in the otic vesicle (ov) (Fig 3, HH 17: A black arrow; HH 19: C.2 white arrow; HH 21: E.2 black arrowhead). Furthermore, the branchial arches (ba) are specifically stained after Axin2 in-situ hybridization (Fig 3, HH 19: C.2 black arrow, HH 21: E.2, HH 22: F.3). Moreover, the brain vesicles express Axin2.

HH 23 to 29 embryos (Fig 4) express Axin2 in similar regions, compared to the earlier developmental stages, with little changes. Axin2 is expressed in brain and otic vesicle (ov) throughout these stages (Fig 4, HH 24: B.1 white arrow). In addition, the branchial arches (ba) show intense staining (Fig 4, HH 24: B.1), which becomes restricted during development and predominantly was observed on the protuberances of the mandibular (Fig 4, HH 27: E black arrowhead, HH 28: F white arrow) and maxillary arch, respectively (Fig 4, HH 29: G white arrow). The expression pattern in the neural tube (nt) changes from two longitudinal lines (as described above) to one central line (Fig 4, HH 26: D.3; HH 27: E.1 white arrows). Another expression site of Axin2 is presented in Fig 3, picture E. Here, the white arrow indicates an Axin2 expression in the facial development. Axin2 is still expressed in the limbs (lb) by stage HH 26 (Fig 4, D.1). Here, it is notable that in further developed stages the future shoulder is heavily stained (Fig 4, HH 26: D.1, HH 28: F and HH 29: G red arrows). The interdigital zones, where programmed cell death occurs, express Axin2 (Fig 4, HH 28: F and HH 29: G black arrows). This observation was continuously found in the development of digits in later stages (Fig 4, HH 31: H black arrow and HH 32: I). Strong Axin2 expression is also visible in these
older stages' shoulders (Fig 4, HH 31: H and HH 32: I). The chicken external ear (ee) expresses Axin2 as well (Fig 4, HH 18: more prominent staining in the lb and in the ba. (C, C.1, C.2 & C.3) HH 19: Axin2 transcripts in the nt (C.1) and dml (C.1, white arrow), in lb (C, C.1), in ov (C.2, white arrow), ba (C.2, black arrow) and in the tip of the tail (C.3, black arrow). (D, D.1, D.2) HH 20: similar expression of cAxin2. (D.1) the wing bud expresses Axin2, white arrow: aer. (D.2) dorsal view: prominent expression in nt (black arrowhead) and isf (white arrow). (E, E.1, E.2, E.3) HH 21: Transcripts in the tip of the tail (E, black arrowhead), in nt and so(E.1), in the otic anlage (E.2, black arrowhead) and in the developing lb (E.3). (F, F.1, F.2, F.3 and F.4) HH 22: consecutive expression of Axin2 mRNA in ba (F, black arrow; F.3), nt (F, 1, black arrow), isf (F, white arrow, F.2, white arrow) and tail (F.4, red arrow). lb-limb buds, ov-otic vesicle, dml-dorso medial lip, ba-branchial arches, nt-neural tube, aer-apical ectodermal ridge, isf-intersomitic furrow, so-somites.

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Fig 3. Expression pattern of cAxin2 from HH stage 17 to 22. (A) HH 17 embryo with Axin2 expression in the brain, lb (red arrows), tail and ov (black arrow). (A.1) dorsal view: expression in the dml (white arrow). (B) HH 18: more prominent staining in the lb and in the ba. (C, C.1, C.2 & C.3) HH 19: Axin2 transcripts in the nt (C.1) and dml (C.1, white arrow), in lb (C, C.1), in ov (C.2, white arrow), ba (C.2, black arrow) and in the tip of the tail (C.3, black arrow). (D, D.1, D.2) HH 20: similar expression of cAxin2. (D.1) the wing bud expresses Axin2, white arrow: aer. (D.2) dorsal view: prominent expression in nt (black arrowhead) and isf (white arrow). (E, E.1, E.2, E.3) HH 21: Transcripts in the tip of the tail (E, black arrowhead), in nt and so(E.1), in the otic anlage (E.2, black arrowhead) and in the developing lb (E.3). (F, F.1, F.2, F.3 and F.4) HH 22: consecutive expression of Axin2 mRNA in ba (F, black arrow; F.3), nt (F, 1, black arrow), isf (F, white arrow, F.2, white arrow) and tail (F.4, red arrow). lb-limb buds, ov-otic vesicle, dml-dorso medial lip, ba-branchial arches, nt-neural tube, aer-apical ectodermal ridge, isf-intersomitic furrow, so-somites.

