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Evolution of the vertebrate claudin gene family: insights from a basal vertebrate, the sea lamprey

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ABSTRACT Claudins are major constituents of tight junctions, contributing both to their intercellular sealing and selective permeability properties. While claudins and claudin-like molecules are present in some invertebrates, the association of claudins with tight junctions has been conclusively documented only in vertebrates. Here we report the sequencing, phylogenetic analysis and comprehensive spatiotemporal expression analysis of the entire claudin gene family in the basal extant vertebrate, the sea lamprey. Our results demonstrate that clear orthologues to about half of all mammalian claudins are present in the lamprey, suggesting that at least one round of whole genome duplication contributed to the diversification of this gene family. Expression analysis revealed that claudins are expressed in discrete and specific domains, many of which represent vertebrate-specific innovations, such as in cranial ectodermal placodes and the neural crest; whereas others represent structures characteristic of chordates, e.g. pronephros, notochord, somites, endostyle and pharyngeal arches. By comparing the embryonic expression of claudins in the lamprey to that of other vertebrates, we found that ancestral expression patterns were often preserved in higher vertebrates. Morpholino mediated loss of Cldn3b demonstrated a functional role for this protein in placode and pharyngeal arch morphogenesis. Taken together, our data provide novel insights into the origins and evolution of the claudin gene family and the significance of claudin proteins in the evolution of vertebrates.

KEY WORDS: *claudin, lamprey, vertebrate primordia, embryo*

Tight junctions are connections between neighbouring epithelial cells that are used to hold sheets of cells together and to control the movement of molecules across the epithelial sheet. Tight junctions are composed of three core components: integral membrane barrier proteins (claudins, TAMPS and JAMs), peripheral scaffolding proteins (ZO, cingulin) and intracellular cytoskeletal proteins (Van Itallie and Anderson, 2013). Claudins are crucial both for the formation of the intercellular seal and for the control of permeability of the tight junction (Van Itallie and Anderson, 2006, Van Itallie and Anderson, 2013).

Electron microscopic studies demonstrated that tight junctions are absent from the tissues of amphioxus (Lane *et al.*, 1987), but are present in various tissues in seven species of ascidians (Lane *et al.*, 1986), the sister group of vertebrates. However, the protein composition of ascidian tight junctions is somewhat different from that of vertebrate tight junctions: no occludins are present in the ascidians (Sasakura *et al.*, 2003). Tight junctions are the main junction type present in vertebrates (Gunzel and Yu, 2013, Lal-Nag and Morin, 2009). These facts suggest that tight junctions originated before the urochordate/vertebrate split, and became further elaborated in the vertebtates.

Interestingly, claudin-like genes significantly predate the origin of tight junctions and appear to trace their origin to the base of metazoan lineage: a member of PMP-22/EMP/MP-20/Claudin superfamily was

Abbreviations used in this paper: Cldn, claudin; Cldns, claudins; E, embryonic day.

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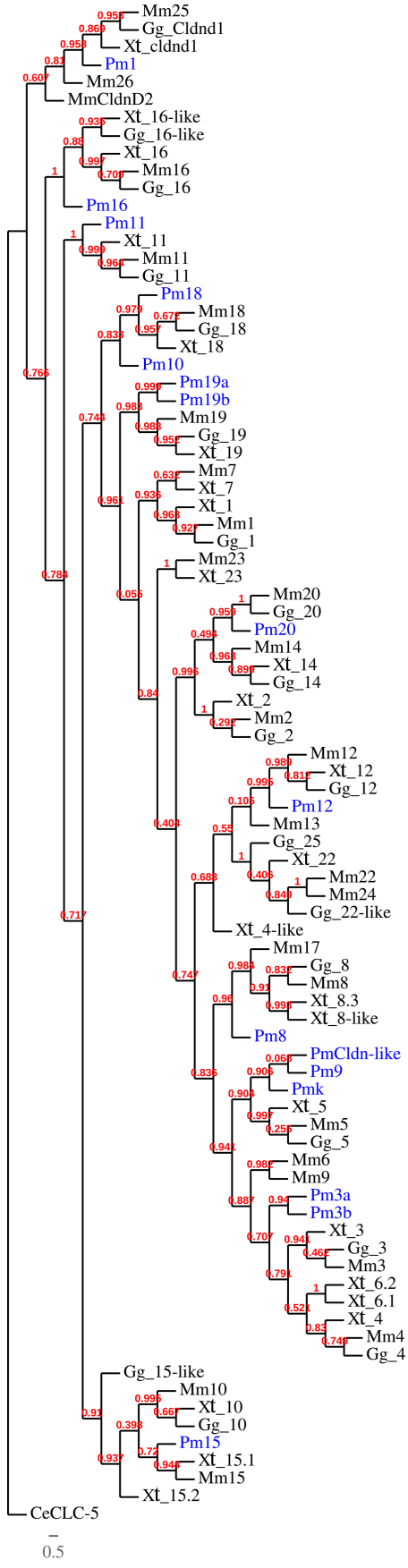

found in the desmosponge *Amphimedon queenslandica* (Fahey and Degnan, 2010) and in two other sponge species (Leys and Riesgo, 2012). Claudin-like proteins have been found in *C. elegans* (Asano *et al.*, 2003, Simske, 2013, Simske and Hardin, 2011) and *Drosophila* (Jaspers *et al.*, 2012), where they form part of a different type of intercellular junction. The *C. elegans* claudin-like proteins (CLC-1 to CLC-5) localize to or near septate junctions and appear to play a role in cell adhesion and epithelial permeability via the paracellular pathway (Asano *et al.*, 2003). *Drosophila* homologues of claudins, *Megatrachea*, *Sinuous* and *Kune-Kune*, localize to the septate junctions (invertebrate homologue of the tight junctions), and their loss results in the disruption of junctional structure, epithelial adhesion and permeability (Behr *et al.*, 2003, Jaspers *et al.*, 2012, Nelson *et al.*, 2010, Wu *et al.*, 2004). Therefore, even though the sequences of the non-chordate invertebrate and vertebrate claudins are highly divergent (Simske, 2013), the non-vertebrate claudin-like proteins appear to play similar roles to their vertebrate counterparts, albeit within different junctional complexes.

The appearance of true tight junctions was accompanied by expansion of the claudin gene family. It has been proposed that diversification of the vertebrates and appearance of some vertebrate characteristics may have been facilitated by this diversification, which allowed for the appearance of many diverse epithelial types with a range of selective permeabilities. However, this hypothesis was difficult to test because of the absence of sequencing or expression data from basal vertebrates and non-vertebrate chordates. For example, *Ciona intestinalis* has at most 11, and possibly as few as 3 true claudins (Sasakura *et al.*, 2003), while there are 20 claudins in *Xenopus tropicalis* (Baltzegar *et al.*, 2013), 17 in the chick (Collins *et al.*, 2013), while mouse and human have at least 27 claudin family members (Mineta *et al.*, 2011). Teleosts are particularly rich in claudin homologues, with 56 claudins having been identified in the pufferfish *Fugu rubripes* (Loh *et al.*, 2004) and 54 were recently annotated in zebrafish (Baltzegar *et al.*, 2013). The abundance of claudins in the fish could be due both to tandem gene duplication events and a whole genome duplication event that occurred in the teleost lineage (Baltzegar *et al.*, 2013, Lal-Nag and Morin, 2009, Loh *et al.*, 2004). It has been proposed that teleosts need a larger variety of claudins in order to facilitate ion exchange with the aquatic environment and to maintain osmotic balance, particularly in aquatic habitats of different salinity (Loh *et al.*, 2004).

The main function of claudins within the tight junctions is to control the junction's ion and solute permeability. Claudins can be separated into two functional groups: "classic" claudins that reduce the permeability of the epithelia ("barrier" claudins), and "non-classic" claudins that promote selective ion permeability ("pore" claudins) (Hou *et al.*, 2013, Krause *et al.*, 2009, Van Itallie and Anderson, 2006). The properties of epithelia/endothelia are thus determined by the combination of claudins that are expressed. This is of particular relevance to epithelial formation during embryogenesis, where differential permeability of epithelia

Fig. 1. Analyis of the phylogenetic relationships among vertebrate claudins. *Maximum likelihood phylogenetic tree constructed using lamprey (Pm),* Xenopus *(Xt), chick (Gg) and mouse (Mm) claudin amino acid sequences. The tree is rooted with* C. elegans *(Ce) claudin CLC-5. Lamprey proteins are highlighted in blue. Statistical support values are indicated on tree nodes.*

often plays a role in morphogenetic processes.

Finally, claudins can interact with intracellular signalling molecules via their PDZ binding domain, thereby linking extracellular transport and intracellular signalling events. For example, chick Claudin 10 plays an essential role in left-right embryo patterning: it appears to transmit left-right patterning cues from Hensen's node to the lateral plate mesoderm (Collins *et al.*, 2015).

