The role of proteoglycans in the initiation of neural tube closure

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Declaration of contribution

I, Oleksandr Nychyk confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in my thesis.

___________________________________________

Oleksandr Nychyk
Abstract

Neurulation is the embryonic process that gives rise to the neural tube (NT), the precursor of the brain and spinal cord. Recent work has emphasised the importance of proteoglycans in convergent extension movements and NT closure in lower vertebrates. The current study is focused on the role of proteoglycans in the initiation of NT closure in mammals, termed closure 1.

In this project, the initial aim was to characterise the ‘matrisome’, or in vivo extracellular matrix (ECM) composition, during mammalian neurulation. Tissue site of mRNA expression and protein localisation of ECM components, including proteoglycans, were then investigated showing their distinct expression patterns prior to and after the onset of neural tube closure.

The expression analysis raised various hypothesis that were subsequently tested, demonstrating that impaired sulfation of ECM proteoglycan chains worsens the phenotype of planar cell polarity (PCP) mutant loop tail (Vangl2Lp) predisposed to neural tube defects. Exposure of Vangl2Lp/+ embryos to chlorate, an inhibitor of glycosaminoglycan sulfation, during ex vivo whole embryo culture prevented NT closure, converting Vangl2Lp/+ to the mutant Vangl2Lp/Lp pathophenotype. The same result was obtained by exposure of Vangl2Lp/+ embryos to chondroitinase or heparitinase. Taken together, it indicated that the PCP pathway functionally interacts with chondroitin and heparan sulfat proteoglycans during initiation of NT closure. In order to investigate the possible role of proteoglycans in mammalian convergent extension, the node of Vangl2Lp/+ embryos was labelled with DiO. The study revealed that the PCP-proteoglycan interaction is mediated independently of convergent extension. The failure of neural fold apposition and reduced Fgfr1 signalling was proposed as potential causative mechanism underlying failure of closure 1.

In fish, the cilia motility is dependent on heparan sulfate chains, but this has not been studied in mammals. The present study identified a novel cellular localisation of cohesin/proteoglycan protein Smc3 and its GAG chains. Both Smc3 and CS-E are expressed in the midbody, primary and motile cilia. For the first time, this study showed the nuclear expression of CS chains in mouse embryo. The coordinated movement of Smc3 and CS-E chains during cytokinesis and ciliogenesis suggests conserved role of this protein in mouse cilia and cytokinetic apparatus.
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<thead>
<tr>
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<tbody>
<tr>
<td>APA</td>
<td>All Positions on Average</td>
</tr>
<tr>
<td>BM</td>
<td>Basement membrane</td>
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<tr>
<td>BMP</td>
<td>Bone Morphogenetic Proteins</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
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<tr>
<td>CE</td>
<td>Convergent extension</td>
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<tr>
<td>CEM</td>
<td>mesoderm caudal to the somites</td>
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<td>Chr.ABC</td>
<td>Chondroitinase ABC</td>
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<td>CRN</td>
<td>Craniorachischisis</td>
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</tr>
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<td>Chondroitin sulfate type E</td>
</tr>
<tr>
<td>CSPG</td>
<td>Chondroitin sulfate proteoglycan</td>
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<tr>
<td>DLHP</td>
<td>Dorsolateral hinge points</td>
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<tr>
<td>DMEM</td>
<td>Dulbecco’s Modified Eagle’s Medium</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
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<tr>
<td>ECM</td>
<td>Extracellular Matrix</td>
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<tr>
<td>FBS</td>
<td>Foetal Bovine Serum</td>
</tr>
<tr>
<td>FGF</td>
<td>Fibroblast Growth Factor</td>
</tr>
<tr>
<td>Fgfr1</td>
<td>Fibroblast growth factor receptor 1</td>
</tr>
<tr>
<td>FPKM</td>
<td>Fragments Per Kilobase of transcript per Million mapped reads</td>
</tr>
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<td>GAG</td>
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<td>MAPK</td>
<td>Mitogen-activated protein kinases</td>
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<td>presumptive spinal cord</td>
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<tr>
<td>RA</td>
<td>Retinoic acid</td>
</tr>
<tr>
<td>RGD</td>
<td>Arg-Gly-Asp</td>
</tr>
<tr>
<td>RPKM</td>
<td>Reads Per Kilobase of transcript per Million mapped reads</td>
</tr>
<tr>
<td>RT</td>
<td>Room temperature</td>
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<tr>
<td>SE</td>
<td>Surface ectoderm</td>
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<tr>
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<td>Sonic Hedgehog</td>
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<tr>
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<td>Whole-mount immunofluorescence</td>
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<tr>
<td>WT</td>
<td>wild type</td>
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<td>YS</td>
<td>Yolk Sac</td>
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1. General introduction

1.1 Neurulation

Neurulation is the embryonic process which results in the formation of the neural tube (NT), the precursor of the brain and the spinal cord. It is conventionally divided into two events, primary and secondary neurulation. In vertebrates, primary neurulation originates with the formation of a neural plate - a thickening of specialized dorsal surface ectoderm which subsequently folds and fuses into the NT (Copp et al., 2003). This is followed by secondary neurulation which forms the NT at low sacral and caudal levels (Schoenwolf et al., 1984; Copp et al., 1989). Secondary neurulation does not involve neural folding and adhesion. Instead, the multipotent population of progenitor cells in the tail bud condense in the dorsal midline to form the secondary NT, the lumen of which is continuous with the rostral primary NT (Schoenwolf et al., 1984).

1.1.1 The primary neurulation sequence: closure sites and timing

In mammals, NT closure is a multi-site process which initiates sequentially at distinct levels along the embryo axis. Mouse NT closure initiates at the hindbrain/cervical boundary at the six to seven somite stage (Fig. 1.1A) (Copp et al., 1994a). Upon completion of the initial closure event (termed closure 1), the neural folds continue to close uni- or bi-directionally to form the NT that eventually extends from forebrain to low spine (arrows in Fig. 1.1B). Two additional closure sites appear in the cranial region at E9: closure 2 at the forebrain/midbrain boundary (arrow and dashed line in Fig. 1.1 C, D), which extends bi-directionally, and closure 3 at the rostral proximity of forebrain (arrow in Fig. 1.1D) which extends in a caudal direction. The hindbrain neuropore (between closures 1 and 2) and the anterior neuropore (between closures 2 and 3) complete closure by E9.5 forming the intact tube in the cranial region. In the spinal region, the neural folds fuse caudally from the closure 1 site and eventually the posterior neuropore (PNP) closes at E10.5, completing the primary neurulation (Copp et al., 2003).

Primary neurulation in humans is similar to mouse neurulation (Fig. 1.3) (O’Rahilly et al., 2002). In humans, bending of neural plate initiates approximately 18 days after fertilisation followed by an event corresponding to closure 1 at approximately 21 days and the completion of PNP closure by 26-28 days post-fertilization (Greene et al., 2014).
FIGURE 1.1

Figure 1.1. The sites of mouse NT closure. Scanning electron micrograph (SEM) images representing the three closure sites in E8.5 mouse embryos. (A, B) Dorsal view of mouse embryos at 6 (A) and 8 somite stages. Closure is initiated at the hindbrain/cervical boundary (Closure 1, arrow in A) and spreads bidirectionally from this site (arrows in B). The hindbrain and posterior neuropores are the open regions in the rostral and caudal parts of the embryo. (C, D) Dorsal/rostral views of the head region at 9 somite stage. Closure 2 is initiated at forebrain/midbrain boundary (arrow and dashed line in C, D) and closure 3 is observed at the rostral proximity of forebrain (arrow in D). The anterior neuropore is clearly demarcated between the closure 2 and 3 sites (D). SEM images were taken by Dr Ana Rolo (A, B) and adapted from (Davidson et al., 2007) (C, D). MB (midbrain), FB (forebrain), ANP: anterior neuropore. Scale bars in A - D = 100 µm.

In the cranial region, the closure 3 event is similar between human and mouse; however, the existence of a closure 2 site in humans is controversial, and cranial closure is likely to be achieved by completion of closure through a single cranial neuropore, between closures 1 and 3 (O’Rahilly et al., 2002).
1.1.2 Neural plate shaping and bending

Before the onset of mouse NT closure, the broad neural plate elongates in the rostro-caudal axis (extension) and narrows in the mediolateral axis (convergence) by intercalating cells at the midline (Goto et al., 2002a). Initially, convergent extension (CE) was described in Xenopus embryos and this process was shown to be depended on a non-canonical Wnt signalling cascade, known as planar cell polarity pathway (PCP) (Goto et al., 2002a; Wallingford et al., 2002a). Functional perturbation of PCP components disrupts CE producing a broad neural plate which prevents NT closure in Xenopus (Wallingford et al., 2002a) and mouse embryos (Ybot-Gonzalez et al. 2007b). The crucial role of the PCP pathway in CE cell movements and NT closure is discussed in more detail in Section 1.2 and Chapter 5.

Once CE is under way, elongating and narrowing the future central nervous system, the margins of the neural plate start to elevate forming the neural folds that eventually meet and fuse at the dorsal midline. Bending of the neural plate differs morphologically across different stages, closure sites and regions. At E8-8.5, the flat neural plate begins to bend medially to create the median hinge point (MHP), overlying the notochord (Moury et al., 1995). This process leads to formation of a V-shaped neural groove along the rostro-caudal axis causing the neural fold elevation and apposition (mode 1, Fig. 1.2C). As neurulation progresses along the future spine, from mode 1 to mode 2 (E9.0-9.5), paired dorsolateral hinge points (DLHPs) arise at the intermediate spinal level, therefore, at this stage the neural plate bends at both MHP and DLHPs (mode 2, Fig. 1.2D). DLHPs form precisely at the point of contact of the surface ectoderm to the outside neural fold. At lower spinal levels, the MHP is lost and bending occurs solely at DLHPs (mode 3, Fig. 1.2E).

In the cranial region, the neural folds are initially biconvex, bulging outwards away from the midline, before switching to a biconcave shape. The mechanisms underlying cranial neural fold bending are not well defined and some studies have suggested a key role for expanding mesenchymal tissue and the actin cytoskeleton in this process (Morriss-Kay, 1981; Ybot-Gonzalez et al., 1999). In contrast, NT closure in the spinal region is able to proceed in the absence of pre-somatic mesoderm (Ybot-Gonzalez et al., 2002).

By comparison to other cranial and spinal levels, the closure 1 site is unique in not having focal bending at MHP and DLHPs; instead, the neural plate displays a ‘horseshoe-type’ morphology in which all parts of the neural plate appear to bend equally (Fig. 1.2B). In addition, the closing neural folds are directly adjacent to the epithelial somites; the site of closure 1 is at the level of 3rd somite when embryos have 6-7 somites in total (Sakai, 1989a). By comparison, at lower spinal levels, the closing neural folds are flanked by unsegmented paraxial mesoderm.
**FIGURE 1.2**

![Diagram showing NT morphogenesis along the spinal region.](image)

**Figure 1.2. NT morphogenesis along the spinal region.** (A) At E8.0 the neural plate is flat, flanked by non-neural ectoderm and unsegmented paraxial mesoderm. (B) At E8.5 (closure 1 region) bilateral neural folds form and elevate. The neural plate is flanked by segmented paraxial mesoderm (somites) laterally and non-neural ectoderm (aka surface ectoderm) dorsally. (C) At the upper spinal region, the neural folds bend at the median hinge point (MHP). (D) At the intermediate spinal level (E9.0-9.5) the neural folds bend at both MHP and paired dorsolateral hinge points (DLHPs). (E) At the lower spinal level (E10.0) the neural folds bend solely at DLHPs. The neural plate is surrounded by unsegmented (presomitic) mesoderm at upper, intermediate and lower spinal levels.

The unique morphology and relationship to somitic mesoderm suggests that the developmental mechanisms underlying closure 1 may differ from those at cranial and spinal levels.

The MHP is highly enriched in wedge-shaped cells, which are wider basally than apically and this cell wedging feature is related to interkinetic nuclear migration. The increase in S-phase length and accumulation of basal S-phase nuclei is correlated with cell wedging in the MHP (Meyer et al., 2011). Studies in the past revealed that induction of the MHP is dependent on signals from the underlying notochord. These studies showed that ablation of the notochord, in both chick and mouse embryos, led to the loss of MHP without affecting NT closure (B. P. Davidson et al., 1999; Ybot-Gonzalez et al., 2002). Further evidence for the lack of requirement of MHP for NT closure comes from sonic hedgehog (Shh), Foxa2 and Gli2 mutant embryos that lack a notochord or floor plate; nevertheless, these embryos are able to complete spinal neurulation (Ang et al., 1994; Chiang et al., 1996; Ybot-Gonzalez et al., 2002). Therefore, while...
the MHP is functionally important for subsequent development of the floor plate, it is not required for NT closure.

The formation of DLHPs is regulated by a balance between inhibitory and inductive signals along different rostro-caudal levels (Ybot-Gonzalez et al. 2007a). Shh from the notochord acts as an inhibitor of DLHP formation. The induction of DLHPs associates with decreasing Shh expression and reduction of Shh signalling as neurulation progresses towards the lower spinal regions, which allows a shift from MHP to DLHPs. The formation of DLHPs can be suppressed by the local release of SHH peptide from implanted beads at lower spinal levels (Ybot-Gonzalez et al., 2002). These results explain the absence of DLHPs during mode 1 when Shh signalling is highly active. BMP2, secreted from the surface ectoderm at the dorsal tips of neural folds, also inhibits DLHP bending. At upper spinal levels, Shh inhibits production of the BMP antagonist noggin, so BMP signalling is unopposed and prevents DLHP formation. In the lower spinal levels, Shh production weakens and noggin is no longer repressed and is able to inhibit BMP dorsally; hence, formation of DLHPs is de-repressed (Ybot-Gonzalez et al. 2007a). In contrast to the MHP, DLHPs appear essential for the progression of spinal neurulation. For example, the absence of DLHPs in Zic2 mutant embryos, leads to the severe spina bifida (Ybot-Gonzalez et al. 2007a).

1.1.3 Fusion of neural folds

The last step of primary neurulation is fusion of the opposing neural folds at the dorsal midline. Fusion and remodelling of the neuroepithelium and surface ectoderm results in an intact tube covered by a layer of future epidermal ectoderm. A number of studies have described protrusions that facilitate the primary contact between opposing neural folds (Pai et al., 2012). The cell-type that adheres first appears to vary with axial levels. Recently, the presence of filopodia-like extensions was reported in the closing hindbrain of live embryos (Pyrgaki et al., 2010). In the spinal region, surface ectoderm cells undergo extensive protrusive activity establishing an initial contact at the leading edge of the neural folds (Rolo et al., 2016a). At early stages of spinal neurulation the predominant type of protrusions are filopodia, whereas at later stages a combination of both filopodia and ruffles appear to be present. Knockout of the small GTPase Rac1 from the surface ectoderm results in the suppression of ruffle protrusive activity, prevention of neural fold fusion and hence spina bifida in mutant embryos (Rolo et al., 2016a). This study has therefore demonstrated a requirement of protrusions for closure, at least in the lower spinal neural tube.
1.1.4 Neural tube defects

NT defects (NTDs) are among the commonest and most severe congenital malformations, with a frequency of 0.5-2 per 1000 established pregnancies worldwide (Juriloff et al., 2012). If NT closure is not completed, the neural tissue remains exposed to the extraembryonic environment leading to neurodegeneration and neuronal deficit (Copp et al., 2010). The severity and the spectrum of open NTDs differ with the level of the neuraxis affected (Fig. 1.3). Failure to initiate closure at hindbrain/cervical boundary (closure 1) leads to the most severe form of NTD, craniorachischisis (CRN), where the NT remains open in the midbrain, hindbrain and the entire spinal region. Incomplete closure in the cranial region leads to a condition termed exencephaly, in which the neural tissue protrudes from the developing brain. As development proceeds, exencephaly progresses into anencephaly due to progressive degeneration of the neuroepithelium exposed to amniotic fluid and failure of the skull vault to form over the open lesion (Copp et al., 2009). Failure of closure 3 is less common but, when present, lead to forebrain anencephaly with a split face. CRN is considered to be rare condition (1 in 100,000), and is incompatible with postnatal life resulting to pre- or perinatal lethality.

Progression of closure 1 caudally in the spinal region may stop at any time leading to open spina bifida (myelomeningocele), in which the size of the defect varies along the body axis, depending on the site where the closure fails. The two main forms of open spina bifida are myelocoele where the neural tissue is in direct contact with amniotic fluid, and myelomeningocele (spina bifida cystica) where the neuroepithelium is covered by a meningeal sac (Copp et al., 2015). These conditions are the most common group of open NTDs and are compatible with life. However, the outcome of myelomeningocele is variable and depends on the severity of the lesion. Patients often require lifelong medical care for various complications that happen as the result of the NTD. These complications are mainly associated with neurological deficit below the lesion, affecting both motor and sensory functions (Oakeshott et al., 2012).

Both genetic and environmental factors contribute to aetiology of NTDs. Several genes have been associated with NTD risk in humans and over 250 genetic mouse models of NTDs are available (Harris et al., 2010). A number of mutations cause NTDs in digenic, trigenic, and oligogenic combinations, an aetiology that likely reflects the nature of genetic diversity among human NTDs. Among the mouse models of NTDs approximately 80% display cranial defects and about 20% spina bifida (Harris et al., 2010).
By contrast, only a small number of mouse mutant strains develop CRN (approximately 14) and the majority of the mutations are associated with the PCP pathway. Non-genetic factors associated with NTDs include valproic acid, maternal obesity, maternal diabetes and reduced folate uptake (Oakeshott et al., 2012)

1.2 The planar cell polarity pathway

1.2.1 Vertebrate PCP genes

Planar cell polarity (PCP) refers to the global coordination of cell behaviour within the plane of the cell sheet, perpendicular to the apicobasal axis. Cell polarization has been extensively studied in the fruitfly Drosophila, especially in the development of the Drosophila wing and compound eye (Strutt, 2003). At the cellular level, tissue polarity was found to be controlled by a ‘core’ set of PCP genes (Tissir et al., 2013).

Core PCP genes are highly conserved from flies to mammals. In Drosophila, the PCP module is composed of transmembrane and cytosolic proteins. The transmembrane receptors are Frizzled (Fz), Strabismus (Van Gogh) and Flamingo (Fmi, also known as starry night (Stan)); and the cytosolic proteins are Dishevelled (Dvl), Prickle (Pk) and Diego (Dgo). By comparison to the
fruitfly, several family members have been identified for many of the vertebrate PCP homologs. For example, there are three CelSR genes (homologous to Drosophila Flamingo), two VanGL (homologous to Drosophila Strabismus) and three DVL genes. The core PCP components interact with each other both across cell membranes and in the cytosol. The signalling cascade is established through interactions between the transmembrane components Frd receptors and intracellular Dvl proteins. Dvl is recruited to the plasma membrane upon activation of the pathway. Other core PCP proteins that associate with Dvl include the transmembrane cadherin CelSR1, VanGL2, a tetraspanin protein with PDZ-binding domain, DlgA (Drosophila Disc Large Tumor Suppressor) and ZO1 (Zonula Occludens-1 Protein), and cytosolic protein Pk. Later studies identified two further proteins that are involved in the vertebrate core PCP module: Scribble (Scrib) and protein tyrosine kinase 7 (Ptk7) (Montcouquiol et al., 2003; Lu et al., 2004). The core proteins of mammalian PCP signalling cascade are shown in Figure 1.4. PDZ-binding domain (postsynaptic density-95 / Discs large / Zonula occludens-1 (PDZ) play important roles in a number of molecular biological events, including protein assembly, localisation, and signal transduction.

In Drosophila, the core PCP signalling molecules display an asymmetrical localisation. This results in the formation of distinct protein complexes at opposite poles of the cell: VanGL-Fmi-Pk complex on one side and an Fz-Fmi-Dsh-Dgo complex on the other (Yang et al., 2015). In vertebrates, the subcellular localisation of PCP proteins has not been studied in detail. The asymmetrical distribution of VanGL2 and Dvl was reported in the hair cells of the mouse cochlea (Wang et al., 2005; Montcouquiol, Crenshaw, et al., 2006).

Among nineteen Wnt ligands, only Wnt5a and Wnt11 have been found to regulate the vertebrate PCP signalling cascade. Knockout of Wnt5a displays severe shortening of the A-P axis and truncation of the limbs (Yamaguchi et al., 1999). Wnt5a facilitates PCP establishment by phosphorylating VanGL2 protein (Gao et al., 2011). In addition, this ligand interacts genetically with VanGL2Lp allele during initiation of NT closure (Qian et al., 2007). Both Wnt5a and Wnt11 have been implicated in CE and neurulisation in lower vertebrates. For example, zebrafish silverblick (wnt11 mutant) develops CE defects in the notochord (Heisenberg et al., 2000). In Xenopus, the expression of a dominant-negative form of Wnt11 leads to CE defects and NTDs (Tada et al., 2000). However, the loss of Wnt11 in the mouse does not result in PCP defects, in contrast to the zebrafish mutant. A recent study generated a double knockout of Wnt5a and Wnt11: deletion of Wnt11 worsened the phenotype of Wnt5a-/- embryos, suggesting functional redundancy of these PCP ligands during axis formation in mouse embryo (Andre et al., 2015).
FIGURE 1.4

Figure 1.4. The mammalian planar cell polarity pathway. Schematic representation of the core PCP proteins in a mammalian cell. Black arrows indicate the signalling pathway, blue arrows indicate known biochemical interactions and green arrows indicate known genetic interactions between family members. Adapted from Dr S. Pryor PhD thesis.

Downstream signalling of the vertebrate PCP genes include small Rho-GTPases (RhoA, Cdc42 and Rac1), that are involved in the cytoskeletal regulation of PCP-dependent processes in zebrafish, *Xenopus* and mice (Jessen *et al.*, 2002; Patricia Ybot-Gonzalez, Savery, *et al.*, 2007b). In mouse, the PCP signalling pathway is essential for many morphogenetic processes, including CE and NT closure, inner ear hair cell polarity, morphogenesis of organs such as kidneys, lungs and heart, left-right asymmetry and neuronal migration.

1.2.2 PCP-dependent NT closure

During late gastrulation, neural plate shaping is largely driven by CE cell movements, converting the elliptical gastrula to a keyhole-shaped neurula (Copp *et al.*, 2003). The role of PCP in CE and NT closure was first demonstrated in studies on *Xenopus* and zebrafish mainly
focusing on cell migration during early vertebrate development. In *Xenopus*, overexpression or down-regulation of *Stbm* or *Dvl* disrupts CE in both neural and mesodermal cells. It results in reduced elongation of the A-P axis and NTDs (Wallingford et al., 2001; Darken et al., 2002). For example, the expression of a mutant form of *Dvl* causes failure of NT closure and abnormally broad, short neural plate at early neurula stages (Wallingford et al., 2002b). One possible explanation of CE defects in PCP knockouts is related to the presence of actin-rich polarized lamellipodia being responsible for the movement of migrating cells. Lamellipodia are not polarized in *Dvl* deficient cells of *Xenopus* embryos resulting in a CE defect (Wallingford, 2006).

The neural plate morphology of early embryos with deficient PCP signalling is very similar to that of *Xenopus* embryos with defective CE. For example, the neural plate of the *loop-tail* (*Lp*) mouse mutant is short and broad, and the neural folds are widely spaced apart (Greene et al. 1998). The *Lp* mouse model has a mutation in *Vangl2*, a core PCP gene, and the homozygous *Vangl2<sup>Δn/s</sup> embryos fail to initiate NT closure and develop CRN (see Section 1.2.4). Strikingly, most of the mutants displaying CRN have been associated with the PCP pathway. A closure 1 defect is observed in other mutants with homozygous mutations of core PCP genes (*Celsr1*, *Scrib*, *Ptk7*), and in mouse models with digenic and oligogenic PCP mutant combinations (*Dvl1/2*, *Dvl2/3*, and *Fz3/6*). Perturbation of the trafficking of PCP proteins to plasma membrane also leads to failure of NT closure (*Sec24b* mutant). A number of mouse mutants of PCP-related genes also exhibit CRN and other PCP phenotypes. For example, the ubiquitin ligases Smurf1 and Smurf2 participate in the PCP pathway through a Par6-Dishevelled complex; the double mutants display CRN and inner ear phenotype (Narimatsu et al., 2009). Other participants of the PCP pathway are *Cdx* transcriptional factors (*Cdx1* and 2) that are involved in the transcriptional regulation of the vertebrate PCP component *Ptk7*. *Cdx1/2* double mutants exhibit widened mediolateral axis and CRN (Savory et al., 2011a). A list of PCP mutants with CRN is provided in the Table 1.1.

PCP mouse mutants with CRN closely resemble the CRN condition in humans raising the possibility that members of PCP pathway may play a role in human NTDs. This hypothesis fits the finding that NTD-promoting gene-gene interactions occur among core PCP genes, with digenic combinations of mutant PCP genes causing spina bifida and exencephaly, as well as CRN (Murdoch et al., 2014). Therefore, mouse PCP genes could be involved in the aetiology of a variety of human NTDs. Most of the human mutations of PCP genes identified so far are heterozygous non-synonymous DNA sequence changes in highly conserved amino acids (Juriloff et al., 2012). A study in our lab identified unique non-synonymous coding sequence alterations in the *CELSR1* and *SCRIB* genes in patients with CRN (Robinson et al., 2012).
<table>
<thead>
<tr>
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<th>References</th>
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<td>(Kibir et al. 2001; Kibir et al. 2001; Murdoch et al. 2001a)</td>
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<td>(Hamblet et al. 2002; Wang et al. 2006a)</td>
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<td><strong>Dishevelled2/3</strong></td>
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<td>? (low) 100</td>
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<td>100</td>
<td>(Savory et al., 2011b)</td>
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Table 1.1. Mouse models of CRN: PCP mutants. Only monogenic-null mutants of vertebrate PCP genes / non-core PCP-related genes and paralogous gene interactions that cause failure of closure 1 (CRN) are listed here. Sfrp1, 2 and 5 are secreted frizzled-related proteins that participate in the PCP signalling cascade during neurulation.

These mutations affect subcellular localisation and the trafficking of the proteins to the plasma membrane. Several studies by other groups reported genetic variants in a number of PCP genes in patients with CRN, spina bifida and anencephaly. However, there is a lack of in vitro and in vivo studies that assess the functional effects of these putative mutations.

1.2.3 The loop-tail mouse model

The Lp mouse model was discovered over 60 years ago and the name of the strain came from the looped tail phenotype (Strong and Hollander, 1949). In addition, heterozygous mice
exhibited ‘shaky’ head behaviour that was found to be associated with defective orientation of hair cells in the inner ear (Montcouquiol et al., 2003). The offspring of heterozygous intercrosses contained pups with severe congenital malformation that closely resembled human CRN (Copp et al., 1994b). Subsequent studies identified Vangl2 as the gene mutated in Lp mice. Two Lp alleles were characterised as semi-dominant mutations, resulting in CRN in homozygous mutants and looped tail, or rare spina bifida, in heterozygotes (Copp et al., 1994b; Kibar, Underhill, et al., 2001). The first missense mutation is characterised by a single nucleotide substitution (G to A) changing serine 464 to asparagine (S464N); the second (T to A) was identified at position 765 substituting aspartate 255 to glutamate (D255E) (Kibar, et al. 2001; Murdoch et al. 2001b). Both of these mutations affect highly conserved amino acids within the intracellular C-terminus of the Vangl2 protein (Fig. 1.5). At molecular level, the S464N and D255E mutations interrupt binding of Vangl2 to Dvl proteins, suggesting this interaction is critical for the establishment of the PCP signalling cascade (Torban et al., 2004). A recent study reported another Lp allele in which a C to T change was found in Vangl2 sequence converting glutamate 449 to a stop codon (Chen et al., 2013). This mutation is predicted to be a loss of function allele that may result in the production of a truncated protein lacking the PDZ-binding domain. All three mutations described above result in CRN in homozygous mice and looped tail defects in the heterozygous individuals.

Analysis of expression has shown that Lp mutations do not alter the transcription of Vangl2 mRNA (Kibar, et al., 2001), but lead to a reduction in Vangl2 protein expression in the early embryo (Guyot et al., 2011). Studies at the subcellular level showed that the Lp mutant protein fails to package into transport vesicles (named COPII) and fails to reach the plasma membrane (Merte et al., 2010b). The same study identified a component of COPII vesicles, called Sec24b that is required for the transport of Vangl2 to the plasma membrane. Strikingly, mice carrying a loss-of-function mutation in Sec24b also develop CRN, strongly suggesting that Vangl2 transport is essential for its function (Merte et al., 2010b). Another study showed that the Vangl2Lp protein is capable of binding the normal wild type copy of Vangl2 and Vangl1, leading to a reduction of both proteins in the cell (Gravel et al., 2010; Song et al., 2010). Furthermore, the Lp protein could alter the activity of other PCP components suggesting a dominant-negative effect of this mutation (Yin et al., 2012) (see more details in Chapter 4).

As described above, homozygous Lp/Lp embryos fail to initiate NT closure and develop CRN (Fig. 1.5D). Similarly to other PCP mutants, Lp/Lp embryos exhibit an abnormally wide and short body axis at E8.5 (Lu et al. 2004; Ybot-Gonzalez et al. 2007b).
Figure 1.5. Failure of NT closure in Vangl2<sup>a/Lp</sup> mice and position of loop-tail mutations. (A, B) SEM images of transverse sections of the posterior region at E8.5. +/+ embryos display a sharp midline bend in the neural plate at MHP (A). The MHP is absent from Lp/Lp embryos which have a broad, flat region in the midline (B). (C, D) SEM images of +/+ and Lp/Lp embryos at E9.5. The NT of +/+ embryos is closed apart from an open posterior neuropore (arrow in C), and the somites are normally developed (arrowheads in C). In contrast, the NT of Lp/Lp embryos fail to close in the hindbrain and throughout spinal region (between the white arrows in D). The somites of mutant embryos have an irregular shape (arrowheads in D). (E) Schematic representation of the Vangl2 protein and Lp mutations associated with the CRN phenotype. All mutations are localised in the C-terminus; asterisks indicate their approximate positions. Scale bar in A, B = 0.4 mm; C, D = 0.035 mm. Images (A-D) adapted from (N. D. Greene <i>et al.</i>, 1998).

Prior to the initiation of NT closure, the neural plate of E8.5 Lp/Lp embryos is abnormally broad and flat at the midline in caudal region when compared to wild type embryos (Fig. 1.7A vs B). In addition, homozygous embryos have irregular shaped somites and an abnormally wide notochord. The abnormal neural plate morphology originates from a CE defects in Lp/Lp embryos (Ybot-Gonzalez <i>et al.</i> 2007b; Williams <i>et al.</i> 2014) (see more details in Chapter 5). In
addition to NTDs, *Lp* mutant embryos exhibit PCP-related defects in other tissues including disrupted polarity of stereociliary bundles in the inner ear, heart defects, defective reproductive system development and other defects (Montcouquiol, Sans, *et al.*, 2006; Vandenberg *et al.*, 2009; Song *et al.*, 2010; Ramsbottom *et al.*, 2014).

Several genetic studies have shown that the *Lp* allele interacts with mutations in other PCP genes to cause defects of NT closure in mice. The predominant phenotype produced by core PCP gene mutations is CRN (for example in *Vangl2*<sup>Lp/</sup>;*Celsr1<sup>Crh</sup>/+; *Vangl2*<sup>Lp/</sup>;*Wnt5a<sup>-/-</sup>; *Vangl2*<sup>Lp/</sup>;*Dvl2<sup>-/-</sup>), although other NTD types such as spina bifida and exencephaly were also reported (Juriloff *et al.*, 2012). In addition, the *Vangl2*<sup>Lp</sup> allele interacts with genes outside the PCP pathway leading to a variety of NTD phenotypes in mice. For example, the genetic interaction between *Vangl2*<sup>Lp</sup> and *Sdc4<sup>-/-</sup>* leads to the spina bifida (Escobedo *et al.*, 2013).

### 1.3 Glycosaminoglycans and proteoglycans

#### 1.3.1 Glycosaminoglycans

Glycosaminoglycans (GAGs) are linear unbranched polyanionic molecules of repeating disaccharides that are covalently linked to core proteins to form proteoglycans. The disaccharide subunits are made of a hexosamine (i.e. glucosamine or galactosamine) and a uronic acid (glucuronic acid or iduronic acid) or galactosamine. GAGs are divided into four different subgroups: hyaluronan or hyaluronic acid (HA), heparin/heparan sulfate (Hep/HS), chondroitin/dermatan sulfate (CS/DS) and keratan sulfate (KS). HA is the most common example of non-sulfated GAGs, which consists of repeats of N-acetylglucosamine (GlcNAc) and glucuronic acid (GlcA). Unlike other GAGs, it is not covalently linked to a core protein, but interacts non-covalently with proteoglycans via hyaluronan-binding motifs.

The remaining GAGs (Hep/HS, CS/DS, KS) contain sulfate groups in the disaccharide subunits at various positions. Hep/HS is composed of repeating disaccharide subunits of N-acetylglucosamine and either iduronic or glucuronic acid (Fig. 1.6). The glucosamine, iduronate and glucuronate residues can be substituted with sulfate groups on the sugar hydroxyls at the C2, C3 and C6 positions. A proportion of GlcNAc can be N-deacetylated and then N-sulfated during biosynthesis of Hep/HS GAGs.

The disaccharide subunit in CS is composed of N-acetylgalactosamine (GalNAc) and glucuronic acid (Fig. 1.6). The galactosamine residues are modified by the addition of sulfate groups at C4 and C6 positions.
FIGURE 1.6

![Diagram of GAGs](image)

**Figure 1.6. Disaccharide subunits of HS and CS chains.** HS GAGs consist of repeating N-acetylg glucosamine (GlcNAc) and iduronic acid disaccharide units polymerized into long chains. HS chains are N-sulfated and sulfate groups are found at O-2 and O-6 positions. CS GAGs consist of repeating N-acetylgalactosamine (GalNAc) and glucuronic acid (GlcA) disaccharide units polymerized into long chains. CS chains are sulfated at O-4 and O-6 positions. Modified from (Varki A et al. 2009).

In DS, some of the glucuronate subunits are epimerised to form iduronate. KS differs from other sulfated GAGs in the lack of hexuronic acid. The disaccharide subunit comprises N-acetylg glucosamine and galactose residues.

The presence of the carboxyl groups of the two hexuronic acids, and the occurrence of sulfate groups on disaccharide subunits, provide a strong negative charge to these GAGs that have a high affinity for water. In addition, the negatively charged GAG chains are capable of binding various cationic molecules in cells and in the extracellular matrix (ECM). Different combinations of GAG sulfation motifs can overlap with one another, generating a large variety of different GAG combinations when interacting with growth factors, chemokines and morphogens during tissue and organ development. During mouse primary neurulation, the main sulfated GAGs are HS and CS (Solursh et al., 1977; Copp et al., 1988; Yip et al., 2002). Therefore, these GAG types form the focus of the remainder of this thesis.

### 1.3.1.1 Biosynthesis of GAG chains

Biosynthesis of the GAG linkage region (a tetrasaccharide containing xylose, galactose and glucuronate) occurs in the endoplasmic reticulum/Golgi compartments. The synthesis is initiated by the transfer of xylose to the specific serine residue of the core protein (Fig. 1.7).
**Figure 1.7.** Biosynthesis of the GAG linkage region. The synthesis of proteoglycans starts with the formation of the core protein chain. This process is followed by the addition of components of the linker tetrasaccharide (xylose, galactose and glucuronate) in stepwise manner by the respective glycosyltransferases shown above. The same set of transferases is used in synthesis of HS and CS proteoglycans. After the completion of synthesis of the linkage region, the biochemical pathway separates to form either HS or CS proteoglycan. Genes coding for glycosyltransferases: Xyl transferase (Xyl1 and Xyl2); Gal transferase I (B4galt7); Gal transferase II (B3galt6); GlcA transferase I (B3gat3). Gal: galactose, GlcA: glucuronate, Xyl: xylose

Following that, galactose and glucuronate are sequentially added by their respective glycosyltransferases. The same set of glycosyltransferases is used for the synthesis of the linkage region for both CS and HS proteoglycans. The first sugar residue that is attached to the tetrasaccharide linkage region determines what type of GAG chain will be made. HS chain synthesis is initiated via addition of the first N-acetylgalactosamine residue to the glucuronate by GlcNAc transferase I (Fig. 1.8). Polymerisation of the chain is then achieved by the HS-polymerase complex, containing the Ext1 and Ext2 enzymes. In the next steps, HS chains undergo a variable amount of sequential modification involving deacetylation, epimerisation, and N-and O-sulfation. The catalytic reactions, enzymes and enzyme-coding genes for HS biosynthetic pathway are shown in Fig. 1.8. Initial studies assumed that HS sulfation patterns were only generated by sulfotransferases. Subsequently, a group of extracellular sulfatases (Sulf1 and Sulf2) were identified (Dhoot, 2001), which are able to remodel 6-O-sulfated levels on the cell surface and in the ECM.
Figure 1.8. Biosynthesis of HS chains. HS synthesis begins with addition of the first N-acetylglucosamine residue to the linker region by GlcNAc transferase I (encoded by ExtI1-3 genes). Following that, the HS chain is elongated by the alternative addition of glucuronate and N-acetylglucosamine. This reaction is catalysed by HS-polymerase complex that possesses both glucuronate transferase and N-acetylglucosamine transferase activity. The enzyme complex is encoded by Ext1 and Ext2 genes. The next step involves removal of N-acetyl group from N-acetylglucosamine and addition of sulfate group to the GlcN by the bifunctional enzyme N-deacetylase N-sulfotransferase (Ndst1-4 genes). Some glucuronate residues are epimerised by glucuronate C5 epimerase encoded by the Glce gene. In the final steps, the sulfate groups are added to C2, C3 and C6 positions of sugar residues by the respective 2-O, 3-O and 6-O sulfotransferases (encoded by Hs2st1; Hs3st1,2, 4-6, 3a1, 3b1; Hs6st1-3 respectively). 3′-phosphoadenylyl-5′-phosphosulfate (PAPs) is the high-energy donor of sulfate groups. GlcA: glucuronate, GlcNAc: N-acetylglucosamine, IdoA: iduronate, NDST: N-deacetylase N-sulfotransferase, OST: O-sulfotransferase.
FIGURE 1.9

**Figure 1.9. Biosynthesis of CS chains.** CS synthesis begins with addition of N-acetylgalactosamine to the linker region by GalNAc transferase I encoded by the Chsy1 gene. Elongation of the CS chain follows with alternating addition of glucuronate and N-acetylgalactosamine (Chsy1 and Chsy2 genes). The GAG chain is then sulfated by 4-O (encoded by Chst11-14 genes) to produce CS-A chain type and 6-O sulfotransferases (Chst2, Chst3 and Chst7) to produce CS-C chain type. Another sulfotransferase (6-OST*, encoded by Chst15 gene) precisely catalyses 6-O-sulfation of the already 4-O-sulfated GalNac (4,6-SO₄) that results in the formation of CS-E chain type. GlcA: glucuronate, GalNAc: N-acetylgalactosamine, IdoA: iduronate, OST: O-sulfotransferase.

In CS synthesis, GalNAc-transferase I adds the first N-acetylgalactosamine residue to the linkage region (Fig. 1.9). The elongation of CS chains is performed by the alternative addition of N-acetylgalactosamine and glucuronate residues. The chains are subsequently modified by 4- and 6-O sulfotransferases.

Addition of sulfate groups to the 4-hydroxyl of N-acetylgalactosamine leads to chondroitin-4 sulfate (also called CS-A) and to the 6-hydroxyl form, chondroitin-6 sulfate (called CS-C). Disulfated CS chains (CS-E) are formed by specific sulfotransferases that catalyse 6-O-sulfation of the already 4-sulfated GalNAc. The catalytic reactions, enzymes and enzyme-coding genes for CS biosynthetic pathway are shown in Fig. 1.9.

Further processing of the core protein could involve the attachment of either O- or N-linked oligosaccharides to the serine and arginine residues respectively of the protein backbone (Kjellén and Lindahl 1991).
1.3.1.2 Impairment of HS biosynthesis

Several *Drosophila* and mouse mutants with defects in GAG biosynthesis has been reported. These mutants have emphasized the requirement for GAGs and proteoglycans during embryonic and postnatal development. The requirement for HS in the Hedgehog (Hh) signalling pathway has been shown in the knockout of *Drosophila* tout-velu, a gene orthologue of the mammalian *EXT* gene family (Bellaiche et al., 1998). The tout-velu mutant embryos exhibit a segment polarity phenotype resembling the phenotypic features of hedgehog mutants. The importance the HS-polymerase complex in HS synthesis has been shown during mouse early embryonic development. Ext1 mutants are arrested at the gastrulation stage, and display defects in mesoderm formation, with failure of elongation of the egg cylinder (Lin et al., 2000). In humans, mutations in *EXT1* and *ETX2* genes are associated with hereditary multiple exostoses type 1 and 2. These disorders are characterised by the formation of cartilage-capped tumours (exostoses), possible caused by hyperactive Indian Hh signalling (Huegel et al., 2013).

Heparan chains are first sulfated by NDST enzymes that transfer sulfate groups to glucuronate. Lack of N-sulfation in both *Drosophila* and mouse mutants has serious implications for their development. A number of signalling cascades, such as Wnt, FGF and Hh pathways, are disrupted in the *Drosophila sugarless* knockouts of NDST enzymes (Bernfield et al., 1999). *Ndst1* mouse mutants die shortly after birth and exhibit defects in lung development cerebral dysplasia, accompanied by a reduction in both N- and O-sulfation of HS chains (G. Fan et al., 2000).

Many studies have emphasised the in vivo importance of O-sulfotransferases (HS) for several key signalling pathways. For example, *Drosophila* mutants lacking both Hs2st and Hs6st enzymes (2- and 6-O sulfotransferases) exhibit major defects in FGF signalling whereas the single knockouts have a normal FGF pathway (Kamimura et al., 2006). This finding suggests that the specific sulfation pattern of HS chains can be bypassed if the overall level of sulfation is not affected. In mice, the absence of HS6ST1 enzyme results in embryonic lethality at late stages of gestation. *Hs6st1* mutants exhibit defective retinal axon guidance due to the impaired Slit/Robo pathway (Pratt et al., 2006). By contrast, HS6ST2-deficient mice develop normally (Nagai et al., 2013). In turn, *Hs2st<sup>-/-</sup>* mouse mutants survive until birth, but die perinatally from kidney failure due to the disrupted signalling between ureteric bud and metanephric mesenchyme (Merry et al., 2002). The HS chains of mutant mice lack 2-O-sulfate groups, however, these chains are extensively modified above wild type levels by 6-O sulfotransferases.
1.3.1.3 Impairment of CS biosynthesis

GlcA transferase I is involved in synthesis of the linkage region for both HS and CS chains. A mouse mutant for GlcA transferase I (B3gat3) exhibits embryonic lethality before the 8-cell stage due to cytokinesis failure, which has been linked to the a deficiency in CS, but not HS chains (Izumikawa et al., 2015). This study has shown that CS but not HS, GAGs are indispensable for embryonic cell division in mammals. Similar results were obtained in the knockdown of the C.elegans ortholog of chondroitin polymerizing factor (pfc-1) and knockout of the nematode ortholog of chondroitin synthase (sqv-5) (Hwang et al., 2003; Izumikawa et al., 2004). Functional knockdown of pfc-1 leads to a marked decline of the nematode non-sulfated form of CS and cytokinetic regression in early embryogenesis (Izumikawa et al., 2004). In mice, deletion of Chsy1 does not result in early embryonic lethality; Chsy1-deficient mice are viable but exhibit defects in bone development such as chondrodysplasia, delayed endochondral ossification and progression of the bifurcation of digits (Wilson et al., 2012). These defects have been linked to impaired Hh signalling in primary chondrocytes which suggests that both HS and CS proteoglycans regulate skeletal development and digit patterning.