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0.3 Axin2 expression during somitogenesis

In transversal sections of in-situ hybridized chicken embryos, Axin2 expression was found during somitic differentiation (Fig 5). Green bars in the whole mount specimens (A, B, C, D, E, F, G) indicate the levels, where the sections have been performed. In the segmented paraxial mesoderm, Axin2 is expressed in the epithelial somites and in the differentiating dermomyotome. At HH stage 15 transcripts are mainly detectable in the medial and medio-dorsal wall of the
epithelial somites (Fig 5, A.1 black arrow). This expression gains intensity in stage HH 16 and 17 as the somite (so) maturates (Fig 5, B.1, C.3). More cranially in HH 17, where somites have maturated even further, deep epithelialization of the somite (so) has begun (Fig 5, C.2). Axin2 expression is relatively strong in the remaining medio-dorsal epithelium (Fig 5, C.2 black arrow) and in the mesenchyme ventrally flanking the neural tube (nt)(Fig 5, C.2 red arrow). Further cranially, where the dermomyotome is almost completely formed (Fig 5, C.1), Axin2 expression was found in the most ventral parts of the forming dml of the dermomyotome (Fig 5,
C.1 black arrow) and in a patch adjacent to the ventral neural tube (nt) (Fig 5, C.1 red arrow). In stage HH 19, when the dermomyotome is fully established, transcripts are visible in the ventrally facing margin of the dml, neighbouring the sclerotome (Fig 5, HH 19: D.1 black arrow). In HH stage 20, at limb level Axin2 expression is detectable also in the ventro-lateral lip (vll) (Fig 5, E.1 wing level). In further development, this expression gets restricted to the dorsal half of the dermomyotome (dm), the epaxial myotome and appears more faintly (Fig 5, E.2 interlimb level).

**Fig 5. Axin2 expression during somitogenesis and in the developing neural tube.** Transversal sections: green bars in overviews indicate sectioning level. (A) HH 15, overview; (A.1) expression in medial epithelial so (black arrow) and faintly all over the nt. (B) HH 16, overview; (B.1) increased expression in the medial somitic epithelium. (C) HH 17, overview; (C.1) Axin2 in dml (black arrow), in the mesenchyme ventrally flanking the nt (red arrow) and predominantly in the dorsal nt. (C.2) Expression in dorso-medial somitic epithelium (black arrow), ventrally in the mesenchyme (red arrow) and all over the nt. (C.3) Expression in the medial epithelial so. (D) HH 19, overview; (D.1) expression in dml and nt; (D.2) expression restricted to dorsal nt (green arrow) and ventrally in the neighbouring tissue (red arrow). (E) HH 20, overview; (E.1) expression throughout the dm; (E.2) Axin2 transcripts in the dorsal nt (green arrow), in the ventro-medially adjacent mesenchyme (red arrow) and weakly in the dml. (F) HH 22, overview; (F.1) Axin2 in the dorsal-most nt (green arrow) and in the overlying ectoderm (black arrow). (G) HH27, overview; (G.1) Axin2 expression in rp, fp (red arrow), drg (black arrow) and subectodermal space (green arrow). (H.1, H.2, H.3) snt of the tail. (H.1) HH 24, faint expression in all parts of the snt; (H.2) HH 27, restricted and intensified expression in the dorsal snt and in overlying subectodermal space; (H.3) HH 28, Axin2 in dorsal most snt and subectodermal space. so-somite, nt-neural tube, dml-dorso medial lip, dm-dermomyotome, rp-roof plate, fp-floor plate, drg-dorsal root ganglion, snt-secondary neural tube.