In this work, we report a detailed phylogenetic and expression analysis of the entire claudin gene family in the most basal extant vertebrate lamprey. Our results demonstrated that about half of all higher vertebrate claudins (12) have at least one ancestral lamprey homologue, and that lineage-specific gene duplications played a significant role in the diversification of this gene family. Our *in situ* hybridization analysis revealed strong and specific expression of claudins in embryonic structures that are characteristic of vertebrates, such as the otic placodes, neural crest and cartilage of the oral cavity and pharyngeal arches. Morpholino-mediated loss of Claudin 3b resulted in abnormal development of the lamprey ear placodes and pharyngeal arches, demonstrating for the first time the functional significance of claudins in the formation of vertebrate primordia.

Results

Phylogenetic analysis of the claudin gene family

We identified 16 individual claudin genes in the lamprey genome. Transcriptome analysis demonstrated that some of the lamprey claudins had multiple alternative splice forms; for example Cldn 18 (2 splice forms affecting the coding region), Cldn 10 (4 splice forms), claudin 19b (4 splice forms with the splicing not affecting the coding region). In order to understand the relationships between lamprey and higher vertebrate claudins, we constructed a maximum likelihood phylogenetic tree using all known lamprey, *Xenopus*, chick and mouse claudin protein sequences (see Table S1 in supplementary information for the list of protein sequences and accession numbers). The tree was rooted with a *C. elegans* claudin CLC-5. The results of our phylogenetic analysis demonstrate that most of the lamprey claudins cluster to the base of an orthologous higher vertebrate claudin lineage (e.g. Cldns 18, 15, 11, 19a and b, 8, 3a and b, 20, 12, 16 and 1), suggesting an ancestral relationship. Interestingly, there appears to be an almost 1 to 1 ratio of lamprey to higher vertebrate claudins, with a few exceptions due to lineage specific duplications. For example, mouse Cldn 24 and 22, 3 and 4, 6 and 9, and 8 and 17 are paralogous pairs that reside next to each other on chromosomes 8, 5, 17 and 16 respectively, and appear to have arisen recently as a result of tandem duplication in the mammalian or possibly tetrapod lineage (Collins *et al.*, 2013, Lal-Nag and Morin, 2009). As expected, only one lamprey orthologue is found in these groups. The presence of two Cldn19 and two Cldn3 genes in the lamprey appears to result from a lamprey specific duplication event that took place after the divergence of the lamprey and gnatostome lineages. Some of the tetrapod claudin groups (e.g., Cldns 1/7) lack a lamprey member, suggesting that these groups may have evolved by additional duplications in the gnatostome lineage (Baltzegar *et al.*, 2013). Alternatively, the lamprey homologues may have been lost within the lamprey lineage over evolutionary time or could have been underrepresented in the genomic and transcriptomic databases used in this study (see Materials and Methods).

Expression of claudins in adult lamprey tissues and whole lamprey embryos

We used RT-PCR to analyse the expression of lamprey claudins during days E8-E18 of embryogenesis, as well as in selected adult tissues. Claudins 3b, 18 (splice form 2), 8, 9, 10, 11, 16 and 19b were expressed throughout all embryonic stages analysed, while Claudins 1, 20, 3a,19a and 18 (splice form 1) were not expressed. Claudin 12 was expressed at all stages analysed except E15, while claudin 15 was expressed at E8 and 9, turned off at E10, and was again amplified from RNA collected at later stages (from E11 onwards) (Fig. 2).

Fewer claudins were expressed in the adult tissues. Claudins 3b, 8, 9, 11, 16 and 18.2 were expressed in the eye, and the same

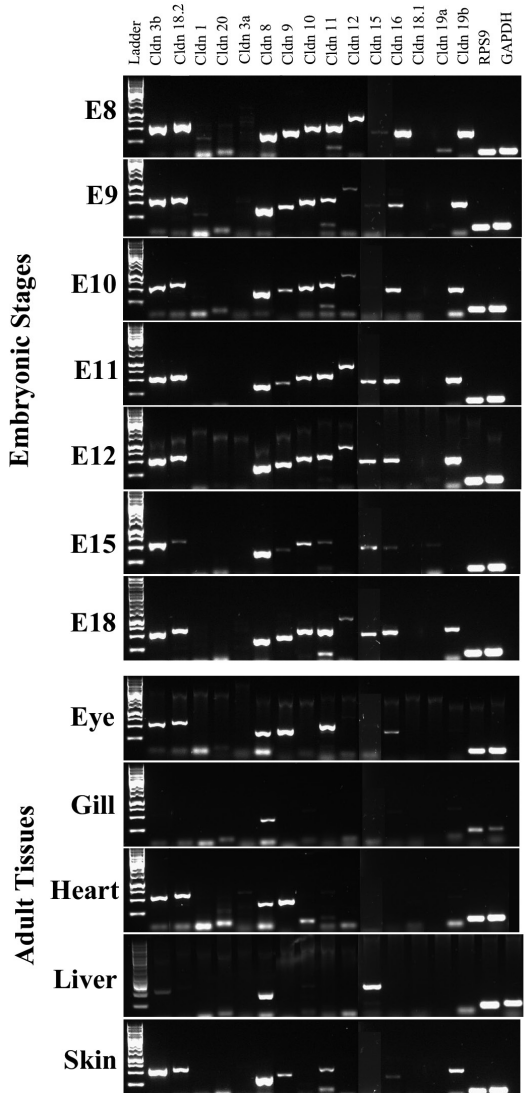

Fig. 2. Expression of claudins in lamprey adult and embryonic tissues. *Analysis of claudin expression by RT-PCR in adult eye, gill, heart, liver and skin and at selected stages of embryonic development. Primers for GAPDH and RPS9 were used as controls. In some instances (certain tissue or developmental stage) non-specific amplification or primer dimer formation was observed. The identity of the band of expected size was in every case confirmed by sequencing.*

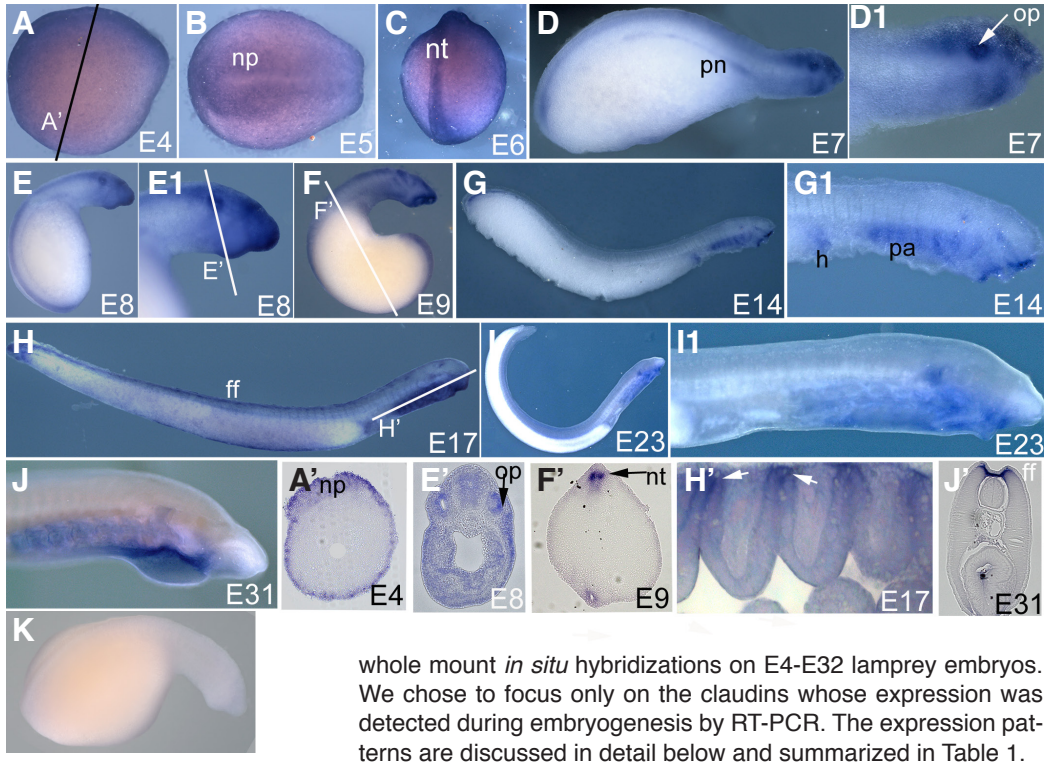

Fig. 3. Claudin 3b expression. *Claudin 3b is initially expressed in the neural plate/neural tube* **(A-C, A', F')**. *From E7, its expression is seen in the otic placode/ear* **(D-J, E')** *and pronephros* **(D)**. *Starting at E10, Cldn3b is expressed in the heart* **(G, G1, H)** *and the endoderm of the pharyngeal arches* **(G-J, H')**. **(K)** *Negative control – an E8 embryo hybridized with an antisense probe to demonstrate specificity of staining. np- neural plate, nt – neural tube, pn – pronephros, op- otic placode, pa – pharyngeal arches, h – heart, ff – fin fold/ dorsal fin. White arrows in J' indicate the areas of highest Cldn3b expression within the pharyngeal arches.*

claudins except 16 were observed in the heart. Skin expressed claudins 3b, 8, 9, 11, 18.2, 19b and 16. Claudin 16 transcripts were amplified less, suggesting lower levels. Gills expressed predominantly claudin 8, while Claudins 3b, 8 and 15 were found in the liver (Fig. 2).

