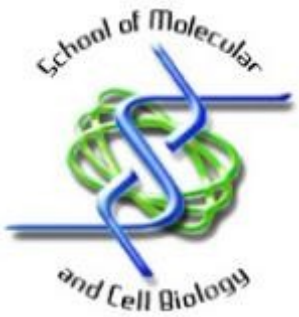

School of Molecular and Cell Biology

University of the Witwatersrand

Johannesburg

Characterisation of Novel Claudin Gene Expression during *Petromyzon marinus* Embryo Development

Master of Science Dissertation

April 2015

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Advisor: Professor Rob Veale

Contents

Declaration

I, Nicholas Dean declare that this Dissertation is my own, unaided work. It is being submitted for the Degree of Master of Science at the University of the Witwatersrand, Johannesburg. It has not been submitted before for any other degree or examination at any other University.

April 2015

Abstract

Claudins are a family of proteins that are conserved amongst all vertebrates, they are integral in the formation and maintenance of the tight junctions between epithelial cells. Claudins are implicated in embryo morphogenesis, vertebrate evolution, solute movement, cell-cell adhesion, designation of cellular and tissue identity, and several diseases when mutated. *Petromyzon marinus* (the sea lamprey) is the most basal extant vertebrate and is a model organism in both developmental and evolutionary biology for this reason. In this study, the expression patterns and functions of novel claudin genes in *P. marinus* were examined with the aim of discovering more about the role of claudins in vertebrate evolution. Presumptive claudin genes in *P. marinus* were compared to all known claudins in the NCBI database. Primers were designed against all known *P. marinus* claudin genes and RT-PCR was performed in order to determine their expression levels at embryonic stages E8 to E18, as well as in adult eye, gill, heart, liver and skin tissues. Probes were designed against *Claudin 1a*, *Claudin 9*, *Claudin 10* and *Claudin 19b* and RNA *in situ* hybridisation was performed on embryos at developmental stages E4 to E31 in order to determine their spatial expression patterns. Areas of common claudin gene expression appear to include the pharyngeal arches, otic placode, neural tube, notochord and ectoderm. *Claudin 1a* is uniquely expressed in the lamprey migrating neural crest. Morpholino-mediated gene knockouts were performed on *P. marinus* embryos and the loss of *Claudin 19b* appears to result in abnormal somite morphogenesis.

Dedication

Thank you to my parents for their constant backing and assistance whenever needed.

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Research Output

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"Claudin family of the lamprey, *Petromyzon marinus*." Manuscript in preparation.

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List of Abbreviations

Cldn - Claudin

dff - Dorsal fin fold

DIG - Digoxigenin

ec - Ectoderm

ECM - Extracellular matrix

EMT - Epithelial-to-mesenchymal transition

es - Eyespot

ff - Finfold

FGF - Fibroblast growth factor

hg - Hypobranchial groove

HH - Hamburger Hamiltion (staging series)

MET - Mesenchymal-to-epithelial transition

ml - Marginal layer (of neural tube)

nc - Neural crest

no - Notochord

np - Neural plate (border)

nt - Neural tube

oh - Oral hood

ot - Otic placode

pa - Pharyngeal arches

pc - Pericardial cavity

ph - Pharyngeal cavity

pk - Presumptive kidney

pl - Presumptive liver

RT-PCR - Reverse transcription PCR

sg - Subpharyngeal gland

so - Somites

st - Stomodeum

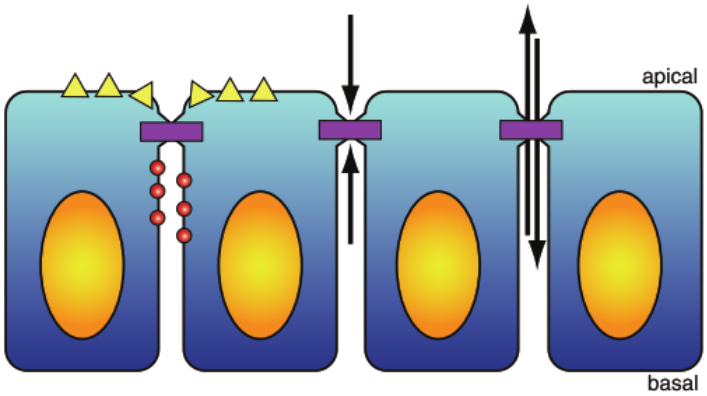
vff - Ventral finfold

1 Introduction

1.1 Claudins

Claudins were first described by Furuse *et al*. in 1998. The name "claudin" is derived from the Latin word "claudere" which means "to close". This name was given to claudins because of the role as a barrier between epithelial cells that they were discovered to have (Furuse *et al*., 1998).

Claudins are a family of proteins that are integral components of tight junctions (Gupta and Ryan, 2010) and are the major structural components of the tight junction (Günzel and Yu, 2013). Claudins comprise a major component of the paracellular barrier and in doing so, control the flow of molecules in the intracellular space that is between the cells of the epithelium (Anderson and Itallie, 2009). Claudins make up both paracellular barriers and pores (small openings in the paracellular matrix) that control the permeability of epithelial as well as endothelial cell sheets (Günzel and Yu, 2013).



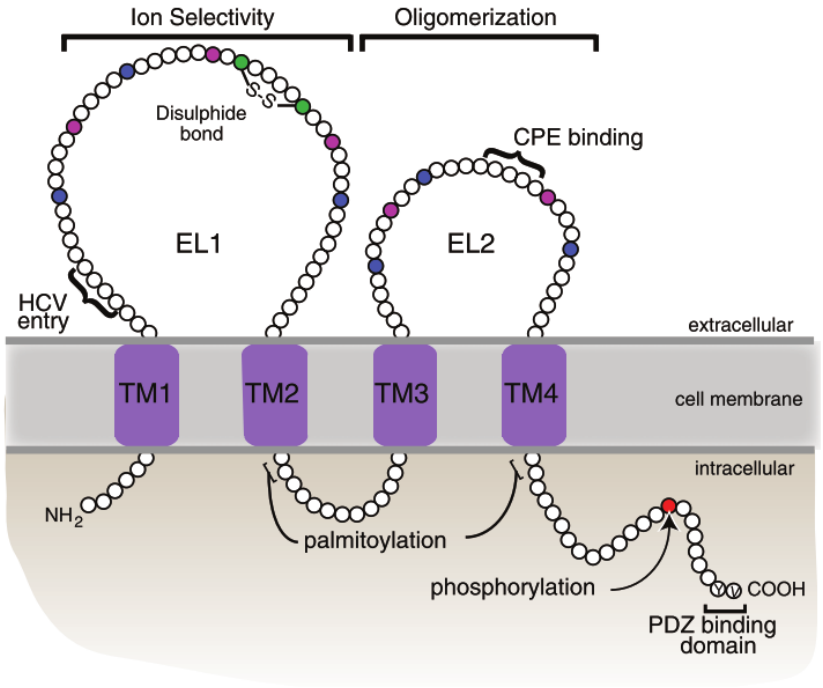
(Gupta and Ryan, 2010)

Figure 1.1: Structure of the tight junction.

The apical position of claudins within the tight junction is indicated by the purple rectangles. Tight junctions can act as barriers that prevent components of the apical and basal cell membrane from mixing (far left) as well as preventing the movement of ions between the paracellular space (center). Tight junctions can also be leaky, in which case they can allow for the movement of certain fluids and molecules through the paracellular space (far right).

Claudins are relatively small transmembrane proteins that range from 20 to 34 kilodaltons in size. They cross the cellular membrane four times with the N and C-terminal ends of the proteins being located in the cytoplasm. The C-terminal end is variable in length and is involved in the localization of claudins to the tight junctions (Anderson and Itallie, 2009).

The N-terminal end is involved in side-to-side oligomerisation of claudins and is therefore implicated in the formation of tight junction strands (Gupta and Ryan, 2010). The cysteins of different claudins can form disulphide bonds and thus join together in this manner (Anderson and Itallie, 2009). Some claudins are able to participate in both heteromeric (between different kinds of claudins) and homomeric (between similar claudins) interactions (Lal-Nag and Morin, 2009). This infers that there is the potential for diversity of the tight junction strands that can be formed between cells due to the interactions between different claudins. This would mean that different claudin combinations could allow for a high degree of variability, complexity and selectivity of the ions that are allowed to move between the cells and through the intracellular space (Gupta and Ryan, 2010). Claudins contain two extracellular loops which exhibit the highest degree of conservation (Anderson and Itallie, 2009). The loops have also been shown to act as receptors for the Hepatitis C virus and the *Clostridium perfringens* enterotoxin (Lal-Nag and Morin, 2009).



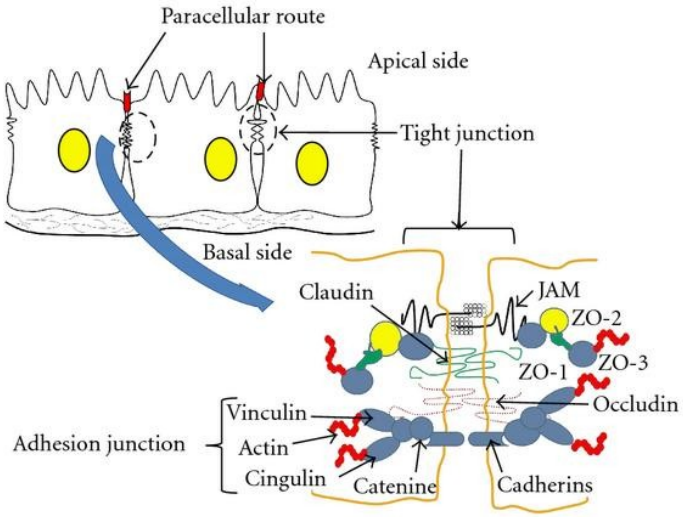
(Gupta and Ryan, 2010)

Figure 1.2: The structure of claudins.

The transmembrane domains (purple rectangles), the extracellular loops (EL 1 and EL 2), PDZ binding domain at the C-terminal end and other structures are illustrated.

Claudins have also been observed to be involved in signal transduction pathways to and from the tight junction. Claudins are also implicated in maintaining cell polarity by separating the

basal and apical membrane surfaces of the cell through the formation of tight junctions (Lal-Nag and Morin, 2009).



(Singh *et al*., 2010)

Figure 1.3: Schematic presentation of tight junction location between the epithelial cells and paracellular transport. Lower part represents tight junction strands and interaction of their major components.

Claudins form the major structural and functional component of the tight junction, however there are also other junctional proteins that are located in the paracellular space. From **Figure 1.3** it is evident that occludins are also located at the tight junction and have been shown to play a similar role to claudins. Similarly to adhesion junctions, tight junctions are made up of a protein complex that includes cadherins, catenins, actin, cingulin, ZO-1, JAM-1 and viniculin along with occludins and claudins (see **Figure 1.3**).

Claudins, however are not always found at the tight junction (Günzel and Yu, 2013). Claudins that are localized in areas other than the tight junction have been implicated in the formation of adhesive cell contacts (Günzel and Yu, 2013). Claudin 1 has been found to mediate contact between dendritic cells and the epidermis and Claudin 2 has been seen to form contacts between metastatic breast cancer cells and hepatocytes (Günzel and Yu, 2013). Claudins have been observed to interact with cell surface receptors and other adhesion molecules (tetraspanins and integrins) (Günzel and Yu, 2013). Claudins have also been found in the nucleus where they may have a direct role in regulating gene expression (Günzel and Yu, 2013). However, these alternative functions of claudins are still poorly understood.

Claudins are found mainly in vertebrates (all vertebrate genomes contain claudins), however they have also been discovered in some invertebrates such as *Caenorhabditis elegans* and *Drosophila melanogaster*. Vertebrate claudins however, differ from the claudin-like proteins that are found in invertebrates in that they are major structural components in the tight junctions, whereas invertebrates do not possess tight junctions between their cells (Anderson and Itallie, 2009). All human claudins, except for Claudin 12 have been found to contain domains that bind to PDZ (Post synaptic density protein, *Drosophila* disc large tumour suppressor, zonula occludens-1 protein) domains of scaffold proteins (Anderson and Itallie, 2009). PDZ domains are structural domains that are found in most living organisms scaffold proteins and are important regulators that are involved in many cell signaling pathways. In this way claudins are able to modulate interactions within signaling pathways and complexes. Claudins through their interactions with scaffold proteins are able to regulate signal transduction and alter the localization of different signaling pathway constituents within the cell. These interactions are believed to link extracellular transport between adjacent cells with intracellular signaling events and in so doing, modulate morphogenesis, cell polarity, cell proliferation and cellular differentiation (Gupta and Ryan, 2010).

More than 20 distinct claudin family members have been described in most vertebrates (Lal-Nag and Morin, 2009). Claudins are distributed evenly throughout the vertebrate genome with several pairs of claudins being closely linked (such as *Claudin*s *3* and *4*, *6* and *9*, *8* and *17*, *10* and *15* and *22* and *24*) and located close together on the same chromosome (Collins *et al*., 2013). For example, Claudins 8 and 17 are located 3 kb apart on chromosome 1 in the chicken (Collins *et al*., 2013). This suggests that gene duplication has been involved in the expansion and diversity of the claudin family (Gupta and Ryan, 2010).

Mutations in claudin genes have been implicated in many different diseases (Gupta and Ryan, 2010). Although claudins are expressed in all adult endothelial and epithelial tissues, claudin gene mutations in human and mouse models have mostly been associated with diseases of the skin, kidney and ear (Gupta and Ryan, 2010). However, mutations in claudin genes have also been implicated in breast (Kramer *et al*., 2000), colon (Resnick *et al*., 2005) and ovarian cancers (Agarwal *et al*., 2005).

Several claudins are expressed in the human epidermis, including Claudins 1 to 7 (Gupta and Ryan, 2010). Mice that have a *Claudin 1* deficiency have dry skin and die within 24 hours due to excessive water loss. These mice still contain tight junctions in the epidermis, however

the barrier function is disrupted (Furuse *et al*., 2002). *Claudin 1* gene mutations in humans have been linked to autosomal recessive neonatal icthyosis and sclerosing cholangitis syndrome. This disorder is very severe and leads to a rapid death in most cases. The dry skin is associated with impaired epithelial barrier function (Hadj-Rabia *et al*., 2004). Transgenic mice that overexpress *Claudin 6* have a thick, disorganized epidermis which indicates that the epidermal terminal differentiation has been disrupted (Turksen and Troy, 2002). From these examples it is evident that several claudins play important roles in the development and maintenance of epithelial tissues.

Mutations in *Claudin 16* and *19* have been associated with the autosomal recessive disorder familial hypomagnesemia with hypercalciuria and nephrocalcinosis (Lal-Nag and Morin, 2009). Claudins 16 and 19 interact with tight junctions in the thick ascending limb of the nephron and form a pore that is cation-selective. Mutations in these genes result in an unregulated flux of sodium, calcium and magnesium ions between the lumen and interstitium (Gupta and Ryan, 2010). So Claudins 16 and 19 seem to play an important role in regulating sodium, calcium and magnesium ion movement between tissues during early human development.

Claudin 14 mutations have been associated with deafness and have been observed in families that suffer from deafness (Wilcox *et al*., 2001). This is yet another example of the importance of claudins in regulating developmental events and ensuring correct development of important mammalian structures.

Claudins are necessary for the formation of tight junctions and therefore in the structure, development and maintenance of epithelial tissues. Mutations or alterations in claudin genes have been shown to affect the development of vertebrate embryos in several different ways, which could result in congenital disorders or an unviable embryo during development.

1.1.1 The Role of Claudins in the Permeability of Tight Junctions

Tight junctions, also known as zonula occludens or occluding junctions are areas between cells where the cellular membranes join together and form a tight barrier. The barrier that is formed by the tight junction is impermeable to fluids and is therefore implicated in preventing the flow and transport of solutes between cells. Tight junctions are found exclusively in vertebrates (Anderson and Itallie, 2009).

Claudins, along with occludins play an important role within the tight junction. Claudins form a network of transmembrane proteins between cells (Anderson and Itallie, 2009) (see **Figure 1.3**). Claudins are the most important proteins within the tight junction (Lal-Nag and Morin, 2009) and are embedded within the plasma membranes of adjacent cells, with extracellular domains joining adjacent claudins together (Anderson and Itallie, 2009) forming claudin strands between cells. Each strand can act independently and in this way, the number of strands between adjacent cells can affect the movement of ions and solutes between the cells (Lal-Nag and Morin, 2009).Therefore the more claudins localized to the intracellular space between cells, the lower the permeability of the intracellular space to fluids and therefore ions and solutes. Claudins are able to associate with different peripheral membrane proteins that are localized on the intracellular side of the plasma membrane. These proteins (such as zonula occludens protein-1) are involved in anchoring the claudin strands to the actin component of the cytoskeleton (Anderson and Itallie, 2009). So in this way, tight junctions are able to join the cytoskeletons of adjacent cells.

In addition to acting as a selectively permeable barrier between different tissues or environments, the tight junction is also able to act as a fence within the cell membrane (Günzel and Yu, 2013). This "fence" function serves to mechanically restrict the movement of proteins and lipids around the lipid bilayer (Günzel and Yu, 2013). This ensures that the different components of the cell membrane remain separate at the tight junction so that apical and basolateral domains can be formed (Gupta and Ryan, 2010). This is important for directional transepithelial transport (Günzel and Yu, 2013). The barrier and fence functions of claudins appear to be independent of each other (Günzel and Yu, 2013).

The transmembrane protein component of claudins seem to be functionally integral to the barrier, pore and fence functions of the tight junction (Günzel and Yu, 2013).

Tight junctions have several important functions that include holding cells together and forming barriers between cells. Tight junctions are able to maintain the polarity of cells by preventing the diffusion of membrane proteins between the apical and basal cellular surfaces. This allows for the cell to have different functions for the basal, apical and lateral surfaces (Anderson and Itallie, 2009). If a molecule is to pass through one of these cells, it has to do so through either diffusion or active transport, so in this way the tight junction aids in the selectivity of molecule/solute transport through specific tissues (an example of this is the blood-brain barrier which is highly selective in terms of its permeability) (Gupta and Ryan,

2010). It has been discovered that tight junctions play an important role in embryonic morphogenesis by holding cells together, regulating the flow of different molecules between different tissues and through modulating the movement of fluids between different areas in the developing embryo.

1.1.2 The Role of Tight Junctions in Epithelia

Epithelium is a basic form of tissue that lines the cavities and surfaces of structures throughout the body of animals. Epithelial cells are generally tightly stacked together with very small intracellular spaces; the cells are attached to each other at multiple areas by tight junctions, desmosomes or adherens junctions. Epithelia require the diffusion of substances through the basement membrane in order to remain nourished and therefore viable. The epithelial basement membrane is implicated in the structure, adhesion and the selective permeability of the cell (Marieb, 1995). An important function of epithelia is to act as a physical and chemical barrier between different tissues or environments (Günzel and Yu, 2013).

The cellular junctions within epithelial tissues are comprised of several protein complexes that allow for contact between adjacent cells and between cells and the extracellular matrix (Marieb, 1995). One of these protein complexes that is integral in joining together epithelial cells is the tight junction. The tight junction is also implicated in forming a paracellular barrier of epithelial cells that aids in the control and selectivity of paracellular transport (Anderson and Itallie, 2009).

Epithelia are designated as being either tight or leaky and this is dependent on the ability of tight junctions to prevent fluid and solute movement between the intracellular spaces. Tight epithelia have tight junctions that prevent the movement of most molecules between cells some examples of tight epithelia include the distal convoluted tubule of the nephron in the kidney and the bile ducts in the liver. Tight epithelia are therefore found in tissues and structures that require an impermeable barrier. Leaky epithelia conversely do not have tight junctions or have less complex tight junctions. For example, the tight junction in the kidney proximal tubule is very simple and is inherently very leaky (Anderson and Itallie, 2009).

Tight junctions are involved in holding epithelial cells together, maintaining the structure and permeability of epithelial tissues during development and in adulthood and are therefore an integral component in vertebrate morphology.

1.1.3 The Role of Claudins in Embryo Morphogenesis

Claudins have been implicated in many different morphological processes during embryogenesis such as gastrulation, neurulation, neural crest migration and somitogenesis. However, claudins are also involved in many other isolated morphological events in the developing vertebrate embryo as well. For example, sertoli cells in *Claudin 11* null mice have been shown to continue to express differentiated cellular markers and later detach from the basement membrane and slough off into the lumen (Morital *et al*., 1999). This demonstrates that Claudin 11 is important in stabilizing the epithelial cell integrity in tight junctions. Therefore, Claudin 11 is necessary for the correct differentiation, development and viability of mouse embryos.

1.1.3.1 Gastrulation

Epithelia are formed early on in embryonic development and play an integral role in the growth and development of the embryo. Epithelial layers play a central role in many embryonic morphogenetic processes; epithelial cells go through extremely coordinated shape changes during the development of the embryo (Quintin *et al*., 2008). During invagination, a sheet of epithelial cells bends inward to form an indent. Invangination of the ectodermal germ layer during gastrulation leads to the formation of the endoderm and mesoderm, which are the integral germ layers in the early developing embryo. Epithelial tissues are derived from all three of the embryonic germ layers (ectoderm, mesoderm and endoderm) (Campbell *et al*., 2008). So, epithelia are essential in the formation and development of all structures in the embryo.

The fact that epithelia play such an integral role in embryo morphogenesis, also implicates the tight junction in embryonic morphogenesis. The tight junction plays an integral role in regulating the permeability of epithelia during early development, therefore modulating the movement of signaling molecules that are involved in the designation of tissues between

different areas in the embryo. Tight junctions also modulate the structure and movement of epithelia during embryo morphogenesis and the formation of new body structures.

The fact that epithelia and therefore tight junctions are so strongly implicated in embryo morphogenesis, also implicates claudins strongly in this process. As claudins are the most important and abundant proteins in the tight junction, they are also responsible for the adherence and permeability of different epithelial tissues during embryo morphogenesis.

Claudins are expressed in epithelial and endothelial tissues both in the adult and in the embryo; most of the time, multiple claudins are being expressed in each cell. During embryogenesis, boundaries of claudin gene expression are formed and these boundaries correspond to sites where inductive interactions are involved in patterning of the embryo. The overlapping areas of claudin expression that have been observed in the chick embryo may designate variably distinct domains of ion permeability within the early epiblast and in ectodermal, mesodermal and endodermal derivatives that could influence embryonic patterning and morphogenesis during development (Collins *et al*., 2013). For example, Collins et al. (2013) discovered that a subset of claudins exhibit a differential expression across the epiblast that corresponds to the boundary between the non-neural ectoderm and the presumptive neural plate in a horseshoe shape at the anterior of the developing embryo. Many boundaries of claudin expression were described in this study and it was discovered that many claudins are co-expressed, which suggests that different claudin expression could be co-regulated by a similar group of transcription factors (Collins *et al*., 2013). Boundaries of claudin expression were observed in the epithelial derivatives of the skull region, feathers and internal organs (Collins *et al*., 2013). A variable combinatorial code of claudins in different tissues is involved in the maintenance of microenvironments that are required for development, morphogenesis and normal patterning of the epithelia early on in chick development (Collins *et al*., 2013).

During morphogenic processes that require epithelial to mesenchymal transition (such as gastrulation and neural crest migration), claudin expression is turned off in cells that leave the epithelium to form the mesenchymal layer (Gupta and Ryan, 2010). This indicates that claudins are strongly implicated in, and indeed necessary for the formation and maintenance of normally functioning epithelial tissue.

During embryonic morphogenesis, cell layers move and extend, while cellular shape and contacts are maintained (Gupta and Ryan, 2010). It has been discovered that during zebrafish

gastrulation, Claudin E is needed in order to maintain the tight junction contacts between the different cell layers that are undergoing migration (known as epiboly) (Siddiquil *et al*., 2009). If the *Claudin E* gene is not present, the initiation and progression of epiboly is delayed (Siddiquil *et al*., 2009). Epiboly is an important cellular movement that occurs in the early embryo at the same time as gastrulation, and is important for the physical restructuring of the embryo and therefore its viability (Campbell *et al*., 2008). So it is evident that Claudin E is integral for the morphogenesis of the early zebrafish embryo.

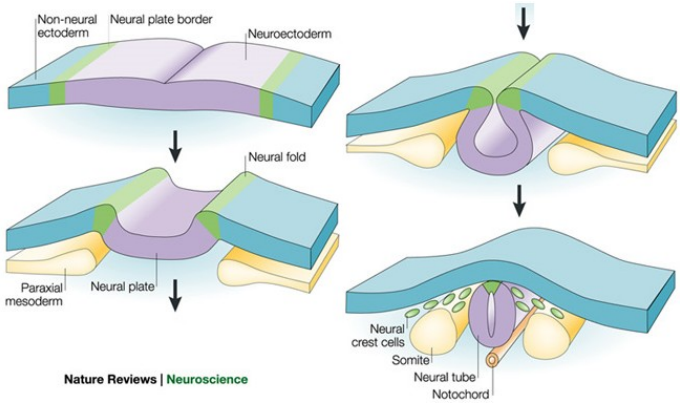
Removal of *Claudin 4* and *Claudin 6* from the trophectoderm of early mouse embryos resulted in the collapse of the blastocyst which prevented any further embryonic development. This indicates that *Claudin* 4 and *Claudin* 6 are strongly implicated in aiding tight junctions in the trophectoderm in maintaining the blastocoel shape through hydrostatic pressure in mouse embryos (Moriwaki *et al*., 2007). The blastocoel is the first cell cavity that is formed as the embryo enlarges and it is essential for the subsequent gastrulation of the embryo (Campbell *et al*., 2008). *Claudin* 4 and 6 are therefore integral in the development and viability of early mouse embryos.

In a study conducted by Collins *et al*. (2013), in an attempt to map the temporal and spatial expression of claudins in the chick embryo it was discovered that all of the screened claudins with the exception of *Claudin 5* were expressed in the epiblast (gives rise to the three germ layers). *Claudins 2*, *4*, *10*, *15*, *17* and *23* were found to be expressed in the mesoderm (Collins *et al*., 2013). *Claudins 1*, *2*, *4*, *8*, *10*, *11*, *14*, *15*, *16*, *17*, *18* and *23* expression was observed in the endoderm (Collins *et al*., 2013). *Claudins 2*, *4*, *10*, *14*, *15*, *16*, *17*, *18* and *23* are expressed in Hensen's node (the organiser for gastrulation) (Collins *et al*., 2013). All screened claudins were seen to be expressed in the extraembryonic membrane during gastrulation (Collins *et al*., 2013). From these findings, it is clear that claudins play a crucial role in the process of gastrulation in the developing chick embryo.

1.1.3.2 Neurulation

Claudins have been implicated in the process of neural plate formation and neurulation during chick development (Collins *et al*., 2013). The notochord is the first organ to form in the developing embryo, followed by the neural plate. There is a primary (neural plate bends inwards until the edges contact and fuse) and secondary (hollowing out of the neural tube)

neurulation (Campbell *et al*., 2008). The ectodermal cells that cover the notochord on the dorsal part of the embryo ultimately form the neural plate. The ectoderm above the notochord is designated identity as the neural plate following signalling from molecules secreted by mesodermal and other tissues (Campbell *et al*., 2008). Primary neurulation occurs in response to signalling from soluble growth factors that are secreted from the notochord. After formation, the edges of the neural plate thicken and hinge upwards to form the neural folds (see **Figure 1.4**). A groove forms between these folds called the neural groove (see **Figure 1.4**). The neural folds move towards each other and fuse, this fusion requires the regulation of cell adhesion molecules (such as claudins). The joining of the neural groove into a cylindrical shape is mediated by this fusion of the neural folds (Campbell *et al*., 2008). This cylinder of cells forms a chord-like structure that later migrates inwards and hollows out to form the neural tube (see **Figure 1.4**). During this process, the presumptive neural crest forms from a thin band of ectodermal cells on either side of the neural plate (neural plate border) (Nikitina *et al*., 2009).



(http://www.nature.com/nrn/journal/v4/n10/fig_tab/nrn1219_F1.html)

Figure 1.4: **The process of neurulation** (the figure has been edited).

Failure of the neural tube to close anteriorly results in anencephaly, which is a condition characterised by forebrain and skull degeneration (Campbell *et al*., 2008). Failure of the posterior neural tube to close is known as spina bifida, which is a disorder that is characterised by failure to form the neural plate (Campbell *et al*., 2008). Less severe deformations are characterised by defects in the meninges and vertebrae over the posterior spinal cord. Severe neural tube defects are almost always fatal (Campbell *et al*., 2008). So loss of certain claudin expression could potentially result in these disorders.

The process of neural tube formation in the zebrafish *Danio rerio* is very similar to amniote and amphibian neurulation, and so it is a good model for studying brain morphogenesis in vertebrates (Zhang *et al*., 2012). The formation of the primary brain ventricle occurs rapidly in two steps (brain ventricle opening and expansion) (Zhang *et al*., 2012). The neural tube first opens into the fore, mid and hindbrain ventricle (Zhang *et al*., 2012). The opening of the neural tube is dependent on the morphogenesis of a polarised neuroepithelium, maintenance of a Na^+, K^+ gradient and increased cell proliferation (Zhang *et al*., 2012). The expansion of the brain ventricle is dependent on heartbeat and circulation (Zhang *et al*., 2012). During the ventricular expansion, the neuroepithelial cells are connected by adhesion and tight junctions (Zhang *et al*., 2012). The junctions join the cells together and form a barrier between the interior and exterior of the neural tube (Zhang *et al*., 2012). Tight junctions are the most important and abundant cell-cell contact structures between the neuroepithelial cells at this stage (Zhang *et al*., 2012). Loss of Claudin 5a in zebrafish embryos resulted in a reduction in tight junction permeability at the neuroepithelial cell layer (Zhang *et al*., 2012). These mutants did not form inflated brain ventricles during early embryogenesis (Zhang *et al*., 2012). The blood-brain barrier was greatly affected in terms of barrier permeability of smaller molecules in these mutants (Zhang *et al*., 2012). Claudin 3 is found in the endocardium and endothelium and together with Claudin 5, it may be involved in maintaining circulation in the developing embryo that is required for the process of brain ventricular expansion (Zhang *et al*., 2012).

Expression of *Claudin 3* and *Claudin 5* is also observed in the endothelial cell junctions of the blood-brain barrier in the mouse and human (Zhang *et al*., 2012). This indicates that these claudins play a similar role following neurulation in mammals as they do in the zebrafish.

During neurulation in the chick embryo, *Claudins 2*, *4*, *8*, *10*, *14*, *15*, *16*, *17*, *18* and *23* are expressed in the neural ectoderm (Collins *et al*., 2013). All screened claudins have been seen to be expressed in the non-neural ectoderm with the exception of *Claudin 5* (Collins *et al*., 2013). *Claudin 1*, *2*, *3*, *4*, *8*, *14*, *15*, *16*, *17*, *18*, *22* and *23* expression is observed in the pharynx (Collins *et al*., 2013). *Claudins 2*, *10* and *14* are expressed in the heart during neurulation in the chick (Collins *et al*., 2013). *Claudin 2* is expressed in the somites, whereas *Claudin 5* is expressed in the vasculature of the neurulating chick embryo (Collins *et al*., 2013). From these findings, it is clear that claudins play a key role in neurulation in the chick embryo.

1.1.3.3 Neural Crest Migration

Following neurulation, the neural plate border cells are specified by neural plate border specifiers (Meulemans and Bronner-Fraser, 2004). Once the neural crest cells have been specified, the neural crest specifiers activate the expression of effector genes, which confer certain properties such as migration and multipotency to the cells (Meulemans and Bronner-Fraser, 2004). Delamination of the neural crest cells then occurs via regulation of cell morphology and adhesive molecules (such as claudins) (Meulemans and Bronner-Fraser, 2004). The delamination process involves the cells undergoing an epithelial-to-mesenchymal transition (EMT), during which the tight junctions are dismantled (Fishwick *et al*., 2012). The downregulation of *Claudin 1* has been implicated in the dismantling of the tight junctions in the apical neuroepithelium of the chick embryo (Fishwick *et al*., 2012). During the EMT, the premigratory neural crest cells depolarise, dismantle cellular junctions and rearrange cytoskeletal properties in order to facilitate the migration process (Fishwick *et al*., 2012). The neural crest cells delaminate and migrate from the dorsal neural tube to different regions of the embryo going on to form different structures in the head, peripheral nervous system and cardiovascular system (Nikitina *et al*., 2009). The cranial neural crest migrates dorsolaterally and goes on to form the craniofacial mesenchyme (Le Douarin and Kalcheim, 1999). The craniofacial mesenchyme differentiates into a range of cranial ganglia and craniofacial cartilage and bones (Le Douarin and Kalcheim, 1999). The cranial neural crest cells enter the pharyngeal pouches and arches where they go on to form parts of the thymus, middle ear and jaw bones, as well as portions of the of the tooth primordia (Le Douarin and Kalcheim, 1999). The trunk neural crest gives rise to the melanocytes which migrate dorsolaterally into the ectoderm, as well as the dorsal root ganglia, sympathetic ganglia, adrenal medulla, and the nerves that surrounding the aorta (Le Douarin and Kalcheim, 1999). The vagal and sacral neural crest cells go on to form the ganglia of the enteric nervous system and the parasympathetic ganglia (Le Douarin and Kalcheim, 1999). The cardiac neural crest develops into melanocytes, cartilage, connective tissue, neurons of some of the pharyngeal arches and parts of the heart (Le Douarin and Kalcheim, 1999).

Claudin 1 expression has been implicated in neural crest specification and emigration in the developing chicken embryo (Fishwick *et al*., 2012). Fishwick *et al*. (2012) doscovered that Claudin 1 may play a role in controlling the EMT in premigratory neural crest cells, as well as their ability to exit the neural tube. The overexpression of *Claudin 1* has been shown to

reduce neural crest cell migration, whereas loss of Claudin 1 results in premature migration of the neural crest (Fishwick *et al*., 2012). Claudin 1 plays a vital role in the tight junctions that link the premigratory neural crest cells (Fishwick *et al*., 2012), and it may also play an important part in the reassembling of the tight junctions once the neural crest cells have reached their respective destinations.

1.1.3.4 Somitogenesis

Claudins have been observed to be expressed in the somites of developing *Xenopus laevis* (Raciti *et al*., 2008) and chick embryos (Collins *et al*., 2013). Somitogenesis is the process whereby somites form. Somites are bilaterally paired blocks of mesoderm that form along the anterior-posterior axis in developing embryos (in segmented animals) (Gomez *et al*., 2008). In vertebrates, the somites develop into skeletal muscle, tendons, cartilage, endothelial cells, parts of the skeleton and the dermis (Gomez *et al*., 2008).

Somites form from the paraxial mesoderm in the neurulating embryo (Gomez *et al*., 2008). The paraxial mesoderm extends as the embryo gastrulates (Gomez *et al*., 2008). The notochord then extends from the anterior end of the embryo and the paraxial mesoderm extends along with it (Gomez *et al*., 2008). As gastrulation continues, the paraxial mesoderm buds off and compacts into discrete blocks this process is periodical and is cyclical and oscillatory (in part due to the negative feedback loop between *Notch* and *Lunatic Fringe*), and new somite blocks are formed periodically due to this (Goldbeter and Pourquié, 2008).

The periodic nature of somite formation is also attributed to a "clock-and-wavefront" mechanism which is maintained by the Wnt signalling pathway (Gomez *et al*., 2008). A Wnt/FGF gradient (posterior) opposes an anterior retinoic acid gradient that moves towards the anterior end of the embryo and somites are formed where these gradients meet (Gomez *et al*., 2008). The somites then undergo a mesenchymal-to-epithelial transition in order to form an outer epithelial layer and an inner mesenchymal layer (Gilbert, 2010). Termination of the somitogenesis process is active and associated with cell death in the tail bud (Gomez *et al*., 2008). The cells within the somites later go on to differentiate and form different structures in the body (Gomez *et al*., 2008).

The changes in cellular shape, migration and adhesion are critical for tissue morphogenesis during embryonic development. The mesenchymal-to-epithelial transition (MET) that occurs during somitogenesis in mammals is important for correct patterning of the axial musculoskeletal system (Rowton *et al*., 2013). The MET establishes the metameric patterning of the axial musculoskeleton, as well as influences the migration of endothelial and neural crest cells (Rowton *et al*., 2013). Signalling from the overlying surface ectoderm triggers the initiation and maintenance of the somitic MET in the paraxial mesoderm of the developing mouse and chick embryo (Rowton *et al*., 2013). This process is independent of the segmentation of the paraxial mesoderm (Rowton *et al*., 2013). *Wnt6* expression in the surface ectoderm initiates the somitic MET, so in this way initiation of MET occurs downstream of the Wnt signalling pathway (Rowton *et al*., 2013). PARAXIS (a transcription factor) is expressed downstream of Wnt signalling and appears to play an important role in inducing MET in the paraxial mesoderm (Rowton *et al*., 2013). MET is induced by the formation of a sheet of cells via the recruitment of primordial cadherin-mediated adherens junctions at the borders of adjacent cells (Rowton *et al*., 2013). While this is happening, the reorganisation of the microtubule cytoskeleton results in the polarisation of these cells and the assembly of tight junctions at the apical surface of the epithelium (Rowton *et al*., 2013). During this process several claudins are recruited at the tight junctions, most notably *Claudin 11* (Rowton *et al*., 2013). The epithelium is stabilised by the formation of focal adhesions between cell receptors and components of the extracellular matrix (ECM) on the basal surface of the epithelial cells (Rowton *et al*., 2013).