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In mice, Axin2 expression was found to oscillate in the segmental plate mesoderm and to occupy a central role for the segmentation of the presomitic mesoderm [50, 78]. We were able to detect Axin2 expression in the posterior psm in chicken from stage HH 09 to HH 16 (Figs 1 and 3). In mice, the expression of Wnt-genes alternates with the expression of FGFs in the PSM [78], indicating a similar mechanism in chicken. Interestingly, Axin2 mutant mice still undergo segmentation with slight to average deviation [55, 78]. Additionally, Axin2 transcripts were found during the maturation of the somites. In this process, a network of many different Wnt-molecules and other signals is described to play a role. The patterning of the somites is controlled by dorsalizing Wnt1 and Wnt3a from the dorsal neural tube [79–81], such as Wnt6 from the overlying ectoderm [82]. Wnt11 was described to maintain the epithelial status of the dml, while Wnt6 from the ectoderm maintains the epithelial ventro-lateral lip (VLL) [83]. Additionally, it was found that Wnt1 and Wnt3a are required for the formation of the dml [81]. Axin2 expression in the dml and its progenitors (Fig 5) indicate a potential role in the proper development of the dml and the deriving dermis. This hypothesis is supported when regarding the expression of Axin2 in the dermal derived feather buds (Fig 4, H.1, I.1).

0.4 Expression pattern in the developing neural tube

Regarding the development of the neural tube, Axin2 is expressed from neurulation to the differentiated mature neural tube (nt) (Figs 2 and 5). In Fig 5, the maturation of the neural tube (nt) is depicted. First, Axin2 mRNA was detected in a sprinkled distribution all over the neural tube (nt) (Fig 5, HH 15: A.1, HH 16: B.1, HH 17: C.1 and HH 19: D.1), with an intensified region at the medio-dorsal neuroepithelium (Fig 5, HH 17: C.1, HH 19: D.1). More cranially in HH 19, this expression appears more intense at the dorsal third (Fig 5, D.2 green arrow), while faint sprinkled expression remains in the ventral half of the neural tube (nt) (Fig 5, D.2). By HH stage 20, predominantly the dorsal expression domain increases even more (Fig 5, E.2 green arrow). Further, the faint expression site in the neighbouring tissue at left an right ventral side of the neural tube (nt) expands dorsally (Fig 5, E.2 red arrow). When maturing, the neural tube (nt) expresses Axin2 strongly in the dorso-medial neuroepithelium (Fig 5, HH 22: F.1 green arrow). Additionally, Axin2 transcripts are found in the overlying ectoderm and the sub-ectodermal mesenchyme flanking the dorsal neural tube (nt) (Fig 5, HH 22: F.1 black arrow). In HH 27, Axin2 expression was observed in the dorsal most part of the neural tube (nt) and in the roof- and floor plate (rp)(fp) (Fig 5, G.1). The black arrow in G.1 (Fig 5) reveals to the tip of the dorsal root ganglion (drg) that heavily expresses Axin2. Further, the dorsal ectoderm and subectodermal space overlying the neural tube (nt) are intensively stained (Fig 5, G.1 green arrow).

Axin2 transcripts were also found in secondary neurulation in the tail bud (Fig 2, C.2, C.3, C.4). After secondary neurulation, the differentiating secondary neural tube (snt) heavily expresses Axin2 (Fig 5, H.1, H.2, H.3). First, this expression is well distributed over the entire neuroepithelium (Fig 5, HH 24: H.1). During maturation, transcripts were observed in HH 27, (Fig 5, H.2) mainly in the dorsal half of the secondary neural tube (snt) as in the overlying sub-ectodermal mesenchyme and ectoderm. By HH 28 the Axin2 is missing in the ventral two thirds of the secondary neural tube (snt), but is expressed intensively in the dorsal third, such as in the ectoderm and subectodermal mesenchyme (Fig 5, H.3).