Spatiotemporal expression pattern of claudins during lamprey embryogenesis

In order to investigate spatial distribution of the various claudin family members during lamprey embryogenesis, we performed whole mount *in situ* hybridizations on E4-E32 lamprey embryos. We chose to focus only on the claudins whose expression was detected during embryogenesis by RT-PCR. The expression patterns are discussed in detail below and summarized in Table 1.

Claudin 3b expression was first observed at E4 throughout both the neural plate and the non-neural ectoderm (Fig. 3A). Cldn3b was transiently expressed in pronephros from E7 to E9. Expression in the otic placodes was initiated at E7 and continued until E23 (Fig.3 I1), after which it gradually declined. Additionally, strong expression was seen in the neural tube from E6 until E11 (Fig. 3 C,E,F'), in the folds of the endoderm of the pharyngeal arches (Fig. 3 H,H'), the fin folds and the heart.

Similarly, expression of Claudin 8 was initially seen throughout the ectoderm of an early (E5 and E6) lamprey embryo (Fig. 4 A,B,B'). By E7, the expression is confined to several distinct do-

TABLE 1

SPATIOTEMPORAL EXPRESSION OF LAMPREY CLAUDINS DURING LAMPREY EMBRYOGENESIS

Anatomical location	Cldn3b	Cldn8	Cldn9	Cldn10	Cldn12	Cldn16	Cldn18	Cldn19b
Ectoderm								
Neural plate/tubez	E5-E11	E8-E17	E6-E23					E7
Eye spot			E16-E23					
Neural crest							E6-E9	
Otic placodes/ears	E7-E31	E7-E23	E6-E23	E7-E31			E6.5-E9	E9-E31
Non-neural ectoderm	E5-E6	E4-E7		E5-E17	E4-E24		E10-E18	
Mesoderm								
Notochord				E10-E19				E7-E18
Somites			E10-E23					
Heart	E10-E17				E24-E31		E10-E14	E14-E31
Pronephros/ kidneys	E7-E9		E9-E19			E7-E24		
Endoderm								
Endostyle			E19-E23					
Liver				E31				
Organs containing derivatives of several germ layers								
Pharyngeal arches	E12-E31 (End)					E10-E18 (1st pa)	E24-E31 (cart.)	E14-E17 (End)
Fin folds	E14-E17		E31	E31				
Stomodeum (mouth)	E7-E31	E23-E31			E12-E24			

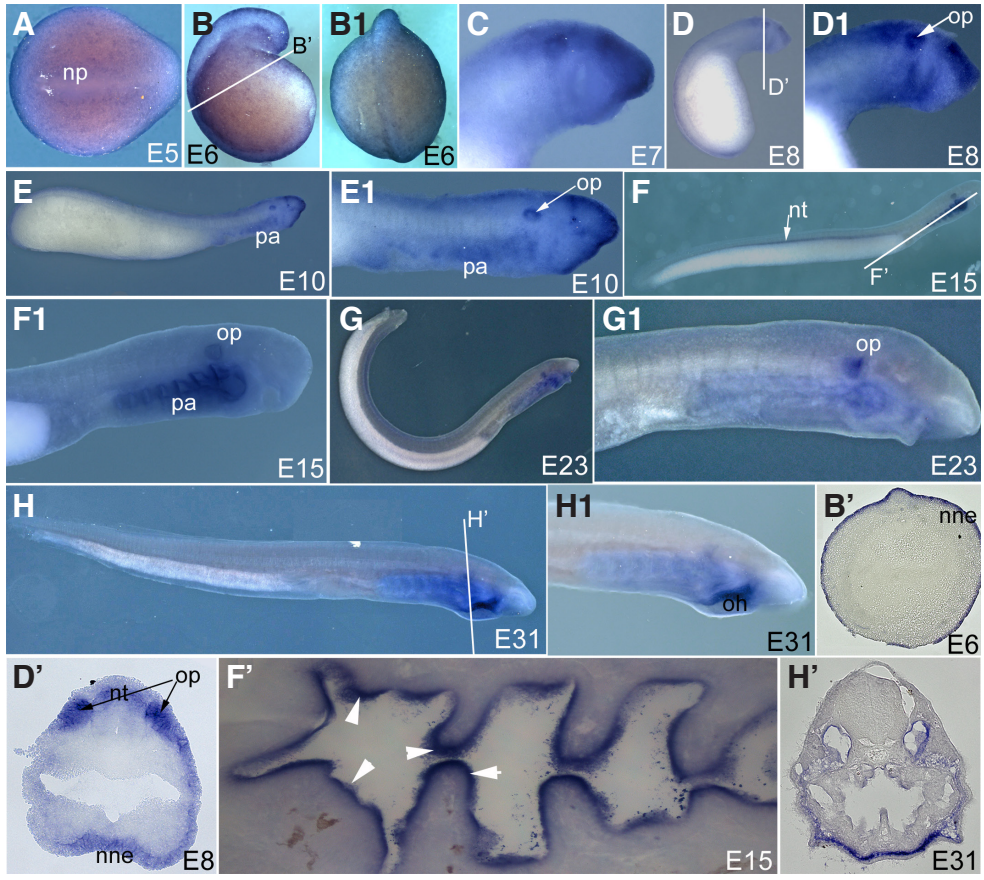

Fig. 4. Claudin 8 expression. *Claudin 8 expression starts around E4 throughout the neural plate and non-neural ectoderm of the embryo* **(A, B, B1, B')**. *Later, Cldn 8 mRNA is seen in the otic placode/ear* **(D-G1, D')**, *the neural tube* **(F, D')** *and oral hood cartilage* **(H, H1, H')**. *np – neural plate, nt – neural tube, nne – non-neural ectoderm, op – otic placode/ear, pa – pharyngeal arches, oh – oral hood. The apparent expression in the pharyngeal arches in F1 is due to the trapping of the stain in the pharyngeal cavity (data not shown).*

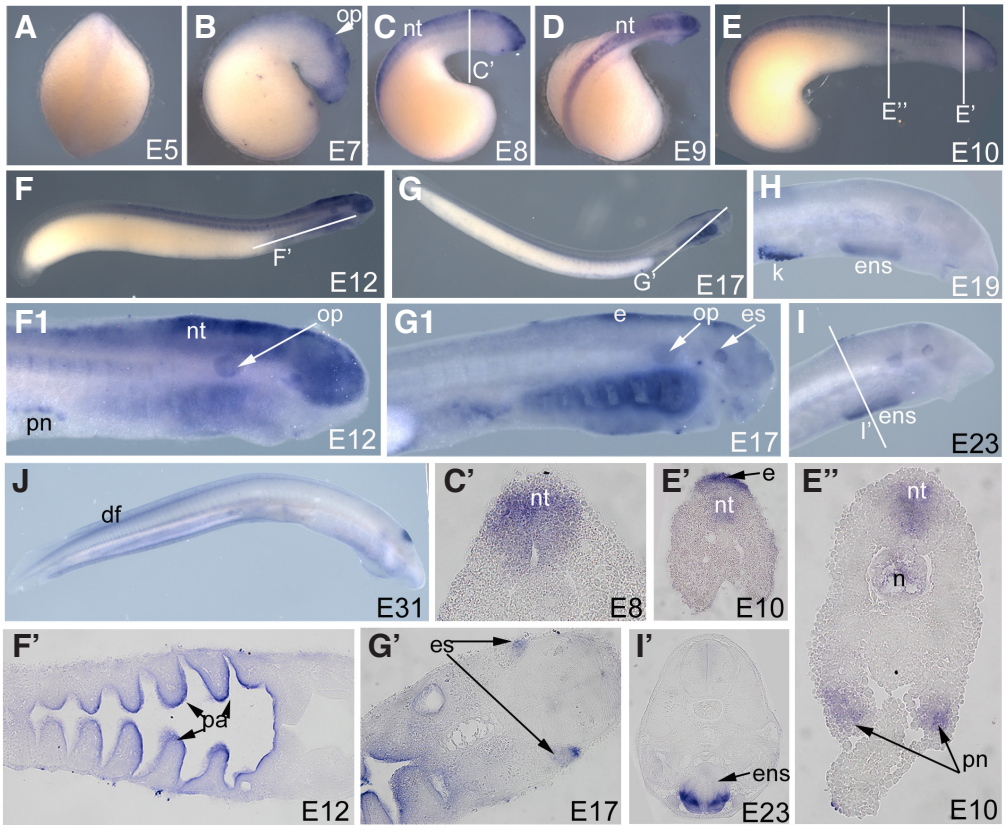

Fig. 5. Expression pattern of Claudin 9. *Claudin 9 expression is seen in the neural tube* **(B-G1, C', E', E'')**, *otic placode/ear* **(B-I, G')**, *notochord* **(E-I, E'')**, *retina/eye spot* **(G1-I, G')**, *pronephros/kidney* **(E-H, E'')** *and endostyle* **(H, I, I')**.*Staining on the inside of the pharyngeal arches* **(F1, G1)** *is an artefact, due to the stain precipitating within the pharyngeal cavity* **(F')**. *nt – neural tube, e – head ectoderm, op – otic placode/ear, pa – pharyngeal arches, ens- endostyle, pn – pronephros, k – kidney, es – eye spot, n – notochord, df – dorsal fin.*