Several studies have highlighted the physiological importance of CS sulfotransferases in different organs and systems. Mice carrying a targeted disruption of 6-O-sulfotransferase (Chst3) have significantly reduced levels of CS-C and CS-D units in spleen and brain, and a decreased number of splenic naïve T lymphocytes (Uchimura et al., 2002). However, no apparent viability differences between wild type and homozygous mutants were observed. Interestingly, unlike 6-O-sulfotransferase deficiency, the mouse mutant of the Chst11 gene (encoding 4-O-sulfotransferase) dies within six hours of birth due to respiratory distress (Kluppel, 2005). These mutants exhibit severe chondrodysplasia with abnormalities in the chondrocyte columns and cartilage growth plates, marked reduction in 4-O-sulfated CS chains, upregulation of TGF-β signalling and down-regulation of the BMP pathway.

1.3.2 Proteoglycans

Proteoglycans are formed by the covalent attachment of GAG chains to a core protein via the linkage region. Proteoglycans are found in the ECM, basement membranes and on cell surfaces. The expression patterns of proteoglycans and their chains are tightly regulated during development. Proteoglycans are classified by the core protein, cellular and subcellular localisation or the predominant GAG species attached. Examples of HS proteoglycans (HSPGs) and CS proteoglycans (CSPGs) are presented in Table 1.2 and Table 1.3 respectively, based on
published classification by (Varki A, et al., 2009). Specific examples of proteoglycans are described Sections 1.3.2.1-3 and in Chapter 3.

1.3.2.1 Syndecans and glypicans

Syndecans are transmembrane signalling proteoglycans that are classified as HSPGs, although some members have both HS and CS chains. In vertebrates, the syndecan family is composed of four members: syndecans 1 to 4 (Iozzo and Schaefer 2015).

<table>
<thead>
<tr>
<th>Gene symbol</th>
<th>Protein name</th>
<th>Number of HS/CS chains</th>
<th>Tissue distribution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hspg2</td>
<td>Perlecan</td>
<td>1–3 HS, 1-3 CS</td>
<td>secreted; basement membranes; cartilage</td>
</tr>
<tr>
<td>Agrn</td>
<td>Agrin</td>
<td>1–3 HS, 1-2 CS</td>
<td>secreted; neuromuscular junctions</td>
</tr>
<tr>
<td>Col18a1</td>
<td>Collagen type XVIII</td>
<td>2–3 HS</td>
<td>secreted; basement membranes</td>
</tr>
<tr>
<td>Sdc1-4</td>
<td>Syndecans 1–4</td>
<td>1–3 HS, 1-2 CS</td>
<td>membrane bound; epithelial cells and fibroblasts</td>
</tr>
<tr>
<td>Tgfrb3</td>
<td>Betaglycan</td>
<td>1 HS, 1 CS</td>
<td>membrane bound; fibroblasts</td>
</tr>
<tr>
<td>Gpc1-6</td>
<td>Glypicans 1–6</td>
<td>1–3 HS</td>
<td>membrane bound; epithelial cells and fibroblasts</td>
</tr>
<tr>
<td>Srgn</td>
<td>Serglycin</td>
<td>10–15 heparin/CS</td>
<td>intracellular granules; mast cells</td>
</tr>
</tbody>
</table>

Table 1.2 Examples of HSPGs. Mouse HSPG genes and core proteins with corresponding number of GAG chains and tissue distribution. Predominant GAG chains are highlighted in bold.

<table>
<thead>
<tr>
<th>Gene symbol</th>
<th>Protein name</th>
<th>Number of CS chains</th>
<th>Tissue distribution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Agrn</td>
<td>Aggrecan</td>
<td>~100</td>
<td>secreted; cartilage</td>
</tr>
<tr>
<td>Vcan</td>
<td>Versican/PG-M</td>
<td>12–15</td>
<td>secreted; connective tissue cells; aorta; brain</td>
</tr>
<tr>
<td>Ncan</td>
<td>Neurocan</td>
<td>1–2</td>
<td>secreted; brain</td>
</tr>
<tr>
<td>Pgb2</td>
<td>Brevican</td>
<td>0–4</td>
<td>secreted; brain</td>
</tr>
<tr>
<td>SLRPs</td>
<td>Decorin</td>
<td>1</td>
<td>secreted; connective tissue cells</td>
</tr>
<tr>
<td>Pgs1</td>
<td>Biglycan</td>
<td>1–2</td>
<td>secreted; connective tissue cells</td>
</tr>
<tr>
<td>Other examples</td>
<td>Leprecan</td>
<td>1–2</td>
<td>secreted; basement membranes</td>
</tr>
<tr>
<td>Pprz</td>
<td>Phosphacan</td>
<td>2–5</td>
<td>membrane bound; brain</td>
</tr>
<tr>
<td>Cd44</td>
<td>CD44</td>
<td>1–4</td>
<td>membrane bound; lymphocytes</td>
</tr>
<tr>
<td>Cspg4</td>
<td>NG2</td>
<td>2–3</td>
<td>membrane bound; neural cells</td>
</tr>
<tr>
<td>Srgn</td>
<td>Serglycin</td>
<td>10–15</td>
<td>intracellular granules; myeloid cells</td>
</tr>
<tr>
<td>Smc3</td>
<td>Smc3, bamacan</td>
<td>3-6</td>
<td>nucleus, basement membranes</td>
</tr>
</tbody>
</table>

Table 1.3 Examples of CSPGs. Mouse CSPG genes and core proteins with corresponding number of GAG chains and tissue distribution. SLRPs = small leucine-rich proteoglycans.
Syndecans contain an ectodomain, a single-pass transmembrane domain and a short cytoplasmic domain. The latter harbours a unique signature that interacts with PDZ-binding proteins. The ectodomain usually possesses 3 to 5 HS chains and sometimes CS/DS chains, making syndecans hybrid proteoglycans. Several studies have shown that syndecans, acting as cell surface co-receptors, are involved in cytoskeleton organisation, cell adhesion, migration and proliferation. Syndecans play an important role in a wide variety of biological processes by interacting with numerous signalling molecules, especially throughout their GAG chains but also through the core protein. One of the studies showed that enzymatic cleavage of HS chains eliminates the proliferation response to FGF2 (Clayton et al., 2001). The importance of GAG-dependent binding of growth factors by syndecan-4 was studied using a competitive inhibitor of GAG sulfation, chlorate. This study showed that the inhibition of GAG sulfation disrupts BMP/noggin signalling (O’Connell et al., 2007). In vivo studies on lower vertebrates revealed the importance of syndecans in a number of key signalling pathways during early embryogenesis. In Xenopus, syndecan-4 is involved in neural induction via ERK and PKC-dependent pathways (Kuriyama et al., 2009). Injection of syndecan-4 transcript led to an induction of neural, but not mesodermal, markers. Slightly later in development, syndecan-4 participates in PCP signalling, and regulates gastrulation and NT closure of Xenopus embryos (Muñoz et al., 2006). In mice, all syndecan knockouts are viable and fertile but display various defects after the exposure to physiological stresses (Alexopoulou et al., 2007). These defects include perturbed angiogenesis, inflammation and wound healing for Sdc1-/- and Sdc4-/- knockouts.

Glypicans are HSPGs that are anchored to the cell surface by a glycosylphosphatidylinositol (GPI) linkage. The glypican family has six members: glypicans 1 to 6. The HS GAGs of glypicans are attached near the juxtamembrane region allowing the chains to span a large area of cell surface, thereby presenting various signalling molecules, such as Wnt, Hh and FGFs, to their receptors. In addition to the family-unique protein core, glypicans also possess a stretch of amino acids in the ectodomain that is similar to the Cys-rich domain of Frizzled proteins. Indeed, a recent study showed that glypican-3 directly interacts with Frizzled and regulates canonical Wnt signalling (Capurro et al., 2014). Functionally, glypicans have been implicated in angiogenesis and tumor growth. In humans, mutations of GPC3 cause the X-linked Symons-Golabi-Behmel syndrome, characterised by cardiovascular anomalies, pre- and postnatal overgrowth and renal dysplasia (Filmus et al., 2014). Studies on Gpc3-deficient mice showed elevated Hh-dependent signalling activity suggesting that glypican-3 acts as an inhibitor of the Hh pathway. Moreover, GPC3-evoked inhibition of Hh signalling is dependent of the degree of heparan sulfation (Capurro et al., 2015).
1.3.2.2 Basement membrane proteoglycans

This group consists of four proteoglycans (perlecan, agrin, collagen XVIII and collagen XV) that are localised in the basement membrane (BM) of many cell types. Both perlecan and agrin contain laminin-like modules and HS chains; the former proteoglycan also can have CS chains making perlecan a hybrid proteoglycan. Perlecan (encoded by the Hspg2 gene) starts to be expressed from pre-implantation and post-implantation stages and its distribution parallels the expression patterns of FGF-2 and FGF4/8 suggesting that perlecan may play a role in regulating FGF signalling (Aviezer et al. 1994; Matsuo and Kimura-Yoshida 2013). However, chimeric studies with HS–deficient embryos supported the hypothesis that cell surface HS GAGs rather than ECM-associated chains are critical for the retention of FGF proteins and subsequent activation of the FGF pathway (Shimokawa et al., 2011). The importance of perlecan HS chains was demonstrated in mouse mutants with deletion of exon 3, which encodes a domain responsible for attachment of HS chains. Mutant mice exhibit impaired angiogenesis, wound healing and suppression of tumor growth (Zhou, 2004). In contrast, the complete mouse knockout of Hspg2 results in early embryonic lethality and defects in BM maintenance characterised by severe chondro- and skeletal dysplasia, defective cephalic development and myocardial cleft (Costell et al., 1999).

Both agrin and perlecan are involved in the final steps of BM assembly by establishing collateral linkages to the cell surface (Hopf et al., 1999; Moll et al., 2001). These linkages allow proper attachment of the collagen type IV and laminin networks to the cell surface receptors. In mammals, the majority of the studies on agrin have focused on the proteoglycan contribution to the regulation of the postsynaptic apparatus at the neuromuscular junction. The N-terminal and central regions of agrin core protein mainly possess HS and rarely CS chains (Winzen et al., 2003). The HS GAGs of agrin bind N-CAM, FGF2, thrombospondin and the protein phosphatase δ (Burgess et al., 2002). Complete agrin knockout is embryonic lethal with the following features: reduced number, density and size of postsynaptic receptor aggregates in muscles; smaller brains; abnormal development of intraneuronal synapses and other neuromuscular associated defects (Sanes, et al., 1998; Gautam, et al., 1996).

Collagens XV and XVIII harbour structural features of proteoglycans and collagens, being substituted with CS and HS respectively (Seppinen et al., 2011).

1.3.2.3 Versican, aggregcan, phosphocan and bamacan

Versican (encoded by the Vcan gene) belongs to the family of aggregating CSPGs distributed primarily within the ECM. Alternative splicing of transcripts coding for GAG chain binding
regions produces four isoforms of versican named V0, V1, V2 and V3. Each isoform contains different lengths of the GAG-binding regions with a variable number of attached GAG chains. V0 and V1 are the most abundant versican isoforms expressed in vascular smooth muscle cells, developing heart and limbs, whereas V2 isoform is mainly detected in the brain (Zimmermann and Dours-Zimmermann 2008). Functionally, versican is implicated in the regulation of cell migration, adhesion and inflammation by interacting with a variety of binding partners including hyaluronan, fibronectin, integrin β1, fibulins and tenascin-R (Wu et al., 2005). Previous studies have shown versican to bind type I collagen and fibronectin and these interactions are responsible for the inhibition of cell adhesion in melanoma cells (Touab et al., 2002; Wu et al., 2005). While collagen, fibronectin and integrins promote cell adhesion, versican blocks cell binding to fibronectin and reduces cell adhesion in several CNS cell types (Braunewell et al., 1995). Versican knockout studies showed that this proteoglycan is involved in a variety of developmental events such as cardiac development, neural crest migration (NCC) and limb development (Nandadasa et al., 2014). For example, versican heart defect (Vcan<sup>hdf</sup>) mutant mice, do not survive beyond E10.5 and develop severe heart abnormalities (Yamamura et al., 1997). Studies in Pax3 Splotch mutant (Pax3<sup>Sp</sup>) have shown that versican is a negative regulator of NCC migration (Tremblay et al., 1995; D J Henderson et al., 1997). Splotch mutants have upregulated expression of Vcan transcripts and exhibit anomalies such as cardiac outflow tract septation defects, pigmentation defects, and absence of dorsal root ganglia: all highlighting compromised NCC function in these mice.

Aggrecan, which complexes with hyaluronan and linker protein, is a load-bearing proteoglycan of cartilage. The large aggregates maintain a stable network and provide mechanical support to cartilage (Wight et al., 2011). The largest GAG-binding domain of aggrecan can contain more than 100 covalently linked CS chains; the presence of KS chains was also reported (Iozzo et al., 2015). The cartilage-related role of aggrecan is underscored by several genetic studies including cartilage matrix deficiency mice (cmd) that harbour C-terminal truncation of the core protein (Vertel, 1995). Aggrecan is almost absent in the cartilage of cmd mutants leading to shortened long bones and early lethality due to the respiratory failure.

Phosphacan is a CSPG that interacts with neural cell-adhesion molecules and neurons and possesses the soluble ectodomain of a Receptor-type protein tyrosine phosphatase β (RPTPβ). Functionally, phosphacan blocks the growth-promoting features of tenastin, axonin-1 and N-CAM (Liu et al., 2006). Bamacan was first discovered as the basement membrane CSPG by John Couchman in late 90’s (Couchman et al., 1996). While subsequent studies discovered its nuclear localisation and its role in the cohesion complex formation (Krasikova et al., 2005; Losada et al., 2005) (see Chapter 5 and 6).
1.4 FGF/FGFR expression and signalling during development

FGF ligands and their receptors (FGFRs) play crucial roles in many cellular processes including proliferation, differentiation, survival and patterning during early stages of embryo development and during organogenesis. The FGF family is composed of secreted FGF and intracellular FGF proteins. The latter serve as cofactors for voltage gated sodium channels. Secreted FGFs share a similar internal core and have high to moderate binding affinity for both heparin and FGFRs. The FGFRs are receptor tyrosine kinases (RTKs) that contain a heparin-binding domain, three extracellular Ig-like domains, a hydrophobic transmembrane domain, and a split intracellular tyrosine domain. The mammalian FGFR family consists of four genes (FGFR1-4) that undergo alternative splicing in their extracellular domains to generate different splice variants of FGFR1-4. The FGF signalling pathway is initiated by the binding of FGF proteins to FGFRs and HS. The GAG chain promotes dimerization of the FGF-bound FGF receptor by binding both proteins directly and stabilizing ternary complex. The activated FGFR is coupled to intracellular signalling cascades including the RAS-MAPK, PLCγ, PI3K-AKT and STAT pathways which regulate multiple developmental processes through effects on cell proliferation and differentiation.

The FGF signalling pathway makes diverse contributions to vertebrate development. The earliest requirement of FGFs was documented in the preimplantation embryo, where Fgf4 is first detected in the morula and then in the epiblast cells of the inner cell mass (ICM). Both ICM proliferation and formation of primitive ectoderm are perturbed in the mouse mutant of Fgf4 (Krawchuk et al., 2013). Fgfr1 and Fgr2 are expressed in the ICM and Fgfr2 is also detected in the embryonic ectoderm (Orr-Urtreger et al., 1991). Both RTKs may serve as receptors for Fgf4 during the peri-implantation period. Indeed, Fgfr2 knockout studies also show defects in outgrowth, differentiation and maintenance of the ICM. Deletion of Fgfr1 or Fgf8 demonstrates a function slightly later in the embryonic development, with phenotypes affecting gastrulation and specification of mesoderm (Yamaguchi et al., 1994; Sun et al., 1999). In gastrulation, the process of cellular organization known as epithelial-to-mesenchymal transition (EMT) takes place upon the formation of the primitive streak. During EMT, epiblast cells ingress through the primitive streak and adopt a mesenchymal phenotype. While Fgfr1 is detected in the embryonic endoderm, Fgf8 is expressed in the primitive streak during gastrulation (Yamaguchi et al., 1992; Lin et al., 2002). Ffgr1−/− embryos show severe gastrulation defects and display retarded migration of mesodermal precursor cells in the primitive streak (Yamaguchi et al., 1994). Similar defects are observed in Fgf8 null mice (Sun et al., 1999).
The induction of neural tissues also occurs during gastrulation representing a fundamental step in generation of the vertebrate nervous system. The FGF pathway regulates neural induction in cells of pluripotent dorsal ectoderm by inhibiting the expression of BMPs. In particular, the expression of Fgf3 and Fgf4 downregulates Bmp4 and Bpm7 in early neural tissues (Wilson et al., 2000; Marchal et al., 2009). FGF signalling is also required for the expression of BMP antagonist Noggin. In addition, activation of FGF signalling results in the inhibitory phosphorylation of Smad transcriptional factors which block their ability to activate the transcription of BMP target genes (Teven et al., 2014).

Later in development, FGFs act as posteriorizing factors during patterning of neuroepithelium and activate the transcription of a subset of posterior neural genes (Doniach, 1995). For example, ectopic expression of FGF ligands in the neural plate upregulates the expression of key posterior Hox genes and leads to inhibition of anterior development (Pownall et al., 1996, 1998).

FGF signalling is essential for limb bud development. FGF ligands and receptors generate a positive feedback loop between epithelial and mesenchymal tissues that initiates and stimulates outgrowth and morphogenesis of the early limb bud. Prior to induction of the apical epidermal ridge (AER), Fgfr1 is detected in the underlying mesenchyme and Fgfr2 is expressed in both the mesenchymal tissue and ectoderm of the future limb bud. Deletion of Fgfr2 from the AER results in the blunt truncations of the limb. In contrast, Fgfr1 plays a role in later stages of limb development. Inactivation of Fgfr1 results in defective patterning of distal limb fields and impaired digit formation (Xu et al., 1998). Mesenchymal Fgf10 signals to overlying ectoderm to initiate formation of the AER (Ornitz et al., 2015). Upon induction, the AER expresses Fgf2, Fgf4 and Fgf8, while Fgf2 and Fgf10 are transcribed in the limb bud mesoderm (Mahmood et al., 1995; Ros et al., 1996; Xu et al., 1998).
1.5 Left-right asymmetry

Although the vertebrate body plan exhibits external bilateral symmetry, the interior features are not symmetrical: the left side contains the stomach, pancreas, spleen and most of the heart, whereas the right side contains the gall bladder and most of the liver (Raya et al., 2006). This left-right (LR) asymmetry is established during embryogenesis by complex interplay between genetic and epigenetic cascades. Initial studies in the mid-1990s discovered that asymmetrical gene expression during early embryogenesis is associated with the development of LR asymmetry of the vertebrate body. One of the studies found that Nodal was asymmetrically expressed in the left side of the node in mouse embryos (Collignon et al., 1996) (Fig. 1.9A). At the same time, Meno and colleagues independently discovered another asymmetrically expressed gene Lefty2 in the mouse embryo (Meno et al., 1996) (Fig. 1.9B). Asymmetrical expression of both Nodal and Lefty2 lasts only several hours, from 2 to 6 somite stage in mouse embryos. Subsequent studies found that Nodal and Lefty2 belong to the TGFβ family proteins and play central roles in the initiation of LR asymmetry (reviewed in Babu and Roy 2013).

In chick and mouse embryos, Nodal starts to be expressed throughout the node, a morphologically distinct group of cells located at the anterior tip of the primitive streak. Following that, Nodal expression becomes restricted to the left side of the node (Levin et al., 1995; Collignon et al., 1996). The asymmetrical expression of Nodal then spreads to the lateral plate mesoderm (LPM), where Nodal promotes its own expression, as well as that of other asymmetrically expressed genes including Lefty2 and Pitx2. Biochemical studies have found that Lefty2 is an antagonist of Nodal. Lefty2 competitively binds to Nodal receptors and limits the influence of Nodal activity on the left LPM (reviewed in Hamada 2008). By contrast, the transcriptional factor Pitx2 is an effector of Nodal signalling (Hamada et al., 2002). Pitx2 regulates the subsequent asymmetric events by controlling the expression of the genes important for left-sided morphogenesis (Fig. 1.9E).

How does the asymmetric expression of Nodal become established in the first place? Studies in the past discovered that motile cilia on the mouse node produce a leftward flow of extraembryonic fluid triggering the onset of LR asymmetric gene expression (Nonaka et al., 1998; Hirokawa et al., 2006). Long before the discovery of the role of node in LR asymmetry, it was reported that each cell of the node has a monocilium projecting from its outward facing surface (Fig. 1.9C, D) (Jurand, 1974). The connection between LR asymmetry and cilia was established from the studies of human patients with rare genetic condition, Kartagener
**Figure 1.9. Nodal pathway activity in the establishment of L–R asymmetry.** (A, B) Whole mount in situ hybridisation of E8.5 wild type mouse embryos showing the expression of Nodal (A, C–E) and Lefty (B) transcripts. *Lefty1* and *Lefty2* are mainly localised to the midline and the left lateral plate, respectively (B). (C, D) Scanning electron microscope images of the node. Each node cell has a monocilium that is shown at high magnification (C). Ventral view of the node in lower magnification (D). Antero-posterior (AP) and Left-Right (LR) orientations are shown. The black arrow indicates the leftward flow. (E) A simplified diagram showing asymmetric expression of Nodal in the node, and the main elements of asymmetric Nodal signalling in the left LPM. A-D adapted from (Hamada, 2008) and E from (Babu et al., 2013).

syndrome (KS) (Afzelius, 1976). Phenotypic features of this disorder include respiratory dysfunction together with *situs inversus*, wherein the major visceral organs are reversed from their normal positions. Subsequent studies identified the causative mutations in genes encoding kinesin proteins.
Kinesins are required for cilia assembly though intraflagellar transport. Mouse knockouts for kinesin genes Kif3a and Kif3b fail to assemble cilia and develop LR defects, resembling patients with KS (Nonaka et al., 1998; Takeda et al., 1999). Direct visualisation of the node in wild type embryos demonstrates that mouse nodal cilia are motile and beat in a clockwise rotary pattern to produce a leftward flow of extraembryonic fluid; by contrast, Kif mutants lose both cilia and directional fluid flow (Nonaka et al., 1998; Okada et al., 1999; Takeda et al., 1999). The importance of cilia-driven nodal flow and their role in LR asymmetry was independently demonstrated in another mouse mutant named inversus viscerus (iv) (Supp et al., 1997). The iv gene encodes left-right dynein, a motor protein that is required for the motility of node cilia. Dynein deficiency has no effect on cilia specification, but makes cilia immotile, resulting in randomised LR asymmetry (Supp et al., 1997; Okada et al., 1999).

After the discovery of nodal flow, two models have been proposed to explain how it functions in establishing LR asymmetry. The ‘morphogen model’ proposes that nodal flow, generated by directed beating of the nodal monocilia, results in the left-sided accumulation of a morphogen (Fig. 1.10a). The asymmetrical distribution of the morphogen then triggers signalling cascades.
that settle the asymmetry in the developing embryo. One of the important questions is the identity of the morphogen itself (reviewed in Babu and Roy 2013). An important clue came from the work of Tanaka and colleagues (Tanaka et al., 2005). This group noticed flowing material, termed nodal vesicle parcels (NVP), inside the node cavity. Authors identified the NVP components, Shh and Retinoic acid (RA), which were released into the nodal flow in a FGF-dependent manner. Despite these findings, the morphogen hypothesis has not been further supported. In fact, genetic analysis of Shh and RA signalling cascades do not corroborate their roles as nodal morphogens (Zhang et al., 2001; Vilhais-Neto et al., 2010).

The two-cilia model proposes that the beating of motile cilia at the centre of the node generates a leftward fluid flow, which is detected by immotile cilia located on the perinodal crown cells (Fig. 1.10b) (reviewed in Babu and Roy 2013). This model was supported by genetic studies on Pdk2 mutant mice. Pdk2 protein forms a Ca$^{2+}$ ion channel essential for kidney tubular transport, and implicated in the causation of polycystic kidney disease in man (Harris et al., 2009). Pdk2-deficient mice exhibit classical LR asymmetry defects, therefore implicating Ca$^{2+}$ signalling in the establishment of LR asymmetry (Pennekamp et al., 2002). Interestingly, Pdk2 is expressed in motile cilia of node cells and in the immotile cilia of perinodal cells. McGrath and colleagues observed an increased influx of Ca$^{2+}$ ions on the left side of the node and this asymmetrical spike was randomised in the iv mutants and was not detected in the Pdk2-deficient mice (McGrath et al., 2003). In summary, nodal flow produced by the centrally located motile cilia is sensed by the peripheral immotile cilia via Pdk2. This results in the influx of Ca$^{2+}$ signalling on the left, which then induces asymmetrical nodal expression and the downstream events of LR asymmetry specification.

In addition to cilia motility, normal nodal flow is dependent on the precise position of cilia on node cells. Several studies revealed that the cilia are posteriorly tilted, allowing them to produce an effective stroke towards the left side of the node (Cartwright et al., 2004; Nonaka et al., 2005). The polarization of cilia position in the epithelium is a type of planar polarity controlled by the PCP signalling cascade (described in Section 1.2). The connection between PCP and cilia position was first described in the context of hair cells in the cochlea. In these cells, the position of immotile cilia is dependent on PCP (Jones et al., 2008). Studies in lower vertebrates demonstrated the requirement of PCP pathway for polarized orientation of the basal bodies. However, mouse PCP mutants, including Vangl2, Scrib, Celsr1 and Fz3/6 do not display obvious defects in the L-R asymmetry (Curtin et al., 2003; Montcouquiol et al., 2003; Murdoch et al., 2003; Lee et al., 2008). Therefore, the signals that control cilia position in mammalian embryos remain to be determined.
1.6 Thesis hypothesis, aims and objectives

**Chapter 3**
The aim of Chapter 3 was to determine the molecular composition of the ECM (matrisome) and the ECM receptors by RNA-seq analysis of the closure 1 region. In particular, the goal was to define the range of proteoglycan genes that are transcribed at the stage of neural tube closure initiation. This study provided a comprehensive list of proteoglycan and GAG biosynthetic/turnover genes that are likely to play a role during mouse primary neurulation. Moreover, it gave indications of the GAG chains that are synthesised at the initial site of mouse neural tube closure, and allowed the mRNA tissue source and the final destination of CS and HS chains, selected proteoglycans, and other ECM molecules to be determined. *In situ* hybridisation and immunofluorescence then revealed three sites of Vangl2 expression at the closure 1 region: neural plate, somites and surface ectoderm.

**Chapter 4**
Chapter 4 aimed to test the following hypothesis:

- Proteoglycans are required for initiation of neural tube closure in mammals.
- GAG chains interact with the PCP pathway, in particular Vangl2, at the initial site of closure.

The specific objective was to evaluate the potential importance of HS and CS chains in closure 1 by exposing *Vangl2*\(^{Lp/+}\) embryos in culture to chlorate treatment, or to GAG digestion by heparitinase or chondroitinase, prior to the initiation of NT closure. The effect of the chlorate treatment was independently tested in a targeted transgenic line *Vangl2*\(^{flox/−}\). The specificity of the treatments was verified by immunofluorescence analysis and a rescue experiment. Functional studies demonstrated that impaired sulfation of GAG chains worsens the phenotype of PCP mutant *loop tail* (*Vangl2*\(^{Lp}\)) embryos, which are predisposed to neural tube defects. Exposure of *Lp/+* embryos to chlorate during *ex vivo* whole embryo culture prevented NT closure, converting *Lp/+* to the mutant *Lp/Lp* pathophenotype. The next objective was to examine the morphological origin of the defect by analysing morphological parameters of the neural plate during the onset of NT closure. This study showed that loss of GAG sulfation results in a change of neural plate morphology in both *+/+* and *Lp/+* embryos.

**Chapter 5**
Chapter 5 aimed to investigate two questions:

- Morphogenetic, cellular and molecular basis of Vangl2-proteoglycan interaction
In this study, it was hypothesised that proteoglycans and GAGs play a role in mouse convergent extension. The hypothesis was tested by labelling of the node and midline of +/+ and Lp/+ embryos prior to closure 1. Node labelling was followed by immediate exposure to chlorate. This study revealed that Lp/+ embryos undergo normal midline extension of DiO-labelled node-derived cells, irrespective of whether they were treated with chlorate. Other potential mechanisms of Vangl2-PG interaction were also investigated in this chapter: cytoskeleton, somite morphology and Fgfr1 signalling.

Several studies demonstrated the role of proteoglycans and GAG chains in L-R asymmetry specification of lower and higher vertebrates. This Chapter aimed to examine the expression patterns of CS-E chains and Smc3 protein by whole-mount immunofluorescence during early mouse embryogenesis. This work revealed the presence of Smc3 and CS-E in the motile and perinodal ciliary axoneme suggesting a potential role of CS-E and Smc3 in the specification of the L-R axis.
2. Materials and Methods

2.1 Mouse strains and genotyping

Generation and maintenance of all mice colonies used in this project was performed by Dawn Savery.

2.1.1 Vangl2lop

Loop-tail mice (Jackson Laboratories, U.S.) were maintained as a heterozygous (Vangl2lop/+ ) colony. Two Loop tail (Jackson Laboratories, U.S.) colonies were kept on the following backgrounds: CBA/Ca (Harlan Laboratories, UK) and C3H/HeH background (MRC Harwell, UK). Both colonies were maintained in heterozygous state (Vangl2lop/+).

2.1.2 Vangl2flox

Vangl2flox/flox mice (C57BL/6J background) were a gift from Prof. Deborah Henderson (Newcastle University). To generate mice for experimental crosses, Vangl2flox/flox were crossed first with β-actincre/+ mice. This produced Vangl2flox/- ; β-actincre/+ , which were backcrossed to Vangl2flox/flox to remove residual Cre. The colony was maintained by crossing Vangl2flox/flox to Vangl2flox/-. 

2.1.3 β-actincre

β-actincre mice were supplied by J.P. Martinez-Barbera and were maintained as a heterozygous colony: β-actincre/+ on a C57BL/6J background.

2.1.4 Wild type

CD1 random-bred, wild-type mice (Charles River Laboratories International, Inc.) were maintained by the staff at the UCL Institute of Child Health animal facility.

BALB/c wild type mice (Jackson Laboratories, U.S.) were maintained by Dawn Savery.

2.1.5 Genotyping

Genotyping of mice and embryos was performed by polymerase chain reaction (PCR) amplification of DNA extracted from ear clips and yolk sacs respectively. The samples were collected into sterile 0.2 ml tubes; yolk sacs were stored at -20 °C prior to genotyping.

2.1.5.1 DNA extraction
DNA extraction for *Vangl2<sup>lp</sup>* and *Cre* PCR was carried out using PCR lysis buffer (VWR). 20 µl of the buffer and 5 µl of proteinase K (Thermoscientific) was added to the tube with yolk sac and the mix was incubated for 3 hrs at 56 °C. Then, the enzyme was heat inactivated at 85 °C for 45 min. The tubes were briefly centrifuged and stored at 4 °C.

DNA extraction for *Vangl2<sup>flox</sup>* PCR was performed using GeneJet Genomic DNA purification kit (K0721, Thermoscientific). Yolk sacs were lysed with 20 µl digestion solution and 5 µl of proteinase K and the mix was incubated for 3 hrs at 56 °C. 180 µl of digestion solution and 20 µl of proteinase K were used to lyse ear clips. After the lysis, the DNA was purified according to manufacturer’s instructions. DNA was eluted in 50 µl of elution buffer and stored at 4 °C.

### 2.1.5.2 *Vangl2* genotyping assays

*Vangl2* genotyping assays were carried out by PCR amplification of the polymorphic *Crp* microsatellite region, that is closely linked to *Lp* on mouse chromosome (Copp *et al.*, 1994b). The PCR reaction mixture and running conditions are provided in Table 2.1 and Table 2.2. On the CBA, BALB/c and C3H backgrounds the *Vangl2<sup>+</sup>* and *Vangl2<sup>lp</sup>* alleles co-segregated with 150 and 140 base pair (bp) fragments, respectively.

For *Vangl2<sup>flox</sup>* genotyping, two PCRs were carried out: 1st to detect the wild type (+) and floxed alleles and 2nd to detect the deleted (-) allele. The PCR reaction mixes and running conditions are provided in Table 2.2 and Table 2.3. PCR products have the following bp lengths: 701 (floxed), 560 (wild type) and 203 (deleted).
<table>
<thead>
<tr>
<th>Reaction component</th>
<th>Volume (µl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10x PCR buffer (Invitrogen)</td>
<td>5</td>
</tr>
<tr>
<td>50 mM MgCl$_2$ (Invitrogen)</td>
<td>1.5</td>
</tr>
<tr>
<td>2 mM dNTPs (Promega)</td>
<td>5</td>
</tr>
<tr>
<td>40 µM Primer mix (Sigma)</td>
<td>0.3</td>
</tr>
<tr>
<td>5 U/µl Taq DNA polymerase (Invitrogen)</td>
<td>0.25</td>
</tr>
<tr>
<td>Genomic DNA</td>
<td>2</td>
</tr>
<tr>
<td>H$_2$O (Sigma)</td>
<td>35.95</td>
</tr>
<tr>
<td><strong>Total volume</strong></td>
<td><strong>50</strong></td>
</tr>
</tbody>
</table>

Primer sequences (5'-3'): Forward: GAGGGAGAAGAATTATGTCTG  
Reverse: AGAATCTGACTTACCAGTG

Table 2.1. PCR reaction mixes for Vangl2$^{Lp}$ genotyping

<table>
<thead>
<tr>
<th>Step</th>
<th>Vangl2$^{Lp}$</th>
<th>Vangl2$^+$ / Vangl2$^{flox}$/Vangl2$^{-}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heating</td>
<td>95 °C, 2 min</td>
<td>95 °C, 2 min</td>
</tr>
<tr>
<td>Denaturation</td>
<td>94 °C, 1 min</td>
<td>95 °C, 30 sec</td>
</tr>
<tr>
<td>Annealing</td>
<td>58 °C, 1 min</td>
<td>67.5 °C, 30 sec</td>
</tr>
<tr>
<td>Extension</td>
<td>72 °C, 1 min</td>
<td>72 °C, 30 sec</td>
</tr>
<tr>
<td>Final extension</td>
<td>72 °C, 10 min</td>
<td>72 °C, 10 min</td>
</tr>
</tbody>
</table>

Table 2.2. PCR running conditions for Vangl2 genotyping

For Lp PCRs, steps 2-4 were repeated for 35 cycles. For wild type/flox and deleted PCRs, steps 2-4 were repeated for 16 cycles, with the annealing temperature decreasing by 0.5 °C per cycle until it reached 60 °C. Steps 2-4 were then repeated for a further 19 cycles with the annealing temperature at 60 °C.
### Table 2.3 PCR reaction mixes for \textit{Vangl2} $^+$, \textit{Vangl2} $^{flox}$ and \textit{Vangl2} $^-$ genotyping

<table>
<thead>
<tr>
<th>Reaction component</th>
<th>Volume (µl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5x GoTaq Flexi buffer (Promega)</td>
<td>2</td>
</tr>
<tr>
<td>25 mM MgCl$_2$ (Promega)</td>
<td>0.8</td>
</tr>
<tr>
<td>2 mM dNTPs (Promega)</td>
<td>2</td>
</tr>
<tr>
<td>10 µM F primer (wild type/flox or deleted)</td>
<td>1</td>
</tr>
<tr>
<td>10 µM common Reverse primer</td>
<td>1</td>
</tr>
<tr>
<td>5 U/µl GoTaq Flexi DNA polymerase (Promega)</td>
<td>0.25</td>
</tr>
<tr>
<td>Genomic DNA</td>
<td>2</td>
</tr>
<tr>
<td>H$_2$O (Sigma)</td>
<td>8.95</td>
</tr>
<tr>
<td><strong>Total volume</strong></td>
<td><strong>18</strong></td>
</tr>
</tbody>
</table>

Primer sequences (5'-3'):  
- Wt/flox forward: CCGCTGGCTTCTGCTGCTG  
- Del forward: TTGACCTCAGTGGACCGCCC  
- Common reverse: TCCTCGCCATCCCACCCCTCG

### 2.1.5.3 Cre genotyping assays

Genotyping of \textit{Cre} recombinase was performed by PCR amplification of a 500 bp fragment. The PCR reaction mixture was as shown in Table 2.1 and running conditions are provided in Table 2.4. Positive and negative control tubes were included for each batch of samples for \textit{Vangl2} and \textit{Cre} PCR.

Primer sequences (5'-3'):  
- \textit{Cre} F: CCCTGATCCTGGCAATTTCGGC  
- \textit{Cre} R: GATGCAACGAGTGATGAGGTTCGC

<table>
<thead>
<tr>
<th>Step</th>
<th>Condition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heating</td>
<td>95 °C, 2 min</td>
</tr>
<tr>
<td>Denaturation</td>
<td>94 °C, 30 sec</td>
</tr>
<tr>
<td>Annealing</td>
<td>63 °C, 30 sec</td>
</tr>
<tr>
<td>Extension</td>
<td>70 °C, 45 sec</td>
</tr>
<tr>
<td>Final extension</td>
<td>72 °C, 5 min</td>
</tr>
</tbody>
</table>

**Table 2.4.** PCR running conditions for genotyping of \textit{Cre} recombinase
2.1.5.4 Agarose gel electrophoresis

PCR products were separated by agarose gel electrophoresis and analysed by comparison to appropriate molecular weight markers using Bioline Hyperladders (ladder V for 25-500 bp bands; ladder I for 200-10,000 bp bands). Gel concentrations were 1-5% weight/volume. Agarose was dissolved in TAE buffer (40 mM Tris acetate and 1 mM EDTA in water) with ethidium bromide dye added (6 µl of dye per 100 ml gel). Orange G (Sigma) solution was used as a loading buffer.

2.2 Mouse embryology

2.2.1 Embryo collection, dissection and storage

Noon of the day a vaginal plug was found was designated embryonic day (E)0.5. Pregnant females were killed by cervical dislocation, the abdomen was surgically opened and the uterus was collected into pre-warmed (37˚C) dissecting medium (Dulbecco’s Modified Eagle’s Medium containing 25 mM HEPES [DMEM, Invitrogen] supplemented with 10% heat-inactivated foetal bovine serum (FBS, Invitrogen).

The uterus was taken to a stereo-microscope (Zeiss SV6 or SV11) for dissection. Embryos were dissected along the mesometrial side using forceps (number 5, Dumont). Each decidua was gently removed from the uterine wall. Then, each decidua was opened and dissected away. Next, the trophoblast layer and underlying Reichert’s membrane were gently peeled away, exposing the embryo within its yolk sac. The ectoplacental cone and yolk sac were left intact if the embryos were used for a culture experiment. Alternatively, the yolk sac and amnion were removed, somites were counted and the dissected embryos were numbered for future reference. Embryos for whole mount in situ hybridisation were fixed in 4% paraformaldehyde in DEPC-H₂O overnight at 4°C, dehydrated to 100% methanol and stored at -20°C. Embryos for immunohistochemistry on cryosections were fixed in 4% PFA in PBS at RT or in 100% methanol or acetone on ice for 1 h. Methanol and acetone fixed embryos were subsequently rehydrated to PBS and stored at 4°C until embedding for cryosectioning. Yolk sacs were washed in cold PBS and stored at -20 °C for genotyping.

2.2.2 Preparation of rat serum for embryo culture

Blood was collected from adult male rats by staff at UCL Biological Services. Animals were anaesthetised using isofluorane, and anaesthesia was confirmed by pinching one of the paws.
The abdomen was surgically opened and, using a syringe, blood was withdrawn from the abdominal aorta until the animal had been exsanguinated. The blood was immediately centrifuged at room temperature (RT) for 5 min (10,000 x g) to separate the serum supernatant from the blood. Once the serum had coagulated, the clot was squeezed with flat forceps, the tubes were centrifuged at 4000 rpm for 5 min at 4°C and the serum was transferred to fresh tubes and centrifuged again to remove contaminating red blood cells. Any serum that remained pink in colour due to the presence of lysed red blood cells was discarded. The serum was pooled in fresh tubes, heat inactivated for 30 min at 56°C, chilled on ice, and aliquoted for storage at -20°C. Immediately prior to whole embryo culture, aliquots were thawed, warmed to 37°C and passed through a Millipore filter (0.45 μm pore size).

2.2.3 Whole embryo culture

After dissection for culture, embryos were transferred to fresh dissecting medium. Meanwhile, rat serum was defrosted and filtered into 30 ml plastic culture tubes (Nunc). The tubes were smeared with silicone grease (Borer Chemie) to create an airtight seal. Then, the rat serum was gassed with 5% O2 / 5% CO2 / 90% N2 for 1 minute and left to equilibrate for 15 min at 37°C in a roller culture incubator (B.T.C Engineering or New Brunswick Galaxy 170S). Embryos were transferred to the serum using a plastic Pasteur pipette (0.4 - 0.5 ml per embryo) and the serum was then re-gassed with 5% O2 / 5% CO2 / 90% N2 for 1 minute. For longer cultures serum was re-gassed every 8 - 12 hrs.

2.2.3.1 Assessment of embryos after the culture

At the end of the culture period, embryos were transferred into a petri dish in a small amount of the culture serum and rated according to yolk sac circulation/shape and heart beat (Table 2.5). Then, embryos were transferred to pre-warmed dissecting medium, dissected and yolk sacs were collected for genotyping. Somite number was counted to assess developmental progression. Embryos were examined to determine whether neural tube closure had been completed at the site of Closure 1 (Copp et al., 1994b). Embryos were imaged in PBS on a stereomicroscope (Leica MZ FLIII). The images were taken blind to genotype and treatment groups. Embryos with a high yolk sac score (2 or 3; Table 2.5) were processed for further analysis. Healthy looking young embryos with a good heart beat and a healthy yolk sac were included in the absence of apparent circulation (score of 1), as yolk sac circulation is usually not present at the earlier stages of development (6-8 somite stage).
<table>
<thead>
<tr>
<th>Rating</th>
<th>Heart beat yolk circulation (YS) and shape</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>Very good heartbeat, round YS with strong circulation</td>
</tr>
<tr>
<td>2</td>
<td>Good heartbeat, round YS with weak circulation</td>
</tr>
<tr>
<td>1</td>
<td>Weak heartbeat, round YS with no circulation</td>
</tr>
<tr>
<td>0</td>
<td>Dead embryo: no heartbeat, wrinkled YS with no circulation</td>
</tr>
</tbody>
</table>

Table 2.5. Scoring categories after embryo culture

2.2.3.2 Chlorate treatment

Inhibition of glycosaminoglycan sulfation was achieved by culturing E8.0-8.5 embryos in the presence of sodium chlorate (Sigma). Chlorate crystals were dissolved in sterile H₂O, aliquoted and stored at -20 °C. Aliquots were thawed before use and any unused solution was discarded. Embryo cultures were stabilized for 1 h, then sodium chlorate was added to a final concentration of 60, 30, 20, 10 or 5 mM (1% volume added) and the same volume of sterile water was added to control cultures. After completion of the dose-response series, 10 mM concentration of chlorate was used in subsequent experiments. Rescue experiments involved culturing embryos with 10 mM chlorate together with 10 mM exogenous sulfate (Sigma). The embryos were randomly distributed to different treatment groups and cultured for the same time length (20-24 hrs). After the culture, the embryos were harvested and processed as described in Section 2.2.2.1.

