Cell dynamics and cell-matrix interactions
during neural tube closure

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Declaration

I, Matteo Amitaba Molè 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 the thesis.

Matteo Amitaba Molè
Abstract

During development the neuroepithelium bends and fuses dorsally to form the neural tube, precursor of the brain and spinal cord. The current study focused on the role of cell-extracellular matrix (ECM) interactions and cell dynamics during neural tube morphogenesis.

ECM-integrin interactions have long been thought to play a role in neural tube closure, but there has been little experimental analysis. In this study, the initial aim was to identify the ECM constituents and integrin receptors expressed during neurulation and to define their tissue source of expression and protein localisation. This revealed a significant upregulation of the major integrins at the site of zippering.

To address the potential role of ECM-integrin interactions at this site, a conditional approach was used to delete the central receptor subunit, integrin β1, from either the dorsal neural tube or surface ectoderm. Deletion of integrin β1 prevented neural tube closure leading to exencephaly and spina bifida. A model based on loss of dorsal anchorage between surface ectoderm and neural tube was proposed as potential causative mechanism underlying failure of neural tube closure.

During neurulation, the formation of dorsolateral hinge points is characterized by a cell number increase in the dorsal neuroepithelium. The hypothesis that neuroepithelial cells may translocate dorsally was tested by vital fluorescent dye labelling. This demonstrated for the first time that cells migrate within the plane of the neuroepithelium from the mid-ventral to the most dorsal regions during the onset of dorsolateral bending. In vivo live imaging confirmed the above pattern of cell dynamics and further showed the presence of two main streams of cell migration, rostro-dorsally and caudally directed, identifying fundamentally different cell behaviours in the neuroepithelium, depending on rostro-caudal position.
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## Abbreviations

<table>
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<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>BM</td>
<td>Basement membrane</td>
</tr>
<tr>
<td>BMP</td>
<td>Bone Morphogenetic Proteins</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>CE</td>
<td>Convergent extension</td>
</tr>
<tr>
<td>CLE</td>
<td>Caudal lateral epiblast</td>
</tr>
<tr>
<td>CNH</td>
<td>Chordo-neural hinge</td>
</tr>
<tr>
<td>DIC</td>
<td>Differential interference contrast</td>
</tr>
<tr>
<td>DLHP</td>
<td>Dorsolateral hinge points</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s Modified Eagle’s Medium</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
</tr>
<tr>
<td>DOC</td>
<td>Deoxycholic acid</td>
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<tr>
<td>ECM</td>
<td>Extracellular Matrix</td>
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<tr>
<td>EGFP</td>
<td>Enhanced Green Fluorescent Protein</td>
</tr>
<tr>
<td>EYFP</td>
<td>Enhanced Yellow Fluorescent Protein</td>
</tr>
<tr>
<td>FAK</td>
<td>Focal adhesion kinase</td>
</tr>
<tr>
<td>FBS</td>
<td>Foetal Bovine Serum</td>
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<tr>
<td>FGF</td>
<td>Fibroblast Growth Factors</td>
</tr>
<tr>
<td>GAG</td>
<td>Glycosaminoglycans</td>
</tr>
<tr>
<td>GEF</td>
<td>Guanine nucleotide exchange factors</td>
</tr>
<tr>
<td>HSPG</td>
<td>Heparan Sulfate Proteoglycan</td>
</tr>
<tr>
<td>ILK</td>
<td>Integrin linked kinase</td>
</tr>
<tr>
<td>KO</td>
<td>Knock-out</td>
</tr>
<tr>
<td>LB</td>
<td>Luria Broth</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen-activated protein kinases</td>
</tr>
<tr>
<td>MHP</td>
<td>Median hinge point</td>
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<tr>
<td>NMP</td>
<td>Neuromesodermal progenitors</td>
</tr>
<tr>
<td>NTD</td>
<td>Neural tube defects</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PCP</td>
<td>Planar Cell Polarity</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>----------------------------------------</td>
</tr>
<tr>
<td>PFA</td>
<td>Paraformaldehyde</td>
</tr>
<tr>
<td>PNP</td>
<td>Posterior Neuropore</td>
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<tr>
<td>PS</td>
<td>Position-specific</td>
</tr>
<tr>
<td>RGD</td>
<td>Arg-Gly-Asp</td>
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<tr>
<td>SEM</td>
<td>Scanning electron microscopy</td>
</tr>
<tr>
<td>SHH</td>
<td>Sonic Hedgehog</td>
</tr>
<tr>
<td>VLA</td>
<td>Very late antigens of activation</td>
</tr>
<tr>
<td>YS</td>
<td>Yolk Sac</td>
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1. General Introduction

1.1 Neural tube morphogenesis

Neurulation is a fundamental process of vertebrate embryogenesis which results in the formation of the neural tube, precursor of entire central nervous system (Colas and Schoenwolf 2001). The rostral part of the neural tube differentiates into the brain (fore-, mid- and hindbrain), while the caudal portion gives rise to the spinal cord.

At the end of gastrulation, the rostral region of the ectoderm layer becomes instructed by signalling emanating from the node during a process known as neural induction (Sander and Faessler 2001; Stern 2005). Thus, the neural plate becomes specified as an apico-basal thickening of the ectoderm germ layer. The broad neural plate undergoes substantial cellular and tissue changes, becoming elongated along the rostro-caudal axis and narrowed medio-laterally through the mechanism of cellular convergent extension (Wallingford et al. 2002, Ybot-Gonzalez et al. 2007). Concomitantly, the flat neural plate starts to invaginate medially at a focal site of bending, termed median hinge point (MHP) (Moury and Schoenwolf 1995). This gives rise to a V-shaped neural groove which runs along the entire rostro-caudal axis, causing the lateral edges of the neural plate to elevate. In the spinal region at E8.5, the neural plate bends exclusively at the MHP (Fig. 1.1 A) (Shum and Copp 1996). As neurulation progresses further caudally, the straight elevated neural folds, in addition to the MHP, undergo bending dorsolaterally at two paired sites named dorsolateral hinge points (DLHPs) at E9.5 (Fig 1.1 B). This step is essential for the folds to come into apposition towards the dorsal midline. DLHPs form precisely at the site where the neuroepithelium exchanges its basal contact from the mesoderm to the surface ectoderm. At late stages of neurulation, the neural tube loses the MHPs and bends exclusively via the DLHPs (Fig 1.1 C). Eventually, the apposed neural folds come into contact and fuse dorsally to re-establish epithelial continuity between the opposing neural folds (Pai et al. 2012).
Originally, closure of the neural tube was thought to be a continuous process initiating in a region halfway along the rostro-caudal axis of the embryo and then progressing rostrally and caudally to close the entire neural tube (Copp et al 2013). Studies in chick (Van Straaten et al. 1996), mouse (Golden and Chernoff 1993; Copp et al. 1990) and human embryos (O’Rahilly and Müller 2002) challenged this idea and introduced the concept that neurulation is a multi-site process with separate initiation points of fusion along the embryo axis.

In mouse, at E8.5 (6 somite stage) the neural folds come into contact and fuse dorsally for the first time in a region located at the boundary between the hindbrain and spinal cord named closure 1 (Fig 1.2). From this site, fusion proceeds bidirectionally in both rostral and caudal directions. Two additional sites of closure appear 12 hours later in the cranial region: closure 2 at the midbrain-forebrain boundary, and closure 3 at the very rostral end of the forebrain (Fig 1.2). The open regions of neural tube between the sites of closure are termed neuropores. The anterior neuropore (between closures 3 and 2) and the hindbrain neuropore (between closures 2 and 1) eventually close by 16 somites, terminating the process of cranial neural tube closure. Simultaneously, spinal neural tube closure is still happening and caudally directed progression of closure 1 eventually seals the posterior neuropore (PNP) at 30 somites, thus completing the process of primary neurulation (Copp et al. 2003).
In humans, neural tube closure similarly initiates at closure 1, at 18 days post-fertilisation, followed by formation of an event corresponding to closure 3 (Copp and Greene 2013). The existence of closure 2 in humans is controversial, and the most detailed studies suggest a model where cranial neurulation progresses by completion of closure within a single cranial neuropore (O’Rahilly and Müller 2002). Eventually, closure of the PNP and thus completion of primary neurulation occur at 26-28 day post-fertilisation.

Completion of primary neurulation is followed by the process of secondary neurulation which forms the neural tube at low sacral and coccygeal regions (Schoenwolf 1984; Copp and Brook 1989). In contrast to primary neurulation where the neural folds fuse dorsally, during secondary neurulation the neural tube forms by canalisation of a medullary cord precursor. This latter originates from a multipotent population of progenitors located in the tail bud region, which originates from internalisation of the remnant of the primitive streak (Cambray and Wilson 2002).

1.2 Neural tube defects

Neural tube defects (NTDs) are severe congenital malformations, affecting 0.5-2 in every 1,000 established pregnancies world-wide (Mitchell 2005), which arise during embryo development due to failure to close the neural tube (Copp and Greene 2013). This can result either from defective initiation of closure at any of the above sites or from perturbation of fusion progression along the embryo body axis, leading to the development of an open defect. Failure to initiate closure 1 leads to the most severe form of NTDs, craniorachischisis where the neural tube remains open along the entire embryo body axis (Fig. 1.2). Faulty cranial closure leads instead to a condition termed exencephaly, where the neural tissue protrudes outside the surface of the embryo (Fig. 1.2). The condition of exencephaly further evolves to become anencephaly due to progressive degeneration of the brain exposed to amniotic fluid and failure of the skull vault to form over the open lesion. Both craniorachischisis and anencephaly are not compatible with postnatal survival leading to pre- or perinatal lethality.

Progression of closure 1 down the body axis may halt at any time during embryogenesis leading to the condition of open spina bifida, with the size of the defect proportional to the axial level where closure stops (Fig. 1.2). The PNP remains open and the vertebral arches of sclerotome origin are unable to cover the neural tube. Due to exposure to the amniotic environment, the neural tissue degenerates giving rise to the condition of myelocele, if directly
in contact with the amniotic fluid, or to myelomeningocele (spina bifida cystica), if encased inside a sac covered by the meninges (Copp et al. 2015). This condition is compatible with postnatal survival but leads to severe neurological impairment from the site of the lesion downward which affects both the somatosensory and motor systems.

**FIGURE 1.2**

![Diagram of neural tube defects](image)

*Figure 1.2. Primary neurulation and neural tube defects.* Closure of the neural tube initiates at the 6 somite stage at the boundary between the hindbrain and spinal cord, at closure 1. The wave of zippering propagates bidirectionally. A second site of fusion is closure 2 which occurs at the boundary between forebrain and hindbrain. Closure 3 forms at the very rostral end of the forebrain. The region of open neural tube between two sites of closure are the neuropores. The anterior neuropore lies between closures 3 and 2. The hindbrain neuropore lies between closures 2 and 1. The posterior neuropore (PNP) is located at the very caudal end of the developing body axis (region in yellow). Cranial closure completes at about 16 somites by sealing of the anterior and hindbrain neuropores. Spinal closure progresses until 30 somites when the PNP is eventually closed. This event marks the end of primary neurulation. Failure to initiate closure 1 leads to craniorachischisis. Failure to either initiate closures 2-3 or to seal the anterior and hindbrain neuropore leads to anencephaly. Failure to complete closure of the PNP leads to open spina bifida.
1.3 Molecular and cellular basis of neural tube closure

1.3.1 Shaping of the neural plate

At the onset of neurulation, the broad neural plate becomes elongated rostro-caudally (extension) and narrowed medio-laterally (convergence) by the mechanism of convergent extension (CE). This process was first described in *Xenopus* embryos and shown to be regulated by non-canonical Wnt signalling (the PCP pathway) (Goto and Keller 2002; Wallingford and Harland 2002). Perturbation of CE, by disruption of PCP signalling, results in a significantly wide neural plate which prevents fold apposition and fusion at the dorsal midline. Similarly, disruption of PCP signalling in mouse, for example in the loop tail mutant (\textit{Vangl2}\textsuperscript{Lp}) (Greene et al. 1998), prevents initiation of closure 1 and leads to the development of craniorachischisis due to defective convergence and extension of the neural plate (Ybot-Gonzalez et al. 2007b). Recent live imaging studies in mouse provided the first evidence \textit{in vivo} that CE results from precise mediolateral cell intercalation governed by a combination of polarised basolateral protrusive activity and apical junction rearrangement (Williams et al. 2014).

1.3.2 Neuroepithelium bending

Bending of the neural plate at the hinge points is another key event during neural tube morphogenesis which ensures elevation and apposition of the folds. For example, the essential role of dorsolateral bending is demonstrated by the extensive spina bifida observed in the Kumba mouse model (loss of function mutation of \textit{Zic2} gene) (Ybot-Gonzalez et al. 2007). In this mutant, the elevated neural folds remain straight and lack DLHPs, disrupting progression of closure at the lower spinal level.