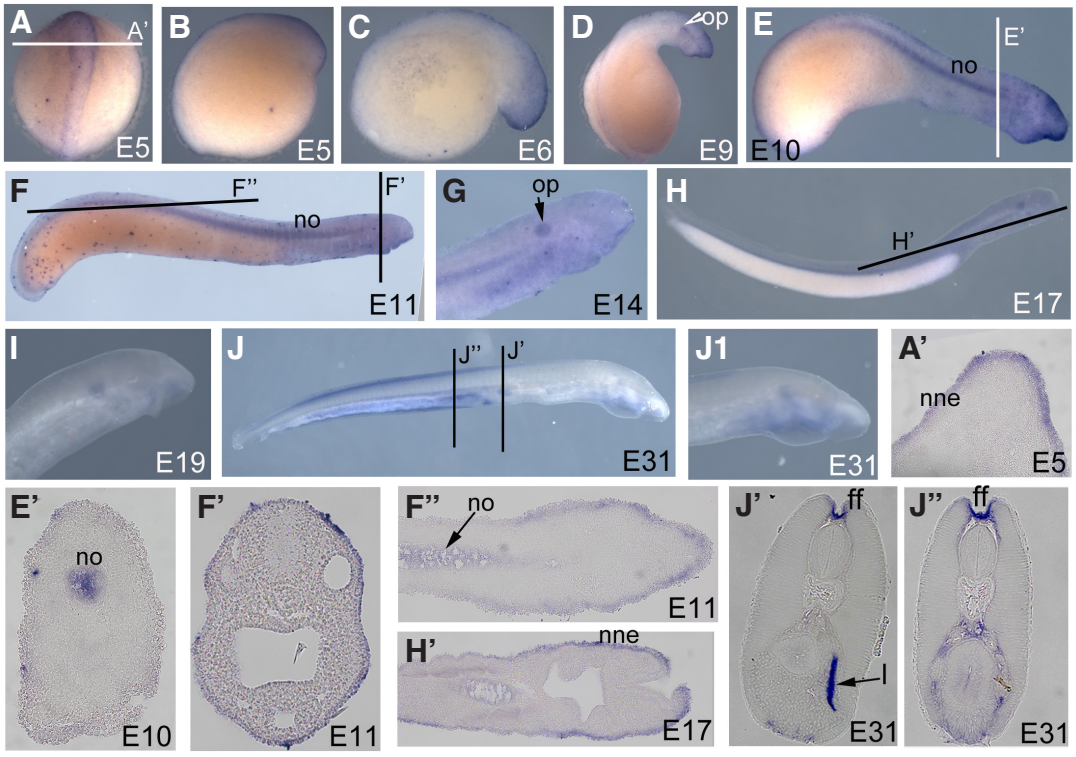

Fig. 6. Expression pattern of Claudin 10. *Claudin 10 is expressed in the non-neural ectoderm, mostly in the head* **(A-H, A', F', H')**, *the notochord* **(E-H, E', F")**, *otic placodes* **(D, G, I, J1)**, *fin folds* **(J, J', J")** *and liver* **(J')**. *op- otic placodes, no – notochord, nne - non-neural ectoderm, ff – fin fold, l – liver.*

mains: the otic placode, neural tube and ventral head ectoderm (Fig. 4 C,D,D'). Expression in the ears/otic vesicles persists until E23 (Fig. 4 G,G1), while the neural tube expression declines by E17 (data not shown). We also observed strong expression of Cldn8 in the cartilage of the oral hood at later developmental stages (Fig. 4 H,H1,H').

Claudin 9 demonstrated an interesting and dynamic expression pattern. Cldn9 transcripts were seen in the neural tube from E6 until about E23, with the highest levels at E12 (Fig. 5F1), in the otic placode/ear from E6 until E23 (Fig. 5 B,F1,G1,I and data not shown) and in the eye spot from E16 until E23 (Fig. 5 G1,G',I). Cldn9 transcripts were also seen in the pronephros/kidneys from E9 until E19 (Fig. 5 E,E",F1,H), and in the endostyle (the lamprey equivalent of the thyroid gland) between E19 and E23 (Fig. 5 H,I,I'). While we observed staining within the pharyngeal arches, stain distribution on the sections through this region suggests that it is likely to be due to trapping (Fig.5F').

Claudin 10 expression was first observed at E6, within the future head ectoderm of the embryo (Fig. 6 A, A'). Expression in the head ectoderm was seen until E17 (Fig. 6 F",H'). Additionally Cldn10 was seen in the otic placodes/ears from E7 until E31 (Fig. 6 D,G,H,I,J1), and throughout the notochord from E10 until E19 (Fig. 6 E,F,E',F" and data not shown). Interestingly, we also observed strong expression of Cldn10 in the liver at E31 (Fig. 6J'), though only a small band could be amplified by RT-PCR in the adult lamprey liver (Fig. 2), suggesting low expression levels.

Claudin 12 expression was seen in the ectoderm of the head from E5.5 until E24 (Fig. 7 B-F), in the heart at E24-31 (Fig. 7 G,G1,H) and the mouth of E12-E24 embryo (Fig. 7 E-G1).

Expression of Claudin 16 was restricted to only two domains:

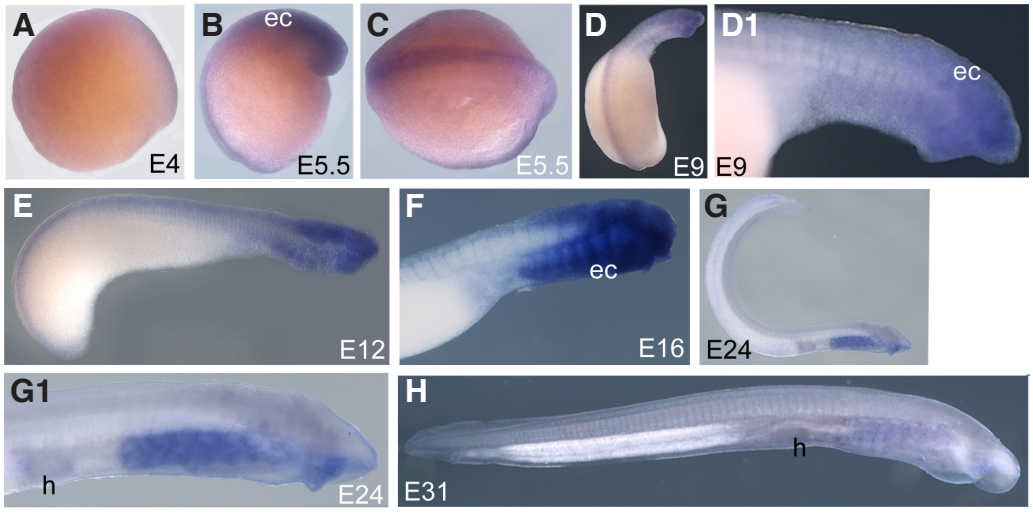

Fig. 7. Claudin 12 expression. *Expression of claudin 12 is seen in the non-neural ectoderm, predominantly of the head* **(A-G1)**, *in the mouth* **(E-G1)** *as well as in the heart* **(G-H)**. *ec – ectoderm, h – heart.*