2.2.3.3 Chondroitinase ABC and Heparitinase III treatment

Enzymatic digestion of HS and CS chains was achieved by Heparitinase III (Hep.III, Sigma) and Chondroitinase ABC (Chr.ABC, Amsbio) treatment respectively. First, the enzymatic digestion was validated on tissue sections (see Section 2.6.2) and then it was applied in embryo cultures. The enzymes were defrosted on ice, pre-warmed at 37°C for 5 min and mixed with 0.1% of FastGreen (1:10 dilution). Immediately, Chondroitinase ABC (2U) or Heparitinase III (5U) or PBS was injected into amniotic cavity of E8.5 embryos. Embryos were held in place with forceps and a surgical thin glass micropipette (mouth controlled) was inserted into the amniotic cavity; one of the solutions (heparitinase III, chondroitinase ABC or buffer; approximately 0.2 µl of one the solutions per embryo) was slowly released into the cavity until the colour of the amniotic fluid was changed from transparent to light green. The injected embryos were randomly distributed into different treatment groups and cultured for 20-24 hrs. Embryos were
transferred into whole embryo culture and maintained for 20-24 hrs. The embryo health parameters and completion of closure 1 were assessed after the culture (see Section 2.2.2.1).

**2.2.3.4 DiO labelling of the node**

Carbocyanine dye used: SP-DiO (green) (D7778, Thermo Fisher). The dye was prepared by dissolving powder in 25 μl DMSO and stored as 5 μl aliquots at -20°C, wrapped in foil. The final working solution was prepared by adding 45 μl of 0.3 M sucrose (sterile-filtered), covering in foil and storage at +4°C.

DiO labelling of the node was done by Matteo Mole and Dorothee Mugele. Embryos dissected for culture (see Section 2.2.1) were transferred to a new dish with fresh dissecting medium. Embryos were held in place with forceps and injections were made into the node by releasing the DiO while slowly withdrawing the needle. A number of embryos were collected at time 0 to verify the injection site. Time 0 embryos were examined under the fluorescence stereomicroscope (Leica MZ FLIII) and successful injection appeared as one mark in the node region. The remaining injected embryos were randomly distributed into different treatment groups (see section 2.2.2.2) and cultured overnight for 20-24 hrs. At the end of the culture the embryos were assessed as described above (Section 2.2.2.1). Fluorescent images were taken on a stereomicroscope to analyse the extension of DiO labelled cells. The localization of DiO was evaluated by viewing the embryo from its ventral side. The embryos were flattened with forceps and then photographed. To further confirm the presence of the dye in the midline, a number of embryos (Time 0 and post culture) were embedded in cryo-blocks for transverse histological sectioning.

**2.2.3.5 Embryonic measurements**

Throughout this study, live embryos were imaged after culture and a number of morphometric measurements were made from photographs using Fiji software. Embryo length was measured as a distance from the forebrain to the tip of the tail, providing an indicator of axial elongation (Fig. 4.6). Distance of midline extension of DiO labelled cells was measured from the node injection site (caudal region) as far as the level of the heart (Fig. 5.1). The presence of the dye in head folds was considered as non-specific staining. Embryo width was measured at the level of 3rd-4th somites (dorsal view). This distance was taken between lateral somite borders, centred at midline (Fig. 5.2). All measurements were made blind to genotype and treatment group.
2.3 RNA-seq

Generation of the library and RNA-sequencing was performed at UCL Genomics. The protocol was provided by staff from UCL Genomics.

2.3.1 Tissue collection for RNA-seq, RNA extraction and quality control

Wild type (BALB/c background) mouse embryos were collected at E8.5 and dissected as described in Section 2.2.1. Extraembryonic tissues were removed. Embryos at the 4 - 6 somite stage (prior to closure 1) were selected for RNA collection; the region (‘cut’) between somite 1 and last somite was isolated; cranial and caudal regions were avoided for tissue collection. ‘Cuts’ were washed in diethylpyrocarbonate treated phosphate buffered saline (DEPC-PBS), frozen in dry ice and stored at -80°C. RNA was extracted using the RNeasy minikit (Qiagen) according to manufacturer’s instruction. The quality control for RNA integrity was performed on the Agilent Bioanalyser. Two samples were used for RNA-seq: sample 1 containing 6 ‘cuts’ (ON1, 75ng/µl) and sample 5 with 3 ‘cuts’ (ONS, 32ng/µl).

2.3.2 Generation of library and sequencing

Sequencing methods were provided by UCL Genomics staff. Samples were processed using Illumina’s TruSeq Stranded mRNA LT sample preparation kit (p/n RS-122-2101) according to manufacturer’s instructions. Deviations from the protocol were as follows: 250 ng total RNA was used as starting material, fragmentation was carried out for 10 mins instead of 8 mins, and 14 cycles of PCR were used.

Briefly, mRNA was isolated from total RNA using Oligo dT beads to pull down Poly-Adenylated transcripts. The purified mRNA was fragmented using chemical fragmentation (heat and divalent metal cation) and primed with random hexamers. Strand-specific first strand cDNA was generated using Reverse Transcriptase and Actinomycin D. This allows RNA dependent synthesis while preventing spurious DNA-dependent synthesis. The second cDNA strand was synthesised using dUTP instead of dTTP, to maintain strand specificity. The cDNA is then “A-tailed” at the 3’ end to prevent self-ligation during the addition of the Adaptors (Adaptor have a complementary “T-tail”). Indexing Adaptors are ligated to the A-Tailed cDNA. The adaptors contain sequences that allow the libraries to be amplified by PCR, bind to the flow cell and be uniquely identified by way of a 6 bp Index sequence. Finally, PCR is carried out to amplify only those cDNA fragments that have adaptors bound to both ends.

Libraries to be multiplexed in the same run are pooled in equimolar quantities, calculated from qPCR and Bioanalyser fragment analysis. Samples were sequenced on the NextSeq 500
instrument (Illumina, San Diego, US) using a 43 bp paired end run. Samples were batched (multiplexed) in a single run, resulting in 16 million reads per sample. Transcript abundance was obtained in Reads Per Kilobase of exon per Million fragments mapped (RPKM). The RPKM value is the normalised expression for each transcript as determined by normalizing for RNA length and for the total read number in the measurement (Mortazavi et al., 2008).

2.3.3 Bioinformatics analysis

DESeq2 algorithm was used for normalisation and quantification (in house pipeline by Dr Vincent Plagnol, UCL). Data were demultiplexed and converted to fastq files using Illumina’s bcl2fastq conversion software v2.16. Fastq files were aligned to the Mouse mm10 (Refseq) genome using the Tophat app in Illumina’s online tool called Basespace. The rest of the analysis were carried out using GeneSpring software. The figures were produced using normalised read counts in log2 scale.

The first step of data analysis was filtering genes based on expression level. The positive (genes known to be expressed at the closure 1 region) and negative (genes not normally expressed at the closure 1 region) controls were identified in order to filter out genes with expression values that were ‘below background’. The negative control set allowed a minimum threshold for signal intensity (number of counts) to be defined. Only transcripts with counts above the threshold were retained in the analysis. This difference in signal distribution (expression value) between positive and negative control group was used to perform a ‘cut-off’ – removal of genes with low count reads. Moreover, the positive control group genes were expected to be represented above threshold in the RNAseq data, and this was indeed confirmed.

‘Genes after cut-off’ were analysed for the presence of the following gene sets:

- Murine matrisome dataset (updated 2014) (Naba et al., 2016)
- ECM receptors, and proteoglycans not listed in the matrisome (bamacan and phosphocan)
- CS/DS biosynthetic genes that are involved in biosynthesis of CS/DS chains (Reactome database) (http://www.reactome.org/content/detail/R-MMU-2022870)
- HS biosynthetic genes and sulfatases that are involved in biosynthesis and processing of HS chains (Reactome database) (http://www.reactome.org/content/detail/R-MMU-2022928)
The above gene sets were imported into GeneSpring and the corresponding genes were extracted from RNA-seq data. Figures were generated by GeneSpring GX software.

**2.3.4 Analysis of the transcriptome of mid-gestation mouse embryos (MGME) from Werber 2014 study.**

The mapping results, read counts and coverage were extracted via the UCSC genome browser ([http://overview.molgen.mpg.de/ucsc.html](http://overview.molgen.mpg.de/ucsc.html)) (Werber et al., 2014). The tissue-specific RNA-seq tracks were converted into log2 scale; read counts were reported per tissue type (PSC: presumptive spinal cord; CEM: caudal end mesoderm; somites; heart and head). The gene expression data were presented in heatmaps and tables. The heatmaps were generated in Fiji using the conversion of read counts into images; red-to-blue LUT was applied to visualise the data with red corresponding to highly expressed and blue to lowly expressed genes.

**2.4 Histology**

**2.4.1 Cryo-embedding**

After fixation, embryos were washed twice for 10 min in PBS on ice. PBS was replaced with sucrose solution (20% sucrose in PBS) and incubated for at least 1 h on ice. Next, the sucrose solution was replaced with melted gelatine (7.5% gelatine in sucrose solution) and the embryos were incubated in a water bath at 37 °C for 30 min. Following that the embryos were placed into a small Petri dish on a pre-set gelatine layer. Fresh melted gelatine was added to the dish and the embryos were oriented for transverse or sagittal sections. The dish was left on ice for 30 min allowing the gelatine to set. The gelatine block containing the embryo was cut out and glued onto a wooden disk. The disk with attached block was drawn into isopentane (chilled down to -65 °C) for a few sec and then was transferred to -80 °C.

**2.4.2 Cryosectioning**

Sections were cut on a cryostat (Leica) at a thickness of 10 µm, placed on SuperFrost Plus slides and stored at -20 °C.

**2.5 In situ hybridization**

Whole mount *in situ* hybridization (WISH) was used to analyse the expression of proteoglycans and Vangl2 at closure 1. Standard RNA probe preparation and WISH protocols were adapted from the thesis of Dr Yun Jin Pai, a former PhD student in the lab.
2.5.1 RNA isolation and cDNA synthesis

Wild type BALB/c E10 embryonic tissue fragments were collected and stored at -80 °C until processing. RNA was extracted using TRizol reagent (Invitrogen). Samples were homogenised in TRizol (500 µl) and incubated for 5 min at RT. Chloroform was added to each sample (100 µl) and the tubes were shaken for 15 sec and incubated for 3 min at RT. The samples were centrifuged at 12,000 x g for 15 min at 4 °C and the upper phase was transferred to a new tube. RNA was precipitated with isopropyl alcohol (250 µl) for 10 min at RT and centrifuged at 12,000 x g for 10 min at RT at 4 °C. The supernatant was discarded and the pellet was washed with 75% ethanol-DEPC-H2O (500 µl), vortexed and centrifuged again at 12,000 x g for 5 min at 4 °C. The ethanol was discarded, and the pellet left to air-dry for 10 min. The pellet was re-suspended in DEPC-H2O (10 µl). To remove contaminating DNA, the RNA sample was treated with DNase treatment and removal kit (Ambion). 2.5 µl of 10X DNase buffer, 1 µl RNase inhibitor and 0.5 µl DNAse I were added to the samples and the mix was incubated for 30 min at 37 °C. DNase Inactivation Reagent (2.5 µl) was added and the samples were incubated for 2 min at RT and spun at 10,000 rpm for 2 min. The supernatant was collected into a new tube and stored at -80°C. cDNA was generated using the Superscript VILO cDNA synthesis Kit (Invitrogen). 5X VILO™ Reaction Mix (4 µl), 10X SuperScript™ Enzyme Mix (2 µl) were added to 2 µg of RNA. DEPC-H2O was used to top up volume to 20 µl. The mixture was incubated for 10 min at 25 °C, then for an hour at 42 °C and finally for 5 min at 85 °C. cDNA was stored at -20 °C and used for in situ probe synthesis. The quality of cDNA was analysed by amplification of the house-keeping gene GAPDH using standard PCR. The PCR product was run on an agarose gel to confirm the correct size of the GAPDH band (177 bp).

2.5.2 RNA probe preparation for in situ hybridization

2.5.2.1 Probe design

Probes were designed to recognise eight proteoglycan transcripts. The primers and corresponding Ensembl gene IDs are listed in Table 2.7. The probes were amplified by RT-PCR using cDNA prepared from wild type embryos. Vangl2 probe was provided by Prof Deborah Henderson (Doudney et al., 2005). The PCR conditions are: 35 cycles of 1 minute at 94 °C, 1 minute at 58 °C, 1 minute at 72°C. The reaction mixture is listed in Table 2.6 (AccuPrime™ Taq DNA Polymerase, Invitrogen). The PCR product was purified using QIAquick PCR Purification Kit (Qiagen) according to manufacturer’s instructions.
<table>
<thead>
<tr>
<th>Reagent</th>
<th>Volume per reaction (µl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10X AccuPrime PCR buffer</td>
<td>5</td>
</tr>
<tr>
<td>Forward primer (10 µM)</td>
<td>1</td>
</tr>
<tr>
<td>Reverse primer (10 µM)</td>
<td>1</td>
</tr>
<tr>
<td>cDNA sample</td>
<td>1</td>
</tr>
<tr>
<td>High Fidelity DNA polymerase (5 U/µl)</td>
<td>0.2</td>
</tr>
<tr>
<td>Distilled, de-ionized water</td>
<td>41.8</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>50</strong></td>
</tr>
</tbody>
</table>

Table 2.6. RT-PCR reaction mix for amplification of proteoglycan cDNAs.
<table>
<thead>
<tr>
<th>Probe</th>
<th>Ensembl gene ID</th>
<th>Forward primer</th>
<th>Reverse primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vcan</td>
<td>ENSMUSG00000021614</td>
<td>5'-GGATGCTGCTGAAAGGGAGT-3'</td>
<td>5'-GCACCGGATAGTTGGAAAGGT-3'</td>
</tr>
<tr>
<td>Smc3</td>
<td>ENSMUSG00000024974</td>
<td>5'-AAGCGGCAAGGAAATTGGA-3'</td>
<td>5'-CATGCACGGCTAGCTCCATA-3'</td>
</tr>
<tr>
<td>Hspg2</td>
<td>ENSMUSG00000028763</td>
<td>5'-GAGTATCGCTGTGACCGGAG-3'</td>
<td>5'-CTCCAGGCTGCTTGACAGAA-3'</td>
</tr>
<tr>
<td>Agrn</td>
<td>ENSMUSG00000041936</td>
<td>5'-CTAGGGGAATCTCCGGTCCC-3'</td>
<td>5'-TGCAACGCGGTTTCTCCTCCTC-3'</td>
</tr>
<tr>
<td>Gpc1</td>
<td>ENSMUSG00000034220</td>
<td>5'-GACTCCCTACCAGACCCCAT-3'</td>
<td>5'-AAAGTTTCCACGACCACCCA-3'</td>
</tr>
<tr>
<td>Gpc3</td>
<td>ENSMUSG00000055653</td>
<td>5'-GCTGCTCGGCCTTTTCTA-3'</td>
<td>5'-TGCGCAGACAGATGGTTCTC-3'</td>
</tr>
<tr>
<td>Gpc6</td>
<td>ENSMUSG00000058571</td>
<td>5'-CCGGCTGGTCACAGACATCAA-3'</td>
<td>5'-CCGACGCTGCAGACAnna-3'</td>
</tr>
<tr>
<td>Sdc1</td>
<td>ENSMUSG00000020592</td>
<td>5'-GGGCAGCTAGTTTGGCAACTG-3'</td>
<td>5'-GCCACAGTCCTTCCAAAGTG-3'</td>
</tr>
</tbody>
</table>

Table 2.7. Primers for RNA probes
2.5.2.2 Ligation of the probe into the vector system

Insert sizes and restriction enzymes used for in situ hybridisation RNA probes are listed in Table 2.8. The PCR product containing the insert of interest was ligated into pGEM®-T Easy Vector System (Promega). The vector contains SP6 and T7 polymerase recognition sites. A ligation mixture containing 5 μl of 2X Rapid Ligation Buffer, 1 μl of pGEM-T Easy Vector, 3 μl of PCR product, and 1 μl of T4 DNA Ligase was incubated for an hour at RT. The amount of PCR product added to the mixture was calculated for optimal ligation efficiency with a 3:1 product / insert to vector ratio.

2.5.2.3 Transformation of competent cells

2 μl of ligation product was added to 50 μl of DH5α™ competent cells and incubated on ice for 20 min. Cells were then heat shocked for 45 sec at 42 °C, and incubated on ice for 2 min. 950 μl of Luria Broth Base (LB-Broth; Invitrogen) was then added to the tube and the mix was incubated for 90 min at 37 °C, shaking. At the end of this period, samples (100 μl) were plated onto an LB plate containing 50 μg/ml of ampicillin, 100 μM of isopropyl β-D-1-thiogalactopyranoside (IPTG), and 0.3% of X-gal (Promega), and incubated overnight (18 hrs) at 37 °C. The following day, white colonies were picked out and inoculated into 10 ml of LB-broth containing 50 μg/ml ampicillin and incubated overnight at 37 °C, shaking. The following day, the broth was centrifuged at 6000 x g for 15 min at 4 °C to obtain the pellet and it was ready for plasmid preparation.

2.5.2.4 Preparation of plasmid and transcription of DIG probe

Plasmid DNA was isolated using the QIAprep Spin Miniprep Kit (Qiagen) according to manufacturer’s instructions. The DNA concentration was obtained using the NanoDrop spectrophotometer. The orientation of the insert was obtained by PCR with probe primers together with T7 and SP6 primers. For example, using this method, the Vcan probe sequence was determined to be 5’ at SP6 end and 3’ at the T7 end, therefore, an antisense probe would be generated by a digestion at the SP6 promoter end followed by RNA synthesis using the T7 RNA polymerase, and a sense probe by digestion at the T7 promoter end followed by RNA synthesis using the SP6 polymerase. For further validation of the probe sequence, an aliquot of the plasmid DNA with insert was sent for sequencing at Source BioScience, UK. All plasmids were linearized, purified and orientation of the insert and corresponding restriction enzymes are listed in Table 2.8. A digoxigenin (DIG)-labelled, single-stranded RNA probe was prepared by transcribing the purified digest in the following mixture: 1 μg of digested plasmid DNA, 2 μl of DIG RNA labelling mix (Roche), 2 μl of transcription buffer (Roche), 0.5 μl of RNase Inhibitor
and 2 μl of the appropriate RNA polymerases. The final volume (20 μl) was obtained by adding DEPC-H$_2$O. The mixture was incubated for 2 hrs at 37 °C. The transcribed DIG probe was purified in a Chroma Spin-100 DEPC-H$_2$O column (Clontech); 40 μl formamide and 1 μl RNAse inhibitor was added to the 50 μl of DIG-labelled, single stranded RNA probe. Probes were stored at -20°C until use.

### 2.5.3 Whole mount in situ hybridization

Wild type embryos were used for whole mount in situ hybridization. The following developmental timepoints were investigated: 0 – 7 somite stage (prior to closure 1); 8-13 somite stage (after closure 1).

#### 2.5.3.1 Pre-treatment and hybridization

Embryos were first rehydrated by a series of washes in 75%, 50%, and 25% methanol/ PBT (PBS containing 0.1% Tween-20) and two subsequent washes in PBT alone. Then, the embryos were bleached with 6% hydrogen peroxide in PBT for 1 h on ice, treated with 5 μg/ml of proteinase K in PBT at RT for 1 min (for E8.5 embryos) or 2 min (for E9.0 embryos). The reaction was stopped by adding 2 mg/ml of glycine in PBT at RT. Before re-fixation, the embryos were washed in PBT for 10 min on ice. The embryos were then re-fixed in 0.2% gluteraldehyde prepared in 4% PFA for 20 min at RT. After fixation, the embryos were washed in PBT, transferred to 1 ml of pre-warmed pre-hybridisation buffer containing 50% formamide, 5X saline sodium citrate (SSC) at pH 4.5, 50 μg/ml yeast RNA, 1% SDS, and 50 μg/ml heparin in DEPC-treated water, and incubated at 70 °C for 2 hrs. 10 μl of each DIG-labelled RNA probe was added per 1 ml of pre-hybridisation buffer and left to incubate overnight at 70 °C.

#### 2.5.3.2 Post-hybridization and DIG antibody detection

Following hybridisation, the embryos were washed in pre-warmed formamide solutions: twice in Solution 1 (50% formamide, 5X SSC, and 1% SDS in DEPC-H$_2$O) for 30 min each at 70 °C, and then twice in Solution 2 (50% formamide, 2X SSC, and 1% SDS in DEPC-H$_2$O) for 30 min each at 65 °C. A TBST wash solution was prepared with 1% Tween-20 in Tris-buffered saline (TBS; made with 50 mM Tris-Cl at pH 7.6 and 150 mM NaCl) and approximately 0.2 g (per 500 ml of TBST solution) of tetramisole hydrochloride (Sigma-Aldrich). The embryos were washed in TBST and blocked with 10% heat-inactivated sheep serum (Invitrogen) in TBST for at least an hour at RT. The blocking solution was replaced with anti-DIG-AP antibody (Roche; 11 093 274 910) at 1:2,000 dilution prepared in TBST containing 1% sheep serum. The embryos were left to incubate on a shaker at 4°C overnight. The following day, the embryos were washed with five,
1 hr washes in TBST. After the last wash, the embryos were left washing in TBST overnight at 4°C, shaking.

2.5.3.3 Development of signal

An NTMT wash solution was prepared containing 100 mM NaCl, 100 mM Tris pH 9.5, 50 mM magnesium chloride (MgCl2), 1% Tween-20, and approximately 25 mg (per 50 ml of NTMT solution) of tetramisole hydrochloride, in distilled water. The colour developing solution was prepared with 4.5 μl/ml 4-nitroblue tetrazolium chloride, NBT (Roche) and 3.5 μl/ml 5-bromo-4-chloro-3-indoylphosphate, BCIP (Roche) in NTMT solution. The embryos were washed twice in NTMT for 10 min each and then incubated with the developing solution at room temperature in the dark. Signal development was stopped after sufficient staining was achieved (dependent on the probe) by making brief PBT washes. Sense and anti-sense probes were developed for the same time period. The embryos were photographed stored at 4 °C in PBT with sodium azide to prevent fungal growth. Prior to sectioning the embryos were re-fixed in 4% PFA overnight at 4°C.

2.5.3.4 Vibratome sectioning

The embryos were washed twice in PBS for 15 min to remove the fixative. Then, embryos were embedded in a gelatine/albumin mixture (0.45% gelatine, 27% albumin, 18% sucrose in PBS) overnight at 4 °C. The next day, 200 μl of embedding medium was pipetted into plastic moulds. 20 μl of gluteraldehyde (0.2%) was added to the medium in each mould and mixed in quickly. The embryos were transferred into the moulds and orientated as quickly as possible. The blocks were left to dry for 30 min, then cut out of the mould and incubated in PBS for at least 1 hr in 4°C. Vibratome sectioning was then performed on the blocks to obtain sections at a thickness of 40 μm. The sections were mounted in 50% glycerol (in distilled H2O) and coverslips sealed with clear nail varnish.
<table>
<thead>
<tr>
<th>Probe</th>
<th>Insert size, bp</th>
<th>Restriction enzyme and RNA polymerase for anti-sense probe</th>
<th>Restriction enzyme and RNA polymerase for sense probe</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vcan</td>
<td>448</td>
<td>SacII, SP6</td>
<td>Sall, T7</td>
</tr>
<tr>
<td>Smc3</td>
<td>499</td>
<td>PstI, T7</td>
<td>Sp6, SacII</td>
</tr>
<tr>
<td>Hspg2</td>
<td>451</td>
<td>SacII, SP6</td>
<td>PstI, T7</td>
</tr>
<tr>
<td>Agrn</td>
<td>428</td>
<td>SacII, Sp6</td>
<td>Sall, T7</td>
</tr>
<tr>
<td>Gpc1</td>
<td>496</td>
<td>SpeI, T7</td>
<td>SacII, SP6</td>
</tr>
<tr>
<td>Gpc3</td>
<td>482</td>
<td>Sall, T7</td>
<td>SacII, SP6</td>
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<tr>
<td>Gpc6</td>
<td>500</td>
<td>PstI, T7</td>
<td>SacII, SP6</td>
</tr>
<tr>
<td>Sdc1</td>
<td>494</td>
<td>SpeI, T7</td>
<td>Ncol, SP6</td>
</tr>
<tr>
<td>Vangl2</td>
<td>554</td>
<td>Apal, SP6</td>
<td>PstI, T7</td>
</tr>
</tbody>
</table>

Table 2.8. RNA probes for whole mount *in situ* hybridisation

2.6 Immunofluorescence

All protocols were performed on 10 μm frozen sections. Washing steps were performed by shaking at RT in plastic troughs. For defrosting, permeabilization, antigen blocking, antibody and DAPI incubation slides were placed horizontally in a humidified chamber protected from light. Slides with frozen sections were placed in a humidified chamber for 30 min. After defrosting, slides were incubated in PBS for 30 min at 37 °C and then washed in PBS for 5 min at RT. Subsequently, slides were blocked with blocking buffer (10% heat inactivated sheep serum [HISS], 0.1% Tween in PBS) for 1 h at RT (200 μl per slides + parafilm). Different blocking buffer was used for IHC of Vangl2 (5% normal goat serum [NGS], 0.1% Triton, 2% bovine serum albumin [BSA] in PBS). The blocking solution was replaced with corresponding primary antibody (see Table 2.9) diluted in blocking buffer and incubated overnight at 4 °C (200 μl per slide + parafilm). The following day, slides were washed three times in PBS and incubated in secondary antibodies (see Table 2.10) at 1:500 dilution in blocking buffer for 1 h at RT. Slides were washed three times in PBS and incubated with DAPI 1:10,000 for 3 min, washed 3 times with PBS and mounted in Mowiol. Imaging was by epifluorescence on an inverted LSM710 or LSM880 with Airyscan confocal system (Carl Zeiss Ltd, UK). Images were processed in Fiji (Image J) software.
2.6.1 Antibodies

The list of primary antibodies is presented in Table 2.9 and secondary antibodies in Table 2.10.

2.6.2 Validation of enzymes on tissue sections

Tissue sections were pre-treated with Heparitinase III (Hep.III) and Chondroitinase ABC (Chr.ABC) that specifically cleave HS or CS chains respectively. Enzymes were diluted in enzyme buffer to: 0.5 U/ml Hep.III (20 mM Tris-HCl, pH7.5, containing 0.1 mg/ml BSA and 4 mM CaCl₂); 0.2 U/ml Chr.ABC (0.1 M NH₄Ac, pH 8.0). The slides were defrosted, incubated in PBS at 37 °C, washed once in PBS at RT. Subsequently, slides were incubated with Hep.III or Chr.ABC at 37 °C for 1 hr (200 µl per slide + parafilm). Control slides were incubated with corresponding enzyme buffer. Slides were washed three times with PBS at RT, blocked with corresponding primary antibodies as described in Section 2.6. It was important to test the ability of enzymes to cleave the GAG chains on tissue sections prior to applying for embryo culture experiments (see Section 2.2.2.3).

2.6.3 Phalloidin staining

Phalloidin was used to stain F-actin. Tissue sections and whole embryos (PFA fixed only) were incubated with phalloidin (1:200, Alexa-Fluor-568–phalloidin, A12380, Life Technologies). Phalloidin was added together with secondary antibody (see Sections 2.6 and 2.7).

2.7 Immunofluorescence of whole embryos

Embryos were collected as described in Section 2.2.1. Embryos were washed in PBT (0.1% Triton in PBS) for 30 min at RT and blocked with blocking solution (5% BSA in PBT) overnight at 4 °C while rocking. Following day the embryos were incubated with primary antibody overnight at 4 °C with rocking. Blocking solution was used to dilute the primary and secondary antibodies to appropriate working concentrations (Table 2.9). Next day the embryos were washed three times in blocking solution for 1 h rocking at RT and incubated with secondary antibody for 2 hrs rocking at RT. Embryos were washed in blocking solution three times for 1 h rocking at RT, then incubated with DAPI 1:15,000 overnight rocking at 4 °C. After washing three times in PBS 30 min at RT, embryos were stored in 0.1% sodium azide in PBS at 4 °C until ready for imaging.
<table>
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<th>Supplier, catalogue number</th>
<th>Source</th>
<th>Fixation/ A/g retrieval</th>
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<th>2-ry A/b dilution for IHC</th>
<th>1-ry A/b dilution for WMI</th>
<th>2-ry A/b dilution for WMI</th>
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<td>1:100</td>
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<td>n/a</td>
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Table 2.9. Primary antibodies. Citric buffer (CB).
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**Table 2.10 Secondary antibodies**
2.8 Imaging and data analysis

Images were analysed using Fiji (Image J) software. Contrast and brightness corrections were adjusted across entire image. These adjustments were performed on all images immunofluorescent images (whole mount and sections). Where specified in the figure legends, images were deconvoluted using Huygens software package.

Isolation of surface ectoderm and apical surface of neural plate:

Images acquired by laser scanning microscopy and processed in the following steps:

1) For each z slice of a thresholded image each x column is scanned from top to bottom until the 1st positive pixel is reached (i.e the top surface), this is recorded to the result table as xy coordinates.

2) Move to the next x column and repeat the process until all x columns are scanned.

3) Any empty columns are skipped, so only xy coordinates of the upper surface of the image in each slice are recorded.

4) The resulting xy coordinates are used to draw a polyline of defined width (the depth of the surface of the tissue to be extracted) on a new image of the same dimensions as the original.

5) This is repeated for every z slice. The resulting image is used as a mask to extract just the surface of the original image.

2.9 Scanning electron microscopy

The protocol was adapted from Rolo et al (2016). Embryos were fixed at 4°C in 2% glutaraldehyde, 2% PFA in 0.1 M phosphate buffer (pH7.4), post-fixed in 1% OsO₄/ 1.5% K₂Fe(CN)₆ in 0.1 M phosphate buffer for 1.5 hours. After washes in distilled water, embryos were dehydrated to 100% ethanol, followed by 1 acetone wash. Embryos were critical point dried using CO₂ and mounted on aluminium stubs. After mounting samples were coated with a layer of Au/Pd (2 nm thick) using a Gatan ion beam coater and imaged with a JEOL 7401 FEGSEM by Dr Ana Rolo. Protrusions were categorised as: ruffles, ruffles and filopodia, as previously reported (Rolo et al. 2016).
2.10 Statistical analysis

Statistical analysis of embryo length was performed in OriginPro software (v. 2016), using the following linear regression (LR) tests: LR analysis against 0 for individual datasets; LR analysis with F-test to compare two datasets.

Statistical analysis of frequency of NTDs in cultured embryos were performed by Fisher exact test.

Statistical analysis of embryo width and midline extension in cultured embryos were performed in Graph Prism software (v. 6) using Two-way ANOVA test.

Statistical analysis of neural plate morphology was performed by mixed model analysis in SPSS (v.24) with four fixed effects (genotype, treatment, position and somite stage) that were calculated for each parameter (distance between NF tips, medial angle or elevation of NF) along the closure 1 region. The first order interactions between fixed effects were computed per parameter. When the fixed effects were significant overall, a post hoc Bonferroni correction was used to identify the individual sites at which the effect was significant (p-value < 0.05).
3. Analysis of matrisome, glycosaminoglycan chains and Vangl2 distribution during initiation of neural tube closure

3.1 Introduction

The extracellular matrix (ECM) is a meshwork of cross-linked proteins and carbohydrates that are critically important for cell proliferation, survival, differentiation and morphogenesis. The composition and organization of ECM components changes during development beginning at the earliest stages of embryogenesis. For example, assembly of the first basement membrane is initiated by laminins in the periimplantation period of mouse development and during gastrulation of fly and nematode with laminin-1 appearing to be essential for this process in mammals. The laminins self-assemble into heterodimers by binding directly to their cell-surface receptor integrins at the basal side of the cells (Li et al., 2003). In contrast, the different proteoglycans are localised on the opposite sides of the epithelial layers, with perlecan restricted to the basal surface and glypicans to the apical compartments of the epithelia (Brown, 2011). The spatiotemporal pattern of proteoglycan expression allows regulation of signalling on both basal and apical surfaces of epithelial cells.

Prior to the initiation of neural tube closure, the neural plate displays ‘horseshoe-type’ morphology and is flanked by somites and overlaid by surface ectoderm (SE) dorsally and visceral endoderm and notochordal plate ventrally (Fig. 3.1A). During the formation of neural plate the neuroepithelial cells polarize, with distinct basal and apical cell surfaces. The basement membrane is deposited and assembled at the basal side of the neuroepithelium creating a mechanical barrier between the neural plate and the mesoderm. Another basement membrane is formed between the basal side of SE and the mesoderm. A number of studies in chick and mouse embryos identified and mapped the main components of the basement membrane around the neural tube, including laminins, collagen IV, fibronectin and proteoglycans (Sternberg and Kimber 1986a; O’Shea and Liu 1987; Sternberg and Kimber 1986b; O’Shea 1987, Copp et al. 2011). Although the distribution of major basement membrane components and GAG chains during spinal neurulation have been studied in the past (Sternberg et al., 1986a, 1986b; O’Shea, 1987; O’Shea et al., 1987; Yip et al., 2002), the expression pattern of those molecules has not been examined specifically at the site of the initiation of neural tube closure. Preliminary studies in our lab revealed the presence of α1, α5, β1, and γ1 subunits of laminins in the matrix between the neural plate and mesoderm, while integrin α3 and α6 are present in the neural folds at the stage preceding neural tube closure (Andrew Copp and Patricia Ybot-Gonzalez, unpublished). Interestingly, integrin α3 α6 
mouse double mutants can develop CRN similarly to that of PCP mutants suggesting that the laminin network is essential for initiation of NT closure in mouse (De Arcangelis et al., 1999).

Richard Hynes and colleagues used bioinformatics and proteomics approaches to characterise genes encoding the mammalian ‘Matrisome’ - the ensemble of normal and tumour extracellular matrix and ECM-associated proteins. 1110 matrisome genes have been identified in the mouse genome (Naba et al., 2012). The murine matrisome is composed of two divisions: the core matrisome, represented by 274 genes, and 778 matrisome associated genes. Core matrisome includes 194 ECM glycoproteins (fibronectin, laminins etc.), 44 collagens and 36 proteoglycans; the matrisome associated division is composed of 165 ECM-affiliated genes, 367 secreted factors and 304 ECM regulators (Hynes et al., 2012; Naba et al., 2012). The generated dataset allows studies of the ECM composition in any given tissue.

Glycosaminoglycans (GAGs) are linear unbranched polyanionic molecules of repeating disaccharides that are covalently linked to core proteins to form proteoglycans (review in Chapter 1). The expression of GAGs is dynamically regulated throughout development (Lindahl, 1999; Oohira et al., 2000; Caterson, 2012). A number of studies showed that the main sulfated GAGs synthesised during primary neurulation in the mammalian embryo are heparan and chondroitin sulfates, while the main non-sulfated GAG synthesised is hyaluronan (Solursh et al., 1977; Copp et al., 1988; Yip et al., 2002). Those studies suggested a potential role of GAGs in regulating neural tube closure.

This chapter examines the molecular composition of the ECM proteins, termed the matrisome, and their receptors and GAG chains at the initial site of mouse neural tube closure (Fig. 3.1B). It provides a baseline for the subsequent studies of the role of ECM/GAGs during initiation of neural tube closure and proteoglycans/Vangl2 interaction at closure 1 (Chapter 4 and 5). The main components of the ECM expressed during primary neurulation are studied by RNA-seq analysis of the closure 1 region of wild type mouse embryos at E8.5. In situ hybridization is used to study the tissue source of expression of proteoglycans and Vangl2. The localisation of the proteins is analysed by immunofluorescence on both whole-mounts and sections. The distribution of chondroitin and heparan sulfate chains is studied using monoclonal antibodies CS-56 and 10E4 respectively.

A large body of evidence suggested that genetic defects in planar cell polarity pathway (PCP) contribute to the etiology of neural tube defects (reviewed in Chapter 1). Our lab and others found that Vangl2, one of the key proteins in PCP signalling, is mutated in the loop-tail mouse (Lp) and the Vangl2Lp/Lp mutants fail to initiate closure 1 (Murdoch et al., 2010). Despite the importance of Vangl2 in primary neurulation, the expression pattern of this gene has not been
investigated in great detail. There is a need to further extend the analysis of Vangl2 expression during initiation of neural tube closure.
3.2 Results

3.2.1 Bioinformatics approach to define the matrisome at closure 1

The complete list of ECM and ECM associated proteins (the ‘matrisome’), needs to be defined in order to analyse the structure and function of extracellular matrices in any given tissue. RNA-seq technology followed by bioinformatic analysis was used to analyse the global composition of ECM at closure 1 region (Fig. 3.1B). Tissue of E8.5 wild type embryos (prior to the initiation of NT closure) was collected and sequenced (Fig. 3.1B). Due to the fact that the main goal of the RNA-seq experiment was to analyse the expression pattern of the genes from the same tissue/genotype a number of filters had to be applied to the data, to define genes with meaningful expression level at the closure 1 region. A positive control set (genes known to be expressed at the closure 1 region), and a negative control set composed of genes known to be expressed only caudally (Cyp26a1, Wnt8a, Spry4, Wnt3a) and rostrally (Tbx2, Cdkn1a, Wnt1, Otx1, Hesx1, Six6), were designed for the filter, in order to define minimum expression values of expressed genes, and maximum values of non-expressed genes. The distribution of expression values of the positive control group (Fig. 3.2D) is clearly separated from that of the negative control group (Fig. 3.2E). This difference in signal distribution between positive and negative control groups was used to perform a ‘cut-off’, in order to remove genes with low count reads that represented ‘background’ expression. The lower ‘cut-off’ level of 5.35 (normalised read counts in log scale) was defined by Cyp26a1 that has the highest expression level within negative control set (Fig. 3.2A). Using this filter, two thirds of the genes (lowest expression values) were removed from the list and the remaining 7782 candidates were considered as ‘truly expressed’ (‘genes after cut-off’) and used for the further analysis (Fig. 3.2C). Notably, most of the variations of expression values between the two replicate samples lie within the lower quartile, in the set of the genes that was removed by the filtering (Fig. 3.2B).

A recent study analysed the transcriptome of mid-gestation mouse embryos (MGME dataset, 3-6 somite stage) (Werber et al., 2014). 5 tissues were micro-dissected: the head; the somites; the heart; the mesoderm caudal to the somites (CEM); presumptive spinal cord (PSC) and the remainder (carcass) (Fig. S3.1B). The mapping results and the coverage are accessible via the UCSC genome browser (http://overview.molgen.mpg.de/ucsc.html, Fig. S3.2).

In the present study, the MGME data source allowed a comparison of gene expression patterns with the transcriptome of the closure 1 region, as determined here.
Figure 3.1. Tissue collection and the pipeline for bioinformatics analysis. (A) Schematic representation of closure 1 region. Dotted line marks the localisation of basement membrane. (B) The tissue from E8.5 embryos (4-6 somite stage, prior to closure 1) were cut between 1st and the last somite excluding cranial and caudal regions. Two pools of samples were collected and used for RNA-seq. Sample 1 (ON1): ‘cuts’ from 6 embryos and sample 2 (ON5): ‘cuts’ from 3 embryos. (C) Normalised RPKM reads were filtered using sets of genes in diamond boxes. Rectangular boxes: number of genes before and after filtering. ON1 used as a reference to do a ‘cut-off’. RPKM: Reads Per Kilobase of transcript per Million mapped reads.
**Figure 3.2. Removal of genes with low read counts.** Genes from positive control are known (published data*) to be expressed at the tissue of interest (‘cuts’); genes from negative control set are normally not present in the middle region (expressed at low levels or absent). (A) Genes with background read count are filtered out by applying positive and negative control sets (red and blue boxes respectively). (B, C) The scatter plots compare the global expression of genes between two samples (ON1, ON5). The gene expression values are presented by normalised intensity value in the log2 scale (Y-axis) per gene. The expression values of most of the genes after ‘cut-off’ (C) fit the straight line confirming the similarity between two samples. (D, E) Genes from positive control (D) show higher expression levels than genes from negative control (E). *Gene expression database at Mouse Genome Informatics was used to generate positive and negative control sets. The colour range is very wide starting from 1.6 (Six6, not expressed) to 9 (Gapdh) in A.

Despite the fact that the two studies used different bioinformatics approaches to analyse the transcriptome of mid-gestation mouse embryos, the pattern of gene expression appears to be similar for a large number of genes (Fig. 3.5, Fig. S3.1). First, the expression of genes from positive and negative control sets was analysed in the MGME data (Fig. S3.1). The lower cut-off
point of 5.6 (normalised read counts in log scale) was arbitrarily defined as the background expression level for MGME dataset. This decision was based on the published tissue site of expression of genes from the negative control set (Mouse Genome Informatics gene expression database). Many genes from the positive control are expressed above the background level in most of the tissues of MGME. Exceptions are the expression of T and Grhl3 which is excluded from somites (Fig. S3.1B and C) that correspond with the published literature (Auden *et al.*, 2006; Savory *et al.*, 2009). It is important to mention that the PSC region included the neural tissue from both closure 1 and the posterior region (Fig. S3.1A). As expected, a number of genes from the negative control set (*Cyp26a1, Wnt8a, Wnt3a* and *FGF8*) are highly expressed in PSC region as they are localised to the posterior neural plate and mesoderm but not expressed in the closure 1 region (Fig. S3.1B and C). The head and the heart transcriptome profiles were included in the analysis to visualise the expression of the genes noted to be present in these specific regions at this developmental stage. For example, *Otx1* and *Hesx1* transcripts are known to be expressed exclusively in the head region whereas *Tbx2* is abundant in the heart tissue during this stage (MGI; Fig. S3.1B and C).

In addition, Werber and colleagues identified 1375 genes that have a tissue-specific expression pattern. For instance, *Celsr1*, one of the key PCP proteins, was shown to be specifically expressed in the PSC region whereas *Dmrt2*, the transcriptional factor important for somite and skeletal system development, was enriched in the somites (Fig. S3.2).

### 3.2.2 Matrisome: the global view on ECM and ECM-associated proteins at closure 1 region

‘Genes after cut-off’ were analysed for the presence of matrisome genes and genes involved in the synthesis/turnover of GAG chains. The 2014 version of the matrisome masterlist (Naba *et al.*, 2016) was applied to extract core matrisome and matrisome-associated proteins (Fig. 3.1B). The analysis revealed that the matrisome of the closure 1 region of E8.5 embryos is composed of 209 genes (out of 1100 murine matrisome genes (Naba *et al.*, 2016)), including 83 core matrisome genes (59 ECM glycoproteins, 20 collagens and 4 proteoglycans) and 126 matrisome-associated genes (37 Secreted factors, 51 ECM regulators and 38 ECM-affiliated genes) (Fig. 3.3A, Table S3.1 and Table S3.2). Both replicate samples have a similar pattern of gene expression confirming the homogeneity of the tissue used for analysis. Hierarchical clustering of six matrisome categories based on the expression values showed that members of all categories are widely distributed. Most of the ECM regulators are localised in the middle and bottom part of the heatmap, whereas members of other categories are widely distributed (Fig. 3.3B). It is important to mention that the proteoglycans are spread across 3 different
categories of the matrisome: ‘Proteoglycans’, ‘ECM-affiliated proteins’ and ‘ECM glycoproteins.’ A separate list of genes (50) was generated in order to include all molecules that are classified as proteoglycans in the literature and used to extract the genes from RNA-seq dataset (Couchman et al., 1996; Jones et al., 2003; Hynes et al., 2012; Iozzo et al., 2015). Fibronectin has the highest expression level among all matrisome genes and it has 8th place in the list of 7782 genes that passed the ‘cut-off’ (ranked highest to lowest).