During the development and maturation of the neural tube, the establishment of a dorso-ventral axis through ventralizing Shh activity versus dorsalizing Wnt-signals has been described [84, 85]. The main Wnt-genes expressed in the dorsal neural tube and roof plate are Wnt1 and Wnt3a [84, 86]. These promote neural proliferation [84, 87]. Therefore, after activation of dorsal Wnt-signalling in the chick, dorso-ventral patterning of the neural tube was
perturbed and mitogenic activity of neural progenitors was increased [88]. Wnt1 and Wnt3a inhibition in mice, besides incomplete closure of the neural folds, displayed phenotypic alterations throughout the neural tube including partially absent basal-, roof- and floor plates [89]. In addition, Wnts have been identified to play a role in ventrally specified neural progenitors [86, 90]. The countless signalling molecules interacting with the Wnt-signalling pathway during neural tube maturation imply that Axin2 expression and its negative-feedback-loop in canonical Wnt-signalling impact this neural development and the basic molecular functions will be of special interest in future research.

0.5 Expression pattern of cAxin2 during limb development

Lim development in chicken starts from an out-bulged ridge of the somatic lateral plate mesoderm by stage HH 15. At HH stage 17 the wing bud heavily express Axin2 predominantly in the dorsal mesenchyme (Fig 6, A.1 black arrow). The hind-limb bud at the same stage is slightly further developed and transcripts of Axin2 are present in the thickened ectoderm, which gives rise to the apical ectodermal ridge (aer)(Fig 6, A.2), as well as at proximo-ventral margin of the lateral plate mesoderm (Fig 6, A.2 black arrow). In stages HH 18 to HH 20 Axin2 is expressed in the dorsal mesenchyme of the rapidly outgrowing limb buds (Fig 6, B.1, C.1, D.1, D.2). Moreover, the apical ectodermal ridge (aer) is heavily stained for Axin2 (Fig 6, B.1, C.1, D.1 black arrow, D.2). By stage HH 23, the transcripts in the dorsal mesenchyme are reduced, though the ectoderm and apical ectodermal ridge (aer) still express Axin2 (Fig 6, E.1 black arrow). This expression was is consistent in further developed stages (Fig 6, HH 25: F.1; HH 26: G.1; HH 27: H.1 black arrow; HH 28: I.1, I.2 and I.3). Moreover, when regarding the developing bones (bo) in HH stage 26 and 28, we verified Axin2 mRNA at the marginal perichondrium (Fig 6, G.1 and I.3 black arrows).

Several members of the Wnt family are expressed in the developing limb ([37] for review). The outgrowth of the limb bud is mediated by the apical ectodermal ridge (aer) [91]. Wnt genes are described to initiate the formation of the limb bud (Wnt2b) from the lateral plate mesoderm as well as the aer (Wnt3a) [92]. The aer in chicken expresses Wnt3a that, by initiating fibroblast growth factor (FGF) expression, mediates the rapid cell proliferation in the mesenchymal progress zone (PZ) underlying the aer [93]. Non-canonical Wnt7a is expressed in the dorsal ectoderm of the chicken limb, being responsible for dorsalization [94, 95]. Its expression site overlaps an additional expression site for Wnt3a in the ectoderm during early limb growth [96]. As Wnt7a target genes are expressed in the mesenchyme underlying the dorsal ectoderm, it was suggested that their signalling ranges as far as the target gene expression [97]. We postulate a similar distance of signalling for the canonical Wnt3a from early dorsal limb ectoderm as a source for early Axin2 expression in the dorsal limb mesenchyme. Mutations of Wnt3a and Wnt7a and FGFs in chicken embryo induced the expression of a gene responsible for a form of polydactyly in human, the Townes-Brock-Syndrome [98]. Later in the limb development, canonical Wnt-signalling is described to promote cell proliferation and the differentiation of connective tissue [99]. Axin2 expression in accordance to our results was reported in the perichondrium of mice [99]. By describing the expression of Axin2 in the chicken developing limb, we want to reveal its presumable function in regulating Wnt-signals that are involved in outgrowth, proliferation and differentiation.