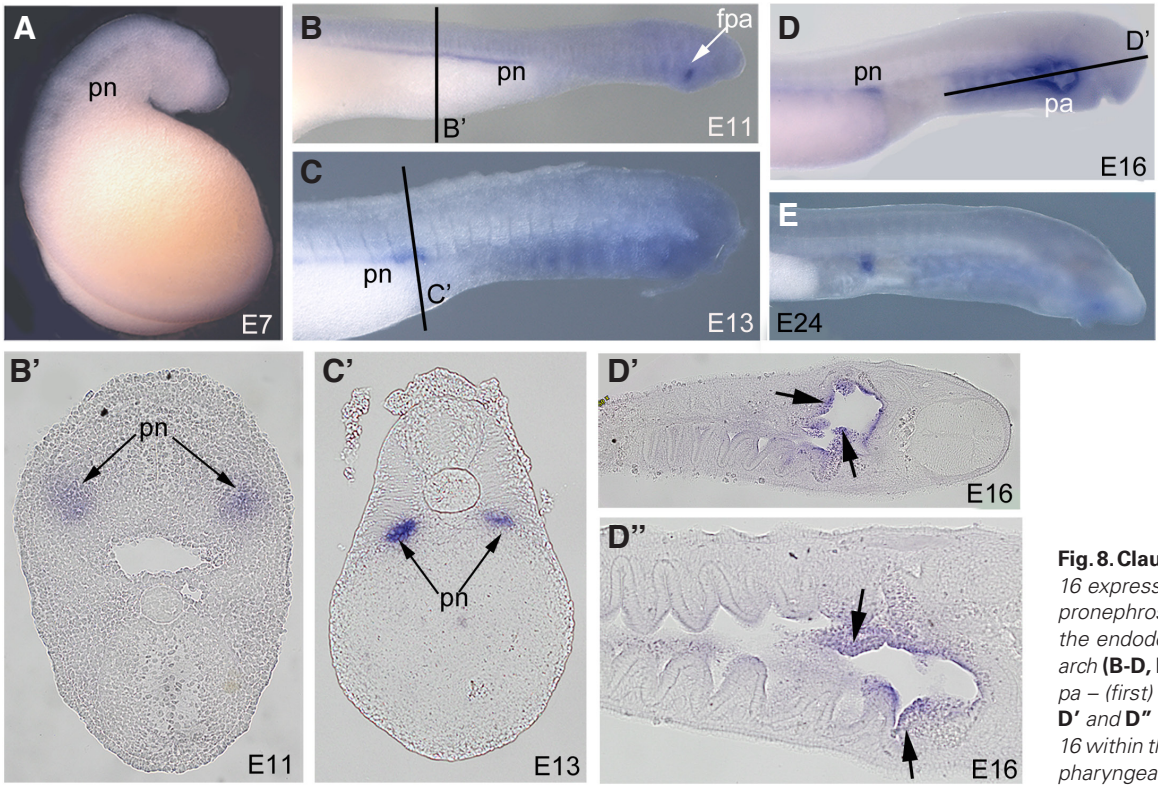

Fig. 8. Claudin 16 expression. *Claudin 16 expression is observed only in the pronephros/kidney* **(A-E, C', B')** *and the endoderm of the first pharyngeal arch* **(B-D, D', D")**. *pn – pronephros, (f) pa – (first) pharyngeal arch. Arrows in* **D'** *and* **D"** *indicate expression of Cldn 16 within the endoderm layer of the 1st pharyngeal arch.*

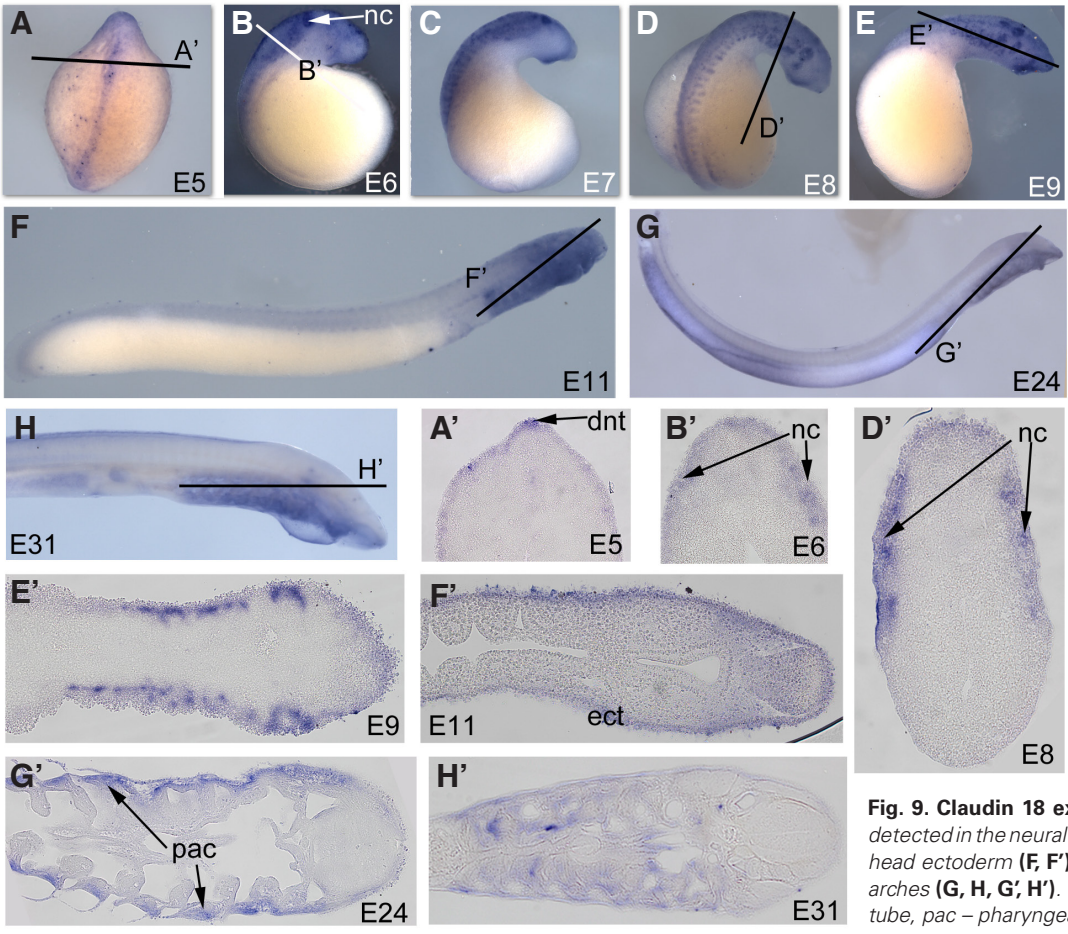

Fig. 9. Claudin 18 expression. *Claudin 18 expression is detected in the neural crest* **(B-E, B'-E')**, *otic placodes* **(B-E)**, *head ectoderm* **(F, F')** *and the cartilage of the pharyngeal arches* **(G, H, G', H')**. *nc – neural crest, dnt – dorsal neural tube, pac – pharyngeal arch cartilage.*

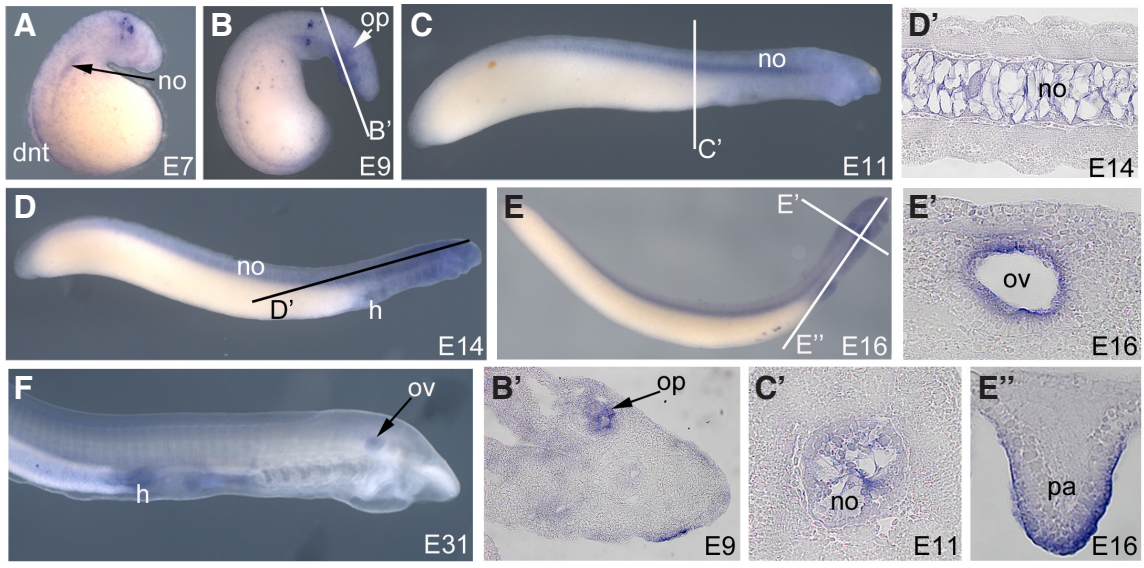

Fig. 10. Claudin 19b expression. *Claudin 19b is expressed in the notochord* **(A-E, C', D')**, *the otic placode/vesicle* **(B-F, B', E')**, *the heart* **(D-F)** *and the endoderm of the pharyngeal arches* **(D, E, E")**. *no- notochord, dnt – dorsal neural tube, op/ov – otic placode/vesicle, h – heart, pa – pharyngeal arches.*

pronephros and the endoderm of the first pharyngeal arch. Expression in the pronephros was seen from E7 until E24 (Fig.8 A-E,C',B'), while pharyngeal arch expression started at E11 and persisted until E18 (Fig.8 B,D,D'D").