The signalling pathways underlying dorsolateral bending in the mouse spinal region have been partially identified. Sonic hedgehog (Shh) signalling was shown to decrease in strength gradually as neurulation progresses towards lower spinal levels, which correlates with the shift of neural plate bending from the midline (MHP) to dorsolateral bending (DLHPs) (Ybot-Gonzalez et al. 2002). Importantly, local release of SHH-N active peptide from implanted beads abolished DLHPs and, on the contrary, suppression of Shh signalling induced formation of premature DLHPs. These results provided solid evidence that Shh, emanating from the notochord, exerts a strong inhibitory effect on dorsolateral bending of the neural plate.
However, the suppression of DLHPs formation by surgical removal of the surface ectoderm directed attention on this tissue as a second signalling centre (Ybot-Gonzalez et al. 2002). One group of key morphogen produced by the surface ectoderm are the Bone Morphogenetic Proteins (BMPs) and these were shown to exercise an inhibitory effect on dorsolateral neural plate bending. Our lab provided evidence that the key mechanism necessary and sufficient for formation of DLHPs is BMP antagonism, and this is achieved by the release of noggin, a BMP antagonist, from the dorsal neural plate (Ybot-Gonzalez et al. 2007). The current model suggests that, at E8.5, Shh suppresses noggin expression. As a consequence, BMP signalling actively inhibits dorsolateral bending causing the neural folds to remain straight. Later, as neurulation proceeds further down the spine, Shh becomes weaker and is not able to repress noggin any more. The latter is now able to inhibit BMP dorsally causing the straight neural folds to bend dorsolaterally, forming the DLHPs.

Bending of the neuroepithelium in the MHP appears to occur by a different mechanism from bending at the DLHPs. The MHP is highly enriched in cells with basally localised nuclei which adopt a wedge shape morphology both in mouse (Smith et al 1994) and chick (Schoenwolf and Franks 1984). In contrast, the site of DLHPs contains cells of different shapes: wedge, spindle (intermediate nucleus) and inverted wedge shape (apical nucleus), which does not differ from non-bending regions of the neuroepithelium. The ability of the nuclei to localise at different positions along the apico-basal axis is due to the process of interkinetic nuclear migration (Spear and Erickson 2012). Each cell of the neural plate in fact makes contact with the basal and apical sides of the monolayer, which is termed a pseudostratified neuroepithelium. The nucleus of each neuroepithelial cell moves along the apico-basal axis as a consequence of cell cycle progression, causing cells to change their shape. In contrast to the asynchronous cell cycle progression of the DLHPs, the MHP is characterised by a relatively long cell cycle due to prolongation of the S-phase which causes nuclei to migrate basally and cells to adopt a wedge shape morphology (McShane et al. 2015). Although the signalling regulating midline bending has not yet been identified, the fact that MHP induction requires proximity to the notochord strongly points towards a potential inducing signalling such as Shh or chordin, which are secreted by notochord cells (Placzek et al. 1990; Patten and Placzek 2002; Ybot-Gonzalez et al. 2002).
1.3.3 Adhesion and fusion

Following elevation and bending, the dorsal tips of the apposing neural folds come into contact and fuse dorsally. Remodelling of the surface ecdoerm and neuroepithelium then results in the formation of a defined closed neural tube covered by a continuous layer of surface ectoderm. The type of cells which mediate initial contact varies along the body axis. In mouse, surface ectoderm cells establish the primary contacts between apposing neural folds in both the midbrain and hindbrain, while neuroepithelial cells mediate the process of fusion in the forebrain (Geelen and Langman 1979; Geelen and Langman 1977). Cellular adhesion in the cranial neural folds appears to be regulated by interactions of cell surface ephrin receptors with ephrin ligands as shown by failure of neural tube zippering in ephrin-A5 null mice (Holmberg et al. 2000).

In the caudal region, primary mediators of adhesion at the site of fusion are the cells of surface ectoderm (Rolo et al. 2016). The site of initial contact between apposing folds is characterised by intense protrusive activity emanating from the surface ectoderm cells. While filopodia type protrusions predominate at early stages of spinal neural tube closure, later stages are characterised by a combination of both filopodia and ruffles (sheet-like lamellipodia). Suppression of protrusions by deletion of Rac1 from the surface ectoderm cells prevents dorsal fusion and leads to spina bifida in these embryos, demonstrating the key requirement of protrusions for zippering (Rolo et al. 2016).

A different mechanism from focal zippering has been recently proposed for fusion of the neural folds in the midbrain. In contrast to a single point of adhesion between apposing folds, multiple sites of contacts have been observed by live imaging of the midbrain, with the surface ectoderm cells extending cellular bridges across the physical gap between the apposing folds (Pyrgaki et al. 2010).
1.4 Basement membrane structure and function

“Membranaceous sheath of the most exquisite delicacy” is the first description of the basement membrane by Bowman during his observations of muscles (Bowman 1840). The basement membrane (BM) is a specialised type of extracellular matrix (ECM), present in all Metazoa, which lines the basal side of epithelia and endothelia and surrounds muscle, fat and Schwann cells (Hynes and Naba 2012; LeBleu et al. 2007; Yurchenco 2011). Thus, it separates epithelial cells from the underlying and surrounding mesenchymal tissue. This thin layer of extracellular components (50-100 nm thick) when viewed by electron microscopy appears to consist of an electron-dense sheet (lamina densa) separated from the cell membrane by an electron-lucent sheet (lamina lucida). These latter form collectively the basal lamina, which together with the lamina reticularis, synthesised by the mesenchyme, constitute the entire structure of the BM which can be observed by light microscopy.

The primary components of the BM architecture are: collagen type IV and laminins which give rise to the two major supramolecular networks, and nidogens and the heparan sulfate proteoglycan (HSPGs) perlecan which act as scaffolds to cross link the network. Additional components to the basic BM backbone such as fibronectin, agrin, fibulin, collagen type XV, collagen type XVIII, usherin, bamacan, nephronectin, papilin, netrins further enrich the composition of the BM increasing its heterogeneity within different tissues.

1.4.1 Laminins

Laminins are one of the major components of the intricate network of molecules constituting the BM. Laminins are long glycoproteins composed of three polypeptide chains (five α, four β and three γ subunits) twisted together to form a cross-like structure. 16 different isoforms exist, due to the combination between different chain types, and they are capable of self-assembly into networks in vitro by non-covalent interactions between trimers via the N-terminal LN module (Durbeej 2010). Specifically, laminin α1β1γ1 and laminin α5β1γ1 are the first constituents of the BM to be expressed during embryogenesis and are thought to be the primary organisers to induce initial BM assembly (Miner 2008).
1.4.2 Collagen type IV

Collagen type IV is another major molecule of the BM, which assembles extracellularly into a flexible network providing structural and tensile strength to the BM (Ricard-Blum 2011a). It is a type of non-fibrillary collagen composed of three α chains which interact covalently via the NC1 domain (C-terminal domain) to form a heterotrimeric helix structure called a protomer. Six different genes exist encoding the 6 alpha chains, α1-α6, but only three major trimeric combinations have been identified: α1α1α2, α3α4α5, α5α5α6. The major variant present in almost every BM is the combination α1α1α2 whereas the other two forms are more tissue- and stage-specific with α3α4α5 being a major component of the glomerular basement membrane and α5α5α6 being present in the neuromuscular junction (Yurchenco 2011). Similar to laminins, collagen type IV protomers are capable of self-assembly. Interactions via the NC1 domains and 7S domains allow polymerisation of the protomers into a 2D branching network.

In addition to collagen type IV, the BM may also contain minor forms of two other types of collagens: collagen type XVIII (Marneros and Olsen 2005) and collagen type XV (Myers et al. 1996). The former is modified by attachment of heparan sulfate groups while the latter contains both heparan and chondroitin sulfate chains. They both seem to specifically localise at the BM-stromal interface to mediate linkage to the stroma.

1.4.3 Nidogens and Perlecan

Essential components for the formation of the basic architecture of the BM are the small glycoproteins nidogen-1 and nidogen-2, also known as entactins (Schymeinsky et al. 2002). Nidogens bind to laminins on one side of the BM, and interact with collagen type IV on the other side, acting as bridge between the two network-forming components. Their role is therefore essential for stabilisation of the overall architecture of the BM, especially when subjected to intense mechanical stresses.

Perlecan (HSPG) is the most abundant heparan sulfate proteoglycan present in the BM. It binds to nidogen, which in turn is connected to the laminin-nidogen-collagen IV axis, and to the cell surface receptors integrins and α-dystroglycan (Hopf et al. 2001; Hopf et al. 1999). Collateral linkages mediated by perlecan ensure proper anchorage of the laminin and collagen type IV networks to the cell surface receptors. Agrin can be also present in the BM and functions similarly to perlecan in establishing collateral linkages to the cell surface (Moll et al. 2001).
1.4.4 Fibronectin

An abundant and ubiquitous component of the interstitial ECM, fibronectin can also be present in the BM alongside its primary constituents (reviewed in Schwarzbauer & DeSimone 2011). Fibronectin is a large dimeric glycoprotein composed of two polypeptide chains joined by disulphide bonds at the C-terminus. Both subunits are encoded by a single Fn1 gene, both in humans and mouse, but alternative splicing of its pre-mRNA can give rise to a variety of different isoforms. Two types of fibronectin are present: plasma fibronectin which exists as a soluble form in the blood plasma and other interstitial fluids, produced by hepatocytes; and cellular fibronectin present in the ECM which is initially secreted in a soluble state, largely by fibroblasts, to eventually polymerise into insoluble fibrils. Fibronectin binds and interacts with integrins via a sequence of three amino acids (Arg-Gly-Asp: RGD motif). Despite being the central site for cell binding, several other motifs exist along the fibronectin molecule which are recognised by different integrins. Fibronectin interacts also with collagens and with heparan and chondroitin sulfate chains, for example on the transmembrane proteoglycans syndecans (Woods et al. 2000).

1.5 Basement membrane assembly and molecular interactions

The assembly of the BM starts on the surface of cells by means of interactions between laminins and active ECM cell surface receptors (integrins, dystroglycan, transmembrane heparan sulphate proteoglycans) through the laminin LG domain (carboxyl terminal globular domain). Single laminin monomers then start to polymerise via their LN domain (amino terminal) giving rise to a bi-dimensional network. Formation of this initial scaffold promotes subsequent accumulation of other ECM molecules, collagen type IV in particular, which also polymerises into a second covalently stabilised network that interacts with the initial laminin network. The small glycosylated nidogen proteins act as a bridge between the laminins and collagen type IV networks. Eventually, perlecanc establishes collateral linkages between nidogen and the ECM receptors stabilising this supramolecular structure into a final mature BM complex (Yurchenco 2011).

Despite the capability of self-polymerisation in vitro, assembly of the BM is facilitated and regulated by bidirectional interactions between the BM components and the receptors expressed on the membrane of cells. The active receptors permit the capture and
concentration of BM constituents at the site of assembly but also the initial accumulation of ECM molecules facilitates recruitment and activation of the corresponding receptors, supporting the idea of a cooperative bidirectional mechanism for BM assembly (Brown 2011).

In contrast to laminins and collagen type IV, assembly of fibronectin is entirely a cell-driven process which requires binding to the integrin receptors (Schwarzbauer and DeSimone 2011). Fibronectin is initially secreted into the extracellular space as dimers which are maintained in a soluble status due to their compact conformation which prevents initiation of fibrillogenesis. Integrin $\alpha_5\beta_1$ is the primary mediator of fibronectin fibrillogenesis, despite several other receptors (integrins $\alpha_4\beta_1$, $\alpha_v\beta_1$, $\alpha_v\beta_3$, syndecan-1 and -2) also contributing to fibronectin assembly. Thus, integrin $\alpha_5\beta_1$ binds to the soluble fibronectin dimers via the RGD motif.

This initial binding promotes clustering of the integrin receptors which accumulate at this site bound to fibronectin dimers, still in a compact conformation. Binding of the dimers also induces linkage of the cytoplasmic tail of the integrin receptor to the internal actin cytoskeleton. Activation of downstream Rho GTPases stimulates contractility of the actomyosin stress fibres. Contractility and tension are thus transmitted to the fibronectin bound dimers externally inducing a conformational change which exposes the cryptic site for fibronectin fibrillogenesis. Focal concentration of dimers bound to integrin facilitates interactions between the mechanically stretched dimers via the 70-kDa fragment assembly sites located at the N-terminus. Thus short fibrils or multimers start to form which are soluble in deoxycholate (DOC) detergent. Fibronectin dimers continue to be added end-to-end thus lengthening the fibrils which are eventually converted into a DOC-insoluble network due to strong non-covalent protein-protein interactions.
1.6 Cell surface receptors interacting with the basement membrane

1.6.1 Integrin receptors

The main cell surface receptors which mediate binding to the BM molecules are the integrins, a vast family of transmembrane glycosylated receptors, composed of α and β subunits, which act as a linkage between the external environment (ECM) and the internal cytoskeleton (either actin or intermediate filaments). Alongside their structural role as mechanical connectors, their signalling influences almost all aspect of cell behaviour: they regulate gene expression, affect cell polarity, survival, differentiation, proliferation and direct cell migration (Barczyk et al. 2010; Schwartz 2010; Lowell and Mayadas 2012a; Campbell and Humphries 2011).

Integrins were first characterised at the molecular level 30 years ago by the seminal work of Erkki Ruoslahti and Richard O. Hynes (Barczyk et al. 2010). The name “integrins” was coined to highlight the crucial role of these receptors in maintaining the integrity of linkage between the outside (ECM) and the inside of the cell (cytoskeleton) (Tamkun et al. 1986). At that time several other groups were working in diverse cell surface receptors, termed position-specific (PS) antigens in Drosophila and very late antigens of activation (VLA) on cells of the immune system, which eventually were recognised to belong to the same integrin family (Barczyk et al. 2010).