A study conducted by Rowton *et al*. in 2013 concluded that PARAXIS regulated genes that are implicated in MET or EMT, as well as ECM formation and stabilisation, cytoskeletal rearrangements via focal adhesion and tight junction formation and the Wnt signalling pathway (Rowton *et al*., 2013). *Paraxis* is expressed downstream of Wnt signalling, it later goes on to regulate the formation of tight junctions in the somitic epithelia in part, by inducing the expression of *Claudin 11*. The fundamental function of PARAXIS is to maintain the spatial order of the somite compartments through the epithelialisation of the somites during embryogenesis, as well as the maintenance of an epithelialized dermomyotome (Rowton *et al*., 2013). Claudin 11 has been observed to be upregulated 2.3 fold in the presomitic mesoderm and somitic tissues following the upregulation of *Paraxis* (Rowton *et al*., 2013). Claudin 11 has been implicated in cellular adhesion and repulsion, as well as the

structural and homeostatic maintenance of the epithelia during, and post MET in the somites of the mouse (Rowton *et al*., 2013).

It has been shown in the past that ECM composition and adhesion proteins (such as claudins) at focal adhesions (such as tight junctions) are important for proper somitogenesis (Rowton *et al*., 2013). It is now clear that claudin gene expression is vital for normal embryological development, especially where epithelial structures are involved in morphological processes. Notably, Claudin11 seems to be important for maintaining the epithelia during and after the MET in vertebrate somitogenesis.

1.2 Evolutionary Expansion and Diversification of Vertebrate Claudins

Claudins are integral to vertebrate development and homeostatsis within the embryo (Baltzegar *et al*., 2013) and therefore have played a crucial role in vertebrate evolution. The full number of claudin genes in vertebrates is still unknown and new more divergent claudins are still being discovered (Baltzegar *et al*., 2013).

It is important to enclose body compartments in animals in order to modulate structure and function. Tight junctions appear to have evolved relatively recently, so there must have been some kind of claudin-like protein that predates vertebrate evolution. Claudins are present in all vertebrates and in invertebrate tunicates, they are however not found in the cephalochordate *Branchiostoma* (Günzel and Yu, 2013). More basal invertebrates have septate junctions, which are a functional alternative to claudins (Günzel and Yu, 2013). Septate junctions are present in cephalochordates, hemichordates and echinoderms with no presence of any claudin genes (Günzel and Yu, 2013). However, there have been discoveries of tight-junction-like structures in arthropods (Günzel and Yu, 2013). So tight-junctions either evolved from septate junctions and the tight-junction-like structures and tight-junctions evolved independently of each other, or septate junctions and tight-junctions evolved completely independently of one another. The previous seems to be more likely due to the absence of true claudins in some cephalochordates and lower invertebrates and the huge diversity of claudins throughout vertebrates.

Teleost fish are the largest and most diverse group of vertebrates that comprise almost 50% of all known living vertebrate species (approximately 27000) (Kolosov *et al*., 2013). Teleost

fish are characterized by the presence of a moving jaw. Fish have an extremely large number of claudins. Fifty-six claudins have been discovered in *Fugu rubripes* (Loh *et al*., 2004), with a total of 63 claudins found in teleost fish (Kolosov *et al*., 2013). Although this number is misleading as it is probably due to a tandem gene duplication event that occurred independently as well as the whole genome duplication event in teleost fish (Loh *et al*., 2004). The diversification of claudin genes in fish may be due to the need for fish to osmoregulate and adapt to different water conditions. Fish that move between fresh and salt water environments have been shown to undergo changes in tight-junction composition especially in the skin, gills, intestine and kidney; as these tissues are subjected to major changes in ionic and osmotic concentration gradients between the two different types of environments (Günzel and Yu, 2013).

1.2.1 Expression and Function of Claudins in Adult Teleost Fish Tissues

Of all of the claudins reported in teleost fish, approximately 70% are found in either the brain or eye (Kolosov *et al*., 2013). This indicates that Claudins play an integral role in development, maintenance and diversification of both brain and eye structures.

Thirty-two claudins are expressed in the gills of *F. rubripes* (Loh *et al*., 2004), which indicates a strong correlation between claudin expression and normal development and maintenance of the gill. The gill is a highly complex organ that is implicated in respiration, osmoregulation, maintenance of homeostasis and waste excretion in fish. The gill epithelium is in direct contact with the external environment, which often differs hugely from the internal environment within the fish. The gill epithelium is therefore an integral barrier against the external environment. Therefore these 32 claudins, in maintaining the barrier between the gill epithelium and the external environment are vital in development, maintenance and diversification of the gill structures in fish.

Twenty-four claudins have been found to be expressed in the intestinal lining of *F. rubripes* (Loh *et al*., 2004). The intestine, especially in salt water fish acts to regulate the salt/water balance. Salt water fish consume and desalinate salt water within the gut to some extent and the presence of these 24 claudins may be to aid the transport of water and the removal of salts from water that is assimilated in the intestine.

Thirty-five claudins have been seen to be expressed in fish kidney tissue (Kolosov *et al*., 2013). Kidneys serve to maintain homeostasis through the elimination of excess water and the reabsorption of ions if necessary. This is particularly applicable in salt water fish, where excess Na^+ and Cl^- ions will be excreted in a highly concentrated urine, whereas fresh water fish will retain these ions and secrete a relatively dilute urine. The kidneys also aid in excretion of waste products. The barrier function of tight-junctions and therefore claudins within the kidney tissues facilitates this process and ensures the maintenance of homeostasis within the fish. For these reasons, it is evident that through evolution, these 35 claudins have moulded the development and structure of the fish kidney so that it is able to adapt to a wide range of environments.

Twenty-five claudins are expressed in the epidermis of *F. rubripes* (Loh *et al*., 2004). The epidermis comes into direct contact with the environment and is vital for the maintenance of homeostasis. The epidermis also plays an important role as a barrier against pathogens and infection. Changes in epidermal claudin mRNA abundance may aid *Tetradon* (a teleost fish) species in adapting to salt water or fresh water conditions by modulating changes in the barrier properties in response to these different environments (Kolosov *et al*., 2013). It is evident that through evolution, these 25 claudins have moulded the development and structure of the fish epidermis so that it is able to adapt to a range of environments.

There are currently 27 known mammalian claudins (Günzel and Yu, 2013), each with a unique expression pattern and function. Similarly to teleost fish, normal claudin expression has been shown to be vital for normal eye, brain, kidney and skin development and function in mammals.

Due to the constant discovery of new genes, claudins are being incorrectly classified and designated (Baltzegar *et al*., 2013). For these reasons, it is difficult to classify and compare claudins from different vertebrate species. However, Baltzegar *et al*. (2013) have made a concerted effort to revise and reorganize the phylogeny of the claudin gene family. Baltzegar *et al*. (2013) drew heavily on data published previously by Loh *et al*. (2004) as this is one of very few fully annotated claudin gene sets in any organism (*F. rubripes*). For these reasons (as well as the closest phylogenetic relationship to *P. marinus* out of any other model organisms that have currently been studied), the data discovered by these two teams has been included as a reference for claudin gene expression. The data is summarized in the **Table 1.1** on the following page.

Claudin genes are almost ubiquitously expressed amoungst *F. rubripes* and *D. rerio* (**Table 1.1**). It has been shown that claudin diversity has been increased due to tandem gene duplication in fish (Baltzegar *et al*., 2013). Whole genome duplication has also had a major impact on claudin diversity in teleost fish (Baltzegar *et al*., 2013; Collins *et al*., 2013). If the tandem gene duplication of claudins occurred before the whole genome duplication events, this would explain the huge diversity within the claudin gene family. For this reason, further observation of claudin genes in more basal fish taxa (such as *P. marinus*) is required in order to understand alternative mechanisms of genomic addition and evolutionary diversification and expansion of the claudin family (Baltzegar *et al*., 2013) .

Claudin gene expression and function appears to vary between vertebrates, even those that are phylogenetically similar such as *F. rubripes* and *D. rerio*. Further study in other vertebrate model systems is required in order to fully understand the role that claudins have played throughout vertebrate evolution.

The expression of claudin genes in adult *Danio rerio* and *Fugu rubripes* tissues is summarised in **Table 1.1** on the following page in order to act as a reference for the localisation of claudin gene expression in jawed fish.

Table 1.1: RT-PCR expression data of claudin genes in *F. rubripes* (Hwee Loh *et al*., 2004) and *D. rerio* (Baltzegar *et al*., 2013) adult tissues.

Claudin Gene	Region of Expression in *Fugu rubripes*	Region of Expression in *Danio rerio*
1	Eye, gill, skin.	Gill, skin.
2	Brain, eye, heart, kidney, liver.	Spleen, testis.
3	Brain, eye, gill, heart, intestine, kidney, liver, skin.	Gill, heart, kidney, spleen, skin, testis.
5	Brain, eye, gill, heart, intestine, kidney, liver, muscle, ovary, skin, spleen, testis, embryo.	Brain, gill, heart, kidney, spleen, skin, testis.
6	Gill, heart, intestine, liver.	---
7	Brain, eye, gill, heart, intestine, kidney, liver, muscle, ovary, skin, testis.	Brain, eye, gill, heart, kidney, spleen, skin, testis.
8	Brain, eye, gill, intestine, kidney, skin.	Brain, gill, kidney, spleen, skin, testis.
10	Eye, gill, intestine, kidney, skin.	Gill, kidney, spleen, testis.
11	Brain, heart, kidney, liver, testis.	Brain, gill, heart, kidney, spleen, skin, testis.
12	Brain, eye, gill, heart, intestine, kidney, liver, muscle, ovary, skin, spleen, testis.	Brain, gill, heart, kidney, spleen, skin, testis.
13	Gill.	---
14	Eye, gill, heart, intestine, kidney, liver, testis.	---
15	Intestine, kidney.	Spleen, testis.
18	---	Kidney.
19	Brain, gill.	Brain.
20	Brain, gill, heart.	Brain, gill, kidney, spleen, testis.
23	Brain, eye, gill, heart, intestine, kidney, liver, muscle, skin, testis.	Brain, gill, heart, kidney,spleen.
25	Eye, intestine.	Brain, gill, heart, kidney, spleen, skin.
26	Brain, eye, gill, heart, intestine, kidney, liver, muscle, ovary, skin, spleen, testis.	---
27	Brain, eye, gill, intestine, kidney, liver, skin, embryo.	---
28	Eye, gill, heart, intestine, kidney, muscle, ovary, skin, testis.	Brain, eye, gill, spleen, skin.
29	Brain, gill, intestine, ovary, testis.	Spleen, testis.
30	Brain, eye, gill, heart, intestine, kidney, liver, muscle, ovary, skin, testis.	Brain, eye, gill, kidney, spleen, skin, testis.
31	Brain, eye, gill, heart, intestine, kidney, liver, muscle, skin, testis.	Brain, skin.
32	Brain, eye, gill, heart, intestine, kidney, liver, muscle, skin, testis.	Brain, gill, heart, spleen, skin, testis.
33	Gill, embryo.	Brain, gill, skin, testis.
35	---	Brain, spleen.
36	---	Spleen, skin.

The table above shows a summarized version of data published by Loh *et al*. (2004) and Baltzegar *et al*. (2013). The *D. rerio* dataset only includes expression data within brain, eye, gill, heart, kidney, spleen, skin and testicular tissue as RNA was only extracted and amplified from these tissues. The data shows many similarities in expression (as is expected), as well as some differences which may infer incorrect classification of gene orthologues or a rapid change in function of these genes through evolutionary events.

1.2.2 *Claudin 1*

Claudin 1, like all other claudin genes encodes a membrane protein that is an integral component of the tight junction strands. *Claudin 1* is expressed in most tissues throughout the body (Günzel and Yu, 2013). *Claudin* 1 is expressed in the skin epidermis where it plays a vital role as a barrier to solute movement (Günzel and Yu, 2013). Claudin1 has been shown to interact with Claudin 5 and Claudin 3 in humans. Claudins have been shown to play an important role in normal vertebrate development and homeostatsis within the developing embryo (Baltzegar *et al*., 2013). The table below shows an outline of *Claudin 1* gene expression during the development of several vertebrate model organisms. It is intended to clarify the areas of expression *Claudin 1* throughout vertebrate evolution.

Table 1.2: *Claudin 1* spatial gene expression patterns in vertebrate model organisms.

Organism	Region of Expression (Stage of Development)	Citation/s
Fugu rubripes	Eye, gill, skin (Adult).	Loh *et al*., 2004
Danio rerio	Gill, skin (Adult).	Baltzegar *et al*., 2013
Xenopus laevis	No data.	---
Gallus gallus domesticus	Epiblast, endoderm, extra-embryonic ectoderm, neural crest (HH4 – HH7), non-neural ectoderm, pharynx (HH8 – HH12), eye epithelium, otic vesicle, nasal epithelium, limb ectoderm, apical ectodermal ridge, surface ectoderm, feather buds, pancreas, lung, kidney (E3 – E10).	Collins *et al*., 2013 Fishwick *et al*., 2012
Mus Musculus	Nervous system including the brain and future spinal cord (E11.5), renal & urinary system and the epidermis (E12.5), epidermis (E13.5), the alimentary system including the submandibular gland primordium, the renal & urinary system including the kidney, epidermis, the nervous system, esophagus, tongue, tongue muscle, teeth, the skeletal system (E14.5), the nervous sytem including the brain and spinal cord, retina, epidermis (E15.5), the submandibular gland, the renal & urinary system, epidermis (E16.5), the renal & urinary system, epidermis (E17.5), the renal & urinary system (E18.5). Expression in the renal & urinary system and the nervous system is exhibited throughout development, into adulthood.	Magdaleno *et al*., 2006. Ohta *et al*., 2006. Troy *et al*., 2007. Traweger *et al*., 2002. Hashizume *et al*., 2004. Diez-Roux *et al*., 2011.

The expression data was determined mainly via RNA in situ hybridization, but immunohistochemistry, Western blot and RT-PCR assays were also used. The *G. gallus* developmental stages are based upon the *Hamburger Hamilton* staging series. The *Mus musculus* developmental stages are based upon the standard mammalian developmental staging series (Taylor Stage Comparison).

Most of the current expression data on claudins (especially in mammalian models) has been due to concentrating on gene expression in specific tissue types or related to specific

pathologies. For this reason, a truly complete claudin expression profile (especially during embryonic development) is not available in any organism. This is compounded by the fact that many claudin genes have yet to be discovered in these organisms. However, from data that we currently have access to, it is evident that *Claudin 1* expression is important in eye, gill and skin tissue in fish. *Claudin1* expression is important in the renal & urinary sytem, nervous system, epidermis and skeletal system in mammals and birds throughout development. It is clear that *Claudin 1* expression is important for facilitating normal development and functioning in a wide range of structures throughout vertebrates.

A loss of *Claudin 1* expression has been seen to result in wrinkled skin and death within one day of birth in the mouse *M. musculus* (Furuse et al., 2002). It can therefore be inferred that the role of *Claudin 1* in normal development and maintenance of epithelial structures in mammals is critical for viability and survival. This is further evident in studies of *Claudin 1* in humans, which have shown that a loss of function mutation results in neonatal octhyosis-sclerotising cholangitis syndrome (a fatal disorder) in which the skin is abnormally thick and unable to retain moisture (Anderson and Itallie, 2009).

Alteration of *Claudin 1* expression has been observed to affect the direction of heart looping in chicken and frog models (Collins *et al*., 2013). This is the first conserved morphological sign of left-right patterning in vertebrates (Collins *et al*., 2013).

Claudin 1 has been shown to be highly expressed in the premigratory neural crest of the developing chick embryo (Fishwick *et al*., 2012). *Claudin 1* is down-regulated in migratory neural crest cells, and its over-expression has been shown to decrease neural crest migration in the chick embryo (Fishwick *et al*., 2012). These findings indicate that *Claudin 1* may be involved in controlling the ability of premigratory neural crest cells to undergo the epithelial-to-mesenchymal transition and later exit the neural tube. Removal of *Claudin 1* expression resulted in the promotion of uncontrolled midbrain neural crest cell emigration, which resulted in the expansion of the migratory neural crest domain in the embryo (Fishwick *et al*., 2012).

It is obvious that *Claudin 1* is an integral gene in vertebrates. The role of *Claudin 1* in vertebrate embryogenesis and the maintenance of epidermal tissues in adulthood are vital. For these reasons *Claudin 1* must have played an important role in vertebrate evolution.

1.2.3 *Claudin 9*

Claudin 9, similar to all other claudin genes encodes a membrane protein that is an integral component of the tight junction strands. *Claudin 9* has been shown to be involved in hearing in humans (Anderson and Itallie, 2009). *Claudin 9* is expressed in the tight junctions of the cochlea, where it acts to separate the high K^+ endolymph from the low K^+ perilymph (Günzel and Yu, 2013). The perilymph covers the outer sensory cells (Günzel and Yu, 2013). *Claudin 9* seems to prevent the sensory cells from exposure to high K^+ concentrations (Günzel and Yu, 2013). The table below shows an outline of *Claudin 9* gene expression during the development of *M. musculus* only, as no expression data is available in any other organism.

Table 1.3: *Claudin 9* spatial gene expression patterns in vertebrate model organisms.

Organism	Region of Expression (Stage of Development)	Citation/s
Fugu rubripes	No data.	---
Danio rerio	No data.	---
Xenopus laevis	No data.	---
Gallus gallus domesticus	No data.	---
Mus Musculus	Endoderm, yolk sac (E8 – E8.5), pancreas epithelium, pituitary gland, cochlea, nasal cavity olfactory epithelium (E14.5), submandibular gland (E16.5).	Hou *et al*., 2007. Hoffman *et al*., 2008. Hashizume *et al*., 2004. Diez-Roux *et al*., 2011.

The expression data was determined mainly via RNA in situ hybridization, but immunohistochemistry, Western blot and RT-PCR assays may also have been used. The *Mus musculus* developmental stages are based upon the standard mammalian developmental staging series (Taylor Stage Comparison).

Claudin 9 expression is visible early on in mammalian development and appears to be important in the normal development and functioning of epithelial, glandular and auditory (cochlea) structures in the embryo.

Mutations in *Claudin 9* result in severe and early hearing loss in humans and mice (Anderson and Itallie, 2009). The hearing loss appears to be associated with rapid degeneration of the outer hair cells and an increased perilymph potassium ion concentration. It is therefore evident that *Claudin 9* is involved in the normal development or auditory structures in mammals.

It is evident that normal *Claudin 9* expression is integral in mammals. The role of *Claudin 9* in vertebrate embryogenesis and the maintenance of epidermal tissues in adulthood are yet to

be elucidated. For these reasons *Claudin 9* must be further studied in order to determine its spatial expression patterns amoungst more basal vertebrates.

1.2.4 *Claudin 10*

Multiple *Claudin 10* transcript variants (variably spliced) have been described in a number of organisms (Günzel and Yu, 2013). The different transcript variants are differentially expressed in different tissues throughout the organism (Günzel and Yu, 2013). However, for the focus of this study, these datasets have been simplified. The table below shows an outline of *Claudin 10* gene expression during the development of several vertebrate model organisms. It is intended to clarify the areas of expression of *Claudin* 10 throughout vertebrate evolution.

Table 1.4: *Claudin 10* spatial gene expression patterns in vertebrate model organisms.

Organism	Region of Expression (Stage of Development)	Citation/s
Fugu rubripes	Eye, gill, kidney, intestine, skin (Adult).	Loh *et al*., 2004
Danio rerio	Gill, kidney, spleen, testis (Adult).	Baltzegar *et al*., 2013
Xenopus laevis	No data.	---
Gallus gallus domesticus	Epiblast, mesoderm, endoderm, extra-embryonic ectoderm, Hensen's node (HH4 – HH7), neural ectoderm, non-neural ectoderm, heart (HH8 – HH12), eye epithelium, otic vesicle, nasal epithelium, limb ectoderm, surface ectoderm, lung, kidney (E3 – E10).	Collins *et al*., 2013
Mus Musculus	Renal & urinary system (E12.5), submandibular gland primordium, renal & urinary system including the metanephrous, nasal cavity olfactory epithelium, pancreas, lungs, vibrissa, esophagus epithelium, oral epithelium, teeth (E14.5), submandibular gland, renal & urinary system (E16.5, E17.5, E18.5). Expression in the renal & urinary system is exhibited throughout development, into adulthood.	Ohta H, *et al*., 2006. Hashizume *et al*. 2004. Diez-Roux *et al*., 2011.

The expression data was determined mainly via RNA in situ hybridization, but immunohistochemistry, Western blot and RT-PCR assays may also have been used. The *G. gallus* developmental stages are based upon the *Hamburger Hamilton* staging series. The *Mus musculus* developmental stages are based upon the standard mammalian developmental staging series (Taylor Stage Comparison).

From the data above, *Claudin 10* expression appears to be important in the eye, gill, kidney, intestine, skin and testis of fish. Expression in *G. gallus* is visible very early on and appears to be integral in early development in the chick. Expression is later visible in the sensory organs, ectoderm, lung and kidney. The conserved expression in the eye, lung, kidney and skin appear to convey a highly conserved function of the gene. Expression in the renal & urinary system, epithelium, lungs and nasal cavity in *M. musculus* appear to further reinforce the conservation of *Claudin* 10 expression and therefore function.

Claudin 10 loss of function mutations result in nephrocalcinosis and hypermagnesemia in the thick ascending limb of the renal tubules in mice (Breiderhoff *et al*., 2012). Abnormalities in renal absorbtion are also associated with *Claudin* 10 loss of function (Breiderhoff *et al*., 2012). This indicates that *Claudin 10* expression is vital for normal kidney development, maintenance and functioning.

The conservation of *Claudin 10* expression and function throughout vertebrates points to *Claudin 10* as an important gene in driving the evolution of vertebrates and vertebrate structures.

1.2.5 *Claudin 19*

Claudin 19 has been strongly implicated in magnesium transport in humans (Günzel and Yu, 2013). The table below shows an outline of *Claudin 19* gene expression during the development of several vertebrate model organisms. It is intended to clarify the areas of expression *Claudin 19* throughout vertebrate evolution.

Table 1.5: *Claudin 19* spatial gene expression patterns in other vertebrate model organisms.

Organism	Region of Expression (Stage of Development)	Citation/s
Fugu rubripes	Brain, gill (Adult).	Loh *et al*., 2004
Danio rerio	Brain (Adult).	Baltzegar *et al*., 2013
Xenopus laevis	Renal & urinary system, including the kidney, eye, somites (NF Stage 35 & 36).	Raciti *et al*., 2008
Gallus gallus domesticus	No data.	---
Mus Musculus	Nervous sytem including the brain (E14.5), renal & urinary system including the metanephrous, renal medullary vasculature (E17.5).	Diez-Roux *et al*., 2011.

The expression data was determined mainly via RNA in situ hybridization, but immunohistochemistry, Western blot and RT-PCR assays may also have been used. *X. Laevis* developmental stages are based upon the staging series published by Nieuwkoop and Faber in 1956. The *Mus musculus* developmental stages are based upon the standard mammalian developmental staging series (Carnegie Stage Comparison).

Claudin 19 is expressed in the brain and gills in fish. This expression is conserved throughout vertebrate evolution, as *Claudin 19* expression is prominent in the nervous system and many parts of the brain in *M. musculus*. *Claudin 19* is expressed in the renal & urinary system (including the kidney) in *X. laevis*, this expression is conserved throughout vertebrate evolution as expression in the renal & urinary system is prevalent during *M. musculus* development.

Mice that are not expressing *Claudin 19* have peripheral neuropathy and exhibit severe behavioral abnormalities (Miyamoto *et al*., 2005). These mice also lack tight junctions on their myelinated Schwann cells and therefore have abnormal nerve conduction (Miyamoto *et al*., 2005). *Claudin 19* mutations have also been shown to affect eye morphology and normal kidney functioning (Günzel and Yu, 2013).

The conservation of *Claudin 19* expression and function throughout vertebrates seems to infer that *Claudin 19* is an important gene in driving the evolution of vertebrates and vertebrate structures such as the brain, eye and kidney.

1.3 Justification for Research

1.3.1 The Importance of Understanding Claudins

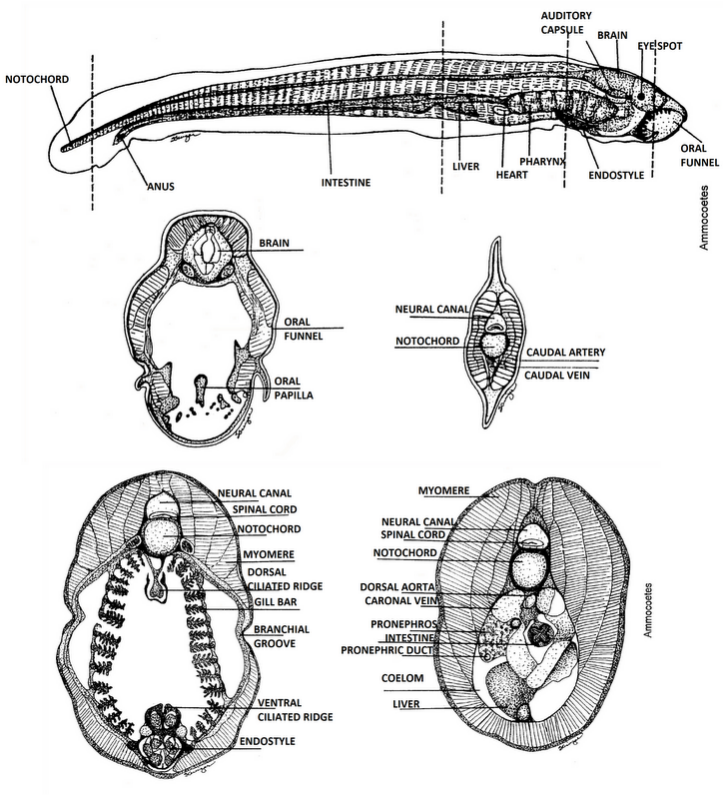
The evolution of vertebrates has been dependant on the ability to form discrete areas within the body with differing form and function via the complex compartmentalization of the embryo. This morphogenesis of vertebrate embryos is mediated by the movement of sheets of cells that are able to form barriers between different embryonic structures which allows for the regulation of the movement of signalling molecules between different parts of the embryo. The compartmentalization of the embryo allows for the formation of the diverse range of vertebrate body structures. These sheets of cells that form these barriers (and therefore the different structures of the embryo) are called epithelia. Epithelia are held together and regulated by tight junctions between the epithelial cells. Tight junctions are formed mainly due to the presence of claudins. So in this way claudins play an integral role in embryo morphogenesis and indeed the formation of vertebrate body structures would not be possible without these proteins.

Mutations in claudin genes have been implicated in several human diseases such as neonatal icthyosis and sclerotising cholangitis syndrome, nonsyndromic deafness (*Claudin 1*), kidney stones, decreased bone mineral density, hypercalciuria, low serum CO_2 (*Claudin 14*), familial hypomagnesemia with hypercalciuria and nephrocalcinosis syndrome (*Claudin 16*), ocular disease, myopia (*Claudin 19*) and several other diseases in humans alone (Gupta and Ryan, 2010). For this reason it is important to study claudins as potential targets for treatments for these congenital disorders. The abnormal expression of claudin genes has also been implicated in the onset and progression of cancer in humans and mice (Gupta and Ryan, 2010) and may also be a potential target for anti-cancer treatments (Lal-Nag and Morin, 2009).

Claudins are heavily involved in embryogenic development and the evolution of vertebrates, they are also implicated in several human diseases. For these reasons it is important to study claudins as furthering the understanding of these genes would be beneficial both in better understanding the process of embryonic development as well as their role in vertebrate evolution and human health.

1.3.2 *Petromyzon marinus* as a Model Organism for Studying Vertebrate Evolution

Petromyzon marinus, otherwise known as the sea lamprey is a parasitic lamprey that can grow up to 90cm in length. *P. marinus* has been studied extensively in the past because of its economic importance as a parasite of edible fish in the North American Great Lakes. However, more recently *P. marinus* has been studied as its embryos are used to make inferences about early developmental patterns and vertebrate evolution (Richardson and Wright, 2003). The genome of *P. marinus* was sequenced in 2013 and has been shown to have an abnormally high GC content and amino acid usage patterns when compared to more complex vertebrates (Smith *et al*., 2013).



(http://1.bp.blogspot.com/-vYaPRxxGEY8/TV394giMsVI/AAAAAAAAACU/wJ7KjJPjla4/s1600/amphioxus+ammocoetes.png)

Figure 1.5: Lamprey larvae anatomy.

The figure above displays the anatomy of a lamprey larva. Juvenile *P. marinus* live in fresh water in the form of larvae (or ammocoetes) where they obtain nutrients via filter feeding before they mature into adults and move into a salt water environment in the open oceans to feed on the blood of marine fish (Campbell *et al*., 2008). *P. marinus* has a cartilaginous skeleton. Unlike the cartilage found in most vertebrates, the lamprey cartilage does not

contain any collagen, it is instead a stiff protein matrix (Campbell *et al*., 2008). The notochord of lampreys remains as the main axial skeleton in adults (Campbell *et al*., 2008). There is a cartilaginous pipe that surrounds the notochord where symmetrical cartilage projections, similar to the vertebrae of more complex vertebrates extends dorsally and partially encloses the nerve cord (Campbell *et al*., 2008).

Following fertilisation of the lamprey egg the blastula forms between 1 (E1) and 2 days (E2) (Tahara, 1988). Gastrulation begins around day 3 (E3) with neurulation initiating by E4 (Tahara, 1988). By E5.5 the blastopore is closed so that the neural folds are visibly raised, it is also around this time that somitogenesis begins (Tahara, 1988). At E6 the neural folds fuse and the delamination and migration of the neural crest occurs following this at E6.5 (Tahara, 1988). By E7 the cheek pouch is visible, the notochord and anterior somites separate from the prechordal plate and the trunk lateral plate forms (Tahara, 1988). At E8 the neural tube and otic placode are visible, whereas the following day at E9, the eyespot and stomodeum are formed (Tahara, 1988). By stage E11, the embryos have started hatching from the egg, the nasal pit, blood cells, brain and renal system have formed (Tahara, 1988). Stage E12 is characterised by a beating heart and tailbud, the larvae are now free living (Tahara, 1988). By E16 there is further heart differentiation, as well as the aggregation of pigmentation (Tahara, 1988). At E18 the eyespot is clearly formed and there are blood vessels in the typhlosole (Tahara, 1988). From E19 onwards, the larvae begin to look more similar to adult lamprey.

Lampreys are part of an ancient vertebrate lineage that diverged from other vertebrates approximately 500 million years ago. *P. marinus* is therefore currently the most basal extant vertebrate (Smith *et al*., 2013). For this reason, *P. marinus* could potentially provide information as to the ancestry of vertebrate genome organization and vertebrate biology.

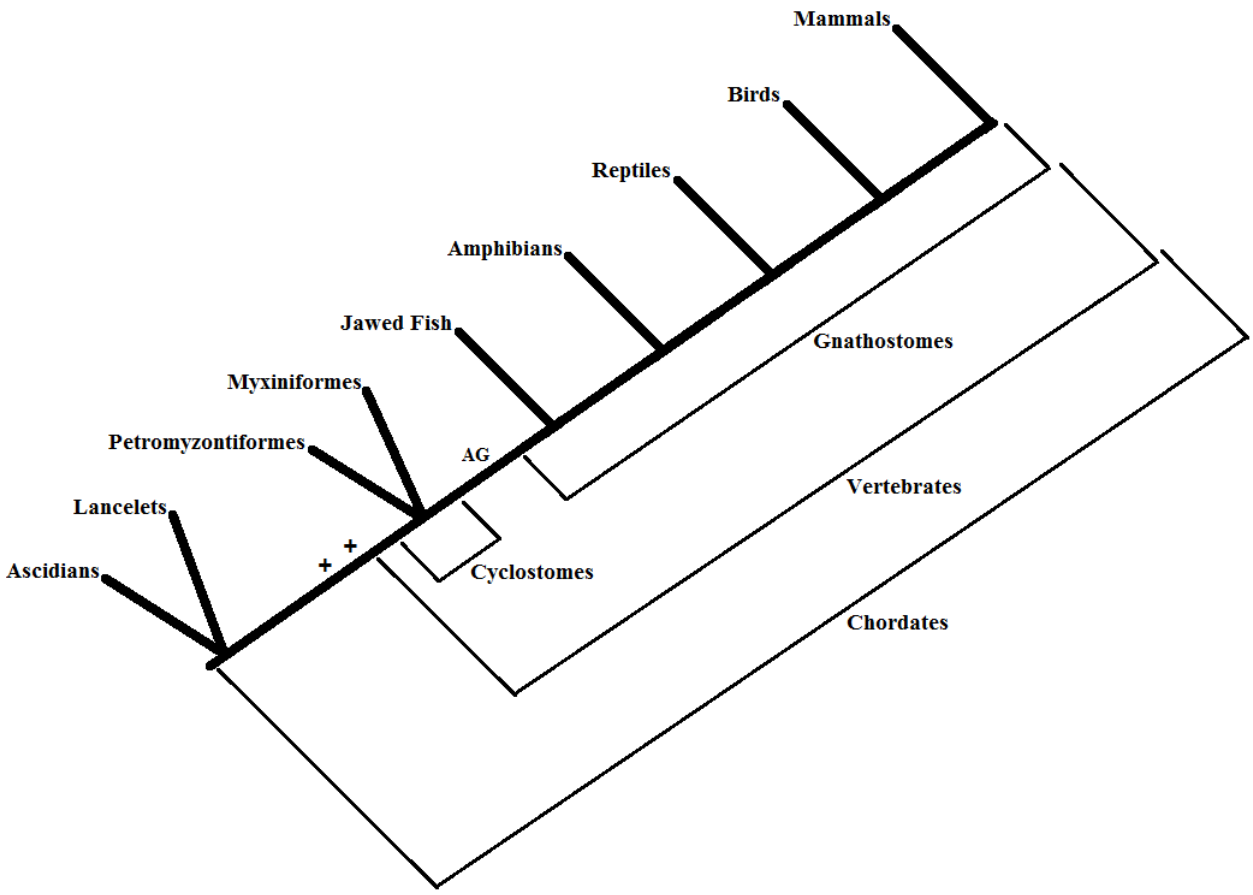


Figure 1.6: Vertebrate evolution.

Vertebrates arose from simple organsisms such as Ascidians and Lanclets, which are grouped together with vertebrates in the phylum Chordata (chordates are animals that possess a notochord, a hollow dorsal nerve chord, an endostyle, pharyngeal slits, and a post-anal tail at some stage of their life cycle). Two complete genome duplications occurred separately (indicated by +). Cyclostomes were the first vertebrates to arise, the only currently extant cyclostomes are Petromyzontiformes (lampreys) and Myxiniformes (hagfish) and they are characterized by their lack of a jaw structure. Therefore lampreys arose before the acquisition of a jaw structure (approximately 500 million years ago). Gnathastomes (jawed vertebrates) later evolved from an ancestral gnathostome (**AG**) several million years later. Gnathostomes include 99% of extant vertebrates such as jawed fish (teleosts), amphibians, reptiles, birds and mammals. **Figure 1.6** illustrates that lampreys are amongst the most basal extant vertebrates (Smith *et al*., 2013; Campbell *et al*., 2008).

Table 1.6: Number of known claudin genes in different chordates.

Organism	Number of Known Claudin Genes
Halocynthia roretzi (Ascidian)	Potentially 3
Lamprey (Jawless Fish)	Unknown
Zebrafish (Jawed Fish)	15
Xenopus (Amphibian)	7 or more
Chicken (Bird)	17
Human (Mammal)	27

Table 1.6 illustrates the number of different known claudin genes in different vertebrates (according to the NCBI database, 10/12/2014). The number of different claudins increases as the complexity of the organism increases (with the exception of fish, however they are known to have undergone several additional gene duplication events, which would account for this) (Smith *et al*., 2013). Several claudins are also believed to have undergone tandem gene duplications in fish. The correlation between an increase in the number of claudin genes and the increase in the complexity of the vertebrate body plan could mean that claudins play an important role in the evolution of an increasingly complex vertebrate body plan.

The number of claudins in the lamprey genome is currently not known. As lampreys are the most basal extant vertebrates, it would be insightful to discover more about claudins in these organisms as it would lead to a better understanding of the role that claudins have played in the evolution of vertebrates.

The lamprey genome is an important resource that can be used to discover clues as to the origins and evolution of vertebrates (Smith *et al*., 2013). The *P. marinus* genome has been observed to have undergone two complete genome duplications (Smith *et al*., 2013) and these genome duplication events have been shown to be a potentially integral part of claudin evolution and diversification in vertebrates (Gupta and Ryan, 2010). For these reasons, *P. marinus* can also be used as a model organism to study the origin, function and evolution of claudins in the vertebrate genome.

The neural crest is a multifunctional tissue, it is important in evolutionary biology because of its hypothesised specificity to vertebrates and significant contribution to the cranial complexity that separates vertebrates from other animals (Shimeld and Donoghue, 2012). Lamprey neural crest is very similar to that of the gnathostomes, this includes its ability to differentiate into a wide range of tissues and structures (Shimeld and Donoghue, 2012).

However, lampreys lack certain anatomical features such as the jaw that is associated with gnathostomes. So, lampreys could be used as a tool to understand how certain features of the vertebrate body plan (such as the jaw) evolved.