Claudin 18 was the only member of the lamprey claudin family that exhibited expression in the premigratory and migrating neural crest from E5 until E9 (Fig. 9 B-E,B',D',E'). Additionally, Cldn 18 expression was also observed in the otic placodes from E6.5 until E9 (Fig. 9 C-E), and later (E10-E18) in the non-neural ectoderm of the head (Fig. 9 F,F' and data not shown). Claudin 18 transcripts were also seen in cartilage of the pharyngeal arches (a neural crest derivative) at E24-E31 (Fig.9 G,H,G',H'). Low levels of expression could also be detected in the heart at E10-E14.

Claudin 19b expression was most prominent in the notochord between E7 and E18 (Fig. 10 A-E,D',C'), as well as in the otic placodes/vesicles throughout most developmental stages examined (Fig. 10 B,D,F,B',E'). Expression was also observed in the endoderm of the pharyngeal arches at E14-E17 (Fig. 10 D,E,E").

Loss of Claudin 3b results in abnormal ear placode and pharyngeal arch morpholgenesis

Claudin 3b is expressed both in ear placodes and pharyngeal arches of lamprey embryos; moreover, expression of claudin 3 homologues in the developing ear is conserved in other vertebrates (Haworth *et al.*, 2005, Kollmar *et al.*, 2001). In order to investigate the function of Claudin3b in lamprey development, we injected a translation-blocking Cldn3b morpholino into one or two-cell stage lamprey embryos. The embryos were collected at E16 and analysed by *in situ* hybridization with SoxE1 probe. We observed defects in the folding of the pharyngeal arches in 61% of morphants and defects in the formation of the ear placode in 32% of morphants (Fig. 11 G,H). In contrast, embryos injected with either the standard control morpholino or the 5-nucleotide mismatch Cldn3b control morpholino were mostly normal, with only a low percentage of mildly abnormal embryos. Chi square test results indicated that the differences between the Cldn3B morphants and control morpholino injected embryos (both standard control and Cldn3b control) were highly statistically significant with p values of <0.01.

Discussion

Claudins are key constituents of the tight junctions, essential both for the establishment of the physical connection between the epithelial cells and for the selective permeability of the junction to ions and small molecules. Even though claudin-like molecules are present in several non-chordate invertebrates, major diversification of claudins occurred only within the vertebrate lineage, simultaneously with the appearance of tight junctions. Claudin-like sequences are present in non-vertebrate chordates amphioxus and ascidians (Sasakura *et al.*, 2003; JGI Amphioxus Genome Browser); however tight junctions are not present in the amphioxus (Lane *et al.*, 1987) and appear for the first time in ascidians (Lane *et al.*, 1986). The ascidian claudin proteins are so highly divergent from their vertebrate counterparts, that phylogenetic analyses conducted by Sasakura *et al.,* were inconclusive as to the origin of claudins and the relationship between the ascidian and the vertebrate claudins (Sasakura *et al.*, 2003). Here, we analysed the entire claudin family from the most basal extant vertebrate the sea lamprey (*Petromyzon marinus*). The results of our phylogenetic analysis demonstrate that the 16 distinct claudin sequences present in the lamprey are orthologous to 12 (out of 26) mammalian claudins (4 out of the 16 represent lamprey-specific gene duplications). Our analysis is in agreement with previously reported lineage-specific gene duplication and gene loss events that played a role in the evolution of this gene family. For instance, mouse claudins 22 and 24, 8 and 17, and 6 and 9, appear to have arisen as the result of tandem duplications in the mammalian lineage, as previously reported (Baltzegar *et al.*, 2013, Lal-Nag and Morin, 2009). However, duplication that produced claudins 3 and 4 must have occurred in the tetrapods, and not in the mammalian lineage (Loh *et al.*, 2004), since claudin 3 and 4 orthologues are found in the chick and *Xenopus*, but no Cldn4 is found in the teleosts (Baltzegar *et al.*, 2013) or the lamprey. Lamprey-specific duplications are seen in the case of PmCldn3a and 3b, and PmCldn19a and 19b, which are ancestral to the Cldn3/4/6 and Cldn19/1/7 paralogous groups, respectively.

In order to gain a better understanding of the relationship between vertebrate and chordate claudins, we also performed

maximum likelihood phylogenetic analysis of all claudin sequences from lamprey, *Xenopus*, chick, mouse, amphioxus and *Ciona*. (18 sequences designated as claudin or claudin-like were retrieved from the amphioxus genome and 14 sequences from *Ciona intestinalis* genome.) However, our analysis was not conclusive: we found that most of the putative claudins from amphioxus and *Ciona* clustered together (Fig. S1), suggesting that the claudins are highly divergent between the three major chordate lineages. It is also possible that our selection of claudin-like sequences from the invertebrate chordates included more divergent members of the PMP-22/EMP/MP20/claudin family in addition to true claudins. This is supported by the fact that four of *Ciona* claudin-like genes and five amphioxus genes demonstrated clear orthologous relationships with different subgroups of vertebrate claudins (Fig. S1); these may represent the only true claudin sequences in these chordates. Further analysis of the genome synteny and protein structures of the claudins from invertebrate chordates is required before the relationships within chordate claudins can be unambiguously resolved. It will also be necessary to determine which of the putative *Ciona* claudin-like proteins localize to the tight junctions.

Several studies examining the roles of claudins during vertebrate embryogenesis have been conducted in recent years; however, our understanding of claudins' role in development is far from complete. The results from studies in zebrafish, chick, mouse and *Xenopus* suggest that claudins are able to influence embryonic morphogenesis through three distinct molecular mechanisms: by effecting changes in the hydrostatic pressure inside the lumens of embryonic structures, by maintaining connections between individual epithelial cells and finally by as yet poorly understood mechanism involving linking the paracellular transport events with intracellular signalling through their interaction with the cytoplasmic plaque (Furuse and Moriwaki, 2009, Gupta and Ryan, 2010).

Claudins influence hydrostatic pressure within the lumens of embryonic structures through their ability to control selective permeability of epithelia. Claudins 4 and 6 were found to be essential for the blastocyst expansion during early mouse development: when Cldn4 and 6 activity was inhibited, blastocoel failed to form (Moriwaki *et al.*, 2007). Morpholino-mediated inhibition of Claudin 15 during zebrafish development resulted in multiple lumens forming in the gut instead of a single lumen, suggesting that cation pore property of Cldn15 is essential for the luminal fluid accumulation (Bagnat *et al.*, 2007). Similarly, Claudin 5a is required in the neuroepithelium of zebrafish hindbrain to promote proper expansion of this neural tube region; in its absence the lumen is reduced and morphologically abnormal (Zhang *et al.*, 2010).