3.2.3 RNA-seq analysis of proteoglycans

16 proteoglycan genes (out of 50 analysed) are expressed in the E8.5 mouse embryo at the closure 1 region (Fig. 10C, 11A) including 3 CSPGs, 10 HSPGs and 3 non-sulfated proteoglycans. Strikingly, three proteoglycans (versican, agrin and perlecan) are present in the top 10 matrisome genes of the E8.5 mouse embryo (Table S3.1). All 3 CSPGs are have high expression values and are located in the upper part of the heatmap. The top hit from the CSPG group is Vcan (versican) that has the highest expression level across all proteoglycans (Fig. 3.4A). Versican is a secreted proteoglycan type that is mainly localised in the mesenchymal tissue and was shown to be expressed at the site of somitogenesis of E9.5 mouse embryos (Deborah J Henderson et al., 1997). The second CSPG is Smc3 gene (bamacan) also known as basement membrane-chondroitin sulfate proteoglycan. Bamacan was originally isolated from Reichert’s membrane and was shown to be a basement membrane (BM) component of Engelbreth-Holm-Swarm tumor matrix, and possibly of the BMs of other tissues (Iozzo et al., 1987; Couchman et al., 1996). It was shown previously that CS chains are localised at the neuroepithelial and SE basement membranes during spinal neurulation (Yip et al., 2002). Another CSPG expressed during initiation of neural tube closure is Ptprz1. The Ptprz1 transcripts are detected in PSC and somites (Fig. 3.5). The Ptprz1 gene encodes a member of receptor-type tyrosine-protein phosphatase family also known as phosphacan. Ptprz contains a carbonic anhydrase domain followed by a fibronectin type III domain and then a stretch of CS chains. The chains are responsible for binding various ligands including pleiotrophin (Maeda et al., 2003).

HSPG genes represent most of the proteoglycans expressed at the closure 1 region. The basement membrane components Agrn (agrin) and Hspg2 (perlecan) are the top hits from HSPG group (Fig. 3.4). These proteoglycans are described in the Section 3.2.6. Four members of the glypican family (GPI-anchored HSPGs) are present in the closure 1 region (Fig. 3.4). Gpc1 (glypican-1), Gpc3 (glypican-3) and Gpc6 (glypican-6) transcripts show similar expression levels in PSC, somites and CEM whereas Gpc2 (glypican-2) is more abundant in the somites (Fig. 3.5).
Figure 3.3. Matrisome of closure 1 region of wild type E8.5 mouse embryo. (A) Definition of matrisome categories. 209 matrisome genes were detected in E8.5 mouse embryos at closure 1 region. Matrisome genes have two divisions: core matrisome and matrisome-associated genes and each division has 3 categories. Core matrisome categories: ECM Glycoproteins, Collagens and Proteoglycans. Matrisome-associated proteins include ECM regulators, secreted factors and ECM-affiliated proteins. (B) The distribution of gene expression values is very similar between the two replicate samples. Trace lines show the expression values from lowest to highest in comparison to average expression (dashed line), each for ON1 and ON5). Hierarchical clustering of six matrisome categories based on the expression values presented by the heatmap. (C) Modified set of proteoglycans include genes from three matrisome categories and also genes added manually. External proteoglycans not included in the matrisome masterlist: phosphacan (Ptrz1), bamacan (Smc3) and Cd44.
One of the members, \textit{Gpc6} has high sequence identity to knypek (kny), the zebrafish glypican that plays an important role in convergent extension cell movements of lower vertebrates (Topczewski \textit{et al.}, 2001). It is important to mention that expression of glypican 4 was not detected above threshold level in the tissue that was used for RNAseq (data not shown). This agrees with the published data showed that glypcan-4 is expressed in the anterior neural ridge and not at the site of initiation of NT closure of E8.5 embryos (Ybot-Gonzalez \textit{et al.}, 2005). All four members of the syndecan family (transmembrane HSPGs) are detected at the closure 1 region with \textit{Sdc1} (syndecan-1, epithelial type) having the highest and \textit{Sdc4} (syndecan-4) the lowest expression level. The expression of syndecan-4 mRNA is higher in the neural plate then in somites or CEM (Fig. 3.5).

Three non-sulfated proteoglycans are also expressed at the closure 1 region (\textit{Hapln1, Nepn} and \textit{Cd44}, Fig. 3.4). The Hapln1 gene (hyaluronan and proteoglycan link protein 1) has the highest level of expression, taking 6th place across all proteoglycans detected in this tissue. Hapln1 was shown to interact with versican in order to stabilise proteoglycan aggregates in the extracellular cartilage (Matsumoto \textit{et al.}, 2003). \textit{Nepn} encodes nephrocan, the earliest definitive endoderm specific and regional marker (Hou \textit{et al.}, 2007). Mochida \textit{et al.} showed that nephrocan acts as an inhibitor of Transforming Growth Factor-β signalling (Mochida \textit{et al.}, 2006). \textit{Cd44} has the lowest expression level among all proteoglycans at closure 1 (Fig. 3.4) and is an adhesion protein that acts as a major receptor for hyaluronan. Wheatley and colleagues showed that the \textit{Cd44} transcripts are present in the somites and heart tissue (Wheatley \textit{et al.}, 1993), this data agrees with MGME dataset (Fig. 3.5A).

3.2.4 RNA-seq analysis of GAG synthetic/turnover genes

Fourteen genes involved in HS chain biosynthesis are detected at the site of closure 1 (Fig. 3.4B). A schematic diagram of HS biosynthetic pathway is presented in Fig. S3.4. HS consists of repeating GlcNAc-GlcN and IdoA disaccharide units polymerized into long chains. The synthesis of HS chains is initiated by the members of the Extl family of glycosyltransferases by adding the N-acetylglucosamine to the non-reducing end of the tetrasaccharide region. Extl3 is proposed to be the main enzyme that catalyses the initiation of HS biosynthesis \textit{in vivo} (Holmborn \textit{et al.}, 2012; Kreuger \textit{et al.}, 2012). Analysis of the closure 1 transcriptome shows that \textit{Extl3} is most highly expressed gene of HS biosynthetic machinery (Fig. 3.4B) and its expression level is similar in closure 1, PSC, somites and CEM (Fig. 3.5B). After the Extl-mediated initiation of HS chains, the polysaccharide is extended by the HS-polymerase complex, containing the Ext1 and Ext2 enzymes, which transfer GlcNAc-GlcA residue to the growing polymer.
Figure 3.4. Proteoglycans and GAG turnover/synthetic genes. Hierarchical clustering of three gene sets (Proteoglycans, A; HS turnover/synthetic genes, B; CS synthetic genes, C) based on expression value. The expression value of each gene is presented as a colour-intensity value in each sample: from red (the highest) to blue (the lowest). (A) The sixteen Proteoglycan gene names are colour coded: CSPGs in green, HSPGs in red and non-sulfated proteoglycans in black. The top proteoglycan hit is Vcan (CSPG) followed by two HSPGs (Agrn, Hspg2). The majority of the expressed proteoglycans are HSPGs. (B) HS sulfatases genes are the most abundant (Sulf2 and Sulf1), followed by glycosyltransferases (Ext group) and sulfotransferases (Ndst). (C) The top CS synthetic genes are chondroitin sulfate synthase (Chsy1) and dermatan-sulfate epimerase (Dse). Genes involved in synthesis of HS GAG chains (14 in B) are more abundant than CS-GAG synthetic genes (7 in C). Note: each set of genes has an independent heatmap range (see legends) and the colour ranges are narrower than in the control sets (Figure 3.3).

Both glycosyltransferases are expressed at similar levels in closure 1 and other tissues of the MGME dataset (Fig. 3.4B, Fig. 3.5B). The next step in synthesis involves the modification of the HS backbone by N-deacetylase/N-sulfotransferase (Ndst). In vitro studies have shown that the length of the N-sulfated domains correlates with concentration of sulfate donor PAPS (Carlsson et al., 2008). Four Ndst genes have been identified and found to have different acetylation and sulfation activity (Aikawa et al., 2001) with Ndst1 and Ndst2 shown to be the most widely expressed genes. Indeed, both Ndst1 and Ndst2 are present in closure 1 and MGME datasets with Ndst1 having a higher expression level than Ndst2 (Fig. 3.4B, Fig. 3.5A and B).
After modification of HS chains by Ndsts, GlcA units are epimerized to IdoA by a single C5-epimerase encoded by the Glce gene. Epimerization is followed by sulfation at the 2-O position through the action of 2-O-sulfotransferase (Hs2st). Hs2st1 is the third most expressed gene of HS biosynthetic machinery and it has a similar expression pattern across different tissues. In contrast, Glce gene has a higher expression level in the somites (Fig. 3.4B, Fig. 3.5A and B).

As the ability of HS chains to interact with secreted factors is dependent on the presence of O-sulfate groups, it is important to note that, although there is only one 2-O sulfotransferase, there are seven 3-O sulfotransferases and three 6-O sulfotransferases. The expression of multiple sulfotransferases allows the generation of functionally specific and diverse HS sequences. Analysis of mRNA transcripts shows that two 6-O sulfotransferase, Hs6st1 and Hs6st2, are present in closure 1 and MGME datasets with Hs6st2 having a higher expression level in the somites (Fig. 3.5A and B). The addition of 6-O-sulfate groups was shown to be essential in the binding of HS to several secreted factors including FGF2 (Lamanna et al., 2006). Addition of sulfate group to the 3-OH position of glucosamine in HS is thought to be the final element of biosynthetic pathway. Only one 3-O sulfotransferase, Hst3stb1, is expressed during initiation of neural tube closure and its expression level is higher in somites (Fig. 3.5A and B).

Two endosulfatases, Sulf1 and Sulf2, are able to modify the sulfation patterns of HS chains by removing the 6-O-sulfate groups from HS in the extracellular environment. Both Sulf1 and Sulf2 genes are expressed at high levels in closure 1 and MGME datasets. These enzymes were originally described in muscle and neural progenitors of quail embryos (Dhoot, 2001). Interestingly, Qsulf1 expression in the neural plate follows a ventro-dorsal gradient that is dependent on Shh signalling. The expression of Sulf1 mRNA in E9-E15 mouse embryos shows a similar gradient of expression whereas Sulf2 is expressed in floor and roof plate at these developmental stages (Kalusz et al., 2009).

A schematic diagram of CS biosynthetic pathway is presented in Fig. S3.5. CS are long-chain polysaccharides consisting of repeating GalNAc-GlcA disaccharide units. CS synthetic genes (6) are less abundant than genes involved in the HS chain biosynthesis at the closure 1 region (Fig. 3.4B vs Fig. 3.4C). This is consistent with the higher abundance of HSPGs in comparison to CSPGs in this dataset (Fig. 3.4A). The assembly of CS chains is initiated by the glycosyltransferases, Chsy1, Chsy2 and Chsy3 that transfer GalNAc to the non-reducing end of GlcA residue. Chsy1 is the most abundant component of CS biosynthetic machinery at closure 1 and it has similar expression levels in PSC and somites (Fig. 3.4, Fig. 3.5A and B). Chondroitin polymerization factor (Chpf1 and Chpf2) is needed for chondroitin polymerizing activity of mammalian chondroitin synthase (Chsy). Chpf mRNA is equally abundant in PSC and somites (Fig. 3.5A).
Figure 3.5

A

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B

Figure 3.5. The comparison of closure 1 and tissue-specific RNA-seq (Werber et al., 2014): proteoglycans, HS synthesis and turnover, CS and DS synthesis, secreted factors. (A) RNA-seq of closure 1: transcript abundance presented in log$_2$ scale Read$\times$Per Kilobase of exon per Million mapped reads (RPKM); read counts are reported as average of two replicates; the lower cut-off is 5.3 RPKM read counts. Tissue-specific RNA-seq (Werber et al., 2014): transcript abundance showed in log$_2$ scale Fragment$\times$Per Kilobase of transcript per Million mapped reads (FPKM). (B) Right to left hierarchical clustering of selected genes based on the expression values in log$_2$ scale presented by the heatmap. Read counts are reported per tissue type. PSC: presumptive spinal cord; CEM: caudal end mesoderm.
In contrast, Chfy2 mRNA is detected at high level only in somites whereas PSC display expression below the threshold. To the present, seven sulfotransferases responsible for the sulfation of CS and DS chains have been discovered. Analysis of the closure 1 transcriptome shows that three sulfotransferases (Chst2, Chst11 and Chst15) are present in closure 1 dataset. Chst2 and Chst15 catalyse 6-O-sulfation and Chst11 catalyses 4-O sulfation of GalNAc residues that allows formation of monosulfated A and C units. Subsequent sulfation of A and C units leads to the formation of disulfated D and E units. It is important to note that dermatan sulfate epimerase (Dse) is the second most highly expressed gene of CS/DS synthetic pathway at closure 1. Dse converts the D-glucuronic acid to L-iduronic acid in DS biosynthesis. However, none of the dermatan sulfotransferases were expressed above the threshold in closure 1 dataset (data not shown), suggesting that dermatan sulfate is not a significant GAG in closure 1.

3.2.5 RNA-seq analysis of ECM-associated secreted factors

Many secreted factors bind to ECM proteins as a part of their regulation. Growth factors can bind to specific domains of ECM proteins. For example, fibronectin interacts specifically with VEGF, HDF and PDGF while follistatin domains, found in variety of ECM proteins, are well known to bind BMP ligands (Wang et al., 2008; Lin et al., 2011; Hynes et al., 2012). Notably, the most common ECM binding partners of secreted factors are GAGs, especially heparan sulfate. In the ECM, as well as at the cell surface, the binding, activation or immobilization of various secreted factors is controlled by GAG chains (Zhang, 2010). In this study, the expression of secreted factors was quantified by RNA-seq.

Of the total secreted factors analysed, thirty seven are expressed during initiation of neural tube closure (Fig. 3.3A, Table S3.2). The present study mainly focused on the secreted factors known to interact with GAG chains and the receptors of associated signalling pathways. The list of GAG-binding growth factors and morphogens was obtained from the KEGG pathway database (http://www.genome.jp/kegg-bin/get_htext#A1) and from published literature (Zhang, 2010). The expression of these proteins was analysed in closure 1 and MGME datasets. Twenty one GAG-binding proteins were identified in the closure 1 region (Fig. 3.6A). These proteins belong to a variety of growth factor signalling pathways including canonical and non-canonical Wnt, FGF, BMP, VEGF, Shh and others. The most highly expressed ligand is Igf2. Igfbp2 protein has two heparin-binding domains and it is known that heparan sulfate specifically targets IGF2/IGBP2 complex to the bone matrix (Lund et al., 2014).
3.2.5.1 Canonical and non-canonical Wnt pathway

Anna Ferrer-Vaquer and colleagues generated a Wnt/β-catenin reporter system that allowed the study of canonical Wnt signalling pathway activity at single cell resolution during mouse development (Ferrer-Vaquer et al., 2010). In the closure 1 region, reporter expression was detected in the neuroepithelium whereas the reporter signal in the somites and SE remained negative. Eight somite embryos, just after completion of closure 1, were used in this study, therefore, the activity of Wnt/β-catenin pathway needs to be validated prior to the initiation of neural tube closure. One canonical Wnt ligand (Wnt6) and one non-canonical Wnt ligand (Wnt5a) are detected in closure 1 dataset (Fig. 3.6). Analysis of the MGME dataset showed that Wnt6 is predominantly expressed in the PSC whereas Wnt5a has similar expression levels in PSC and somites (Fig. 3.6B). Heparan sulfate chains are required for proper activity of canonical Wnt proteins and have an impact on their distribution and receptor binding (Fuerer et al., 2010). In contrast, chondroitin sulfate chains may have the opposite effect on Wnt signalling. For instance, CS-E chains act as inhibitors of specific outcomes of Wnt3a signalling (Willis et al., 2012). Syndecan 1 and syndecan 4 are known to regulate the Wnt5a signalling pathway during development and cancer (Muñoz et al., 2006; O’Connell et al., 2009).

It is important to mention that the expression of Wnt11, another PCP ligand, is below the threshold in the closure 1 dataset. GAG chains are also known to interact with Wnt antagonists such as frizzled-related proteins, two of which (Sfrp1, Frzb) are expressed in the closure 1 dataset (Fig 3.6A). In addition, two other members of the secreted frizzled-related protein family, Sfrp2 and Sfrp5, are expressed in the closure 1 region. Inactivation of Sfrp1, Sfrp2 and Sfrp5 results in defective convergent extension and somitogenesis suggesting that Sfrps function is important for both canonical and non-canonical Wnt signalling pathways (Satoh et al., 2008). The frizzled receptor family is composed of 10 members, two of which are receptors for PCP signalling (Fzd3, Fzd6); the remaining proteins mainly bind the ligands of canonical Wnt signalling. Both Fzd3 and Fzd6 are detected in the closure 1 dataset with Fzd3 having a much higher expression level than Frz6 (Fig. 3.6). Interestingly, Fzd6 is expressed above threshold only in the PSC sample whereas Fzd3 is expressed at similar levels in PSC and somites. The most highly expressed frizzled receptors of canonical Wnt signalling are Fzd7, Fzd2 and Fzd10. In addition to frizzled receptors, significant high levels of Lrp6 and Lrp5, another family of Wnt co-receptors, were also detected (Fig. 3.6).
3.2.5.2 FGF pathway

GAGs positively and negatively regulate the FGF signalling pathway by presenting or sequestering the ligands from the FGF receptors, promoting dimerization and stabilising the receptor-ligand complexes. Two FGF ligands (FGF13 and FGF15) are detected above threshold level in the closure 1 dataset. FGF13 belongs to the intracellular subfamily of FGF proteins whereas FGF15 is a component of the endocrine subfamily. Interestingly, FGF15 has very low affinity for heparan sulfate, whereas signalling of canonical FGFs such as FGF1, FGF2 and FGF8 is highly dependent on HS and CS chains (Guan et al., 2016). Analysis of the MGME dataset revealed that FGF15 is mainly detected in the PSC while FGF13 has similar expression levels in somites and PSC (Fig 3.6A). As mentioned above, Werber and colleagues identified 1375 genes that are differentially expressed between the six tissue samples. For example, Fgfbp3 was shown to be exclusively expressed in the PSC sample and the expression pattern of this gene was confirmed by in situ hybridisation assay (Werber et al., 2014). One of the studies showed that Fgfbp3 (FGF binding protein 3) prevents binding of Fgf2 to heparin and inhibits immobilisation of this ligand on ECM GAGs, allowing the release of Fgf2 and subsequent activation of FGF receptor signalling (Zhang et al., 2008). The expression of Fgfbp3 is detected in the closure 1 dataset (Fig. 3.6A). The most highly expressed FGF receptors are Fgfr1 and Fgfr2. Both receptors are equally abundant in the somites and PSC samples. Moderate levels of Fgfr3 were also detected (Fig. 3.6).

3.2.5.3 Sonic hedgehog pathway

A number of studies show that sonic hedgehog (Shh) interacts with proteoglycans in vitro and in vivo. For example, heparan sulfate chains of glypican bind Shh, to allow anchoring and increasing concentration of the ligand on the pericellular membrane and subsequent presentation of Shh to its receptor patched (Vaillant et al., 2009). Other studies show that Shh could act be upstream of proteoglycans; for example, sonic hedgehog induces the mRNA expression of the chondroitin sulfate proteoglycan, versican, during development of the chick enteric nervous system (Nagy et al., 2016). Shh transcripts are detected at very low levels in the closure 1 dataset and are only present above threshold level in the PSC tissue sample (Fig. 3.6A). The low levels of Shh mRNA could be explained by the restricted expression of this gene which is only detected in the notochordal and floor plate at E8.5 (MGI gene expression database). Nevertheless, the receptor of Shh signalling, Ptc1 and its binding partner Smo, are expressed at very high levels in PSC and somites (Fig. 3.6A and B).
3.2.5.4 Pleiotrophin, midkine, BMP and TGF-β

Pleiotrophin (Ptn) and midkine (Mk) are two neurotrophic factors that are able to bind GAGs. Ptn and Mk can simultaneously bind CS and HS chains of syndecan-1 and syndecan-4, producing a ternary complex that translocates the secreted factors to the corresponding receptors (Deepa et al., 2004). Protein tyrosine phosphatase receptor type Z (Ptprz) is the main receptor for both neurotrophic factors. CS chains of Ptprz are needed for high affinity binding of Ptn and Mk, and removal of the GAG chains from the receptor dramatically decreases the downstream signalling of the ligands (Kuboyama et al., 2016). Ptn and Mk are expressed at high levels in the closure 1 dataset. Mk is more abundant in the somites whereas Ptn has similar expression level in both PSC and somites (Fig. 3.6A). Notably, growth factor-binding experiments showed that HS and CS/DS GAGs bind midkine and pleiotrophin at high affinity during amphibian neurulation (Yamada et al., 2009). Furthermore, the expression of Ptn was first detected in mice at E8.5 in the neuroepithelium cells, whereas Mk started to be expressed in the embryonic ectoderm as early as E5.5 (Q. W. Fan et al., 2000).

Ligands and receptors of the TGF-β and BMP signalling pathways are detected in the closure 1 dataset. These include Tgfb2 ligand, Tgfbpr1 and Tgfbpr3 receptors, the Bmp5 and Bmp7 secreted factors, and their receptor Bmpr1a. (Fig. 3.6).

3.2.6 RNA-seq analysis of basement membrane components and integrin receptors

The major protein components of metazoan basement membranes (BMs) are collagens, laminins, perlecan and nidogen (Hynes, 2012). All of these components are expressed at high levels during initiation of neural tube closure (Fig. 3.7, Fig. S3.1).

Collagen type IV is present in all basal laminas where it forms a two-dimensional reticulum. It is encoded by six genes that give rise to a number of hetero-trimeric combinations (Yurchenco, 2011). Analysis of the closure 1 transcriptome demonstrates that the most highly expressed collagen type IV genes are Col4a1 and Col4a2 (Fig. 3.7C). The combination of these molecules gives rise to the collagen type IV α1α1α2, the most common hetero-trimeric variant in all BMs. Col4a5 and Col4a6 genes are expressed at much lower levels, the assembly of which leads to the formation of α5α5α6 hetero-trimer. The second most abundant BM component of closure 1 region is collagen XVIII (Col18a1) (Fig. 3.7C). This protein contains collagen multiple triple-helix domains that are interrupted by non-collagenous domains with attached HS chains. Mutations in collagen XVIII has been associated with Knobloch syndrome (Kliemann et al., 2003). The main features of this disorder include retinal abnormalities and encephalocele, leading to the suggestion that collagen type XVIII may play a role in retinal development.
**Figure 3.6**

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### B

**Secreted factors**

- **Closure 1**
  - PSC
  - Somites
  - CEM

### C

**Receptors**

- **Closure 1**
  - PSC
  - Somites
  - CEM

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Laminins are hetero-trimers composed of one of five α chains, one of three β chains and one of three γ chains, connected through a long coiled-coil domain (Hohenester et al., 2013). Analysis of the closure 1 RNA-seq dataset reveals that the most highly expressed laminin genes are Lama5, Lamb1, Lamc1 and Lama1 which form the α5β1γ1 and α1β1γ1 trimers (Fig. 3.7C). The presence of genes that encode these trimeric combinations was detected at both mRNA and protein levels during spinal neurulation (Copp et al., 2011). Lamb2 gene is expressed at lower levels; it encodes β2 laminin that can form α5β2γ1 and α1β2γ1 trimers, suggesting that these variant laminins may also be present during neurulation.

Two HSPGs, agrin and perlecan, are expressed at high levels during initiation of neural tube closure (Fig. 3.7C). These proteoglycans are mainly localised in the basement membrane providing collateral linkage to the cell surfaces. Agrin and perlecan play related roles in basement membrane assembly by binding to laminin and nidogen, respectively, as well as contributing to cell surface interactions through binding to α-dystroglycan, and α2β1 integrin (Yurchenco, 2011). The expression of nidogen 1 and nidogen 2 encoding genes, Nid1 and Nid2 respectively, is also detected in RNA-seq dataset (Fig. 3.7C). Nidogens are necessary for the formation of the stable bridge between the coiled-coil domain of laminin and type IV collagen (Yurchenco, 2011).

Specific interactions between cells and the ECM proteins are mediated by ECM receptors. The major receptors are integrins, each composed of an α and a β subunit. The integrin family is highly diverse, with 24 different αβ heterodimeric members that mediate the attachment of the cells to the ECM (Barczyk et al., 2010). In the present study, the expression of α and β subunits was investigated by RNA-seq. It allowed the identification of functional heterodimeric combinations that are likely to be present in the closure 1 region.

Integrin β1 (Itgb1) is the most highly expressed subunit with an average RPKM of 10.24 (Fig. 3.7E). This subunit can bind multiple α partners and plays a key role in assembly of many different integrin receptor combinations. It is not surprising, therefore, that Integrin β1 is expressed at high level. The analysis of closure 1 transcriptome detected the expression of
three integrin subfamilies including those interacting with laminins, RGD motif and leukocytes, whereas collagen-interacting integrin receptors were not expressed at this stage (Fig. 3.7E). The most highly expressed dimeric combinations are α3β1 representing the major integrin receptor that recognises laminin (Fig. 3.7B and E) and another laminin-interacting integrin, α6β1, which is also expressed at high level. In addition to integrins, another major laminin-binding receptor is dystroglycan. This receptor connects the external laminin basement membrane to the F-actin cytoskeleton through dystrophin interactions (Yurchenco, 2011). Dystroglycan is encoded by a single gene Dag1 which is expressed at high level in the closure 1 dataset (Fig. 3.7B and E). Therefore, the laminin network connects to cells via interaction with integrins α3 and α6, as well as through binding to dystroglycan.

In addition to laminin-binding integrins, the fibronectin receptor, α5β1, is also strongly expressed (Fig. 3.7A and E). Consistent with this, moderate levels of integrin β5 were also detected, a subunit that forms the fibronectin-binding integrin αvβ5. However, αv is expressed only at low levels (Fig. 3.7A and E) suggesting a minor role for this integrin.
Figure 3.7. RNA-seq expression analysis of ECM receptor/ligand complexes at closure 1.

(A) Hierarchical clustering of the gene sets is based on expression value. The genes sets are: (A) Fibronectin and receptors; (B) Laminin ligands and receptors; (C) Basement membrane core genes. Integrin β1 is the most highly expressed gene among all integrin subunits. (D) Catalogue of integrin subunit combinations arranged by expression and ligand specificity, as described in Barczyk et al., 2010 and Lowell et al., 2012. (E) Table of normalised RPKM reads. Read counts are reported for each of the two replicates with average expression values. Each set of genes has an independent heatmap legend in A-C. The lower cut-off is 5.3 read counts.

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<p>| INTEGRINS &amp; DYSTROGlyCAN: hierarchical expression of genes, RPKM normalised counts |
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To determine the tissue site of expression, the mRNA of selected proteoglycans was analysed by whole mount in situ hybridisation and the protein localisation was examined by immunofluorescence. Gpc1 mRNA exhibits a ubiquitous expression pattern before and after the initiation of neural tube closure with expression in the paraxial mesoderm, SE and neuroepithelium at all body levels (Fig. 3.8A and B). At the closure 1 site, the strongest expression is detected in the neural plate and SE, with continued expression in the same domains after the completion of closure 1 (Fig. 3.8A-iv and Fig. 3.8B-iv respectively).

Glypican3 (Gpc3) showed the highest expression level among the four glypicans detected in the RNA-seq dataset (Fig 3.4A). However, in situ hybridisation analysis showed that Gpc3 transcripts are mainly localised to the caudal region prior to onset of neural tube closure. At the closure 1 site, the gene is transcribed in the dorsal neuroepithelium, notochordal plate and in the fully formed somites (Fig. 3.8D-i-v). The gene starts to be more ubiquitously expressed after the completion of closure 1 (Fig. 3.8E-i, ii). Transverse sections reveal that the Gpc3 is strongly expressed in the somites and that weaker staining is detected in recently closed neural tube. At the posterior region, the gene continues to be expressed in paraxial mesoderm flanking the open PNP, whereas the expression in the neural plate follows a ventro-dorsal gradient (Fig. 3.8E-i-v). SE appears largely negative for Gpc3 mRNA.

Syndecan1 (Sdc1) is the most highly transcribed member of the syndecan family detected in the closure 1 RNA-seq dataset (Fig. 3.4A). At early somite stages, Sdc1 transcripts are detected in the neuroepithelium, mesoderm and SE (Fig. 3.8F). During the onset of neural tube closure, Sdc1 mRNA continues to be expressed in the neuroepithelial cells, somites and paraxial mesoderm flanking the open PNP (Fig. 3.8H). The neural plate is strongly stained at all body levels. It is interesting to note that the expression of Sdc1 gene is downregulated in the most medial SE and tips of the closing neural folds (arrows in Fig. 3.8H-iv).

Consistent with the Sdc1 transcript expression pattern, syndecan-1 protein localises to the cell membranes of neuroepithelium, SE, and mesoderm, including the somites (Fig. 3.11B). This includes the dorsal SE where syndecan-1 protein is present at the basolateral cell junctions, despite the finding of downregulated Sdc1 mRNA. This is also evident from whole mount immunostaining of the closure 1 region where the basolateral surface of the closing neural folds is enriched for syndecan-1 protein (arrow in Fig. 3.11A-iv). Syndecan-1 considered to be a hybrid proteoglycan as both HS and CS chains are attached to the core protein (Iozzo et al., 2015).
Figure 3.8. GPI-anchored and transmembrane proteoglycans at closure 1. *In situ* hybridisation analysis of *Gpc1* (A, B), *Gpc3* (D, E) and *Sdc1* (F, H) at E8.5. Expression is shown prior to (5 somite stage) and after the onset of neural tube closure (7/8 somite stage). All three genes are expressed at multiple sites, as explained in the text. (C, E, G) Sense probe controls for *Gpc1* (C), *Gpc3* (E) and *Sdc1* (G). Transverse sections through embryos are shown at the levels indicated by the dashed lines, to display sections at brain/heart, closure 1 and posterior levels. Min. number of embryos for each probe: 5 for anti-sense and 3 for sense.
Previous studies reported the expression of syndecan-1 on the basolateral surfaces of neuroepithelial and mesodermal cells at E8.5 (Sutherland et al., 1991), in agreement with the present findings.

**Bamacan (Smc3)** exhibits a distinct and dynamic expression pattern during initiation of neural tube closure (Fig. 3.9). At the 2 somite stage, expression in whole mounts appears to be ubiquitous whereas transverse sections reveal that, in addition to weak expression in all tissues, Smc3 transcripts are localised intensely to the basal surface of neuroepithelial cells (except the midline which is negative) at all axial levels, both prior to and at the onset of neural tube closure (arrows in Fig. 3.9A, B and C). This basal enrichment of Smc3 transcripts could be related to a basal protein localisation and, indeed, it was shown previously that many tissues express Smc3 as a BM component (Wu et al., 1997).

**Versican (Vcan)** is the most highly expressed proteoglycan in the closure 1 RNA-seq dataset (Fig. 3.4A). During the onset of neural tube closure, Vcan transcripts are found in the neuroepithelium, somites, heart tissue and mesoderm (Fig. 3.9E). Expression appears especially intense in the fully formed somites flanking the neural plate. In the neural plate, Vcan expression exhibits cranio-caudal variation: at hindbrain level the staining is excluded from the neural plate whereas, in the closure 1 and posterior regions there is a strong expression throughout the neuroepithelium. At the 12 somite stage, the gene continues to be expressed in the neural tube, heart tissue and mesoderm. Rostral somites are strongly stained whereas more caudal somites have lower levels of the transcripts (Fig. 3.9F). Importantly, intense Vcan staining also occurs in the tailbud region, where neuro-mesodermal progenitors are located (arrow in Fig. 3.9F-v). The SE is negative for Vcan at E8.5 and E9.0.

Immunofluorescence analysis on sections reveals that versican protein localises to the paraxial mesoderm flanking the neural plate (Fig. 3.11C) and is subsequently enriched at somite borders, in agreement with previously published protein expression in chick embryos (Landolt et al., 1995). In addition to mesoderm expression, Vcan protein is detected at the basal surface of the neuroepithelium, at basolateral junctions in the SE and around visceral endoderm cells (arrows in Fig. 3.11C-iv). Since the SE is negative for Vcan mRNA, the protein is probably secreted from mesodermal cells.
Figure 3.9. Expression of CSPGs during initiation of neural tube closure. (A-C) In situ hybridisation analysis of Smc3 at E8.5. Expression is shown before the initiation of neural tube closure (2 and 5 somite stages; A, B) and during the onset of closure (7 somite stage; C). Smc3 expression appears ubiquitous in whole mounts although sections show strong, specific localisation of transcripts to the basal neuroepithelium. (D) Smc3 sense probe control. (E, F) In situ hybridisation to detect expression of the GAGβ domain in V0 and V2 Vcan isoforms at E8.5 and E9.0. Vcan expression analysed just prior initiation of closure 1 (6 somite stage; E) and during spinal neurulation (12 somite stage; F). Transcripts are detected in the paraxial mesoderm and neuroepithelium, whereas SE is negative. (G) Vcan sense probe control. Transverse sections through the embryos are shown at the levels indicated by the dashed lines, displaying sections at brain/heart (ii in A and iii in B, C, E, F) closure 1 (iv in B, C, E, F-) and posterior levels (v in B, C, E, F). Min. number of embryos for each probe: 5 for anti-sense and 3 for sense.
Agrin (Agrn) is the second most highly expressed proteoglycan by RNAseq at the site of closure 1 (Fig. 3.4A). At the 4 somite stage, expression is mainly detected in the neural plate and SE (Fig. 3.10A). Mesoderm is weakly stained anteriorly and is negative more caudally. During the onset of neural tube closure, Agrn is expressed throughout the neuroepithelium, SE and visceral endoderm (Fig. 3.10B). The neural plate and SE are strongly stained at the site of neural fold fusion whereas the mesoderm remains negative in this region (Fig. 3.10B-iv). Notably, the ventral midline neuroepithelium is negative at the closure 1 site. More caudally, intense staining is detected in the neuroepithelium, SE and visceral endoderm while mesoderm is only weakly stained (Fig. 3.10B-v).

Perlecan (Hspg2) expression, in contrast to agrin, is detected most intensely in the SE and mesoderm (Fig. 3.10D). Expression in the somites was present in the MGME dataset (8.15 RPKM in log scale). The Hspg2 probe also detects strong signal in the developing heart and blood vessels (arrows in Fig. 3.10D-iv, v), consistent with findings that Hspg2 knockout mice develop severe heart defects and die at E10-E12 (Arikawa-Hirasawa et al., 1999). The BM is absent in perlecan-null cardiomyocytes suggesting that this protein is critical for BM integrity during cardiac development (Costell et al., 1999; Sasse et al., 2008). At the closure 1 site, Hspg2 continues to be expressed in the SE and mesoderm while the neural plate remains largely negative. Posteriorly, transcripts are detected only in the SE and visceral endoderm.

Immunofluorescence analysis reveals that perlecan localises in the BMs of the SE and neuroepithelium, showing co-localisation with other BM components such as laminin and collagen (Fig. 3.11D and Fig. 3.15A and E respectively). Perlecan precisely defines the BMs on the basal surface of the neuroepithelium and SE and together with other BM components allows the separation of these tissues from mesoderm.
Figure 3.10. Expression of basement membrane HSPGs at closure 1. (A) In situ hybridisation analysis of Agrn at E8.5. Expression analysed prior to (4 somite stage) and during the onset of closure 1 (7 somite stage). The transcripts are mainly detected in the SE and neuroepithelium. (C) Sense probe control. (D) Hspg2 mRNA localises to the SE, paraxial and lateral mesoderm at 5 somite stage. The signal is not detected in the neural plate. (E) Sense probe control. Transverse sections through the embryos shown at the levels indicated by the dashed lines displaying sections at brain/heart (A-ii, B-iii, D-iii,v), closure 1 (B-iv, D-iv) and posterior levels (B-v, D-vi). Min. number of embryos for each probe: 5 for anti-sense and 3 for sense.
Figure 3.11. Syndecan-1, versican and perlecan distribution. Immunofluorescence on whole-mounts (A) and cryosections (B, C, D). Dorsal view (A i-ii) and re-sliced sections (A iii-iv) shows that SE cells express syndecan-1 at basolateral junctions (arrows in A-iv). Syndecan-1 protein is localised to the membrane of neuroepithelial and mesodermal cells. (B) Versican protein localises to the basal surface of the neuroepithelium, in the matrix between mesoderm cells and at the basolateral junctions of SE cells; weak staining is observed in the neuroepithelial cell membrane (C). Perlecan localises in the basement membranes underlying the neural plate and SE (D). Images were acquired by laser-scanning confocal microscopy and processed by single z-plane. Min. number of embryos for each antibody: 3.
3.2.8 Expression pattern of heparan and chondroitin sulfate chains

The expression of heparan and chondroitin sulfate chains has previously been studied in detail during spinal neurulation (Yip et al., 2002). However, the distribution of the GAG chains was not analysed prior to the onset of neural tube closure. In the present study, immunofluorescence analysis was performed to determine the expression pattern of sulfated GAGs at the closure 1 site. Wild type embryos were collected prior to the initiation of neural tube closure, and sections at various levels were analysed for the presence of heparan and chondroitin sulfate (HS and CS) using the 10E4 and CS-56 antibodies respectively. 10E4 recognises N-sulfated GAG residues present on the HS chains (David et al., 1992) while CS-56 reacts specifically with CS types A and C but not with type B (Avnu et al., 1984; Sobue et al., 1989).

To confirm that the anti-HS and anti-CS antibodies recognise their respective GAGs, tissue sections were pre-treated with either Heparitinase III (to degrade HS chains), Chondroitinase ABC (to degrade CS chains) or buffer solution, before applying the primary antibodies. Pre-incubation with enzyme buffer led to strong staining with both anti-CS and anti-HS antibodies (Fig. S3.3A and B respectively). In contrast, heparitinase III treatment dramatically reduced the staining of HS chains while the enzyme had no effect on the expression of CS chains (Fig. S3.3E and F respectively). A similar reduction of staining was observed for CS after the treatment with Chondroitinase ABC (Fig. S3.3B), although, this enzyme did not affect the expression pattern of HS chains (Fig. S3.3F). Hence, the 10E4 and CS-56 antibodies are highly specific for their respective GAG chains in embryonic tissues.

3.2.8.1 Distribution of heparan sulfate chains at E8.5

Transverse sections of the closure 1 region reveal strong staining of HS chains in the BM of the neural plate, SE and visceral endoderm (Fig. 3.12A). SE and neural plate share the HS chains located at the SE/NP interface in the most dorsal region (arrow in Fig. 3.12A-iii). Moderate staining is observed at the basolateral junctions of the SE cells (arrow in Fig. 3.12D-iii) and in the membrane of mesodermal cells including within the somites (Fig. 3.12B). In addition to the membrane staining, HS chains are also deposited around cells of paraxial mesoderm. Neuroepithelial cell membranes are weakly stained by 10E4 antibody. Strikingly, nuclear HS staining was observed in the most dorsal neuroepithelial and
Figure 3.12. Expression pattern of heparan sulfate (HS) chains at closure 1 region. Immunofluorescence staining on cryosections of wild type embryos (10E4 antibody). HS chains localise to the basement membrane of neuroepithelium and SE in the closure 1 region (arrow in A-iii). Strong staining is present at the lateral junctions of SE cells (arrow in D-iii) and weak staining of the membranes of mesodermal and neuroepithelial cells (arrows in B-iii and C-i). The chains also show nuclear localisation in the mesodermal cells, dorsal neuroepithelium and SE cells at the site of fusion (arrows in B-iii, C-iii and D-iii). Images acquired by laser-scanning microscopy using oil immersion, deconvoluted post-acquisition and processed by single z-plane in B-D and z-projection in A. Number of embryos for 10E4 antibody: 4.
SE cells and in some mesodermal cells (Fig. 3.12B, C and D). The nuclear staining is not a bleed through from DAPI as the images were acquired in separate channels and the expression was detected outside of nucleoli staining.

Transverse sections of the caudal region show that HS chains continue to be expressed at the BM of neural plate, SE and visceral endoderm (Fig. 3.14C and D). HS chains are also detected in the presomitic mesoderm, neuroepithelial cell membranes and SE. Immunofluorescence analysis on sagittal sections reveal that somite borders do not express HS chains (Fig. 3.14D).

**3.2.8.2 Distribution of chondroitin sulfate chains at E8.5**

Immunofluorescence analysis on tissue sections reveals the presence of CS chains in the BM underlying the neural plate, SE and visceral endoderm (Fig. 3.13A). The lateral neuroepithelial BM, where the neuroepithelium lies adjacent to the SE, stains more intensively than the ventro-medial BM. CS chains are also present in the ECM network around mesodermal cells and connect these cells with adjacent BM around neural plate. (arrow in Fig. 3.13A-vii). Neuroepithelial cell membranes are weakly stained by CS-56 antibody. Sagittal sections throughout the closure 1 site show strong staining of CS chains at the somite borders and weak staining within the somites (arrow in Fig. 3.13B-iii).

At the caudal region, CS chains continue to be expressed in the BM of neural plate, SE and visceral endoderm (Fig. 3.13B, Fig. 3.14A). The chains are also expressed in presomitic mesoderm. Whole mount immunostaining (WMI) reveals the presence of chains in the basolateral junctions of SE cells (Fig. 3.13C, Fig. 3.14B). Digital re-slicing of WMI z-stack detects intense staining in the ventral surface of the primitive notochord (arrow in Fig. 3.14B-ii).
Figure 3.13. Expression of chondroitin sulfate (CS) chains during initiation of neural tube closure. (A, B) Immunohistochemical analysis on transverse sections shows the presence of CS chains in the basement membrane of SE and neuroepithelium at closure 1 (A-i). The area between the dorsal neural plate and SE is especially strongly stained (arrow in A-vi). Sagittal sections reveal strong expression of the chains at somite borders at closure 1 site (arrow in B- iii, iv). (C) Whole-mount immunofluorescence analysis shows that CS chains localise to lateral junctions of SE cells (optical re-slice, dorsal view, arrow in C-i). Images acquired by laser-scanning microscopy and deconvoluted post-acquisition. Number of embryos for CS-56 antibody: 4.
Figure 3.14. Immunostaining for CS and HS chains through the posterior region of E8.5 embryos. (A) Immunofluorescence analysis on transverse sections shows that CS chains are expressed at the basal surface of the neural plate and SE; strong staining is also present in paraxial mesoderm. (B) Whole-mount immunofluorescence analysis of resliced sections reveals that CS chains surround the cells of the early-formed notochord (dashed circles). (C, D) HS chains localise to the basement membrane of the neuroepithelium and SE as well as in the membrane of neuroepithelial and mesodermal cells. (D) Sagittal sections show that somite borders are negative for HS chains, in contrast to the abundant CS chains (compare with Figure 3.13B). Images acquired by laser-scanning microscopy. Min. number of embryos for each antibody/region: 3
3.2.8.3 Co-localisation of GAG chains with laminin and expression of fibronectin and collagen IV at closure 1

Transverse sections of the closure 1 region reveal the presence of laminin in the basement membranes underlying SE and neural plate (Fig. 3.15A). Double immunofluorescence analysis on cryosections show that both CS and HS chains co-localise with laminin-containing basement membrane of SE and neural plate. HS staining shows an almost complete overlap with laminin whereas CS chains are also detected outside the laminin-expressing domain (Fig. 3.15A and B).

Fibronectin is detected in stromal ECM within the somites flanking the neural plate (Fig. 3.15C), as well as at the boundary zone between the somites (Fig. 3.15D). Fibronectin also localises to the BM of the SE and neural plate, where it is present within a dense fibrillary BM at the SE/NP interface, similar to CS chains.