The functional receptor exists as a heterodimer composed of an α and β subunit which associate by non-covalent interactions (Hynes 2002). In vertebrates, 18 α and 8 β subunits have been identified which give rise to 24 different functional integrin receptors (Fig. 1.3), with each subunit encoded by a single gene. Dimerization between the two subunits occurs intracellularly, where usually the β is present in excess and the α subunit is limiting (Humphries 2000). The α subunit therefore determines both the dimeric combination and the amount of functional receptor which will be eventually transported on the cell surface. Individual α or β subunits cannot be present freely on the surface of the cells.

Each subunit contains a large extracellular domain which regulates binding to the ECM ligand, a single spanning transmembrane domain and a short cytoplasmic tail which mediates indirect binding to the actin cytoskeleton via scaffolding proteins (Zent and Pozzi 2010). The globular N-terminal domain of the α subunit together with the domain of the β subunit determine cooperatively ligand specify where the ligand sits at the interface between the extracellular domains of the α and β subunits. However, some α subunits (leukocyte-specific and collagen-
interacting) contain an additional I-domain (Johnson et al. 2009). In this case binding to the ECM ligand is mediated exclusively by the I-domain of the α subunit and the β subunit does not take part in ligand recognition. The cytoplasmic tail is usually very short, and the β subunit tail contains the sequences for binding of talin and kindlins, to mediate anchorage to the cytoskeleton.

1.6.2 Dystroglycan complex

Dystroglycan is another ECM receptor, composed of a transmembrane β subunit which interacts with the cytoskeleton (dystrophin-utrophin to F-actin) and an extracellular α subunit that connects with the extracellular matrix through long carbohydrate chains (Ervasti and Campbell 1993; Michele and Campbell 2003). In some tissues, such as skeletal muscle and Reichert's membrane, dystroglycan is thought to be the primary organiser for anchorage of laminins and BM assembly (Williamson et al. 1997; Henry and Campbell 1998). However, it has not been extensively studied in other embryonic processes, and so the role of dystroglycan during non-muscle development is poorly understood.
**Figure 1.3. Integrin heterodimeric combinations.** The integrin combinations are grouped according to ligand specificity. Presence of α domain is reported. Table based on the following references: Lowell and Mayadas 2012b; Barczyk et al. 2010b.

<table>
<thead>
<tr>
<th>Integrin</th>
<th>αl</th>
<th>Ligands and recognition sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>αβ1(CD49c, VLA3)</td>
<td>α1</td>
<td>Laminins (LN-511 &gt; LN-332 &gt; LN-211), Thrombospondin, uPAR</td>
</tr>
<tr>
<td>αβ1 (CD49f, VLA6)</td>
<td>α1</td>
<td>Laminins (LN-511 &gt; LN-332 &gt; LN-411), Thrombospondin, ADAM, Cyr61</td>
</tr>
<tr>
<td>αβ4</td>
<td>α1</td>
<td>Laminins (LN-332 &gt; LN-511)</td>
</tr>
<tr>
<td>α7β1</td>
<td>α7</td>
<td>α7Xβ1: (LN-511 &gt; LN-211 &gt; LN-411 &gt; LN-111), a7Xβ1: (LN-111 &gt; LN-211 &gt; LN-511).</td>
</tr>
<tr>
<td>avβ1(CD51)</td>
<td>AV</td>
<td>RGD: Fibronectin, Vitronectin, Osteopontin, Latent TGF-β.</td>
</tr>
<tr>
<td>avβ3</td>
<td>AV</td>
<td>RGD: Fibronectin, Vitronectin, Fibrinogen, Thrombospondin, vWF, Cyr61, fibrillin, tenascin, PECAM-1, Osteopontin, ADAM, MMP, uPAR, uPA, ICAM-4.</td>
</tr>
<tr>
<td>avβ5</td>
<td>AV</td>
<td>RGD: Vitronectin, Osteopontin, Latent TGF-β.</td>
</tr>
<tr>
<td>avβ6</td>
<td>AV</td>
<td>RGD: Fibronectin, Latent TGF-β, Osteopontin, ADAM.</td>
</tr>
<tr>
<td>avβ8</td>
<td>AV</td>
<td>RGD: Vitronectin, Latent TGF-β.</td>
</tr>
<tr>
<td>α1β3(CD41, Gpllb)</td>
<td>α1β3</td>
<td>RGD: Fibrinogen, Fibronectin, Vitronectin, Thrombospondin, vWF, Cyr61, ICAM-4, CD40.</td>
</tr>
<tr>
<td>α5β1 (CD49e, VLA5)</td>
<td>α5β1</td>
<td>Fibronectin (RGD), Osteopontin, Fibrillin, Thrombospondin, ADAM, COMP.</td>
</tr>
<tr>
<td>α8β1</td>
<td>α8β1</td>
<td>RGD: Fibronectin, Vitronectin, Nephroectin, Osteopontin, Latent TGF-β.</td>
</tr>
<tr>
<td>α1β1(CD49a, VLA1)</td>
<td>x</td>
<td>Collagen IV &gt; collagen I (GFOGER); Collagen IX. Semaphorin 7A</td>
</tr>
<tr>
<td>α2β1(CD49b, VLA2)</td>
<td>x</td>
<td>Collagen IV &gt; collagen I (GFOGER); Collagen IX. Tenasin, E-Cadherin.</td>
</tr>
<tr>
<td>α10β1</td>
<td>x</td>
<td>Collagen IV &gt; collagen VI &gt; collagen III (GFOGER); Collagen IX</td>
</tr>
<tr>
<td>α11β1</td>
<td>x</td>
<td>Collagen I &gt; collagen IV (GFOGER); Collagen IX</td>
</tr>
<tr>
<td>αβ2(CD11d)</td>
<td>x</td>
<td>ICAM-3 and VCAM-1, Fibrinogen, Plasminogen.</td>
</tr>
<tr>
<td>αβ2(CD11a)</td>
<td>x</td>
<td>ICAM-1, -2, -3, -5</td>
</tr>
<tr>
<td>αMβ2(CD11b)</td>
<td>x</td>
<td>Fibrinogen, ICAM, IC3b, Heparin, Factor X.</td>
</tr>
<tr>
<td>αXβ2(CD11c)</td>
<td>x</td>
<td>Fibrinogen, IC3b, Heparin, Collagen, Plasminogen, ICAM-4.</td>
</tr>
<tr>
<td>αβ7(CD103, HML-1)</td>
<td>x</td>
<td>E-Cadherin</td>
</tr>
<tr>
<td>α4β1 (CD49d, VLA4)</td>
<td>x</td>
<td>Fibronectin and VCAM-1, Thrombospondin, ICAM-4, MadCAM.</td>
</tr>
<tr>
<td>α4β7</td>
<td>x</td>
<td>MadCAM-1, Fibronectin, VCAM-1</td>
</tr>
<tr>
<td>α9β1</td>
<td>x</td>
<td>Tenascin-C, VEGF-C, VEGF-D, VCAM-1, Osteopontin, ADAM.</td>
</tr>
</tbody>
</table>
1.7 Integrin signalling

Integrins have a dual role: they establish a mechanical anchorage, connecting to the cytoskeleton via their cytoplasmic tail, and to the ECM via their extracellular domains. This linkage bi-directionally regulates cytoskeletal organisation and cell motility on the one hand, and ECM architecture externally on the other. Alongside their structural role, integrins also sense the external environment and transduce responses intracellularly via activation of various downstream signalling pathways which regulate polarity, survival, differentiation and proliferation (Barczyk et al. 2010; Schwartz 2010; Lowell and Mayadas 2012a; Campbell and Humphries 2011). The ability of integrins to transduce signalling intracellularly is known as “outside-in signalling”.

1.7.1 Inside-out signalling

During some specific physiological situations, such as coagulation, induction of inflammatory responses or regulation of motility during morphogenesis, integrins can exist in an inactive or low affinity state which prevents binding of cells (e.g. platelets and leukocytes) to the freely circulating ECM ligands, present for example in the blood. In these situations, integrin activation needs to be tightly regulated to ensure adhesion only in response to activation of the inflammatory response or to prevent bleeding (Shattil et al. 2010). The process of integrin activation from intracellular signals is known as “inside-out signalling” and has been extensively elucidated for the integrin αIIbβ3 which is present on the surface of platelets (Wegener et al. 2007).

Two of the major players in integrin activation are talin and kindlins (Moser et al. 2009; Calderwood et al. 2013). The former is an actin-binding protein which recognises a specific motif in the cytoplasmic tail of β subunit. Binding of talin induces activation of integrins by destabilising the integrin salt bridge which exists between the cytosplamic tails of the α and β subunits. Kindlins, despite being not sufficient for integrin activation alone, cooperate with talin to bind distinct regions of the cytoplasmic tail of the β subunit.

Upon binding and breakage of the salt bridge, the cytoplasmic tails of the α and β subunits become separated and reoriented leading to a conformational change of the extracellular domain to a high affinity state. The extracellular domain of integrins thus shift from a bent
low-affinity conformation to an extended active conformation which unravels the binding sites and induces binding to the ECM ligand. According to the “switchblade model” only integrins in an open conformation can engage in ligand binding (Luo et al. 2007). In contrast, the “deadbolt model” predicts that integrin activation occurs only after ligand binding has occurred and serves to increase ligand binding affinity (Xiong et al. 2003).

1.7.2 Outside-in signalling

Upon binding to the ligand, integrins start to cluster and a preliminary adhesion between the ECM and the internal cytoskeleton is established, with talin mediating the first anchorage between the actin cytoskeleton and the cytoplasmic tail of the β subunit (Fig 1.4) (Harburger and Calderwood 2009). This initial anchorage further matures into a focal complex by the recruitment of additional cytoskeletal and signalling molecules which reinforce the ECM-integrin-actin cytoskeleton axis and initiate downstream signalling. This gives rise to a multiprotein complex at the cytoplasmic tail of integrins with 156 cross-interacting components which collectively form the “integrin adhesome” (Zaidel-Bar et al. 2007; Zaidel-Bar and Geiger 2010).

The anchorage between integrins and the actin cytoskeleton is further reinforced by outside-in signalling. Following the initial integrin-F-actin binding mediated by talin, vinculin is recruited to the nascent adhesion and acts as a linker to stabilise the talin-actin interactions (Fig. 1.4) (Humphries et al. 2007). α-actinin promotes cross-linkage of actomyosin stress fibres and interacts with both talin and vinculin, reinforcing the cytoskeleton adhesion (Sjöblom et al. 2008). Paxillin recruitment acts as a docking site for binding or kinases and actin-binding protein at the site of nascent adhesion (Turner 2000). Another major scaffolding protein is the integrin linked kinase (ILK) which further stabilises the connection to the actin cytoskeleton via its binding partner, parvin and PINCH (Fig. 1.4) (Legate et al. 2006). However, it is still unknown if ILK functions also as a kinase alongside its role as scaffolding protein.

At the heart of the integrin adhesome lies the central focal adhesion kinase (FAK) (Mitra et al. 2005). Recruitment and activation of FAK at the site of adhesion induces phosphorylation of the kinase Src, which in turns phosphorylates FAK, further increasing the catalytic activity and promoting the full activation of the two kinases. The FAK-Src complex (Fig. 1.4) acts as a central node within the integrin adhesome which integrates downstream signalling deriving from the outside-in integrin pathway with signalling deriving from growth factors which cross-
talk with the integrin receptors (Mitra et al. 2005). Multiple extracellular inputs thus converge onto the FAK-Src complex which acts as an integrator to modulate an output response by the activation of several downstream signalings.

The FAK-Src complex plays a crucial role in the regulation of the actin cytoskeleton assembly and contractility. It coordinates formation and disassembly of the stress fibres and cortical actin at the site of focal adhesion by activating α-actinin, which crosslinks actomyosin stress fibres, and by regulating the action of the Rho family small GTPases (Fig. 1.4) (Mitra et al. 2005). The FAK-Src complex associates with guanine nucleotide exchange factors (GEFs) or with GTPase-activating proteins (GAPs), modulating indirectly the suppression or activation of the Rho family small GTPases. Motility of the cell at the leading edge is therefore regulated by the FAK-Src complex which coordinates the suppression of actomyosin contractility by RhoA inhibition, which controls the state of myosin light chain phosphorylation (MLC) via ROCK, and concomitantly stimulates lamellipodia and filopodia formation by inducing Rac1 and Cdc42 respectively (Huveneers and Danen 2009). Moreover, the FAK-Src complex promotes polarisation by modulating microtubule stabilisation at the leading edge of motile cells via the Rho GTPase effector Diaphanous (mDia) (Palazzo et al. 2004).