Claudin-linked epithelial sheets are essential during diverse

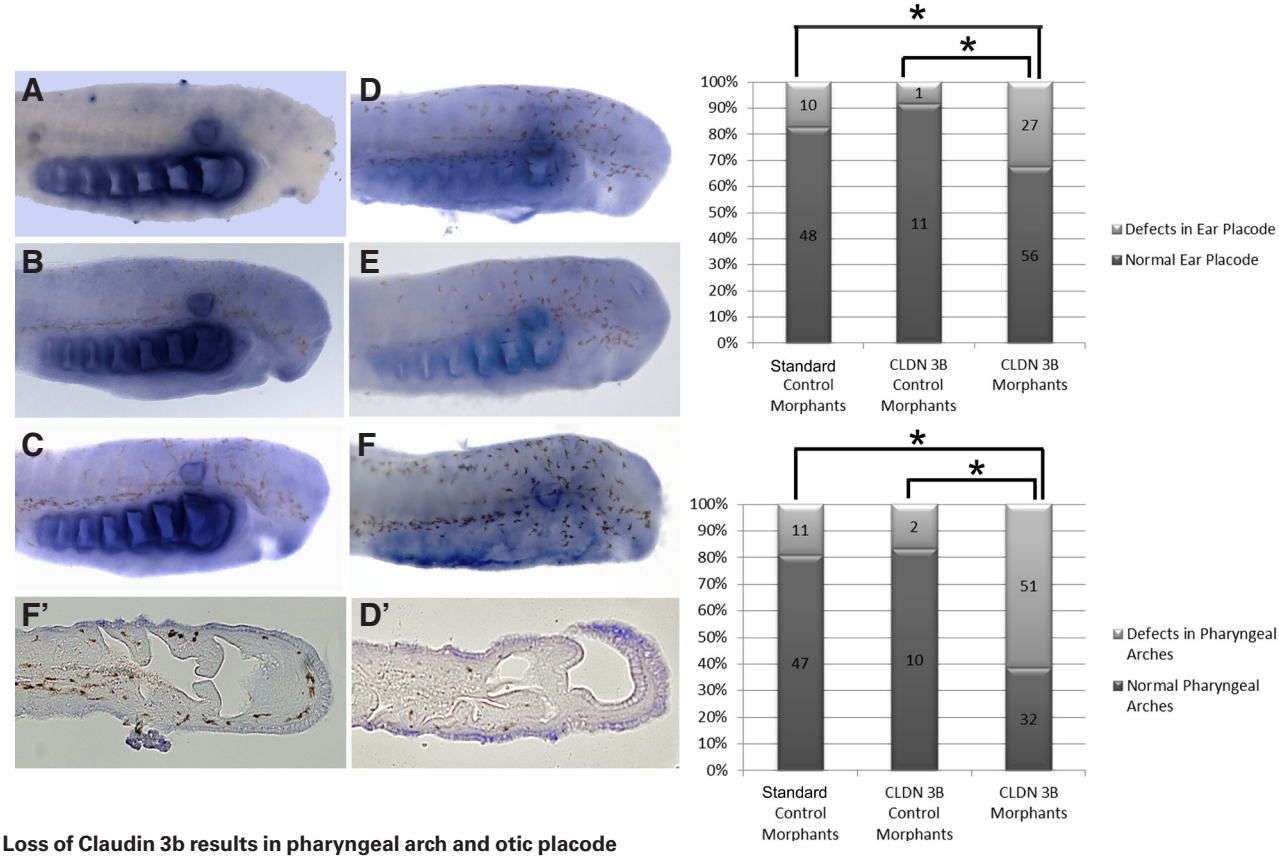

Fig. 11. Loss of Claudin 3b results in pharyngeal arch and otic placode defects. *Cldn3b and control morpholino injected lamprey embryos were allowed to develop to E16 and stained with a SoxE1 probe.* **(A)** *Uninjected lamprey embryo showing normal ear and pharyngeal arch development.* **(B)** *Lamprey embryo injected with a standard control morpholino.* **(C)** *Lamprey embryo injected with a Clnd3b 5-mismatch control morpholino.* **(D-F)** *Embryos injected with a Cldn3b translation blocking morpholino, exhibiting defects in both the otic placodes and the branchial arches* **(D)**, *just the otic placodes* **(E)** *or only branchial arches* **(F)**. *The numbers of the embryos demonstrating the phenotypes are shown in the graphs. Asterisk indicates significant differences ($p<0.01$) between control and experimental morphants by chi-square test.*

morphogenetic events. Zebrafish Claudin E is required for the initiation of epiboly; if it is absent, epiboly is delayed due to reduced tension between the cell layers (Siddiqui *et al.*, 2010). Loss of Claudin 1 in mice results in disruption of skin epithelial barrier, excessive transdermal water loss and death within 24hrs from birth (Furuse *et al.*, 2002).

Our spatiotemporal analysis of the claudin expression throughout lamprey embryogenesis and in early larval stages revealed that many of the claudins are expressed in the developing vertebrate primordia in strong and specific manner. These primordia include both the evolutionary novelties of the vertebrates such as the neural crest, neurogenic placodes and endoskeleton, as well as morphological features that characterize all chordates, such as the notochord, somites, endostyle and pharyngeal arches (Satoh *et al.*, 2014, Shimeld and Holland, 2000). For instance, Cldn18 expression was observed in the premigratory and migrating neural crest. This was surprising, because Cldn18 has not been reported to be expressed during neural crest development in any other vertebrate. Claudin 18 knockout mice are viable and exhibit changes in cell architecture and differentiation of alveolar epithelium, but no neural crest phenotype, suggesting that it is not involved in neural crest development in mice (LaFemina *et al.*, 2014, Li *et al.*, 2014). In chick, claudin 18 expression has been reported in Hensen's node, epiblast, neural and non-neural ectoderm, pharynx, eye and limb ectoderm, but not in the migrating neural crest (Collins *et al.*, 2013). So far, there has only been a single report of any involvement of a claudin (Cldn 1) in neural crest development (Fishwick *et al.*, 2012). Claudin 1 is expressed in chick premigratory neural crest, but is downregulated during the neural crest migration. Morpholino-mediated loss of Cldn1 expression resulted in earlier and more extensive neural crest migration, consistent with the proposed role of Cldn1 is stabilizing the intercellular tight junctions in the dorsal neural tube (Fishwick *et al.*, 2012). The significance of Cldn18 expression in the lamprey migrating neural crest is not clear at present and requires further investigation. It is unlikely that tight junctions are maintained during lamprey neural crest migration, rather Cldn18 might be regulated at the protein level. It is also possible that Cldn18 plays a different role, independent of its function in the tight junctions: several claudins, including Cldn18, have been reported to regulate gene expression and cell differentiation, however the mechanism whereby they are able to do this is still unknown (Gonzalez-Mariscal *et al.*, 2014).

Several lamprey claudins (Cldn3b, Cldn8, Cldn9, Cldn10, Cldn18 and Cldn19b) were co-expressed in the otic placode/developing ear of lamprey embryos. An overlapping subset of claudin genes (Cldn 1, 3, 4, 8, 10, 11, 12, 14, 15, 16) was reported to be expressed during otic development in the chick embryo (Collins *et al.*, 2013, Haworth *et al.*, 2005, Simard *et al.*, 2005). In zebrafish, claudin j, recently reclassified as Cldn8-like (Baltzegar *et al.*, 2013), is expressed during otic placode and vesicle development, and mutation or morpholino-mediated downregulation of Cldnj expression results in deafness due to severely reduced otoliths (Hardison *et al.*, 2005). Mutations in human claudins 1, 14, 16 and 19 and loss-of-function mutations in mouse claudins 9, 11 and 14 are associated with deafness (Gupta and Ryan, 2010). Cldn3, Cldn4 and Cldn6 are expressed during otic vesicle development in E9.0-9.5 mouse embryos (Kollmar *et al.*, 2001). Even though the currently available published data on the embryonic expression and function of claudins is incomplete for most model vertebrate species, comparing our findings to the published data brings us to the conclusion that expression and function of a subset of claudin genes (Cldn 3, 8, 9, 10 and 19) in the otic placode/vesicle is conserved to the base of the vertebrate lineage.

Lamprey claudins 3b, 8 and 9 are expressed in different subsets of neural tube cells during development. In zebrafish, Claudin5a is expressed throughout the midbrain, hindbrain and spinal cord region, and plays a crucial role in brain ventricle expansion by sealing the tight junctions of the neuroepithelial layer and maintaining the hydrostatic pressure within the brain ventricles, which causes their expansion (Zhang *et al.*, 2010). In lamprey, the neural tube initially forms as a solid rod, and the dilated ventricles are not seen until about E10-E12. Judging from the expression pattern, lamprey Cldn9 might play a role in ventricle expansion, while the other two claudins might have other roles in the development of the neurons or glia of the neural tube.

We observed expression of several Cldns in different subsets of embryonic lamprey cartilage. Cldn16 was seen in the intermediate domain of the first pharyngeal arch, Cldn18 in the branchial and hypobranchial cartilage, and Cldn3b and 8 were observed in the mucocartilage of the mouth (lower lip). This observation supports the presence of multiple distinct types of cartilage in the lamprey head (Cattell *et al.*, 2011), and identifies these Cldns as potential novel molecular markers for different subsets of lamprey cartilage. It is of interest that Cldn18 function is skeletogenesis appears to be conserved with higher vertebrates: claudin 18 knockout mice exhibit increased bone resorption and osteoclast differentiation (Linares *et al.*, 2012). To the best of our knowledge, claudin expression during embryonic cartilage development has not been studied in any model vertebrate organism, which makes it difficult for us to make comparisons.

Three of the lamprey claudins (Cldn 3b, Cldn 9 and Cldn16) were expressed during pronephric kidney development. Claudin 3, 9 and 16 expression has not been examined during zebrafish pronephros development (McKee *et al.*, 2014), but *Xenopus* Cldn3 and Cldn16 orthologues are expressed during pronephros development (Raciti *et al.*, 2008). Similarly, Cldn3 is expressed in the developing meso- and metanephric kidney in chick embryos (Collins *et al.*, 2013, Haddad *et al.*, 2011, Haworth *et al.*, 2005). Cldn 3 transcripts and protein were expressed in the developing mouse metanephric kidney, and overexpression of Cldn3 in *in vitro* model of kidney tubule formation promoted tubulogenesis (Haddad *et al.*, 2011). Claudin 16 and Claudin 9 expression was observed in mouse embryonic and neonatal kidney (Abuazza *et al.*, 2006, Khairallah *et al.*, 2014). Additionally, Cldn16 knockout mice and humans with mutations in Cldn16 gene exhibit kidney disease (Gupta and Ryan, 2010). Taken together, this suggests that the original kidney specific functions of Cldn3, 9 and 16 is conserved throughout the vertebrates. However, higher vertebrates recruited a wide range of additional claudin family members that are expressed both during kidney morphogenesis and in the adult kidney. For instance, zebrafish pronephros expresses Cldn8 and Cldn15a (McKee *et al.*, 2014), Cldn4, 6, 8, 14 and 19 are expressed in the embryonic pronephros of *Xenopus* (Raciti *et al.*, 2008, Sun *et al.*, 2015). Cldn 1, 4 and 10 are expressed in the chick mesonephric kidney (Collins *et al.*, 2013). Cldn 7 and 19 are strongly expressed in the developing mouse nephron (Khairallah *et al.*, 2014). Additionally, kidney abnormalities observed in Cldn 2, 4, 7 and 10 knockout mice suggest that these claudins are also

important either for embryogenesis or for the adult kidney function (Gupta and Ryan, 2010). Thus, diversification of the claudin family might have contributed to the evolution of the more complex forms of kidney characteristic of the amniotes.