Analogous to laminin and perlecain, collagen IV localises to BMs of the neural tube and SE, where it is strikingly absent from the site of the neural fold fusion. The presence of laminin, collagen IV and perlecain in the BMs of the closure 1 region confirms that these BMs are fully assembled mature structures.
Figure 3.15. Co-localisation of HS and CS chains with laminin, and expression of fibronectin and collagen IV. (A, B) Double staining immunohistochemistry: anti-laminin together with anti-HS or anti-CS antibodies. Laminin co-localises with HS and CS chains at the BM of neural plate and SE. (C, D) Fibronectin localises to the basement membrane of neural plate and SE; sagittal sections show strong staining in the apical (inner) part of the somitic cells and weak staining at somite borders. (E) Collagen IV is detected in the basement membrane underlying the neural tube and SE. Images acquired by laser-scanning microscopy and deconvoluted post-acquisition. Min. number of embryos for each antibody: 3
3.2.9 Expression pattern of Vangl2 before and after initiation of neural tube closure

The Planar Cell Polarity (PCP) pathway, a non-canonical Wnt signalling cascade, is required for convergent extension (CE) cell movements that shape the gastrulation-stage embryo (Williams et al., 2014). The present study analysed the expression of Vangl2, one of the key proteins of PCP signalling cascade, before and after initiation of neural tube closure, using in situ hybridisation and immunofluorescence on sections and on the whole embryo.

Previous studies suggested that at E8.0 Vangl2 mRNA is restricted to the future hindbrain and upper spine in the closure 1 region (Ybot-Gonzalez et al. 2005; Ybot-Gonzalez et al. 2007), before being expressed in the closed neural tube and the hindgut (Murdoch, Doudney, et al., 2001; Torban et al., 2007). In agreement with published work, Vangl2 transcripts were indeed found to be expressed at high level at the site of closure 1 (Fig. 3.16A). Moderate signal was also detected in the midbrain and caudal regions (Fig. 3.16A). Transverse sections reveal strong expression in the neural plate at hindbrain and closure 1 levels, whereas the signal was reduced in the posterior region. In addition, somite cells and the cells of presomitic and lateral mesoderm express Vangl2 mRNA at the 6 somite stage and, strikingly, transcripts are also detected in the SE and visceral endoderm at all body levels (arrows in Fig. 3.16A-iii-v). At the 9 somite stage, the strongest signal is detected in the closed neural tube and the expression weakens at the level of the brain and open PNP (Fig. 3.16B). Transverse sections show that the neuroepithelium remains the main site of Vangl2 expression at the closure 1 level whereas at the most caudal region the gene is ubiquitously expressed at lower levels. At the 14 somite stage, Vangl2 mRNA continues to be strongly expressed in the closed neural tube, with intense staining also detected in the open midbrain and forebrain (Fig. 3.16C). The neural plate signal becomes weaker in the open PNP. Paraxial mesoderm and SE are also positively stained by the Vangl2 probe.

The expression of Vangl2 protein was subsequently examined by immunohistochemistry (Fig. 3.16D-F). The sections of closure 1 region (five to six somite stage, wild type and Lp mutant) were stained with Vangl2 antibody. Immunofluorescence on transverse sections confirmed that Vangl2 protein is strongly expressed in the membranes of neuroepithelial and SE cells and in cells of the paraxial mesoderm. Immunofluorescence intensity profile of Vangl2 was plotted for neural plate and paraxial mesoderm. Neuroepithelial cells have a stronger intensity than the cells of paraxial mesoderm (Fig. S3.6A, B). Membrane staining was absent in the Lp mutants (Fig. 3.16F). In order to confirm Vangl2 expression in SE cells, the sections were counter-stained with E-cadherin, a known SE marker. E-cadherin is expressed almost exclusively in the adherens junctions of SE and visceral endoderm (Fig. 3.16E-ii).
Figure 3.16. Expression of Vangl2 before and after initiation of the neural tube closure. (A-C) Vangl2 transcripts are strongly expressed by neuroepithelial cells from E8.5 to E9.5. The expression in neural plate weakens at the PNP level. In addition to neural plate expression, mRNA is detected in the SE and, much less intensely, in visceral endoderm and paraxial mesoderm at all body levels. Immunofluorescence on cryosections show that Vangl2 protein is expressed in the neuroepithelium, SE and mesoderm before, during and after closure 1 (D-E) \( (n = 4) \). Double staining with E-cadherin confirms the presence Vangl2 in the SE (E-iv). (F) The membrane staining is lost in all tissues in \( Lp \) mutant embryos \( (n = 3) \). Vangl2 antibody codes: 2G4.
Vangl2 protein co-localises with E-cadherin at the site of closure 1 (Fig. 3.16E-iv and F-iv).

Although, immunofluorescence on sections provides detailed knowledge about expression of the proteins in 2D, it does not allow optimal visualisation of the single cell layer of SE on sections and it is hard to study large regions of the apical surface of neuroepithelial cells. In contrast, whole mount immunofluorescence (WMI) provides a 3D picture of the distribution of an antigen in tissues. WMI protocols were developed in our lab to study the expression of proteins during primary neurulation.

The anti-Vangl2 2G4 was used in all WMI studies and it allowed 3D whole mount analysis of the protein before and after initiation of neural tube closure (Fig. 3.17-3.19). The neural folds come very close to each other at the closure 1 region (square box in Fig. 3.17A) while being far apart in the hindbrain and posterior regions. Consistent with the results of in situ hybridisation and IHC, Vangl2 is detected in the SE and neuroepithelial cells of the closure 1 and posterior region (dorsal view in Fig. 3.17A and B). The protein is expressed in the lateral junctions of the SE and throughout the neuroepithelial cell membranes. Notably, the maximum projection of the ventral surface reveals an increase of Vangl2 expression in the node and the midline (Fig. 3.17C and D). A single z-plane through the closure 1 region highlights the expression of the protein in the membrane of somitic cells and ventral neuroepithelium (Fig. 3.17D and E). Vangl2 is also detected in the lateral junctions in the visceral endoderm cells. WMI study confirmed the loss of membrane staining in the neural plate in Lp mutants (Fig. S3.6D).

Double WMI with E-cadherin was used to verify the presence of Vangl2 in SE during neural tube closure. At the 5 somite stage, E-cadherin is expressed in the lateral junctions of SE cells which wrap the neural folds dorsally at the closure 1 region (Fig. 3.18A-ii, B-ii). Overall, E-cadherin-labelled SE cells are localised laterally to the dorsal neuroepithelium. Vangl2 is expressed in both neuroepithelial and SE cells (Fig. 3.18A-i, B-i). Since Vangl2 is detected in neuroepithelium, SE and mesoderm cells, digital separation of the tissues was needed to obtain the expression of the protein in a single cell layer and to remove the signal from underlying tissue such as mesoderm. In-house Fiji script allowed Vangl2 expression to be ‘isolated’ in a single layer: e.g. SE (see Section 2.8 in Methods). The processed images display the signal from SE cells and the apical surface of the neural plate (Fig. 3.18C). These images clearly highlight the expression of Vangl2 in the lateral junctions of SE cells and its co-localisation with E-cadherin signal (Fig. 3.18C-iv-vi). In addition, the apical surface of neural plate is positive for Vangl2 and negative for E-cadherin signal (Fig. 3.18C-iii, vi).
Figure 3.17. Whole-mount immunofluorescence analysis of Vangl2 at E8.5. 3D reconstruction of the closure 1 region (dorsal view in A) and posterior region (dorsal view in B, ventral view in C): Vangl2 is expressed in the SE and visceral endoderm cells. Ventral view shows that the protein is upregulated in the midline and at the node (arrow in –ii and C-iii). Optical re-slice of the closure 1 region from the ventral surface (single z-plane in E) demonstrates the presence of Vangl2 in the somites and floor plate (arrow in E-i) and somites marked as 1s, 2s and 3s in E-iii. Images acquired by laser-scanning confocal microscopy. Min. number of embryos for Vangl2 antibody: 3.
Figure 3.18. Co-localisation of Vangl2 and E-cadherin prior to closure 1. Whole-mount immunofluorescence of Vangl2 and E-cadherin at E8.5. (A, B, C) Dorsal view of the closure 1 region in a 5 somite stage wild type embryo shows that Vangl2 co-localises with E-cadherin in the SE. Expression of E-cadherin is restricted to SE whereas Vangl2 is also expressed in the neural plate. (C) The SE (SE) and apical surface of neural plate (NP) was “isolated” virtually using in-house macros (see Section 2.8). Images acquired by confocal laser-scanning microscopy. Min. number of embryos for each antibody: 3
At E9.5, Vangl2 continues to be expressed in the neuroepithelium and SE cells as shown by 3D whole-mount analysis of the PNP region (Fig. 3.19). Despite the low expression of mRNA in the PNP, Vangl2 protein seems to be strongly expressed in the same region. Vangl2 is detected in neuroepithelial cell membranes and in the lateral junctions of SE cells in both closed and open posterior regions (Fig. 3.19A). In the open PNP, Vangl2 co-localises with E-cadherin in the adherens junctions at the border between the neural fold tips and the most dorsal SE cells (Fig. 3.19B). At the zippering point, both proteins continue to be localised to the adherens junctions of SE cells, where E-cadherin stains intensely, while Vangl2 is also expressed in the neuroepithelial cell membranes (Fig. 3.19C).
Figure 3.19. Double whole-mount immunofluorescence of Vangl2 and E-cadherin during spinal neurulation. (A-C) Dorsal view of 17 somite wild type embryo reveals that Vangl2 is co-localised with E-cadherin in both the closed and open posterior regions. Both proteins are expressed at the lateral junctions of SE cells. Additionally, Vangl2 is expressed in the neuroepithelial cells of recently closed and open neural plate (green in B-i and C-iii). The expression of E-cadherin is upregulated at the zippering point (arrow in C-ii). Images acquired by laser-scanning microscopy and processed by z-projection (A, B) and single Z-plane (C). Min. number of embryos for each antibody: 3.

3.2.10 The morphology of cell protrusions during initiation of neural tube closure

Alongside growth factor signalling, proteoglycans and their chains control the protrusive activity at focal adhesion sites during cell migration by regulating the activity of the small GTPases Cdc42 and Rac1 to induce the formation of filopodia (Couchman, 2010; Lin et al., 2010). For example, syndecan-2 was shown to induce filopodia formation via the neurofibromin–PKA–Ena/VASP pathway (Lin et al., 2007). In turn, knockout of syndecan-4 in fibroblasts results in highly delocalised Rac1 activity that leads to loss of directional migration in response to the ECM (Bass et al., 2007). The presence of protrusive activity including filopodia and ruffles has been described in mouse embryos at the site of neural fold fusion in the spinal region after completion of closure 1 (Rolo et al., 2016b). This study showed that these protrusions are regulated by Rac1 and Cdc42 and the genetic ablation of the former downregulated ruffle-based protrusive activity and resulted in failure of neural tube closure.

To investigate the presence of protrusive activity at the site of closure 1, scanning electron microscopy (SEM) was carried out by Dr Ana Rolo on wild type embryos before, during the onset and after the initiation of neural tube closure. At the 6 somite stage, protrusions consist mainly of membrane ruffles on the edges of neural folds and SE; filopodia are present to a lesser extent in the same region (arrows in Fig. 3.20A-iii). During the onset of neural tube closure (seven somite stage), the protrusions are predominantly filopodial, projecting from both sites (Fig. 3.20B-iii). These protrusions reach across connecting the opposed neural folds. At the time of neural fold fusion, a mixture of filopodia and ruffles are present in the closure 1 site (Fig. 3.20C-iii). The membrane ruffles and filopodia are observed at the surface while the complex filopodial cross-network is seen between the fusing neural folds. Upon the completion of closure 1, protrusions contain mainly finger-like filopodia at the site of neural fold fusion in posterior region (Fig. 3.20D-iii).
Figure 3.20. The localisation of cell protrusions at the closure 1 site (A-D) SEM images showing a dorsal view of the closure 1 region of 6ss (A) and 7ss embryos (B, C), and the PNP region of an 8ss embryo (D). One neural fold is depicted in A and both folds are shown in B-D. The point of neural fold apposition (closure 1) at 6ss exhibits ruffles on the SE cells (top arrow in A-iii) and filopodia emanating from the neuroepithelial cells (lower arrow in A-iii). At 7ss, the region of recently closed neural tube has pulled apart following fixation, and shows filopodial protrusions bridging the midline deep between the folds (white arrow in B-iii), while filo-lamellipodial protrusions are visible more superficially crossing the midline (arrow in C-iii). The zippering point in the posterior neuropore at 8ss (D-iii) is characterised by abundant filopodia, as previously described for early PNP closure (Rolo et al, 2016). Red hot pseudo-colour LUT used in A iii'- D iii'. ss: somite stage. Scale bars: Ai-Di: 100 µm; Aii-DiO: 10 µm; Aiii-DiiOI: 5 µm.
3.3 Discussion

In the last few decades, a better characterisation of ECM composition, structure and function has revealed that the ECM microenvironment plays a critical role in providing chemical and mechanical cues that regulate cell growth, differentiation and morphogenesis. The current work aimed to provide a comprehensive catalogue of ECM molecules and ECM-associated factors (the matrisome), ECM receptors and GAGs during initiation of neural tube closure. A range of techniques including RNA-seq, in situ hybridisation and immunofluorescence analysis allowed the study of expression patterns for specific ECM molecules and Vangl2, one of the key PCP proteins, for which mutant mice are studied in the later chapters of this thesis. The analysis has provided a list of proteoglycan and GAG biosynthetic/turnover genes that may play a role during mouse primary neurulation. Studying gene and protein expression at the site of closure 1 allowed the identification of mRNA tissue sources and the final destination of selected proteoglycans and other ECM molecules. Importantly, it is also enabled the mapping of HS and CS chain expression at the tissue and cellular level. Overall, the study has defined the ECM molecular composition and morphological characteristics of the closure 1 region of wild type mouse embryos.

Several distinct basement membranes (BMs) are present during elevation of the neural folds at the closure 1 region (Fig. 3.21). The first lies between the neural plate and the paraxial mesoderm separating the neuroepithelial cells from the somites. Further BMs localise to the basal surfaces of SE dorsally, and visceral endoderm ventrally. A novel basement membrane is deposited during closure, as the dorsal neuroepithelium changes its basal contact from paraxial mesoderm to SE. This BM becomes shared between two epithelia, the NP and SE, which is an unusual situation as BMs are normally interposed between epithelial and mesenchymal tissues. Immunofluorescence analysis identifies the major components of three BMs (text box in Fig. 3.21). The main network-forming components (laminin, collagen IV and fibronectin) are detected at the basal side of neuroepithelium, SE and visceral endoderm forming a continuous layer of basement membrane. Among HSPGs, perlecan, an essential protein for stabilising the binding of laminin to cells, is also expressed in the BMs at the initial site of neural tube closure. The presence of perlecan in the BMs of closure 1 region emphasises that the matrix is structurally mature since the key role of this protein is the final stabilisation and assembly of the BM (Yurchenco, 2011). This study shows that HS chains are highly expressed in the neuroepithelial and SE BMs and this expression pattern could be associated with the presence of perlecan, and possibly agrin in this tissue. In addition to HS chains, CS epitopes also co-localise to the laminin-
FIGURE 3.21

Basement membranes (BMs) separate the neural plate from mesoderm (BM in blue) and underlie the SE dorsally and visceral endoderm ventrally (BMs in pink). A further BM arises between the basal surface of the neuroepithelium and the SE, in the dorsal neural folds as they become elevated during closure (BM in red). The ECM components of the BMs that were identified by immunofluorescence analysis are listed in the text box. In summary, the BM around the neural plate and underlying surface ectoderm and visceral endoderm is structurally mature and is enriched for laminin, fibronectin and collagen IV; the BM also contains HS-bearing proteoglycans agrin and perlecan and, to a minor extent, the CS-bearing proteoglycan versican. SE: surface ectoderm.

rich BM, and are highly expressed at the interface between dorsal SE and NP. The BM-associated expression of CS chains reflects the localisation of versican at the basal side of neuroepithelial cells.

Although, versican is not considered to be a typical BM component, previous studies showed that this protein is an ECM component in the basement membranes of venules and is actively processed during pathological angiogenesis (Bode-Lesniewska et al., 1996; Fu et al., 2011). Recent study used proteomic approach to detect the BM-associated proteins in human eye tissue and it revealed the presence of Smc3 peptides in the BM of human retinal blood vessels, lens capsules and inner limiting membranes (Uechi et al., 2014). Nevertheless, Smc3 is well known for its role in the cohesion complex and chromosome maintenance (Sun et al., 2013).
Therefore, it remains to be determined whether the basal enrichment of Smc3 mRNA is associated with final destination of the protein being a part of BM or cohesion complex/cell cycle. Bamacan may be a core protein that carries CS chains in the basement membrane of E8.5 mouse embryos.

In summary, BM-associated proteoglycans, GAGs and other components of basal lamina act as a mechanical barrier and a scaffold to which the neuroepithelial and mesodermal cells adhere. Interestingly, CS chains are highly expressed at the somite borders at the site of closure 1, while HS chains are not detected in the matrix between the somites. The ECM becomes deposited at the somite borders after the segmentation from the anterior presomitic mesoderm. Mutations in fibronectin and their α5 integrin receptors affect mesoderm development and the segmentation process in mouse embryos (Watt et al., 1994). However, the role of CSPGs in the in the segmentation of paraxial mesoderm has not been studied in the past.

In addition to the structural BM architecture, this study also identified the main ECM receptors that may regulate BM assembly at E8.5. Here, two integrin subfamilies have been studied in more detail: laminin- and fibronectin-interacting receptors (Fig. 3.22). Among the former, integrins α3β1 and α6β1 are the central receptors that mediate the interaction with the laminin BM, in the closure 1 region. Interestingly, studies in our group showed that integrin β1 is expressed in both SE and NP BMs whereas α3 receptor is localised to the SE and α6 to the NP BMs (Matteo Mole, unpublished data). RNA-seq analysis revealed that the main fibronectin receptors are integrins α5β1 and αvβ1; they are known to be present in the mesoderm and

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**FIGURE 3.22**

**Figure 3.22. Summary of ECM receptor/integrin ligand combinations during the onset of neural tube closure.** Diagram of α- and β-subunit combinations which define the four main integrin sub-families: laminin-, collagen-, fibronectin/RGD- and leukocyte-interacting. The summary is based on the analysis of RNA-seq data for the closure 1 region in the present study.
neuroepithelium respectively (Matteo Mole, unpublished data). Overall, integrin β1 is the most highly expressed subunit that pairs with specific α subunits to facilitate the interaction with fibronectin or laminin in the BMs.

Integrin/ECM ligand cell-matrix interaction may be required for convergent extension cell movements. Previous studies of notochord morphogenesis in the ascidian, *Ciona*, revealed that PCP signalling and the ECM function in parallel to regulate convergent extension (CE). Mutation in the orthologues of Prickle, a component of PCP pathway, or laminin α3/4/5 results in partial loss of CE whereas double mutants display a more severe CE defect (Veeman *et al.*, 2008). Importantly, double knockout mice for α3 and α6 integrins have been observed to display a craniorachischisis phenotype that closely resembles that of PCP mutants (De Arcangelis *et al.*, 1999).

Besides having a structural role, GAGs and corresponding core proteins may play a role in the initiation of neural tube closure by interacting with secreted factors that are involved in the neurulation process. During the onset of neural tube closure the members of the glypican and syndecan families of proteoglycans are expressed together with the ligands of canonical and non-canonical Wnt, FGF, BMP and Shh pathways, known to play a role in primary neurulation (Ybot-Gonzalez *et al.*, 2002; Hoch, 2006; Patricia Ybot-Gonzalez, Gaston-Massuet, *et al.*, 2007; Andre *et al.*, 2015). Among glypicans, Gpc3 is the most highly expressed gene that is mainly detected in the dorsal neuroepithelium and somites. In contrast, Gpc1 mRNA is more ubiquitously expressed during the onset of neural tube closure. Glypicans are GPI-anchored proteoglycans, therefore, the mRNA-producing cells should express the protein on their surface. The HS staining at the neuroepithelial and mesodermal cell membranes could be associated with expression of glypicans in these tissues. Recent work showed that glypican-3 binds to Frizzled receptors 4, 7 and 8, therefore acting directly in the regulation of canonical Wnt signalling (Capurro *et al.*, 2014). On other hand, glypican-1 was shown to play an important role in early neurogenesis by regulating FGF17 signalling (Fuerer *et al.*, 2010). Gpc1<sup>−/−</sup> mice display a significant reduction in brain size that is detected very early during neurogenesis (E8.5-E9.5).

Syndecan–1 is the most highly expressed member of the syndecan family. It is a hybrid proteoglycan that can have both HS and CS chains attached to the core protein. Therefore, the expression of CS chains in the cell membrane of neuroepithelium, SE and mesoderm could be associated with the presence of syndecan-1 in these cell types. A new role for syndecan-1 is associated with its ability to reach the nuclei in a variety of cell types. One study showed that the transport of syndecan-1 to the nucleus is regulated by a mechanism that needs HS chains as this process is inhibited by chlorate (Stewart *et al.*, 2015). Although, syndecan-1 is not
detected in nuclei at the closure 1 region, nevertheless HS chains were found to be expressed in the nuclei of most dorsal cells of the neuroepithelium, SE, and mesoderm.

The identification of HS in the nucleus was reported as early as 40 years ago although, this unusual localisation of HS alone, or as a part of core protein, became accepted only recently (Kovalszky et al., 2014). A number of studies verified the presence of proteoglycans in the nucleus of various tumor cells and non-cancerous cell lines and tissues. For instance, syndecan-1 has been found in the nuclear compartment of a wide range of cancer types including breast carcinoma, chondrosarcoma, neuroblastoma and multiple myeloma (Schrage et al., 2009; Kovalszky et al., 2014). In turn, glypicans have been found in the cell nucleus of neurons and glioma cells (Liang et al., 1997). The nuclear HS staining could be associated with other proteoglycans such as syndecan-2, glypican-1 and glypican-3 all of which were shown to be expressed in the nuclei of neurons and other brain cell types (Kovalszky et al., 2014). The functions of nuclear proteoglycans and their HS chains are mainly attributed the interaction of HS with secreted factors and various nuclear structures. For instance, nuclear HS chains regulate the activity of histone acetyl transferase and DNA Topoisomerase I (Buczek-Thomas et al., 2008).

Mutant and targeted gene deletions that cause failure of closure 1 in mouse embryos have been mapped to components of the PCP pathway (Juriloff et al., 2012). Beside their importance in CE and neural tube closure, the expression analysis of PCP genes has not been performed in great detail especially at the protein level. This study analysed the expression pattern of the core PCP component Vangl2 before and after the initiation of neural tube closure. Previous studies showed Vangl2 mRNA is mainly expressed in the neural plate, with weak staining also detected in the caudal mesoderm (Murdoch, Doudney, et al., 2001; Torban et al., 2007). In the present work, expression analysis revealed the presence of Vangl2 protein throughout neuroepithelial cell membranes at both closure 1 and more posterior axial levels. Vangl2 mRNA and protein were also detected in SE cells and in the somites. Vangl2 co-localises with SE marker E-cadherin at the closure 1 site (E8.5) and at the PNP level (E9.5) (Fig. 3.16E-F; Fig. 3.18 and Fig. 3.19). In fact, a recent study showed that Vangl2 co-immunoprecipitates with E-cadherin and regulates cadherin internalization in embryonic kidney cells (Nagaoka et al., 2014). An interesting question in this context is the role of Vangl2 and other components of PCP pathway in the SE. This tissue was shown to be essential for the formation of dorso-lateral hinge points during spinal neurulation (Ybot-Gonzalez et al., 2002), however, the requirement of SE for closure 1 has not yet been addressed. In addition to the neural plate and SE expression, Vangl2 protein is also detected in the membranes of paraxial mesoderm cells.
FIGURE 3.23

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**Figure 3.23. Summary of immunofluorescence analysis of GAGs, proteoglycans and Vangl2 expression at the closure 1 site.** The results are based on immunofluorescence analysis of sections and whole mounts. Expression patterns were analysed for HS and CS chains, perlecan, versican, syndecan-1 and Vangl2 protein prior to the initiation of neural tube closure. Colour code: dark green – expressed at high/moderate level; light green – expressed at low level; red – not present. BM: basement membrane; CM: cell membrane; NS: nuclear staining; LJ: lateral junctions; SB: somite borders; SE: SE; NP: neural plate.

This tissue undergoes CE that is dependent on the PCP pathway in zebrafish, *Xenopus* and mouse (Ninomiya et al., 2004; Yen et al., 2009). In summary, RNAseq, in situ hybridisation and immunofluorescence analysis (Fig. 3.23) have all revealed that Vangl2, proteoglycans and their GAG chains are co-expressed in the neuroepithelium, paraxial mesoderm and SE of the closure 1 region.

Hence, there is great potential for PCP-PG and PCP-GAG interactions during the morphogenetic events of closure 1. In the next chapter, the potential role of Vangl2-GAG interactions in closure 1 are tested using whole embryo culture of *Vangl2* mutant embryos in the presence of inhibitors of GAG sulfation and of proteolytic enzymes that cleave GAG chains.

The present study analysed protrusions during the onset of NT closure and found that the predominant protrusions are filopodial, projecting from both sites. It is important to note, that neuroepithelial cells appear to become apposed prior to the SE cells meeting at the midline during the onset of neural tube closure (arrow in Fig. 3.16E-iv). It suggests that the first point of contact may come from neuroepithelial cells rather than cells of the SE. Upon neural fold fusion, SE completely covers the closed neural tube dorsally (arrow in Fig. 3.16F-iv).
3.4 Supplementary material
Table S3.1
CORE MATRISOME: hierarchical expression of genes, RPKM normalised counts
Category
ECM Glycoproteins
Collagens
Collagens
Proteoglycans
ECM Glycoproteins
ECM Glycoproteins
ECM Glycoproteins
Proteoglycans
Collagens
ECM Glycoproteins
ECM Glycoproteins
ECM Glycoproteins
ECM Glycoproteins
ECM Glycoproteins
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Collagens

Gene
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Lama5
Hspg2
Col4a2
Pxdn
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Lamc1
Lama1
Igfbp5
Nid2
Col2a1
Fbln1
Col5a1
Hapln1
Fras1
Col4a5
Ntn1
Smoc1
Col1a1
Col1a2
Col3a1
Fbn1
Ltbp1
Igfbp4
Thbs4
Svep1
Nid1
Sparc
Col5a2
Reln
Col27a1
Slit2
Vwa9
Hmcn1
Lamb2
Thbs3
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Nepn
Spon1
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Tnc
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Ddx26b
Ltbp4
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Col4a6
Col9a1
Egflam
Col11a1
Col11a2
Vtn
Igfbp2
Creld2
Slit1
Lama4
Emilin2
Crim1
Vwa2
Fbln2
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Ensembl gene ID

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Table S3.2

MATRISOME-ASSOCIATED GENES: hierarchical expression of genes, RPMK normalised counts
Table S2. Table of normalised read counts of matrisome-associated RNA-Seq analysis. Transcript abundance obtained by quantification and normalisation of raw counts and presented in log2 scale. Read counts per Kilobase of exon per Million fragments mapped reads (RPKM). Read counts are reported for each of the two replicates with average expression values. The lower cut-offs 3 read counts.
Figure S3.1. The comparison of closure 1 and tissue-specific RNA-seq (Werber et al., 2014): positive and negative control sets. (A) Schematic of the tissues dissected from 3-6 somites mouse embryos and schematic of cell lineages derived from the epiblast; dissected tissues are framed (adapted from Werber et al., 2014). (B) RNA-seq of closure 1: transcript abundance presented in log2 scale Reads Per Kilobase of exon per Million fragments mapped reads (RPKM); read counts are reported as average of two replicates; the lower cut-off is 5.3 RPKM read counts. Tissue-specific RNA-seq (Werber et al., 2014): transcript abundance showed in log2 scale Fragments Per Kilobase of transcript per Million mapped reads (FPKM); read counts are reported per tissue type. PSC: presumptive spinal cord; CEM: caudal end mesoderm. (C) Right to left hierarchical clustering of positive and negative control sets based on the expression values presented by the heatmap.
Figure S3.2. Schematic representation of browser window for 5 genes from Werber et al., 2014. PSC, somites and CEM RefSeq annotations are visualised via UCSC browser. The x-axis shows the coverage of RNA-seq tracks and the y-axis display the number of reads counts in linear scale. The reads are reported per tissue type. FPKM: Fragments Per Kilobase of transcript per Million mapped reads.
Figure S3.3. Validation of GAG proteolytic enzymes and primary antibodies against CS and HS chains in vitro. Cryosections were pre-treated with enzyme buffer prior to IHC against CS or HS chains. (A) CS chains show normal expression pattern after the treatment with enzyme buffer. (B) The staining of CS chains is strongly reduced after the treatment with Chondroitinase ABC. (C) Heparitinase (Hep.III) does not affect the CS chain distribution. (D) HS chains display normal expression pattern after the buffer treatment. (E) Heparitinase treated section show strong reduction of HS staining. (F) Chondroitinase ABC does not affect the expression pattern of HS chains. Min. number of embryos for each antibody with or without enzymatic treatment: 4.
FIGURE S3.4

Figure S3.4. Biosynthesis of HS chains during initiation of NT closure. HS synthesis begins with addition of the first N-acetylgulcosamine residue to the linker region by GlcNAc transferase I (encoded by Extl3 genes). Follows that, the HS chain is elongated by the alternative addition of glucuronate and N-acetylgulcosamine. This reaction is catalysed by HS-polymerase complex that possesses both glucuronate transferase and N-acetylglucosamine transferase activity. The enzyme complex is encoded by Ext1 and Ext2 genes. The next step involves removal of N-acetyl group from N-acetylgulcosamine by bifunctional enzyme N-deacetylase N-sulfotransferase (Ndst1 and Ndst2 genes). Some glucuronate residues are epimerised by glucuronate C5 epimerase encoded by Glce gene. In the final steps, the sulfate groups are added to C2, C3 and C6 positions of sugar residues by the respective 2-O, 3-O and 6-O sulfotransferases (encoded by Hs2st1; Hs3st3b1; Hs6st1 and Hs6st2 respectively). The genes that encode catalytic enzymes are depicted in red. GlcA: glucuronate, GlcNAc: N-acetylglucosamine, IdoA: iduronate, NDST: N-deacetylase N-sulfotransferase, OST: O-sulfotransferase.
Figure 3.5. Biosynthesis of CS chains during initiation of NT closure. CS synthesis begins with addition of N-acetylgalactosamine to the linker region by GalNAc transferase I encoded by Chsy1 gene. Elongation of CS chain follows with alternating addition of glucuronate and N-acetylgalactosamine (Chsy1, Chpf and Chpf2 genes). The GAG chain is then sulfated by 4-O (encoded by Chst11 gene) and 6-O sulfotransferases (Chst2) to produce CS-A and CA-C chains respectively. Another sulfotransferase (encoded by Chst15 gene) precisely catalyses 6-O-sulfation of the already 4-O-sulfated GalNac (4,6-SO₄) to produce CS-E chain type. GlcA: glucuronate, GalNAc: N-acetylgalactosamine, IdoA: iduronate, OST: O-sulfotransferase.
Figure S3.6. Vangl2 intensity profile and comparison of protein expression in wild type and Lp mutant.
(A) Immunofluorescence of Vangl2 protein on tissues sections. (B) Vangl2 fluorescence intensity profiles in neural plate (NP) and mesoderm (ME). The ME intensity is lower than NP intensity (n = 3 for each genotype). (C, D) Single z-plane from WMI of Vangl2 shows staining in the neuroepithelial membrane in wild type embryo (cranial region). The membrane staining is lost in the matched region of Lp mutant embryo. Scale bar: 100 µm in C, D.
4. The requirement for sulfated GAG chains during initiation of NT closure

4.1 Introduction

Neurulation is the series of embryonic events that gives rise to the closed neural tube (NT), the precursor of the brain and spinal cord. In vertebrates, this process originates with the formation of a neural plate - a thickening of the dorsal surface ectoderm which subsequently folds and fuses into the NT. In the mouse closure initiates at the hindbrain/cervical boundary at the 6-7 somite stage (Copp et al., 1994b). Upon completion of the initial closure event (termed closure 1), the neural folds continue to close uni- or bi-directionally to form the NT that eventually extends from forebrain to low spine. Brain and spine closure both progress by a discontinuous series of ‘zippering’ events, so that the fusion process takes place as an extension of a recently closed region. At lower spinal levels, the closing neural folds are flanked by unsegmented mesoderm. In contrast, closure 1 is a *de novo* event that occurs mid-way along the open neural groove at the level of the 3rd somite (Sakai, 1989b). Initiation of NT closure happens at very similar stage and somite level in human (O’Rahilly et al., 2002) and chick embryos (Van Straaten et al., 1996). The closure 1 site differs morphologically from closure at all other body levels. During cranial and spinal neurulation, the closing neural plate bends focally at the midline (median hinge point, MHP) and/or dorsolaterally (dorsolateral hinge points, DLHPs) (Ybot-Gonzalez, et al., 2002; Ybot-Gonzalez, et al., 2007). In contrast, the closure 1 site does not show focal bending at MHP and DLHPs; instead, the neural plate displays a ‘horseshoe-type’ morphology in cross section. In addition, the closing neural folds are directly adjacent to epithelial somites, not unsegmented mesoderm as at other body levels. Hence, the unique neural plate morphology and mesoderm relationship of the initial closure site suggests that the developmental mechanisms underlying the closure 1 may differ from those at cranial and spinal levels.

Initiation of NT closure requires signalling via a non-canonical Wnt cascade, termed the PCP pathway. This signalling cascade regulates convergent extension cell movements that shape the embryo during gastrulation (Skoglund et al., 2010a). NT closure is disrupted in mice homozygous for mutations in the PCP pathway making PCP the only known signalling cascade required for initiation of NT closure (Wallingford, 2012). A similar phenotype is observed in double heterozygotes of core PCP gene mutations demonstrating the essential nature of multiple protein components within this pathway. *Vangl2*°/°, the mutant heterozygote, is usually normal except for occasional spina bifida and a looped tail (Juriloff et al., 2012). However, many genetic combinations of the *Lp* mutation with other PCP-gene mutants result in NTDs, including craniorachischisis (CRN) (Murdoch et al., 2014). Furthermore, the *Lp* allele
has also been used to identify genes outside the PCP pathway that may participate in the PCP signalling and NT closure. For example, the Lp mutation interacts genetically with Syndecan 4 (Sdc4) loss of function to disrupt spinal neurulation (Escobedo et al., 2013).

In general, all eukaryotic sulphation reactions require active sulfate donor 3'-phosphoadenosine 5'-phosphosulfate (PAPS). The addition of sulfate group to various biomolecules is thought to be high affinity/low capacity conjugation system. It can be understood that the supply of the PAPS precursor is rate-limiting and therefore, sulfation process could be controlled in general by the availability of PAPS.

Proteoglycans possess chains of sulfated GAGs that bind and regulate the activity of many secreted factors during development; these interactions are dependent on the degree of sulfation, epimerisation and acetylation of the chains (Gorsi et al., 2007; Iozzo et al., 2015). All GAGs are O-sulfated, with the exception of hyaluronan which is unsulfated. HS chains and heparins are, in addition, N-sulfated on many of their GAG residues. The sulfate donor for GAG sulfation is 3'PAPS.

The donor is synthesised by two sequential enzymatic reactions using ATP sulfurylase in the first reaction and adenosine-5’phosphosulfate kinase in a second reaction. In mammals, the bifunctional enzyme PAPS synthetase catalyses both reactions (Humphries et al., 1988).

\[
\text{Reaction 1: } \text{ATP} + \text{SO}_4^{2-} \rightarrow \text{APS} + \text{PP}_i
\]

\[
\text{Reaction 2: } \text{APS} + \text{ATP} \rightarrow \text{PAPS} + \text{ADP}
\]

Chlorate is a competitive inhibitor of PAPS synthesis. The inhibitor acts as sulfate analogue and competes with SO\(_4^{2-}\) in Reaction 1 that results in the production of 3'-phosphoadenosine 5'-phosphochlorate (PAPC). Accordingly, as the concentration of chlorate in the cells increases, the amount of PAPS is reduced. Under these conditions, the sulphotransferase reactions proceed more slowly. Sulfation of GAGs takes place in the trans-Golgi and is the final step in the processing of GAGs in the secretory pathway. Therefore, when the cells are treated with chlorate, changes in the sulfation of secreted GAGs can be seen within a few minutes, and the full effect of chlorate treatment is usually observed in about 30 minutes (Conrad, 2001).

Previous studies showed that heparan and chondroitin sulfate are the main sulfated glycosaminoglycan chains synthesised at the time of primary neurulation (Solursh et al., 1977; Copp et al., 1988; Yip et al., 2002). The present study confirms that HS and CS chains are expressed in the BM and on the cell surfaces of neuroepithelium and surface ectoderm, as well as in the adjacent mesoderm, at both the closure site 1 and posterior neuropore region (Chapter 3 and Yip et al., 2002). Previous studies of wild type embryos showed that HS chains
are essential for cranial and spinal neurulation: degradation of these chains by heparitinase resulted in inhibition of cranial NT closure (Tuckett et al., 1989). Moreover, spinal neurulation was affected by treating embryos with the competitive inhibitor of GAG sulfation, chlorate (Yip et al., 2002). Treated embryos displayed accelerated PNP closure along with suppression of neuroepithelial bending. On the other hand, the role of CS chains in primary neurulation remains unclear.

Although sulfated GAGs are known to be required for cranial and spinal neurulation, their role in the initiation of NT closure has not been previously examined. This chapter aims to address the potential importance of HS and CS chains in closure 1 by exposing Vangl2^{Lp/} embryos in culture to chlorate treatment, or GAG digestion by heparitinase or chondroitinase, prior to the initiation of NT closure. The effect of the chlorate treatment is independently tested in a targeted transgenic line Vangl2^{floox/}. The effects of these treatments on NT closure and other morphological parameters are studied in whole embryo culture, while the specificity of the treatments is verified by immunofluorescence analysis and rescue experiment.
4.2 Results

4.2.1 Inhibition of GAG sulfation disrupts NT closure in Vangl2\textsuperscript{Lp/+} embryos

4.2.1.1 Titration of chlorate concentration

Chlorate is a competitive inhibitor of GAG chain sulfation and is commonly used to investigate the role of sulfate groups in proteoglycan function (Conrad, 2001). Cell exposed to chlorate produce low or unsulfated GAG chains (Fig. 4.2A). Chlorate has been added to both cell and embryo cultures at concentrations up to 30 mM to inhibit sulfation with no other apparent adverse effects on GAG or protein synthesis or cell viability (Greve \textit{et al}., 1988; Humphries \textit{et al}., 1988; Yip \textit{et al}., 2002).

Titration of chlorate concentration in whole embryo culture was performed in order to identify a minimum concentration that would affect closure 1 in Vangl2\textsuperscript{+/+} and Vangl2\textsuperscript{Lp/+} embryos without toxic effects on embryonic growth and development. E8.5 wild type embryos (BALB/c genetic background) at the 0-5 somite stage (prior to closure 1) were cultured for 24 hours in the presence of different concentrations of chlorate, or water as a control (Fig. 4.1). The embryos were examined at the end of culture for the following health parameters: yolk sac circulation and shape, and heart beat (see Chapter 2, Section 2.2.2.1). Comparison of yolk sac circulation among embryos exposed to 0, 1, 5, and 10 mM chlorate showed no statistical differences between groups (Fig. 4.1A). Moreover, embryos that were exposed to chlorate at 10 mM or less had no adverse effects on heart beat or yolk sac shape (Fig. 4.1A-C). In contrast, 20 and 30 mM concentrations of chlorate significantly reduced yolk sac circulation (p < 0.001).

In addition, embryos treated with 30 mM displayed sub-epidermal blebs and moderate waviness of the NT (data not shown). Previous study in our lab showed that 30 mM chlorate had no effect on embryo (E9.0-E9.5) health parameters during whole embryo culture (Yip \textit{et al}., 2002). However, in this study younger embryos (E8.0-E8.5) were used for the culture experiments and hence, these individuals could be more sensitive to the higher concentration of chlorate than the older embryos. 60 mM chlorate severely affected all health parameters and was classified as toxic (Fig. 4.1C). It is important to note that a previous study used 15 mM chlorate in whole embryo culture experiments on E8-8.5 mouse embryos without affecting embryo health parameters (Oki \textit{et al}., 2007). Chlorate concentrations of 20 mM or less were used in subsequent culture experiments on Lp/+ embryos.
Figure 4.1. Effect of chlorate concentration on embryo health parameters during embryo culture. E8.5 mouse embryos (0-5 somite stage) were cultured for 24 hrs in the presence of 0, 1, 5, 10, 20, 30 or 60 mM chlorate. Embryo health parameters were measured after the culture by applying scores to: YS (yolk sac) circulation (3=very good, 2=good, 1=present, 0=absent); heart beat (1=present, 0=absent); YS shape (round shape – 1, wrinkled - 0). (A) Mean (+ SEM) YS circulation score and the results of independent t-test that show a significant difference between 0 mM and 20 mM, and between 0 mM and 30 mM (**p < 0.001). (B) The percentage of the embryos with good heart beat. (C) Summary table representing the effect of chlorate on the embryo health parameters. Red arrows indicate adverse effects of chlorate concentration on embryo health parameters. YS circulation and heart beat were not detected in the 60 mM group.

4.2.1.2 Severe NT defects develop in chlorate treated Vangl2<sup>Lp/+</sup> embryos

Wild-type and Lp/+ embryos at 0-5 somite stage (tested separately on Balb/c and CBA genetic backgrounds) were used in whole embryo culture experiments. E8.5 embryos at 0-5 somite stage (prior to closure 1) were cultured for 24 hours in the presence of 20 mM, 10 mM, 5 mM chlorate or vehicle (water) (Fig. 4.2). To ensure quality of data, the embryos were allocated randomly to different treatment groups. Analysis of embryos after culture was performed blind to genotype and treatment. Treated embryos were examined for the completion of closure 1 after the end of culture (Fig. 4.2B). Both +/- and Lp/+ embryos cultured in the presence of water completed closure 1.
FIGURE 4.2

Figure 4.2. The effect of chlorate on the initiation of NT closure in +/- and Lp/+ embryos.

(A) Experimental setup of chlorate treatment: E8.5 littermate embryos at 0-5 somite stage (prior to closure 1) were cultured for 24 hours with or without chlorate, the competitive inhibitor of GAGs sulfation. (B) The chlorate concentration was titrated on +/- and Lp/+ embryos from two genetic backgrounds: BALB/c on left and CBA on the right. The percentage of open NT in different treatment groups was analysed after culture with 20, 10, 5mM of chlorate or H2O. Treatment with 20 and 10 mM (BALB/c and CBA) and 5 mM chlorate (CBA only) significantly affected NT closure in Lp/+ compared to +/- embryos. Water treatment had no significant effect on NT closure on both +/- and Lp/+ embryos. (C) Rescue experiment (CBA background): embryos treated with H2O or 10 mM chlorate or chlorate plus sodium sulfate (SO4). Number of embryos is shown per genotype/treatment. *p-value < 0.05; **p-value < 0.01; ***p-value < 0.001; n/s: not significant. P-values calculated by Fisher exact test.
NT closure was significantly affected (open NT) in the Lp/+ embryos treated with 20 mM, 10 mM and 5 mM of chlorate, whereas closure 1 of wild type embryos was not significantly affected by the same inhibitor concentrations (Fig. 4.2B). Importantly, the effect of chlorate concentration on closure 1 showed a similar trend in Lp/+ embryos on two different genetic backgrounds (Balb/c and CBA). The chlorate concentration of 10 mM had no adverse effects on embryo health parameters and this concentration was used in all subsequent culture experiments on Lp/+ embryos (CBA background).