Besides the ability to regulate the cytoskeleton, the FAK-Src complex also plays a crucial role in control of cell cycle progression and cell survival. Signalling from growth factors and integrins are integrated downstream of the FAK-Src complex with the activation of the Ras-MEK-MAPK pathway, leading to the phosphorylation of MAPK and ERK2 (Fig. 1.4) (Chiarugi and Giannoni 2008). Cell survival is also regulated by the activation of Akt via recruitment of PI-3-kinase by FAK to the site of focal adhesion (Fig. 1.4) (Miranti and Brugge 2002a).
Figure 1.4. Outside-in signalling. Upon integrin binding to the ligand (a molecule of laminin is depicted) and receptor clustering, talin mediates the first anchorage between the actin cytoskeleton and the cytoplasmic tail of the β subunit. Vinculin binds talin and F-actin. α-actinin crosslinks actomyosin stress fibers and connects both talin and vinculin to the cytoskeleton. The FAK-Src complex is recruited to the site of the nascent focal adhesion and is activated by cross-phosphorylation. The FAK-Src complex receives signals both from activation by growth factors and from adhesion of integrins to the substrate. The FAK-Src complex modulates the cytoskeleton via α-actin and via the activation of the Rho-family small GTPases. Activation of the Ras-MEK-MAPK signalling downstream of the FAK-Src complex regulates cell survival, proliferation and cell cycle progression. The downstream Akt-PI3-kinase signalling regulates cell survival.
1.8 Genetic analysis of ECM constituents and integrins in vivo: phenotypes of knock-out (KO) mice

Studies of KO mice have provided some insights into the in vivo role of the adhesome during embryo development. Deletion of integrins (Fig. 1.5) produces a range of different phenotypes which encompass severe conditions of early embryonic lethality, in the case of the major fibronectin interacting receptors such as integrin β1, α5 and αv, and milder defects compatible with postnatal survival, when the group of the major collagen interacting integrins are inactivated.

1.8.1 Integrin β1 mutants

The most severe phenotype arises from deletion of integrin β1 which causes simultaneously the loss of 12 different integrins due to the obligatory requirement for this receptor to dimerise with 12 α subunits. Despite normal fertilisation and normal pre-implantation development, removal of this key subunit leads to arrest of embryo development at peri-implantation stage at E5.5 with deterioration of the inner cell mass from E4.5, despite normal attachment of the trophoblast cells to the decidual tissue (Fassler and Meyer 1995; Stephens et al. 1995). One possible explanation proposed for the above phenotype is that integrin β1 adhesion at this stage may be essential for survival and growth of the inner cell mass. An alternative hypothesis is that deletion of integrin β1 prevents correct assembly of the BM and that lack of BM would eventually lead to early lethality. Further insights on the in vivo role of integrin β1 during peri-implantation development come from a recent study which proposed a new model of epiblast morphogenesis based on integrin-BM interactions (Bedzhov and Zernicka-Goetz 2014). According to this model, deposition of the laminin BM and activation of signalling through integrin β1 are essential for the initial establishment of polarity of the epiblast. Perturbation of integrin β1 causes the epiblast to fail in polarisation and lumen formation preventing further development of the epithelium into a polarised rosette like structure.
### FIGURE 1.5

<table>
<thead>
<tr>
<th>Gene</th>
<th>Receptor</th>
<th>Phenotype</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Itgb1</td>
<td>Integrin β1</td>
<td>Embryonic lethality at peri-implantation stage</td>
<td>(Fassler and Meyer 1995; Stephens et al. 1995)</td>
</tr>
<tr>
<td>Itga3</td>
<td>Integrin α3</td>
<td>Perinatal lethal. Skin blistering. Defects in kidney and lung development.</td>
<td>(Kreidberg et al. 1996)</td>
</tr>
<tr>
<td>Itga6</td>
<td>Integrin α6</td>
<td>Perinatal lethal. Skin blistering. Defects in brain cortex organisation</td>
<td>(Georges-Labouesse et al. 1996)</td>
</tr>
<tr>
<td>Itga7</td>
<td>Integrin α6</td>
<td>Perinatal lethal. Muscular dystrophy, defects in vasculature and placenta formation</td>
<td>(Mayer et al. 1997)</td>
</tr>
<tr>
<td>Itga1</td>
<td>Integrin α1</td>
<td>Viable and fertile. No phenotype.</td>
<td>(Gardner et al. 1996)</td>
</tr>
<tr>
<td>Itga2</td>
<td>Integrin α2</td>
<td>Viable and fertile. Mild phenotype: reduced branching morphogenesis mammary glands.</td>
<td>(Chen et al. 2002; Holtkötter et al. 2002)</td>
</tr>
<tr>
<td>Itga10</td>
<td>Integrin α10</td>
<td>Viable and fertile. Mild chondrodysplasia.</td>
<td>(Bengtsson et al. 2005)</td>
</tr>
<tr>
<td>Itga11</td>
<td>Integrin α11</td>
<td>Viable and fertile. Mild defect incisor eruption.</td>
<td>(Popova et al. 2007)</td>
</tr>
<tr>
<td>Itga5</td>
<td>Integrin α5</td>
<td>Embryonic lethal: shortened body axis, defects in mesodermal derivatives.</td>
<td>(Yang et al. 1993a)</td>
</tr>
<tr>
<td>Itgav</td>
<td>Integrin αv</td>
<td>Embryonic lethal (80% penetrance) due to vascular defects.</td>
<td>(Bader et al. 1998)</td>
</tr>
<tr>
<td>Itga8</td>
<td>Integrin α8</td>
<td>Perinatal lethality. Defective kidney morphogenesis.</td>
<td>(Müller et al. 1997)</td>
</tr>
<tr>
<td>Itgb3</td>
<td>Integrin β3</td>
<td>Viable and fertile. Platelets abnormalities, bone defects.</td>
<td>(Hodivala-Dilke et al. 1999)</td>
</tr>
<tr>
<td>Itgb5</td>
<td>Integrin β5</td>
<td>Viable and fertile. No phenotype.</td>
<td>(Huang et al., 2000)</td>
</tr>
<tr>
<td>Itgb8</td>
<td>Integrin β8</td>
<td>Perinatal lethality due to vascular defects of placenta.</td>
<td>(Zhu et al. 2002)</td>
</tr>
<tr>
<td>Itgb1bp1</td>
<td>Integrin β binding protein 1</td>
<td>Skull and skeleton abnormalities due to reduced osteoblast proliferation and delayed bone mineralisation.</td>
<td>(Bouvard et al. 2007)</td>
</tr>
</tbody>
</table>

Fig. 1.5. Phenotypes of the integrin KO mice. Summary of the KO phenotypes for each integrin subunit, grouped by ligand binding affinity: laminin-interacting integrin subunits in red, collagen IV-interacting subunits in blue, fibronectin-interacting subunits in green.

#### 1.8.2 Laminins and integrin interacting receptor mutants

The observed peri-implantation lethality of integrin β1 KOs is consistent with the phenotype observed in laminin γ1 KO embryos which also die at E5.5 despite normal development before implantation (Smyth et al. 1999). Removal of laminin γ1 was shown to disrupt correct assembly of the BM and as a consequence to prevent normal morphogenesis of the embryo with a significant increase in cell death. The two main laminin isoforms expressed at the peri-implantation stage are laminin α1β1γ1 and laminin α5β1γ1 which localise in two main BMs present at this stage: the BM between the epiblast and visceral endoderm, and the BM which gives rise to Reichert’s membrane. Consistent with the laminin γ1 phenotype, ablation of laminin β1 also prevents normal BM assembly and leads to early lethality at E5.5 since no functional laminin trimers can be assembled (Miner et al. 2004).
On the contrary, removal of laminin α1 has a milder effect: null embryos for laminin α1 succeed to assemble a BM, due to the presence of laminin α5 which can act as a compensatory subunit, and initiate cavitation (Miner et al. 2004). However, failure of assembly of Reichert’s membrane leads eventually to lethality and arrest of embryogenesis at E6.5-7. Interestingly, deletion of dystroglycan, a laminin receptor, also results in disruption of Reichert’s membrane which prevents embryo development beyond the egg cylinder stage (Williamson et al. 1997). This shows the critical role of dystroglycan as a receptor for Reichert’s membrane BM assembly and is consistent with an early requirement for integrin receptors in the assembly of epiblast/primitive endoderm BMs.

Embryos deficient for the laminin α5 subunit progress successfully beyond the implantation stage, likely due to compensation from the α1 subunit, and instead start to develop significant morphological defects at later stages of embryo development (Miner et al. 1998). At E12.5, null embryos fail to separate the digits (syndactyly) of the forelimbs and show a severe dysmorphogenesis of the placental labyrinth which leads to lethality at E14-17. Interestingly, a high percentage (60%) of mutant embryos fail to complete cranial neural tube closure which results in exencephaly encompassing the mid- and hindbrain regions but does not affect the caudal neural tube. Analysis of the ultrastructure of the ECM shows that both the BM underlying the SE and the one beneath the NE are structurally intact except for the region of SE in close proximity to the dorsal neural folds. The authors (Miner et al. 1998) speculate that impairment of this BM would affect the capability of the overlying SE to generate lateral forces essential for medial convergence and thus for neural tube closure.

In contrast to laminin α1β1γ1 and α5β1γ1 which are the main isoforms involved during early embryo development, the other laminin isoforms appear to have a specific role at later stages and when mutated to show a phenotype only around or after birth. KO of laminin α2 leads to peripheral neuropathy and muscular dystrophy (Xu et al. 1994; Sunada et al. 1994), a phenotype that is also caused by deletion of its major integrin receptor integrin α7β1 (Mayer et al. 1997). Laminin α4 (Patton et al. 2001) and β2 (Noakes et al. 1995) are important in neuromuscular synapses and no phenotype has been overserved for KO of laminin γ3 subunit (Dénes et al. 2007). Genetic KO of laminin α3 (Ryan et al. 1999), β3 (Kuster et al. 1997) and γ2 (Meng et al. 2003) subunits die all few days after birth because of severe skin blistering defects.

Laminin α3β3γ2 interacts specifically with integrin α6β4 and, in contrast to the majority of integrins which mediate interactions with the actin cytoskeleton, integrin α6β4 instead connects the laminin BM to the intermediate filament cytoskeleton. This gives rise to a
specialised type of adhesion named hemidesmosomes on the basal side of stratified epithelia such as the keratinocytes of the skin. Deletion of either integrin α6 or integrin β4 leads to loss of hemidesmosomes and severe blistering of the skin (Dowling et al. 1996; Georges-Labouesse et al. 1996; van der Neut et al. 1996), as observed in KO mice of laminin α3, β3 and γ2 subunits. Despite integrin α6β4 being the major receptor, integrin α3β1 also takes part in the early maturation of the pre-hemidesmosomal structure and facilitates the recruitment of integrin α6β4 at this site (Wickström et al. 2011). Mutations in several components of this integrin-intermediate filament-laminin axis results in a skin blistering conditions in humans known as epidermolysis bullosa (Uitto 2009).

KO of the another major laminin receptor, integrin α3, leads to defects of kidney and lung morphogenesis due to disruption of epithelial BM assembly (Kreidberg et al. 1996). Interestingly, doubly homozygous mutants for integrin α3 and integrin α6 develop NTDs, including some embryos that were reported to fail to close the entire neural tube. This led to development of craniorachischisis from the midbrain to the spinal level with deformation of the most caudal axis (kinked tail) (De Arcangelis et al. 1999). However, breeding of similar mice in our lab failed to reproduce this finding, with only occasional double homozygotes displaying hindbrain exencephaly (Carvalho and Copp, unpublished).

1.8.3 Collagen IV and integrin interacting receptors mutants

Collagen type IV is an essential component of the BM together with laminins. Ablation of the major isoform of collagen type IV (α1α1α2) allows embryo development and BM formation until E9.5 (Pöschl et al. 2004). After this stage, collagen type IV is essential to maintain integrity and stability of the BM under increasing mechanical demand. KO Col4a1/2 embryos die at E10.5-11.5 because of structural abnormalities of the BM and failure of Reichert’s membrane to expand.

Loss of the major collagen IV interacting integrins (α1, α2, α10, α11) does seem to have a major impact to the development of the embryo. Abolition of either of integrin α1 (Gardner et al. 1996) or integrin α2 (Chen et al. 2002; Holtkötter et al. 2002) does not produce any observable phenotype, with viable and fertile offspring. Loss integrin α10 produces a mild chondrodysplasia (Bengtsson et al. 2005) while deletion of integrin α11 leads to a mild reduction in size and body weight (Popova et al. 2007). The observed phenotype is not associated with defects affecting the skeletal system. It is instead caused by malnutrition due
to delay in incisors eruption. Supplementation of soft diet was in fact able to rescue the defect and to normalise the mice body weight. Clearly there must be functional redundancy between these collagen IV-binding integrin α chains.