Starting from the most basal extant example of a vertebrate, we can begin to construct an evolutionary tree of vertebrate claudins from the bottom up and therefore further our understanding of these integral genes within the vertebrate genome.

1.4 Aim and Objectives

Aim:

To determine the expression patterns and functions of several novel claudin genes in the *P. marinus* embryo.

Objectives:

1. Perform reverse transcription PCR on *P. marinus* embryo and adult tissue samples.

2. Clone DNA probe templates (for *in situ* hybridization).

3. Produce RNA probes (from DNA templates) for *in situ* hybridization.

4. Perform *in situ* hybridization of *P. marinus* embryos at different stages of development.

5. Perform morpholino-mediated gene knockouts in *P. marinus* embryos.

6. Analyse the morphants using *in situ* hybridization.

2 Materials and Methods

The experimental methods that were carried out in this project are summarised in the figure below and are later explained in more detail.

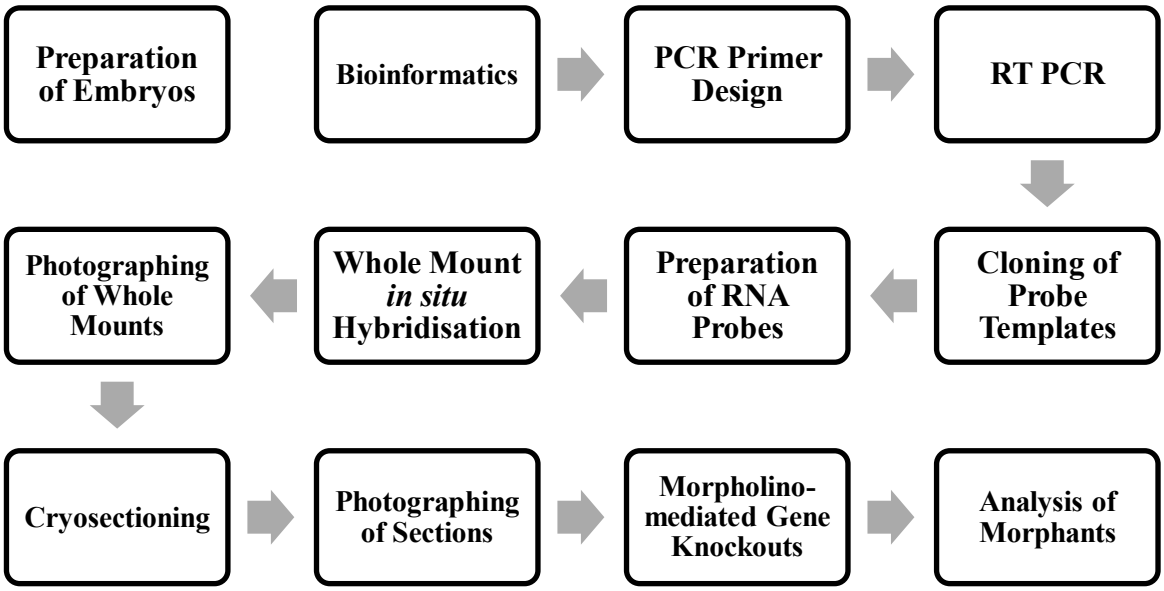


Figure 2.1: Summary of methodology carried out.

2.1 Preparation of Embryos

The protocols described by Nikitina *et al*. (2009) were followed in order to prepare the *Petromyzon marinus* embryos. All procedures carried out at the California Institute of Technology were performed by Natalya Nikitina, Christian Mukendi and Thembekile Zwane and the procedures that they followed are described below. *P. marinus* breeding specimens were reared at the Division of Biology at the California Institute of Technology (Pasadena, California, USA). Eggs and sperm were obtained from gravid females and spermiating males respectively. The eggs and sperm were mixed in filtered spring water and allowed to fertilise for at least fifteen minutes. Fertilised eggs were incubated in MMR (20 mM $CaCl_2 \cdot 2H_2O$, 1 mM EDTA, 50 mM HEPES, 20mM KCl, 10 mM $MgSO_4$, 1 M NaCl) at 18°C until the required developmental stage had been reached, after which they were collected (embryos were collected between day four and day thirty-one post-fertilisation). Collected embryos were placed in MEMFA (MOPS, EGTA, $MgSO_4$, 4% formaldehyde) for sixty minutes (in order to euthanize the embryos and fix the tissues) after which they were washed in a 0.1X

PTW (1X PTW is 23 mM NaH_2PO_4, 1.54 M NaCl, 80 mM $Na_2HPO_4 \cdot 7H_2O$, 0.1% Tween-20) solution. The fixed embryos were then dehydrated through a methanol/PTW series until a 100% methanol concentration had been reached. This dehydrated state (in methanol) serves to preserve the embryonic tissues as well as the embryonic RNA. Dehydrated embryos were then transported to the University of the Witwatersrand (Johannesburg, South Africa) where they were stored at -20°C.

Embryos at stages E4 to E9 were rehydrated through a methanol/PTW series until a 100% 0.1X PTW concentration had been reached. The chorions were removed from these embryos under a dissecting microscope using extremely fine forceps. The removal of the chorion allows for the RNA *in situ* hybridisation probe to penetrate into the embryonic tissues and bind to the target mRNA unimpeded by the chorion (which acts as a barrier). Once the chorions were removed, the embryos were dehydrated again prior to *in situ* hybridisation.

2.2 Bioinformatics

The sequences of several presumptive *P. marinus* claudin genes were shared with us by Professor Jeremiah Smith (Department of Biology, University of Kentucky, Lexington, Kentucky, USA). The sequences were first searched against all known sequences in the National Center for Biotechnology Information (NCBI) database using the blastx tool (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi?PROGRAM=blastx&PAGE_TYPE=BlastSearch&LINK_LOC=blasthome), this allows the user to search protein databases using a translated nucleotide query. This was done in order to eliminate potential duplicates and to ensure that complete coding sequences are used as well as to designate identities to unique genes within the data set. Genes were designated identity based on their sequence similarity and homology to known claudin gene sequences in the NCBI database. These sequences were then subjected to a multiple alignment using the Clustal Omega tool (http://www.ebi.ac.uk/Tools/msa/clustalo/) on the European Bioinformatics Institute (EMBL-EBI) website, and further refined using the DNASTAR computer software (Lasergene) in order to obtain the full gene sequence and ensure that separate genes are selected as the sequences may have been incomplete.

2.3 PCR Primer Design

PCR primers were designed for all unique *P. marinus* caludin cDNAs using the Primer 3 primer designing tool online software (http://primer3.ut.ee/). Primers were designed to amplify segments within the 3` or 5` untranslated regions where possible, rather than the coding regions, as this would increase the specificity of the primers as well as aid in constructing high quality RNA probe templates for down-stream applications such as *in situ* hybridisation (there appears to be a high level of sequence homology in *P. marinus* claudin genes within the coding region, possibly compounded by the high GC content of the *P. marinus* genome). Primer sequences were sent to Inqaba Biotechnical Industries (Pretoria, South Africa) where the PCR primers were synthesized.

Table 2.1: PCR primer sequences.

Gene	Primer Sequences	PCR Product Size (Base Pairs)
Claudin 1a	F: CGAGGCAAACAATCGGGAAT R: CTTCGCTGGGTTTGGTTTGG	586
Claudin 1b	F: GAGAGAGGCGGTGGAGGT R: AAGAAACGTCGACCAGCCG	516
Claudin 2	F: GAACACTCGCCTGCAACTG R: CAAAACCCAGGTACAGCGAG	500
Claudin 3a	F: ATCGAGGAGGAGGAGACCAA R: CTGAGTCTCAATGGGCCTCA	501
Claudin 3b	F: TCCATCGTCTTCCACACTCC R: GGGCACTATGGGGTTGTAGA	528
Claudin 5	F: GTTGCTGTGTAGGAGGAGGT R: AGCACTTTCCCCTCTCCATC	411
Claudin 8	F: ATTGTGGGAGGAGAGGGTTG R: CTCCCTTCCTCTCCGCTTAG	509
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
Claudin18	F:ATGCACGTCGTTGGGTTTG R:CCGAACGAGAACCTGGTGTA	521
Claudin 19a1	F: CCTGGAGGCAGTGGTATAGG R: CACTCGCCACATACAGAAGC	541
Claudin 19a2	F: TCGGCCAACATTCAATGCAA R: CACACACGGAAACAAAACGC	582
Claudin 19b	F: CCTGGAGGCAGTGGTATAGG R: CACTCGCCACATACAGAAGC	541
GAPDH	F: CCGTGCAAAAGGAAGACATT R: CTTCCCATCCTCAACCTTCA	134
RPS9	F: GTGGCGTGTCAAGTTCACC R: CATCTTGGACTCATCCAGCA	148

PCR primers for all known *P. marinus* claudin genes are listed in the table. The positive control PCR primers are also included. The respective PCR product sizes are indicated alongside.

2.4 Reverse-Transcription PCR

RNA was extracted from *P. marinus* embryos at embryonic stages E7, E8, E9, E10, E11, E12, E15 and E18 using the RNAqueous® Total RNA Isolation Kit (Life Technologies) as per the manufacturer's description. The embryonic RNA was extracted from several different embryos at each stage and then was pooled together in order to increase the overall concentration. RNA was also extracted from notochord, intestine, blood, muscle, brain, testis, eye, gill, heart, liver and skin tissue harvested from an adult male *P. marinus* using the RNAqueous® Total RNA Isolation Kit (Life Technologies) as per the manufacturer's description. The RNA was then dehydrated using GENTegra RNA columns (integenX) as per the manufacturer's protocol. RNA extraction was performed at the Division of Biology at the California Institute of Technology (Pasadena, California, USA) by Natalya Nikitina, Christian Mukendi and Thembekile Zwane. The lyophilised RNA was later transported to the University of the Witwatersrand (Johannesburg, South Africa) for further analysis.

The RNA was rehydrated in sterile, nuclease-free water to a concentration of 500 ng/µl. A minimal volume of the RNA was resolved on a 1% agarose gel containing 1X GR Green nucleic acid stain for fifty minutes at eighty Volts. The gel was photographed using a Bio-Rad Gel-Doc system (Bio-Rad) and the BioRad Image Lab software (version4.0).This was done in order to determine RNA integrity and quality. The samples that were found to contain RNA of a reasonable quality were selected for reverse-transcription (RT) PCR. RNA was first subjected to a *DNase* treatment in order to ensure that no residual genomic DNA (potentially not visible on gel) had been eluted during the extraction process. This involved treating 1 µg of RNA with 1 µl of *DNase1* as described by the manufacturer (Thermo Scientific) at 37°C for 30 minutes. 1 µl of 50 mM EDTA was then added to the reaction and incubated for ten minutes at 65°C in order to inactivate the *DNase* enzyme. The *DNase*-treated RNA (1 µg) was then subjected to reverse-transcription using the Thermo Scientific RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific) as per the manufacturer's protocol for synthesising GC-rich DNA using oligo-dT primers. The newly synthesised cDNA was subjected to standard PCR (see **Table 2.2**) using KAPA Taq ReadyMix (KAPA Biosystems) as per the manufacturer's description for a standard 20 µl reaction using all newly designed novel claudin gene PCR primers as well as *P. marinus* GAPDH and RPS9 PCR primers as positive controls.

Table 2.2: Thermocycler conditions for PCR.

Step	Temperature (°C)	Time (minutes)	Number of Cycles
Initial Denaturation	95	3	1
Denaturation	95	0.5	30
Primer Annealing	58	0.5	30
Extension	72	1	30
Final Extension	72	10	1

The PCR products were then resolved on a 1% agarose gel as described previously.

2.5 Cloning of Claudin Probe Templates

Claudin 1a, *Claudin 9*, *Claudin 10* and *Claudin 19b* PCR amplicons were first purified using the GeneJet PCR Purification Kit (Thermo Scientific) as per the manufacturer's description in order to remove any contaminants that may interfere with the ligation reaction. Each PCR product was then added to an individual standard 10 µl ligation reaction respectively as described in the pGEM®-T and pGEM®-T Easy Vector Systems protocol (Promega, see **Figure 6.1** in the **Appendix**) in which a 3:1 insert:vector ratio of PCR amplicon (with T overhang from PCR amplification with Taq polymerase) and pGEM®-T Easy Vector were added to 2X Rapid Ligation Buffer, T4 DNA Ligase and sterile nuclease-free water. The ligation reaction was mixed and allowed to incubate at 4°C for sixteen hours in order to ensure that the maximum number of successful transformants was achieved. The ligated plasmids were transformed into high efficiency JM109 competent cells (Promega) as per the pGEM®-T and pGEM®-T Easy Vector Systems protocol (Promega). One hundered µl of the transformed JM109 cells were plated on LB/ampicillin/IPTG/X-Gal plates and incubated at 37°C for 16 hours. White colonies (those that had successfully incorporated the insert DNA into the multiple cloning site) were selected and incubated in 10 ml of ampicillin-containing LB broth at 37°C for 16 hours with shaking at 155 rpm. Five millilitres of each of the respective broth cultures were then centrifuged at 8000 rpm for 5 minutes in order to pellet the transformed bacterial cells. The plasmid DNA was extracted from the bacterial pellet using the GeneJET Plasmid Miniprep Kit (Thermo Scientific) as per the manufacturer's protocol for extracting high-copy number plasmids. The plasmid DNA was analysed for purity and concentration using a NanoDrop™ ND-1000. DNA extracts of high quality and

concentration were subjected to restriction enzyme digestion using *EcoRI* (as this would remove the insert from the multiple cloning site within the pGEM®-T Easy Vector (Promega)). The *EcoRI* digestion was carried out as per the manufacturer's description (Thermo Scientific Fast Digest) at 37°C for 60 minutes. 250 ng of the digests were resolved on a 1% agarose gel. Plasmids containing inserts of the expected size, were sent to Inqaba Biotechnical Industries (Pretoria, South Africa) where they were subjected to DNA sequencing in the direction of the T7 promoter on the pGEM®-T Easy Vector (Promega). DNA sequences were aligned with the original claudin gene sequences and checked in order to confirm the identity of each insert. Plasmids containing the correct inserts of each respective claudin gene segment were used for preparation of RNA probes for *in situ* hybridisation.

A full length *Claudin 19b* clone, as well as a *Twist A* and a *Prdm1* clone were previously acquired from the California Institute of Technology (Pasadena, California, USA) and were later transported to the University of the Witwatersrand (Johannesburg, South Africa) for further analysis. These clones were contained in a pCMV•SPORT6 Vector (Life Technologies, see **Figure 6.1** in the **Appendix**) and were subjected to the same treatment as described above with the exception of a *SalI-NotI* double digest being performed in order to excise the insert as opposed to the *EcoRI* digest with the pGEM®-T Easy Vector (Promega). The gel containing these digests was resolved for 3,5 hours under the same conditions as previously stated due to the similarity in the sizes (approximately 4000 bp) of these three inserts with the pCMV SPORT6 Vector (Life Technologies) itself.

2.6 Preparation of RNA Probes

Ten µg of pGEM®-T Easy Vector (Promega) containing *Claudin 1a*, *Claudin 9*, *Claudin 10* and *Claudin 19b* gene fragments in the anti-sense direction (so that the probe is able to bind to complementary mRNA) were each restricted with *SalI* at 37°C for 16 hours in order to linearise the plasmids and maintain the connection between the T7 promoter region and the inserts. Ten µg of pCMV SPORT6 Vectors (Life Technologies) containing the full length *Claudin 19b* clone, *TwistA* and *Prdm1* inserts in the anti-sense direction respectively were each restricted with *NotI* at 37°C for 16 hours in order to linearise the plasmids and maintain the connection between the T7 promoter region and the inserts (the *TwistA* probe was prepared by Christian Mukendi and the *Prdm1* probe was prepared by Natalya Nikitina). A

concentration higher than that recommended by the manufacturer (3 µl per reaction) was used in order to ensure that no undigested plasmid remained (as this could interfere with the probe synthesis process). 250 ng of the digests were resolved on a 1% agarose gel. The plasmid digests were purified using the GeneJet PCR Purification Kit (Thermo Scientific) as per the manufacturer's description in order to remove any contaminants that may interfere with the probe synthesis reaction. 1 µg of the purified, linear plasmid DNA was then added to 5X Transcription Buffer (Thermo Scientific), 10X Digoxigenin RNA Labelling Mix (Roche), RiboLock RNase Inhibitor (Thermo Scientific), T7 RNA Polymerase (Thermo Scientific) and sterile nuclease-free water to make up the probe synthesis reactions. The reactions were mixed and incubated for 60 minutes at 37°C, after which 1 µl of additional T7 RNA Polymerase was added to the reactions. The reactions were then incubated for a further 60 minutes, after which 1 µl of the reaction was removed and resolved on a 1% agarose gel containing for 15 minutes at 100 Volts to check for the presence of a clear RNA band. Successful reactions were treated with 2 µl of 10X *DNase* Buffer and *DNase* (Thermo Scientific) respectively and incubated at 37°C for 60 minutes in order to remove the plasmid DNA from the reaction (as it may interfere with the RNA purification process). 1 µl of the reaction was removed and resolved on an agarose gel as described before in order to ensure the RNA quality and purity. The newly synthesised DIG-labelled RNA probes were purified using the NucleoSpin® RNA Purification Kit (Macherey-Nagel) as per the manufacturer's description to remove any unincorporated DIG-labelled nucleotides that might cause background staining during subsequent *in situ* hybridisation. The probes were then diluted in 1,5 ml of Hybridisation Mixture (50% formamide, 1.3X SSC, 5 mM EDTA, 200 µg/ml tRNA, 0.1% Tween 20, 0.5% CHAPS Hydrate, 100 µg/ml Heparin) to make a 5X probe stock and stored at -20°C in order to preserve the RNA probes.

2.7 Whole Mount *in situ* Hybridisation

The *in situ* hybridisation protocol described by Nikitina *et al*. (2009) was closely followed during the experimental procedure. *P. marinus* embryos at stages E4 to E31 were rehydrated through a methanol/PTW series until a 100% PTW (phosphate buffered saline, Tween 20) concentration had been reached. The embryos were then bleached in a solution of 0.5% SSC, 10% H_2O_2, 5% formamide. The embryos in the bleaching solution were placed in a light box for 10 minutes in order to speed up the reaction. The bleaching was performed in order to

lighten the pigmentation of the ectoderm so that the expression signal (precipitated stain) was clearer. The bleaching solution was then washed out with PTW and the embryos were treated with 14-22 µg/ml of proteinase K (Roche) for 10 minutes. This was done in order to permeabilise the ectoderm of the embryos so that the RNA probe was able to penetrate and bind to all complementary mRNA within the embryo. The proteinase K activity was inactivated with a 2 mg/ml glycine solution and washed out with PTW. The embryos were then fixed in a 4% paraformaldehyde, 0.2% glutaraldehyde solution for 10 minutes in order to preserve the embryo morphology. Following fixation, the embryos were equilibrated into a Hybridisation Mixture solution.

The embryos were later pre-hybridised in fresh Hybe Mix at 70°C for 3 hours in order to prepare the embryos for hybridisation with the probe. After pre-hybridisation, the embryos were added to a 1X solution of the DIG-labelled RNA probe and hybridised at 70°C for a minimum of 16 hours. During this process, the DIG-labelled RNA probes would bind to any complementary mRNA within the embryo with a high level of specificity

Following the overnight incubation period, the probe was removed and the embryos were thoroughly washed with Hybe Mix at 70°C to remove any unbound probe. The embryos were then equilibrated into an MABT (0.4 M maleic acid, 150 mM NaCL, 0.2 M Tris base, 0.1% Tween 20) solution. The MABT solution was then replaced with a solution of MABT containing 3% Bovine Serum Albumin (blocking solution), in which the embryos were incubated for at least 60 minutes to allow for increased antibody binding specificity. The blocking solution was then changed to a 1:1000 dilution of Anti-DIG-Alkaline Phosphatase antibody in blocking solution. The embryos were incubated in the antibody at 4°C for at least 16 hours with gentle shaking. This is to allow the antibody to bind to the DIG conjugated to the hybridised probes within the embryos.

The following day, the antibody solution was removed and the embryos were washed extensively with MABT to remove any unbound antibody. Next, the MABT was replaced with NTMT (100 mM NaCl, 100 mM Tris-HCl, 50 mM $MgCl_2$, 0.1% Tween 20). The NTMT was replaced after 1 hour with either a BM Purple (Roche) solution (centrifuged at 12000 rpm and filtered with a 0.2 µm filter) or an NTMT solution containing NBT/BCIP (filtered with a 0.2 µm filter). The BM Purple or NBT/BCIP act as a substrate for the alkaline phosphatase enzyme that is conjugated to the anti-DIG antibody. Cleavage of these substrates by alkaline phosphatase yields a blue/purple precipitate which marks the regions where the

probes have bound to complementary mRNA and therefore where the gene of interest is expressed. The embryos were left in the colour solution until a clear signal had developed. Once a clear signal had developed, the colour solution was washed out with PTW and the embryos were again fixed for 2 hours in a 4% paraformaldehyde solution in order to improve their integrity and inactivate the alkaline phosphatase enzyme. Following fixation, the embryos were washed with PTW and dehydrated through a methanol series of increasing concentration as previously described. The embryos were left overnight in methanol at 4°C to wash off any background staining, thus yielding a cleaner signal. After washing in methanol, the embryos were rehydrated (as previously described) and equilibrated into a 25% glycerol solution for photographing (the viscous solution makes it easier to manipulate the embryos).

The embryos were photographed at varying magnifications on a thin 0,5% agarose bed using a Zeiss Axio Zoom.V16 Camera Microscope.

2.8 Cryosectioning

The stained embryos were washed in PTW in order to remove any glycerol. They were then fixed in a 4% paraformaldehyde solution for 60 minutes to further preserve the tissues and later, washed again with PTW. The embryos were then incubated in a 5% sucrose solution for 6 hours and further equilibrated into a 15% sucrose solution at 4°C for at least 16 hours in order to cryoprotect the embryos (to prevent ice crystals from forming in the embryonic tissues) during the freezing process. Following cryoprotection, the embryos were incubated in a 7,5% gelatine, 15% sucrose solution at 37°C for 16 hours in order to allow for the gelatine to penetrate into the embryonic tissues. The following day, the embryos were equilibrated into a 20% gelatine, 15% sucrose solution at 37°C for 3 hours before embedding. The embryos were then embedded in moulds in different orientations using fine forceps to manipulate them under a dissecting microscope. The gelatine was allowed to solidify before the moulds were flash-frozen in liquid nitrogen. The frozen gelatine blocks (containing the embryos in different orientations) were removed from the moulds and stored at -20°C.

The embryos were sectioned at -30°C in a Leica CM1510 S Cryostat at a thickness of 20 µm. The sections were adhered onto positively charged Thermo Scientific™ SuperFrost™ Plus microscope slides. The slides were then incubated at room temperature overnight in an upright position in order to allow the sections to adhere strongly before mounting.

The next day, the slides were incubated at 42°C for 10 minutes in PTW in order to remove the gelatine from the sections. The slides were later rinsed in PTW several times at room temperature in order to clean them further. Excess PTW was carefully removed from the slides and two drops of CC Mount (Sigma-Aldrich) permanent aqueous mounting media was added to the slides. The slides were then covered completely with 60 mm glass cover-slips. The slides were stored in an upright position overnight in order to allow the mounting media to dry; following this the slides were carefully sealed with clear nail polish in order to prevent the mounting media and sections from drying out.

The sections were photographed at varying magnifications using an Olympus Provis AX70 Research Camera Microscope.

2.9 Morpholino-Mediated Gene Knockouts and Analysis of Morphants

Anti-fluorescein isothiocyanate labelled morpholinos were designed against *Claudin 1a*, *Claudin 10* and *Claudin 19b* by Natalya Nikitina. The morpholinos were designed to have specific sequence similarity to each of these individual mRNA species. The morpholino binds to the mRNA and in so doing inhibits the binding of the translational machinery and therefore the expression of the gene of interest.

Table 2.3: Morpholinos designed against target claudin mRNAs.

Target mRNA	Morpholino Sequence
Claudin 1a	5`-ACAGCGCAAACCCAACGACGTGCAT-3`
Claudin 10	5`-GCTTGGAGCCCTTCTGCAAAGAAGC-3`
Claudin 19b	5`-CCATCCTCGCCTCGCTTGAAACTTC-3`
Standard Negative Control	5`-CCTCTTACCTCAGTTACAATTTATA-3`

The morpholino-mediated translation inhibition protocol described by Nikitina *et al*. (2009) was closely followed during the experimental procedure. *Claudin 19b* morpholino injections were carried out at the Division of Biology at the California Institute of Technology (Pasadena, California, USA) by Natalya Nikitina in July 2013. *Claudin 1a* and *Claudin 10* morpholino injections were carried out at the Division of Biology at the California Institute of

Technology (Pasadena, California, USA) by Christian Mukendi in July 2014. Multiple *P. marinus* embryos were collected at both one-cell and two-cell stages. Viable embryos were selected and placed in an injection dish. The embryos were injected with 6 to 10 µl of each respective morpholino separately. The two-cell stage embryos were injected only in one cell (one side). Following injection, the embryos were incubated at 18°C in 0.1X MMR and collected at stages E10, E15, E16 and E17. Only embryos that had incorporated the morpholino were collected (visualised using a fluorescent microscope at 521 nm for presence of the morpholino). This same procedure was carried out on embryos with a non-binding negative control morpholino, this standard control morpholino (Gene Tools, LLC) targets a human beta-globin intron mutation that causes beta-thalassemia, it has been shown to cause little change in phenotype in any known system except for human beta-thalessemic hematopoetic cells and is widely used as a negative control. A 5 base pair mismatch (also non-binding) morpholino was also included as an additional negative control. The embryos were then collected and prepared as described in **2.1**. The prepared embryos were later transported to the University of the Witwatersrand (Johannesburg, South Africa) for further analysis.

The morphants were subjected to *in situ* hybridisation as previously described, using probes designed against *TwistA* to stain the otic placode and *Prdm1* to stain the somites (Nikitina *et al*., 2011). The morphants were photographed as previously described. Wild-type embryos were also included as an additional control.

Morphants were analysed by observing any morphological or phenotypic effects that the knockdown of the genes by the morpholinos may have caused.

3 Results

3.1 Reverse-Transcription PCR Reveals Expression of Most Claudins at All Stages of *P. marinus* Embryonic Development With a More Localised Expression in Adult Tissues

RNA extracts (extracted by N. Nikitina, C. Mukendi and T. Zwane) from embryonic stage E7 and notochord, intestine, blood, muscle, brain and testis from an adult male *P. marinus* did not yield RNA of a sufficient quality to perform RT PCR. RT PCR was performed on RNA extracted from embryonic stages E8, E9, E10, E11, E12, E15 and E18 as well as from adult eye, gill, heart, liver and skin tissue successfully. Primers designed against all known *P. marinus* claudin genes (see **Table 2.1**) were used to amplify the cDNA under the same cycling conditions. The *GAPDH* and *RPS9* primer sets act as a control for both RNA quality and scientific technique for amplification. The agarose gels showing the PCR amplicons from RT PCR are shown in **Figure 3.1A** and the summarised results are shown in **Figure 3.1B**.

The identity of the bands (*Claudin 1a*, *Claudin 3b*, *Claudin 5*, *Claudin 8*, *Claudin 9*, *Claudin 10*, *Claudin 11*, *Claudin 12*, *Claudin 15*, *Claudin 16* and *Claudin 19b*) was confirmed by subsequent cloning and DNA sequencing at Inqaba Biotechnical Industries (Pretoria, South Africa). See **Figure 6.2** in the **Appendix** for the results of *Claudin 1a*, *9*, *10* and *19b* sequencing. The *Claudin 3b*, *Claudin 5*, *Claudin 8*, *Claudin 12*, *Claudin 15* and *Claudin 16* clones were prepared and sent for sequencing by other members of the research team within the lab. The low molecular weight bands in some of the lanes are primer dimers, and the large molecular weight bands of the wrong size may be due to non-specific amplification.

Claudin 3b, *Claudin 5*, *Claudin 9*, *Claudin 10*, *Claudin 11*, *Claudin 16* and *Claudin 19a1* are expressed at all embryonic stages screened, with *Claudin 3b* and *Claudin 5* exhibiting the strongest levels of expression. *Claudin 19a2* is only expressed at stage E15, this may reveal true expression of *Claudin 19a2*, but is most probably attributed to a poor binding and amplification of the *Claudin 19a2* primer set used to the Claudin 19a2 cDNA. *Claudin 5* is the only gene that is expressed in all adult tissues.

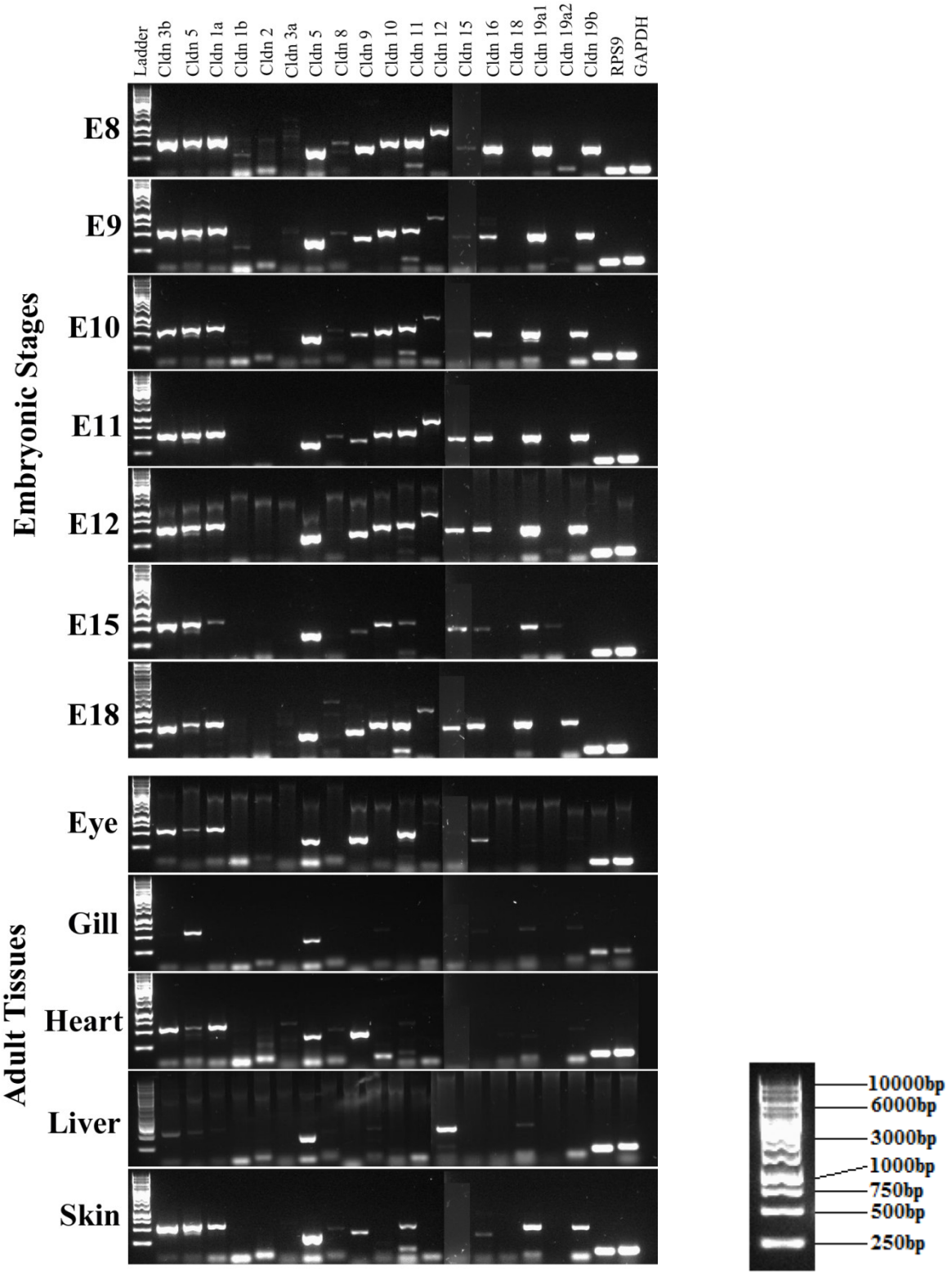


Figure 3.1A: Reverse-transcription PCR performed with all known claudin PCR primer sets performed on RNA extracted from *P. marinus* embryos and adult tissues.

DNA ladder is GeneRuler 1kb DNA Ladder (Thermo Scientific) and is annotated to the lower left of the figure.

	E8	E9	E10	E11	E12	E15	E18
Cldn 1a							
Cldn 1b							
Cldn 2							
Cldn 3a							
Cldn 3b							
Cldn 5							
Cldn 5							
Cldn 8							
Cldn 9							
Cldn 10							
Cldn 11							
Cldn 12							
Cldn 15							
Cldn 16							
Cldn 18							
Cldn 19a1							
Cldn 19a2							
Cldn 19b							
RPS9							
GAPDH							

	Skin	Eye	Liver	Heart	Gill
Cldn 1a					
Cldn 1b					
Cldn 2					
Cldn 3a					
Cldn 3b					
Cldn 5					
Cldn 5/6					
Cldn 8					
Cldn 9					
Cldn 10					
Cldn 11					
Cldn 12					
Cldn 15					
Cldn 16					
Cldn 18					
Cldn 19a1					
Cldn 19a2					
Cldn 19b					
RPS9					
GAPDH					

Figure 3.1B: Summarised results of reverse-transcription PCR.

Gene expression is indicated by highlighting the corresponding box. White boxes indicate that there is no gene expression**.** RNA extracts from different embryonic stages are in the summarised figure above and RNA extracts from adult tissues are summarised in the figure below.

3.2 Plasmid Digests of pGEM-T Easy and pCMV SPORT6 Vectors Reveal Successfully Cloned Inserts of Comparable Size to Expected Claudin Gene Fragments

The PCR fragments from RT-PCR (**Figure 3.1A**) were ligated into pGEM-T Easy Vectors, transformed into *E. coli* JM109 and the plasmid DNA was later extracted from the JM109 cells. The images in **Figure 3.2** show plasmids subjected to enzymatic digests to remove the inserts resolved on agarose gels. This plasmid DNA was restricted with *EcoRI* to check for insert size. From **A**, it is visible that the *Claudin 1a* , *Claudin 9* and *Claudin 10* inserts were successfully cloned into the plasmids. The *Claudin 19b* insert was successfully ligated into the plasmid as is visible in **B** where 1 to 5 are all replicates (only 2 was unsuccessful). The *Claudin 1a* insert size is 586 bp, the *Claudin 9* insert size is 408 bp, the *Claudin 10* insert is 599 bp and the *Claudin 19b* insert is 582 bp. The full length *Claudin 19b* clone was subjected to *SalI* digestion (a, c, e, g, i, k, m) and a *SalI-NotI* double digestion (b, d, f, h, j, l, n) as seen in **C** and the inserts were all of the expected size (3900 bp). The full length *Claudin 19b* clone was included simply to test the difference between an RNA *in situ* hybridization probe made from a full length clone and a shorter clone of only the non-coding region of the gene in subsequent *in situ* hybridization experiments. This is because the full length clone has considerable sequence similarity with other claudins in every region except for the UTR (sub-cloned probe template).

Please see the **Appendix** for full sequences of clones as determined by DNA sequencing at Inqaba Biotechnical Industries (Pretoria, South Africa), as well as the full length *Claudin 19b* sequence. The corresponding vector maps are also included in **Figure 6.1** in the **Appendix**.

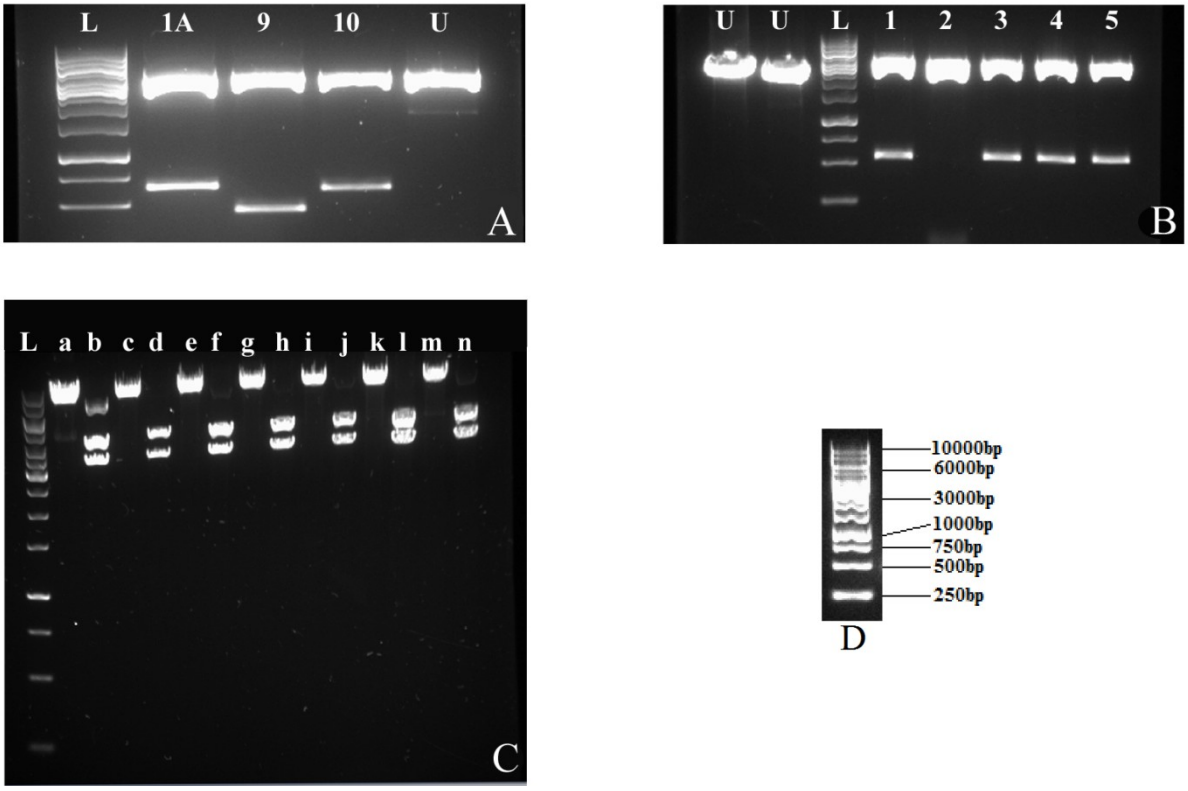


Figure 3.2: Plasmid digests of pGEM-T easy and pCMV SPORT6 vectors reveals successfully cloned inserts of claudin gene fragments.