Having found the minimum concentration of inhibitor that was required to perturb NT closure in Lp/+ embryos, the data were then validated by functional rescue experiments (Fig. 4.2C). Embryos were cultured in the presence of 10 mM chlorate alone, chlorate and sodium sulfate (SO₄) or water. All +/- and most Lp/+ embryos completed closure 1 in the control group. A small proportion of +/- failed to initiate NT closure after the exposure to chlorate, however, this effect was not significantly different from the genotype-matched embryos of the water control group. In contrast, chlorate significantly affected the initiation of NT closure in Lp/+ embryos in comparison to controls (p < 0.001). Strikingly, addition of sulfate significantly reduced the number of Lp/+ embryos with open NT. Notably, the percentage of Lp/+ embryos with open NT did not differ significantly between the sulfate rescue and water control groups.

The morphological appearance of chlorate treated embryos is shown in Fig. 4.3. Both +/- and Lp/+ embryos from the water control group undergo normal closure 1 and have an open posterior neuropore as expected at this stage (Fig. 4.3A and B). +/- embryos treated with 20, 10 mM of chlorate have a wavy NT, irregular shaped somites and incomplete axial rotation; however, closure 1 is completed (Fig. 4.3C and E). In contrast, Lp/+ embryos fail to initiate NT closure and develop CRN (entirely open NT) after exposure to 20, 10 and 5 mM of chlorate (Fig. 4.3 D, F and I). Additionally, the somites of these embryos have an irregular shape and structure. Notably, embryos treated with a low concentration of chlorate (5 mM) have a less severe phenotype. Importantly, both +/- and Lp/+ embryos from sulfate rescue group complete closure 1 and do not display other adverse effects on embryo morphology (Fig. 4.3L, M).
Figure 4.3. Inhibition of GAG sulfation by chlorate induces CRN in Lp/+ embryos (CBA background). E8.5 embryos (prior to closure 1) cultured for 24 hours with 20, 10, 5, 1 mM of chlorate (CL), H2O or 10 mM of chlorate plus 20 mM of sodium sulfate (SO4). (A, B) Side view of +/+ and dorsal view of Lp/+ embryos after culture without chlorate. Both +/+ and Lp/+ (arrows in B) embryos from control group complete closure 1 and have an open posterior neuropore as expected at this stage. (C-K) Side views of +/+ and dorsal views of Lp/+ embryos cultured in the presence of 20, 10, 5 and 1 mM of chlorate respectively. +/+ embryos have wavy NT (black arrow in insert E) and incomplete axial rotation (20 and 10 mM CL) after exposure to chlorate. However, these embryos undergo normal closure 1. In contrast, Lp/+ embryos treated with 20, 10 and 5 mM of chlorate fail closure 1 and develop CRN (asterisks in D, F and arrows in I). (L, M) Side view of +/+ and dorsal view of Lp/+ embryos cultured in the presence of chlorate and sulfate. Both genotypes complete closure 1. Scale bar: 0.5 mm in A-M.

4.2.1.3 Chlorate reduces the sulfation of GAGs during whole embryo culture

To confirm the effect of chlorate treatment on sulfated GAGs, immunofluorescence analysis was carried out on the cultured embryos. Transverse sections of the closure 1 region were immunostained with anti-chondroitin sulfate (CS-56) and anti-heparan sulfate antibodies (10E4) (Fig. 4.4 and Fig. 4.5 respectively). For CS chains, wild-type and Lp/+ embryos cultured in the presence of H2O show strong CS staining that is similar to the immunostaining of wild type embryos prior to closure 1 (Chapter 3, Section 3.2.8). CS chains are strongly expressed in the BM around the NT and underlying the surface ectoderm (Fig. 4.4A, D).
Figure 4.4. Chlorate reduces sulfation of CS chains in cultured embryos. Immunofluorescence analysis of CS chain distribution of embryos cultured in the presence of H₂O (control) (A, D), 10 mM chlorate alone (B, E) or 10 mM chlorate plus sodium sulfate (C, F). (A, D) Sections from control +/+ and Lp/+ embryos have normal distribution of CS chains. The chains are localised to the BM underlying surface ectoderm and NT; the staining is detected around mesenchymal cells, notochord and in the neuroepithelium and surface ectoderm. (B, E) Chlorate treatment dramatically reduces the staining of the chains in both genotypes and leads to failure of closure 1 in Lp/+ embryo. (C, F) Staining of CS chains in +/+ and Lp/+ embryos from the rescue group (chlorate plus sulfate) appears to be similar to the control group and both embryos have completed closure 1 (A and D vs C and F). Asterisk: open NT. Minimum three embryos analysed per culture condition/genotype. Scale bar: 50 µm.

Moderate staining is also detected in the paraxial mesoderm and notochord and weak staining is present in the neuroepithelium. In contrast, the staining of CS chains is dramatically reduced in the chlorate treated +/+ and Lp/+ embryos (Fig. 4.4B and E). Immunofluorescent staining of CS GAGs is reduced in intensity and displays a patchy appearance at the basement membrane around the NT and beneath the surface ectoderm. CS appears almost absent from mesenchymal tissue. Notably, the NT remains open in the Lp/+ embryos treated with chlorate; the neural plate of these embryos has convex shape (asterisk in Fig. 4.4E-ii). Addition of sulfate re-establishes the expression pattern of CS chains in the chlorate treated +/+ and Lp/+ embryos (Fig. 4.4C and F). Furthermore, the addition of sulfate rescues the closure 1 failure in Lp/+ embryos (Fig. 4.4F).
Figure 4.5. Chlorate reduces sulfation of HS chains in cultured embryos. Immunofluorescence analysis of HS chain distribution in embryos cultured in the presence of H$_2$O (control) (A, D), 10 mM chlorate alone (B, E) or 10 mM chlorate plus sodium sulfate (C, F). (A, D) Both $^{+}/^{+}$ and $^{Lp}/^{+}$ embryos show normal distribution of HS chains after culture with water addition. HS is strongly expressed in the BMs of NT, surface ectoderm and notochord, and within the mesenchyme. (B, E) Chlorate reduces the immunofluorescent staining of HS chains in all of these locations and leads to failure of NT closure in $^{Lp}/^{+}$ embryos (asterisk in E-ii). (C, F) The staining of HS chains in the $^{+}/^{+}$ and $^{Lp}/^{+}$ embryos from the rescue group (chlorate plus sulfate) is similar to the water control group. Notably, both $^{+}/^{+}$ and $^{Lp}/^{+}$ embryos have completed closure 1 (A and D vs C and F). A minimum of three embryos analysed per culture condition/genotype. Scale bar: 50 µm.

For HS chains, $^{+}/^{+}$ and $^{Lp}/^{+}$ embryos from control group show strong staining in the BM around the NT and notochord, and beneath the surface ectoderm (Fig. 4.5A and D). HS chains are also present in the paraxial mesoderm and visceral endoderm. Previous study showed a reduction of total sulfated HS chains (by 3G10 antibody against non-reducing end of HA chains) in $^{Lp}/^{+}$ and absence of these chains in $^{Lp}/^{Lp}$ at E14.5 (Escobedo et al., 2013). However, this study did not observe a reduction of HS epitopes in $^{Lp}/^{+}$ embryos at E9.0 stage. These differences could be explained by different strategies (antibodies and methods of detection) and embryonic stages used in the studies. Chlorate dramatically reduces the immunostaining of HS chains in both $^{+}/^{+}$ and $^{Lp}/^{+}$ embryos (Fig. 4.5B and E). The immunostaining in BM has
patchy appearance whereas mesenchymal tissues has completely lost staining of HS chains. Addition of sulfate rescues the expression pattern of HS chains in the chlorate treated +/+ and Lp/+ embryos (Fig. 4.5C and F).

4.2.1.4 Chlorate reduces embryo length of Vangl2+/+ and not Vangl2Lp/+ embryos.

To determine if removal of sulfate groups from GAGs has an effect on body elongation, embryo length vs somite number was compared among +/+ and Lp/+ embryos cultured in the presence of vehicle (H2O) or chlorate (Fig. 4.6). $r^2$ measures how close the data are to a fitted regression line. There is no linear correlation in the data when $r^2 = 0.0$ and there is a strong linear correlation when $r^2$ close to 1. In vehicle treated +/+ embryos, $r^2 = 0.86$ (p-value of the slope < 0.001) indicating a strong positive linear relationship between embryo length and somite number, which predicts an increase in embryo length over time during normal developmental progression (Fig. 4.6B). Chlorate reduced the positive linear relationship between embryo length and somite stage in +/+ embryos ($r^2 = 0.52$, $p < 0.001$) (Fig. 4.6B). Comparison of the slopes of the two regression lines shows a significant difference between vehicle and chlorate treated +/+ embryos (p-value of the slopes = 0.016). Confidence intervals (CIs) start to diverge from the 12 somite stage with chlorate treated +/+ embryos having a smaller length than +/+ controls.

The length of vehicle treated Lp/+ embryos does not change significantly from 10 to 16 somites. $r^2 = 0.05$ indicates no linear relationship between embryo length and somite number ($p = 0.21$) (Fig. 4.6C). Linear regression analysis of length shows that embryo length of vehicle treated Lp/+ embryos is statistically different from +/+ littermates in the same treatment group. Comparison of the slopes of the two regression lines shows a significant difference between vehicle treated +/+ and Lp/+ embryos (p-value of the slopes < 0.001). Confidence intervals (CIs) start to diverge from 12 somite stage with Lp/+ control embryos having a smaller length that +/+ littermates (Fig. 4.6C). In chlorate treated Lp/+, $r^2 = 0.42$ shows a positive linear relationship between embryo length and somite stage (p-value of the slope = 0.0015) (Fig.4.6D). At the 0.05 significance level, the slopes of vehicle and chlorate treated Lp/+ embryos are not different significantly (p-value of the slopes = 0.24). The length of chlorate treated Lp/+ embryos was significantly smaller than length of +/+ littermates from the same treatment group (p-value of the slopes = 0.03).

In summary, embryo length of chlorate treated +/+ embryos is significantly reduced compared with vehicle treated +/+ littermates, whereas the length of chlorate and vehicle treated Lp/+ embryos does not differ significantly.
Figure 4.6. Analysis of embryo length after culture. (A) +/- (WT) and Lp/+ (LpHet) embryos were imaged after 24 hour culture (chlorate or water) using bright field stereoscope. Length was measured around the dorsal surface of the embryo, from forebrain to tailbud in Fiji. (B-E) Linear regression analysis of embryo length with 95% confidence intervals (CI) and a best fit line. Data are arranged pairwise, to facilitate comparisons. (B) WT H2O (vehicle) vs WT CL (chlorate); (C) WT H2O vs LpHet H2O; (D) LpHet H2O vs LpHet CL; (E) WT CL vs LpHet CL. (B) Embryo length of WT embryos follows a positive linear correlation pattern with increasing somite number ($r^2 = 0.86$, p-value of the slope is $< 0.001$). Reduction of positive linear correlation between the embryo length and somite stage is observed in chlorate treated WT individuals ($r^2 = 0.52$, p = 0.21). At the 0.05 significance level, the slopes of WT and LpHet control datasets are statistically different (p value of the slopes < 0.001). (D) Chlorate treatment has no effect on embryo length in LpHets; the slopes do not differ significantly between water and chlorate treated embryos. (E) At the 0.05 significance level, the slopes of WT and LpHet datasets from the chlorate group are statistically different (p value of the slopes = 0.03). The length of chlorate treated LpHet embryos is shorter than the length of chlorate treated WT individuals.
4.2.2 Inhibition of GAG sulfation leads to failure of NT closure in Vangl2<sup>flox/-</sup> embryos

To extend the work on the Vangl2<sup>Lp</sup> allele, Vangl2<sup>flox/-</sup> mice (a gift from Prof Deborah Henderson) were bred to generate Vangl2<sup>flox/flox</sup>, Vangl2<sup>flox/-</sup> and Vangl2<sup>-/-</sup> littermates. The present study used a previously generated floxed allele of Vangl2 (Vangl2<sup>flox</sup>) to generate a null allele (via β-actin Cre recombination, prior to the study). First, the penetrance of CRN was analysed after the completion of primary neurulation. Embryos were collected at E10.5-11.5 and the frequency of closure 1 failure was recorded (Fig. 4.7). Both Vangl2<sup>flox/flox</sup>, Vangl2<sup>flox/-</sup> embryos complete primary neurulation (NT is closed) (Fig. 4.7A, D and B, E respectively), whereas Vangl2<sup>-/-</sup> littermates fail to initiate closure 1 and develop the severe neural tube defect, CRN (Fig. 4.7C and F). Therefore, global loss of Vangl2 using Vangl2<sup>flox</sup> allele recapitulates the Vangl2<sup>Lp/Lp</sup> pathophenotype. This agrees with the previously published finding of CRN (100%) in the same knockout (Ramsbottom et al., 2014) (Fig. 4.7G).

Next, embryos from the Vangl2<sup>flox/-</sup> x Vangl2<sup>flox/-</sup> cross were collected at E8.5 (0-5 somite stage) and were used in whole embryo culture experiments. E8.5 embryos at 0-5 somite stage (prior to closure 1) were cultured for 24 hours in the presence of 10 mM chlorate or vehicle (water) (Fig. 4.8). To ensure quality of data, the embryos were allocated randomly to different treatment groups. Analysis of embryos after culture was performed blind to genotype and treatment. Treated embryos were examined for completion of closure 1 after the end of culture (Fig. 4.8A). Both Vangl2<sup>flox/flox</sup>, Vangl2<sup>flox/-</sup> embryos cultured in the presence of water (H<sub>2</sub>O) completed closure 1 (Fig. 4.8B and C), whereas Vangl2<sup>-/-</sup> littermates failed to initiate NT closure in both treatment groups (Fig. 4.8D and G). NT closure was significantly affected (open NT) in the Vangl2<sup>flox/-</sup> embryos treated with 10 mM of chlorate (Fig. 4.8F), whereas closure 1 of wild type embryos was not significantly affected by the same inhibitor concentration (Fig. 4.8E). The penetrance of CRN in chlorate treated Vangl2<sup>flox/-</sup> embryos is significantly lower than the penetrance of this NT defect in chlorate treated Vangl2<sup>Lp/Lp</sup> embryos (Fig. 4.8A). Interestingly, chlorate treated Vangl2<sup>-/-</sup> embryos have a broader neural plate in comparison to the mutants from water-treated control group (Fig. 4.8 D vs G). Chlorate did not change the frequency of CRN in Vangl2<sup>-/-</sup> embryos (100% in both cases).
**Figure 4.7.** *Vangl2*^-/-* mutant embryos fail to initiate NT closure and develop CRN. Normal development at E10.5 and E11.5 in wild type embryos (*Vangl2*^-/^-) and heterozygous embryos (*Vangl2*^-/-) (A, D and B, E respectively). Mutant embryos (*Vangl2*^-/-) display an open NT from midbrain to low spine (arrows in C and F). The midbrain and the forebrain are closed in the mutants. (G) The frequency of failure of closure 1 in the three genotypes.
FIGURE 4.8

Chlorate treatment induces failure of closure 1 in \textit{Vangl2}^{flox/-} embryos. E8.5 embryos (prior to closure 1) cultured for 24 hours with 10 mM of chlorate or H2O. (A) Chlorate significantly affects NT closure in \textit{Vangl2}^{flox/-} embryos (p-value < 0.05). The penetrance of closure 1 failure (open NT) in chlorate treated \textit{Vangl2}^{flox/-} embryos is significantly lower than in chlorate treated \textit{Vangl2}^{Lp/+} embryos (p-value < 0.001). (B-D) Both \textit{Vangl2}^{flox/-} and \textit{Vangl2}^{flox/-} embryos complete closure 1 while \textit{Vangl2}^{+/-} embryos fail to initiate NT closure in the water control group. (E-G) \textit{Vangl2}^{flox/flox} embryos treated with chlorate complete closure 1 and have wavy NT irregular shaped somites, but achieve closure 1, whereas a proportion of \textit{Vangl2}^{flox/-} embryos fail to initiate closure 1 after exposure to chlorate. Note the exceptionally broad neural plate in the \textit{Vangl2}^{+/-} embryo exposed to chlorate. P-values calculated by Fisher exact test. Number of embryos is shown per genotype/treatment. *p-value < 0.05; **p-value < 0.001. Scale bar: 0.5 mm in B-H.
4.2.3 Enzymatic cleavage of GAG chains recapitulates the phenotype of chlorate treated Vangl2Lp/+ embryos

The previous work in this chapter showed that inhibition of GAG sulfation disrupts NT closure specifically in Vangl2Lp/+ and Vangl2flox/flox embryos. Chlorate affects the sulfation of both HS and CS chains, so it was not possible to distinguish what types of GAG chain are required for the initiation of NT closure. In order to address this question, +/- and Lp/+ embryos at 0-5 somite stage were cultured in the presence of specific GAG degrading enzymes: heparitinase III (Hep.III) for HS or chondroitinase ABC (Chr.ABC) for CS. Enzyme buffer was added as control (Fig. 4.9). The enzymes or buffer were injected into amniotic cavity prior to the culture and the injection was repeated after four hours of the culture (Fig. 4.9A). The double injection was necessary to achieve the maximum effect of the enzymatic treatment prior to closure 1 as the single injections were found not to be effective. Single injection of Chr.ABC: 8/8 +/- and 6/7 Lp/+ embryos completed closure 1. Single injection of Hep.III: 7/7 +/- and 5/6 Lp/+ embryos completed closure 1. To ensure quality of data, the embryos were allocated randomly into different treatment groups. Analysis of embryos after culture was performed blind to genotype and treatment. The enzymatic treatment had no adverse effects on embryo health parameters (data not shown). Treated embryos were examined for the completion of closure 1 at the end of culture with Hep.III, Chr.ABC or enzyme buffer (Fig. 4.9B). Both +/- and Lp/+ embryos cultured in the presence of the buffer completed closure 1, whereas NT closure was significantly affected (open NT) in Lp/+ embryos treated with Hep.III or Chr.ABC. A small number of wild type embryos failed in closure 1 after treatment with either Hep.III or Chr.ABC, although the frequency of NT closure failure was not significantly different from wild type buffer-treated controls.

The morphological appearance of embryos treated with enzymes is shown in Fig. 4.9C-H. Both +/- and Lp/+ embryos injected with buffer underwent normal closure 1 and had an open posterior neuropore as expected at this stage (Fig. 4.9C, F). +/- embryos treated with Hep.III had a wavy NT, irregularly shaped somites and incomplete axial rotation; however, closure 1 was completed (Fig. 4.3D). In contrast, Lp/+ embryos failed to initiate NT closure and developed CRN (entirely open NT) after exposure to Hep.III (arrows in Fig. 4.9G). Chr.ABC treated +/- embryos achieved closure 1 and did not display any other abnormalities (Fig. 4.9E). In contrast, Chr.ABc treatment led to failure of closure 1 in Lp/+ embryos (arrows in Fig. 4.9H).
Figure 4.9. Enzymatic cleavage of CS and HS chains induces CRN in Lp/+ embryos.

(A) Experimental setup of enzymatic treatment of E8.5 embryos at 0-5 somite stage (prior to closure 1). Enzyme buffer, heparitinase III (Hep.III, to cleave HS chains, 2U/ml) or chondroitinase ABC (Chr.ABC, to cleave CS chains, 4U/ml) were injected into amniotic cavity at time 0 and injection was repeated at 4 hours of culture. Embryos were cultured for 24 hours in total. (B) Both +/- and Lp/+ embryos from the control group (enzyme buffer) completed closure 1. Both Hep.III and Chr.ABC significantly affected NT closure (open NT) in Lp/+ embryos whereas +/- embryos remained largely unaffected. Number of embryos is shown per genotype/treatment. (C, F) Embryos from the control group developed normally and completed closure 1. (D, E) Hep.III and Chr.ABC treated +/- embryos underwent normal closure 1 but displayed a wavy NT (Hep.III only). (G, H) Hep.III and Chr.ABC treated Lp/+ embryos failed to initiate NT closure in most cases (arrows). The somite stage is indicated for each embryo. Scale bar: 0.5 mm in C-H. *p-value < 0.05; **p-value < 0.01; ***p-value < 0.001. P-values calculated by Fisher exact test.
To confirm the effect of enzymatic treatment on GAG chains, immunofluorescence analysis was carried out on the cultured embryos. Transverse sections of the closure 1 region were immunostained with anti-chondroitin sulfate (CS-56) and anti-heparan sulfate antibodies (10E4) (Fig. 4.10A-D and E-H respectively).

For CS chains, +/+ and Lp/+ embryos cultured in the presence of enzyme buffer show strong staining that is similar to the immunostaining of embryos cultured in the presence of water (Fig.4.4A, D). CS chains are strongly expressed in the BM around NT and underlying the surface ectoderm (Fig. 4.4A and D). Moderate staining is also detected in the paraxial mesoderm and notochord and weak staining is present in the neuroepithelium. In contrast, the staining of CS chains is almost absent in the Chr.ABC treated +/+ and Lp/+ embryos (Fig. 4.10B and D). Immunofluorescence of CS GAGs display a patchy appearance at the apical surface of neural plate that may resulted from the trapping of cleaved CS chains. Notably, the NT remains open in the Lp/+ embryos treated with Chr.ABC (asterisk in Fig. 4.10D-ii). In contrast, +/+ embryos complete closure 1 even in the absence of CS chains (Fig. 4.10B-ii).

For HS chains, +/+ and Lp/+ embryos injected with enzyme buffer show strong staining of HS chains in the BM around the NT and notochord, and beneath the surface ectoderm (Fig. 4.10E and G). Hep.III dramatically reduces the immunostaining of HS chains in both +/+ and Lp/+ embryos (Fig. 4.10F and H). The immunostaining has a patchy appearance in the BM underlying the surface ectoderm whereas the BM around NT has completely lost HS staining. However, Hep.III treated +/+ embryos complete initiation of NT closure (Fig.4.10F-ii) whereas Lp/+ littermates fail in closure 1 and develop CRN upon the enzymatic cleavage of HS chains (Fig.4.10H-ii).

In summary, the enzymatic cleavage of HS and CS chains by Hep.III and Chr.ABC respectively recapitulates the phenotype of chlorate treated Lp/+ embryos suggesting that both chain types are required for the initiation of NT closure.
FIGURE 4.10

Figure 4.10. Immunofluorescence analysis of embryos cultured with chondroitinase ABC and heparitinase III. (A, C) Both +/+ and Lp/+ embryos from the control group (buffer) show a normal distribution of CS chains after culture. Strong staining is detected in the BMs of the NT and surface ectoderm, and moderate expression in the paraxial mesoderm. (B, D) Chr.ABC injection dramatically reduces the staining of the CS chains in both genotypes and leads to failure of NT closure in Lp/+ embryo (asterisk in D-ii). (E, G) HS chains are normally distributed in embryos injected with enzyme buffer. (F, H) +/+ and Lp/+ embryos injected with Hep.III display very weak staining of HS chains. Hep.III treatment leads to the failure of closure 1 in Lp/+ embryo (asterisk in H-ii). A minimum of three embryos were analysed per culture condition/genotype. Scale bar: 50 µm.
4.2.4 Determining the somite stage at which chlorate treatment is effective in causing NT defects

In the preceding experiments, somite stage was recorded at the end of the culture, but initial somite stage (at the start of treatment) was not recorded for individual embryos. However, an important question arises: is there a developmental ‘window’ (in terms of somite stage) during which chlorate treatment must be delivered in order to cause failure of closure 1? That is, how close to the stage of closure 1 does GAG chain suppression remain effective in blocking closure 1 in Lp/+ embryos? To address this question the somite stage at the start of culture was calculated by subtracting the number of somites formed during the culture period from the number of somites recorded at the end of treatment (Fig. 4.11). Since one somite is added approximately every 2 h, the 24 h culture period was assumed to represent the addition of 12 somites in each case. Embryos were pooled into three somite groups (0-1, 2-3, and 4-5 somites at the start of culture) and the percentage of embryos with open vs closed NT was analysed in each genotype/treatment combination (Fig. 4.11A).

The analysis showed that the small proportion of +/- embryos that fail to initiate closure 1 after chlorate treatment had either 0-1 or 4-5s at the start of treatment, whereas embryos beginning treatment at 2-3s were unaffected (Fig. 4.11B). In contrast, the majority of Lp/+ embryos that failed in NT closure when exposed to chlorate had 0-1s or 2-3s at the start of treatment, while embryos were somewhat less affected when treatment began at 4-5s. Therefore, this analysis indicates that chlorate must be present in the culture environment at least 2 somite stages (4 h) before closure 1 occurs, in order to be maximally effective in blocking closure in Lp/+ embryos. Similar analysis of enzymatic treatment was not performed due to the small number of replicates per somite group/treatment condition.
Figure 4.11. Correlation between somite stage at start of treatment, and closure 1. (A) Schematic to show how somite stage (s) at the start of chlorate was calculated by subtracting the number of somites formed during the culture (2 h = 1 somite; 24 h = 12 somites) from the number of somites recorded at the end of the treatment. Embryos were pooled into three ‘starting’ somite groups: 0-1, 2-3, 4-5. The number of replicates is indicated in each bar. (B) Percentage of open/closed +/+ and Lp/+ embryos is shown for the calculated somite stages at the start of chlorate or water treatment.

4.2.5 Inhibition of GAG sulfation affects neural plate morphology in the closure 1 region of Vangl2+/+ but not Vangl2Lp/+ embryos

4.2.5.1 Analysis of neural plate shape

In order to investigate the embryonic mechanisms leading to failure of NT closure in chlorate treated Lp/+ embryos, the morphology of mid-axial tissues was examined during the onset of closure 1. Cultures were started at the 0-4 somite stage (prior to closure 1) and were continued for 8 hours, in the presence of 10 mM chlorate or vehicle (water). Embryos were
allocated randomly to different treatment groups, and somite stage was recorded at the end of culture. Embryos were fixed and exposed to CellMask™, which stains membranes non-specifically, then imaged using confocal microscopy. Images were processed in Fiji to obtain transverse sections of the closing NT and measurements were made blind to genotype and treatment (Fig. 4.12).

Neural plate morphology of the closure 1 region was examined in 5, 6, 7 and 8 somite stage +/+ and Lp/+ embryos treated with chlorate or water (Fig. 4.12C-F). Confocal microscopy revealed a ‘horseshoe-type’ morphology of the neuroepithelium in +/+ embryos of the water-treated control group; all dorso-ventral regions of the neural plate appear to bend equally in embryos with 5-7 somites (Fig. 4.12C). The distance between the neural fold tips decreases with increasing somite stage and the folds eventually fuse dorsally at the 7 somite stage. In contrast, Lp/+ littermates from the same treatment group exhibit a ‘V’-shaped neural plate with sharper bending at the midline at the 5 and 6 somite stages (Fig. 4.12E-i, ii). The neural folds of Lp/+ embryos elevate and come into contact along the ventro-dorsal axes, and eventually fuse at the 8 somite stage.

Chlorate affects neural plate morphology at the closure 1 region of both +/+ and Lp/+ embryos. The neuroepithelium of chlorate treated +/+ individuals loses the normal ‘horseshoe-type’ morphology, and embryos display a sharp bend in the midline of the neural plate at the 5 and 6 somite stage (Fig. 4.12D). The neural folds of these embryos come in contact and appear to fuse below the dorsal most tips at the 8 somite stage. The neural plate of chlorate treated Lp/+ embryos has a similar morphology to Lp/+ embryos from the control group at 5 and 6 somite stage. However, the distance between the neural fold tips is larger in the chlorate treated versus control Lp/+ embryos, especially at 7 somite stage (Fig. 4.12E vs F). The neural folds become convex in Lp/+embryos treated with chlorate by the 7 somite stage and fail to fuse at the 8 somite stage (Fig. 4.12F-iii-iv).
Figure 4.12. Chlorate effects on neural plate morphology of the closure 1 region in Lp/+ and +/- embryos. +/- (WT) and Lp/+ (LpHet) littermate embryos were cultured for 8 hrs in 10 mM chlorate or vehicle (H2O), fixed, stained with CellMask™ and imaged using confocal microscopy for morphological analysis. The images were re-sliced in Fiji to obtain transverse sections of the closure 1 region, located at the level of the third somite. (A) The distance between the neural folds (NF), medial angle and NF elevation were measured at 10 sequential positions 20 µm apart, moving rostrocaudally along the closure 1 region. (B) The number of embryos analysed per somite stage/genotype/treatment condition. (C-F) Representative images of re-sliced closure 1 regions at different somite stages (5-8 somites) are shown for each condition. (C) The neural plate of WT embryos from the water-treated control group is concave inwards and the NFs contact and fuse at the dorsal region. (E) In contrast, the neural plate of LpHet water-treated embryos is ‘V’ shaped and the NFs appear to first come into contact at a more ventral position than in +/- embryos. Nevertheless, the fused Lp/+ neural tube (8 somites) has a similar morphology to +/- controls. (D) The neural plate of WT embryos from chlorate group loses its concave shape and the NFs appear quite straight at 5 to 7 somite stage. The NFs fuse by the 8 somite stage. (F) The neural plate of chlorate treated LpHet embryos has a convex shape, in striking contrast to the concave shape of +/- control embryos (C) and the NFs fail to fuse, even by 8 somites.
4.2.5.2 Analysis of distance between neural folds tips, medial angle and elevation of neural folds

After analysis of neural plate morphology, a series of measurements were compared: the distance between the neural fold (NF) tips, medial angle and NF elevation (Fig. 4.12A). Measurements were taken at 10 sequential positions rostrocaudally, each 20 µm apart and a minimum of four embryos were used per somite stage/genotype/treatment condition (Fig. 4.12B). Statistical comparison was performed by mixed model analysis in SPSS (see Section 2.9). First, the overall effects of genotype, treatment, position or somite stage (fixed effects) were analyzed for each measurement along the closure 1 region. After that, the first order interactions between fixed effects were computed per parameter. Non-significant interactions were removed from the model and subsequent tests were carried out. When the fixed effects were significant overall, a post hoc Bonferroni correction was used to identify the individual rostrocaudal positions at which the effect was significant (p-value < 0.05). Analyses of embryos at different somite stages (5-7 somites) are shown for all parameters in Fig. 4.13A, the results of statistical tests are displayed in Fig. 4.13B, and the pairwise analyses for each parameter are presented in Fig. 4.14-4.16.
Figure 4.13. Chlorate treatment affects the distance between neural folds and medial angle but not neural fold elevation. +/- (WT) and Lp/+ (LpHet) littermate embryos were cultured for 8 hrs in chlorate (CL) or vehicle (H2O), fixed and imaged using confocal microscopy for morphological analyses. The distance between the neural folds (NF) (A), medial angle (B) and NF elevation (C) were calculated at 10 sequential positions, each 20 µm apart, centred around closure 1. (A-C) Analyses of embryos at different somite stages (5-7 somites) are shown for each parameter. (D) Summary table of fixed effects (genotype, treatment, position and somite_stage) and two-way interactions (Genotype * Treatment, Genotype * Somite_stage, Treatment * Somite_stage) with corresponding p-values for distance between the neural folds, medial angle and NF elevation. Chlorate treatment significantly affects the distance between NFs and medial angle but not NF elevation. Points represent the mean +/- SEM. P-values derived by mixed model analysis.
4.2.5.3 Distance between neural fold tips

In +/- embryos treated with vehicle the distance between NFs decreased between the 5 and 6 somite stage (absolute change across All Positions on Average in µm (APA) 5s vs 6s = 87.2, p < 0.001) and between the 6s (s for somite) and 7s (APA = 30.3, p < 0.001) (Fig. 4.13A). Chlorate significantly increased NF distance in +/- embryos at all somite stages tested overall (APA for 5s = 34.7 p < 0.001; 6s = 20.5, p = 0.014 and 7s = 49.9, p < 0.001), and this difference was only significant at individual sites of 7s embryos following the post hoc adjustments (asterisks in Fig. 4.14A, p < 0.05). NF distance decreased significantly between 5s and 6s in +/- embryos treated with chlorate (APA = 34.7, p < 0.001), but chlorate prevented the reduction in NF distance between 6s and 7s (APA = 20.6, p = 1).

The NF distance of vehicle treated Lp/+ embryos decreased significantly between each of the somite stages tested (APA 5s vs 6s = 81.1, p < 0.001; 6s vs 7s = 47.5, p < 0.001). NF distance was always significantly greater in Lp/+ than somite stage matched +/- embryos overall (APA for 5s = 39.5; p < 0.001; 6s = 39.6, p < 0.001; 7s = 22.5, p = 0.005), but this difference was not significant at individual sites following the post hoc adjustments (4.14B). As seen in +/- embryos, chlorate treatment significantly increased NF distance in Lp/+ embryos at all somite stages tested overall (APA 5s = 42.5, 6s = 69.2, 7s = 89.6 with p < 0.001 for each stage). The effect of chlorate in Lp/+ embryos was significant at most individual positions at 6s and 7s (asterisks in Fig. 4.14C, p < 0.05). NF distance decreased between each somite stage in chlorate treated Lp/+, indicating chlorate did not arrest NF apposition in these embryos (APA for 5s vs 6s = 54.4, p < 0.001; 6s vs 7s = 27.1, p = 0.002).

NF distances in chlorate treated Lp/+ embryos were significantly greater than +/- at each somite stage overall (APA 5s = 47.4, 6s = 88.3, 7s = 62.2, p < 0.001 for each stage), and were significantly different at most positions tested at 6s and 7s (asterisks in Fig. 4.14D, p <0.05). These differences suggest a ‘worsening’ of the effect of chlorate treatment in Lp/+ vs +/- embryos (Genotype*Treatment interaction, p < 0.001). Consistent with this interaction, the increase in NF width in chlorate vs vehicle treated embryos was greater in Lp/+ (APA 5s = 42.5, 6s = 69.2, 7s = 89.6 with p < 0.001 for each stage) than +/- embryos (absolute increase APA 5s = 34.7, p< 0.001; 6s = 20.6, p = 0.014, 7s = 49.9, p < 0.001) (Fig. 4.14A vs 4.14C).
FIGURE 4.14

Distance between neural fold tips

Transverse sections of closure 1 (position 1 to 10 = 200 µm)

Figure 4.14. Chlorate treatment delays neural fold apposition to a greater extent in LpHet than WT embryos. Pairwise analysis of cultured embryos. +/- (WT) and Lp/+ (LpHet) littermate embryos were cultured for 8 hrs in chlorate (CL) or vehicle (H2O), fixed and imaged using confocal microscopy for morphological analyses. The distance between the neural folds was calculated at 10 sequential positions, 20 µm apart, centred around closure 1. (A-D) Analyses of embryos at different somite stages (5-7 somites) are shown. (A) Comparison of vehicle and chlorate-treated WT embryos indicating chlorate significantly increases neural fold distance selectively at the 7 somite stage. (B) Comparison of vehicle-treated WT vs LpHet embryos indicating no significant differences between genotypes at any somite stage. (C) Comparison of vehicle and chlorate-treated LpHet embryos indicating chlorate significantly increases neural fold distance at both the 6 and 7 somite stages. (D) Comparison of chlorate-treated WT and LpHet embryos indicating significantly greater neural fold distances in the LpHet embryos at caudal positions at the 5 somite stage and across all positions tested at the 6 and 7 somite stages. Points represent the mean +/- SEM, n = min 4. * p < 0.05 by mixed model analysis with post-hoc Bonferroni correction.
4.2.5.4 Medial angle

In +/- embryos treated with vehicle the medial angle decreased between 5s and 6s (APA = 40.9, p < 0.001) and between 6s and 7s (APA = 18.5, p < 0.001) (Fig. 4.15A). Chlorate significantly increased the medial angle in +/- embryos at all somite stages tested overall (APA for 5s = 14.2, p < 0.001; 6s = 12.3 p = 0.001; and 7s = 22.7, p < 0.001), and this difference was only significant at individual sites of 7s embryos following the post hoc adjustments (asterisks in Fig. 4.15A, p < 0.05). Medial angle decreased significantly between 5 and 6 somite stage in +/- embryos treated with chlorate (APA = 43, p < 0.001), and to lesser extent between the 6 and 7s (APA = 8, p = 0.006).

The medial angle of vehicle treated Lp/+ embryos decreased significantly between each of the somite stages tested (APA 5s vs 6s = 31.5, p < 0.001; 6s vs 7s = 16.9, p < 0.001). Medial angle was significantly greater in 6s and 7s Lp/+ than somite stage matched +/- embryos overall (APA 5s = 4.3, p = 0.27; 6s = 13.8, p < 0.001; 7s = 15.4, p < 0.001), but this difference was not significant at individual sites following the post hoc adjustments (4.15B). As seen in +/- embryos, chlorate treatment significantly increased medial angle in Lp/+ embryos at all somite stages tested overall (APA 5s = 25.5, 6s = 30.1 and 7s = 37.9, p < 0.001 for each stage). The effect of chlorate in Lp/+ embryos was significant at individual positions at 5s and most individual positions at 6 and 7 somite stages (asterisks in Fig. 4.15C, p < 0.05). Medial angle decreased between 5 and 6 somite stages (absolute decrease APA for 5s vs 6s = 26.8, p < 0.001) and to a lesser extent between 6 and 7 somite stages in chlorate treated Lp/+ (6s vs 7s = 9, p = 0.02). Hence, chlorate did not arrest the reduction of medial angle in these embryos.

Medial angles in chlorate treated Lp/+ embryos were significantly greater than +/- at each somite stage overall (absolute increase APA 5s = 15.5, 6s = 31.7, 7s = 30.7, p < 0.001 for each stage) and were significantly different at all individual positions tested at the 6 and 7ss (Fig. 4.15D). These differences suggest a ‘worsening’ of the effect of chlorate treatment in Lp/+ vs +/- embryos as seen for NF distance (Genotype*Treatment interaction, p < 0.001). Consistent with this interaction, the increase in medial angle in chlorate vs vehicle embryos was greater in Lp/+ (absolute increase APA 5s = 25.5, 6s = 30.1, 7s = 37.9 with p < 0.001 for each stage) than +/- embryos (absolute increase APA 5s = 14.2, p <0.001; 6s = 12.3, p = 0.001; 7s = 22.7, p < 0.001) (Fig. 4.15A vs 4.15C).
Figure 4.15. Chlorate treatment increases medial angle to a greater extent in LpHet than WT embryos. Pairwise analysis of cultured embryos. +/- (WT) and Lp/+ (LpHet) littermate embryos were cultured for 8 hrs in chlorate (CL) or vehicle (H2O), fixed and imaged using confocal microscopy for morphological analyses. The medial angle was calculated at 10 sequential positions, 20 μm apart, centred around closure 1. (A-D) Analyses of embryos at different somite stages (5-7 somites) are shown. (A) Comparison of vehicle and chlorate-treated WT embryos indicating chlorate significantly increases medial angle selectively at the 7 somite stage. (B) Comparison of vehicle-treated WT vs LpHet embryos indicating no significant differences between genotypes at any somite stage. (C) Comparison of vehicle and chlorate-treated LpHet embryos indicating chlorate significantly increases medial angle at caudal positions at 5 somite stage and across all positions tested at the 6 and 7 somite stages. (D) Comparison of chlorate-treated WT and LpHet embryos indicating significantly greater medial angle in the LpHet embryos at both 6 and 7 somite stages. Points represent the mean +/- SEM, n = min 4. * p < 0.05 by mixed model analysis with post-hoc Bonferroni correction.
4.2.5.5 Elevation of neural folds

In +/+ embryos treated with vehicle and chlorate the elevation of NFs does not change significantly between the 5 and 6 somite stages (vehicle: APA = 3.43, p = 0.1; chlorate: APA = 2, p=0.15) but significantly increases between the 6s and 7s (vehicle: APA = 20.3, p < 0.001; chlorate: APA = 14.1, p <0.001) (Fig. 4.16A). Chlorate had no significant effect on NF elevation in +/+ embryos at any somite stage tested.

The NF elevation of vehicle treated Lp/+ embryos did not change significantly at any of the somite stages tested (5s vs 6s p = 0.48; 6s vs 7s p = 0.27). NF elevation was significantly greater in 5 and 6s and lower in 7s Lp/+ than somite stage matched +/+ embryos overall (APA 5s = 15.9, p < 0.001; 6s = 19.4, p < 0.001; 7s = - 8.84, p =0.005) and this difference was only significant at individual sites of 5 and 6s embryos following the post hoc adjustments (asterisks in Fig. 4.16B). Chlorate significantly decreased NF elevation of Lp/+ embryos at 5s only (overall APA = 20.9, p < 0.001) and this difference was significant at caudal positions of 5s embryos following the post hoc adjustments (asterisks in Fig. 4.16C, p < 0.05). Elevation of NFs increased significantly between 5 and 6 somite stage (absolute increase APA for 5s vs 6s = 19.1, p < 0.001) and there was no significant change of NF elevation between 6 and 7 somite stages in chlorate treated Lp/+ (p = 0.81).

NF elevation in chlorate treated Lp/+ embryos was significantly greater than +/+ only at 6 somite stage (APA = 16.0, p < 0.001) but there were no significant differences at any individual positions tested (Fig. 4.16D). Treatment had no significant effect on NF elevation in +/+ embryos. The reduction of NF elevation in chlorate vs vehicle embryos was only observed in 5s Lp/+ (APA 5s = 20.9, p < 0.001) and not in +/+ (p > 0.05) (Fig. 4.16A vs 4.16C).
Figure 4.16. Chlorate treatment has no effect on neural fold elevation in either WT or LpHet embryos. Pairwise analysis of cultured embryos. +/- (WT) and Lp/+ (LpHet) littermate embryos were cultured for 8 hrs in chlorate (CL) or vehicle (H2O), fixed and imaged using confocal microscopy for morphological analyses. The neural fold elevation was calculated at 10 sequential positions, 20 μm apart, centred around closure 1. (A-D) Analyses of embryos at different somite stages (5-7 somites) are shown. (A) Comparison of vehicle and chlorate-treated WT embryos indicating no significant differences of neural fold elevation at any somite stages. (B) Comparison of vehicle-treated WT vs LpHet embryos indicating significantly greater elevation of neural folds in LpHet embryos at caudal positions at 5 and 6 somite stage. (C) Comparison of vehicle and chlorate-treated LpHet embryos showing significantly greater elevation of neural folds in vehicle treated LpHet at caudal positions at 5 somite stage. (D) Comparison of chlorate-treated WT and LpHet embryos indicating no significant differences between genotypes at any somite stage. Points represent the mean +/- SEM, n = min 4. * p < 0.05 by mixed model analysis with post-hoc Bonferroni correction.
4.3 Discussion

A major advance in the understanding of aetiology of closure 1 failure comes from the genetic studies on PCP mutants and PCP-associated genetic effects (Savory et al. 2011; Wallingford 2012; Murdoch et al. 2014). Although, these studies highlight a critical requirement of the PCP pathway for initiation of NT closure, it remains likely that other genes and signalling cascades also play a role in the initial closure event in mammals.

This chapter has examined the role of sulfated GAGs in the initiation of NT closure in mouse embryos. An *ex vivo* approach was taken to study the requirements of GAG chains for the initial closure event using chlorate, a competitive inhibitor of GAG sulfation. Functional studies based on the expression analysis demonstrated that impaired sulfation of GAG chains worsens the phenotype of PCP mutant *loop tail* (*Vangl2*<sup>LP</sup>) embryos, which are predisposed to neural tube defects. Exposure of *Lp/+* embryos to chlorate during *ex vivo* whole embryo culture prevented NT closure, converting *Lp/+* to the mutant *Lp/Lp* pathophenotype. Previous studies on genetic interactions between PCP genes demonstrated a variable penetrance and severity of the NT defects between different double-heterozygous combinations (Murdoch *et al.*, 2014). However, this work shows a similar penetrance of CRN between chlorate treated *Lp/+* embryos from two different genetic backgrounds. The specificity of the treatment was verified by immunofluorescence and a functional rescue experiment. Chlorate effectively reduced the sulfation of both HS and CS GAGs during the culture and this effect on the chains was successfully rescued by supplementation of the culture medium with sulfate. More importantly, the frequency of closure 1 failure was significantly reduced in *Lp/+* embryos supplemented with sulfate. The results of the rescue of HS and CS expression pattern agrees with the previously published studies showing a reversible effect of chlorate on GAG sulfation during cell, tissue, organoid and embryo cultures (Humphries & Silbert 1988; Yip *et al.* 2002; Shannon *et al.* 2003). Exogenous sulfate groups compete with chlorate intracellularly and increase the availability of the sulfate donor (PAPS) to sulfotransferases that subsequently results in the production and secretion of normally sulfated GAGs (Safaiyan *et al.*, 1999).