Morpholino-mediated translation inhibition of Cldn 3b demonstrated that this junctional protein is involved in the development of pharyngeal arches and ear placodes in lamprey embryo. Given that Cldn3b is expressed in a section of pharyngeal endodermal epithelium that is tightly folded to join together individual pharyngeal arches, it is likely that disruption of Cldn3b expression results in loss of tight junctions in that region, and consequent abnormal pharyngeal arch architecture. However, Cldn3b morphants also exhibited lower levels of SoxE1 expression in both pharyngeal arches and ear placodes, suggesting that Cldn3b might play a role in regulation of SoxE1 expression. Further experiments are necessary in order to understand the mechanism of Cldn3b action during lamprey embryo development.

Taken together, our phylogenetic and expression data provides interesting insights into the role of the claudin gene family in vertebrate evolution. Further experiments using non-vertebrate chordates (amphioxus, *Ciona*) are needed to fully understand the significance of tight junctions and their integral proteins claudins in the origin of vertebrate/ chordate characteristics. It would be also interesting to explore the roles of other claudins in the morphogenesis of vertebrate primordia at the functional level. Further work will also focus on resolving the mechanism whereby claudins are able to regulate gene expression.

Materials and Methods

Lamprey claudin clones

Six clones were obtained by heterospecific screening of a directional high quality full-length cDNA library prepared from embryonic day 2-12 lampreys using Superscript Plasmid System from Invitrogen (Carlsbad, CA) (Sauka-Spengler *et al.*, 2007) per standard methods. Three of these (Claudin 3b, Claudin 8 and Claudin 19b) were confirmed to be true lamprey claudins by sequencing. Their full length sequences were deposited into GeneBank with the following accession numbers: KP677499, KP677500, KP677501.

P. marinus claudin sequences and RNAseq data generated as part of the lamprey genome project (Smith *et al.*, 2013) were used to design primers to amplify all known lamprey claudins; these were used as templates for *in situ* probe preparation. Previously published RNAseq data from early embryonic stages (SRX110029-35) (Smith *et al.,* 2013) and testes (SRX104180) (PMID: 22818913) were assembled using trinity (PMID: 21572440).

RNA isolation and RT-PCR

RNA was extracted from 20-50 *P. marinus* embryos at E8, E9, E10, E11, E12, E15 and E18 using the RNAqueous® Total RNA Isolation Kit (Life Technologies) as per the manufacturer's instructions. RNA was also extracted from eye, gill, heart, liver and skin tissue harvested from an adult male *P. marinus* following the same procedure. The RNA was then dehydrated using GENTegra RNA columns (integenX) for transport and storage.

For RT-PCR, the RNA was rehydrated in sterile, nuclease-free water to a concentration of 500 ng/μl, DNase-treated to remove any residual genomic DNA, reverse-transcribed using RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific) and oligo-dT primers (Inqaba Biotechnology, Pretoria, South Africa). The cDNA was subjected to standard PCR (denaturation at 95°C for 3 minutes, followed by 30 cycles of 95°C for 30 seconds, 58°C for 30 seconds and 72°C for 1 minute) using KAPA Taq ReadyMix (KAPA Biosystems). The claudin primers used, as well as positive control primers (RPS9 and GAPDH) (Nikitina *et al.*, 2008) are listed in Table 2. In some cases we observed amplification of weaker additional bands. Every band was excised and sequenced to confirm its identity, and only the band of the expected size (Table 2) corresponded to the correct product.

Lamprey embryo culture

Adult lampreys (*Petromyzon marinus*) were obtained from Hammond Bay Biological Station, Millersburg, MI, USA. The animals were housed in 12°C circulating water tanks at Caltech lamprey facility as previously described (Nikitina *et al.*, 2009a). Lampreys were matured by expanding their daylight cycle and raising the temperature by 1°C per day until 18°C was reached. Eggs and sperm were harvested from gravid females, and *in vitro* fertilization was performed in 100-200ml of 18°C spring water (Sparkletts). After 6 hours when the embryos had undergone the first division, they were transferred to 0.1X MMR (Marc's Modified Ringer's solution) media for long-term culture. For *in situ* hybridization embryos (E4-E32) were fixed with MEMFA (4% formaldehyde, 0.1M MOPS (pH 7.4), 1 mM MgSO4, 2 mM EGTA), dehydrated, and stored in MeOH at -20°C.

In situ *hybridization and sectioning*

In situ hybridization was performed as previously described (Nikitina *et al.*, 2009c). Antisense probes were used as negative controls. After sufficient colour development, the embryos were cryoprotected through sucrose series of increasing concentration, transferred to 7.5% gelatin/15% sucrose/PBS, then mounted in 20% gelatin/PBS and frozen in liquid nitrogen. Cryosections (12-20μm) were collected on Super Frost Plus slides (Thermo Scientific).

Phylogenetic analysis

Protein sequences for all known lamprey, mouse, chick and *Xenopus tropicalis* caludins were aligned using Muscle multiple sequence alignment tool. PhyML was used to build a maximum likelihood phylogenetic tree. The

TABLE 2

PCR PRIMER SEQUENCES

Gene	Primer Sequences	PCR Product Size (Base Pairs)
Claudin 1	F: GAGAGAGGCGGTGGAGGT R: AAGAAACGTCGACCAGCCG	516
Claudin 3a	F: ATCGAGGAGGAGGAGACCAA R: CTGAGTCTCAATGGGCCTCA	501
Claudin 3b	F: TCCATCGTCTTCCACACTCC R: GGGCACTATGGGGTTGTAGA	528
Claudin 8	F: GTTGCTGTGTAGGAGGAGGT R: AGCACTTTCCCCTCTCCATC	411
Claudin 9	F: GTGCACGACTCCATGCTG R: CTGGAGGCCGTAAAAGGG	408
Claudin 10	F: TGTATGGTGATGCTGCGTTG R: TTTCAACCTTGCCGTCACTG	599
Claudin 11	F: GAGTCTCAGCGAGGGGATC R: GTAGCCAAAGTTCACCACCC	580
Claudin 12	F: TCGTGCAAAGGGGTACTCAT R: CGATTCTAGTGCAACCCGTG	946
Claudin 15	F: ACTACTGGAAGGTGTCGACG R: TGTGGTCTCTCTGCGTAGAC	507
Claudin 16	F: CGAATCAAGACCCGCATCTG R: GGTGGTGGTCGTGTTAATGG	447
Claudin 18.1	F: ATGCACGTCGTTGGGTTTG R: CCGAACGAGAACCTGGTGTA	521
Claudin 18.2	F: CGAGGCAAACAATCGGGAAT R: CTTCGCTGGGTTTGGTTTGG	586
Claudin 19a	F: TCGGCCAACATTCAATGCAA R: CACACACGGAAACAAAACGC	582
Claudin 19b	F: CCTGGAGGCAGTGGTATAGG R: CACTCGCCACATACAGAAGC	541
Claudin 20	F: GAACACTCGCCTGCAACTG R: CAAAACCCAGGTACAGCGAG	500
GAPDH	F: CCGTGCAAAAGGAAGACATT R: CTTCCCATCCTCAACCTTCA	134
RPS9	F: GTGGCGTGTCAAGTTCACC R: CATCTTGGACTCATCCAGCA	148

tree was rooted using a distantly related claudin CLC-5 from *Caenorhabditis elegans*. The tree statistical support was calculated using approximate likelihood ratio test, and the final tree was visualized using TreeDyn.

Morpholino injection

FITC-labeled translation inhibiting morpholino antisense nucleotides were purchased from GeneTools (Philomath, OR, USA).The sequences used were as follows:

Standard control morpholino: 5'- CCTCTTACCTCAGTTACAATTTATA-3'
Claudin 3b morpholino: 5'- AGCTGCATGCCCGTGCTCGCCATG-3'
Claudin 3b 5-mismatch control morpholino: AcCTcCATGCCg-GTcCTCcCCATG

The morpholinos were injected into a single blastomere at one or two-cell stage, and the embryos were cultured and collected as previously described (Nikitina *et al.*, 2009b). Embryos were cultured until they reached the desired stages, then fixed, dehydrated and processed as described above. The numbers of embryos displaying normal morphology and abnormalities in the ear placode and pharyngeal arches were counted and chi-square test was performed on the final results.

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