0.6 Expression pattern of Axin2 during chicken nephrogenesis

The kidney development in birds and mammals takes place in three generations of nephric precursors [100]. In this study, an Axin2 expression in mesonephric development is described (Fig 7). In stage HH 19 the mesonephric duct (md) at leg level faintly expresses Axin2 (Fig 7,
A.1 black arrow). By HH 20 at interlimb level, the staining expands to the overlying coelomic epithelium (coe) (Fig 7, B.1 black arrow).

At leg level in HH 20, intense Axin2 expression in the nephric duct (md-mesonephric duct) and coelomic epithelium (coe) is observed (Fig 7, B.2 black arrow). When further differentiating,
transcription of \textit{Axin2} decreases, but is still detectable in mesonephric duct (md) and overlying coelomic epithelium (coe) (Fig 7, HH 23: C.1 black arrow). In addition, Fig 7 shows transversal sections of the cloaca, where \textit{Axin2} is expressed predominantly in the coelomic epithelium (Fig 7, D.1, E.1 black arrows). Pictures D.1 and E.1 demonstrate detectable transcripts in the cloacal ectoderm (HH 26: D.1, HH 28: E.1 black arrows). md-mesonephric duct.

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\textbf{Fig 7. Expression of \textit{Axin2} in nephric duct.} \textit{Axin2} mRNA expression in the \textit{md} (HH 19: A.1, leg level, black arrow; HH 20: B.1, interlimb level, black arrow and B.2, leg level; HH 23: C.1, caudal interlimb level, black arrow). The overlying thickened ectoderm strongly expresses \textit{Axin2} from HH stage 20 (B.1 & B.2, black arrows). Pictures D.1 and E.1 demonstrate detectable transcripts in the cloacal ectoderm (HH 26: D.1, HH 28: E.1 black arrows). md-mesonephric duct.

The role of \textit{Wnt} in the developing kidney has been extensively studied in the past. \textit{Wnt4} and \textit{Wnt9b} were described to be expressed in the nephric duct and coelomic epithelium [101–103]. The initiation of tubulogenesis of the developing kidney requires canonical \textit{Wnt4} and \textit{Wnt9b} signals from the nephric duct [101, 102]. Later in development both \textit{Wnt}-ligands were
described to act through the PCP and the Ca2+-dependent pathway as well [104–107]. As the Wnt-ligands partially activate different intracellular responses in the course of kidney development, the research faces a challenging aim in understanding this network. In Xenopus a model mediating the switch from canonical to non-canonical Wnt-signalling during nephrogenesis was proposed [108, 109]. However, canonical Wnt-signalling is known to mediate not only nephron induction, but also its orientation, cell proliferation, specification and differentiation [107, 110–113]. Alterations in canonical and non-canonical Wnt-signalling are known to cause polycystic kidney diseases [114, 115]. Taken together, we suggest that Axin2 might impact kidney development by regulating Wnt-signalling as indicated, through its expression in the nephric duct and coelom epithelium. The Axin2 expression in the coelomic epithelium could possibly hint a role for Axin2 in the development of the derived Mullerian-duct that develops to form the female genitals. As male gonads develop from the nephric or Wolffian-duct, Axin2 might be involved in this developmental process as well.