**FIGURE 1.6**

<table>
<thead>
<tr>
<th>Gene</th>
<th>ECM component</th>
<th>Phenotype</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lama1</td>
<td>Laminin α1</td>
<td>Embryonic lethal due to failure of assembly of the Reichert membrane.</td>
<td>(Miner et al., 2004)</td>
</tr>
<tr>
<td>Lama2</td>
<td>Laminin α2</td>
<td>Peripheral neuropathy and muscular dystrophy.</td>
<td>(Sunada et al., 1994)</td>
</tr>
<tr>
<td>Lama3</td>
<td>Laminin α3</td>
<td>Perinatal lethality due to skin blistering.</td>
<td>(Ryan et al., 1999)</td>
</tr>
<tr>
<td>Lama4</td>
<td>Laminin α4</td>
<td>Neuromuscular junction defect.</td>
<td>(Patton et al. 2001)</td>
</tr>
<tr>
<td>Lama5</td>
<td>Laminin α5</td>
<td>Perinatal lethality. Syndactyly, dysmorphogenesis of the placental labyrinth, cranial NTDs.</td>
<td>(Miner et al. 1998)</td>
</tr>
<tr>
<td>Lamb1</td>
<td>Laminin β1</td>
<td>Embryonic lethal due to failure of BM assembly.</td>
<td>(Miner et al. 2004)</td>
</tr>
<tr>
<td>Lamb2</td>
<td>Laminin β2</td>
<td>Neuromuscular junction defect.</td>
<td>(Noakes et al., 1995)</td>
</tr>
<tr>
<td>Lamb3</td>
<td>Laminin β3</td>
<td>Perinatal lethality due to skin blistering.</td>
<td>(Kuster et al., 1997)</td>
</tr>
<tr>
<td>Lamc1</td>
<td>Laminin γ1</td>
<td>Embryonic lethal due to failure of BM assembly.</td>
<td>(Smyth et al. 1999)</td>
</tr>
<tr>
<td>Lamc2</td>
<td>Laminin γ2</td>
<td>Perinatal lethality due to skin blistering.</td>
<td>(Meng et al. 2003)</td>
</tr>
<tr>
<td>Lamc3</td>
<td>Laminin γ3</td>
<td>No Phenotype</td>
<td>(Dénes et al. 2007)</td>
</tr>
<tr>
<td>Col4a1/2</td>
<td>Collagen IV</td>
<td>Structural integrity of the BM</td>
<td>(Pöschl et al. 2004)</td>
</tr>
<tr>
<td>Hspg2</td>
<td>Perlecan</td>
<td>Defect in BM maintenance: myocardium cleft, defective cephalic development, severe chondro- and skeletal dysplasia</td>
<td>(Arikawa-Hirasawa et al. 1999; Costell et al., 1999)</td>
</tr>
<tr>
<td>Nid1</td>
<td>Nidogen 1</td>
<td>Normal BM and normal embryo development.</td>
<td>(Murshed et al. 2000)</td>
</tr>
<tr>
<td>Nid2</td>
<td>Nidogen 2</td>
<td>Normal BM and normal embryo development.</td>
<td>(Schymineksy et al. 2002)</td>
</tr>
<tr>
<td>Nid1, Nid2</td>
<td>Nidogen 1 &amp; 2</td>
<td>Perinatal lethality due to defective BM.</td>
<td>(Bader et al. 2005)</td>
</tr>
<tr>
<td>Fn1</td>
<td>Fibronectin</td>
<td>Embryonic lethal due to defective mesodermal derivatives: lack of somites, notochord, shortened rostro-caudal axis, defects in yolk sac vasculature.</td>
<td>(George et al., 1993; Georges-Labouesse., 1996)</td>
</tr>
</tbody>
</table>

**Figure 1.6. Phenotypes of the BM KO mice.**

**1.8.4 Perlecan and nidogen mutants**

Perlecan is a ubiquitous constituent of the BM. However, deletion of Hspg2, coding for perlecan, does not prevent BM assembly. Instead it affects maintenance of BM stability over time especially in tissue subjected to high mechanical stresses (Costell et al. 1999). Embryos null for Hspg2 display defects in the myocardium leading to a small cleft, a defect in the brain vesicles which protrude out resembling exencephaly (50% penetrance), and severe defects in cartilaginous tissues with skeletal dysplasia with 40% of the embryos, which die at E10.5 (Costell et al. 1999; Arikawa-Hirasawa et al. 1999).

Nidogens, together with perlecan, are essential components that contribute to the stabilisation of the BM acting as a bridge between the collagen type IV and laminin networks. However, loss of either nidogen 1 (Murshed et al. 2000) or nidogen 2 does not prevent BM
assembly or interfere with normal embryonic development, possibility due to the ability of each to compensate for the other. Loss of both nidogen 1 and 2 affects the architecture and stability of the BM, in particular the cardiac tissue and lungs, leading eventually to perinatal lethality (Bader et al. 2005).

1.8.5 Fibronectin and integrin interacting receptor mutants

In contrast to laminin, mouse embryos deficient for fibronectin develop beyond the peri-implantation stage, and proceed though gastrulation without impairment of cell migration, but arrest at initiation of neurulation (George et al. 1993; Georges-Labouesse et al. 1996). Loss of fibronectin appears to affect in particular the mesodermal derivatives in the trunk region. Embryos lack notochord and posterior somites and display a significantly shortened rostro-caudal axis at E8.5. The kinked neural tube is probably a secondary consequence due to the defect in elongation of the body axis. Defective yolk sac vasculature is probably the leading cause of growth retardation and early lethality in these embryos at E8.5.

Deletion of integrin α5, a key fibronectin-interacting receptor, similarly affects mesodermal tissue derivatives in the posterior region: embryos display a significantly shortened body axis, defects in vasculogenesis and mesoderm defects (Yang et al. 1993). However, embryos progress further than the fibronectin KO and die at E10-11. Surprisingly, fibronectin fibrillogenesis proceeds normally and cell migration does not seem to be affected by removal of the receptor. Together, this points to a vital role for another major fibronectin receptors. Removal of the integrin αv subunit, and consequently ablation of all the five αv integrins (integrin αvβ1, αvβ3, αvβ5, αvβ6, αvβ8), leads to a milder defect (Bader et al. 1998). 80% of these embryos die at E10 due to placental defects while 20% survive postnatally with defective vasculogenesis. Similar to integrin α5 KO, loss of αv does not prevent fibronectin fibrillogenesis. The milder defect in the latter suggests that the role of αv in mesoderm formation is compensated by the α5. To pinpoint the role of these receptors in fibronectin fibrillogenesis, the RGD motif was replaced with an inactive RGE sequence, thus abolishing the ability to interact concomitantly both with integrin α5 and αv (Takahashi et al. 2007; Girós et al. 2011). Mice deficient for the RGD motif recapitulate closely the phenotype of the fibronectin and integrin α5 KO embryos, with shortened rostro-caudal axis and mesodermal defects but surprisingly are able to assemble normally fibronectin, pointing towards another potential motif for regulation of fibronectin fibrillogenesis. This predicted fibronectin receptor is yet to be identified.
1.9 Thesis overview

The current study investigates two aspects of neural tube morphogenesis:

- The *in vivo* role of integrin-BM interactions during neural tube closure.
- The cellular dynamics of neuroepithelial cells during neural tube morphogenesis.

Chapter 3

In chapter 3 the molecular composition of the ECM is initially examined by RNA-seq analysis of the caudal region of the embryo which reveals *Fn1*, coding for fibronectin, as the most significantly expressed gene within the matrisome collection. Tissue source of expression and protein localisation are then investigated by *in situ* hybridisation and immunofluorescence analysis respectively, showing a differential composition of the BMs surrounding the neural tube. The integrin subunit combinations expressed during neurulation are identified initially by RNA-seq. *In situ* hybridisation and immunofluorescence analyses reveal a focal upregulation of the majority of the integrin subunits at the site of zippering.

Chapter 4

The central integrin β1 subunit is conditionally ablated in the dorsal neural folds by targeting either the dorsal neuroepithelium (*Pax3*-cre) or the dorsal surface ectoderm (*Grhl3*-cre). Deletion of the subunit from this latter tissue leads to failure of caudal neural tube closure and development of open spina bifida at high frequency (78%). To examine the molecular origin of the defect, developmental features that are likely to rely on outside-in pathways downstream of integrin signalling activation are investigated: cytoskeleton, contractility, cell protrusions, and apoptosis. Fibronectin fibrillogenesis is confirmed to occur normally despite removal of the central integrin subunit. Medio-lateral neural fold recoil following laser ablation shows no differences compared to the wild type strain response.

Chapter 5

Neuroepithelial cell fate during neural fold elevation is investigated by vital cell labelling in whole embryo culture. This demonstrates a trend for neuroepithelial cells to translocate towards the dorsal part of the neural folds during neural tube closure. This pattern is further confirmed in vivo by live imaging of the PNP region via optimisation of a time-lapse protocol for 3D analysis of cell dynamics in the mouse neural tube. The study shows the presence of two main streams of cell migration, rostro-dorsally and caudally directed, identifying fundamentally different cell behaviours in the neuroepithelium of the PNP, depending on rostro-caudal position.
2. Materials and Methods

2.1 Mouse strains

Animal studies were performed according to the UK Animals (Scientific Procedures) Act 1986 and the Medical Research Council’s Responsibility in the Use of Animals for Medical Research (July 1993). Mouse strains were the following:

- Inbred BALB/c mouse line for immunofluorescence, in situ hybridisation and vital carbocyanine dye labelling studies
- Floxed line: \( \text{Itg}\beta1^{+/f} \) kept on a C57Bl/6 background (Potocnik et al. 2000) (gene symbol: Itgb1\(^{tm1}\text{Ref}\), MGI: 1926498)
- Floxed line: \( \text{Rosa}26^{\text{EYFP/EYFP}} \) (Srinivas et al. 2001) (gene symbol: Gt(ROSA)26Sor\(^{tm1}\text{.1(EYFP)}\text{cos}\), MGI: 2449041)
- Floxed line: \( \text{Rosa}26^{\text{mTmG/mTmG}} \) (Muzumdar et al. 2007) (gene symbol: Gt(ROSA)26Sor\(^{tm4}(\text{ACTB-tdTomato,EGFP})\text{uo}\), MGI: 3716464)
- Cre line: \( \text{Pax3}^{\text{Cre/+}} \) kept on a C57Bl/6 background (Engleka et al. 2005) (gene symbol: Pax3\(^{tm1}\text{crejoe}\), MGI: 3573783)
- Cre line: \( \text{Grhl3}^{\text{Cre/+}} \) kept on a C57Bl/6 background (Camerer et al. 2010) (gene symbol: Grhl3\(^{tm1}\text{crejgh}\), MGI: 4430902)
- Cre line: \( \text{Nkx1.2}^{\text{CreERT2/+}} \) kept on a C57Bl/6 background (Rodrigo et al. 2016)

Mice were mated overnight and checked the following morning for the presence of a copulation plug, designated as E0.5. Embryos were collected at different stages between E8.5 and E15.5, dissected from the uterus in 37°C warm Dulbecco’s Modified Eagle’s Medium (DMEM) supplemented with 10% heat inactivated Foetal Bovine Serum (FBS). Embryos were then rinsed in ice-cold phosphate buffered saline (PBS) and immersed in fixative solutions.
2.2 Tamoxifen injections

0.1 g of Tamoxifen (Sigma T5648) and 0.025 g of Progesterone (Sigma P8783) (it can be omitted, mainly used for late stage of pregnancy or postnatal, maximum limit of 2 mg of progesterone per mouse injection) were dissolved in 500 µl ethanol. Then, 4.5 ml of corn oil were added to reach a final volume of 5 ml. Final solution: 20 mg/ml Tamoxifen, 5 mg/ml Progesterone in corn oil. Dissolved by rotating overnight at +4°C, protected from light. Tamoxifen solution was delivered by single intraperitoneal injection. Maximum volume injected per mouse: 200 µl as stated by the Home Office regulation. Minimal recombination for live imaging studies was achieved by injecting 0.2 mg tamoxifen per 40 g mouse weight (from 2 mg/ml tamoxifen stock), 24 hours prior to embryo collection. Maximal recombination by injecting 5 mg tamoxifen per 40 g body weight (from 20 mg/ml tamoxifen stock).

2.3 Embryo culture

For culture, embryos were dissected from the trophoblast and Reichert’s membrane, preserving the yolk sac, amniotic membrane and ectoplacental cone and were cultured in 100% rat serum as previously described (Copp et al. 2000). Briefly, rat serum was filtered (0.45 µm filtered), transferred to a 30 ml plastic culture tube (Nunc), sealed by silicone grease (Borer Chemie) and gassed with 5% O₂, 5% CO₂, 90% N₂ for 1 minute (for stage E8.5), or 20% O₂, 5% CO₂, 75% N₂ (for stage E9.5) at 37°C in a roller culture incubator (B.T.C Engineering or New Brunswick Galaxy 170S). Embryos were gently transferred into the serum, re-gassed for 1 minute and incubated rolling at 37°C up to a maximum of 48 hours. Gassing was performed every 12 h. At the end of the culture period, embryos were assessed for yolk sac circulation. Embryos with good heartbeat, round YS morphology and visible YS circulation were used for further analysis. In the case of suboptimal parameters such as lack of heart beat, wrinkled appearance of the YS and low YS circulation, embryos were discarded and excluded from analysis.
2.4 Fixation

**Methanol fixation**: embryos were immersed in -20°C cold DMSO:MeOH (1:5), incubated for 1 hour at 4°C. The procedure causes fixation and concomitant dehydration of the embryos. Samples stored in 100% MeOH at -20°C.

**Acetone fixation**: embryos were immersed in -20°C cold 100% acetone, incubated for 30 min at 4°C. The procedure causes fixation and concomitant dehydration of the embryo. Samples stored in 100% MeOH at -20°C.

**PFA fixation**: embryos were immersed in +4°C cold 4% PFA (in PBS) (pH 7.4), incubated overnight at +4°C. Embryos are then dehydrated through a graded scale to 100% MeOH and stored in 100% MeOH at -20°C.

2.5 Genotyping

DNA was extracted from yolk sac (stage < 10.5 somites) or from the limb bud (stage > 10.5 somites). Samples for DNA extraction were rinsed in PBS and stored at -20°C. 24 µl of the DNA lysis buffer (peqlab Cat No 31-102-T) and 1 µl of proteinase K (10 mg/ml peqlab, 04-1071) were added to each sample and incubated at 55°C for 3 hours up to 12 hours. Proteinase K inactivation was performed by incubating the samples at 85°C for 45 minutes. 2-3 µl from the above solution were used for PCR genotyping.