A: 1A - *Claudin 1a*, 9 - *Claudin 9*, 10 - *Claudin 10*, L - 1 kb GeneRuler DNA Ladder (Thermo Scientific), U - unsuccessful or undigested plasmid, **B**: 1-5 = *Claudin 19b* replicates, **C**: Full length *Claudin 19b* clone was subjected to *SalI* digestion (a, c, e, g, i, k, m) and a *SalI-NotI* double digestion (b, d, f, h, j, l, n), **D**: 1 kb GeneRuler DNA Ladder (Thermo Scientific). *Claudin 1a*, *9*, *10* and *19b* inserts were cloned in pGEM-T Easy by A-T cloning whereas the full length *Claudin 19b* gene was cloned in pCMV SPORT6 by directional cloning (see vector maps in **Figure 6.1** the **Appendix**).

3.3 RNA Probe Synthesis Reactions Proved Successful for *Claudin 1a*, *Claudin 9*, *Claudin 10* and *Claudin 19b*

Figure 3.3 shows the progress of probe synthesis reactions for *Claudin 1a*, *Claudin 9*, *Claudin 10*, the *Claudin 19b* PCR amplicon insert (from a UTR segment of *the Claudin 19b* gene) and the *Claudin 19b* full length clone. Linearisation of the plasmids was successful for all the probe synthesis reactions with *SalI*. The probes were synthesized successfully and the RNA quality was good (single, high intensity band). After *DNase* treatment the presence and quality of the RNA probe was adequate for *in situ* hybridization use. The resolution of the RNA gels is poor due to the gels being resolved at a high voltage for a very short time period (in order to maintain RNA integrity as it degrades rapidly) and it is therefore difficult to determine the exact sizes of certain bands.

Table 3.1: Summary of RNA *in situ* hybridization probes used in this study.

Target mRNA	Target Region	Probe Length	Synthesised by
Claudin 1a	5` UTR	586 bp	Nicholas Dean
Claudin 9	Partial CDS	408 bp	Nicholas Dean
Claudin 10	3` UTR	599 bp	Nicholas Dean
Claudin 19b	3` UTR	541 bp	Nicholas Dean
Claudin 19b	Entire Gene	3900 bp	Nicholas Dean
Prdm1	Entire Gene	~ 3500 bp	Natalya Nikitina
TwistA	Entire Gene	~ 3500 bp	Christian Mukendi

All probes were tested successfully on a sample of *P. marinus* embryos at a range of developmental stages to ensure that the probes were binding specifically before experimental use.

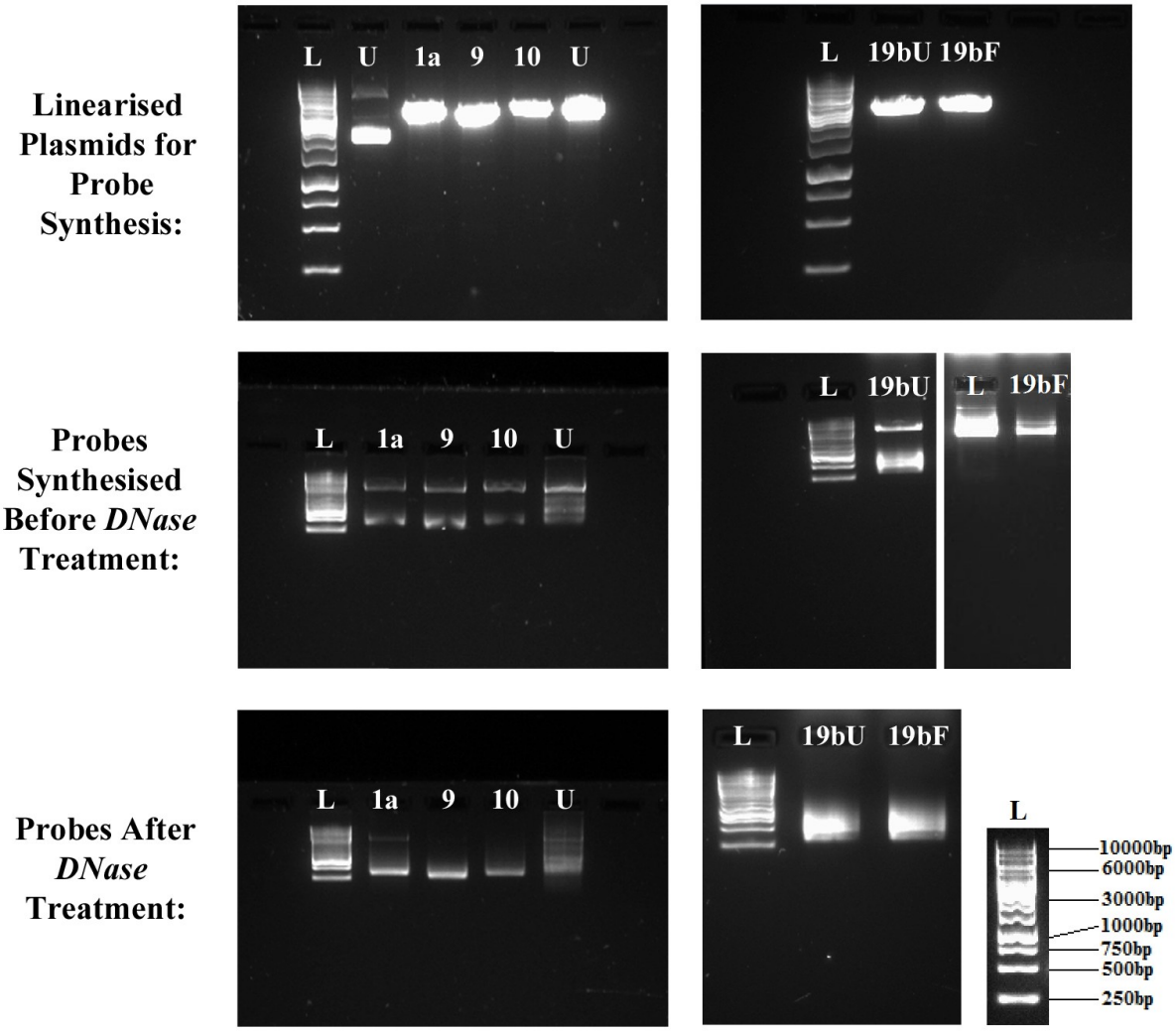


Figure 3.3: RNA probe synthesis reactions for *Claudin 1a*, *Claudin 9*, *Claudin 10* and *Claudin 19b*.

L - 1 kb GeneRuler DNA Ladder (Thermo Scientific), U - undigested plasmid or an unsuccessful reaction, 1a - *Claudin 1a*, 9 - *Claudin 9*, 10 - *Claudin 10*, 19bF - *Claudin 19b* Full Length (pCMV SPORT6 full length clone), 19bU - *Claudin 19b* UTR (pGEM-T Easy PCR amplicon clone).

3.4 *Claudin 1a* is Expressed Most Notably in the Migrating Neural Crest

The spatial expression pattern of *Claudin 1a* is shown in **Figure 3.4A** (results of whole mount *in situ* hybridisation of *P. marinus* embryos at stages E5 to E12 with *Claudin 1a* probe) and **Figure 3.4B** (results of whole mount *in situ* hybridisation of *P. marinus* embryos at stages E14 to E31 with *Claudin 1a* probe). Up to 3 replicates of each developmental stage was performed in order to ensure consistency of the expression pattern. Areas of blue/purple staining indicate where the probe has bound and therefore where *Claudin 1a* is expressed. *Claudin 1a* expression is visible at stage E5 (A, B, C) in the neural plate border and migrating neural crest, stage E6 (D) and E7 (E) in the otic placode, stage E8 (F) and E9 (G) in the otic placode, somites, migrating neural crest and stomodeum, stage E10 (H) in the stomodeum and otic placode, stage E11 (I) and E12 (J) in the stomodeum, fin fold and pericardial cavity, stage E14 (K) in the oral hood, pharyngeal arches, head ectoderm and fin fold, stages E15 (L), E16 (M), E17 (N) and E18 (O) in the ectoderm, pharyngeal arches, oral hood and fin fold, stages E19 (R), E24 (P, Q) and E31 (S) in the pharyngeal arches, oral hood, fin fold and oral aperture.

The whole mounts were sectioned at angles indicated by the corresponding lines on the figures. The section figures reveal that expression is visible at stage E5 (A`) in the neural plate border, stage E6 (D`) in the ectoderm, stage E8 (F`) in the ectoderm and migrating neural crest, stage E10 (H`) in the ectoderm and otic placode, stage E12 (J`) in the ectoderm and the marginal layer of the neural tube, stage E19 (R`, R``, R```, R````) in the pharyngeal cavity, dorsal fin fold and the ectoderm of the pharyngeal arches, stage E31 (S`) in the endoderm of the pharyngeal arches.

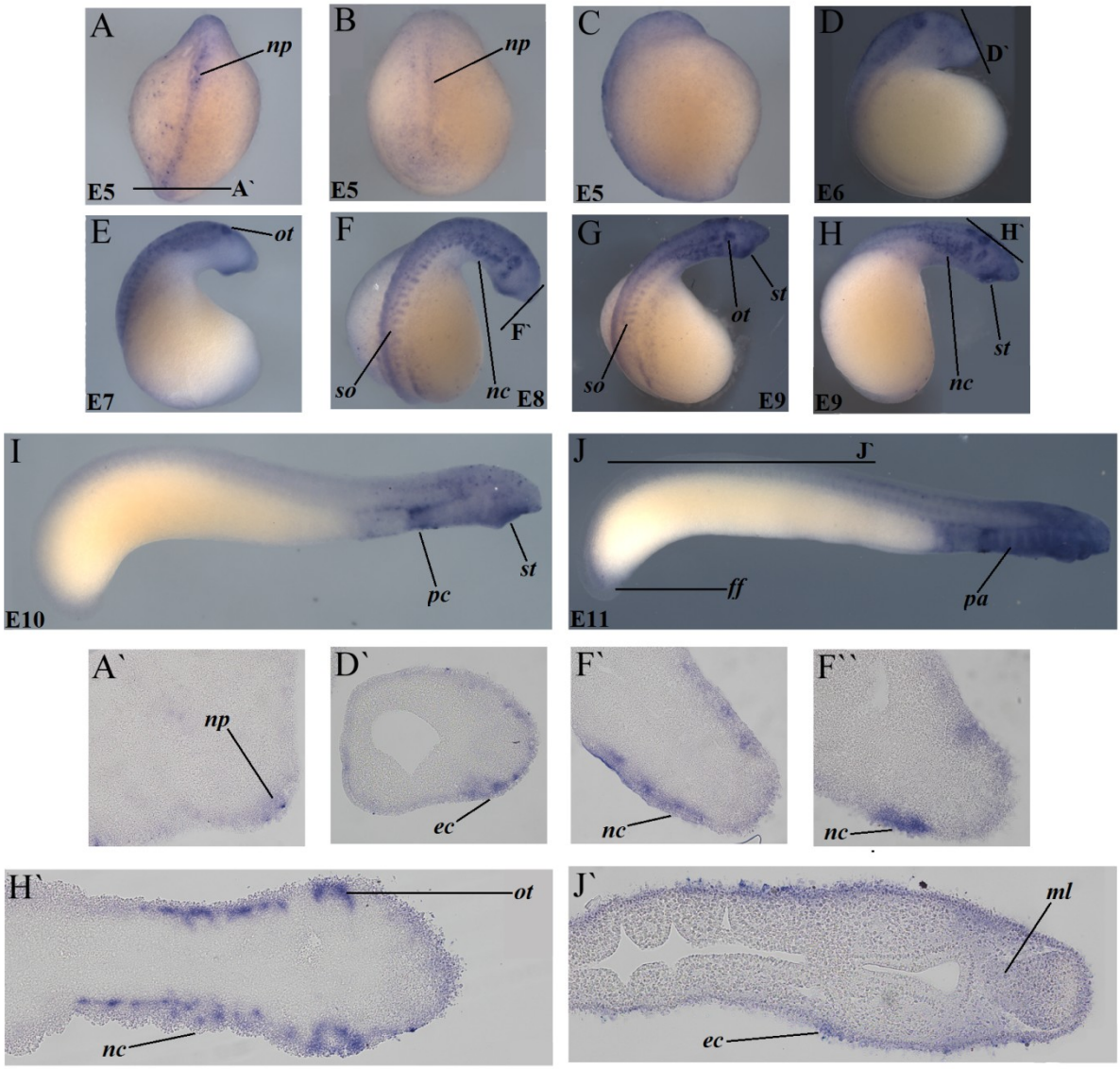


Figure 3.4A: Expression profile of *Claudin 1a* during *P. marinus* embryogenesis at stages E5 to E12.

Claudin 1a is expressed in the neural plate border (A-C, A`), otic placode (D-H, H`), migrating neural crest (F-H, F``), somites (E-G), stomatodeum (G-I), pericardial cavity (I), fin fold (J), ectoderm (D`, F`, H`, J`), marginal layer of the neural tube (J`). *ec* – ectoderm, *ff* – fin fold, *ml* – marginal layer of neural tube, *nc* – neural crest, *np* – neural plate border, *ot* – otic placode, *pc* – pericardial cavity, *so* – somites, *st*- stomatodeum.

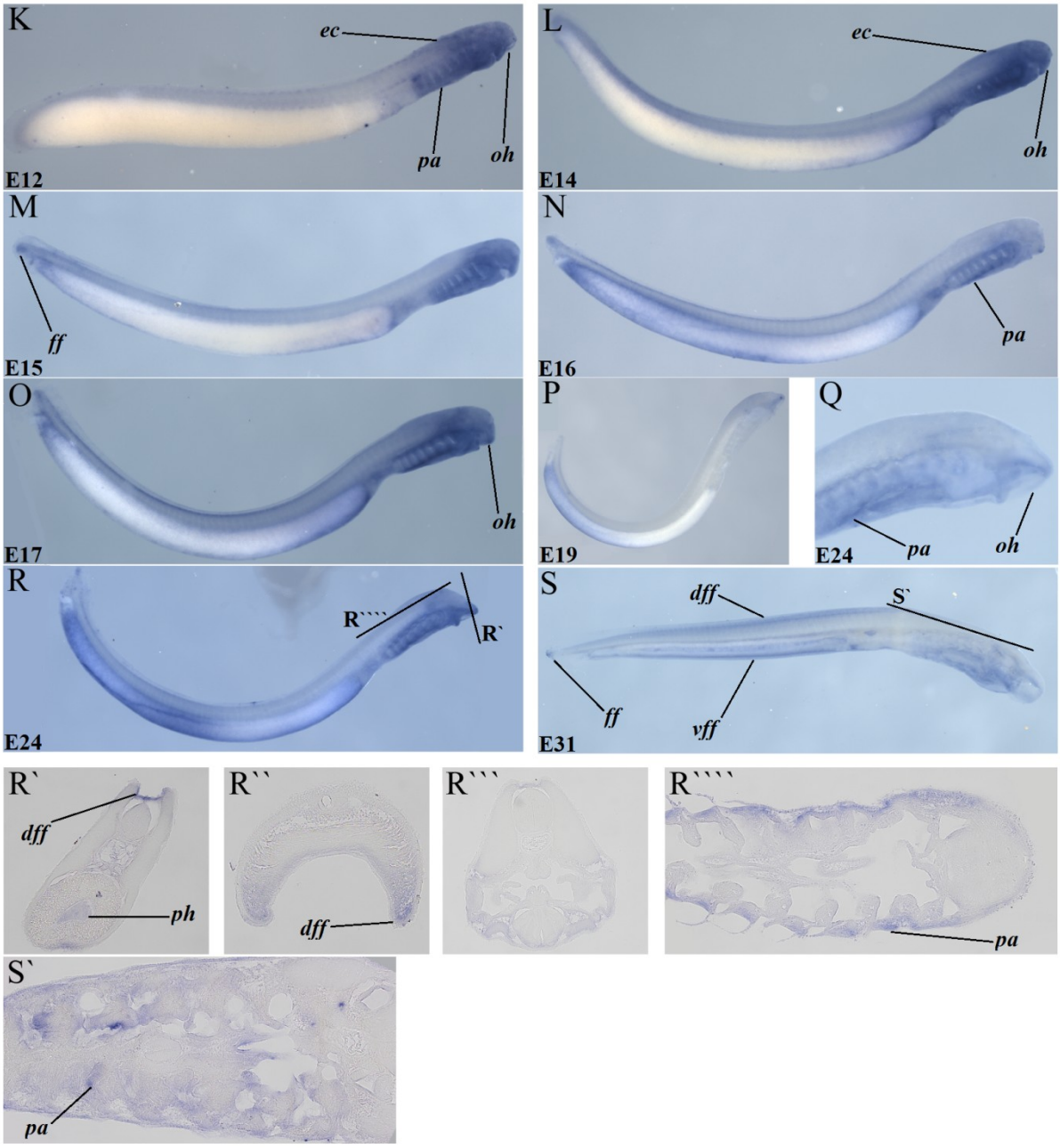


Figure 3.4B: Expression profile of *Claudin 1a* during *P. marinus* embryogenesis at stages E14 to E31.

Claudin 1a is expressed in the head ectoderm (K-O), pharyngeal arches (K-S, R````, S`), oral hood (K-Q), fin fold (K-S), dorsal fin fold (S, R`, R``) and ventral fin fold (S). *dff* – dorsal fin fold, *ec*- ectoderm, *ff*- fin fold, *oh* – oral hood, *pa* – pharyngeal arches, *vff* – ventral fin fold.

3.5 *Claudin 9* is Expressed in the Presumptive Kidneys, Subpharyngeal Gland, Neural Tube and Eyespot

The spatial expression pattern of *Claudin 9* is shown in **Figure 3.5A** (results of whole mount *in situ* hybridisation of *P. marinus* embryos at stages E5 to E12 with *Claudin 9* probe) and **Figure 3.5B** (results of whole mount *in situ* hybridisation of *P. marinus* embryos at stages E14 to E31 with *Claudin 9* probe). Up to 3 replicates of each developmental stage was performed in order to ensure consistency of the expression pattern. Areas of blue/purple staining indicate where the probe has bound and therefore where *Claudin 9* is expressed. *Claudin 9* expression is visible at stage E5 (A) in the neural plate border, stage E6 (B) and E7 (C, D) in the neural tube, stage E8 (E), E9 (F) and E10 (G) in the neural tube, stage E11 (H) in the stomodeum, and pharyngeal arches, stage E12 (I, J) in the ectoderm, pharyngeal arches, oral hood and otic placode, stage E14 (K) to E18 (P) in the pharyngeal arches, otic placode and eyespot, stages E19 (Q, R) and E 24 (S, T) in the otic placode, eyespot, subpharyngeal gland and pericardial cavity (E19 only), stage E31 (U) in the head ectoderm, dorsal fin fold and fin fold.

The whole mounts were sectioned at angles indicated by the corresponding lines on the figures. The section figures reveal that expression is visible at stage E8 (E`, E``) in the neural tube and head ectoderm, stage E10 (G`) in the neural tube, notochord and presumptive kidneys, stage E12 (I`, I``, I```, I````) in the pharyngeal arches endoderm, pharyngeal lumen, neural tube, notochord and pharyngeal cavity, stage E14 (K`, K``, K```) in the pharyngeal arches, and neural tube, stage E18 (P`, P``) in the pharyngeal arches, otic placode, eyespot, neural tube, pharyngeal cavity and pharyngeal lumen, stage E21 (S`, S``) in the subpharyngeal gland and neural tube.

There is evidence that there may be some signal trapping in the lumen of the pharyngeal arches at E18 (**Figure 3.5B**, P`). This may not indicate true expression of the gene in this region.

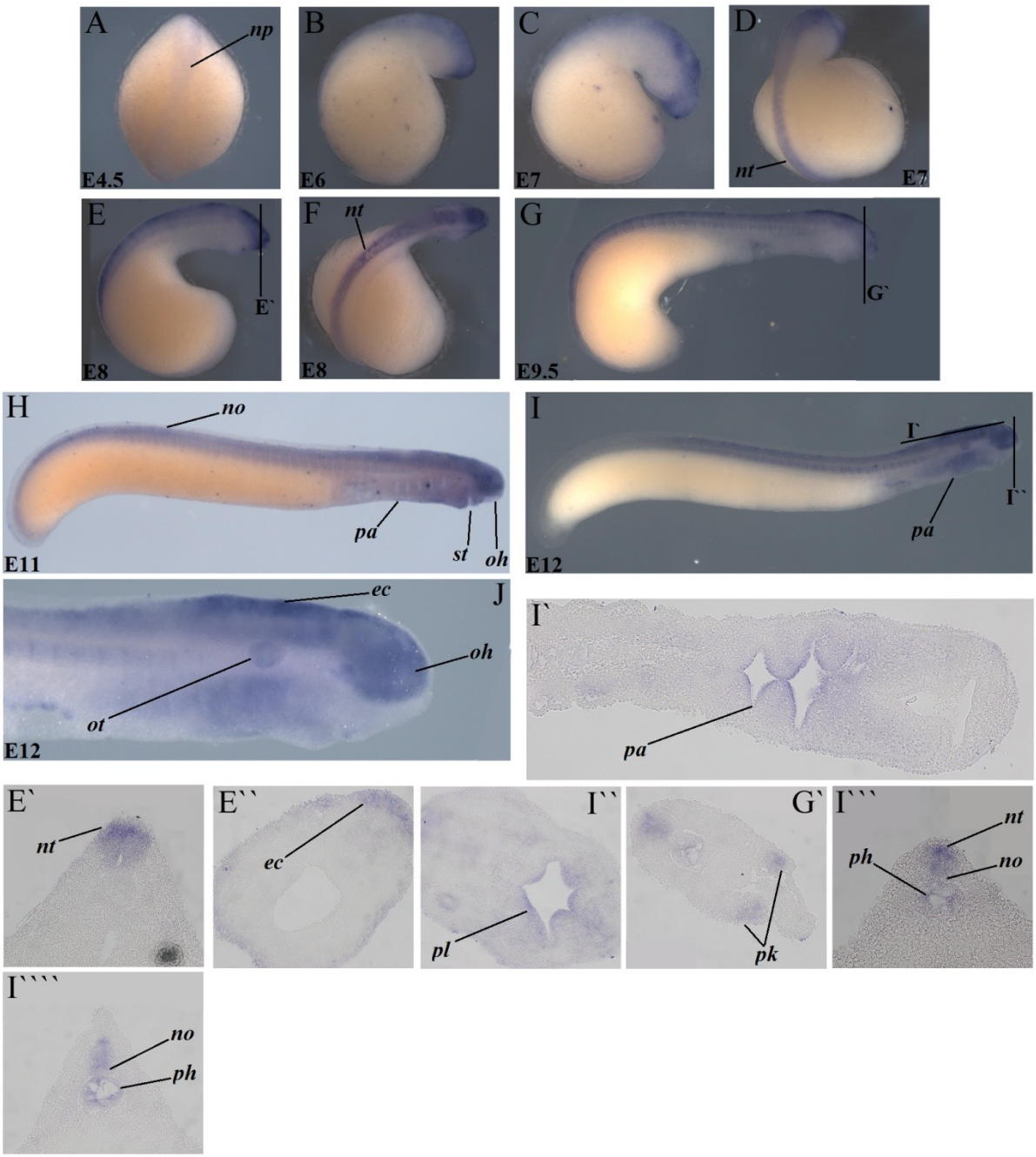


Figure 3.5A: Expression profile of *Claudin 9* during *P. marinus* embryogenesis at stages E5 to E12.

Claudin 9 is expressed in the neural plate border (A), neural tube (D-I, E`, I```), notochord (H, I```, I````), pharyngeal arches (H-I, I`), stomatodeum (H), oral hood (H-I), head ectoderm (J, E``), pharyngeal lumen (I`, I``), presumptive kidneys (G`) and pharyngeal cavity (I```, I````). *ec* – ectoderm, *no* – notochord, *np* – neural plate border, *nt* – neural tube, *oh* – oral hood, *pa* – pharyngeal arches, *ph* – pharyngeal cavity, *pk* – presumptive kidneys, *pl* – pharyngeal lumen *st* – stomodeum.

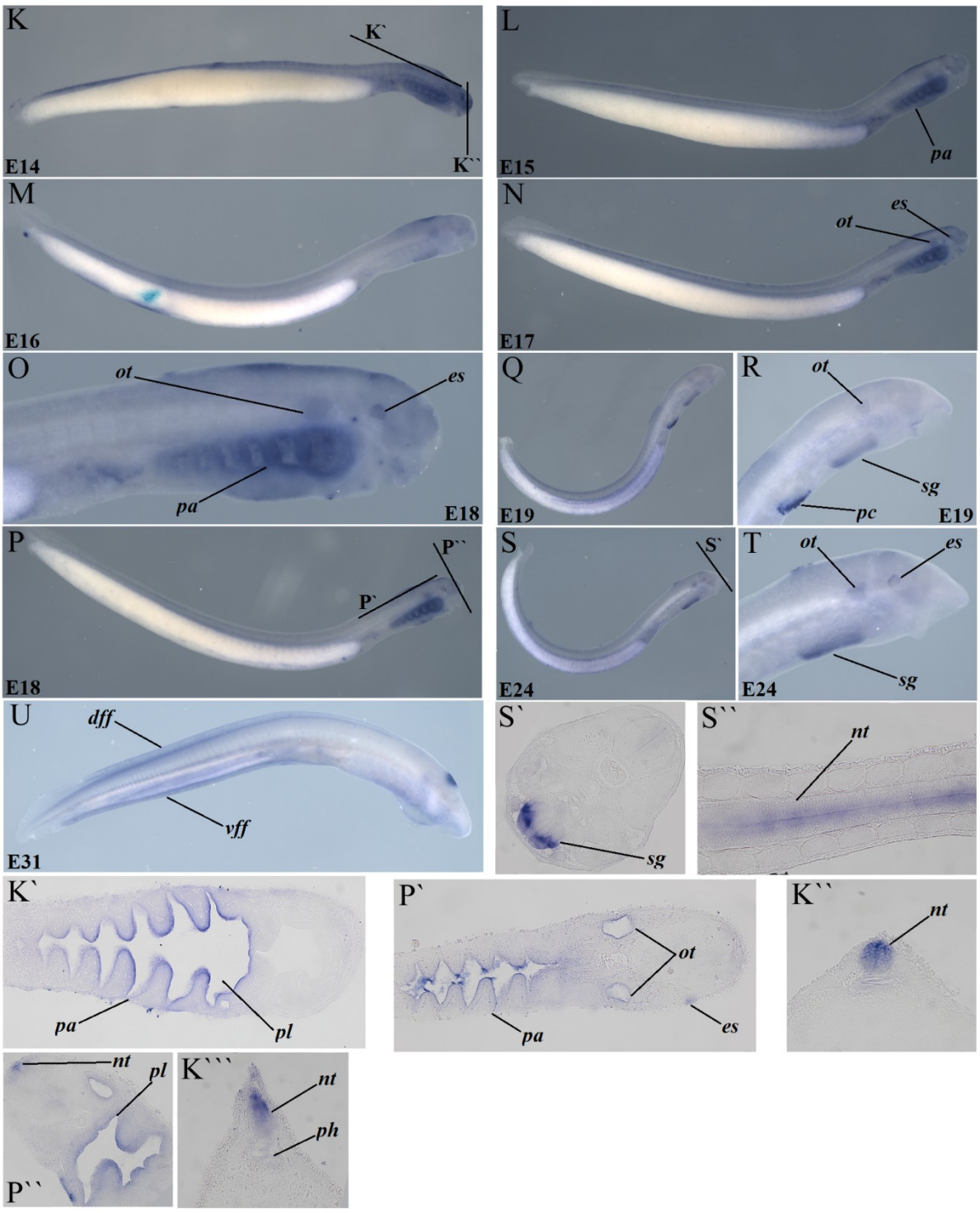


Figure 3.5B: Expression profile of *Claudin 9* during *P. marinus* embryogenesis at stages E14 to E31.

Claudin 9 is expressed in the pharyngeal arches (K-O, K`, P`), otic placode (K-T, P`), eye spot (K-T, P`), subpharyngeal gland (Q-T, S`), pericardial cavity (Q-R), dorsal fin fold (U), ventral fin fold (U), neural tube (S``, K``, P``) and pharyngeal lumen (K`, P`, P``). *dff* – dorsal fin fold, *es* – eye spot, *nt* – neural tube, *pl* – pharyngeal lumen, *ot* – otic placode, *pa* – pharyngeal arches, *pc* – pericardial cavity, *sg* – subpharyngeal gland, *vff* – ventral fin fold.

3.6 *Claudin 10* is Expressed in the Otic Placode, Notochord and Presumptive Liver

The spatial expression pattern of *Claudin 10* is shown in **Figure 3.6A** (results of whole mount *in situ* hybridisation of *P. marinus* embryos at stages E5 to E12 with *Claudin 10* probe) and **Figure 3.6B** (results of whole mount *in situ* hybridisation of *P. marinus* embryos at stages E14 to E31 with *Claudin 10* probe). Up to 3 replicates of each developmental stage was performed in order to ensure consistency of the expression pattern. Areas of blue/purple staining indicate where the probe has bound and therefore where *Claudin 10* is expressed. *Claudin 10* expression is visible at stage E5 (A, B) in the neural plate border, stage E6 (C) and E7 (D) in the ectoderm, stage E8 (E) and E9 (F) in the ectoderm, and stomodeum, stage E10 (G) in the ectoderm, stomodeum, and notochord, stage E11 (H) in the ectoderm and notochord, stage E12 (I) in the ectoderm, notochord, stomodeum, oral hood and fin fold, stage E14 (J), E15 (M), E16 (N), E17 (O) and E18 (P) in the pharyngeal arches, ectoderm (E14 only), otic placode and notochord, stage E19 (R, S) and E 24 (T, U) in the otic placode, stage E31 (U) in the dorsal fin fold.

The whole mounts were sectioned at angles indicated by the corresponding lines on the figures. The section figures reveal that expression is visible at stage E5 (A`, A``) in the neural plate border and ectoderm, stage E6 (C`), E8 (E`) and E9 (F`) in the ectoderm, stage E10 (G`) in the notochord, stage E12 (I`, I``, I```) in the ectoderm, pharyngeal cavity and notochord, stage E16 (N`, N``) in the pharyngeal arches, otic placode and ectoderm, stage E18 (P`) in the ectoderm and notochord, stage E31 (W`, W``) in the presumptive liver and dorsal fin fold.

There is evidence that there may be some signal trapping in the lumen of the pharyngeal arches at E16 (**Figure 3.6B**, N``). This may not indicate true expression of the gene in this region.

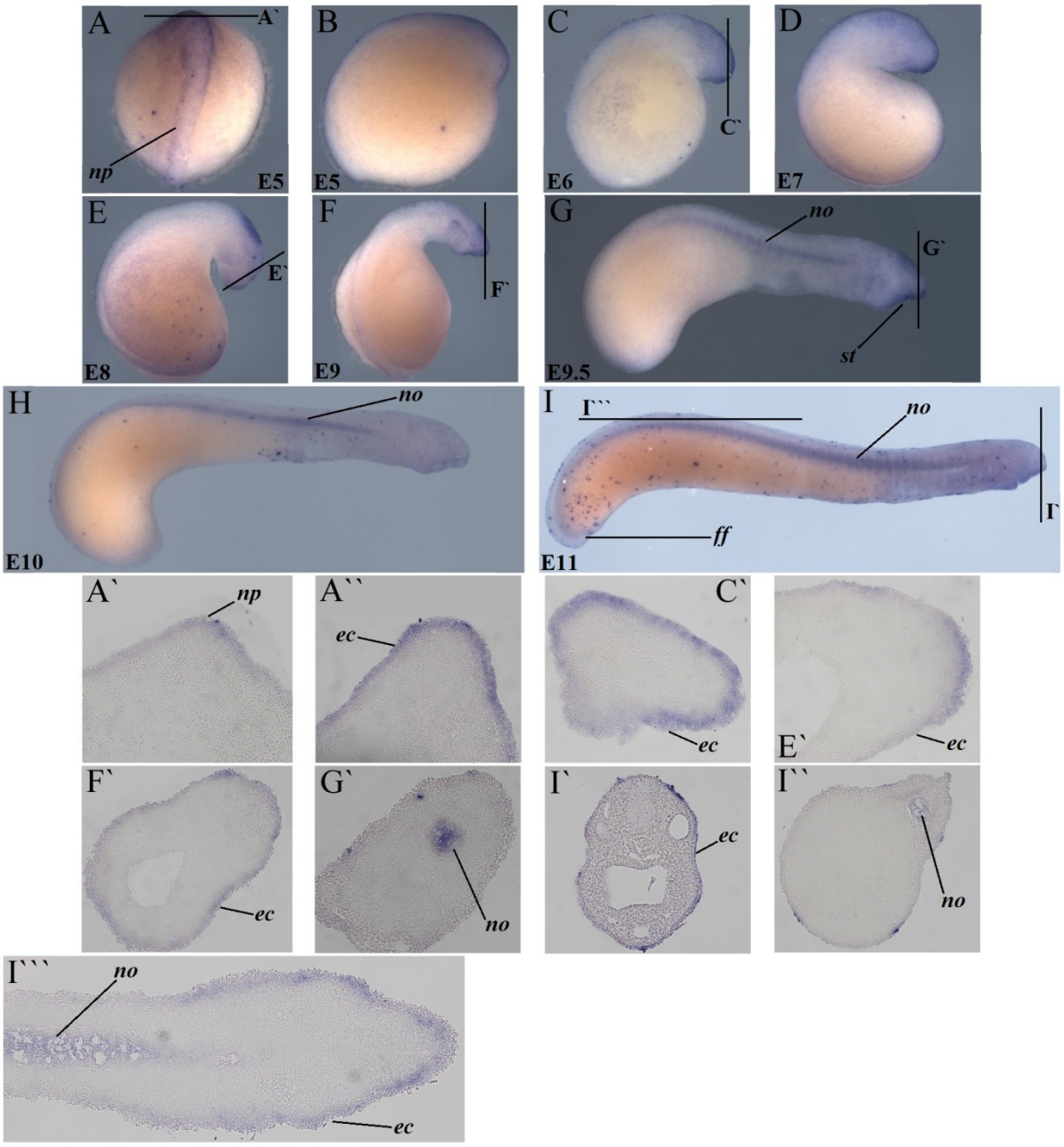


Figure 3.6A: Expression profile of *Claudin 10* during *P. marinus* embryogenesis at stages E5 to E12.

Claudin 10 is expressed in the neural plate border (A, A`, A``), notochord (G-I, G`, I``, I```), stomatodeum (F-G), fin fold (I) and ectoderm (A``, C`, E`, F`, I`, I```). *ec* – ectoderm, *ff* – fin fold, *no* – notochord, *np* – neural plate border, *st* – stomodeum.