### 0.7 cAxin2 expression in developing chicken eye

The chicken eye initially develops, as the prosencephalon out-pockets and the optic vesicle (ov) invaginates to the head mesenchyme. Axin2 in this process is expressed in the proximal layer of the bi-layered optic vesicle (ov) (Fig 8, HH 15: A.1, black arrow). By stage HH 16 the lens vesicle (lv) has formed from the ectoderm (Fig 8, B.1). Axin2 transcripts are still detectable mainly in the proximal layer of the optic cup (oc) (Fig 8, B.1, HH 17: C.1 black arrow). An additional expression in the subectodermal mesenchyme overlying the optic cup (oc) and surrounding the lens vesicle (lv) is established at stage HH 18 (Fig 8, D.1 black arrow). While the lens vesicle (lv) expresses little Axin2 in the inner lens epithelium (Fig 8, HH 19: E.1 and HH 20: F.1, F.2, F.3 red arrows), transcripts in the optic cup (oc) are found in both proximal and distal layer at the epithelial margins facing towards the vesicular space (Fig 8: HH 19: E.1 black arrow, HH 20: F.4 black arrow). In the following observed stages, the proximal layer of the optic cup (oc) has formed the retinal pigmented epithelium (rpe), whereas the distal layer differentiates into the retina [116]. Axin2 expression was found only in the lens (Fig 8, HH 24: H.3 red arrow), ectoderm and subectodermal mesenchyme covering the eye (Fig 8, HH 24: H.1 and H.2 black arrows). Regarding the formation of the optic nerve (on) and optic chiasm (och), Axin2 expression is observable in the approaching and fusing neuroepithelial layers (Fig 8, HH 25: I.1, HH 26: J.1, HH 27: K.1 black arrows). Further, Axin2 is expressed in the future cornea covering the eye (Fig 8, HH 25: I.1, HH 26: J.1, HH 27: K.2 black arrow, HH 28: L.1 and L.2 black arrows) and in the posterior lens epithelium (Fig 8, HH 26: J.2 and J.3; HH 27: K.1 and K.2 red and green arrows).

Anteriorly expressed inhibitors of canonical Wnt signals are required for the initiation of the eye as described in zebrafish [117, 118]. Later, Wnt2b is expressed in the proliferative lens epithelium [119], retinal pigmented epithelium (rpe) and periphery of the optic cup [120–122]. Further, Wnt3 and Wnt11 were found to be expressed in the outer layer of the chicken optic cup [122]. Wnt2b was described to be responsible for maintaining the proliferative state of neural progenitors in the retina in chick [123]. Previous studies have reported a depigmentation of the retinal pigmented epithelium (rpe) after disruption if Wnt2b signalling in the chicken eye [120]. Our observed Axin2 expression in the lens overlaps with regions of increased cell proliferation, which express Wnt-ligands as well [122, 124, 125]. The chicken developing cornea and corneal stroma cells express Wnt3a and Wnt9b [126]. Interestingly, a subgroup of the disease familial adenomatous polyposis coli (FAP), which is caused by a truncation in APC or Axin2, the Gardner syndrome, includes a congenital hypertrophy of the rpe [127]. Additionally, some cases of tetra amelia, which is the result of homozygous Wnt3 mutations, exhibit optic malformations [128].
Conclusion

In the present study, we describe the expression pattern of avian Axin2 during embryonic development. We found a dynamic, temporally and spatially restricted expression pattern in many developing structures and tissues. In the early development of the chick, Axin2 was
expressed in the primitive streak and underlying mesoderm, in the neural folds and in the head fold. It was additionally expressed during secondary neurulation in the tailbud mesenchyme. Here, the pre-somitic mesoderm as well transcribes Axin2. We were able to detect such expression in the posterior psm and during the maturation of the somites in its medial epithelium and in the dml. By this developmental stage, transcripts were also detectable in the brain and differentiating neural tube. In the developing limb a dynamic expression was found. Furthermore, we detected Axin2 mRNA in the nephric duct and coelomic epithelium. Regarding the head of the chicken embryo, Axin2 was expressed in branchial arches and sensory anlagen. Later in development, expression in feather buds, interdigital spaces, external ear and scleral ossicles on the eye was observed.

The expression of Axin2 in mice was previously found in the primitive streak, head folds, neural tube, branchial arches I and II (maxillary and mandibular arch), psm and dml, tailbud, limbs, kidney and brain [45, 78].

These findings are mainly consistent to the expression we found in the chick. Additionally, we were able to show Axin2 expression in the developing eye and in the otic vesicle. With this study we want to point out the often neglected impact of Axin2 in many Wnt-dependant developmental processes. While Wnt-ligands are extensively studied, investigating their regulation through Axin2 in the respective tissues might help understanding the interactions of different signalling factors.

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