**PCR protocol**:

<table>
<thead>
<tr>
<th>Stock concentration</th>
<th>Final concentration</th>
<th>Volume per sample</th>
</tr>
</thead>
<tbody>
<tr>
<td>Buffer 10x</td>
<td>1x</td>
<td>5 µl</td>
</tr>
<tr>
<td>dNTPs 2 mM</td>
<td>0.2 mM</td>
<td>5 µl</td>
</tr>
<tr>
<td>MgCl2 50 mM</td>
<td>1.5 mM</td>
<td>1.5 µl</td>
</tr>
<tr>
<td>F 10 µM</td>
<td>0.5 uM</td>
<td>2.5 µl</td>
</tr>
<tr>
<td>R 10 µM</td>
<td>0.5 uM</td>
<td>2.5 µl</td>
</tr>
<tr>
<td>Taq 1 U</td>
<td>0.25 µl</td>
<td></td>
</tr>
<tr>
<td>Sigma water</td>
<td>31.25 µl</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>Final vol: 48 µl</td>
<td></td>
</tr>
</tbody>
</table>
**Gene: Itgβ1**

To be able to distinguish the floxed allele from the + allele, primers were designed as illustrated in chapter 4 (Fig. 4.1 E).

*Forward:* CTTTGCGTTGTCAGCATGGG  
*Reverse:* ACACTGCCATCTGCGTCTT

*Cycles:* 95 °C 3 min. 35 cycles: 95 °C 30 sec, 53 °C 30 sec, 72 °C 1 min. 72 °C 5 min.

*Band products (2.5% agarose gel):* 500 bp (floxed allele), 300 bp (wild type allele), 500+400+300 (floxed + wild type alleles).

**Gene: Pax3 Cre vs. +**

Primer 1: CTGCACTCGGTGTACAGG  
Primer 2: AAGCGAGCACAGTGCGGC

Primer 3: GAAACAGCATTGCTGTCACTTGGTCGTGCG

*Cycles:* 94 °C 2 min. 32 cycles: 94 °C 30 sec, 60 °C 30 sec, 72 °C 45 sec. 72 °C 5 min.

*Band products (2.5% agarose gel):* 600 bp (cre allele), 350 bp (wild type allele).

**Gene: Grhl3 Cre vs. +**

*Forward:* ACCCTGATCTGGCAATTTCGGC  
*Reverse:* GATGCAACGAGTGATGAGGTTCGC

*Cycles:* 94 °C 2 min. 30 cycles: 94 °C 30 sec, 63 °C 30 sec, 72 °C 45 sec. 72 °C 5 min.

*Band products (2.5% agarose gel):* 500 bp (cre allele), no band (wild type allele).

**Gene: Nkx1.2 Cre vs. +**

Primer 1: ACGTCCAGACACAGCATAGG  
Primer 2: TCACTGAGCAGGTTCAGG

Primer 3: CAAGGTTATGGTAGCCTGG  
Primer 4: TGAGCCAGCTAGGTTTGG

*Cycles:* 94 °C 2 min. 32 cycles: 94 °C 30 sec, 60 °C 30 sec, 72 °C 45 sec. 72 °C 5 min.

*Band products (2.5% agarose gel):* 300 bp (cre allele), 175 bp (wild type allele).
2.6 *In situ* hybridisation

Transcript sequences (cDNA sequences) of the genes of interest were downloaded from Ensembl Genome Browser, *Mus musculus* database. Sequences were uploaded in Primer-BLAST (NCBI) in order to design primer pairs which amplify products of about 500 bp length and with high GC content (> 50%) and low self-complementarity.

Fibronectin: *Fn1* gene ENSMUSG00000026193, Transcript ID ENSMUST00000055226 which correspond to the full length transcript (8315 bp, NM_010233).

Integrin β1: *Itgb1* gene ENSMUSG00000025809, Transcript ID ENSMUST00000090006 full length transcript (3815 bp, NM_010578).

Integrin α5: *Itga5* gene ENSMUSG0000000555, Transcript ID ENSMUST0000023128, only one transcript (4379 bp, NM_010577).

Integrin αv: *Itgav* gene ENSMUSG0000027087, Transcript ID ENSMUST0000028499, full length transcript (7054 bp, NM_008402)

Integrin β5: *Itgb5* gene ENSMUSG00000022817, Transcript ID ENSMUST00000015028, full length transcript (3090 bp, NM_001145884)

Integrin α3: *Itga3* gene ENSMUSG00000001507, Transcript ID ENSMUST0000001548, full length transcript (4861 bp, NM_013565).

Integrin α6: *Itga6* gene ENSMUSG0000027111, Transcript ID ENSMUST0000028522, full length transcript (4310 bp, NM_008397).

Dystroglycan: *Dag1* gene ENSMUSG00000039952, Transcript ID ENSMUST00000191899, full length transcript (5662 bp, NM_001276486).

**Primer sequences (5′->3′):**

Fibronectin: forward (GCATCAGCCGGATGTTAGA), reverse (GGTTGGTGATGAAGGGGTC). This primer pair amplifies a 498 bp product which targets all the 7 slice variants of *Fn1* gene in mouse (Transcript variant 1, 2, 3, 4, 5, X1, X2).

Integrin β1: forward (GCTGGGTTTCACTTTGCTGG), reverse (CCCATTCCCTCATGGC). Product size: 609 bp.
Integrin α5: forward (GCTCCTCCATTTGGCATGT), reverse (TAGCCGAAGTAGGAGGCCAT).
Product size: 535 bp.

Integrin αv: forward (GCACGTCTCCAGGATGTTTCT), reverse (TTCTGCCACTTGCGAAT).
Product size: 485 bp.

Integrin α3: forward (ACTTCCAGAAAGGTGCGGG), reverse (CACTGTGCCCAAAAGAAGC).
Product size: 512 bp.

Integrin α6: forward (ATGAAAGTCTCCTGGTGCTT), reverse (CTCGAGAACCTGTGTTGGCT).
Product size: 542 bp.

Dystroglycan: forward (CGTCACTACCACAACTCGG), reverse (GTGAATGTCATTCACCACCG).
Product size: 551 bp.

AccuPrime Taq DNA Polymerase High Fidelity (Invitrogen by Life Technologies, Cat no. 12346) was used for high fidelity amplification by PCR reaction. cDNA template extracted from Balb/C WT embryos (stage 32-34 som), caudal region.

<table>
<thead>
<tr>
<th>Reagents (stock concentration)</th>
<th>Final concentration</th>
<th>Volume per sample</th>
</tr>
</thead>
<tbody>
<tr>
<td>Accu Prime PCR Buffer I (10 x)</td>
<td>1x</td>
<td>5 µl</td>
</tr>
<tr>
<td>Forward primer (10 µM)</td>
<td>0.5 µM</td>
<td>1 µl</td>
</tr>
<tr>
<td>Reverse primer (10 µM)</td>
<td>0.5 µM</td>
<td>1 µl</td>
</tr>
<tr>
<td>Template cDNA</td>
<td>0.1 pg-20 ng</td>
<td>1 µl</td>
</tr>
<tr>
<td>AccuPrime Taq High Fidelity</td>
<td>Around 1 U</td>
<td>0.2 µl</td>
</tr>
<tr>
<td>Sigma water</td>
<td></td>
<td>to 50 µl final volume</td>
</tr>
</tbody>
</table>

PCR programme: Initial denaturation 94°C 2 min. 35 cycles: Denaturation 94 °C 30 sec, Annealing 60°C 30 sec, Extension 68°C 30 sec. Final extension 68°C 5 min. The amplified products were visualised by gel electrophoresis and quantified by using NanoDrop spectrophotometer (Thermo Scientific) after purification of the PCR reaction by QIAquick PCR purification Kit Protocol (Cat No. 280106).

Ligation reaction: The amplified products were then ligated into the vector pGEM-T easy (Promega Kit Ref A137A). Incubation 1 hour at room temperature.
<table>
<thead>
<tr>
<th>Reagents (stock concentration)</th>
<th>Final concentration</th>
<th>Volume per sample</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rapid Ligation Buffer (2X)</td>
<td>1x</td>
<td>5 µl</td>
</tr>
<tr>
<td>pGEM-T Easy vector (50 ng)</td>
<td>5 ng</td>
<td>1 µl</td>
</tr>
<tr>
<td>PCR product</td>
<td>optimised insert : vector molar ratio</td>
<td>X µl</td>
</tr>
<tr>
<td>T4 DNA Ligase (3 Units/ µl)</td>
<td>0.1 pg-20 ng</td>
<td>1 µl</td>
</tr>
<tr>
<td>Sigma water</td>
<td></td>
<td>to 10 µl final volume</td>
</tr>
</tbody>
</table>

**Transformation:** The plasmid DNA was then transformed in DH5α competent cells by heat shock. 2 µl plasmid DNA were transferred in 50 µl aliquot of competent cells on ice, incubated on ice 20 min, heat shocked 50 sec 42°C and then grown in LB medium at 37°C on shaking for 1 hour. Cells were then plated into Luria Broth agar plate containing 50 µg/ml Ampicillin and 100 mM IPTG and 50 mg/ml X-Gal for white/blue screening, incubated over night at 37°C. Single white colonies were isolated and grown in 10 ml LB medium (50 µg/ml Ampicillin) over night at 37 °C with shaking. Cells were harvested by centrifugation at 6800 x g 20 min 4°C. Plasmid DNA was then extracted by using the QIAprep Spin Miniprep Kit (250, Cat No.27106) protocol and resuspended in sigma water.

**Nanodrop quantification and sequencing:** Concentration of the plasmid was quantified by NanoDrop spectrophotometer (Thermo Scientific). Plasmid DNA was sent to SourceBioscience (www.lifesciences.sourcebioscience.com) for sequencing by both T7 and Sp6 polymerases (pGEM-T easy vector). The quality of sequencing and directionality of the insert were assessed by using Chroma software.

**Plasmid linearization by restriction enzyme digestion:** Plasmid was then linearized by restriction enzymatic digestion. Initially the sequence of the insert was checked by WebCutter to exclude the presence of any internal site recognised by RE. Reactions were incubated for 2 hours at 37°C. Success of linearization was assessed by gel electrophoresis and the product was then purified by QIAquick PCR purification Kit Protocol (Cat No. 280106).
**Transcription:** Digoxigenin-labelled single-stranded RNA probe was transcribed from the purified digest in the following mixture: 1 μg of linearized plasmid, 2 μl of DIG RNA labelling mix (Roche), 2 μl of transcription buffer (Roche), 0.5 μl of RNAse Inhibitor (Roche), and 2 μl of the appropriate RNA polymerases (T7 or Sp6) up to a final volume of 20 μl by addition of DEPC-H₂O. Incubation for 2 hours at 37°C. The transcribed DIG probe was purified in a Chroma Spin-100 DEPC-H₂O column (Clontech). 40 μl formamide and 1 μl RNAse inhibitor were added to the purified DIG-labelled single stranded RNA probe for final storage at -20°C.

**Whole mount in situ hybridisation:** embryos were initially fixed in 4% PFA at 4°C overnight and dehydrated to 100% MeOH. Embryos were rehydrated from 100% MeOH to PBT and bleached with 6% hydrogen peroxide in PBT for 1 hour, shaking on ice. After washes in PBT, embryos were incubated in proteinase K (10 mg/mL stock) at room temperature as follows:

<table>
<thead>
<tr>
<th>Stage</th>
<th>Concentration</th>
<th>Incubation Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>E7.5</td>
<td>2.5 μg/mL</td>
<td>1 min</td>
</tr>
<tr>
<td>E8.5</td>
<td>5 μg/mL</td>
<td>1 min</td>
</tr>
<tr>
<td>E9.5</td>
<td>5 μg/mL</td>
<td>2 min</td>
</tr>
<tr>
<td>E10.5</td>
<td>5 μg/mL</td>
<td>7 min</td>
</tr>
<tr>
<td>E11.5</td>
<td>10 μg/mL</td>
<td>8 min</td>
</tr>
</tbody>
</table>

Permeabilisation reaction was stopped by glycine solution: 2mg/mL of glycine in PBT, 5 min. After washes in PBT, embryos were post-fixed in 0.2% gluteraldehyde in 4% PFA (in PBS), 20 minutes at room temperature, followed by washes. Hybridisation mix was prepared as follows: (50 % formamide, 5x SSC pH 4.5, 50 μg/mL yeast RNA, 1% SDS, 50 μg/mL heparin). 1 mL of pre-warmed hybridisation mix (70°C) was added to each embryo and incubated at 70°C for 2 hours. DIG-labelling probe was added to the hybridisation mix and embryos were incubated overnight at 70°C (hybridisation step). The following day embryos were washed 3x 30 min in solution 1 at 70°C (50% formamide, 5X SSC, 1% SDS), 2x 30 min in solution 2 at 65°C (50% formamide at 70°C, 5X SSC, 1% SDS), and finally 3x 5 min in TBST (1X TBS, 1% Tween-20, Tetramisole) at room temperature. Embryos were blocked by using 10% sheep serum in TBST for 60-90 minutes at room temperature and incubated overnight with anti-DIG AP antibody in TBST + 1% sheep serum at 4°C. The following day embryos were washed in in TBST, followed by NTMT (100 mM NaCl, 100 mM Tris HCl pH 9.5, 50 mM MgCl₂, 50 mM MgCl₂, 1% Tween-20, Tetramisole hydrochloride) for initial equilibration. NBT (4-Nitroblue tetrazolium chloride) (4.5 uL/mL) and BCIP (5-Bromo-4-chloro-3-indoyl-phosphate) (3.5 uL/mL) were diluted in NTMT. Embryos were incubated in the above solution in dark at room temperature till the colour is fully developed. Reaction stopped in PBT. Embryos were post-fixed in 4% PFA and imaged using a LEICA DFC490 camera on a light microscope (ZEISS Stemi SV11). Embryos were
embedded in gelatin-albumin and sectioned by vibratome (Leica VT1000S) (40 μm thickness). Images of sections were acquired in Zeiss AxioCamMr brightfield microscope by differential interference contrast (DIC), Nomarski.