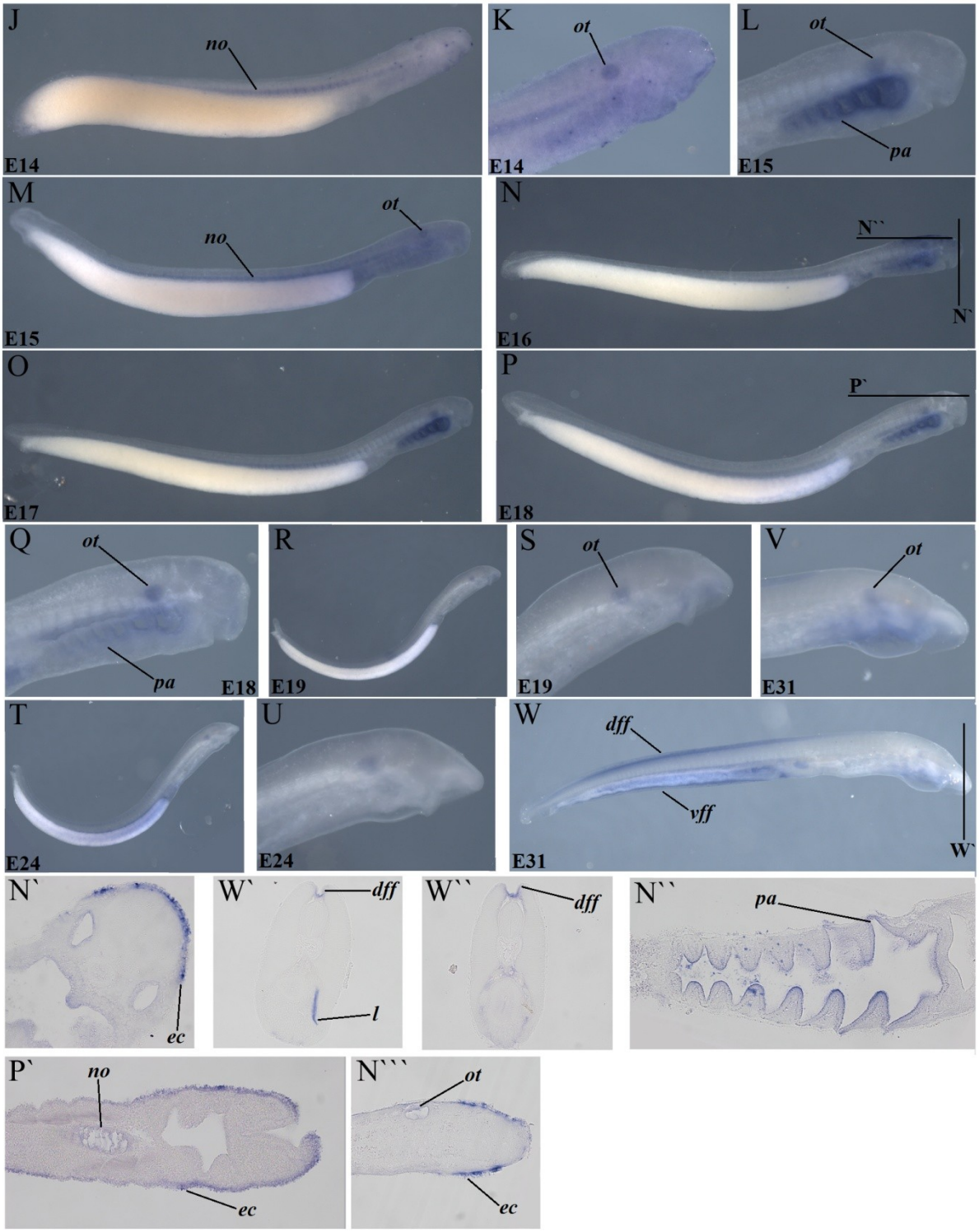


Figure 3.6B: Expression profile of *Claudin 10* during *P. marinus* embryogenesis at stages E14 to E31.

Claudin 10 is expressed in the notochord (J-P, P`), otic placode (J-W, N```), pharyngeal arches (L-Q, N``), dorsal fin fold (W, W`, W``), ventral fin fold (W) and ectoderm (N`, P`, N```). *dff* – dorsal fin fold, *ec* – ectoderm, *no* – notochord, *ot* – otic placode, *pa* – pharyngeal arches, *vff* – ventral fin fold.

3.7 *Claudin 19b* is Expressed in the Otic Placode and Somites

The spatial expression pattern of *Claudin 19b* is shown in **Figure 3.7A** (results of whole mount *in situ* hybridisation of *P. marinus* embryos at stages E5 to E12 with *Claudin 19b* probe) and **Figure 3.7B** (results of whole mount *in situ* hybridisation of *P. marinus* embryos at stages E14 to E31 with *Claudin 19b* probe). Up to 3 replicates of each developmental stage was performed in order to ensure consistency of the expression pattern. Areas of blue/purple staining indicate where the probe has bound and therefore where *Claudin 19b* is expressed. *Claudin 19b* expression is visible at stage E5 (A, B, C) in the ectoderm, stage E6 (D) and E7 (E) in the ectoderm, stage E8 (F) in the ectoderm and notochord, E9 (G) in the ectoderm, notochord and stomodeum (st), stage E10 (H) in the ectoderm and notochord, stage E11 (I, J) in the ectoderm, oral hood, notochord, and otic placode, stage E12 (K) in the ectoderm, notochord, oral hood, otic placode and pharyngeal arches, stage E14 (L) and E15 (M) in the pharyngeal arches, otic placode and notochord, stage E16 (N, O) in the pharyngeal arches, otic placode, notochord and pericardial cavity, E17 (P) in the pharyngeal arches, otic placode, notochord and pericardial cavity, stages E18 (Q) to E24 (R) in the pharyngeal arches, otic placode and pericardial cavity, stage E31 (T, U) in the pericardial cavity and otic placode. Interestingly, there appears to be a difference in the level of expression of *Claudin 19b* in the ectoderm on opposing sides of the embryo at stage E5 (see B and C).

P. marinus embryos were also hybridized with a full length *Claudin 19b* probe, showing a similar expression profile (see **Figure 6.4A** and **Figure 6.4B** in the **Appendix**). However, this probe yielded slightly lower signal clarity than the *Claudin 19b* probe targeting the untranslated region of the gene.

The whole mounts were sectioned at angles indicated by the corresponding lines on the figures. The section figures reveal that expression is visible at stage E8 (F`) in the notochord and ectoderm, stage E9 (G`, G``, G```) in the ectoderm, otic placode, and pharyngel lumen, stage E11 (J`, J``) in the notochord and pharyngeal cavity, at stage E14 (L`, L``, L```) in the ectoderm, notochord and the marginal layer of the neural tube, stage E16 (O`) in the pharyngeal arches, otic placode and pharyngeal cavity, stage E17 (P`, P``)

in the hypobranchial groove, otic placode and somites (although expression in the somites is visible as early as E9).

There is evidence that there may be some signal trapping in the lumen of the pharyngeal arches at E16 (**Figure 3.7B**, O`). This may not indicate true expression of the gene in this region.

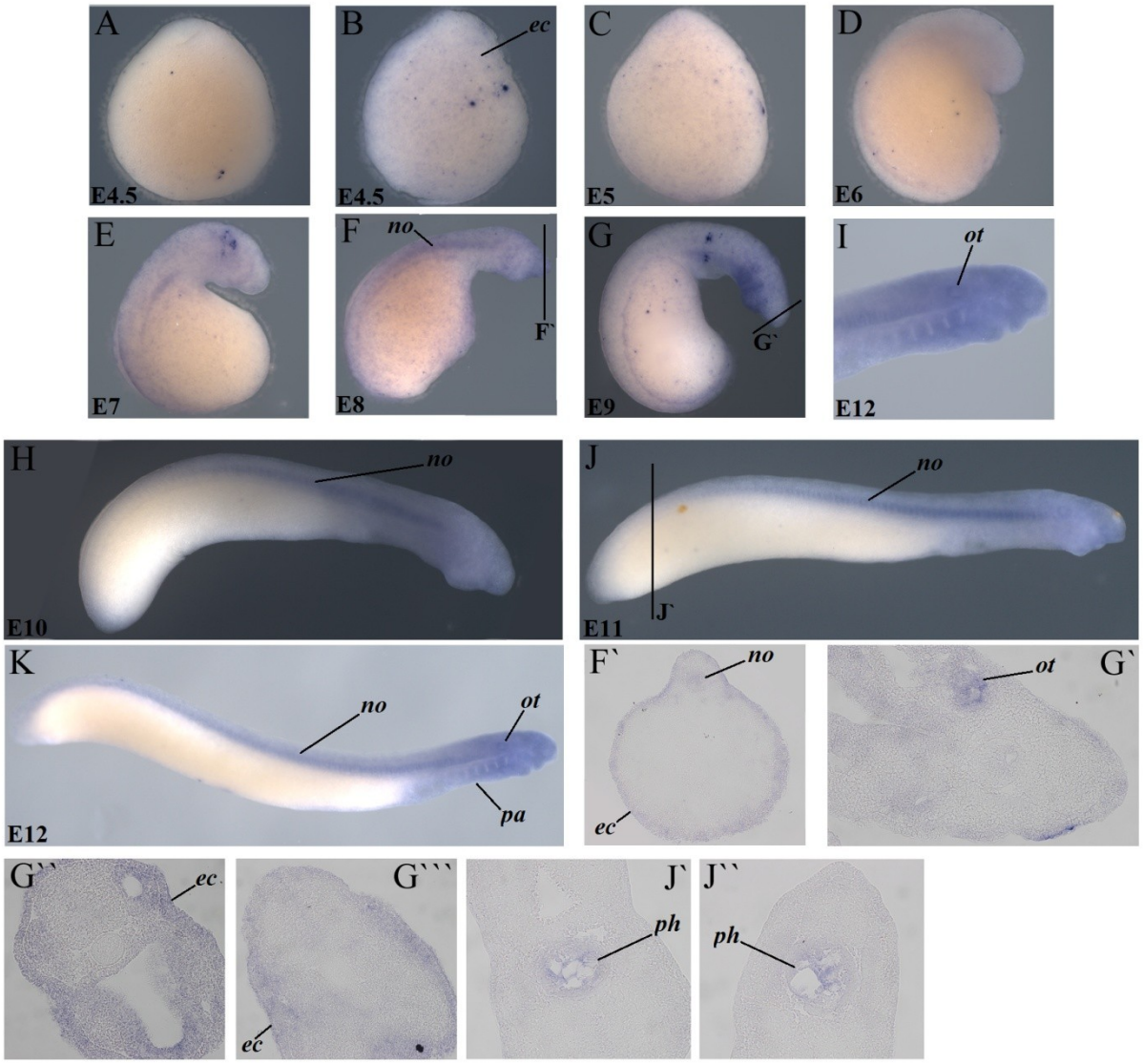


Figure 3.7A: Expression profile of *Claudin 19b* during *P. marinus* embryogenesis at stages E5 to E12.

Claudin 19b is expressed in the ectoderm (B-K, F`, G``, G```), notochord (F-K, F`), otic placode (I-K, G`), pharyngeal arches (I, K) and the pharyngeal cavity (J`, J``). *ec* – ectoderm, *no* – notochord, *ot* – otic placode, *pa* – pharyngeal arches, *ph* – pharyngeal cavity.

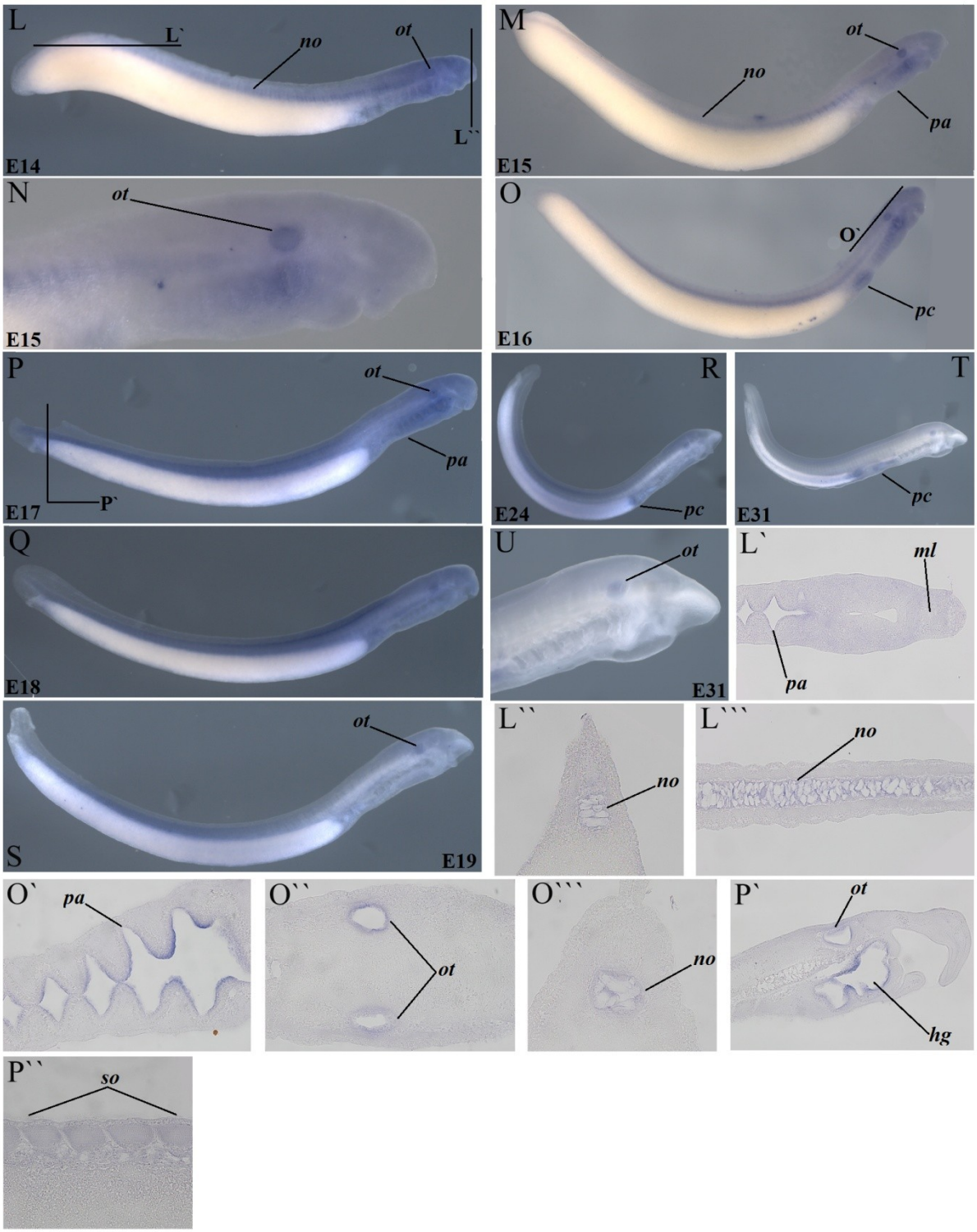


Figure 3.7B: Expression profile of *Claudin 19b* during *P. marinus* embryogenesis at stages E14 to E31.

Claudin 19b is expressed in the notochord (L-R, L``, L```, O```), otic placode (L-U, O``, P`), pharyngeal arches (L-Q, L`, O`), pericardial cavity (O-T), marginal layer of the neural tube (L`), hypobranchial groove (P`) and somites (P``). *hg* – hypobranchial groove, *ml* – marginal layer of the neural tube, *no* – notochord, *ot* – otic placode, *pa* – pharyngeal arches, *pc* – pericardial cavity, *so* – somites.

3.8 *Claudins* Appear to be frequently Co-Expressed in the Same Structures during *P. marinus* Embryonic Development.

All four observed claudin genes are expressed in the ectoderm, pharyngeal arches and otic placode at at least one developmental stage. Other common regions of claudin expression appear to be the dorsal fin fold, neural plate border and notochord, which show expression of least three claudins in at least one developmental stage. (See **Table 3.2**)
There are some regions within the embryo that appear to express many different claudins, and others, which express only a few. Certain tissues may require more complex pore or barrier properties within their epithelia (such as those tissues that are exposed to the external environment and therefore are required to maintain osmotic and ionic homeostasis under more challenging conditions), leading to a greater number of claudins being recruited to these areas. The differing migratory properties of dissimilar tissues during embryonic development may also result in differing requirements for claudin expression.

All of the claudins studied also demonstrate unique expression patterns in specific embryonic domains. For example, expression of *Claudin 9* is seen in the eyespot, pharyngeal lumen, presumptive kidneys, subpharyngeal gland and neural tube, which is not observed in any of the other three genes. Expression of *Claudin 19b* is observed in the hypobranchial groove, which is not seen in any of the other 3 genes. *Claudin 10* expression is seen in the presumptive liver, which is not visible with any of the other genes. *Claudin 1a* is expressed uniquely in the migrating neural crest. (See **Table 3.2**)

Table 3.2: Summary of claudin gene expression throughout *P. marinus* embryonic development.

Embryonic Structure	*Claudin 1a*	*Claudin 9*	*Claudin 10*	*Claudin 19b*
Dorsal Fin Fold	E31	E31	E31	
Ectoderm	E6–E31	E6–E31	E6–E31	E5–E14
Eyespot		E14–E24		
Fin Fold	E11–E31		E11–E14	
Hypobranchial Groove				E17
Marginal Layer of the Neural Tube	E12			E14
Neural Crest	E8–E9.5			
Neural Plate Border	E5	E5	E5	
Neural Tube		E6–E24		
Notochord		E11–E18	E10–E18	E8–E18
Oral Hood	E14–E31	E11–E14		
Otic Placode	E7–E9.5	E12–E24	E14–E24	E11–E31
Pericardial Cavity		E16–E24		E16–E31
Pharyngeal Arches	E12–E31	E12–E17	E14–E15, E17–E18	E12–E15, E17–E18
Pharyngeal Cavity		E19		E11–E18
Pharyngeal Lumen		E12–E18		
Presumptive Kidneys		E10		
Presumptive Liver			E31	
Subpharyngeal Gland		E19–E24		
Somites	E8–E9			E9–E18
Stomodeum	E9–E11	E11		

The stages of embryonic development at which the genes are expressed are indicated.

3.9 Analysis of Mutants Reveals that Loss of *Claudin 19b* Expression Results in Abnormal Somitogenesis.

Twenty-two *P. marinus* embryos, staged at approximately E10 were injected with a morpholino designed specifically to inhibit *Claudin 19b* expression. Fifteen embryos, staged approximately E10 were injected with a scrambled (5 base mismatch) *Claudin 19b* morpholino as controls. An additional control set of twelve E16 embryos injected with a non-specific morpholino and eleven wild-type E10 embryos (untreated) were included as additional controls. All embryos were subjected to *in situ* hybridisation using a *P. marinus Prdm1* probe in order to visualise the somites (Nikitina *et al*., 2011). The wild-types were included as a reference for an unaffected somite phenotype. The results of this analysis can be seen in **Figure 3.9A**.

Prdm1-hybridised morphants were analysed and designated as either **Non-Affected**, **Mildly-Affected** or **Severely-Affected** and scored according to the following criteria:
Non-Affected: No effect on phenotype. Similar phenotype to wild-type embryos.
Mildly-Affected: One or two consecutive somites that are either uneven (asymmetrical) on either side of the embryo, misshapen, shorter anteriorly, fused/forked or disrupted (weaker staining.)
Severely-Affected: Three or more consecutive somites that are either uneven (asymmetrical) on either side of the embryo, misshapen, shorter anteriorly, fused/forked or disrupted (weaker staining.)
The morphants were analysed, scored and the scores are summarized in the histogram in **Figure 3.9A**.

In the E16 control set, there were 10 (83%) unaffected and 2 (17%) severely affected embryos. In the E10 control set, there were 9 (60%) non-affected, 4 (27%) mildly affected and 2 (13%) severely affected embryos. In the experimental set, there were 5 (23%) non-affected, 8 (36%) mildly affected and 9 (41%) severely affected embryos. The histogram in **Figure 3.9A** shows number of embryos (and their designation) versus the set that they are included in. **Figure 3.9.1A** shows the percentage of embryos affected in each set.

The fused/forked phenotype is indicted by ***f*** on **Figure 3.9A** in which adjacent somites appear to be conjoined at some point and have not separated. The uneven somite phenotype is

indicated by ***u*** on the figure and is an asymmetry between the position or size of the somites on either side of the bilateral axis of the embryo. The misshapen phenotype, indicated by ***m*** shows an apparent loss of the characteristic somite "block" shape. The disrupted phenotype (***d***) is illustrated by a weakened signal or loss of somite presence entirely.

These morpholino experiments are preliminary and further work is required in order to clearly resolve the role of *Claudin 19b* in *P. marinus* somite development

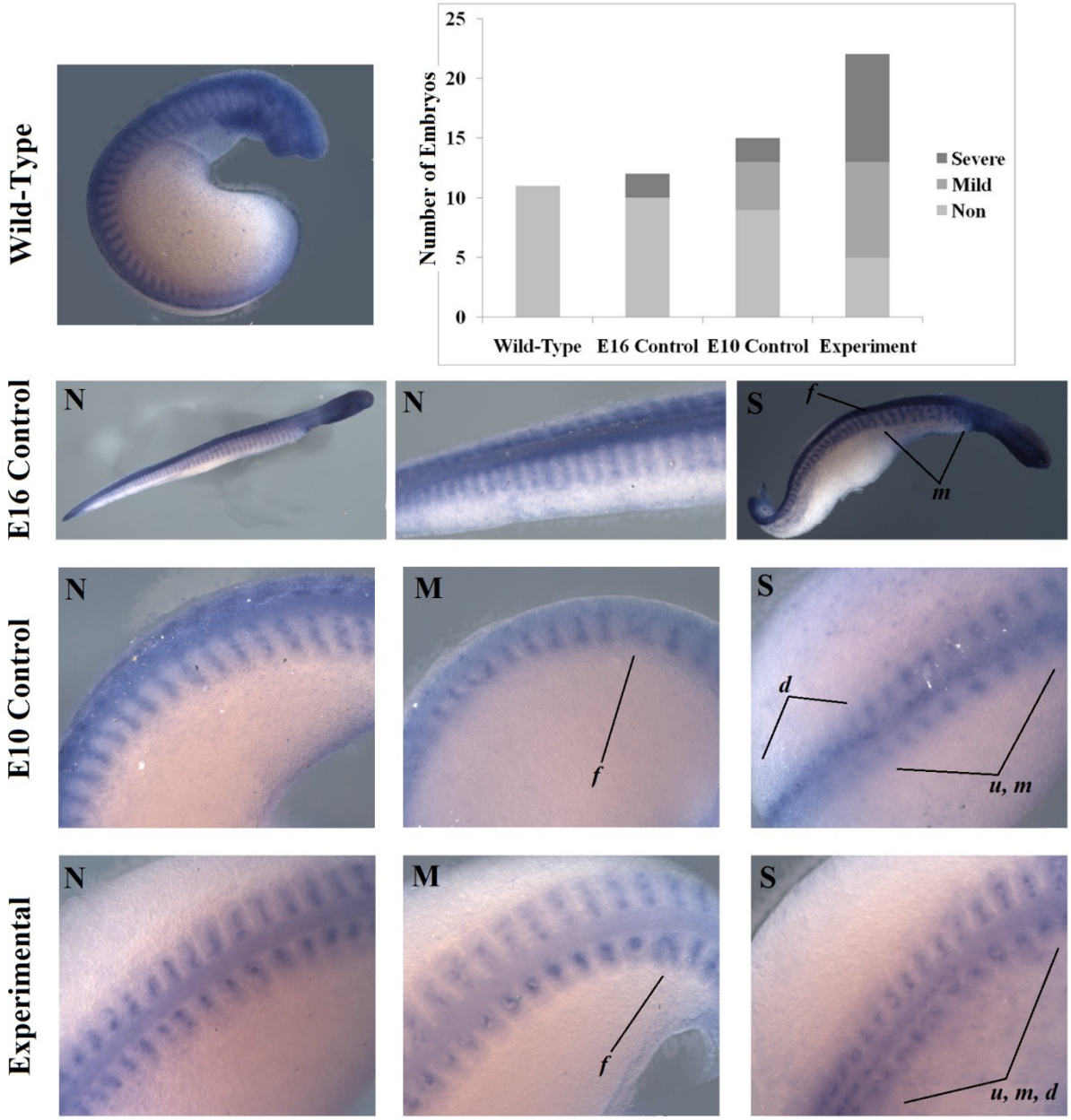


Figure 3.9A: Analysis of *Claudin 19b* mutants via *Prdm1* probe hybridisation and staining.

N – Non-affected, M – Mildly affected, S – Severely affected, *f* – fused/forked, *u* – uneven/asymmetrical, *d* – distorted, *m* – misshapen.

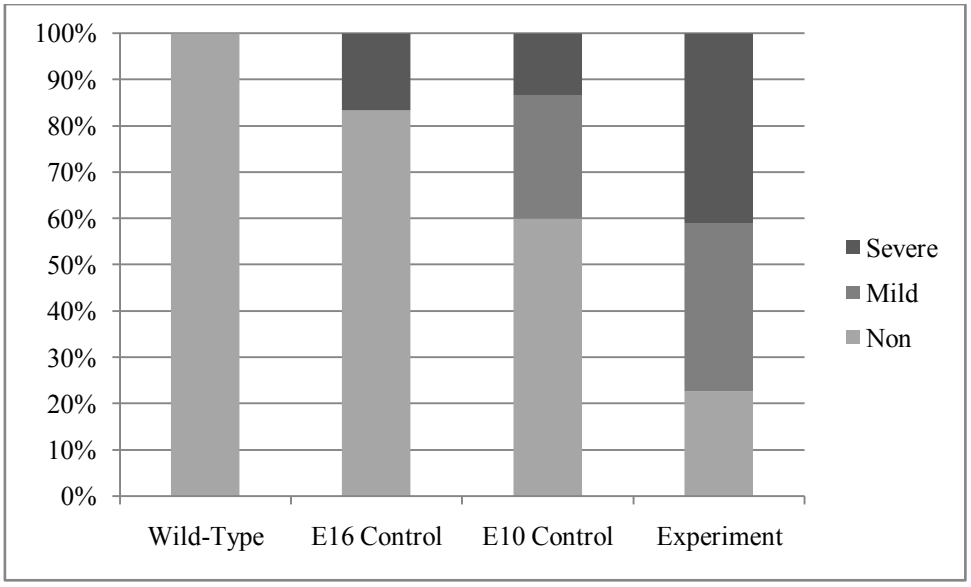


Figure 3.9.1A: Analysis of *Claudin 19b* mutants via *Prdm1* probe hybridisation and staining.

The figure shows the percentage of non-affected, mildly affected and severely affected somite phenotype in each of the treatments.

Several wild-type and *Claudin 19b* mutant (morpholino-injected) *P. marinus* embryos at stages E15, E16 and 17 were were subjected to *in situ* hybridisation using a *P. marinus TwistA* probe in order to visualise the otic placode. The staining was ineffective and the morphology of the otic placode was not clear. The results of this experiment can be seen in **Figure 3.9B**. The *TwistA* expression is clear in the wild-type embryos in the notochord, pharyngeal arches and most importantly in the otic placode, however *TwistA* expression is not clear in the experimental set. The experimental set of embryos had varying ranges of phenotypic malformations (as shown in **Figure 3.9B**) which may be attributed to the injection of the morpholino.

The hybridistion of the *TwistA* probe was carried out in order to resolve a clear visual signal from the ear placode (as has been previously achieved). However, this was only successful when *in situ* hybridisation was performed on the wild-type embryos (in order to test the probe). The experimental embryos did not have a clear signal in the otic placode due to decreased ectodermal integrity. This may have occurred due to the strong expression (and key

role) that *Claudin 19b* exhibits in the ectoderm of *P. marinus*. This decreased ectodermal integrity may have affected the precipitation of the stain or even the RNA integrity through the processing of the embryos.The otic placode was selected to study the effect of loss of Claudin 19b expression on ear development and morphology. The ear is an important, vertebrate specific development and further work on the role that *Claudin 19b* plays in the ear development in lamprey may elucidate the role of claudins in vertebrate ear development and evolution.

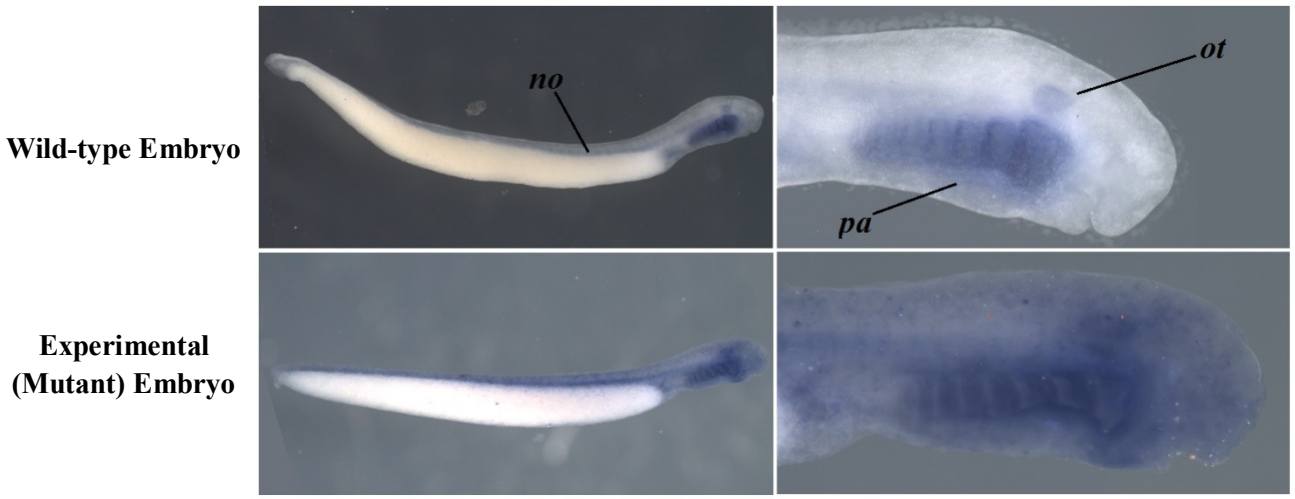


Figure 3.9B: Analysis of *Claudin 19b* mutants via *TwistA* probe hybridisation and staining.

The wild-type embryo, exhibiting a clear signal is above. The representative experimental embryo below exhibits an unclear signal. *no* – notochord, *ot* – otic placode, *pa* – pharyngeal arches.

Claudin 1a and *Claudin 10* morpholino injections were carried out in July 2014 by Christian Mukendi at the California Institute of Technology (Pasadena, California, USA) and proved unsuccessful. Following the morpholino injection, all embryos were unviable. This may be attributed to a particularly weak batch of embryos as the successfully acquired *Claudin 19b* morphants were injected and collected by Natalya Nikitina at Caltech in July 2013 (the previous year). Other factors may include toxicity of the probe or incorrect injection practices (as the embryos are very young and fragile at the stage that they are injected).

4 Discussion

4.1 *Claudin 1a* Gene Expression

The results of RT PCR with the *Claudin 1a* primer set performed on embryonic and adult tissue RNA extracts (**Figure 3.1B**), as well as the results of *in situ* hybridization with the *Claudin 1a* probe (**Figure 3.4A** and **Figure 3.4B**, summarized in **Figure 3.1B**) have been taken into account during the assembly of the figure (**Figure 4.1**) below. The figure summarizes any similarities and differences between the spatial expression pattern of *Claudin 1* that was observed in *P. marinus* (in this study) compared to that observed in higher vertebrates (other published data).

	Species					
	Region of Expression	Mouse	Chicken	*P. marinus*	*F. rubripes*	*D. rerio*
Adult Tissues	Eye	---	---	**Yes**	**Yes**	No
	Gill	---	---	No	**Yes**	**Yes**
	Heart	---	---	Yes	No	No
	Liver	---	---	Yes	No	No
	Skin	---	---	**Yes**	**Yes**	**Yes**
Embryonic Tissues	Ectoderm	**Yes**	**Yes**	**Yes**	---	---
	Esophagus	Yes	No	No	---	---
	Eye	**Yes**	**Yes**	No	---	---
	Mouth Opening/Structures	Yes	No	Yes	---	---
	Nervous System/Brain	Yes	No	No	---	---
	Neural Crest	No	Yes	Yes	---	---
	Neural Ectoderm	No	No	Yes	---	---
	Neural Tube	No	No	Yes	---	---
	Nose/ Nasal Structures	No	Yes	No	---	---
	Otic Placode/Ear	No	**Yes**	**Yes**	---	---
	Pancreas	No	Yes	No	---	---
	Pharynx/Pharyngeal Structures	No	**Yes**	**Yes**	---	---
	Skeletal System/Notochord	Yes	No	No	---	---
	Somites	No	No	Yes	---	---
	Urinary System/Kidney	**Yes**	**Yes**	No	---	---

Figure 4.1.1: Comparison of *P. marinus Claudin 1* gene expression to that of higher vertebrates. The expression of *Claudin 1* throughout embryonic development and in adult tissues is compared between *P. marinus* and that of all available expression data from higher vertebrates. Certain liberties have been taken, in that homologous structures between *P. marinus* and those of higher vertebrates have been included as "Similar Expression" between the organisms as the gene probably serves an analogous purpose in

these structures and has been conserved for this reason. The homologous structures have been indicated alongside each other to avoid confusion. "Unknown" expression (indicated by "---") may be attributed to the lack of comparative structures in the organism, or due to the lack of data either in this study or in any other comparative studies. Body structures that are not comparable between the lamprey and higher vertebrates have been excluded for simplicity's sake. The figure includes data previously published by Loh *et al*., 2004, Baltzegar *et al*., 2013, Collins *et al*., 2013, Fishwick *et al*., 2012, Magdaleno *et al*., 2006, Ohta *et al*., 2006, Troy *et al*., 2007, Traweger *et al*., 2002, Diez-Roux *et al*., 2011 and Hashizume *et al*., 2004, as well as this study.

The results of RT-PCR performed on adult *P. marinus* RNA extracts (**Figure 3.1B**) reveal that *Claudin 1a* is expressed in the skin, eye, liver and heart tissues. The expression of *Claudin 1* is similarly observed in the skin and eye of *F. rubripes* (a saltwater fish) as well as the skin of *D. rerio* (a freshwater fish), this indicates that *Claudin 1* expression in adult skin and eye tissue is a feature that has been conserved throughout vertebrate evolution up until the arrival of Teleost fish. Claudin expression is known to be important in the skin of fish in order to maintain an osmotic and ionic balance in the epithelia of these tissues (Loh *et al*., 2004). The eye is a structure that is also exposed to the aqueous environment that fish are surrounded by and the maintenance of *Claudin 1* expression in the eye probably plays a similar role to that in the skin. It is therefore evident that the maintenance of *Claudin 1* expression in the adult eye and skin tissues throughout the evolution of Teleost fish indicates that *Claudin 1* expression is vital for the maintenance of these tissues in the aqueous environment (be it either salt or freshwater) in which these fish are found. *Claudin 1* expression is however seen in the liver and heart of *P. marinus* which appear to be features of *Claudin 1* expression that have not been maintained in Teleost fish. This could imply that *Claudin 1* expression is not integral in these structures in Teleost fish (or is in fact disadvantageous) in terms of the epithelial properties that it confers in these tissues. It is also possible that a different claudin gene has been modified in some way in order to accommodate for the loss of *Claudin 1* expression in these tissues. *Claudin 1* expression has been observed in the gills of these Teleost fish, however not in the gills of *P. marinus*. We know that claudin expression is integral in the maintenance of osmotic and ionic regulation as well as the maintenance of homeostasis in the gill epithelia of Teleost fish (Loh *et al*., 2004). So it could be possible that this maintenance process is more complex in Teleost fish compared to lamprey and it requires the recruitment of a larger contingent of claudins. It is also possible that this proposed complexity required Claudin 1 to be modified in some way in order to aid in the maintenance of osmotic, ionic and homeostatic balance in the more highly evolved and complex Teleost gill epithelia.

The results of *in situ* hybridization with the *Claudin 1a* probe (**Figure 3.4A** and **Figure 3.4B**, summarized in **Figure 3.1B**) reveal that *Claudin 1a* is expressed in the migrating neural crest,

pharyngeal arches, otic placode, ectoderm, marginal layer of the neural tube, neural plate border, somites and the stomodeum during the course of embryonic development. Many of the cross sections exhibit a blue/purple signal on the edge of the sections. This may be attributed to a strong expression in the ectoderm. A strong ectodermal expression of the claudins is to be expected as claudins are integral components of the tight junctions, which are vital for the maintenance and development of epithelia in vertebrates. Though some sections show a strong ectodermal expression, this signal is absent in other sections both between different embryonic stages and differing claudin genes. This is a specific expression pattern and is clearly not surface trapping.

It is known from studies in higher vertebrates that claudins are important for the development and maintenance of structures in the ear (otic placode) (Anderson and Itallie, 2009), pharyngeal structures and ectoderm (Loh *et al*., 2004). The role of *Claudin 1a* in these structures can be inferred from this data. *Claudin 1* expression is probably involved in the development and maintenance of the epithelial component of the otic placode, pharyngeal arches, ectoderm, neural plate border and neural crest. It may aid in the cellular adhesion of these tissues as well as in the migration of these tissues during embryonic development. What we can state with a high degree of certainty is that Claudin 1a most probably plays a role in maintaining the osmotic, ionic and homeostatic balance within the epithelia of these structures. The role of Claudin 1a in the neural tube, somites and stomodeum however is somewhat more difficult to infer. It probably also plays an important role in the maintenance of the epithelia that are associated with the structures.

The role of *Claudin 1a* expression in the neural plate border and the neural crest however, is a much more interesting prospect. The expression of *Claudin 1a* in *P. marinus* is seen in the neural plate border (A, B, A` in **Figure 3.4A**) and its expression is maintained throughout the migration of the neural crest from stages E8 to E10 (F, G, H, F`, F``, H`). This observation has not been recorded in any other vertebrate before. In higher vertebrates such as the chicken, *Claudin 1* has been shown to be down-regulated in migratory neural crest cells (Fishwick *et al*., 2012). In fact, the over-expression of *Claudin* 1 has been observed to reduce neural crest migration in the developing chicken embryo (Fishwick *et al*., 2012). This is summarised in **Figure 4.1.2** below.