Previous studies have investigated the effects of various concentrations of chlorate on the degree of GAG sulfation. One study showed that low concentrations of chlorate (up to 10 mM) significantly affect the sulfation of CS chains at positions 4, 6 and 0 and only affect heparan sulfation at the 6-O position (Humphries *et al.*, 1988; Safaiyan *et al.*, 1999). In turn, higher concentrations of chlorate reduce chondroitin sulfation up to 90% and heparan sulfation up to 65%. All these studies were performed in cell cultures and may not reflect what happens in cultured embryos. One study from our group investigated the effect of chlorate on the
sulfation of CS and HS chains under whole embryo culture conditions. This work showed that 30 mM chlorate concentration completely abolishes sulfation of CS chains while having a milder effect on heparan sulfation (46% reduction) (Yip et al., 2002). During the present study, titration experiments allowed identification of the minimum concentration of chlorate (5 - 10 mM) required to disturb NT closure of Lp/+ embryos. It could be hypothesized that the chlorate concentration used in current embryo culture experiments mainly affect the sulfation of CS chains, with a lesser and probably more selective effect on heparan sulfation. Chromatography methods and immunofluorescence analysis with antibodies against specific sulfated motifs of HS and CS chains could be used in order to address this hypothesis.

A recent study compared the phenotypes of Vangl2 null embryos with loop-tail mutants and showed that loop-tail is a dominant negative allele. A Vangl2 null allele (Vangl2Δ) was generated by targeting exons four and five, a region that corresponds to Vangl2 transmembrane domains. This study found that embryos with Vangl2Δ did not display classical phenotypic features of the loop-tail allele such as tail defects, imperforate vagina and cochlear polarity defects (Song et al., 2010). Similar results were reported in another study with an independent Vangl2 null allele (Vangl2ΔTMs) (Yin et al., 2012). The penetrance of CRN was only 74% in Vangl2ΔTMs/ΔTMs while Vangl2Δflox/+ mutants had a 100% penetrance of this severe NT defect. The penetrance of CRN was 100% in Vangl2flox/− embryos (from in this study) corresponding with previously published frequency of the CRN in the same knockout (Ramsbottom et al., 2014). This strongly suggests that global loss of Vangl2 using the Vangl2flox allele recapitulates closure 1 failure of Lp/Lp mutants. However, the penetrance of CRN in chlorate treated Vangl2flox/− embryos was less than half the frequency of this NT defect in Vangl2Δflox/+ individuals cultured with chlorate (37% vs 82% respectively). The high penetrance of CRN in Vangl2Δflox/− embryos could be explained by dominant negative feature of loop-tail allele. Hence, the loop-tail mutant protein has an adverse effect on wild type Vangl2, Vangl1 and other PCP proteins resulting in a more profound disturbance on PCP signalling than loss of Vangl2 alone in the null mutant (Yin et al., 2012).

Lp/+ and +/+ embryos respond differently to the treatments (chlorate or enzymes) in the following parameters: the frequency of CRN, the somite stage at which the treatment remains effective and the effect of the treatment on embryo morphology (chlorate only). Interestingly, only a small proportion of +/+ embryos (12-15%) failed closure 1 after exposure to chlorate, chondroitinase or heparitinase. Lp/+ embryos remain sensitive to chlorate from 0 to 5 somite stage suggesting that even a short exposure to the treatment may lead to the failure of closure 1. In contrast, +/+ embryos are mainly sensitive to the treatment at early somite stage and only a small proportion of embryos develop NT defect at the end of the culture. The frequency
of CRN in +/+ embryos and the stage at which the treatment remains effective suggest that sulfated GAGs are important but not essential for the initiation of NT closure in these embryos. This agrees with previously published studies that did not report the failure of closure 1 in +/+ embryos cultured in the presence of heparitinase, chondroitinase or β-D-xyloside, a substance which inhibits proteoglycan synthesis (Morriss-Kay et al., 1982, 1989; Tuckett et al., 1989). The main NT defects reported in these studies were associated with spinal or cranial neurulation.

Apart from closure 1 failure, Lp/Lp mutant embryos have reduced length and increased width in comparison to somite-matched +/+ littermates (Patricia Ybot-Gonzalez, Savery, et al., 2007b). Lp/+ individuals gave intermediate results for both parameters. The changes of length/width in Lp/Lp and Lp/+ littermates are associated with convergent extension defects in these embryos. Notably, chlorate treatment did not significantly reduce the length of Lp/+ embryos. In contrast, the elongation of +/+ littermates was significantly reduced upon the exposure to the inhibitor. It is not clear whether the effect of chlorate on +/+ embryo elongation could be associated with convergent extension or other mechanisms. This question will be addressed in the next chapter.

Previous studies showed that NT closure initiates at the 6-7 somite stage in +/+ embryos (at the level of the third somite) and that closure is delayed in Lp/+ littermates (Golden, et al., 1993; Ybot-Gonzalez, et al., 2007). Furthermore, Vangl2 Lp mutants have an abnormal neural plate, notochord and somites around the stage of the initiation of NT closure (Greene et al. 1998). Chlorate treated Lp/+ embryos develop CRN, a phenotype that is not normally seen in these embryos whereas it is 100% penetrant in Lp/Lp mutants. Therefore, it was necessary to analyse the effect of acute chlorate treatment on neural plate morphology during the onset of NT closure. The neural plate of Lp/+ embryos (without chlorate) differs from +/+ littermates in a number of morphological parameters including neural plate curvature, and the site and the time of neural fold fusion. The latter parameter agrees with a previously published delay in onset of closure 1 in Lp/+ embryos (Gerrelli et al., 1997). This study revealed a change in neural plate shape in chlorate treated +/+ and Lp/+ embryos. Interestingly, chlorate treated wild type embryos lose the normal ‘horse-shoe’ morphology of neuroepithelium in the closure 1 region and this change in shape is similar to that of untreated Lp/+ embryos. The other similarities between these embryos are the delay in NF apposition (distance between NFs) and increased medial angle during the onset of NT closure. In contrast, the inhibition of GAG sulfation by chlorate has a more profound effect on the neural plate morphology of Lp/+ embryos. The treatment worsens the effects on neural fold apposition and medial angle and to the smaller extent affects NF elevation at early somite stages. These adverse effects on neural plate morphology result in the failure of closure 1 in Lp/+ embryos. It is important to mention that
the neural plate of chlorate treated $Lp/+\,$ embryos does not display an enlargement of the floor plate, as previously seen in $Lp/Lp\,$ mutants (Greene et al. 1998).
5. Mechanism of Vangl2-PG interaction and expression pattern of CS-E and Smc3 during early embryonic development

5.1 Introduction

5.1.1 Convergent extension and neural tube closure

Convergent extension (CE) is a key mechanism that triggers body axis elongation, whereby a tissue undertakes simultaneous narrowing and extension. One of the best-studied examples of CE is body axis elongation during gastrulation (reviewed in Keller et al., 2000). In general, CE is characterized by the collective cell movements towards the midline, accompanied by cell intercalations along their axis of movement. Both of these processes result in the narrowing of the body axis mediolaterally (convergence) and elongation along its anterior-posterior axis (extension) (Tada et al., 2012). In frogs, mediolateral cell intercalation underlies CE of the neural plate (Jacobson, 1994) and this process is accomplished by polarized protrusive activity of neural cells (L. A. Davidson et al., 1999). Similar cell behaviours drive CE of the neural keel in zebrafish embryos (Harrington et al., 2010) and CE of mesoderm in frogs, fish and mice (Glickman et al., 2003; Yin et al., 2008; Williams et al., 2014). In all of these studies, the polarity of intercalation is regulated by PCP signalling (Wallingford et al., 2002c; Ciruna et al., 2006; Yen et al., 2009). Therefore, PCP signalling links CE cell movements of neural plate and NT closure and both of these processes fail when the PCP pathway is perturbed. In both Xenopus and mouse embryos, loss of PCP signalling results in the failure of neural CE and neural tube defects (Goto et al., 2002b; Patricia Ybot-Gonzalez, Savery, et al., 2007b). Nearly all mouse models of CRN, which involve almost complete failure of NT closure, result from homozygous mutations of PCP genes (reviewed in Chapter 1). A recent study analysed the CE movements in the $\text{Vangl2}^{\text{Lp/Lp}}$ mutant using a live imaging approach and showed that these mutant embryos maintain tissue polarity but are deficient in apical neighbour exchange in comparison to wild type (Williams et al., 2014). This study showed that $\text{Vangl2}^{\text{Lp/Lp}}$ mutants have affected apical cell behaviour whereas the intercalation of neuroepithelial cells at the basal side remains normal.

Many studies showed an integration of ECM and PCP signalling during neurulation of lower vertebrates. In addition to the role of the PCP signalling in mediolateral cell intercalation, it also coordinates deposition of extracellular matrix (ECM), in particular fibronectin, on the tissue surfaces (Dzamba et al., 2009). Furthermore, the evidence from Xenopus and zebrafish emphasised the importance of cell-ECM interactions for properly oriented and polarised cell intercalation, thus for CE movements (Skoglund et al., 2010b). In turn, integrins, the main class
of ECM receptors, play an essential role in PCP-dependent cell polarization. For instance, perturbation of fibronectin (FN)-integrin α5β1 interaction leads to the failure of CE and formation of short, wide embryos in fish, frog and mouse (Watt et al., 1994; Davidson et al., 2006). In contrast, Vangl2 regulates ECM remodelling through effects on matrix-metalloproteinases (MMPs), notably MMP14. Loss-of-function of Vangl2 leads to upregulation of MMPs that results in the degradation of ECM (Williams et al., 2012). Important lessons about matrix polarization were learned from studies of ascidian notochord morphogenesis that undergoes CE by mediolateral intercalation. The prickle and laminin α3/4/5 orthologue double mutants completely fail CE, while single mutants have less severe phenotypes suggesting that PCP and ECM may normally complement one another in this process (Skoglund et al., 2010b).

In this study, the potential role of PGs and GAGs in mouse CE is investigated by labelling of the node and midline of +/+ and Lp/+ embryos prior to closure 1. Node labelling is followed by immediate exposure to chlorate. This experiment provides an opportunity to study the cellular behaviour underlying CE in Lp/+ embryos with affected GAG sulfation, compared with these cellular events in the wild type situation and in Lp/+ embryos without chlorate treatment. Other potential mechanisms of Vangl2-PG interaction are also examined in this chapter.

5.1.2 Ciliogenesis

Cilia are microtubule-based organelles that project from the surface of many eukaryotic cells. They have emerged as key organelles in a large number of physiological and developmental processes. Cilia are involved in cellular motility, fluid transport and a variety of signalling cascades. Cilia conventionally classified as ‘primary’ or ‘motile’ based on ultrastructural and functional differences (Ishikawa et al., 2011). Primary cilia are immotile and short but they can detect biochemical and physical extracellular signalling (Singla et al., 2006). Motile cilia are localised to epithelial cell surfaces, for example in the oviduct and trachea, where they beat in wave-like patterns to produce fluid movements. Defects in ciliogenesis are implicated in a variety of human diseases including retinal degeneration, polycystic kidney disease and hydrocephalus (Ishikawa et al., 2011). Although, hundreds of ciliary proteins have been identified in the past twenty years, in most cases their function remains elusive.

The primary cilium consists of a microtubule-based structure named the axoneme that is surrounded by a ciliary membrane connected to the plasma membrane. The axoneme is composed of nine parallel doublet microtubules (outer doublets) that elongate from the basal body (Ishikawa et al., 2011). In addition to the nine microtubules, a motile cilium has a single pair of central microtubules in the axoneme that allows generation of motion. Ciliogenesis
occurs through an ordered number of distinct steps. Initially, a basal body forms from an existing centriole and migrates to the cell surface where it attaches to the actin-rich cortex. The orientation and position of the basal body controls the alignment of the resulting cilium. In the next step, the outer doublets of the axoneme extend from the microtubules of basal body, giving rise to the cilium. Elongation of the axoneme relies on intraflagellar transport (IFT), with import and transport of ciliary proteins to the tip. IFT is driven by molecular motors composed of kinesin and dynein motor proteins. Disruption of either basal body proteins or IFT motors leads to the impaired cilia assembly (Santos et al., 2008).

Ciliogenesis is tightly synchronised with the cell cycle. In most cells, cilia typically form during G1 phase and disassemble when mitosis starts. During the G1 or G0 phase, the mother centriole migrates to the cell cortex and nucleates a cilium. When ciliary growth is completed, the cilium functions as a cellular antenna, sensing extracellular signals. Cilia are removed prior to cell division by two mechanisms: cleavage of the cilium away from centriole or resorption of the cilium from the tip (Ishikawa et al., 2011). Removal of the cilium allows centriole pairs to detach from the cortex and dock to the spindle poles for mitosis. Upon the completion of cell division, the centrioles proceed to ciliary re-assembly in G1 phase (Santos et al., 2008).

Previous studies have emphasized the role of motile cilia in the establishment of left-right asymmetry (LR) in vertebrate embryos (reviewed in Chapter 1). Long before these studies it was discovered that in the mouse embryo each node cell has a single cilium projecting from its apical surface (Jurand, 1974). Node cilia are long: at E7.5 they are ~1.5 µm long and grow to 3-5 µm in length by E8.0. The formation of normal node cilia is regulated by a number of transcription factors some of which control cilia motility, while others regulate cilia length. For example, in the absence of Foxj1, cilia become immotile but cilia length is not affected (Chen et al., 1998). In contrast, Rfx3 mutant embryos have short node cilia but their motility is not perturbed (Bonnafe et al., 2004). Both mutants show randomised LR situs, emphasising the need for correct ciliary structure and motility for function.

In the present study, a particular chondroitin sulfate chain type (CS-E) was found to localise specifically to the nodal cilia of mouse embryos. This provided an opportunity to initiate studies of GAGs, particularly CS-E, in ciliary structure and function, a topic that has not previously been explored in the literature.
5.2 Results

5.2.1 Investigating the mechanisms of Vangl2-PG interaction during initiation of neural tube closure

5.2.1.1 Chlorate does not affect convergent extension in +/+ and Lp/+ embryos

Previous studies showed that Lp/Lp embryos exhibit defective convergent extension (CE) prior to closure 1 (Patricia Ybot-Gonzalez, Savery, et al., 2007b; Williams et al., 2014). Ybot-Gonzalez and colleagues labelled the midline of Lp/Lp mutants and showed that these embryos fail CE in both axial mesoderm and neuroepithelium prior to the onset of neurulation (Patricia Ybot-Gonzalez, Savery, et al., 2007b). In this study, the same labelling technique was used to investigate the effect of chlorate treatment on CE. Lp/Lp mutants were used as a ‘positive control’, i.e. these embryos were expected to show reduced CE, even under normal culture conditions.

To determine whether CE is compromised in chlorate-treated +/+ and Lp/+ embryos during initiation of NT closure, the midline was labelled by the focal injection of DiO into the node at E8.5 (Fig. 5.1A). The labelling was performed in collaboration with Dorothee Mugele and Matteo Mole and was undertaken blind to embryo genotype. Some embryos were collected at time 0 to verify the injection site. Analysis of tissue sections from time 0 revealed that both node and the floor plate were successfully labelled with DiO across three genotypes (Fig. 5.1B-D). The remaining embryos were allocated randomly to different treatment groups (chlorate or water), and cultured for 24 hours. The somite stage was recorded at the end of culture. Immediately after culture, the embryos were imaged using a fluorescence microscope and the extension of DiO labelled cells was analysed using in Fiji software (Fig. 5.1A). In addition to the analysis of whole mount images, midline extension (MDE) of labelled cells was confirmed in tissue sections.

Both +/+ and Lp/+ embryos from the water-treated control group exhibit rostrally-directed MDE of DiO-labelled cells (Fig. 5.2A and B respectively). By contrast, Lp/Lp embryos display very limited MDE (Fig. 5.2C), with labelled cells located at the site of node injection in most mutant embryos. Chlorate-treated +/+ and Lp/+ embryos exhibit rostrally-directed MDE of DiO-labelled cells similarly to corresponding littermates from the control group (Fig. 5.2D and E respectively). Chlorate treatment had no discernible effect on the MDE of DiO-labelled cells in Lp/Lp embryos (Fig. 5.2F). However, since axial extension is largely absent from untreated Lp/Lp embryos, it is unclear whether a detrimental effect of chlorate could be detected.
Figure 5.1. Experimental setup of chlorate treatment/node labelling. (A) The node of E8.5 littermate embryos at 0-5 somite stage (prior to closure 1) was labelled with fluorescent dye (DiO) (see Section 2.2.3.4). Following that, embryos were randomly allocated to different treatment groups (chlorate or water) and cultured for 24 hours. After culture, live embryos were imaged by fluorescence microscopy and the extension of DiO labelled cells was measured and computed in Fiji software. (B-D) Embryos (n = 3 for each genotype) were collected at time 0, fixed and sectioned to verify injection site. Immunofluorescence on tissue sections shows the site of DiO injection in +/+; Lp/+ and Lp/Lp embryos. The node and the floor plate are successfully labelled with DiO in all three genotypes (arrows in B-II, C-II and D-II). Scale bar in B-D 100 µm.
Figure 5.2. Chlorate does not affect MDE of +/+ and Lp/+ embryos. Analysis of DiO injected embryos after culture with/without chlorate addition. (A–F) Ventral view (rostral to the top) and transverse sections (trunk region) of DiO injected embryos, cultured for 24 hours with chlorate or water (control). +/+ and Lp/+ embryos from control and chlorate group (A, D and B, C respectively) exhibit MDE of DiO labelled cells detected on both whole mount and in sections. Lp/Lp embryos from both treatment groups (C, F) display very limited MDE of DiO labelled cells. DiO labelling of the cranial region is non-specific, due to release of DiO into the amniotic cavity during the labelling process. Scale bar in A–i – F–i = 0.5 mm, A–ii,iii – F–ii,iii = 50 μm.
Transverse sections demonstrate that node injection predominantly labels the notochord and midline neural plate and that, following 24 h culture, labelled cells can be detected extending along the midline, from the caudal region to upper trunk. There is no detectable difference in this MDE in water or chlorate treated +/+ and \( Lp/+ \) embryos (Fig. 5.2A-ii B-ii and D—ii E-ii respectively). In contrast, midline extension of DiO labelled cells is minimal in the notochord and floor plate of \( Lp/Lp \) embryos, with both treatment groups giving a similar appearance (Fig. 5.2C-ii and F-ii).

After visualisation of migration of DiO-labelled cells in whole embryos and sections, MDE was quantified among the different genotypes and treatment conditions (Fig. 5.3). The rostro-caudal extent of MDE was measured on whole mount images, blind to the genotype and treatment group. Statistical comparison was performed by Two-way ANOVA analysis in SPSS. First, the overall effects of genotype and treatment were analyzed, using MDE measurement as dependent variable. Then, the first order interaction between these fixed effects was computed. When the fixed effects were significant overall, a pairwise comparison was performed for each parameter using Tukey’s multiple comparison test to identify the statistical differences in MDE (p-value < 0.05) between the specific genotype/treatment conditions (Fig. 5.3B).

The difference in MDE between genotypes is greater than expected by chance after allowing for effects of differences between treatment groups (Fig. 5.3B, p < 0.001). In contrast, treatment group does not significantly affect MDE overall (p = 0.92). Furthermore, there is no statistically significant interaction between genotype and treatment (two-way interaction, p = 0.21). The pairwise comparison procedure showed that MDE does not differ significantly between +/+ and \( Lp/+ \) genotypes (p = 0.41), whereas MDE of +/+ and \( Lp/+ \) embryos differs significantly differ from that of \( Lp/Lp \) littermates (p < 0.001 for both +/+ vs \( Lp/Lp \) and \( Lp/+ \) vs \( Lp/Lp \)).

In summary, chlorate treatment does not disrupt CE of +/+ and \( Lp/+ \) embryos whereas \( Lp \) mutants exhibit defective CE in both treatment groups.

5.2.1.2 Chlorate does not change width of +/+ and \( Lp/+ \) embryos

Previous studies showed that in addition to CE defects, \( Lp \) mutants exhibit reduced length and increased width prior to the onset of NT closure (Patricia Ybot-Gonzalez, Savery, \textit{et al.}, 2007b). In Chapter 4 it was shown that Chlorate reduces embryo length of \( Vangl2^{+/+} \) and not \( Vangl2^{Lp/+} \) embryos. To determine whether chlorate affects embryo width, the embryos that were labelled by node injection in the CE study were measured after 24 h culture. Measurements were taken at the level of third-fourth somites, blind to genotype/treatment condition.
Statistical comparison was performed by Two-way ANOVA analysis in SPSS. The statistical workflow was the same as for MDE analysis.

Chlorate treatment does not significantly affect embryo width overall (p = 0.28) whereas there was a highly significant difference in width between genotypes. There was no statistically significant interaction between genotype and treatment (two-way interaction, p = 0.27). Pairwise comparisons showed that the embryo width does not significantly differ between +/+ and Lp/+ embryos treated with chlorate or water (p = 0.98) whereas the width of +/+ and Lp/+ embryos was significantly reduced compared with Lp/Lp littermates (p < 0.001 for both +/+ vs Lp/Lp and Lp/+ vs Lp/Lp).

In summary, chlorate treatment has no significant effect on embryo width of +/+ and Lp/+ littermates whereas Lp/Lp mutants exhibit increased width in both treatment groups.
Figure 5.3. Defective MDE in Lp/Lp embryos. (A) MDE was measured after 24 hour culture with chlorate or water. Embryos from 8 to 16 somites were used for the measurements. Points represent extension of DiO labelled cells along the rostro-caudal axis in individual embryos; mean +/- SEM are also plotted per treatment/genotype group. Lp/Lp embryos have reduced MDE in both treatment groups. (B) Results of statistical analysis by 2-way ANOVA. MDE was compared between different treatment groups and genotypes. The effect of the Treatment is not significant whereas the effect of Genotype is highly significant. The effect of different levels of Genotype does not depend on what level of Treatment is present. MDE does not differ significantly between +/- and Lp/+ embryos from both treatment groups. The MDE of Lp/Lp embryos is significantly reduced compared with +/- and Lp/+ littermates. Asterisk: p-value < 0.001.
Figure 5.4. Chlorate does not change width of +/- and Lp/+ embryos. (A-D) Embryo width was measured at the level of third-fourth somite in embryos cultured for 24 h with chlorate or water. Embryos from 8 to 16 somites were used for the measurements. Lp/+ and Lp/Lp embryos are only shown. (E) Points represent the width of individual embryos; mean +/- SEM are plotted per treatment/genotype group. Lp/Lp embryos have increased width in both treatment groups. (B) Results of statistical analysis by 2-way ANOVA. Width was compared between different treatment groups and genotypes. The effect of the Treatment is not significant and the effect of Genotype is highly significant. The effect of different levels of Genotype does not depend on what level of Treatment is present. Width does not differ significantly between +/- and Lp/+ embryos from both treatment groups. The width of Lp/Lp embryos is significantly greater than in +/- and Lp/+ littermates. Asterisk: p-value < 0.001. Scale bar in A-D = 0.5 mm.
5.2.1.3 Analysis of somite morphology in cultured embryos

In Chapter 4, inhibition of GAG sulfation was found to affect a number of morphological parameters of the neural plate at the closure 1 region. Here, the effect of the chlorate treatment on somite morphology was investigated in the same embryo cohort. Measurements were taken during the onset of NT closure (six to seven somite stage) at the level of third and fourth somite. A minimum of eight embryos was used per genotype/treatment condition (Fig. 5.5), with measurement of somite length (rostro-caudal direction) and somite width (mediolateral direction). In addition, the somite length/width (L/W) ratio was obtained per single somite and the mean of L/W ratio was computed for four somites per embryo. Statistical comparison was performed by Two-way ANOVA analysis in SPSS.

Pairwise comparisons showed that somite length does not differ significantly between +/+ and Lp/+ embryos treated with chlorate or water (Pairwise comparison*Genotype) (Fig. 5.6A, D). Similarly, somite length does not differ significantly between +/+ or Lp/+ embryos from different treatment groups (Pairwise comparison*Treatment). The only significant difference was observed between embryos in the +/+ control and Lp/+ chlorate groups (p = 0.02).

The difference in the somite width mean values among the different levels of Genotype is greater than would be expected by chance after allowing for effects of differences in Treatment. There is a statistically significant difference (p = 0.015) (Fig. 5.6D). Comparison of water and chlorate-treated +/+ vs Lp/+ embryos indicates significantly greater somite width in Lp/+ embryos in both treatment groups (Fig. 5.6B).

Next, L/W somite ratio was analysed in +/+ and Lp/+ embryos in both treatment groups. There is a statistically significant interaction between Genotype and Treatment (p = 0.033). Chlorate does not affect L/W ratio of +/+ embryos (p = 0.99) but reduces the L/W ratio in Lp/+ embryos (p = 0.017) (Fig. 5.6C). The L/W ratio of +/+ and Lp/+ littermates from control group does not differ significantly (p = 0.71). In contrast, L/W ratio in chlorate treated Lp/+ embryos is significantly lower than the ratio of +/+ from the same treatment group (p < 0.001) (Fig. 5.6C). Higher L/W somite ratio (> 1) indicates the greater length and smaller width and the smaller L/W somite ratio indicates smaller length and greater width (<1). Water and chlorate treated +/+ embryos as well as water treated Lp/+ embryos exhibit L/W somite ratio bigger than 1, suggesting that somites of these embryos are elongated in rostro-caudal direction (Fig. 5.5A-ii, and Fig. 5.6C). By contrast, L/W ratio of chlorate treated Lp/+ embryos is close to or lower than 1, indicating that the somites of these embryos have similar length and width (ratio = 1) or elongated in mediolateral direction (ratio <1).
Figure 5.5. Analysis of somite morphology during onset of NT closure. +/- and Lp/+ littermate embryos were cultured for 8 hrs in 10 mM chlorate or H2O (control), fixed, stained with CellMask\textsuperscript{tm} and imaged using confocal microscopy for morphological analysis of somites. (A-i) Z-projection of +/- embryos at 7 somite stage from control group. The somite pairs are indicated in yellow. The images were re-sliced in Fiji (from dorsal to ventral surface) to obtain sections through the somites. (A-ii) Single z-plane through the closure 1 region. The measurements were taken at the level of third/fourth somite. Length (rostrocaudal orientation) and width (mediolateral orientation) measurements were taken half way though the somite. Two somite pairs (4 individual somites) were measured per embryo. L: length, W: width. Scale bar: 50 µm. Pairwise comparison procedure showed that somite width differs significantly between +/- or Lp/+ embryos from the same treatment groups (Pairwise comparison*Genotype, p = 0.034 for control and p = 0.002 for chlorate group) (Fig. 5.6B and D). In summary, Lp/+ embryos exhibit significantly wider somites than +/- littermates in both treatment groups (Fig. 5.6B).
FIGURE 5.6

A

B

C

D

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Figure 5.6. Chlorate treatment reduces somite width in Lp/+ but not in +/- embryos. The somite length (A), width (B) and somite length/width ratio (C) were analysed in the control and chlorate treated +/- and Lp/+ littermate embryos. Six to seven somite stage embryos of the same genotype/treatment were used for the analysis. The individual points on the graph A, B and C show the average length, width and average somite length/width (L/W) ratio respectively for four somites per embryo. Asterisks indicate the significant differences (p-value < 0.05) between genotypes for each parameter. (A) Water and chlorate treatments do not significantly affect somite length in +/- and Lp/+ embryos. (B) Comparison of water and chlorate-treated +/- vs Lp/+ embryos indicating significantly greater somite width in Lp/+ embryos in both treatment groups (p = 0.034 and p = 0.002 respectively). (C) Chlorate significantly reduces somite L/W ratio in Lp/+ but not in +/- embryos. (D) Summary table of fixed effects (genotype, treatment), two-way interaction (Genotype * Treatment) and pairwise comparisons with corresponding p-values for somite length, width and somite length/width ratio. Significance at p < 0.05 is depicted in red. Points represent the mean +/- SEM. P-values for fixed effects and interactions derived by two-way ANOVA analysis. Tukey’s test used to obtain p-values for pairwise comparisons. Number of embryos per genotype/treatment group =8-9. Measurements were made blind to genotype and treatment.

5.2.1.4 Chlorate does not perturb integrity of actin cytoskeleton

Apical actomyosin contraction is regulated through RhoA/Shroom 3 proteins localised to the apical actomyosin complex (Hildebrand, 2005; Escuin et al., 2015). PCP proteins, including Celsr1 and Vangl2, have been also implicated in the regulation of actin cytoskeleton during NT closure. For example, Celsr1 cooperates with Dvl and DAAM1 to upregulate Rho kinase that results in the actomyosin-dependent contraction (Nishimura et al., 2012). Moreover, inhibition of actin cytoskeleton leads to the failure of NT closure in Lp/+ embryos after exposure to ROCK inhibitor (Patricia Ybot-Gonzalez, Savery, et al., 2007b).

The possibility that inhibition of GAG sulfation by chlorate may have an effect on cytoskeleton integrity in treated embryos was examined by phalloidin staining on whole-mount (8 h culture) and sections (24 h culture) of the closure 1 region. Whole-mount phalloidin staining of +/- and Lp/+ embryos from the control group revealed an organised actin network with intense signal at the apical surface (arrows in Fig. 5.7A, B). Short exposure to chlorate had no effect on the apical enrichment of actin cytoskeleton in either +/- or Lp/+ embryos (arrows in Fig. 5.7C, D). Phalloidin staining of neuroepithelial sections demonstrated an actin enrichment at the apical surface and aligned microfilaments within the neuroepithelium in the +/- and Lp/+ embryos from control group (Fig. 5.7E, F). In addition to the apical neural plate enrichment, actin is enriched in the apical part of the somites in the same treatment group. Prolonged exposure to the chlorate did not perturb actin network within neuroepithelium and somites in either +/- or Lp/+ embryos (Fig. 5.7G, H).
FIGURE 5.7

A

Control, 8 h

Phalloidin

B

Control, 24 h

C

Chlorate, 8 h

D

Chlorate, 24 h

E

+/+

Phalloidin

F

+/+

DAPI

G

+/+

Phalloidin

H

Lp/+
Figure 5.7. Analysis of F-actin distribution in cultured embryos. (A-H) F-actin staining (Phalloidin) of +/- and Lp/+ embryos cultured with water or chlorate for 8 or 24 hrs. (A-D) Despite the wider neural plate in chlorate treated +/- and Lp/+ embryos the actin cytoskeleton does not appear to be disrupted. F-actin is enriched at the apical surface of neural plate in both treatment groups. (E-F) Longer exposure to chlorate does not affect F-actin distribution in both +/- and Lp/+ embryos. F-actin is enriched at the apical surface of neural plate and somites, and aligned microfilaments within neuroepithelium under both treatment conditions. Note the failure of NT closure in Lp/+ embryos (asterisk). Number of embryos analysed per genotype/treatment condition: 4. (A-D) Whole mount immunofluorescence, (E-H) immunofluorescence on tissue sections. Scale bar in A-H = 100 µm.

5.2.1.5 Chlorate disrupts Fgfr1 and ERK1/2 signalling during initiation of NT closure

A large number of studies have emphasised the importance of GAGs, in particular heparan sulfate, in FGF signalling (Matsuo et al., 2013). HS chains facilitate stable binding of FGF ligand/receptor complex that results in dimerization/phosphorylation of the FGF receptor (FGFR) and subsequent activation of the FGF signalling cascade (Yayon et al., 1991; Pellegrini et al., 2000; Schlessinger et al., 2000). Inhibition of GAG sulfation by chlorate perturbs FGF signalling during early embryonic development (ex vivo) and in cell culture experiments (Quarto et al., 1994; Oki et al., 2010). The mammalian FGFR family is composed of four genes (FGFR1-4), all of which are expressed during initiation of NT closure (see Chapter 3). Each gene encodes a single-pass transmembrane receptor. Interestingly, only Fgfr1 has been implicated in the NT closure (Hoch et al., 2006).

In this study, whole mount immunohistochemistry (WMI) was performed using an antibody against the active dimerized form of Fgfr1 (pFgfr1). The expression pattern of pFgfr1 was determined in uncultured embryos (Fig. 5.8) and the effect of chlorate was determined in cultured embryos (Fig. 5.9). The expression analysis revealed the presence of pFgfr1 in the membranes of SE and NE cells at the closure 1 and PNP regions; pFgf1 is enriched in the adherens junctions of both cell types (Fig. 5.8A, B-iii) and it becomes translocated to the cytoplasm upon cell division (right side in Fig. 5.8B-iv). In addition, dimerized Fgfr1 is found in the nucleus of NE, SE and mesodermal cells (Fig. 5.8A, B-iv). Nuclear translocation of pFgfr1 has been previously described by a number of studies and it was named ‘integrative nuclear FGFR1 signalling’ (Bryant, 2004; Stachowiak et al., 2007).

Due to the fact that GAGs mainly interact with FGF ligands and receptors at the cell membrane, it was necessary to analyse the effect of chlorate on the membrane and nuclear expression domains separately. Immunofluorescence analysis revealed that chlorate had no effect on the dimerization of Fgfr1 in the nucleus. The intensity of nuclear pFgfr1 is similar between +/- and Lp/+ embryos from both control and chlorate groups (Fig. 5.9A-ii, C-ii vs B-ii, D-ii). However, chlorate dramatically reduced the activation of Fgfr1 in the membrane of both
+/+ and Lp/+ embryos. This study must be considered preliminary, as only 2 embryos of each genotype/treatment combination were studied. An increased number of embryos will be needed to enable an intensity analysis of pFgfr1 staining in both expression domains.

In view of this apparent finding of downregulation of pFgfr1 in chlorate treated embryos, the next experiment addressed the Fgfr1 downstream target ERK1/2 (extracellular regulated kinase), which belongs to the mitogen-activated protein kinase (MAPK) pathway known to be one of the downstream targets of Fgfr signalling cascade. The phosphorylation status of ERK1/2 was examined in cultured embryos (Fig. 5.10). WMI analysis revealed the presence of active/phosphorylated ERK1/2 at the tips of neural folds and lateral SE in +/+, Lp/+ and Lp/Lp embryos from the control group (Fig. 5.10A, B, C-i). pERK1/2 is expressed in the cytoplasm of NE cells at the most dorsal region of the closure 1 site. In contrast, ERK1/2 became hyper-phosphorylated in the whole neural plate and SE of chlorate treated Lp/+ embryos (Fig. 5.10D).

This finding appears to conflict with the downregulated Fgfr1 phosphorylation in the same embryonic tissues (Fig. 5.9). It is generally accepted that activation of Fgfr1 leads to the upregulation of MAPK/ERK pathway (Ornitz et al., no date; Hensel et al., 2012). Further work would be needed to resolve this issue, and particularly to study the phosphorylation of ERK1/2 in chlorate treated +/+ embryos, which was not performed due to the time limitations.
Figure 5.8. Expression of pFgfr1 at closure 1 and PNP regions. (A, B) Whole mount immunofluorescence images of phosphorylated Fgfr1 (pFgfr1) in uncultured wild type embryos. pFgfr1 is localised to the cell membrane, cytoplasm and nucleus of SE and NE cells at the closure 1 region and in NE cells of PNP region. Z-projection in A, B-i-ii, scale bar 100 µm; 5 z slices in A, B-iii-iv, scale bar 50 µm. NE: neuroepithelium; SE: surface ectoderm. Min. number of embryos per region antibody: 3.
Figure 5.9. Chlorate inhibits phosphorylation of Fgfr1 in the membrane but not in the nucleus. (A-D) Whole mount immunofluorescence images of embryos cultured for 8 h with water (+/+, n = 2; Lp/+, n = 2) or chlorate (+/+ = 2; Lp/+ n = 2). Expression domains (membrane/cytoplasm vs nuclear) were isolated in ImageJ using the Image Calculator tool. Membrane/cytoplasmic staining were separated from nuclear staining by subtracting DAPI staining from pFgfr1. Nuclear staining was isolated by adding DAPI + pFgfr1. (A, C) +/- and Lp/+ from control group express pFgfr1 at the cell membrane/cytoplasm and nucleus of NE and SE cells. (B, D) pFgfr1 staining is reduced in the membrane/cytoplasm domain but not in the nuclear domain in chlorate treated +/- and Lp/+ embryos. Scale bar in A-D= 100 µm.
FIGURE 5.10

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Figure 5.10. Inhibition of GAG sulfation results in the upregulation of p-ERK1/2 in Lp/+ embryos. (A-D) Whole mount immunofluorescence images of embryo cultured for 8 h with water (+/+; n = 3; Lp/+, n = 3; Lp/Lp, n = 2) or chlorate (Lp/+, n = 3). (A-D-i-ii) z-projections and (A-D-i’-ii’) single z-plane of closure 1 region. (A-C) +/+, Lp/+ and Lp/Lp embryos from the control group express p-ERK1/2 at the neural fold tips (arrows) and surface ectoderm. (D) Chlorate treatment results in the activation of EKR1/2 in the whole neural plate; strong staining is also detected in the surface ectoderm. Scale bar in A-D-i, ii= 100 µm, A-D-i’, ii’ = 50 µm.
5.2.2 Expression pattern of chondroitin sulfate type E and Smc3 during initiation of left-right asymmetry and NT closure.

5.2.2.1 Expression pattern of chondroitin sulfate type E

Analysis of CS biosynthetic machinery by RNA-seq suggested that three types of CS chains could be present at the closure 1 site (see Section 3.2.4). Subsequently, immunofluorescence analysis confirmed the presence of CS types A (CS-A) and C (CS-C) chains by staining with the CS-56 antibody (see Section 3.2.8.2). Here, the expression pattern of CS type E (CS-E) was analysed using an anti-CS-E antibody developed in Prof Hsieh-Wilson's laboratory. This antibody specifically recognises the CS-E epitope (sulphated at 4-O and 6-O position) and it does not cross react with other CS chain types (Tully et al., 2006; Cheung et al., 2017). Immunofluorescence analysis was performed on wild type embryos at E7.5-8.5.

Whole-mount immunofluorescence staining revealed the presence of CS-E chains in surface ectoderm (SE) and neuroepithelium (NE) prior to and during the onset of NT closure (Fig. 5.11). CS-E chains are detected at the apical surface of both SE and NE cells (arrows in Fig. 5.10A-i,v, B-i,v); the chains form a fibrillary network on the surface of SE and NE cells. Furthermore, CS-E chains are co-localised with ZO-1, an adherens tight junction-associated protein (Tornavaca et al., 2015), in the SE cells (arrows in Fig. 5.11C-iii). In addition to the surface and junctional staining, CS-E chains exhibit a single punctate focus of staining at the centre of the SE and NE cells resembling the staining of primary cilia (arrows in Fig. 5.11A-ii, B-iii). Strong staining is also detected between the cells resembling midbody-like structures (arrows in Fig. 5.11A-iii, B-v). Interestingly, CS-E chains are localized to the fusion point of the neural plate where they are present on cellular bridges that appear to unite the fusing neural folds (arrow in Fig. 5.11B-iii).

Double WMI with Arl13B was used to verify the presence of CS-E chains in the primary and motile cilia (Fig. 5.12). Arl13B belongs to the ADP-riboosylation factor family of GTPases and it is required for ciliary structure. Arl13B is localised to the ciliary membrane and its expression does not overlap with the basal body (Larkins et al., 2011). WMI analysis showed that Arl13B localised to the membrane of motile and primary cilia (Fig. 5.12A-ii, B-ii) and that CS-E co-localizes with Arl13B in the primary cilia of surface ectoderm and motile cilia of the node (Fig. 5.12 A-ix, B-iii). Therefore, the CS-E epitope localises to the ciliary membrane of E8.5 mouse embryos.

To further confirm the cilia- and midbody-associated expression of CS-E chains, double WMI with γ-tubulin antibody or Aurora B was performed (Fig. 5.13). γ-tubulin is known to localise to the basal body of non-dividing cells and its expression is typically excluded from ciliary
membranes (Hagiwara et al., 2000). In addition, this protein is expressed in the centrosomes during mitosis. Analysis of γ-tubulin expression showed that the protein localises to the basal bodies of SE and NE cells in interphase of the cell cycle, and to the centrosomes during mitosis (Fig. 5.13A-ii, B-ii). CS-E chains are not expressed in the basal bodies, instead, the CS-E epitope is detected in the ciliary membrane of both SE and NE cells (Fig. 5.13A-iv-v). During mitosis, the expression of CS-E chains is associated with spindle formation; the chains appear to be attached to the centrosomes labelled with γ-tubulin (Fig. 5.13B, C). The CS-E spindle-associated staining is present in both NE and SE cell types (Fig. 5.13B-iv, C-iv).

Aurora B belongs to the Aurora family of serine/threonine protein kinases and it plays an important role in chromosome segregation (Krenn et al., 2015). This protein is expressed in the midbody, a transient structure that joins two daughter cells at the end of cytokinesis. CS-E chains co-localise with Aurora B in the midbody of SE and NE cells (Fig. 5.13D-iii).
Figure 5.11. Expression pattern of CS-E chains at the closure 1 site. Whole-mount immunofluorescence of CS-E (A, B) and CS-E/ZO-1 at E8.5 (C). Images show dorsal views of closure 1 in 5 and 7 somite wild type embryos. CS-E chains are expressed at the apical surface of neuroepithelium and surface ectoderm (arrows in A-i, B-i and A-ii, B-vi respectively). The staining is more intense at the 7 somite stage. CS-E epitopes are localized to the fusion point of neural plate, where CS-E positive strands appear to unite the fusing neural folds (arrow in B-iv). A single punctate focus of staining is detected at the center of each surface ectoderm and neuroepithelial cell, resembling primary cilia staining (arrow in A-ii-iii, B-iii), and between the surface ectoderm cells resembling midbody staining (arrow in A-iii, B-v). (C) CS-E co-localizes with ZO-1 at tight junctions of SE cells. Scale bar A, B-i: 100 µm; A-ii-iii, B-v-vi: 20 µm; B-iii-iv: 50 µm; C: 10 µm. Images acquired by confocal laser-scanning microscopy. Min. number of embryos for per somite stage: 3.
FIGURE 5.12

Figure 5.12. CS-E chains co-localise with Arl13B in primary and motile cilia. Whole-mount immunofluorescence of CS-E and Arl13B at E8.5 (n = 3). (A) Ventral view of the mouse node (enclosed by dashed lines in A-iii) at the 5 somite stage. Arl13B staining is detected in the motile cilia of the node and the primary cilia of visceral endoderm cells (large cells surrounding the node). Intercellular junctions are also positive for Arl13B antibody. CS-E chains co-localise with Arl13B in both node cilia and primary cilia of visceral endoderm (VE) cells. (B) CS-E and Arl13B are co-expressed in the primary cilia of surface ectoderm cells at the 5 somite stage. Scale bar A-i-iii: 100 µm; A-iv-vi: 20 µm; B-i-iii: 20 µm. Images acquired by confocal laser-scanning microscopy. Min. number of embryos for each antibody: 3.
5.2.2.2 Expression pattern of Smc3 protein

The discovery of cilia-, midbody- and mitotic spindle-associated expression of CS-E chains led to the next research question: what type of core protein could be associated with this expression pattern? In total, thirteen proteoglycans (three CSPGs and ten HSPGs) are expressed during initiation of NT closure, and some of them are hybrid proteoglycans having both CS and HS chains attached to the core protein (see Chapter 3). Cildb (cilia database, http://cildb.cgm.cnrs-gif.fr/) was used to find evidence of cilia-associated proteoglycans expressed at E8.5. Cildb is a ‘knowledgebase’ developed by Paramecium lab at Gif-sur-Yvette, France and is dedicated to proteins involved in cilia, flagella, centrioles, centrosomes and basal bodies of eukaryotes (Arnaiz et al., 2014). The database includes only one proteoglycan, Smc3, with a known role in ciliary structure or function (http://cildb.cgm.cnrs-gif.fr/v3/cgi/protein?organism=Mmusculus&uniquename=CILDB_MMUS_012275) (Khanna et al., 2005; Liu et al., 2007). Smc3 has been detected in the proteome of the photoreceptor sensory cilium complex (Liu et al., 2007). In addition, Smc3 is also detected in the mitotic spindle proteome (Sauer et al., 2005).