2.7 Whole-mount TUNEL staining

Reagents from ApopTag TdT enzyme kit (Millipore, catalogue no: S7107). Procedure as previously described (Martinez Barbera et al. 2002). Embryos were initially fixed in 4% PFA overnight at 4°C and dehydrated through a graded scale to 100% MeOH. Embryos were rehydrated in PBT (0.1% Tween in PBS) and incubated with proteinase K solution (10 μg/ml) for permeabilisation of the tissues according to the developmental stage: E8.5 (1 min), E9.5 (4 min), E10.5 (8 min) at room temperature. The reaction was stopped by glycine solution (2 mg/ml, 2 min) and embryos were post-fixed first in 4% PFA (20 min, room temp) followed by a mixture of ethanol and acetic acid (2:1) on ice (10 min). After incubation in equilibration buffer (ApopTag TdT enzyme kit) (1 hour, room temp), embryos were incubated overnight at 37°C in working strength TdT enzyme (80 μl TdT enzyme, 160 μl reaction buffer, 0.7 μl Triton). The reaction was stopped by incubation in stop/wash buffer (3 hours). Embryos were incubated in blocking solution (5% heat-inactivated sheep serum, 2 mg/ml bovine serum albumin (BSA), in PBT) for 60 min followed by the addition of anti-digoxigenin AP-conjugated Fab fragments antibody (Roche) and incubated overnight, at 4°C. The following day, embryos were initially washed in BSA, equilibrated in NTMT (3x5 min) and incubated protected from light in developing solution (NTMT+NBT/BCIP, as previously described for in situ hybridisation) until colour develops, for few minutes. Reaction was stopped by PBT, followed by fixation in 4% PFA. Images were acquired using a LEICA DFC490 camera on a light microscope (ZEISS Stemi SV11).

2.8 Immunofluorescence

Whole mount: After dissection, embryos were rinsed in PBS and fixed with the appropriate fixative solution, as described in the Table below. Permeabilisation of the tissues was achieved using a minimum of 0.025% Tween (for the integrin receptors) up to a maximum of 0.1% Triton X-100 in PBS (PBT solution), for 1 hour at room temperature with gentle agitation. Embryos were then blocked overnight in 5% BSA/PBT solution (filtered by a 0.45 μm filter prior to use)
at 4°C. The next day, the blocking solution was replaced by the primary antibody diluted at the appropriate concentration in fresh blocking solution. Usually a volume of 150 µl solution was used for each individual embryo. For double or triple immuno, the primary antibodies were combined together in the same solution. Embryos were then washed 3 x 1 hr in blocking solution, to remove excess of the primary antibodies, and incubated for 2 hours at room temperature in Alex Flour-conjugated secondary antibodies diluted 1:500 (see table below) in blocking solution. Phalloidin stained was performed together with the secondary antibodies. Excess secondary antibody was removed by washing for 1 hour in blocking solution and further 2 x 1 hour in PBT at room temperature. Finally, embryos were incubated for 1 hour at room temperature in DAPI diluted 0.5 µg/ml in PBT. After 2 x washes in PBT embryos were stored at + 4°C in PBS with 0.1% sodium azide to prevent fungal or bacterial growth. Stained embryos were positioned and immobilised by using a 2% agarose dish in PBS and imaged on a Zeiss LSM880 confocal microscope using either a 10x/NA0.5 W-Plan Apochromat dipping objective (WD 3.7mm) or a 20x/NA1.0 Plan Apochromat dipping objective (WD 2.4mm).

**Sections (frozen):** Embryos were immersed in 20% sucrose in PBS for 2 hour at 4°C for cryo protection of the tissues. Then incubated in 7.5% gelatine (in 20% sucrose in PBS) at 37°C for 15 minutes to allow penetration of the medium and embedded into the block of gelatine after solidification. Blocks were snap frozen with -70°C isopentane and stored at -80°C until processing. Blocks were sectioned by cryostat (Leica) at 10 µm thickness and slices mounted in Superfrost Plus slides (Thermo Fisher). Removal of gelatin and rehydration of the tissues were achieved by immersing the slides in PBS for 20 min at 37°C. Slides were then covered by a volume of 200 µl per slide of 10% sheep serum, 2 % BSA (filtered) and 0.025% Tween (up to 0.1% Triton) in PBS by applying parafilm for even spreading of the solution, and incubated for 1 hour at room temperature inside a humidified chamber. The above step ensures permeabilisation and blocking of the tissue. Primary antibody was then applied after dilution in the same solution and slides incubated overnight at 4°C. The following day, excess of the antibody was removed by 3 x washes in PBT, 5 min each. Slides were then incubated 1 hour at room temperature protected by light in secondary antibody diluted 1:500 in the same solution of the primary. After washing, slides were incubated in DAPI diluted 0.5 µg/ml in PBT for 10 min for nuclear visualisation and finally mounted in Mowiol and sealed by a 24 x 60 mm # 1.5 coverslip.

**Post-acquisition processing:** Raw files were processed and analysed by Fiji software (Schindelin et al. 2012): brightness adjustments, cropping, outlier removal, quantifications, drift correction. Deconvolution was performed using Huygens software. Quantification of
fluorescence staining in sections was performed on confocal Z projections using Fiji: mean grey values were quantified along the basal perimeter of the neural tube and normalised by subtraction of the average background intensity of the neuroepithelium.

<table>
<thead>
<tr>
<th>Target</th>
<th>Antibody details</th>
<th>Host species/isotype</th>
<th>Fixation</th>
<th>Dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Collagen IV</td>
<td>Ab 19808 Polyclonal</td>
<td>Rabbit IgG</td>
<td>PFA</td>
<td>1:200</td>
</tr>
<tr>
<td>Perlecan</td>
<td>Ab 17848 Monoclonal</td>
<td>Rat IgG2a</td>
<td>Acetone</td>
<td>undiluted</td>
</tr>
<tr>
<td>Nidogen-1</td>
<td>Ab 14511 Polyclonal</td>
<td>Rabbit IgG</td>
<td>Acetone</td>
<td>1:50</td>
</tr>
<tr>
<td>Fibronectin</td>
<td>Ab 23750 Polyclonal</td>
<td>Rabbit IgG</td>
<td>PFA</td>
<td>1:200</td>
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<tr>
<td>Dystroglycan</td>
<td>Millipore 05-593 Monoclonal IIH6C4</td>
<td>Mouse IgM</td>
<td>PFA</td>
<td>1:200</td>
</tr>
<tr>
<td>Integrin α6</td>
<td>MAB 1378 Monoclonal</td>
<td>Rat IgG2a</td>
<td>Methanol</td>
<td>1:50</td>
</tr>
<tr>
<td>Integrin α3</td>
<td>non-commercial (courtesy from Jacob Ross, Prof. Muntoni Lab)</td>
<td>Rabbit IgG</td>
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<td>1:200</td>
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<tr>
<td>Integrin β1</td>
<td>BD Biosciences 553715. Clone 9EG7</td>
<td>Rat monoclonal IgG2a, κ.</td>
<td>PFA or Acetone</td>
<td>1:50</td>
</tr>
<tr>
<td>E-cadherin</td>
<td>BD Biosciences 610181</td>
<td>Mouse</td>
<td>Any fixative</td>
<td>1:200</td>
</tr>
<tr>
<td>pMLCII (Ser19)</td>
<td>Cell Signalling 3671</td>
<td>Rabbit</td>
<td>PFA</td>
<td>1:100</td>
</tr>
<tr>
<td>Phalloidin-568</td>
<td>Thermo Scientific A12380</td>
<td>-</td>
<td>PFA (not dehydrated tissues)</td>
<td>1:200</td>
</tr>
</tbody>
</table>

### 2.9 Scanning electron microscopy

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% K₄Fe(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. Analysis and scoring of protrusion was carried out blindly from genotype by Prof Andrew Copp and Dr Ana Rolo by analysis of the site of PNP fusion point at 2000x magnification, as previously reported (Rolo et al. 2016). Protrusions were categorised as: ruffles (when predominantly or solely of membrane ruffles), ruffles and filopodia (when a mixture of both types of protrusions was present with filopodia emanating from ruffles, or ruffles with microspikes). Presence of solely filopodia or absence of protrusions were not observed.
2.10 RNA-seq analysis

RNA extraction: The caudal region from the 13\textsuperscript{th} - 14\textsuperscript{th} somite boundary was collected from 3 wild type replicates (non-mutant embryos from a cross that was segregating the Zic2\textsuperscript{Ko} mutant allele) at the 20 somite stage. Sex of the embryos was confirmed by genotyping to ensure the presence of both sexes in the analysis. PCR sex genotyping for the Y-chromosome SRY sequence: forward primer (CCGCTGCCAATTCTTTGG), reverse primer (TGAAGCTTTTGGCTTTGAG). After washing in DEPC-PBS the samples were snap frozen in dry ice and stored at -20°C until processing. RNA was extracted by using the RNeasy Mini Kit, Cat no 74104 and eluted in RNase-free Sigma water. RNA samples were then treated to remove any DNA contamination by using the Kit RNA Ambion AM1906. Quality controls of RNA integrity were successfully passed for all the samples with a final RIN value of 9.9-10.0 purity (out of a maximum of 10.0)

Library preparation: 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 minutes instead of 8 minutes, 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 uniquely identified by way of a 6 bp index sequence. Finally a PCR is carried out to amplify only those cDNA fragments that have adaptors bound to both ends.

Sequencing: Libraries to be multiplexed in the same run were pooled in equimolar quantities, calculated from qPCR and/or 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 >15million reads per sample.
**Data Analysis:** Run 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 (https://basespace.illumina.com). Raw read counts were quantified and normalised in StrandNGS Software as **Reads Per Kilobase of per Million** mapped reads (**RPKM**). This provided a means of comparing expression levels of genes between the three samples, by normalising for the length of the RNA transcripts and for the total number of reads from the sample (Mortazavi et al. 2008). A lower cut-off level of 1.5 on normalised values was applied to define the boundary of gene expression level considered to be significant. This was based on RPKM values for known caudally-expressed genes (**T** (Brachyury), **Cyp26A1**, **Nkx 1.2**: RPKM > 4), genes expressed rostrally at the boundary with the caudal region (**Sox1**, **Efna**: RPKM ~ 2) and genes expressed exclusively in the cranial region but excluded from the caudal axial level (**Tbx5**, **Otx1**, **Six6**, **Hesx1**, **Foxg1**: RPKM < 1). The most recent version of the matrisome (v2.0), released in 2016 (Alexandra Naba et al. 2016), was used and only the core matrisome was analysed (273 genes collected).

### 2.11 Laser ablation

For ablation studies, embryos were dissected out of the amnion, positioned in wells cut out of 4% agarose gel made up in DMEM (Thermo Fisher Scientific), submerged in dissection medium (10% FBS in DMEM) and maintained at 37°C throughout imaging. Fine microsurgical swaged needles from 11-0 Mersilene (TG140-6, Ethicon) and 10-0 Prolene (BV75-3, Ethicon) were used to hold the embryos in place for a dorsal view of the open PNP. Images were captured on a Zeiss Examiner LSM880 confocal microscope using a 10x/NA0.5 W-Plan Apochromat dipping objective (WD 3.7mm). The PNP was imaged before and after ablation by reflection using HeNe 633 (2% power), with Z-step of 4.92 μm, (speed = 8, bidirectional imaging, 1024x1024 pixels, averaging: 2). Laser ablation was performed by Dr Gabriel Galea on a Zeiss Examiner LSM880 confocal microscope using a 10x/NA0.5 W-Plan Apochromat and a MaiTai laser (800 nm wavelength, 100% laser power, 131 μs pixel dwell time, 1 iteration). A 300-500 μm long region of fused neural tube was ablated by sequential 15-20 steps of intermittent ablations from the site of fusion towards the rostral closed neural tube. Pre- and post-ablation 3D images were re-oriented and resliced by Imaris. Distance between the tips of the neural folds (PNP width) was measured in resliced z-stacks by Imaris along 200 μl length from the fusion point along the rostro-caudal axis.
2.12 X-gal staining

After dissection, embryos were washed in PBS and fixed in freshly prepared 0.2% glutaraldehyde solution in PBS, on ice with shaking according to the embryonic stage: 30 min (E8.5), 50 min (E9.5), 1 hour (E10.5), 1h 30 min (E11.5). Embryos were then washed 3 x 5 min in 0.1% Tween PBS solution, in ice. A lacZ solution was prepared as follows: 10 mM Potassium Ferrocyanide (K₃Fe(CN)₆), 10 mM Potassium Ferrocyanide (K₄Fe(CN)₆· 3 H₂O, 2 mM MgCl₂, 20 µl Nonidet P40 and 20 mM Trsi HCl pH 7.5 in PBS. X-Gal (5-Bromo-4Chloro-3Indolyl-β-D-Galactopyranoside Sigma B4252) was dissolved in DMSO (Dimethyl Sulfoxide) to a concentration of 100 mg/ml of X-Gal in DMSO. This latter was eventually diluted in the lacZ solution to a final concentration of 1 mg/ml of X-Gal. LacZ solution with X-Gal was pre warmed to 37°C to dissolve X-Gal and then passed through a 0.22 µm filter to eliminate any X-Gal precipitates. 1 ml of pre-warmed LacZ-X-Gal solution was then used per embryo, incubated at 37°C overnight, under rotation, protected from light. Embryos were post-fixed in 4% PFA and sectioned by cryostat as described in Section 1.8.