In the neurulating chicken embryo, *Claudin 1* is strongly expressed in the premigratory neural crest cells (Fishwick *et al*., 2012). However, as the neural crest cells undergo the epithelial-

to-mesenchymal transition (EMT), the expression of *Claudin 1* is switched off (Fishwick *et al*., 2012). The switching off of *Claudin 1* expression coincides with the dismantling of the tight junctions (an important process in order to allow for the EMT) (Fishwick *et al*., 2012). As claudins are strongly expressed in the epithelia and are implicated in joining epithelial cells together, the maintenance of the tight junctions would inevitably impede both the EMT as well as the migration of the neural crest cells. Following migration of the neural crest cells, they then go on to form parts of the cardiovascular system, the peripheral nervous system and the skull.

The presence of *Claudin 1* mRNA is maintained in the migrating neural crest of *P. marinus*, so there must be some other mechanism regulating the expression of *Claudin 1* in order to ensure that the neural crest can undergo the EMT as well as emigrate from the site of the dorsal neural tube. It is possible that the Claudin 1 protein is down-regulated or degraded and its expression is modulated in this way. The reasons behind this maintained expression of *Claudin 1* in the lamprey is unclear. It is a process that has evolved in these early vertebrates in order to facilitate the migration of the neural crest (an important vertebrate-specific cell population), but the differences between the level of gene regulation between lamprey and higher vertebrates seems strange. Perhaps the down-regulation of the expression of *Claudin 1* mRNA is a process that evolved in higher vertebrates as a means to save energy. It seems far more practical to prevent the transcription of mRNA (where possible) rather than degrading a protein (with no clear function in the migrating neural crest cells) that has been painstakingly synthesised. Perhaps lamprey, being the most primitive of vertebrates has not developed this more energy efficient method to regulate Claudin 1 and therefore facilitate the dismantling of the tight junctions during the EMT. Another possibility is that the lamprey *Claudin 1* mRNA is silenced at the post-transcriptional level by RNA interference (RNAi). During this process in plants, small RNA molecules (endogenously produced miRNAs) usually bind to target mRNA with perfect complementarity and label it for degredation (Humphtrys *et al*., 2005; Saumet and Lecellier, 2006). The perfect or near perfect complementarity of the miRNA for its mRNA target induces the cleavage of the mRNA by the RISC complex (Humphtrys *et al*., 2005; Saumet and Lecellier, 2006). However in animals, miRNAs exhibit a more variable sequence and therefore more frequently result in translational repression (Humphtrys *et al*., 2005; Saumet and Lecellier, 2006). The miRNAs bind with a high, but non-perfect sequence similarity. The translational repression has been linked with the steric inhibition of translation initiation factor binding with the mRNA polyadenine tail (Humphtrys *et al*., 2005). The

binding of certain small RNA molecules can also prevent the translation of the target mRNA by preventing the translational machinery from binding to it. This can occur via steric hindrance from the binding of the complementary small RNA molecule or via the recruitment of a complex that physically prevents the binding of the translational machinery. If the *Claudin 1 in situ* hybridisation probe is complementary to a region of the mRNA where the inhibitory miRNA/complex does not bind, it is possible that the probe could bind to and pick up on the presence of the *Claudin 1* mRNA. This post-translational silencing could allow for the re-expression of the mRNA in the neural crest cells once they have reached their destinations and need to reassemble the tight junction. It is possible for the small RNA molecules to remove themselves and unbind from the target mRNA at a beneficial point, thus allowing for translation to occur again. This is a more energy efficient mode of post-translational gene silencing than degradation of the mRNA. There are several possibilities that could explain this difference in *Claudin 1* gene expression in the neural crest between the lamprey and that of higher vertebrates, however further functional genetic research will need to be conducted in order to discover which is truly correct.

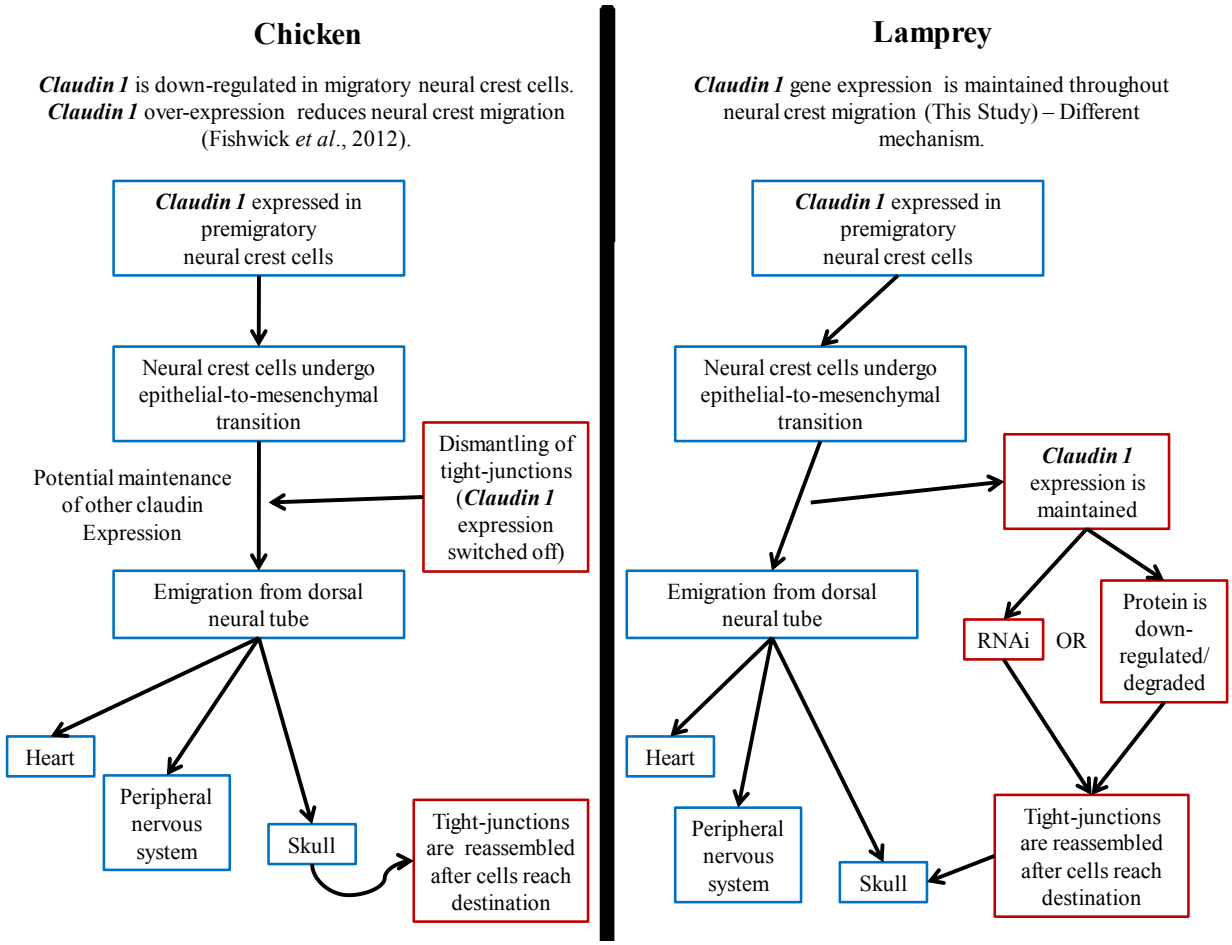


Figure 4.1.2: Expression of *Claudin 1* in the neural crest of the lamprey *P. marinus* compared to its expression in the chicken (Summary of Findings by Fishwick *et al*., 2012 and This Study).

Claudin 1 is expressed in the future spinal cord in the mouse, this is somewhat similar to the expression in the marginal layer of the neural tube in *P. marinus* (**Figure 4.1.1**). This indicates a highly conserved role of Claudin 1 in the development of the vertebrate neural/tube spinal cord. *Claudin 1* has been shown to be expressed in the parts of the skeletal system of the mouse, this could be homologous with the expression of *Claudin 1* in the somites of the early lamprey embryo (the somites differentiate to form portions of the skeletal system). More research needs to be performed on mouse somitogenesis in order to determine if *Claudin 1* has a conserved role here. From **Figure 4.1.1** it is visible that *Claudin 1* is similarly expressed in the epidermis of the developing mouse embryo, this is analogous to the expression of *Claudin 1* that we have observed in the ectoderm of the developing *P. marinus* embryo. This ectodermal expression is similarly seen in the chick embryo. From this

information, we can infer that the expression of *Claudin 1* is vital for the development and maintenance of the skin structures and this function has been maintained throughout vertebrate evolution. It has been shown in mice and humans, that loss of *Claudin1* expression results in lethal skin disorders (Anderson and Itallie, 2009). So we can see that *Claudin 1* is probably one of the most important components of the epidermis in vertebrates. Claudin 1 is vital for normal vertebrate skin development and maintenance of epidermal osmotic conditions (Anderson and Itallie, 2009) and this function is highly conserved over hundreds of millions of years of evolution. We see in **Figure 4.1.1** that *Claudin 1* expression has been observed in the neural crest, pharynx, otic vesicle/placode and lung in the chick embryo, this expression is similarly observed in the *P. marinus* embryo. The chicken lung may be equated to the analogous structure of the gills in the lamprey in that both structures are involved in gas exchange. Expression of *Claudin 1* in the respiratory organs of vertebrates seems to be conserved. So we see that expression in these areas is highly conserved throughout evolution and the presence of claudins in the epithelia of these structures must therefore play a vital role both during development (cell adhesion and movement) and in the maintenance of homeostasis in the epithelia of these tissues. Claudin gene expression in general has been shown to be important in the development of the ear and in maintaining the Na^+/K^+ concentration in the perilymph of the sensory cells in the ear (Anderson and Itallie, 2009). So this function appears to be maintained from the very point of vertebrate evolution, and therefore it is critical.

Expression of *Claudin 1* has been observed in the non-neural ectoderm, eye epithelium, nasal epithelium, pancreas and kidney in the chick embryo, as well as in the nervous system (including the brain), renal & urinary system including the kidney, esophagus, tongue, teeth, skeletal system and retina of the mouse embryo. So *Claudin 1* expression appears to have been altered through vertebrate evolution in order to play a role in the development and maintenance of the epithelia in structures such as the nasal cavity, esophagus, tongue, teeth, skeletal system, pancreas and brain. *Claudin 1* is expressed in the kidney and eye in both the chick and mouse embryo. This infers the evolution of a role for *Claudin 1* in the development and maintenance of the eye and kidney in higher vertebrates. *Claudin 1* expression is seen in the eye tissue of adult *P. marinus*, so this function has potentially been modified to play an important role in the development of eye epithelia in higher vertebrate embryogenesis. Claudin expression in the kidney is very common (Anderson and Itallie, 2009). This makes a lot of sense in that the kidneys serve to maintain homeostasis through the elimination of

excess water and the reabsorption of ions if necessary (maintenance of Na^+/Cl^- concentration) as well as the excretion of waste products. The barrier function of tight-junctions and therefore claudins within the kidney tissues facilitates this process and ensures the maintenance of homeostasis. The role of *Claudin 1*, therefore has been modified in higher vertebrates to also include the maintenance of homeostasis in the renal system (a very important process).

The spatial expression pattern of *Claudin 1* has been mapped extensively in the chick embryo, however it has not been mapped in jawed fish and only portions of the mouse embryo have been targeted. Extensive mapping of the spatial expression of *Claudin 1* throughout vertebrate and fish development needs to be performed so that we can have a clear vision of the role that this widely expressed gene has played throughout vertebrate evolution, as well as its roles in adult tissues.

Morpholino-mediated gene knockouts of *Claudin 1a* were unsuccessful and future work could involve a functional analysis of *Claudin 1a* in the *P. marinus* embryo in order to further elucidate the role that it plays in neural crest migration as well as other processes during development.

It is evident that *Claudin 1* plays an important role in lamprey development as well as the maintenance of tissue epithelia in adult lamprey. *Claudin 1* is implicated in neurulation and neural crest migration, as well as being expressed in a wide variety of embryonic and adult tissues. However, the role of *Claudin 1* appears to have evolved and diversified significantly in higher vertebrates. This signifies the diversity and importance of *Claudin 1* both in embryonic development and throughout vertebrate evolution.

4.2 *Claudin 9* Gene Expression

The results of both RT-PCR (**Figure 3.1B**) and whole mount *in situ* hybridisation of *P. marinus* embryos with the *Claudin 9* probe (**Figure 3.5A** and **Figure 3.5B**) is summarised and compared to the expression of *Claudin 9* that has been observed in previous studies in the developing mouse embryo in **Figure 4.2** below. It must be noted that the spatial mapping of *Claudin 9* expression in the mouse is not complete.

	Species		
	Region of Expression	Mouse	*P. marinus*
Adult Tissues	Eye	---	Yes
	Heart	---	Yes
	Skin	---	Yes
Embryonic Tissues	Ectoderm	No	Yes
	Eye	No	Yes
	Endoderm	Yes	No
	Heart	No	Yes
	Mouth Opening/Structures	No	Yes
	Nervous System/Brain	No	No
	Neural Crest	No	No
	Neural Ectoderm	No	Yes
	Neural Tube	No	Yes
	Nose/Nasal Cavity	Yes	No
	Otic Placode/Ear	**Yes**	**Yes**
	Pancreas	Yes	No
	Pharynx/Pharyngeal Structures	No	Yes
	Pituitary Gland	Yes	No
	Skeletal System/Notochord	No	Yes
	Thyroid	No	Yes
	Urinary System/Kidney	No	Yes
	Yolk Sac	Yes	No

Figure 4.2: Comparison of *P. marinus Claudin 9* gene expression to that of higher vertebrates. The expression of *Claudin 9* throughout embryonic development and in adult tissues is compared between *P. marinus* and that of all available expression data from higher vertebrates. . Certain liberties have been taken, in that homologous structures between *P. marinus* and those of higher vertebrates have been included as "Similar Expression" between the organisms as the gene probably serves an analogous purpose in these structures and has been conserved for this reason. The homologous structures have been indicated alongside each other to avoid confusion. "Unknown" expression (indicated by "---") may be attributed to the lack of comparative structures in the organism, or due to the lack of data either in this study or in any other comparative studies. Body structures that are not comparable between the lamprey and higher vertebrates have been excluded for simplicity's sake. The figure includes data previously published by Hou *et al*., 2007, Hoffman *et al*., 2008, Hashizume *et al*., 2004 and Diez-Roux *et al*., 2011, as well as this study.

We observed that *Claudin 9* was expressed in the skin, eye and heart tissue of an *adult P. marinus*. There is no comparative expression data of *Claudin 9* in adult tissues for any other organism. However, the expression that we observed in the skin and eye is consistent with general claudin expression. The role that *Claudin 9* plays in the skin and eye is probably to maintain the osmotic, ionic and homeostatic balance of the epithelia of these external structures that are in constant contact with the varying external aqueous environment (as has been previously discussed). The role that *Claudin 9* plays in the heart is a bit more difficult to deduce, but it probably plays a similar role in the heart epithelia. Interestingly, claudin expression in general seems to be fairly consistent in the *P. marinus* heart.

The only vertebrate embryo in which expression data of *Claudin 9* is available is that of the mouse. This data on its own is quite limited in that an extensive mapping of claudin gene expression throughout embryonic mouse tissues has not yet been performed. All expression data has been acquired from studies targeting gene expression in specific tissues or expression related to specific pathways in the developing mouse embryo. The lack of *Claudin 9* expression in other vertebrates seems strange. The presence of Claudin 9 in the lamprey, as well as the mouse indicates that it is possible that *Claudin 9* is yet to be discovered in intermediary vertebrates such as jawed fish and the chicken. The lack of data relating to *Claudin 9* could also be due to the constant discovery of new claudin genes and the incorrect classification and designation of these genes, which has been observed in the past (Baltzegar *et al*., 2013). Some claudins are designated with a different nomenclature which makes comparisons difficult to achieve. For these reasons, it is difficult to classify and compare claudins from different vertebrate species.

Expression of *Claudin 9* is observed in the otic placode, ectoderm, eyespot, neural plate, neural tube, notochord, oral hood, pericardial cavity, pharyngeal arches, pharyngeal cavity, pharyngeal lumen, presumptive kidneys, subpharyngeal gland and stomodeum during *P. marinus* development (see **Figure 3.5A** and **Figure 3.5B**). The role of claudin expression in the otic placode and ectoderm has been explained previously and this common expression indicates the importance of claudin expression in these tissues. *Claudin 9* expression in the presumptive thyroid gland (subpharyngeal gland) indicates that it may play a role in the regulation and excretion of hormones from the thyroid, if not purely a role in the development and maintenance of the thyroid epithelia in lamprey. The expression of *Claudin 9* in the neural plate and its maintenance in the neural tube indicate that *Claudin 9* is involved in the process of neurulation and its expression is maintained throughout the development of

the neural tube. Expression in the pericardial cavity and eyespot seems to be very important, not just in the development of these structures, but also in the maintenance of the adult tissues that they predate. *Claudin 9* expression is maintained throughout the development of the eyespot, into the adult eye. This indicates that *Claudin 9* probably plays an integral role in the maintenance of the eye epithelia in lamprey. The maintenance of expression in the pericardial cavity through to the development of the adult heart indicates that *Claudin 9* also plays a vital role in the maintenance of the heart epithelia. Expression in the oral hood and notochord also seems to be important. *Claudin 9* expression in what appear to be the presumptive kidneys (G` in **Figure 3.5A**) appear to indicate the genes involvement in the development of the kidney which, as has been stated previously plays a vital role in maintenance of osmotic homeostasis as well as waste excretion in the lamprey, similarly to other vertebrates.

There is a shared similar expression of *Claudin 9* in the otic placode (presumptive ear) of the lamprey and the cochlea (innear ear structure) of the mouse embryo. *Claudin 9* expression is therefore highly conserved in the ear structures of vertebrates. *Claudin 9* has been shown to be expressed in the tight junctions of the cochlea, where it acts to separate the high K^+ endolymph from the low K^+ perilymph (Günzel and Yu, 2013). The perilymph covers the outer sensory cells (Günzel and Yu, 2013). *Claudin 9* seems to prevent the sensory cells from exposure to high K^+ concentrations (Günzel and Yu, 2013). Loss of Claudin 9 has been shown to result in hearing loss in mammals. So it is evident that Claudin 9 is highly conserved throughout the evolution of the vertebrate ear over hundreds of millions of years. This points towards Claudin 9 being integral in the proper functioning and development of the vertebrate ear all throughout evolution.

The expression of *Claudin 9* in the lamprey ectoderm, eyespot, neural plate, neural tube, notochord, oral hood, pericardial cavity, pharyngeal arches, pharyngeal cavity, pharyngeal lumen, presumptive kidneys, subpharyngeal gland and stomodeum is not seen in the mouse embryo. However the spatial expression patterns of *Claudin 9* gene expression in fish and avian models (as well as a more comprehensive screening of the developing mouse) are required in order to make inferences as to why *Claudin 9* expression has ceased in these structures during the course of vertebrate evolution.

Claudin 9 is expressed in the endoderm, yolk sac, pancreas epithelium and pituitary gland of the developing mouse embryo, which is not observed in the lamprey. Expression is also seen in the mouse nasal cavity olfactory epithelium and submandibular gland for which there are

no analogous structures in the lamprey. Expression in the endoderm, yolk sac and nasal cavity olfactory epithelium indicates that *Claudin 9* has gained new function in mammals and is involved in the regulation of different epithelial structures in higher vertebrates when compared to lamprey. The expression in the pancreas epithelium, pituitary gland and submandibular gland could prove that Claudin 9 played a primitive role in hormone excretion from gland epithelia. Claudin 9 is expressed in the presumptive thyroid gland in lamprey, so it is possible that it has been modified in order to maintain the excretion of hormones from the gland epithelia of a wider range of organs in the mouse.

From this data, we can see that *Claudin 9* is expressed in a wide range of tissue types. It is the most versatile claudin that has been included in this study. Its roles may range from maintenance of homeostasis in the ear, heart, kidney and eye to the regulation of hormone excretion from certain gland epithelia in vertebrates. Claudin 9 is also clearly involved in the development and maintenance of an even wider range of structures in the vertebrate body. However, more data concerning the spatial expression of this gene and the reason for its apparent absence (if it is truly absent) in other vertebrate system needs to be produced in order to better understand the role that it has played throughout vertebrate evolution.

4.3 *Claudin 10* Gene Expression

The data generated from RT-PCR from embryonic and adult tissue RNA extracts (**Figure 3.1B**) as well as the spatial expression patterns of *Claudin 10* as determined by *in situ* hybridisation (**Figure 3.6A** and **Figure 3.6B**) have been summarised and compared to expression data from other vertebrate systems that have previously been studied in **Figure 4.3** below.

	Species					
Adult Tissues	**Region of Expression**	Mouse	Chicken	*P. marinus*	*F. rubripes*	*D. rerio*
	Eye	---	---	No	Yes	No
	Gill	---	---	**Yes**	**Yes**	Yes
	Liver	---	---	Yes	No	No
	Skin	---	---	No	Yes	No
Embryonic Tissues	Ectoderm	**Yes**	**Yes**	**Yes**	---	---
	Esophagus	Yes	No	No	---	---
	Eye Structures	No	Yes	No	---	---
	Heart	No	Yes	No	---	---
	Liver	No	No	Yes	---	---
	Mouth Opening/Structures	Yes	No	No	---	---
	Neural Ectoderm	No	**Yes**	**Yes**	---	---
	Otic Placode/Ear Structures	No	**Yes**	**Yes**	---	---
	Pancreas	Yes	No	No	---	---
	Pharynx/Pharyngeal Structures	No	No	Yes	---	---
	Skeletal System/Notochord	No	No	Yes	---	---
	Urinary System/Kidney	**Yes**	**Yes**	No	---	---

Figure 4.3: Comparison of *P. marinus Claudin 10* gene expression to that of higher vertebrates. The expression of *Claudin 10* throughout embryonic development and in adult tissues is compared between *P. marinus* and that of all available expression data from higher vertebrates. . Certain liberties have been taken, in that homologous structures between *P. marinus* and those of higher vertebrates have been included as "Similar Expression" between the organisms as the gene probably serves an analogous purpose in these structures and has been conserved for this reason. The homologous structures have been indicated alongside each other to avoid confusion. "Unknown" expression (indicated by "---") may be attributed to the lack of comparative structures in the organism, or due to the lack of data either in this study or in any other comparative studies. Body structures that are not comparable between the lamprey and higher vertebrates have been excluded for simplicity's sake. The figure includes data previously published by Loh *et al*., 2004, Baltzegar *et al*., 2013, Collins *et al*., 2013, Ohta H, *et al*., 2006, Hashizume *et al*. 2004, Diez-Roux *et al*., 2011,as well as this study.

Claudin 10 has been shown to be expressed in the liver and gill tissue of an adult *P. marinus* via RT PCR. *Claudin 10* has been shown in the past to equally be expressed in the gill tissue of adult *F. rubripes* and *D. rerio*. This once again, indicates the importance of yet another

claudin in the maintenance of osmotic homeostasis in the gill epithelia of these Teleost fish, as well as the conservation of this gene's function throughout vertebrate evolution. The expression of *Claudin 10* in the liver of the lamprey, however is a feature that has not been conserved in Teleosts. Expression of this gene orthologue is also observed in the eye and skin of *F. rubripes*, but not in lamprey. This once again indicates the modification of this gene's expression in order to perform a maintenance function in the eye and skin tissue epithelia in this more complex fish.

Claudin 10 expression is observed in the neural plate, otic placode, ectoderm, pharyngeal arches, notochord and presumptive liver of the developing *P. marinus* embryo (see **Figure 3.6A** and **Figure 3.6B**). This implicates yet another claudin in the process of neurulation (generation and maintenance/migration of the neural ectoderm) as well as in the development and maintenance of the otic placode, pharyngeal arches and notochord. Expression in the pharyngeal arches is maintained through to the development of the adult gill structures. This indicates that Claudin 10 is vitally important in not only the generation, but the maintenance of the gill tissue in lamprey. This once again can be related to the importance of maintaining an osmotic balance in the gill epithelia. Expression is maintained in what appears to be the presumptive liver (W` in **Figure 3.6B**) through to the development of the adult liver. This implicates Claudin 10 strongly in the development and maintenance of the liver tissues (it is probably also implicated in maintaining the liver epithelia).

Claudin 10 is expressed in the *P. marinus* embryonic ectoderm and pharyngeal arches, similarly to the ectoderm and lung structures in the mouse and chick embryo. This indicates that *Claudin 10* is highly conserved and implicated in the development and maintenance of epithelia in the skin and respiratory organs of the vertebrate. The functions of claudins in these tissues have been previously discussed in-depth. *Claudin 10* is also expressed similarly in the chick and lamprey embryonic neural ectoderm and otic vesicle. This implicates the maintenance of *Claudin 10* in the neural plate throughout vertebrate evolution and therefore strongly in the process of neurulation in vertebrates. *Claudin 10* therefore must play an important role in the development and maintenance of the neural ectoderm during early neurulation in vertebrates. The conservation of expression in the otic placode implicates yet another claudin in the maintenance of the vertebrate ear further validating the importance of claudin expression in the maturation and preservation of this structure.

Claudin 10 expression is observed in the lamprey embryonic notochord and presumptive liver, which is not seen in higher vertebrates. This may reveal a modification of this gene's function in the epithelia of higher vertebrates. *Claudin 10* has been shown to be expressed in the embryonic heart, eye, kidney, pancreas, esophagus, oral epithelium and teeth of these higher vertebrates, but not in lamprey. This could argue for the diversification of *Claudin 10* expression in higher vertebrates in order to take on new roles in the development of these structures in higher vertebrates. Although different claudin expression has been observed in lamprey heart, eye, kidney and oral epithelia, this poses yet another question. Why are the roles of some claudins modified in higher vertebrates to resemble the roles of other claudins in the lamprey? Why are the roles of these claudins not maintained instead of modifying the functions of said other claudins? This could be explained by the fact that in most tissues, there is an expression of multiple claudin genes (Anderson and Itallie, 2009). This relates to the need for differing barrier and pore forming properties at the tight junctions in different epithelial tissues. The combinations of different claudins can confer these different properties. So the expression of different claudins in the same tissues between vertebrates could be due to the requirement for different conditions in the epithelia of the different organisms. This is very possible as vertebrates live in a wide range of environments, ranging from open ocean to dry land and these different environments and differing conditions confer different selective pressures on the epithelia and therefore on the combinations of claudins found in these epithelia. As has previously been mentioned, the fact that there are many more claudin genes in aquatic vertebrates compared to the land vertebrates appears to support this hypothesis. So this difference between specific claudin expression in similar vertebrate tissues could be attributed to these factors.

Morpholino-mediated gene knockouts of *Claudin 10* were unsuccessful and future work could involve a functional analysis of *Claudin 10* in *P. marinus* in order to further elucidate the role that it plays during embryonic development.

Claudin 10 is, like most other claudins expressed in the ectoderm, pharyngeal arches and otic placode. It appears to be vital for maintenance of the lamprey liver and gill tissues, and the role it plays in other vertebrates seems to have diversified considerably. It does however have a consistent and maintained expression in many embryonic vertebrate tissues such as the neural ectoderm, otic placode, ectoderm and respiratory organs. This shows that Claudin 10 is probably, like many other claudins very important in the development and evolution of these structures in vertebrates as a whole.

4.4 *Claudin 19b* Gene Expression

The expression pattern of *Claudin 19b* in *P. marinus* was mapped in both adult tissues and through embryonic development using RT-PCR (**Figure 3.1B**) and whole mount *in situ* hybridisation (**Figure 3.7A and Figure 3.7B**). This data is summarised in **Figure 4.4** below, along with the comparable expression of *Claudin 19* that has been discovered in other vertebrates.

	Species					
	Region of Expression	Mouse	*Xenopus*	*P. marinus*	*F. rubripes*	*D. rerio*
Adult Tissues	Brain	---	---	---	**Yes**	**Yes**
	Gill	---	---	**Yes**	**Yes**	No
	Heart	---	---	Yes	No	No
	Liver	---	---	Yes	No	No
	Skin	---	---	Yes	No	No
Embryonic Tissues	Ectoderm	No	No	Yes	---	---
	Eye	No	Yes	No	---	---
	Heart	No	No	Yes	---	---
	Nervous System/Brain	Yes	No	No	---	---
	Neural Tube	No	No	Yes	---	---
	Otic Placode/Ear	No	No	Yes	---	---
	Pharynx/Pharyngeal Structures	No	No	Yes	---	---
	Skeletal System/Notochord	No	No	Yes	---	---
	Somites	No	**Yes**	**Yes**	---	---
	Urinary System/Kidney	**Yes**	**Yes**	No	---	---

Figure 4.4: Comparison of *P. marinus Claudin 19* gene expression to that of higher vertebrates. The expression of *Claudin 19* throughout embryonic development and in adult tissues is compared between *P. marinus* and that of all available expression data from higher vertebrates. . Certain liberties have been taken, in that homologous structures between *P. marinus* and those of higher vertebrates have been included as "Similar Expression" between the organisms as the gene probably serves an analogous purpose in these structures and has been conserved for this reason. The homologous structures have been indicated alongside each other to avoid confusion. "Unknown" expression (indicated by "---") may be attributed to the lack of comparative structures in the organism, or due to the lack of data either in this study or in any other comparative studies. Body structures that are not comparable between the lamprey and higher vertebrates have been excluded for simplicity's sake. The figure includes data previously published by Loh *et al*., 2004, Baltzegar *et al*., 2013, Diez-Roux *et al*., 2011 and Raciti *et al*., 2008, as well as this study.

Expression of *Claudin 19b* was observed in the skin, liver, heart and gill tissue of an adult *P. marinus*. This expression is somewhat different to that which has been observed in Teleost fish. *F. rubripes* expresses *Claudin 19* in the gills, similarly to *P. marinus*, however it does not exhibit any expression in the skin, liver or heart tissues. *Claudin 19* has been shown to be expressed in the brain tissue of *F. rubripes* and *D. rerio*, indicating that the role of Claudin 19

in higher vertebrates appears to be more localised in the brain and gill epithelia as opposed to the wide range of tissues that it is expressed in in the adult lamprey.

Claudin 19b is expressed in the ectoderm, marginal layer of the neural tube, notochord, otic placode, pericardial cavity, pharyngeal arches, pharyngeal cavity and somites of the developing *P. marinus* embryo. Expression is maintained in the ectoderm through to the development of the adult skin. Expression is also maintained in the pericardial cavity through to the development of the adult heart. This indicates the importance of Claudin 19b in both the skin and heart tissues of the lamprey. Expression in the neural tube implicates all four of the claudins discussed so far in either the process of neurulation or the maintenance of structures formed during neurulation. Expression in the notochord, otic placode, pharyngeal arches and pharyngeal cavity also appear to be important features of claudin expression as previously discussed.

Expression of *Claudin 19* is observed in the somites of the developing *Xenopus* embryo as well as the somites of *P. marinus*. This conservation of *Claudin 19* expression implicates it strongly in the development of, as well as potentially in the evolution of vertebrate somites and the somite derived tissues.

Claudin 19 is expressed in the renal & urinary system (including the kidney) in both the developing *Xenopus* and mouse embryo. This strongly implicates Claudin 19 in the development of the renal structures in higher vertebrates as well as in the maintenance of renal tissues and processes (as has been previously discussed). Expression is evident in the eye of the *Xenopus* embryo, as *Xenopus* are amphibians, Claudin19 could act to regulate the osmotic balance when the eye is exposed to an aqueous environment as it does in Teleost fish. *Claudin 19* is expressed in the brain of the mouse embryo, as it is in the brain of adult teleost fish. This implicates Claudin 19 in the maintenance to brain epithelia in higher vertebrates, a function that has developed over the course of vertebrate evolution.

Claudin 19b appears to be vital for the development and functioning of *P. marinus* skin and heart tissues, as well as in a range of other tissues that are commonly associated with claudin expression in this lamprey species. *Claudin 19* expression has evolved to play a pivotal role in the renal system and brain in higher vertebrates. The expression of *Claudin 19* in the somites is persistently conserved through vertebrate evolution as it is also observed in the *Xenopus* embryo. Claudin 19, like all of the other claudins discussed in this study so far either

plays a role in neurulation or is expressed in the structures derived during neurulation, is important in the ear, skin and pharyngeal structures in the lamprey.

4.5 General Claudin Gene Expression in *P. marinus* reveals Diversification of Claudin Function through Vertebrate Evolution Along with the Conservation of Several Key Roles

From **Table 3.2** (in which the spatial expression patterns of *Claudin 1a*, *9*, *10* and *19b* are summarized) it is evident that the four genes are continuously expressed throughout *P. marinus* embryonic development in the ectoderm, otic placode and pharyngeal arches. These tissues appear to be characteristic areas of claudin gene expression in the lamprey. The neural tube, neural plate border and notochord also seem to be tissues in which claudin gene expression is relatively important. In adult *P. marinus* tissues, claudin gene expression is most notably important in the skin and heart, but it is also highly expressed in the eye, liver and gill tissues (**Figure 3.1** and **Figure 3.1B**).

The general localization of claudin expression in *P. marinus* has some parallels with observed claudin expression in higher vertebrates. The ectoderm and otic placode have proven to be tissues with concentrated claudin expression during embryonic development in other vertebrates such as the chicken and mouse. Expression of claudins in adult skin, eye, liver and gill is also very common in other vertebrates (**Table 1.1**) and expression in the heart is also sometimes observed. However, claudins appear to play a more global role in the development of the renal & urinary system (including the kidney) and the nervous system and brain in higher vertebrate embryos. These systems are highly intricate and their increased complexity in higher vertebrates may lend to the requirement for more complex barrier and pore attributes in their epithelia, and therefore the requirement for the recruitment of a more intricate claudin network. The processes involved in the formation of these structures and therefore the processes involving tissue migration in the developing embryo may also be somewhat more complex and therefore require this increased intricacy of the claudin complex. The expression of *P. marinus* claudins in the adult kidney tissue, unfortunately was not completed in this study and this may be a target for future research in terms of better understanding the evolution of the vertebrate kidney and associated structures. The expression of many claudins in the brain of higher vertebrates lends to the requirement for rapid signal transduction and precise control of signal molecule and substrate movement between the cells of the nervous system. This is a potential gain of function role for claudins in the brain of vertebrates, further indicating the diversity and importance of these proteins throughout vertebrate evolution.

Claudins have been implicated in the process of neurulation and neural crest migration in the chicken (Fishwick *et al*., 2012), however they seem to be more consistently expressed in the lamprey. All four of the genes were expressed at some point in either the neural plate border, neural plate, neural crest or the neural tube. This indicates that claudins probably play a very important role in the process of neurulation and neural crest migration in the lamprey. For this reason, further work should be conducted involving the role of claudins in neurulation in higher vertebrates. Expression of all four of the studied claudin genes in the pharyngeal arches and otic placode point to the importance of these genes in maintaining the osmotic, ionic and homeostatic balance in these tissues in the lamprey. Frequent expression in the notochord, it appears is also characteristic of lamprey claudin expression.

Claudins play an important role in lamprey development and maintenance of tissues. Many of the roles that claudins play in the lamprey have been conserved throughout vertebrate embryonic development, but the role of claudins seems to have diversified considerably as the vertebrate body plan has become more complex.

4.6 Loss of *Claudin 19b* Gene Expression Prevents Normal Somitogenesis in *P. marinus*

Figure 3.9A shows that the loss of *Claudin 19b* expression results in an abnormal somite phenotype. The wild-type embryos indicated in the figure were included as an example of the non-affected (normal) somite phenotype. The E16 control embryos were injected with a non-specific (non-binding) morpholino in order to act as a control for the *Claudin 19b* morpholino. There weren't any embryos available that were injected with the non-specific control at the correct stage (E8-E10), so the E16 controls were used instead. The E10 control embryos were injected with a 5 base pair mismatch of the *Claudin 19b* morpholino, which is also non-binding due to this decreased sequence similarity. The E10 controls also acted as a control for the effects of the *Claudin 19b* morpholino as well as the experimental procedure and injection process. The experimental set was injected with the *Claudin 19b* morpholino (binds specifically to region of the *Claudin 19b* mRNA) and the morpholino was successfully incorporated into the embryos (as checked by fluorescence microscopy).

As expected, none of the wild-type embryos were affected in any way. 17% of the twelve E16 control embryos were designated as being severely affected, however this may be attributed to the physical process of injecting the embryos, which can damage their morphology and subsequent development. This is another reason to include these controls. 40% of the fifteen E10 control set were affected, but the majority (27%) were mildly affected. The vast majority (77%) of the twenty-two experimental embryos in which *Claudin 19b* had been knocked out were affected, with most of these (41%) being severely affected. This data is summarized in the bar graph in **Figure 3.9A**. It is therefore clear that the loss of *Claudin 19b* expression has an effect on somite morphology and that Claudin 19b probably plays an important role in *P. marinus* somitogenesis.