Following the analysis of Smc3 mRNA expression at the site of closure 1 (Chapter 3), in this Chapter, protein localisation was studied in E7.5–8.5 wild type mouse embryos by WMI (Fig. 5.14-5.17). Immunofluorescence revealed the presence of Smc3 protein at the apical surface of NE, SE, nodal and VE cells (Fig. 5.14A-i-ii, B-i-ii). The staining is detected in a single strong punctate focus at the center of the surface ectoderm cells resembling primary cilium staining, as well as between surface ectoderm cells, resembling midbody staining (arrows in Fig. 5.14A-iii). In addition, the protein localizes to the nucleus of SE, NE, VE and node cells (arrowheads in Fig. 5.14 A-iii-iv, B-iii-iv). The nuclear localization has been previously shown by antibody supplier (Abcam, ab9263). Double WMI with acetylated tubulin (Actub) was used to verify the presence of Smc3 in primary and motile cilia. Actub is found in the microtubule-based structure called the axoneme, and WMI analysis revealed the presence of Actub in the axoneme of node cilium (Fig. 5.15A-iii, B-iii). Smc3 is co-expressed with Actub in the motile cilia of the node at E7.5-8.5.
(Fig. 5.15A-iv, B-iv). In addition, Smc3 localised to the nucleus of node cells at both developmental stages (Fig. 5.15A-ii, B-ii).

Next, WMI was used to determine whether Smc3 and CS-E chains co-localise (Fig. 5.16 and Fig. 5.17). Both epitopes co-localise to the apical surface of VE cells, surrounding the node, forming a fibrillary-like structure (Fig. 5.16A-iii, B-iii). The apical surface of SE cells was also double-positive for CS-E/Smc3 staining (data not shown). Most significantly, Smc3 and CS-E chains co-localise to the motile cilia of the node at E7.5-8.5 (Fig. 5.16A-vi, B-vi). Interestingly, Smc3 is also expressed in ring-like structures on the nodal cells whereas the CS-E epitope is excluded from these structures (arrowheads in Fig. 5.16A-vi). Smc3-positive ring-like structures could be endocytic rings that may be involved in the trafficking and recycling of the proteoglycan without the GAG chains attached to the core protein.

In addition to the co-expression in the node, the Smc3/CS-E distribution was analysed in the developing brain (Fig. 5.17). Both epitopes co-localise in the nucleus of mitotic and interphase NE cells (Fig. 5.17B-i-ii). Moreover, the midbody, primary cilia and apical surface of NE cells are co-stained by the Smc3 and CS-E antibodies (Fig. 5.17C-iii).
FIGURE 5.14

Figure 5.14. The expression pattern of Smc3 at E8.5. Whole-mount immunofluorescence of Smc3 at the closure 1 region (A) and the node (B) (n = 3). Smc3 localises to the nucleus and apical surface of NE, SE, nodal and VE cells (z-projection in A, B-i-ii). The staining is detected in the nucleus of mitotic and interphase cells (single z-plane, arrow heads in A, B-iii-iv). In addition, Smc3 staining is detected in the motile cilia of the node, primary cilia and midbody of SE, VE and Ne cells (arrows in A, B-iii-iv). Scale bar A, B-i-ii: 50 µm; A, B-iii-iv: 20 µm. Images acquired by confocal laser-scanning microscopy. Min. number of embryos per region: 3.
Figure 5.15. Smc3 is co-expressed with Actub in the motile cilia of the node. (A, B) Whole-mount immunofluorescence of Smc3 and Actub (acetylated tubulin) at E7.5 (n = 2) and E8.5 (n = 2). Actub is expressed in the membrane of motile cilia. Smc3 co-localises with Actub at E7.5 and E8.5 (arrows in A-iv and B-iv respectively). Scale bar A, B-i: 50 µm; A, B-ii-iv: 10 µm. Images acquired by confocal laser-scanning microscopy. Min. number of embryos per region/stage: 3.
Figure 5.16. Smc3 co-localises with CS-E during establishment and maintenance of LR asymmetry. (A, B) Whole-mount immunofluorescence of Smc3 and CS-E chains at E7.5 (n = 2) and E8.5 (n = 2). Both Smc3 and CS-E are co-localised to the motile cilia of the node at E7.5 and E8.5 (arrows in A-vi and B-vi respectively) and to the apical surface of VE cells (A, B-iv). In addition, Smc3 localises to ring-like structures on the nodal cells, whereas CS-E staining is excluded from this area (arrow heads in A-iv, vi). Scale bar A, B-i-iii: 50 µm; A, B-iv-vi: 10 µm. Images acquired by confocal laser-scanning microscopy. Min. number of embryos per region/stage: 3
Figure 5.17. Smc3 co-localises with CS-E in the developing brain. Whole-mount immunofluorescence of Smc3 and CS-E chains at E8.25 (n = 2). (A) Dorsal view of cranial region at 4 somite stage. (B, C) Zoomed view of NE cells showing the expression of Smc3 (green) and CS-E (red) at the apical surface (C) and within the nucleus (dashed box in B-i). (B) Both Smc3 and CS-E are localised to the nucleus of dividing cells and cells in the interphase (B-i-ii). Smc3 and CS-E are co-expressed in the primary cilia, midbody and apical surface of NE cells (C). Scale bar A: 100 µm; B, C: 10 µm. Images acquired by airyscan. Min. number of embryos: 2.
5.3 Discussion

5.3.1 Cellular and molecular basis of Vangl2-PG interaction

It is well established that the PCP pathway is required for CE cell movements that shape the gastrulation-shaped embryo (Skoglund et al., 2010c). During CE, cells intercalate in the midline, narrowing and lengthening the embryo, prior to the onset of NT closure. This process is thought to be essential for closure initiation, by bringing the neural folds into proximity across the midline. Most homozygous PCP mouse mutants display failure of closure initiation, leading to craniorachischisis, making PCP the only known signalling cascade required for closure (Wallingford, 2012). In Chapter 4, the requirements of sulfated GAG chains for closure was demonstrated by the exposure of Lp/+ embryos to chlorate, an inhibitor of GAG sulfation, which led to the failure of NT closure in these embryos. This chapter has examined the basis of the Vangl2-PG interaction during initiation of NT closure.

Studies on lower vertebrates described the role of PGs in neural CE cell movements and NT closure (Ohkawara et al., 2003; Muñoz et al., 2006). The integration of PCP and PGs was demonstrated by Munoz and colleagues in 2006 when they showed that the recruitment of Dsh to the cell membrane is dependent on FN-integrin and xSyndecan-4/FN interactions. Syndecan-4, a transmembrane type of HSPGs, functionally interacts with Wnt receptor Frz7 and Dsh and is necessary for activation of the PCP pathway during early frog development (Muñoz et al., 2006). Glypican 4 also participates in PCP signalling and CE cell movements. For example, zebrafish glypican 4 (knypek) mutants display CE defects (Topczewski et al., 2001); moreover, double knypek/trilobite (Vangl2) mutants have a more severe PCP phenotype than the single knockouts (Yin et al., 2007). In this chapter, the potential role of GAG chains in mammalian neural CE was investigated. Interestingly, inhibition of GAG sulfation had no effect on CE cell movements in +/+ or Lp/+ embryos. Both +/+ and Lp/+ embryos exhibited rostrally-directed extension of the labelled cells, even when treated with chlorate to abolish GAG sulfation. Moreover, the width of these embryos was not affected by the treatment, unlike embryos in which CE is defective, as exemplified in this study by Lp/Lp mutants. Furthermore, chlorate did not worsen the CE defect of Lp/Lp mutants, although this could reflect the severe abnormality already present in untreated Lp homozygotes. These results suggest that the Vangl2-PG interaction is independent of neural CE in mammalian embryos.

Mesoderm also undergoes CE and this process is regulated by PCP signalling (Wallingford et al., 2001). Failure of CE in zebrafish results in the lateral expansion of somites due to the defective convergent movements of presomitic mesoderm (Jessen et al., 2002). The somites of mouse PCP mutants (e.g. Lp/Lp) display an irregular shape, however, the morphology of somites in
mutant embryos has not been described in detail (N. D. Greene et al., 1998). This study showed that Lp/+ embryos have increased somite width in comparison to +/- littermates and this difference becomes more significant in the chlorate treated group. In contrast, the somite length remains unchanged. As a result, chlorate treatment reduces the L/W ratio of Lp/+ embryo, converting their somites into more rounded structures. It could be argued that the increased width (e.g. lateral expansion) could be due to a CE defect in presomitic mesoderm as was previously seen in zebrafish PCP mutants (Jessen et al., 2002). This study did not investigate mesodermal CE. Alternatively, the changes in somite shape in chlorate treated Lp/+ embryos could be associated with a loss of somite integrity due to the inhibition of GAG sulfation adversely affecting BM integrity or other ECM effects.

In addition to PCP-dependent CE, the PCP pathway regulates tissue morphogenesis by directing cytoskeletal dynamics (Pierangeli et al., 1969). The downstream PCP effectors RhoA and Rac1 control cadherin-mediated cell-cell adhesion. RhoA is involved in the attachment of cells to the substrate and it regulates the formation of stress fibres (Nobes et al., 1999). In turn, Rac1 has been implicated in the epithelial cell-cell adhesion (Braga et al., 1997). Moreover, proteoglycans and GAG chains have also been implicated in cytoskeletal regulation. For instance, syndecan-4 signals through a RhoA/ROCK-I pathway and regulates myosin phosphorylation, focal adhesion assembly and microfilament contraction (Xian et al., 2010). Despite the dramatic change in the overall architecture of the closure 1 region of chlorate treated Lp/+ embryos, failure of NT closure in these embryos does not appear to result from perturbation of the actin cytoskeleton network at the tissue level: no gross defects in actin expression or localisation were detected. However, analysis at the cellular level (NE and somites) is required to completely rule out a role for the actin cytoskeleton in the Vangl2-PG interaction. For example, the distribution of cortical actin and the quantification of apical area of NE cells could be studied to address this question.

Many in vivo and ex vivo studies demonstrated a role for GAGs in regulating FGF signalling during early embryonic development. The Izme (‘lazy mesoderm’) mouse model has a mutation which targets UDP-glucose dehydrogenase, one of the key enzymes in the GAG biosynthetic pathway (García-García et al., 2003). Izme mutants are arrested during gastrulation with defects in migration of endoderm and mesoderm. This phenotype is similar to that of Fgf8 and Fgfr1 mutants (Yamaguchi et al., 1994; Sun et al., 1999). Molecular analysis revealed blockade of FGF pathway in Izme mutant embryos (García-García et al., 2003). Moreover, FGF signalling has been implicated in the NT closure. For example, deletion of the Frs2/3 binding site on Fgfr1 results in the failure of spinal NT closure and tail defects (Hoch et al., 2006). Interestingly, these authors showed that the mutant embryos have normal MAPK
responses to FGF. In contrast, the complete Fgfr1 knockout (Fgfr1\(^{-/-}\)) has defective MAPK pathway activation.

Furthermore, Fgfr1\(^{-/-}\) embryos do not develop somites and fail to initiate NT closure (Hoch et al., 2006). Preliminary results of the present study showed that inhibition of GAG sulfation during embryo culture reduces activation of Fgfr1, as judged by immunofluorescence staining intensity of the phosphorylated receptor. It could be speculated that defective FGF signalling may lead to the failure of NT closure in chlorate treated Lp/+ embryos, but further experiments are required to test this hypothesis (discussed in Chapter 6).

5.3.2. Distribution of CS-E and Smc3 during ciliogenesis and cell cycle

Ciliogenesis and cytokinesis are vital cellular processes that require tight coordination of microtubule organisation and directed trafficking of related proteins. Despite the fact that these processes have been studied in detail, there is little evidence that the regulatory machinery could be shared between them. For the first time, this study showed that Smc3 (a ‘cohesin’ protein) and its CS-E GAG chains localize in specific patterns during ciliogenesis and cytokinesis in the mouse embryo (Fig. 5.18). Originally, Smc3 was discovered as a basement membrane chondroitin sulfate proteoglycan (bamacan) (Couchman et al., 1996), while subsequent studies discovered its nuclear localisation and its role in the cohesion complex formation (Krasikova et al., 2005; Losada et al., 2005). The cohesin complex is composed of four major subunits (Smc1, Smc3, Scc1, and either SA1 or SA2) and is involved in chromosome segregation, DNA repair and genome integrity (Peters et al., 2008). Although the cohesin-associated role of Smc3 has been extensively studied in recent years (Brooker et al., 2014), the extracellular and GAG-related functions of this protein have not been examined since the original study (Couchman et al., 1996). In the present work, the expression analysis uncovered a likely sequence of events in Smc3/CS-E trafficking during cytokinesis and ciliogenesis. As was discussed previously, ciliogenesis is tightly regulated with cell cycle. During mitosis, the Smc3/CS-E complex is localised to the mitotic spindle (metaphase) and it is subsequently relocated to the cytoplasm and midbody at the end of the cell cycle. Upon completion of cell division, the complex translocates to the axoneme of primary cilia. In addition, Smc3 and CS-E generate a fibrillary network on SE and NE cells. Once the cell division is resumed, the complex returns to the nucleus.

Only one study has reported the expression of Smc3 in the primary cilia. Smc3 and Smc1 proteins were detected in the cilia of retinal photoreceptors and Madin-Dardy canine kidney cells (Khanna et al., 2005). In addition, this study showed that Smc3, together with Smc1, interacts with retinitis pigmentosa GTPase regulator (RPGR) protein. Mutations in the RPGR
gene account for 20% of patients with retinitis pigmentosa, a ciliopathy with severe inherited retinal degeneration (Fujita et al., 1997). Taken together, the results of our study provide novel evidence for the possible involvement of Smc3/CS-E in the formation of primary cilia during early embryogenesis.

Studies in zebrafish and mouse embryos have described the potential role of proteoglycans and GAG chains in regulating LR asymmetry (Kramer et al., 2002; Oki et al., 2010; Neugebauer et al., 2013). In the present study, the expression pattern of specific GAG chains and core proteins was investigated at the stage of initiation and maintenance of LR asymmetry in mouse embryos. For the first time, this study reports the presence of Smc3/CS-E in the motile cilia of the node. The core protein/GAG complex is localised to the axoneme of node cilia during initiation and the maintenance of LR asymmetry. The potential role of Smc3/CS-E is further discussed in the Chapter 6.
6. General discussion

This project provides novel evidence on the role of proteoglycans and GAG chains during mouse NT morphogenesis. Before this PhD study began, the molecular composition of the ECM proteins, termed the matrisome, had not been analysed at the start of neurulation. Individual ECM molecules had been characterised by immunostaining, but no systematic survey had been performed. To begin, therefore, this work used RNA sequencing to characterise the matrisome at the initial site of mouse NT closure (Chapter 3). During onset of neurulation, two integrin subfamilies, laminin-interacting receptors (integrins α3β1 and α6β1) and fibronectin-interacting receptors (integrins α5β1 and αvβ1) are expressed at the closure 1 region and may regulate BM assembly during closure 1. This study revealed the spatial distribution of the main BM components and defined the structural architecture of the closure 1 region. HS and CS chains and corresponding core proteins are found to be present in the closure 1 region, just prior to closure onset.

The critical role of PCP pathway in regulating CE and NT closure has been well established in mice; however, the cellular localisation of PCP proteins has not previously been analysed in detail. This study identified a novel site of Vangl2 expression during primary neurulation: Vangl2 is expressed in surface ectoderm and paraxial mesoderm suggesting a potential role of Vangl2 and other PCP components in these tissues. Moreover, functional studies showed that both HS and CS chains are required for the initiation of NT closure, in a PCP-dependent way (Chapter 4). Pharmacological inhibition of GAG sulfation and enzymatic cleavage of HS or CS chains results in the failure of NT closure in Vangl2<sup>Lp/+</sup> embryos and development of craniorachischisis, whereas this response is not seen in wild-type littermate embryos. This finding indicates the functional interaction between the PCP signalling cascade and HS/CS proteoglycans during initiation of NT closure. The PCP/PG interaction is mediated independently of convergent extension (Chapter 5). Preliminary findings suggest the failure of NT closure may result from impaired Fgfr1 signalling and failure of neural fold elevation.

A further aspect to the present study concerns a novel cellular localisation of the cohesin/proteoglycan protein Smc3 and its GAG chains. Both Smc3 and CS-E are found to be expressed in the midbody, primary and motile cilia. For the first time, the study showed the nuclear expression of CS chains in mouse embryo. The coordinated movement of Smc3 and CS-E chains during cytokinesis and ciliogenesis suggests a conserved role of this protein in the mouse cilia and cytokinetic apparatus. This work brings together the large literature on Smc3, as a cohesion protein essential for chromatin structure, with bamacan, a previously identified ECM proteoglycan that appears to share its core protein with Smc3.
6.1 Mechanisms of neural tube closure initiation in higher vertebrates: more questions than answers

Formation of vertebrate central nervous system begins with development of the NT, through the embryonic process of neurulation. Initiation of NT closure (aka closure 1) is an essential step in neurulation comprising de novo folding and fusion events and failure of this process leads to the severe human NTD craniorachischisis. There is very little evidence on the morphological, cellular and molecular mechanisms of the initial closure event in higher vertebrates. Gaining insight into the developmental mechanisms by which the NT begins its closure will be an important step towards identifying the causes of NTDs.

Several ECM components and their receptors are expressed in the rostral and caudal regions of chick and mouse embryos from the earliest stages of primary neurulation. A small number of genetic studies suggested a potential role of these proteins in mammalian neurulation. The present study identified the tissue source and final destination of selected ECM components, proteoglycans and GAG chains at the closure 1 site (Chapter 3). These findings indicated the possible requirements for proteoglycans and GAG chains in the initiation of NT closure (see below) and provided insight into the developmental mechanisms of closure 1 (Chapter 4 and 5).

The gene expression analysis revealed that BM-associated proteoglycans and GAG chains serve as mechanical barrier and a scaffold to which the neuroepithelium and mesodermal cells adhere. In turn, laminin- and fibronectin-interacting receptors (α and β integrins) may regulate BM assembly during initiation of NT closure. Perturbation of BM assembly or deletion of receptors was shown to severely affect the closure of epithelial gap in various systems causing severe congenital abnormalities (Leptin et al., 1989; Hutson et al., 2003). Future experiments, such as deletion of basal lamina components or their receptors at E8.5, could address the requirement of these molecules during initiation of NT closure.

Mouse embryos heterozygous for Lp and null alleles of the core PCP gene Vangl2 usually complete closure 1. The present study found that inhibition or removal of sulfated GAG chains from proteoglycans leads to the failure of closure 1. These results suggest that PCP signalling functionally interacts with proteoglycans and their GAG chains during initiation of neurulation. The potential mechanisms underlying this PCP/PG interaction are discussed below.

In zebrafish, *Xenopus* and mouse embryos, the axial tissues, including the neural plate, elongate rostro-caudally and converge mediolaterally (Tada et al., 2012). This process of convergent extension is responsible for neural plate shaping prior to the onset of NT closure.
and it is regulated by PCP signalling (Chapter 1). The PCP mouse mutants display severe NTDs caused by defective CE that are characterised by a broad non-extended body axis with neural folds being too widely spaced to fuse (Greene, et al., 1998; Ybot-Gonzalez, et al., 2007).

ECM proteins and their receptors play a role in CE in lower vertebrates and perturbation of ECM components results in defective CE (Chapter 5). PCP signalling and ECM integrate to regulate CE during neurulation of lower vertebrates. Moreover, PGs also play a role in CE in both zebrafish and *Xenopus* embryos. Studies in these model organisms revealed PCP-PG interaction at both genetic and protein levels (Henry et al., 2000; Muñoz et al., 2006). There is very little evidence about PCP-PG interaction in higher vertebrates. Initially, it was hypothesised that the failure of closure 1 in GAG-deficient *Lp/+* embryos resulted from a defect of CE. However, experiments in this thesis show that this is not the case. *Lp/+* embryos undergo normal midline extension of DiO-labelled node-derived cells, irrespective of whether they were treated with chlorate. In contrast, PCP-compromised embryos (*Lp* mutants) showed greatly reduced midline extension, confirming that defective CE is a major defect in the absence of Vangl2 function. Furthermore, morphological features (reduced embryo length and increased width) of chlorate treated *Lp/+* embryos are not present, unlike in classical PCP-compromised embryos. The data described above allowed us to reject the hypothesis that a lack of GAG sulfation worsens a mild CE defect in *Lp/+* embryos (P. Ybot-Gonzalez et al., 2007).

Initiation of NT closure in higher vertebrates differs morphologically, and probably mechanically, from closure events at other body levels. Closure 1 is a de novo event that happens mid-way along the open neural groove, at the level of third somite. The closure 1 site does not have focal bending at MHP and DLHPs: in transverse sections, all parts of the neural plate appear to bend. Therefore, the developmental mechanisms underlying closure initiation may differ from those at rostral and caudal regions. This unique morphology suggests unique developmental mechanisms underlying initiation of NT closure.

Inhibition of GAG sulfation changes the morphology of the closure 1 region suggesting that proteoglycans may be involved in regulation of neuroepithelial bending at this site (Chapter 4). The effect of chlorate on neural plate morphology is observed in both *+/+* and *Lp/+* embryos, arguing that some part of the GAG sulfation requirement is PCP-independent. The *+/+* individuals lose the normal ‘horse-shoe’ morphology of the neuroepithelium, and show delayed neural fold apposition and closure. Despite this, *+/+* embryos are able to complete closure in most cases. By contrast, *Lp/+* embryos display more severe abnormalities of neural plate morphology prior to the onset of NT closure that could arise due to a mild CE defect. However, other mechanisms, independently of CE, for example Rho-GTPase regulation, may also be required for closure 1, so that inhibition of GAG sulfation worsens the effect on neural
fold apposition and other morphological parameters in \( Lp/+ \) embryos, ultimately leading to failure of NT closure in \( Lp \) heterozygotes.

Apical constriction of neuroepithelial cells due to cytoskeletal actomyosin contraction is often viewed as the ‘principal motor’ that drives neural plate bending and closure. However, mouse embryos exhibit marked variation among closure sites in their dependence on actomyosin contraction for primary neurulation. Brain closure is sensitive to disassembly of actin microfilaments by Cytochalasin D. In contrast, closure in the low spinal region is not sensitive to this drug. Embryo culture with Cytochalasin D (function in disassembly of actin microfilaments) prevents initiation of closure 1 indicating the role for actin in this process (Ybot-Gonzalez et al., 1999). At the closure 1 site, actin is enriched at the apical surface of neuroepithelium and somites. Currently, it is not clear in which tissue (i.e. neural plate or somites) cytoskeletal function is needed; furthermore, does this function require actomyosin assembly/contractility or disassembly/turnover or both. The WAVE complex regulates the actin cytoskeleton by coupling extracellular signals to polarised cell movement. Nap1 is a regulatory component of the WAVE complex and it is expressed in both mesoderm and neural plate. Nap1-deficient mouse embryos display heart defects, delays in mesoderm migration and somitogenesis and, importantly for this discussion, the mutants fail to initiate NT closure (Rakeman, 2006). It would be interesting to investigate in which tissue Nap1-dependent cytoskeleton function is required for NT closure: mesoderm or neural plate. Work in Chapter 5 showed that inhibition of GAG sulfation does not result in gross perturbation of the actin network in the neural plate and somites. However, cellular analysis of the actomyosin network will be required to fully assess the role of the cytoskeleton in the PCP-PG dependent initiation of NT closure.

Another unique morphological feature of the closure 1 site is the presence of epithelial somites directly adjacent to the closing neural folds. Closure initiation is consistently adjacent to early somites in mouse, human, chick, rabbit and pig embryos (Greene, et al., 1998; Peeters, et al., 1998; van Straaten, et al., 2000), whereas closure at more cranial and caudal levels is adjacent to unsegmented cranial or presomitic caudal mesoderm. Cranial mesoderm has been implicated in the formation of the convex cephalic neural folds, and the expansion of HA-rich (hyaluronic acid) matrix is proposed to drive elevation of neural folds in the cranial region (Morriss, 1980; Zohn et al., 2012). Culture of rodent embryos in hyaluronidase, an enzyme that degrades HA, leads to the delayed elevation of the neural folds (Morriss-Kay et al., 1986). Studies in living chick embryos have described a possible similar biomechanical role for somites, since ‘buttoning’ closure occurs in register with the somites suggesting that the somites may be involved in forcing elevation and apposition of the neural folds (Van Straaten
Carboxyl and sulfate groups of GAGs trap water between their chains, generating a Donnan osmotic equilibrium that was shown to be responsible for the compressive stiffness of the cartilage tissue (Katta et al., 2009). In somites, CS proteoglycans may play a mechanical role by maintaining somite integrity and structure. Chlorate treatment affects somite morphology in Lp/+ embryos which could be associated with inhibition of chondroitin sulfation at somitic borders. This may affect somite morphology and, in turn, elevation and apposition of neural folds. In the published literature, only a few mutants that lack the most rostral somites (i.e. those adjacent to the closure 1 site) have been described (Lawlor, 2002; Hoch et al., 2006). The potential role of rostral somites in the initiation of NT closure could be tested by genetic somite ablation prior to closure 1, for example using inducible diphtheria toxin production (Ivanova et al., 2005).

Completion of NT closure involves adhesion and fusion of the opposing neural folds to create the neural tube. In vertebrates, neural fold fusion requires the formation of cellular protrusions along the midline gap and these actin-rich structures make the first points of attachment of the neural folds. In the low spinal region, the initial points of contact are made by surface ectoderm cells and ablation of Rac1 (regulator of lamelliform cellular protrusions) from these cells results in low spina bifida (Rolo et al., 2016b). The results of the present study suggest that neuroepithelial cells make the first points of contact at the closure 1 region by generating filopodial-like protrusions (Chapter 1). As was described earlier, proteoglycans, for example Syndecan-2, have been implicated in the formation of cellular protrusions in different cell types (Lin et al., 2007). Agrin’s GAG chains regulate actin-based protrusions through the activation of Cdc42 and Rac1 (Lin et al., 2010). Ablation of the GAG chains from agrin reduces filopodia-like retraction fibres in hippocampal neurons. It is possible that sulfated GAG chains may play a role in the formation of cellular protrusions at the closure 1 region, although time limitations prevented a direct study of this question in the present work. Nevertheless, it seems unlikely that failure of NT closure in these embryos is due to defective neural fold fusion. Cellular protrusions only make contact across the midline when the neural folds come into close proximity: e.g. when the folds are within ~50 µm of each other (Dr Ana Rolo, unpublished), whereas chlorate treated Lp/+ embryos exhibit an inter-fold distance that is much larger (~100-130 µm) (Chapter 4). Therefore, it seems probable that an earlier event, such as neural fold elevation, is defective in chlorate treated Lp/+ embryos, leading to failure of closure 1.

The neural plate is overlaid by SE cells dorsally at the closure 1 region and these cells express Vangl2, HS and CS chains and several proteoglycans. Hence, this expression pattern suggests that SE could be involved in mediating the effect of the Vangl2-PG interaction on NT closure.
For example, Vangl2 and syndecan-4 are co-expressed in the SE of posterior neuropore region (Dr Galea, unpublished). Furthermore, double heterozygotes between the Vangl2<sup>Lp</sup> and Sdc4-null alleles develop closure defects during spinal neurulation (Escobedo <i>et al.</i>, 2013) Escobedo. However, deletion of Vangl2 from SE cells does not result in closure 1 failure; instead, mutant embryos develop low spina bifida (Galea <i>et al.</i>, 2018). This suggests that SE-associated proteoglycans and Vangl2 and GAG chains are not required for initiation of NT closure. Additional evidence comes from another group of SE-expressed genes that are required for NT closure: Grainyhead-like 2/3 (<i>Grhl2/3</i>) double knockouts fail to close their posterior and cranial neuropores whereas closure 1 is initiated normally (Rifat <i>et al.</i>, 2010).

In this work, gene expression analysis revealed that ten HSPGs and three CSPGs are expressed during closure 1. The important question that needs to be addressed is: which proteoglycan interacts with <i>Lp</i> allele during initiation of NT closure. The genetic approach, such as CRISPR/Cas9 mutagenesis, could be used to answer this question. CRISPR/Cas9 genome modulation system allows to generate conditional knockouts of multiple genes in mouse (Chen <i>et al.</i>, 2017). For example, multiple proteoglycan genes could be targeted in mouse embryonic stem cells heterozygous for <i>Lp</i> allele. This strategy will allow to screen for neural tube defects in double heterozygous lines and identify the key proteoglycan genes that interact with Vangl2 during neural tube closure.

A key question that needs to be answered is: what are the signalling pathways downstream of the PCP-PG interaction? HS and CS chains bind various signalling molecules and present them to receptors, activating downstream pathways (Yan <i>et al.</i>, 2009). At the closure 1 site, members of the glypican and syndecan families are expressed together with the ligands of canonical Wnt, BMP, Shh, PCP and FGF signalling pathways. The first three pathways are known to play key roles in spinal and/or cranial closure, but there is no evidence for their involvement in closure initiation. The PCP pathway is essential for initiation of NT closure through the regulation of CE, and probably the cytoskeleton, while FGF signalling has been implicated in both spinal and cranial closure (Hoch <i>et al.</i>, 2006). A number of mouse genetic studies suggests a potential role for the FGF pathway in closure initiation. <i>Fgfr1</i> complete knockout results in the failure of somitogenesis and lack of closure 1 (Hoch <i>et al.</i>, 2006). Moreover, mutants of downstream effectors in the FGF pathway, including p38 and PDK1, also show similar defects (Lawlor, 2002; Zohn <i>et al.</i>, 2006). Additional experiments are needed to test this hypothesis. For example, a genetic approach can be applied by generating <i>Fgfr1<sup>−/−</sup></i> with <i>Vangl2<sup>−/−</sup></i> double mutants to evaluate if FGF pathway downregulation reproduces the PCP-PG effect on failure of closure 1. Alternatively, embryo culture experiments with chlorate
plus FGF ligand or FGF receptor inhibitors can be used to test whether the ligand could rescue, and inhibitors could worsen, the NT defect in Lp/+ embryos.

The present study found that Fgfr1-ERK1/2 signalling is misregulated in chlorate treated Lp/+ embryos suggesting a potential role of this pathway in closure initiation. The activation of downstream FGF effector ERK1/2 was perturbed, although the effect appeared to be increased activation, not decreased as might be expected from a reduction in Fgfr1 activation. In addition to FGF, other pathways may contribute to the activation of ERK1/2. For example, Aoki and colleagues observed that the generation of stochastic ERK1/2 pulses requires basal activities of EGFR, PI3K, Ras, and Rac1 and intact actin cytoskeleton (Aoki et al., 2013). Interestingly, all tested inhibitors of actin cytoskeleton (Brebstatin, Jasplakinolide, Cytochalasin D and Latrunculin A) triggered robust ERK1/2 activation in a short period of time (within an hour) and this activity was propagated to the neighbouring cells. Therefore, we could speculate that hyperactivation of ERK1/2 in chlorate treated Lp/+ embryos may be associated with changes in actin cytoskeleton network. Embryo culture experiment with inhibitors of actin cytoskeleton can be used to test whether these inhibitors could trigger ERK1/2 activation similarly to chlorate.

I would like to propose here a two-hit model of PCP-PG interaction during initiation of NT closure (Fig. 6.1). I predict that sulfated HS and CS chains regulate neural plate bending, NF apposition and somite morphology via the Fgfr1 pathway. HS and CS chains are present in the neural plate and somitic mesoderm. Under normal conditions, these sulfated GAGs bind growth factors, such as FGF ligands, and present them to the corresponding receptors, such as Fgfr1 (Fig. 6.1A). GAG/ligand/receptor complexes activate the FGF pathway that is required for normal neural plate and somite morphology. Less sulfated and unsulfated GAGs are incapable of binding the ligands, so the pathway is not activated (Fig. 6.1B). Reduction of Fgfr1 signalling leads to loss of the normal ‘horse-shoe’ morphology of neuroepithelium in the closure 1 region and delay of NT closure in wild type embryos (see Chapter 4). These changes of the neural plate morphology could be associated with changes in proliferation of neuroepithelial. Mild CE defects and aberrant morphology of the closure 1 region predispose Lp/+ embryos to failure of NT closure (first hit) (Fig. 6.1D, left side). Reduction of GAG sulfation and Fgfr1 signalling worsens the morphology of closure 1 region without affecting CE in Lp/+ embryos (second hit) (Fig. 6.1D, left side). Hence, the coexistence of these two hits (partial PCP defect and reduced GAG sulfation) ultimately leads to closure initiation failure and development of craniorachischisis.

The model described above fits well with known additive effects of PCP gene mutations predisposing to NTDs in mice, and with well-established modifying effect of Lp allele.
Moreover, it is particularly relevant to the findings of present study for functional interaction between Vangl2 and proteoglycans. As described earlier, several studies identified rare non-synonymous deleterious sequence variants of PCP genes in human NTD cases (Chapter 1). However, most of the PCP mutations described until now are heterozygous. Proteoglycans and GAG biosynthetic genes therefore represent candidate genes that could contribute to risk of human NTDs. It will be interesting to investigate if rare variants of PCP and PGs are found to be associated with NTD cases in the same patients. Recently, array-based comparative genomic hybridization of a cohort of 189 cases of with non-syndromic lumbar-sacral myelomeningocele among Caucasian and Hispanic patients identified heterozygous deletions of glypican genes GPC5 and GPC6 (Bassuk et al., 2013), as a significant risk factor for the development of NTDs.
FIGURE 6.1

**A** Control embryos

![Control embryos diagram]

**B** Chlorate-treated embryos

![Chlorate-treated embryos diagram]

**C** Wild type

![Wild type diagram]

**D** LpHet

![LpHet diagram]

*Figure 6.1. Two-hit model of PCP-PG interaction during initiation of NT closure.* (A) Under normal culture conditions, sulfated GAGs bind ligands (e.g. FGF) and present them to their receptors (e.g. Fgfr1). This leads to dimerization and phosphorylation of the receptor and subsequent activation of the FGF pathway. (B) In chlorate treated embryos, under- or unsulfated GAGs are synthesised. These GAG chains cannot bind the ligands, and as a result Fgfr1 is not activated. (C) The neural plate of wild type embryos from the water-treated control group is concave inwards and the NFs contact and fuse at the dorsal region. (D) In contrast, the neural plate of Lp/+ water-treated embryos is 'V' shaped and the NFs appear to first come into contact at a more ventral position than in +/+ embryos. I predict that reduction of Fgfr1 signalling affects the morphology of neural plate and somites in chlorate treated Lp/+ embryos: the neural plate has a convex shape, in striking contrast to the concave shape of +/+ control embryos; moreover, the somitic length/width ratio is decreased in Lp/+ in comparison to littermates from the control group. These morphological changes result in failure of NT closure in Lp/+ embryos. NF: neural fold.
6.2 Potential roles of CS-E and Smc3 in LR axis formation and ciliogenesis

At E7.5, LR axis formation is initiated by nodal flow, a leftward movement of extraembryonic fluid on the ventral surface of the node. The flow is generated by the directional beating of motile cilia on the node cells (Babu and Roy, 2013). The flow triggers production of Nodal protein in the node crown cells. Then, Nodal is translocated to the lateral plate mesoderm (LPM) to initiate its own expression on the left side. LR asymmetry is established in the LPM through the reaction/diffusion of Nodal and Lefty that is followed by asymmetric organogenesis (reviewed in Chapter 1). A number of studies have emerged on the role of PGs and GAGs in formation of LR asymmetry.

In zebrafish, HS O-sulfotransferases (OST) regulate LR patterning through control in Kupffer’s vesicle, an analogue of the mouse node. This study showed that 3-OST-5 functions in the FGF pathway to control cilia length and 3-OST-6 regulates cilia motility via cilia arm dynein assembly and kinesin motor molecule expression (Neugebauer et al., 2013). A number of studies have described the role of GAGs in transmission of the Nodal signal from the node to the LPM. Early work showed that xyloside, an inhibitor of GAG biosynthesis, prevents proper cardiac looping in Xenopus embryos (Yost, 1990). A more recent study demonstrated that intact sulfated GAGs in the ECM facilitate the long distance movement of Nodal1 in Xenopus embryos (Marjoram et al., 2011). Furthermore, pharmacological inhibition of GAG synthesis with xyloside resulted in a reduced Nodal LPM signal and planar movement. This study proposed that CSPGs provide directional transport cues for Nodal1 in the left LPM. Oki and colleagues obtained similar results in mouse embryos (Oki et al., 2007). Inhibition of GAG biosynthesis by chlorate or xyloside prevents Nodal expression in the LPM. Although, chlorate treatment affects both HS and CS chains, xyloside was more effective against CS chains (Oki et al., 2007). Oki and colleagues propose that the Nodal/GAG interactions happen at the BM between the node and LPM in the mouse embryo.

In the current study, I discovered a novel localisation of CS-E chains and Smc3 protein during early mouse embryogenesis. Smc3 and CS-E co-localise to the motile and perinodal cilia axoneme suggesting a potential role of CS-E and Smc3 in specification of the LR axis. In contrast, the studies described above were focused on the role of BM- and LPM-associated GAG chains and their role in the propagation of Nodal signalling in LPM. However, the present study proposes that GAG chains, in particular CS-E, could be involved in the initial steps of LR axis determination. CS-E chains on the motile cilia of nodal cells, and on the immotile cilia of peri-nodal cells, could potentially bind and transport Nodal to the left side of LPM. In fact, one study reported that Nodal preferentially interacts in vitro with CS chains, but not with HS GAGs.
Nevertheless, the expression pattern of CS-E requires further validation. For example, Chondroitinase ABC treatment could be performed prior to the addition of anti-CS-E antibody. Chromatography methods could be applied to isolate the nuclear and extracellular fractions of CS-E chains.

The FGF pathway plays essential roles in LR axis formation in vertebrates. Nodal expression in the node and LMP is absent in hypomorphic Fgf8 mutants (Meyers et al., 1999). Furthermore, pharmacological inhibition of Fgfr1 results in similar LR asymmetry defects. Work in lower vertebrates revealed that Fgfr1 controls cilia length and directional fluid flow by regulating expression of the intralagellar transport gene ift88 and two ciliogenic transcription factors, foxj1 and rfx2 (Neugebauer et al., 2009). In vitro studies identified specific molecular interactions of CS-E chains with various FGF ligands (Deepa et al., 2002). It could be hypothesized that CS-E may bind FGF ligands and serve as a co-receptor for the Fgfr1 signalling pathway required for LR axis formation. Genetic and pharmacological intervention studies may address the role of CS-E and Smc3 in LR axis formation and ciliogenesis. The requirement of CS-E for cilia motility could be studied by imaging the node cilia of chlorate treated embryos. Due to the fact that chlorate inhibit sulfation of all GAGs, a more selective approach is needed for manipulating GAG sulfation in various biological systems. For example, Prof Linda Hsieh-Wilson and colleagues at Caltech developed an inhibitor of the sulfotransferase required for sulfation of CS-E chains (Cheung et al., 2017). This sulfotransferase inhibitor was proven to be more selective than chlorate in in vitro systems. Embryo culture experiments with sulfotransferase inhibitor could be used to specifically target CS-E chains during initiation and maintenance of LR asymmetry. Mutations in Smc1 and Smc3 proteins has been associated with Cornelia de Lange syndrome, a severe heterogeneous developmental disorder characterized by growth and cognitive retardation, facial dysmorphia, gastrointestinal abnormalities, microcephaly and upper limb malformations (García et al., 1989). It was proposed that these mutations affect the dynamic association between SMC proteins (cohesins) and DNA during chromosome segregation. Recently, a mouse model of Smc3 was developed by the International Mouse Phenotyping Consortium (MGI:1339795). Heterozygous embryos display a wide range of phenotypic features including decreased body weight, abnormal coat/hair pigmentation, decreased total body fat and decreased bone mineral content. Homozygous individuals exhibit pre-weaning lethality (at E14.5) with complete penetrance, although the mutant phenotype has not been described so far. It would be interesting to compare the phenotypic features of Smc3 mutants with mouse models of ciliopathies and address the functional requirement of Smc3 in ciliogenesis.
Both cytokinesis and ciliogenesis require strict coordination of microtubule organisation and directed trafficking of related proteins. One study showed that several spindle/midbody proteins such as PRC1 and MKLP-1, are localised to the basal bodies in vertebrate ciliated epithelial cells (Smith et al., 2011). The mutants of these proteins display defects in both cilia function and cilia morphology. Another study showed that Smc1 is localised to the basal body and centrosomes suggesting a possible role of this protein in centrosome/basal body related functions (Guan et al., 2008). Bioinformatic comparisons of cilia and midbody proteomes reveal a highly significant degree of overlap (Smith et al., 2011). Taken together, these studies suggest the conserved reuse of a large number of proteins between the cilia and the cytokinetic apparatus.
7. Bibliography


the putative tumour suppressor EXT-1 and is needed for Hh diffusion.’, *Nature*, 394(6688), pp. 85–8. doi: 10.1038/27932.


Fu, Y., Nagy, J. A., Brown, L. F., Shih, S.-C., Johnson, P. Y., Chan, C. K., Dvorak, H. F. and


outflow tract development, somite segmentation and neural tube closure.


neural tube, heart and lung development and abnormal planar cell polarity in the ear.’, BMC developmental biology, 10, p. 87. doi: 10.1186/1471-213X-10-87.


Schrage, Y. M., Hameetman, L., Szuhai, K., Cleton-Jansen, A.-M., Taminiau, A. H. M.,


Ybot-Gonzalez, P., Savery, D., Gerrelli, D., Signore, M., Mitchell, C. E., Faux, C. H.,
signalling and initiation of mouse neural tube closure.’, Development (Cambridge,

Ybot-Gonzalez, P., Savery, D., Gerrelli, D., Signore, M., Mitchell, C. E., Faux, C. H.,
signalling and initiation of mouse neural tube closure.’, Development (Cambridge,

Yen, W. W., Williams, M., Periasamy, A., Conaway, M., Burdsal, C., Keller, R., Lu, X. and
Sutherland, A. (2009) ‘PTK7 is essential for polarized cell motility and convergent
extension during mouse gastrulation.’, Development (Cambridge, England), 136(12),

of polarized cell intercalations drives convergence and extension of presomitic
mesoderm during zebrafish gastrulation’, The Journal of Cell Biology, 180(1), pp. 221–
232. doi: 10.1083/jcb.200704150.

the specification and fate maintenance of zebrafish slow muscle precursors’,

phenotypes between different vangl2 mutants demonstrates dominant effects of the
Looptail mutation during hair cell development.’, PloS one, 7(2), p. e31988. doi:
10.1371/journal.pone.0031988.

2109–2119.

Yost, H. J. (1990) ‘Inhibition of proteoglycan synthesis eliminates left-right asymmetry