The potential stages during which Claudin 19b could be involved in somitogenesis are highlighted in **Figure 4.6** below. The process of somitogenesis is fairly well understood and has been previously described. During the migration of the paraxial mesoderm, Claudin 19b could be involved in maintaining the cell-cell adhesions and therefore implicated in the migration process. The loss of *Claudin 19b* expression may therefore in some way affect the migration of paraxial mesoderm, influencing somitogenesis in this way. Another possible explanation at this stage is that *Claudin 19b* may be implicated in maintaining the retinoic acid or Wnt/FGF concentration gradient through its barrier or pore forming properties

(control the movement of these signalling molecules in the presomitic mesoderm) and loss of its expression therefore affects somitogenesis in this way. If Claudin 19b were to be implicated at this stage of somitogenesis, it may explain the "uneven" phenotype where the somites are asymmetrical on either side of the embryonic bilateral axis. Disruption of somitogenesis in this way could affect the regulation of the periodic, oscillatory progression of the wave/determination front on either side of the embryo, thus leading to an asymmetrical somite phenotype (see **Figure 1.5**).

The loss of *Claudin 19b* expression could potentially affect the "budding off" of paraxial mesoderm, once again due to cell-cell adhesions maintained by Claudin 19b. Another possibility is that *Claudin 19b* could be a downstream determinant in the *Notch* signalling cascade. This could explain the fused/forked somite phenotype due to the improper formation of discrete somite bocks (see **Figure 4.6**).

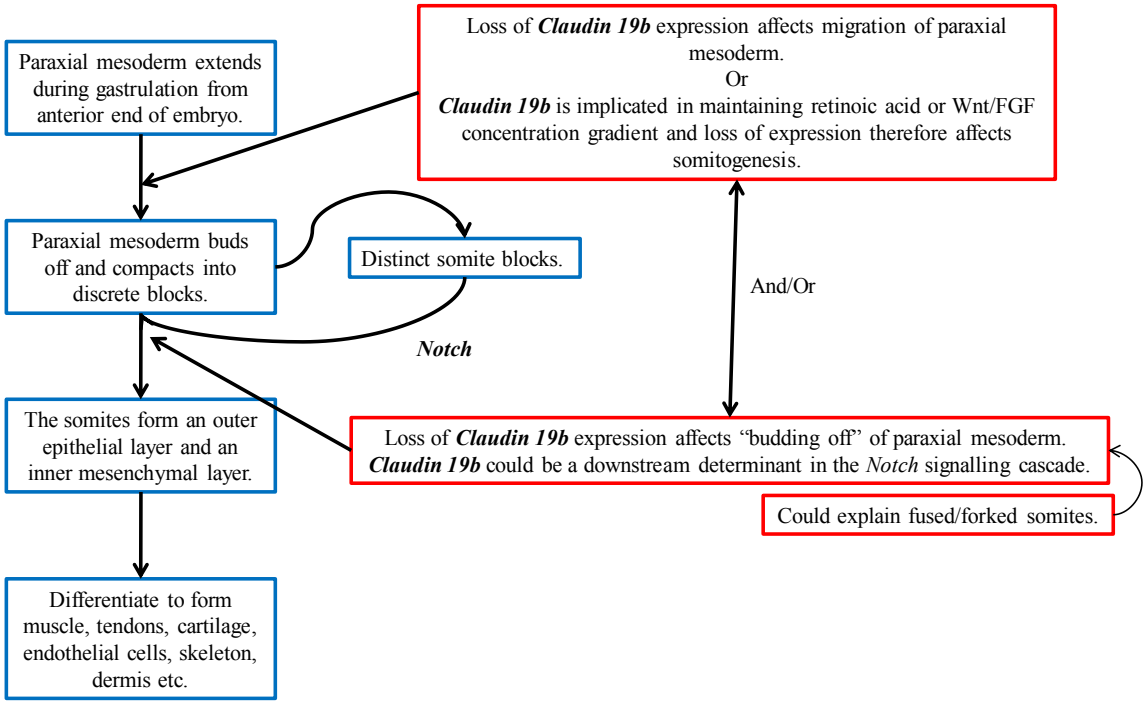


Figure 4.6: Potential roles of Claudin 19b in *P. marinus* somitogenesis.

The most likely explanation however, is that the loss of *Claudin 19b* expression affects the formation of the epithelial layer of the somites (**Figure 4.7**). It is well known and

documented that claudins play a vital role in the development and maintenance of epithelia within the developing vertebrate embryo. Claudins are also imperative for the maintenance of homeostasis in the vertebrate epithelia. So for these reasons, it seems probable that Claudin 19b could be involved in the formation and maintenance of the epithelial layer of the somites in *P. marinus* during somitogenesis. Loss of the epithelial layer may affect the shape or even presence/expression of certain genes in the somite tissues. The mesenchymal-to-epithelial transition (MET) that occurs during somitogenesis is fundamentally initiated by the expression of *Paraxis* downstream of Wnt signalling (Rowton *et al*., 2013). PARAXIS could result in the eventual upregulation of Claudin 19b expression in *P. marinus*, similarly to what Rowton *et al*. (2013) observed with Claudin 11 in the mouse and chicken. The loss of Claudin 19b expression could therefore prevent the formation of tight junctions during the MET and therefore affect the formation of the outer epithelial layer of the somites. Loss of *Claudin 19b* expression would therefore result in the absence of an epithelial layer surrounding the somites which then leads to an observable abnormal somite morphology. This could possibly quite neatly explain the misshapen and disrupted phenotypes.

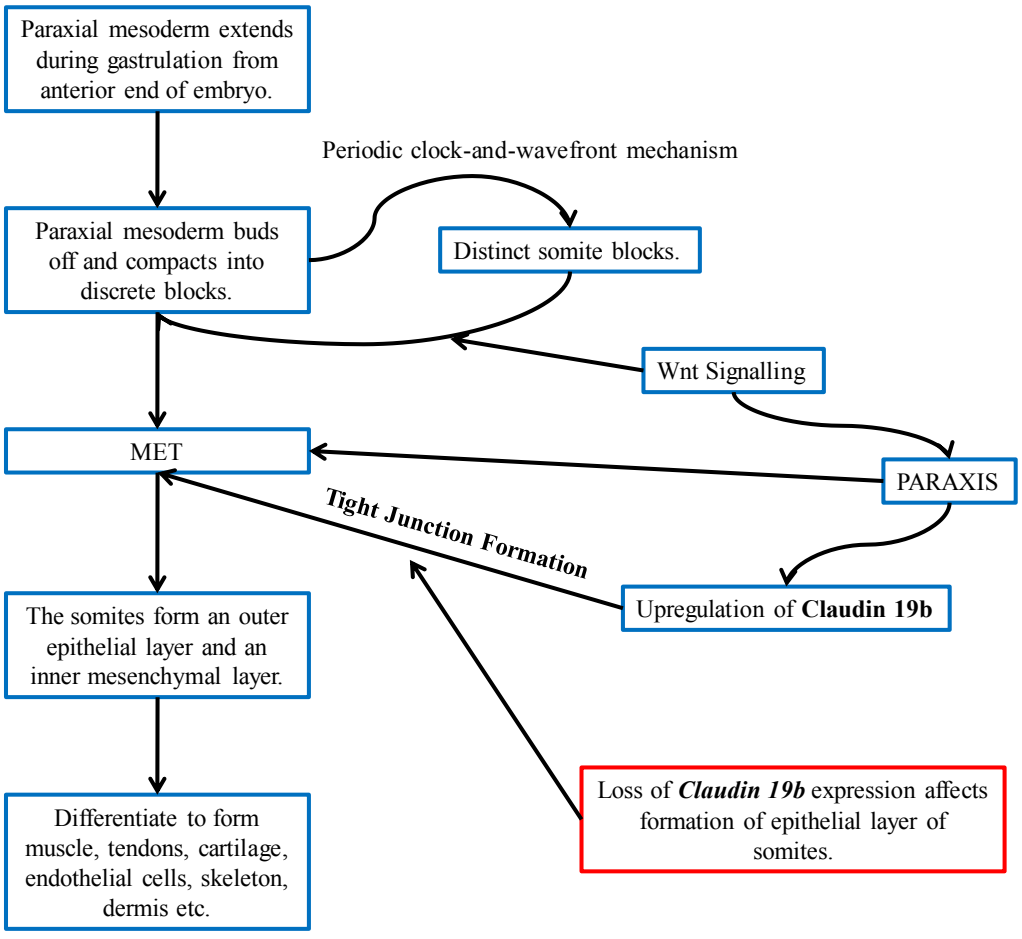


Figure 4.7: Most likely Role of Claudin 19b in *P. marinus* somitogenesis.

It is evident from this data that *Claudin 19b* expression is vital for normal somite formation and somitogenesis in *P. marinus*. Claudin 19 may also play an important role in somitogenesis in other vertebrates such as *Xenopus* (Raciti *et al*., 2008).

4.7 Conclusion

Claudins are a diverse family of proteins that are involved in vertebrate embryogenesis and the maintenance of epithelial structures in both the developing embryo and adult tissues. The roles of some claudins have been strongly conserved throughout vertebrate evolution, but there is also a considerable diversification in the roles that several claudins have gained in higher (more complex) vertebrates. This gain or function may be attributed to the diversification of the vertebrate body plan through evolution. This factor, coupled with the conservation of the function of many claudins indicates that they are an extremely important factor in vertebrate evolution.

Most claudins are expressed throughout *P. marinus* embryonic development, with a more specific, localized expression observed in lamprey adult tissues. All four claudins that were studied were found to be expressed in the ectoderm, otic placode and pharyngeal arches of the developing *P. marinus* embryo. Other structures that exhibited common claudin expression in the lamprey embryo include the neural plate/border, neural tube, fin folds and the notochord. This implicates claudins heavily in the formation, development and maintenance of these structures in *P. marinus*. Out of the claudins that were studied, *Claudin 9* was most frequently expressed in the widest range of embryonic tissues.

Claudin 1a is expressed in the migrating neural crest, of the lamprey (this study), a feature that is not seen in higher vertebrates (Fishwick *et al*., 2012). This points to the development of a different mechanism of *Claudin 1* expression in the neural crest of higher vertebrates to that which was observed in the lamprey. It can be stated that Claudin 1 has played an important role in neural crest formation and migration throughout vertebrate evolution.

Claudin 19b is involved in the development of somites in *P. marinus*. This feature appears to be quite well conserved through vertebrate evolution, as the expression of *Claudin 19* is also observed in the somites of *Xenopus* embryos (Raciti *et al*., 2008). Loss of Claudin 19b expression results in an abnormal somite phenotype in *P. marinus* embryos, which includes fused/forked, asymmetrical, disrupted and misshapen somites. Claudin 19b probably serves to aid in the formation and maintenance of the epithelial layer of the somites during somitogenesis in the lamprey (as has been observed with Claudin 11 in other species in the past), and could serve a similar purpose in higher vertebrates.

Future work surrounding *P. marinus* claudins could involve a more functional analysis of *Claudins 1a*, *9* and *10* via morpholino-mediated gene knockouts. Additional work has been performed on other lamprey claudins (by other colleagues within the laboratory) and this data is in the process of being analysed. A comprehensive manuscript detailing the claudin family in the lamprey is being prepared and will contain the findings of this study as well as that of other studies performed within the laboratory.

Claudins are a diverse group of proteins that serve many roles in the developing *P. marinus* embryo, as well as in the epithelia of adult lamprey tissues. The roles of many claudins are highly conserved, and at times considerably diversified throughout vertebrate evolution. Claudins are vital for the normal development of the *P. marinus* embryo as well as the maintenance of osmotic, ionic and homeostatic balance (due to their barrier and pore forming attributes) in the epithelia of lamprey tissues, as they are in higher vertebrates.

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6 Appendix

The information and figures contained in this section are considered as additional accompaniments to the text and figures contained in the **Methods and Materials** and **Results** section of this paper.

Figure 6.1: pGEM-T Easy and pCMV Sport 6 vector maps.

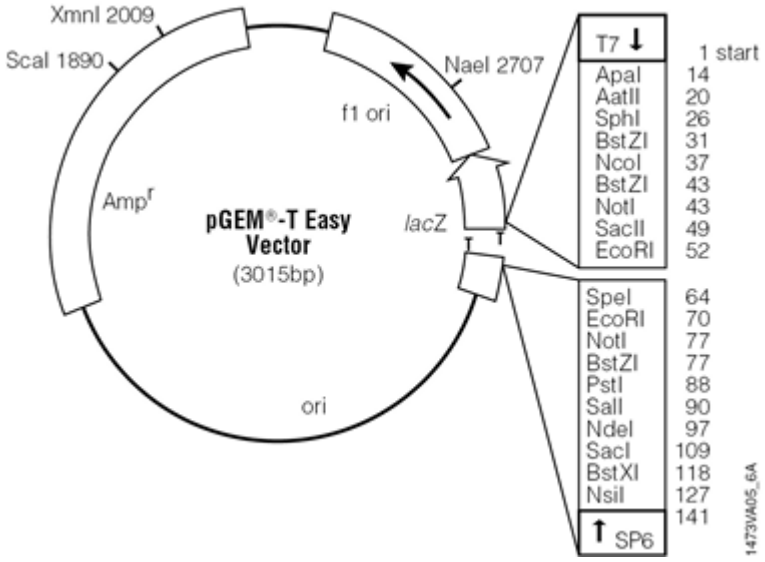

Figure 6.1A: pGEM-T Easy vector map (Promega).

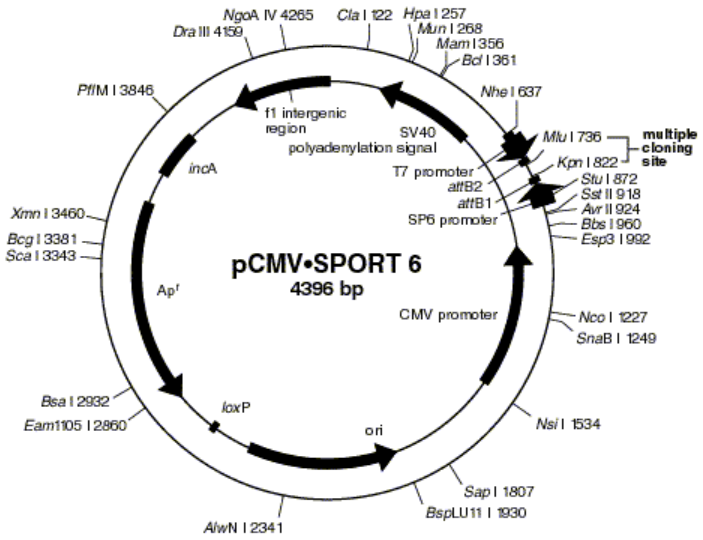

Figure 6.1B: pCMV Sport 6 vector map (Life Technologies).

Figure 6.2: Claudin probe template sequences.

The probe templates below were sequenced by Inqaba Biotechnical Industries (Pretoria, South Africa).

Where necessary, the sequences have been reverse-complemented, so that the CDS is in the correct direction.

Claudin 1a

GCTCCCGGCCGCCATGGCGGCCGCGGGAATTCGATTCTTCGCTGGGTTTGGTTTGGTGGGCGGGGC
GGGGCATGACCCACTGGGCTTGGTGGTCGTGACCGGGAGGCCTGGTTTTGCTCCCGGTGGCCTCGG
CAAAAAGCCGGCCGACCGATCCGTGGGTTTGGCTGCCGTGTCGGCGCGGTTTTCGCCGTTCCCGGA
AGCCGTGGGTCGCTTTGGCGGTGGTGGGCGTTTGGGCCTCGCCGGGGACACGGTTTCTTCCGTCGC
ATCGACCTCGGCGCCCATGGCGCCGAGTGGCACCGCGTGGCCCGGCTCCGCGCCCTTTCCCGGCAG
GGCAGCAGAGTTGCCATTGGCCGTCCTCGTCTTGGGCCCCGACTCGTCCTCGCACTCGGCGCCATC
CGCCAAAGGCTTGCCGCGTTGACGAGTGGGCCGTAGGTGACTGCGCAGATTTCCGATGTCTATTTG
ATTTCCCTGCGATTCCTCGAGCTTGGCGGCGCTAGCGGCGACAGAAGGGGGCATCGCTGGCCGCG
GTTTCACTGACGGCTTGGGGCGGATGGGTGGGTGGTTGGGCCCCGTAATTGCTTTGGTTTCACTCTT
TGCTTCAGATTCCCGATTGTTTGCCTCGAATCACTAGTGAATTCGCGGCCGCCTGCAGGTCGACCA
TATGGGAGAGCTCCCAACGCGTTGGATGCATAGCTTGAGTATTCTATAGTGTCACCTAAATAGCTT
GGCGTAATCATGGTCATAGCTGTTTCCTGTGTGAAATTGTTATCCGCTCACAATTCCACACAACAT
ACGAGCCGGAAGCATAAAGTGTAAAGCCTGGGGTGCCTAATGAGTGAGCTAACTCACATTAATTG
CGTTGCGCTCACTGCCCGCTTTCCAGTCGGGAAACCTGTCGTGCCAGCTGCATTAATGAATCGGCC
AACGCGCGGGGAGAGGCGGTTTGCGTATTGGGCGCTCTTCCGCTTCCTCGCTCACTGACTCGCTGC
GCTCGGTCGTTCGGCTGCGGCGAGCGGTATCAGCTCACTCAAGGCGGTAATACGGTTATCCACAGA
TCAGGGATACGCAGGAAGAACATTGTGAGCAAAGGCAGCAAAGGCCAGAACGTAAAAGCCGSCG
TGCTGCGATTTTCGATAGCTTCCGCCCCCCCTGGAASGGAGCAMT

Claudin 9

GTYCATKCTCCGGCCGCCATGGCGGCCGCGGGAMTTCGATTCTGGAGGCCGTAAAAGGGCCGGGG
CCGACCAGGCTCTGGTCGTGGCCACGAACAGCACAGCAGCCCCCCGCCGAGAAACAGCAGCGTCG
CCGCCCCCCAGCCGACGTACAGGGCCGAGCCGAGCTCCCGCTTGAGGGTGACGGGGACGCCGACG
TCGTAGAAGTCGCGCACGATGTTGTGCGCCGTCCACGACACGGGCGCGAGCATTAGGAGGCCGCA
CAGCGCGAACCCGACGCCTCCCGTGGCCGTGAGACGGCCCTTGGCCGAGCCGTCCTCGTCGCCGC
ACGTCGTGCAGCGCATGCCCGTGGCCGCCACGCCGACCGCGAGCAGGGCGAGGAGGTTGGCGAGC
ACCGTCATCGCGCGAGCCGCCTGCAACTCGCCGTTGAGCGCCAGCATGGAGTCGTGCACAAATCA
CTAGTGAATTCGCGGCCGCCTGCAGGTCGACCATATGGGAGAGCTCCCAACGCGTTGGATGCATA
GCTTGAGTATTCTATAGTGTCACCTAAATAGCTTGGCGTAATCATGGTCATAGCTGTTTCCTGTGTG
AAATTGTTATCCGCTCACAATTCCACACAACATACGAGCCGGAAGCATAAAGTGTAAAGCCTGGG
GTGCCTAATGAGTGAGCTAACTCACATTAATTGCGTTGCGCTCACTGCCCGCTTTCCAGTCGGGAA
ACCTGTCGTGCCAGCTGCATTAATGAATCGGCCAACGCGCGGGGAGAGGCGGTTTGCGTATTGGG
CGCTCTTCCGCTTCCTCGCTCACTGACTCGCTGCGCTCGGTCGTTCGGCTGCGGCGAGCGGTATCAG
CTCACTCAAAGGCGGTAATACGGTTATCCACAGAATCAGGGGATAACGCAGAAAGAACATGTGAG
CAAAAGGCCAGCAAAAGGCCAGGAACCGTAAAAAGGCCGCGTTGCTGGCGTTTTTCCATAGGCTC
CGCCCCCTGACGAGCATCACAAAAATCGACGCTCAAGTCAGAGTGGCGAACCCGACAGACTATAA
AGATACCAGGCGTTCCCCTGAAGCTCCCTCGTGCGCTCTCTGGTCGACCTGCGCTACGGATACTGT
CGCTTTCTCCTCGGAAAGCTGACGCTTTCTCATAGCTCACSCCTGTAGGAATTCTTCG

Claudin 10

GTCCATGCTCCCGGCGCCATGGCGGCCGCGGGAAYYCGATTTTTCAACCTTGCCGTCACTGTCCTT
CTGGTATCTGTGTGCAGTCAATGCACACAGGCCTCGAGTAATGAAGGTTGAGTGTACACGTCATTT
GAAGTCCTATTTATTTAACACTCCGTACGGATACCCAACCCCTGACTAAAGGCATGTCTCTAGATA
AGGTTGATTGACGGGTGTTGAATCTATGCTTGGTCAAGTTTCAGGTTAAGGAACTCACTGAATGGC
AAAATGCATGAGCCACGTATAATGATTTGGCTACCTAAGCAGACGCTTCTGAATTGGAGGGAATA
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AATTACAATTTGTTTCCCTTTTAAAGCCGGTGCCGTTGACGAATGCAAGTGGATTTTTTTAGTTTTT
TGTTGTTCTGTGCCTACGCGCCACTGCCAACGCAGCATCACCATACAAATCACTAGTGAATTCGCG
GCCGCCTGCAGGTCGACCATATGGGAGAGCTCCCAACGCGTTGGATGCATAGCTTGAGTATTCTAT
AGTGTCACCTAAATAGCTTGGCGTAATCATGGTCATAGCTGTTTCCTGTGTGAAATTGTTATCCGCT
CACAATTCCACACAACATACGAGCCGGAAGCATAAAGTGTAAAGCCTGGGGTGCCTAATGAGTGA
GCTAACTCACATTAATTGCGTTGCGCTCACTGCCCGCTTTCCAGTCGGGAAACCTGTCGTGCCAGC
TGCATTAATGAATCGGCCAACGCGCGGGGAGAGGCGGTTTGCGTATTGGGCGCTCTTCCGCTTCCT
CGCTCACTGACTCGCTGCGCTCGGTCGTTCGGCTGCGGCGAGCGGTATCAGCTCACTCAAGGCGGT
AATACGTATCCACAGATCAGGGATACGCAGAAAGAACATGTGAGCAAAGCAGCAAAGGCCAGAT
CGTAAAAGCCGCGATGCTGCGTTTTCAWAGCTCGCCCCCCTKKAMGRARCCATCA

Claudin 19b

TGCTYCCCGGCCGCCATGGCGGCCGCGGGAAYYCGATTCACTCGCCACATACAGAAGCATTTGAA
AACTARRSGCAGCTGTGGCCCGTAGCCAGAATTTTAGTGAATTGCCATTTACTTTTAGAAGAAAAT
ATAAACTGCTGCTATATTGTGGATCGATAATGTCTTTCTGATGTAATATAATAGCACTGAGTAATG
TTTATTCGGGTGTTGTCTTCCATTGTCTCCAATAACTTTCCATATAAATTTTTTGAGCATTTCACATG
CCTAATAATGAAMAAAGCTTGCACAAGAAAGAATCTTATGATAAATCTTCAGTGAAAATCATTAA
CTTTGATATTATGCATTGGCTAAGCAGTTGTTTTGTTAATGAAGGATTTGATTCAAAAGTGTTAGTG
GAATTTTCTGCAGATTTTTGATAGAAATGGCCCAATCTGTAACTAGTCATGGACACCCGGCCACTT
TTTCACCAATTACTGACATTTTTACCAGAACATCAATCAGACTTAAATGAATAACTAATAAACATG
AATACACCAATTGCATGATAACAGCTTATTCCTATACCACTGCCTCCAGGAATCACTAGTGAATTC
GCGGCCGCCTGCAGGTCGACCATATGGGAGAGCTCCCAACGCGTTGGATGCATAGCTTGAGTATTC
TATAGTGTCACCTAAATAGCTTGGCGTAATCATGGTCATAGCTGTTTCCTGTGTGAAATTGTTATCC
GCTCACAATTCCACACAACATACGAGCCGGAAGCATAAAGTGTAAAGCCTGGGGTGCCTAATGAG
TGAGCTAACTCACATTAATTGCGTTGCGCTCACTGCCCGCTTTCCAGTCGGGAAACCTGTCGTGCC
AGCTGCATTAATGAATCGGCCAACGCGCGGGGAGAGGCGGTTTGCGTATTGGGCGCTCTTCCGCTT
YCCTCGCTCACTGACTCGCTGCGCTCGGTCGTTCGGGCTGCGGCGAGCGGTATCAGCTCACTCAAA
GGCGGTATTMCGGTTTATCCACAGAATCAGGGATACSGCAGGAAAGAACCATGTGAGCAAAGCCA
GCAAAAGGCCAGGACCGTAAAAGGCCGCGTTTGCTTGGCGTTTTCATAGCTCCGCCCCCTGACGAA
GCATCACAAAATATCGAACGYCCTCTCAC

Figure 6.3: Claudin coding sequences.

CDS marked with < (beginning) and > (end) of the sequence. PCR Primer binding sequences are marked in **bold**.

Where necessary, the sequences have been reverse-complemented, so that the CDS is in the correct direction.

Claudin 1a (comp290902_c0_seq2): Full CDS

GGCGAAGGGGGGGCGGGGGGGGGCCTTGTCGCCAGAGCCAAGATGCAGCTCCGGGAAGTGAAGA
GGGACGACCGCAAGAGCAGAGCTCTGAGCCCCACGCGACCCCCGCCGCCGATTAAAACCAAGCAG
GCGCCCGTGGCCGCTGCCGCCGCACCCTCGGCGGGAGCCGTCGAGAAGCCGGCGTTTCCCAGCGG
GAAGAAGCCTGGGCCGCCCCTGCCGGACGGAAGGCTGGGCCGCAAGCCCAAGCCAGAGGAGTGG
AAAAAGCCGGGAGCCGCGCCGGTGCCTGCGGAAGTGGGGGACGAGAGGGCGGCAAATGCGGCGC
CCTGCAACGGGTCACCAAAATTGCCGCCCAGGCTGCCGCTTAAATCGAAACTGGAGAAGTGCCCG
ACGAGCGCGGACACGGAGAAGGGCGGGGCCAGCGCGCCCGAAGTCGGCCTGCCCGTCAAGGCCT
TGGTCGGGGACCTGATGAATGCTTTTGCAGGCTTCAAGCCGGCGGCACGGAA**CGAGGCAAACAA**
TCGGGAATCTGAAGCAAAGAGTGAAACCAAAGCAATTACGGGGCCCAACCACCCACCCATCCGC
CCCAAGCCGTCAGTGAAACCGCGGCCAGCGATGCCCCCTTCTGTCGCCGCTAGCGCCGCCAAGCTC
GAGGAATCGCAGGGAAATCAAATAGACATCGGAAATCTGCGCAGTCACCTACGGCCCACTCGTCA
ACGCGGCAAGCCTTTGGCGGATGGCGCCGAGTGCGAGGACGAGTCGGGGCCCAAGACGAGGACG
GCCAATGGCAACTCTGCTGCCCTGCCGGGAAAGGGCGCGGAGCCGGGCCACGCGGTGCCACTCGG
CGCCATGGGCGCCGAGGTCGATGCGACGGAAGAAACCGTGTCCCCGGCGAGGCCCAAACGCCCAC
CACCGCCAAAGCGACCCACGGCTTCCGGGAACGGCGAAAACCGCGCCGACACGGCAGCCAAACC
CACGGATCGGTCGGCCGGCTTTTTGCCGAGGCCACCGGGAGCAAAACCAGGCCTCCCGGTCACGA
CCACCAAGCCCAGTGGGTCATGCCCCGCCCCGCCCA**CCAAACCAAACCCAGCGAAG**GCCTCCGG
CCCCAAGGCAGTCTTCAGCGTGCCGGCAGCAGCAGCGGGCAAAAATGTGCTTCGGGCCTTTGGCC
GGGAAGGTGACGGCGGTGGCGGCGGTGGCGGCGGGGTCACCTTGGCC<ATGCACGTCGTTGGGTT
TGCGCTGTCCACGCTTGGCTTTGGCCTCACGGTGATGGTCATCTATCTCAACTGCTGGAGCATCTAC
AGCCACGGGCCCGGCACCTACATGCAGAGCGGCCTGTGGAACATCTGCGTGAGGAATGCCAGCAC
GGAGCGCGGCGAGGTCTGCAGGCAGTTCTACACGGCGCTGTCACAGCCCAGGTTCTTCCAGGTGG
TGCGTGCGCTGATGCTCATGGCCGTGGCGGTGGGCGCCATCGCGCTGTTCGTTTCCACGCTGGGCA
CGGCGTGCGCCCGCCGCTTCAGCGAGCACGCCAGCACCAAGACCAAACTGACCTGCGCTGGCGGC
GCCCTGTGGCTTCTCACGGGTCTCTGCGTGATGCTGGCCGTGTCCCTTTACGCCAACTTCGTCGTGA
GCCAGCTGTACAACCCGCTGGAGCCCTACGGCGTTCGGTTCTCGTTCGGCGCTGCGCTGTACACCG
GCTGGCTGGCCGGCGCCTTCATCGTCATCGGCGGCGCGCTCATGGTCGCTGTGTTCTTCAGGAAAA
ATTCCCTCCGGACGACGTACAGATTCGTCATCACTGACATCGTCGCGTCCCCTTGA>GGAGCTGGC
GACCCGACAGCCCACGCTCGCAAGAGGACACTTCTAGACCGGGCGGAGAGAGGTTCAAGTTCAAG
GTCACACGCTCATCCAACCCCCGTCCTCAAAGGCGGCTAAGGGAGCAGCAATGCCGCCTTAATCGT
GCGGCAAACGCAGAGCACCATGGGAATTTGGTTGTTTACCGCCGGCCTCGGTGAGCGTGGTCCTTC
CAACCCACCCGTCTGTTCGTGACTTGGAATGCCACGGATTCATTTGAACTCTTTCCCTTTGCCGGGG
CACCAGCAATTTGCAGGGTGTGTGGGGGGGGGGGGGTACATTAAACGACCACCCCCCC

Claudin 1b (comp270469_c0_seq1): Partial CDS

ACCAAACCGGACCGTCAGCGTCGC**GAGAGAGGCGGTGGAGGT**GGAGACACGGTG<ATGATCGG
AGGGGTGAATGGGACGAGAGTCCCCGTGTCGTCCCTCATTGAGGGATTTGCCGAGCCGGACGCCG

ATGAGAATGCGGTGATGATGGCTCTCTGTGCCCTGGGAGGTCACACCGGACTGTGGGGACGGTGT
CTGAACCTCCCGTCACACCGGGGCGAGCTTTGTCTGACTCCCGGCTTCCTGCAGGAATGGGCTCCG
AGGTTCTCCGAACCTGGGAATCACAATTCTCAACTCGACCCAGTGCGGACCGCGCTGTGCAGGGCT
CAGCTGCTGTTGCCACTCGTGGCCCTGACGCTGCTGCTGCTGACCCTGGCGCTGGCTCTGATTGGCT
GTGCCAGCAACTCGTCACTGGCCCTGATCCCCTGTGCCATACTCACCTTACTCGCTGGCCTGTGCAC
CCTCACCACGGCCTCCTCCGTGTCTCTCTCCTCCCTGCCCTGCGTCGATGCCTCCTCTTCGCC**CGGC**
TGGTCGACGTTTCTTGCCCTGGCCTCGGCCTGCCAGGACATCGCCGCCACGGGGCTGCTGATCTG
GGCGAGGGCGGCGAGCCTCAGACACTACCGCTCCGTGCTC>

Claudin 2 (genomic): Predicted Full CDS

<ATGGTGTCCACGGGACTCCAGATCGTGGCCCTGTGCCTGGCCATCGTGGGTCTGCTGGGCACGCT
GGCGGCCACCATCCTCCCACACTGGCAGGTGACGGCTCACGTGGGCACCAGCATCATCACGGCCA
CGGGGCAGATGCGCGGTCTGTGGATGGAGTGCGCCTGGATGAGCACGGGATTCTTCCAGTGCCGC
CCCTTCTACTCCATCCTCGCAATGAACCCGGCTCTCAAGGCCGCGCAGGCCATGATGGTCATCTCG
TGCGTGCTCACGGCGGCGGGCATCGGCGCGGCCACGGTGGGCATGAAGTGCACCCTGCTGCTGGG
CCCATCCTCCCGCCGCAGCAAGTCGCTGGTGGCCGTGTCGGGCGGCCTCTGCCTCATCGTCTCCGC
CCTCTGCACGCTCGTGCCTGTCGCGTGGAACACCCACAACACCGTGCGCCAGTTCTACGACCCGTG
GTACCCGAGTCCCGTCAAGTACGAACTCGGCGCCGCCATCTACCTGGGCTACGCCGCCGCCATCCT
CACCATGGCCGCCGGCGTAATGATGGCCCTGTCGTGCCCCGAGAAGCGGCGACGTGCCGGGCCGC
CACGCCACCTTCGCCCGCGCCGGGATGGGCCGCCGCGTGGGGTGGAGTCCGCGGGGACCGGGACT
CCCGAGGTCCACACGGGCATGAGTTGGGTG>

Claudin 3a (comp287291_c3_seq2): Partial CDS missing about 60aa of 5'end (start)

<AGCACGGGCCAGATGCAGTGCAAGGTCTACGACTCGATGCTGGCCCTGGGGCAGGACCTGCAGG
CCGCGAGAGCCCTGACGGTGGTGGCCGTGGTCGCGGCCGCGGCGGCCCTCCTCGTGGCCATCGCG
GGGGCCAAGTGCACCACGTGC**ATCGAGGAGGAGGAGACCAA**GGCGCGCGTGTCGATAGCGGCG
GGCGCGGGATTCGTGCTGGCGGGCCTCCTGGAGCTCGTGCCCGTGTCCTGGTCGGCCAACTCGGTC
GTCCGCGACTTCTACAACCCGCTGGTGCCCGACTCGACCAAGCGCGAGATCGGCGCCGCCATGTTC
ATCGGCTGGGCCGCGGCCACGCTGCTTGTGCTCGGCGGGGCGCTGCTCTGCTGCTCGTGCCCGCCC
AAGCAGAGGAAATACGCGGCGCCGCCGAAAGGGGGCGGTGGAGGAGGCAGTGGTGGAGGCGGA
GGCGGTGGTGATGATGGAGACGACCGCAAGGGAAAGTAG>CTCCGGTGATGGAGGAAGGAGGAG
GGAGGAAGGAGGAGGAAGGAGGAAGGAGGAGGAATGAGGAGGAAGGATGAAGGGAGGAGGAA
GGAAAGGGAAGGAATGAGGAAGGAGGAGGAGGTAGAGAGTTGTTGGGGAGTCT**TGAGGCCCAT**
TGAGACTCAGGCGACGGAACGGGGACAGCCTCATGGGCGTCGGAGCGGCGATGGCGGCGGCGAT
GATG

Claudin 3b (comp237618_c0_seq1): Full CDS

CGTCAGGAGGACGAAGATGGGGTCAGAAGTCGCTGGTTCTCGACGAGAGAGATACGCCTCCAAAA
ACCCCATCTTCGTCCTCCTGACGCTTGGGCTGTGACGAGGAGAACTTCTATACCAAGGGGCGTGCG
AGGCCTTTTGCGCGTTTTGGCAGTTCCATAATTCTCCTCGAATCCCAACATCGTCCGAATCATCTCG
AATCCGTCAGGCCCGAAATCCGTTCTCCAAATCCATCTTCCAAATCCTCCCCCACCCCGACCTCAA
TTCTCTCCGGCCATCTCCTCCCCCAAGCTCCACTCTCTCTCCTTCCGCTCCACGTCCCCCCGCCCG**T**
CCATCGTCTTCCACACTCCCGTTTTTTTTCCCGCCCGCCAAGCGCGCGCTCCGCAACTCCACAGTG
CCATGGCGAGCACGGGC<ATGCAGCTGCTGGGCCTGGCGCTGTGCATCGTGGGCTGGCTGGGCGC

CATCGTGACGTGCGCCCTGCCCATGTGGAAGGTGTCCGCGTTCATCGGCAACAACATCGTGGTGGC
TCAGATTGTCTGGGAGGGCATCTGGATGAACTGCGTGGTGCAGAGCACGGGCCAGATGCAGTGCA
AGATGTACGACTCGATGCTGGCCCTGGGCCAGGACCTGCAGGCAGCGCGAGCCCTCTCGGTCATC
GCCATCGTCCTCACCGTGCTGGGCATCCTCGTCAGCATCGTGGGCGCCAAGTGCACCACCTGCATC
GAGGACGAGACCACCAAGGCGCGCATCACAGTCGGCTCGGGCATCATCTTCATCCTGTCGGGCGT
CATGGAGCTGATCCCGGTGTCGTGGGCCGCCAACACCATCATCCGCGACT**TCTACAACCCCATAG**
TGCCCGACGCCATGAAGCGCGAGATCGGCGCCTCCATGTACATCGGCTGGGCCGCGGCCGCCCTG
CTCATCCTGGGCGGCGCCCTGCTCTGTTGCCAGTGCCCCCCCAAGCAGGAGAAGTACATGGGGCCT
CGGTCGGTCGCGCCGCCACGCTCCGTGGCCACGGCGAGAAGCAACTACGTGTGA>GGGTGGTTGG
TGGTGGTGTGTGTGCGTGTGTGGGTGGCTGTGGGTGGTGTGGGTGGGTGAGGGAGGTCCTGGGGT
GGGGGTGGAAAAGTAGAATCTGGTCG

Claudin 3b: Partial missing about 35aa from the 3' end of CDS

CCACGCGTCCGGAGAGCGATACGCCTCCAAAAACCCCATCTTCGTCCTCCTGACGCTTGGGCTGTG
ACGAGGAGAACTTCTATACCAAGGGGCGTGCGAGGCCTTTTGCGCGTTTTGGCAGTTCCATAATTC
TCCTCGAATCCCAACATCGTCCGAATCATCTCGAATCCGTCAGGCCCGAAATCCGTTCTCCAAATC
CATCTTCCAAATCCTCCCCCACCCCGACCTCAATTCTCTCCGGCCATCTCCTCCCCCAAGCTCCACT
CTCTCTCCTTCCGCTCCACGTCCCCCCGCCCG**TCCATCGTCTTCCACACTCC**CGTTTTTTTTCCCGC
CCGCCAAGCGCGCGCTCCGCAACTCCACAGTGCC<ATGGCGAGCACGGGCATGCAGCTGCTGGGC
CTGGCGCTGTGCATCGTGGGCTGGCTGGGCGCCATCGTGACGTGCGCCCTGCCCATGTGGAAGGTG
TCCGCGTTCATCGGCAACAACATCGTGGTGGCTCAGATTGTCTGGGAGGGCATCTGGATGAACTGC
GTGGTGCAGAGCACGGGCCAGATGCAGTGCAAGATGTACGACTCGATGCTGGCCCTGGGCCAGGA
CCTGCAGGCAGCACGAGCCCTCTCGGTCATCGCCATCGTCCTCACCGTGCTGGGCATCCTCGTCAG
CATCGTGGGCGCCAAGTGCACCACCTGCATCGAGGACGAGACCACCAAGGCGCGCATCACAGTCG
GCTCGGGCATCATCTTCATCCTGTCGGGCGTCATGGAGCTGATCCCGGTGTCGTGGGCCGCCAACA
CCATCATCCGCGACT**TCTACAACCCCATAGTGCCC**GACGCCATGAAGCGCGAGATCGGCGCCTC
CATGTACATCGGCTGGGCC>

Claudin 5: Full CDS

GTCGACCCACGCGTCCGCTCACCTGCGGCCAGAGCACCACGCTCCTGTTGAGACATCTCATTGAAC
ACCATCGCGGCGATAGGTTTTTTTTTCTCCTTCTCAAATTAACCGGGCTCCATTGTTTGATCAGCTC
ATTCAATTGCCCCCCACCCATCCCAAGTAATTTCGGACGCAGGTGGAATTAATTGGTAGGGGGGAG
GGGGGGGTCAGGTGACAAAAATAAAAAAAAGAATAGAGTTATTTTTTTAAATCGCACTCGGAGGT
TCTCG**CTAAGCGGAGAGGAAGGGAG**AGGGGACAGGAGGAGGAGGAGGGGCGGGCAAAGGTGC
AAGAGGACATCAGCCACCTAGCCGAGAGCGAACGCCGTAGAACCAACTCGTCTACCCGTGGACCC
TAAACCCATCCCGTCCAACACCCCGAG<ATGGCGAACGCCGCTCTGCAGATCGCCGGAATCGCCCT
GGGAATCCTGGGCTGGATCGGCAGCATAGCCGTGACGGTTCTGCCGCAGTGGCGCGTCACCGCCTT
CCTGCCCAACTCGGCCAACATCGTGGTCGGCCAGGTGTTCTGGGAGGGCATTTGGATGCAGTGCGT
CTCGCAGGCAACGGGCCAGCAGCAATGCAAGGTCTACGACTCTCTGCTGGCGCTCACCCCGGACA
TGCAGGCGGCGCGCGGCCTCATGTGCGTGAGCGTGGCCCTGGGCACCCTGGCCATGCTCATCGCG
ACCCTGGGCATGCAGTGCACGCACTGCGTCGAGAACGAACGCGCCAAGGCGTACATCGCCATGTC
GGGCGGCTTCCTCTTCATCATTGCCGCCCTCATCGTCCTCATCCCCGTGTGCTGGACGGCCAACGCC
ATCATCCGCGACTTCTACAACCCCAGCGTGCCCGAGGCCCTCAAGCGTGAGCTCGGGGGAGCTCTC
TACCTCGGCTGGATCTCGGCCTTCATGCTGGTGGCCGGGGGGGCGCTGCTCGCGTGCTCGTGCCCC
CCCAAGGACGAGCCCGGGTACCGCCCGGCGAAGAGCGCCTACGCGGTGTCGGCGCCGCGCACCGT
GCAAGACACGCACCAGCCTCGGAGGGCCATGAGTACCACCTACTCTATGAAGGAGTTTGTGTAA>
GGGTGGTAGGGGGGGTAGACAGGCGGGTTGTTTTGGGGGGGGGTAGACAGGCGGGTTGTTTTTGG

GGGGGTAGACAGGCGGGTTGTTTTTTTTTTGGGGGGGGGGGCGACAAATGCAGTGGCGGTCGTGG
TTTCGAGTTTTTAAAAATAATATCAGAATTTTAGTGTGTTTAAAAAAAAAATGGAAACAGACGGGT
CACGCGTGGCGTTCTAGTGTCTCCTGCACCGAGAGGGTTCCTCCACGCGTCCACGGTGGCGGGCGA
CGCACGGACGCCGCTTAAACTCCACTCGACCGCCCTACTCTAGGCGGTGTCATTCTGGGGGGGGAC
TTGATTATCAAGTCCAAGAC**GTTGCTGTGTAGGAGGAGGT**AAGAGGAGGAAGGGGATGGGGGG
GGGATTATGTCGAGGTGACGTTTATATCAGGATTGGGACAAATGTACAATCTCTCGTTCACGTGAC
ACGCAGCAGAGAATTATCCCTTCGGTGTGCTTTTTTTTTTTGGGAGGGGGGTGATTGCCCAACGTTT
CACGCTCACGTGCTGTGGGAGCTTCTTTGGGGAAACAAGCTCACGTGGTGCGAGACCTGAAAGAA
TGGACAACAGAGGAGCTAATTTGTTTTAATGGTGAAGATGGTTCGTGATGCTGTTGGAGATCAACG
TGTATACCAGGAATTAAATTTCTCAATTAATATGTTCAGAGTCGTTGGGAGTAGGTCGTGGTATGT
GACTGGGTAGCAGCCACG**GATGGAGAGGGGAAAGTGCT**GGATGTGGTGTGCCGTGTACTGGAA
ACATTGTTTTGCTTGTCACCGACCCGGCTGCCAATGTTAGCTCTGCTTGTATATCGCAACTGAGTAG
TAGTAGTCACCGCCATAGCGAGGGGTATACTTGATGTAGTGTGCCCGGGTGGTGAGCTGTATATCG
GAGCTGGCCGTCGTGCGCACGTGAAGTTCGCTTTTGATCCTCGCTGGTCACGAGAGGCGCGAGTCG
ATTCTTCCCACACTCACACACGTGATAGTATTTCTTTTACTGCTGTACTTTTAACGTTTTTCAGCTGT
TTTATTTTTATAAAACTACGTACACGTTTATTTCTGGGTGGTTTTTAAAGGGGGGCGGGATATTTTT
TTGTTGATATTAATATTACTGCTACGACAATTTTTTTTTTTTTATATTATCCGCTCTGGTCATTATC
TCCTGAACTCGGTCGGAAGTTCACAAATCCGGAGTGTGAGAGTTGTGCGTGCGGCTGGGACCATC
ATCATCATCATCGCATCTGTACGGTCCGATAAAATGAATGCCGCTTCCTTGATGAGCGAGGAGGGG
GGGGGGAGAAAAATAATGAACCGGAGGCTCGTATTTGTTTTGGATGTTACTGGGTTCCCGCCATAA
GAATGGGGAATTGCTATCGCACGCCCGGGTGTTCTTTTGGGTTCGGGATGACACTTGGGCATGGCG
AGCACCAGAATCTTTCGTTGTGGCTGATCTGTTTCCCACCCAGCATCGCGATCCACCGACCTGTGC
CTTTTTTGTACTCGTGGCTCATTCCGTGTAAATAATAATAACAATAAAACTTTCTTTCATTTTTCCG
GAAAAAAAAAAAAAAAGGGCGGCCGC

Claudin 8 (comp266504_c0_seq1): Partial cds, missing about 75aa from the 3' end

GAATGTGTGGGTGGGTG**ATTGTGGGAGGAGAGGGTTG**GCGAGGTGTGTGTGTGGAGGGGTGGTT
ACACGCGGCTGTGTGCGTTTGCGCGGTTGGGGGTGAGCCAATTAAACTCAGGACGCCAGCCAATC
GCGAGTTGCGCCCGGCTCGACCCCCACACCGCGTCCACGCGAGGGGTGGTGGCTCAGGTTACGGC
TTCTCGAAGCGGACAGAGCGAGAGAGATAGAGAGACACCCCCGGATTCCCGCTCACTCGCTCACT
CACCTGCGGCCACGAGCACCACGCTCCTGTTGAGACATCTCATTGAACACCATCGCGGCGATAGGT
TTTTTTCTCCTTCTCAAATTAACCGGGCTCCATTGTTTGATCAGCTCATTCAATTGCCCCCCACCCAT
CCCAAGTAATTTCGGACGCAGGTGGAATTAATTGGTAGGGGGGAGGGGGGGTCAGGTGACAAAA
ATAAAAAAAGAATAGAGTTATTTTTTTAAATCGCACTCGGAGGTTCTCG**CTAAGCGGAGAGGAA**
GGGAGAGGGGACAGGAGGAGGAGGAGGGGCGGGCAAAGGTGCAAGAGGACATCAGCCACCTAG
CCGAGAGCGAACGCCGTAGAACCAACTCGTCTACCCGTGGACCCTAAACCCATCCCGTCCAACAC
CCCGAG<ATGGCGAACGCCGCTCTGCAGATCGCCGGAATCGCCCTGGGAATCCTGGGCTGGATCG
GCAGCATAGCCGTGACGGTTCTGCCGCAGTGGCGCGTCACCGCCTTCCTGCCCAACTCGGCCAACA
TCGTGGTCGGCCAGGTGTTCTGGGAGGGCATTTGGATGCAGTGCGTCTCGCAGGCAACGGGCCAG
CAGCAATGCAAGGTCTACGACTCTCTGCTGGCGCTCACCCCGGACATGCAGGCGGCGCGCGGCCT
CATGTGCGTGAGCGTGGCCCTGGGCACCCTGGCCATGCTCATCGCGACCCTGGGCATGCAGTGCAC
GCACTGCGTCGAGAACGAACGCGCCAAGGCGTACATCGCCATGTCGGGCGGCTTCCTCTTCATCAT
TGCCGCCCTCATCGTCCTCATCCCCGTGTGCTGGACGGCCAACGCCATCATCCGCGACTTCTACAA
CC>

Claudin 9 (comp283491_c0_seq2): Partial CDS, missing 57aa from 5' end and 23aa from 3' end

<CACGGGCCAGATGCAGTGCAAG**GTGCACGACTCCATGCTG**GCGCTCAACGGCGAGTTGCAGGC
GGCTCGCGCGATGACGGTGCTCGCCAACCTCCTCGCCCTGCTCGCGGTCGGCGTGGCGGCCACGGG
CATGCGCTGCACGACGTGCGGCGACGAGGACGGCTCGGCCAAGGGCCGTCTCACGGCCACGGGAG
GCGTCGGGTTCGCGCTGTGCGGCCTCCTAATGCTCGCGCCCGTGTCGTGGACGGCGCACAACATCG
TGCGCGACTTCTACGACGTCGGCGTCCCCGTCACCCTCAAGCGGGAGCTCGGCTCGGCCCTGTACG
TCGGCTGGGGGGCGGCGACGCTGCTGTTTCTCGGCGGGGGGCTGCTGTGCTGTTCGTGGCCACGAC
CAGAGCCTGGTCGGCCCCGG**CCCTTTTACGGCCTCCAG**CCGGGCGGGTTGGCCATGCACGGGAC
CGGCCCGAACGCTCCCGGGGCCCCGTTGCCGCGGATGCACTCGGCGGCGGCTGCGGCGGCGGCGC
>

Claudin-10 (genomic)_tr var 1: predicted full cds

<ATGAGCGGCACCTGCCTACAGGTGCTGGGCACGATCATCGCGTGCCTGGGTTGGGTCGGTGCCAC
GGTGGCGACAGCCATGAACGAGTGGCGCGTGACGAGCCGAGCCTCCTCTGTCATCACCGCCACCT
GGGTCTTCCAGGGCCTGTGGATGAACTGTGCCGGCAATGCCCTGGGCGCTGTGCACTGCCGTCCCC
ACCTCACCGTCTTCAAGCTGGAGAACTATGTGCAGGCTTGCAGGGCATTCATGATAACCTCCGTAT
TCTTGGGGTGTTTGGCCATGATCTGCTCTCCGTTCGGCATGGCGTGCATAAGGATCAGCTTCGTGA
GTGACAAAGCAAAGAGAAACATCACATTCATCACCGGCTTCATCTGCATTCTACAAGGAAAGCCC
CGAAGGCTAATGGGACTTACATACTCTGGTAATAGCGTTACTGTCGAGTACTACGATCCCACCATC
ATCGGGGCAAAGTACGAACTTGGCACTGCCTTATTTATTGGATGGGCGAGTGCCTTGCTCACACTG
ACTGGAGGGACCATCATTGTCTGTTCAGGTGTTGGACGATCATACAAGCAGCCCAGGCCGTGGCG
AGCGCTGCTCCCGTCTCCTTGGCGACGTTTCACC>

Claudin 10 (comp293971_c0_seq13)_ tr var 3: Full CDS

CTCTTCCGATCTCAATGCACACGCGCGCGGGTTACTAACAAGGACGAGCATTTTCTGCGGTCAGTG
AATGTTCTTCGAGCATAAAGTTTGCTTCTTTGCAGAAGGGCTCCAAGCTCGGTTGAG<ATGAGCGG
CACCTGCCTACAGGTGCTGGGCACGATCATCGCGTGCCTGGGTTGGGTCGGTGCCACGGTGGCGAC
AACCATGAACGAGTGGCGCGTGACGAGCCGAGCCTCCTCTGTCATCACCGCCACCTGGGTCTTCCA
GGGCCTGTGGATGAACTGTGCCGGCAATGCCCTGGGCGCTGTGCACTGCCGTCCCCACCTCACCGT
CTTCAAGCTGGAGAACTATGTGCAGGCTTGCAGGGCATTCATGATAACCTCCGTATTCTTGGGGTG
TTTGGCCATGATCTGCTCTCCGTTCGGCATGGCGTGCATAAGGATCAGCTTCGTGAGTGACAAAGC
AAAGAGAAACATCACATTCATCACCGGCTTCATCTGCATTCTACAAGGCCTGCTGATTGTGAGCGC
TGTGTCGTGGTATGCGTACCGCGTTACTGTCGAGTACTACGATCCCACCATCATCGGGGCAAAGTA
CGAACTTGGCACTGCCTTATTTATTGGATGGGCGAGTGCCTTGCTCACACTGACTGGAGGGACCAT
CATTGTCTGTTCAGGTGTTGGACGATCATACAAGCAGCCCAGGATTGCTCTGTCAAATCCACACAC
ATCTCCGATGCTGCCAAGGACGTTCAAGAGGCAGCCTTCAGAGCTAAGTGCCAAGAATTACAAGG
CCAACATGTACGTCTGA>AACATGTTGGACTCAAGTCAAGGGGCCTCTCATTCTCTGTTCAACGTTT
TATCAAACTTGGCAGAAGTCCACATTGTGAGTCCAGTTAGCCCTCCGGTGTGTTCTCAGATAGGTG
CCACGTGTTTGTATGGAATGATATCCGATGAAAAGTGTCCACGTGCTGAGAGCATCTTCAACGCGT
GTTGGTATCTTGTTCAGCACATTGAGCGAAACGTCGGACACCATGCTAATCGGCATGTGTTACAAC
CCGAACACAGCATCGGAGTGCTATGAAACACGAGTGTGTATTAAAGCGTAAATGGTAAGCATTTC
TAGATTGGTGTGTGTAGTATTAAAATGTATAACGGGTCTGCTTATGAAAGGAAACGTGTGCACAGG
GGACTTTGCTTACGTGACCGTTCTCCGCATTCTAGCGAGATAAAGTGTATATCTTTAGAAGCAAAT
TATTAGTCATGAATATAATCTGTGCATAAACCTATCGACGGCGTACTTGCTGTATATTTACTGATGT
TCACGTGATGTTACACAGTGCTCATGTTAAAGTAGACATTAATTTATTATTGATCACATGAAATTGT
TACATTAAGCACGCAATTTCGCTCGGTGTTAGTAGCTTCAGATAATTTACAAGCAAAGTTTTAAAC
GAGAGCCGACGGTTGCGTACAGACATCGGCATTACGCTTGTTCTTCTGCAGCGGATGACAAAAAA

ATATAAAAGCTCTTACCGGAAAATGAATAAAATGCACAATTAAGTAGTAATTACAACAACAAAAA
CCCATCAAGCAAATAACTGAACGGCTGTTAAGTTACGGCCGATAATGGAGCAATCACAAAACGAG
AACTAGAAGAGGATAATGAGAAGACGGGGAGATCCCCTCCCCAGCAAAACGATGTTATTCATATT
AAAATGTATCATTTCTATTCGTTTATTAAACATTTTCTTACAATTCAACGAACG**TGTATGGTGATG**
CTGCGTTGGCAGTGGCGCGTAGGCACAGAACAACAAAAAACTAAAAAAATCCACTTGCATTCGT
CAACGGCACCGGCTTTAAAAGGGAAACAAATTGTAATTGGCTAACGTTTCGGGCAAGGGCGCTTC
TGCATTTGTTTACTTGCTGTGCCTTTTATATATTCTTTCTTTTAACACGGGGTTTTTGACGAACGTTT
TTTTTTTATCCATCCTTACTGGTTTGAAGCTGAATTAGCACCCATCACTGGGGATTAAAATTGACAA
TCGCAGTGATAGAAACAAAAATCTCACACGGAACATATTCCCTCCAATTCAGAAGCGTCTGCTTAG
GTAGCCAAATCATTATACGTGGCTCATGCATTTTGCCATTCAGTGAGTTCCTTAACCTGAAACTTGA
CCAAGCATAGATTCAACACCCGTCAATCAACCTTATCTAGAGACATGCCTTTAGTCAGGGGTTGGG
TATCCGTACGGAGTGTTAAATAAATAGGACTTCAAATGACGTGTACACTCAACCTTCATTACTCGA
GGCCTGTGTGCATTGACTGCACACAGATACCAGAAGGA**CAGTGACGGCAAGGTTGAAA**AATATC
CACTACTTGCGCCAGGTTGGTGCTGCTAAGACGCTGCTGGTAACGGATGCGTGTAGTTTGCAGGCT
GTTTTGAGGTTGTGCACCCCGTGTGGCTGCCGGTCGTATGAACTGAAGTTGTTGGTCTGTATGGGA
CCCACGTGACTGTCGAGATCGGAAGAGCG

Claudin 11 (comp279768_c0_seq4): Partial CDS - about 10aa missing from 3' end

AGTCCCACCCAG**GAGTCTCAGCGAGGGGATC**TCAAGCGCGCCGCGGCCGTGCAAAGAGGAGGG
AGGGTGCAGGGAGCTTCTGTAGGGGTCCCCTGGGCGAGGTCCAGCCAAAGCAGGCATGGGCGCTT
CGTGCTTCCAGCTGCTGGGGGTGTCGCTGGGCCTCCTGGGCTGGGTGGCCCTGGTGGTGGCCACGG
CCACCAACGACTGGGTCCTGCTCTGCTCCTACGGCTTCACGGCGTGCGTGCGCTTCGAGGACATGG
GCAGCAAGGGCCTCTGGGCTCAGTGCATCAGCAACAACGGCATCGACCACTGCAAGCCCTTCGCT
GAGATATTAGCCCTACCAGTGTATATACAGGTGTGCCGGGCACTCATGGTGGTGGCCTGCGTGCTG
GGCCTGCCGGCCATCCTGCTCGCCAGCACGGGCCTCAAGTGCATCCGCCTGGGCGAGGAGACGGC
CTCCGAGAAGGCCCAGAGGGTGGTGGCCGGCGGAGTGATGCTCATCCTCGTGGGCCTGTGCACCC
TGGTGGCCACCACGTGGTTCCCCGTGGGCACGCACAAGGAGAGCCTCAAGGG**GGTGGTGAACTT**
TGGCTACTCGCTGTTCGCGGGCTGGGTGGGCACTGCGCTGGCCATCGTGAGCGGCGCGCTGCTCT
GCTGCTGCTCGGGCAGCAAGGAGGAGCGCTACTACGCCTACCCGGCCACCGCCAGCGCCGCCGCC
GCCG>

Claudin 12 (comp 293493_c0_seq3): Full CDS

GCACGGTATCCACGCCGCAGCCTCAGCTCCCCCCTCTGAATTAAACCGTTACCTTCCCCCCTCCATT
TACCTTCCACTTGGTGTCGTCTAAAACTCGCACCACGTGATGCGATCGGGCCAATCGTGGCGCCGC
GTTGAGGGGGCTTCACGTTACCGCGGGTCTCAGCTTTCGCTCTTTATTTTCGCTTTGAAACCCGTAC
GCTTATTTTACAAATTTAACGTTTGCTTGACGTCCTTTATTTTATTTTTTTCCGCGTCGCGTTTGTGT
TCCAAGCGATCGATTAATATTCTAAGTTTGAACGAAGGTGAAAACAAGCCAGGCGGGAACGCGCG
TCGCTACTGTCGGCCAGACTAGCGCGGGCCCGAGAGTGGAGGTTTTTTTTTATTTATTCTGAGGGA
CATCGTGGCCACCTCACTGATTGACTGACGGTGACGTCGACTGGATCCCATCTCCCACCCGTGCGT
GGCATTTCACTCCCGGTTGCCAACAACGGACAGCAACAAGCAGCACTCATTCAAAGTTGTGGTTTT
ACCGTCGTTGCTGTTGATTCTACGATGATAACGTGACGGTGACGGGGGGGGGAGGGGGGTGGAGG
GCATTCGTACTGAGCAGGGCAGTGTGGGGTAGTGGGAATTGAACCTCCTGTCTCCGACCTAAGCG
GCCAGTTCACAAGCGCCGTGACAGTCCGGCTGTGTCGTGAACAGCACGCACAGCCACCGATCAGC
AGCCAGCCAGCCATCTGTCCTCTCAGCACCTTGCTCGTTCTTGTGTATGCATACATTTTTTTTTAAA
TATAAAGCGACTCGGATGTACCGGTCCTACTACAGAATTACCAAAGTCTTTTATGATATATCTCTT
GCGCGTACATAACACGTGTTTTAAAAGTCTTCACACGGCCATGAACCGCCGCTGGCTGATCTCCAG
AAGTGCAGACTCCTGTCTTGACTATGAAATGAGATCCCTGATCATAAAGCCATGGATCCCCCGATA

GCGCCTGTTTTAGCCACAGAGAGCAGTGGTGCATGGCCAGCAGTGATGGGAGCTCATAGCCACCG
TGATTAGTGACAGGGGGCACGCGTTTTTTTGTTTTGTTTTTGTGAGTTAATCTGCCCAGGGCAGTTT
CCTGGATCTAGCCATAACTAAGGGAGGAGTAGTTGAGGTGGTTTTATCCCCCCCCCCTCGCCTTTTT
TCGACAGCGTCATTAAGGCGTCACAATTCATTTTAGTTGCTTTAGAACACTTACTCTGACCTGCACA
TTTTTTAAAAAGAAAATACTAACGCTGAATCCGTCAGAAGCTTTTGACTGCAGTCTAAATGTTTTC
ATGCAGCAAAGCAGCCATTGATGTTTAATTCGGATGAGAAGGTTTTAAATCAGGAATCTCAGAAC
CGAGCGGCGGGGAAATTTAATTTGCTACAAACACAACTCGACCGAGTTGGTCCCTCTGAGACGTA
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AAATAAAACGAAACCACCGTCTCATCAATCAGATCTCAGCCAGGCTGAGCCCCGGTCGCCTTCCG
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CGCCTTGGCCGCCGCGACG<ATGTCGTGTGGCGGCTGCTTCCTGGTGCTGTGGTCGCTGTGCGGCG
CCGCGGCACTCTGCTTGCTCGTCGCCGCCGCCCTCATGCCGTCGTGGCGCGACCTGATACTCGTGG
ACCCGACGAAGCGCTCCATCGTGGTGCGCCACGGCCTCTGGGTTCGCTGCGCCGGGTACGAGGGC
GACGTCCCCGGCGGGGTGGTGTCGTGGGCGGCCGCGTGGTTTGGCCCCGACAGCGGAAAGGAGGG
CGTGGATGGCGGAGGGAGCCCGGGCTGCGTGGTCCGCGACTCCGCGTGGCCGATAGCAACCGACT
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TAATTGGGTACAGTCTGCTCTATAACGTGGTAGTTGCGTTCCTGAAAACCACCCCGTGGTGAAAAA
AATCGCGATACAATAAATATCAAGCACAGTCCATGATAGCACGTGAGATGCGTATGCAGGCTGCA
CAAACACCGAGGTGCGTGGGTAGCACCTAGAAACATCCAGCAGCATCCCACCT

Claudin 15 (comp2832378_c0_seq1): Full CDS

CCACCCACCCCACCCACACCGCCCACAACCGCAGCCACCGGGCTCCGCGAGC<ATGGCCCTGGAC
GTCGTTGGCACGTTGCTGTGCATCGCCGGGTGGACTCTGGTGGGCACGATCATCACCAACC**ACTAC**
TGGAAGGTGTCGACGGTGGCGGGCAGCGTCATCACCACCAACACCATCTACGAGAACCTGTGGC
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CTGGCCACGTGCAGGCGTGCCGGGCTCTGGTCATCACGGCGATCGTGCTCGGATTCTTCGGCACGC
TGCTCGCCTTCCTGGGCATGCGCTGCACCAACATCGTGAGCAGCGACGCCGAGGTGAAGGGTCGC
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TACGCGGCGCAGGTGGTTGCGGAATTCTTCGACCCCTTCTACGCCGGCACAAAGTATGAGTTGGGG
CCGGCTCTGTACATGGGCTGGGCCGGCTCGGGTCTGGCCATCTTAGGCGGCGGCTTGCTGTGCTGC
GTCTGCTGTACTGGGGCCCGTTC**GTCTACGCAGAGAGACCACA**CGTTCAAGTACTCGGCGGCGCG
TGCGGCGACCCCGATCAGCACTGCCACCCCACGGACGATCCTCGACATGGAGGGGCAGACGTACG
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Claudin 16 (comp280178_c0_seq2): Full CDS

ACCACCACCACACCACCACCACCTTGACCAGCTCCGCC<ATGATCACTGCACTTCAGCTCCTCGCC
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GCCTTCGATGGCATCCGCACCTGTGACCAGTTCGACTCTATACTGGCAGAGCACCCACTGAAGATC
GTGCTGTCCCGCTCCCTCATCCTCACCGCTGACATCCTAGCCTGCTTCGCCTGCATAATCCTCATCC
TGGGCCTCGACTGCGTGCGAGTGCTCAGCAATGAGCCC**CGAATCAAGACCCGCATCTG**TTACAC
GGCCGGCTTCATCTACCTCCTCGCCGGCATCCCCGGCATGGTGGGGTCCGTGTGGTACGCCGTGGA
CGTGTACGTGGAGCGCTCCACGCTGGTGCAGCAGAACGTCTTCCTGGGCATACAGTACGAGTTTGG
GTGGTCCTGCTGGCTGGGCATGGCCGGCTCCATGGCCTGCATCCTGGCCGGGATCGTCCTCACCTG
CTGCGTCTACCTGTTCAGGGATGCGAACGCCGCCCGCCTGCACAGATCCCCGTACGTCGCTCCACG
CACCTCCCTGGGCAAGATGTACGCCATGGCTTCCCACGTGTGA>CCTCACTTCCTGTGCGGGGTGA
CGTCACTTCCTGTCATTGCTGCTGCCACTCCCCAATCTCCAAGCGAGGGGAAAATGGCCGCCGTGT
GCTCCT**CCATTAACACGACCACCACC**ACCACTCCTACACCACCACCACTCCTA

Claudin 18 (genomic): Full CDS

GGGGTCACCTTGGCC<ATGCACGTCGTTGGGTTTGCGCTGTCCACGCTTGGCTTTGGCCTCACGGTG
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GGCGCTGTCACAGCCCAGGTTCTTTCAGGTGGTGCGTGCGCTGATGCTCATGGCCGTGGCGGTGGG
CGCCATCGCGCTGTTCGTTTCCACGCTGGGCACGGCGTGCGCCCGCCGCTTCAGCGAGCACGCCAG
CACCAAGACCAAACTGACCTGCGCTGGCGGCACCCTGTGGCTTCTCACGGTGCTATGCTCTCGCGT
AGTATGTTCCTCCTTCGGGGTCAGCCTCGTCACGGAGTTTCCAGGGTGGCTTCGCGTTTCTCCAACC
TCGCCAGGGGGGGGGGTTGGGGGGTTGTCTTCCTTCGGCGAGCTTCAGACATTGTACACCAGGTTC
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ATG>

Claudin 19a var 1 (comp 291939_c0_seq10): Full CDS

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ACCGCTCCCTTTGTTATACAATGTAAATTATTACATCATTTATTTTTTAAGGGTTTATTGTTAAGTAT
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GTTTGCCCAGATATACACCTGGGACTTTAGTTGGTGCCAGCTCAGTGCTCTGATGCAGCTATGATG
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Claudin 19a var 2 (comp 291939_c0_seq5): Full CDS

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ATGCCTCCATGGATTGGTTGGAAAAACCTAACATTTAATTATAATATACTGTATTTAAAAACCGTT
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AACGTCGTACTACTCAAATTGTAAATGTTGTGGAGTAGTAACAAATTG**GCGTTTTGTTTCCGTGT**
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Claudin 19b: Full CDS

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Claudin 19a (genomic 1107):

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Claudin 19b (genomic 7974):

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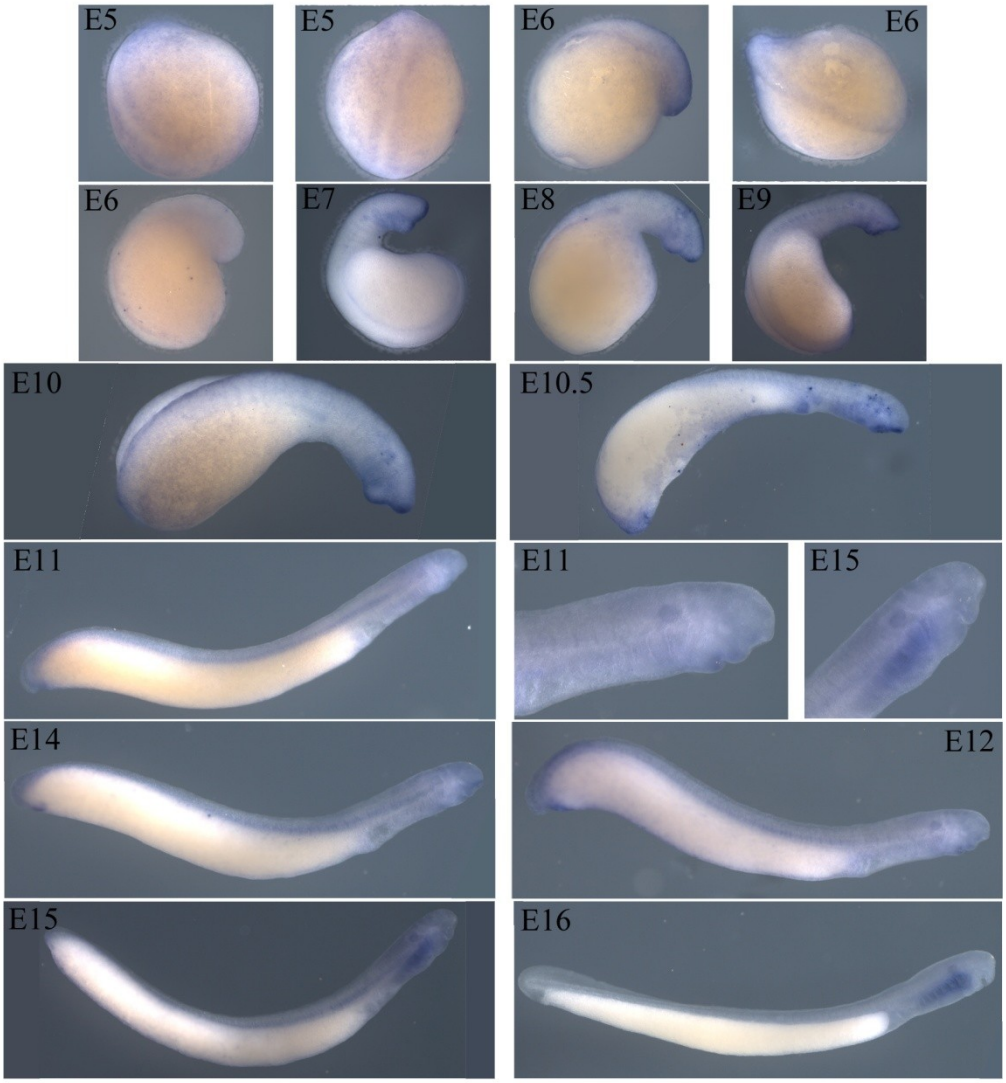

Figure 6.4A: Expression profile of *Claudin 19b* full length probe during *P. marinus* embryogenesis at stages E5 to E16.

The expression pattern of embryos hybridized with the full length *Claudin 19b* probe are similar to those of the probe targeting the untranslated region of the gene (**Figure 3.7A**). The latter have a clearer signal, so they were used for analysis of the *Claudin 19b* spatial gene expression pattern.

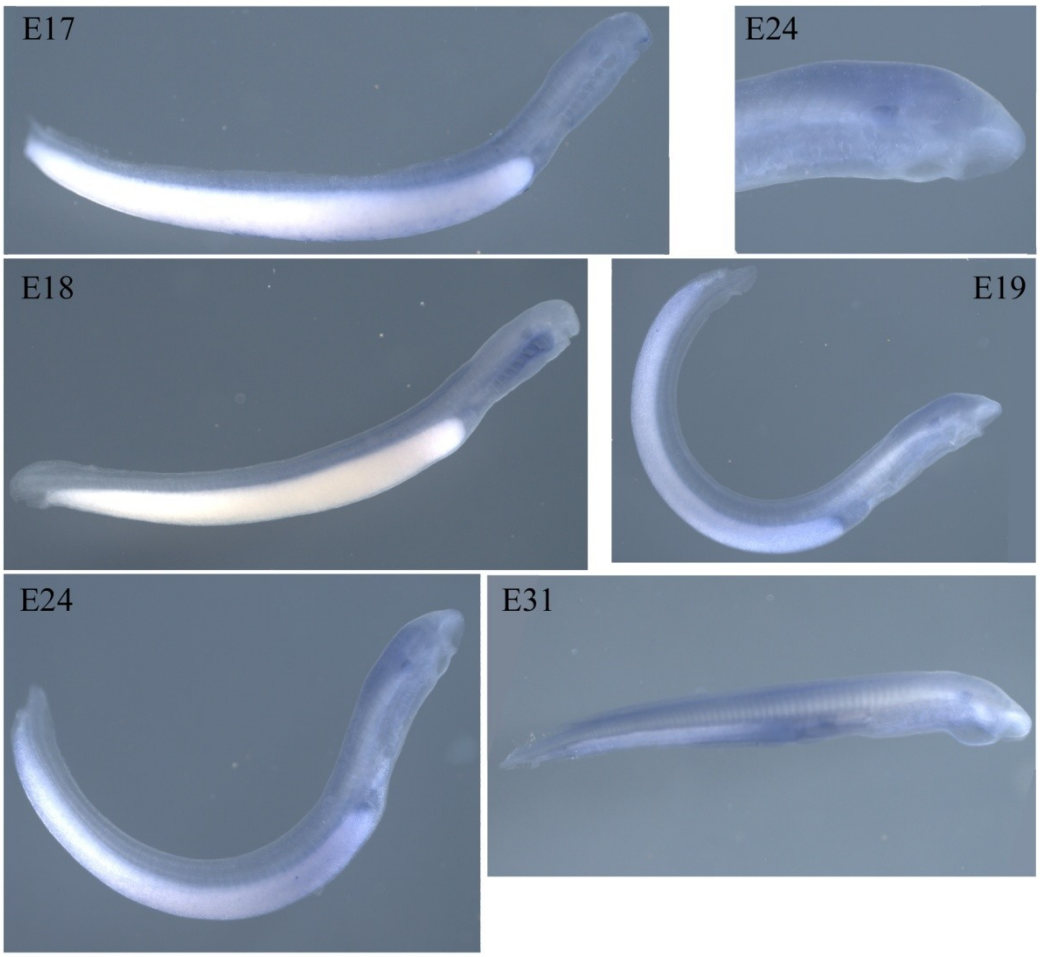

Figure 6.4B: Expression profile of *Claudin 19b* full length probe during *P. marinus* embryogenesis at stages E17 to E31.

The expression pattern of embryos hybridized with the full length *Claudin 19b* probe are similar to those of the probe targeting the untranslated region of the gene (**Figure 3.7B**). The latter have a clearer signal, so they were used for analysis of the *Claudin 19b* spatial gene expression pattern.