2.13 Carbocyanine Dil and DiO labelling

Balb/c wild type embryos were collected at E8.5, at the 6 somite stage for double labelling, and at 8 somites for single Dil labelling. Carbocyanine dyes used: CM-Dil (red) (C7001, Cell Tracker) and SP-Dio (green) (D7778, Thermo Fisher). The dye in powder was initially dissolved in DMSO to a final concentration of 40 mg/ml. This latter was then diluted in 0.3 M sucrose in PBS to a final concentration of 4 mg/ml, and stored at + 4°C protected from light. A surgical thin glass micropipette (mouth controlled) was inserted into the neural folds at the mid-level of the open PNP. The dye was then released upon withdrawal to label the entire thickness of the neuroepithelium. The success of the injection was assessed for each embryo on a fluorescence stereoscope prior to culture. In each litter, half the embryos were assigned blindly to the time 0 group, thus immediately fixed, and half were cultured for 18-20 hours. After culture, embryos were fixed overnight in 4% PFA and sectioned by cryostat for preservation of the fluorescent dyes. Quantification of the degree of dorsal translocation along the ventro-dorsal axis was performed in sections by ImageJ using a spline fit segmented line of the hemi-neural fold.
2.14 Live imaging

For live imaging, embryos were dissected in an intact yolk sac as if intended for long term embryo culture and fixed in place on a filter paper using a similar mounting protocol as previously described (Massarwa and Niswander 2013). Embryos were kept at optimal conditions for static embryo culture: temperature 37°C, medium 100% rat serum, constant gassing at 20% O₂, 5% CO₂ and 90% N₂ in a custom-made humidified chamber. In the first live imaging, a small aperture in the yolk sac was created to expose the region of open PNP while the amniotic membrane was kept fully intact. Images were acquired by confocal microscopy using an inverted confocal Zeiss LSM 710 with a 10x Plan Apochromat dry objective of numerical aperture (NA) 0.45 and working distance (WD) 2000 μm. Image acquisition was every 7 min for a total time of 5 h 57 minutes. 2D cell tracking was performed by Icy software. The second live imaging video used for 3D analysis was acquired by multiphoton microscopy on a Zeiss Examiner LSM880 confocal microscope using a 10x/NA 0.5 W-Plan Apochromat dipping objective (WD 3.7 mm), 0.7x zoom. Imaging was performed by penetration of the laser through the intact yolk sac and amnion. This used a Mai Tai multiphoton laser: 940 nm, power 15.0 % (optimal for EGFP fluorescence). Image acquisition was every 27 min for a total time of 1 h 22 minutes. Resolutions: x: 1.186 μm, y: 1.186 μm, z: 5.000 μm (optimised for 3D tracking) (1024 x 1024, 8-bit, 4 averaging, Z: 102 slices). Pixel dwell: 0.76 μs. Z-stack were corrected for 3D drift by ImageJ and yolk sac fluorescence was removed in ImageJ by a macro designed by Dr Gabriel Galea. 3D cell automatic tracking was performed in Imaris software.

2.15 Statistical analysis

Statistical analysis and graphing were performed in GraphPad Prism 7 software. Comparison of the distribution of the genotypes against expected Mendelian ratios was by Chi-square test. Incidence of different phenotypes, including protrusion categories, between genotypes was tested by Fisher’s exact test. Normality was tested by D’Agostino-Pearson omnibus test. Comparison of two groups in Dil experiment was performed by non-parametric Mann-Whitney test (equivalent to Student’s unpaired t-test). Comparison of multiple groups in Dil/DiO analysis was by non-parametric Kruskal-Wallis test (equivalent to one-way ANOVA) with post-hoc Dunn’s test for multiple comparisons. Comparison of multiple groups by two factors in Dil and DiO analysis was by two-way ANOVA with post-hoc Bonferroni correction for multiple comparisons. In this last case, the non-parametric version of the two-way ANOVA could not be
performed. Linear regression was performed for analysis of PNP length and width, and recoil after laser ablation. All the analyses above were performed blind prior to genotyping. P < 0.05 was considered statistically significant.
3. Basement membranes and integrins during neurulation

3.1 Introduction

During embryogenesis, basement membranes begin to appear as early as the pre-implantation stage (E4) upon specification of the main lineages. The first embryonic basement membrane starts to form on the basal side of the epiblast and separates the embryonic epithelium from the underlying primitive endoderm layer. Synthesis of laminins has been reported since early cleavage stages (Wu et al. 1983; Dziadek and Timpl 1985), probably due to initial maternal mRNA contribution, followed by nidogens, collagen IV (Adamson and Ayers 1979) and fibronectin (Wartiovaara et al. 1979) which are sequentially deposited into the developing ECM. A second BM also starts to be secreted on the basal side of the extra-embryonic trophectoderm which acts as a substrate for migration of the parietal endoderm and synthesis of the thick BM structure which will give rise to the Reichert’s membrane (Hogan et al. 1980).

The development of a new epithelium in embryogenesis always coincides with the formation and assembly of a novel basement membrane. For example, as the neural plate becomes specified and differentiates from the ectoderm germ layer, a novel BM begins to be synthesised basally and bidirectional contacts between the epithelium and the underlying BM are established. Upon initiation of neurulation, two main BMs are present: one on the basal side of the neuroepithelium where it contacts the paraxial mesoderm, and another lining the basal side of the surface ectoderm (Fig. 3.1). As neural fold elevation begins, and particularly as dorsolateral hinge points are formed, the dorsal neuroepithelium changes its basal contact from mesoderm to the surface ectoderm and a novel BM starts to be deposited and organised at the neural fold-surface ectoderm interface (Fig. 3.1, red BM). The BM is usually synthesised locally by the tissues it contacts (Brown 2011). During neurulation, neural plate cells contribute one set of matrix molecules and paraxial mesoderm contributes others and, as soon as the flat epithelial cells of the surface ectoderm come into contact with the dorsal neuroepithelium, new components are secreted at the interface generating heterogeneous BMs around the neural tube.

Studies on the composition of the BMs during neural tube morphogenesis started as early as the mid-1980s. The major components of the BM: laminins, fibronectin, collagen type IV and
proteoglycans were all confirmed to be expressed and to localise around the neural tube during primary neurulation in both chick and mouse (O'Shea and Liu 1987; Sternberg and Kimber 1986a; Sternberg and Kimber 1986b; O'Shea 1987). Furthermore, a pioneering study by Martins-Green (Martins-Green 1988) started to investigate the dynamics of delamination of the surface ectoderm from dorsal neural tube and reported for the first time the synthesis of a developing BM at their dorsal interface. According to this view, the newly synthesised basement membrane would allow the two epithelia to remain anchored to each other until neural fold fusion, when the two opposing BMs coalesce and full separation of the two tissues occurs.

However, despite these initial insights, the role of the BMs during neural tube morphogenesis has remained elusive. More recently, our lab decided to return to the study of the ECM and further investigate the potential role in vivo of BM assembly and interactions in the context of neural tube closure. Initial work from Andrew Copp and Patricia Ybot-Gonzalez examined the mRNA expression and protein localisation of the trimeric laminin variants (Copp et al. 2011). They showed that laminin α1β1γ1 and α5β1γ1 are the two major isoforms present in the BMs in the caudal neural tube and suggested a potential role of these two variants for the initial assembly of the matrix during neurulation. Expression of additional laminin isoforms and the receptors integrin α3 and α6 was also reported in the ventral ectoderm ridge at E9.5 suggesting that laminin-integrins interactions may be essential for its differentiation from the surface ectoderm (Lopez-Escobar et al. 2012).

The present study aims to further extend the analysis of the molecular composition of the BM ligands and receptors during mouse neural tube closure. Initially the main constituents of the BM were examined by RNAseq analysis of the caudal region of wild type embryos at E9.5. Fibronectin appeared to be the most highly expressed component of the ECM together with the receptor integrin β1. In addition, analysis of mRNA by in situ hybridisation helped to identify the tissue sources of expression and revealed a dramatic up-regulation of integrin subunits at the site of dorsal fusion. Even though transcriptome analysis represents a useful tool to identify the main genes expressed from a specific tissue source, it does not always correlate with the protein levels eventually present in the tissue. While a single mRNA in some instances can give rise to less than a thousand protein copies, for other genes a single mRNA molecule can instead generate around a million protein copies (Edfors et al. 2016). Moreover, a poor correlation between transcript levels and absolute protein copies was found in a recent study of the ECM molecules expressed during mouse lung injury (Schiller et al. 2015). ECM protein levels were shown to be downregulated even though transcript levels appeared to be
significantly upregulated by RNA-seq. Therefore protein abundance and localisation were further investigated by immunofluorescence analysis both on sections and by confocal 3D reconstruction of whole mount processed samples. This analysis confirmed the significant up-regulation observed at mRNA levels of integrin receptors in the dorsal fusing neural folds where a fibronectin-rich BM becomes assembled, suggesting a potential interaction between integrins and fibronectin at the dorsal site of zippering.

**Figure 3.1. Basement membranes during neural tube morphogenesis.** Initially at E 8.5, neural plate cells interact with a basement membrane interposed between the neural plate and the paraxial mesoderm (BM in blue). A second basement membrane underlies the basal side of the surface ectoderm (BM in green). As neural fold elevation and bending begins, the dorsal neuroepithelium changes its basal contact from mesoderm to surface ectoderm and a novel basement membrane starts to be deposited and organised at the neural fold-surface ectoderm interface (BM in red).
3.2 Results

3.2.1 RNA-seq analysis of the core matrisome

To examine the composition of the basement membranes at the stage of neural tube closure, transcripts levels of the genes encoding the “matrisome” have been quantified by RNA-Seq analysis. The “matrisome”, initially coined in 1984 by Martins and colleagues (Martin et al. 1984), refers to the complex of genes coding for the structural component of the ECM, referred as the core matrisome, together with all the proteins which interact with and remodel the ECM, referred to as matrisome-associated proteins. This bioinformatic collection of genes has been assembled and made publicly available by the Matrisome Project (http://matrisomeproject.mit.edu/) led by the laboratory of Richard Hynes (Naba et al. 2012; Naba et al. 2016). Here in this analysis, the most recent version of the matrisome (v2.0), released in 2016 (Naba et al. 2016), has been used and only the core matrisome has been analysed (273 genes in total), excluding the dataset of proteins which interact with the ECM but are not themselves structural components of matrix.

RNA used for the above analysis was extracted from three replicates of the wild-type caudal embryonic region, isolated by making a cut at the 13th-14th somite boundary, in embryos with 20 somites. Raw read counts were quantified and normalised as Reads Per Kilobase of transcript per Million mapped reads (RPKM). This provided a means of comparing expression levels of genes between the three samples, by normalising for the length of the RNA transcripts and for the total number of reads from the sample (Mortazavi et al. 2008). A lower cut-off level of 1.5 on normalised values was applied to define the boundary of gene expression level considered to be significant. This was based on RPKM values for known caudally-expressed genes (T (Brachyury), Cyp26A1, Nkx 1.2: RPKM > 4), genes expressed rostrally at the boundary with the caudal region (Sox1, Efna: RPKM ~ 2) and genes expressed exclusively in the cranial region but excluded from the caudal axial level (Tbx5, Otx1, Six6, Hesx1, Foxg1: RPKM < 1).

Out of a total of 273 genes, 81 components of the core matrisome were confirmed to be expressed in the caudal region of the embryo at the 20 somite stage (Fig. 3.2). The four major components (laminins, collagen type IV, nidogens and perlecan) which contribute to the formation of the basic architecture of the basement membranes (LeBleu, Macdonald, and Kalluri 2007) were all highly expressed at this stage.
Collagen type IV is encoded by six genes (α1-α6) which give rise to different hetero-trimer combinations (Yurchenco 2011) (Ricard-Blum 2011b). The analysis showed that the most highly expressed collagen type IV genes are Col4a1 and Col4a2 which form the most common variant present in all BMs: collagen type IV α1α1α2 (Fig. 3.2 and 3.3, blue highlights). The

Figure 3.2. RNA-Seq analysis of the core matrisome. 81 out of 273 genes expressed are shown on the left column next to a zoom-in of the most highly expressed components (RPKM > 4). Fibronectin (Fn1) is the top gene of the entire matrisome dataset. Collagen type IV isoform α1α1α2 together with Laminin isoform α5β1γ1 are the most highly expressed constituents of the basement membrane in the caudal region of the embryo at the 20 somite stage.
tissue-specific trimeric form α5α5α6 appears also to be present due to the expression of \textit{Col4a5} and \textit{Col4a6} genes, even though at lower levels than α1α1α2, while the variant α3α4α5 was not detected (Fig. 3.3).

Laminins are composed of three polypeptide chains (α, β and γ) twisted together to form a cross-like structure (Durbeej 2010). Five different α (α1-α5), three β (β1-β3) and three γ subunits (γ1-γ3) have been identified so far, each encoded by a single gene. Combination of different subunits types gives rise to the large heterogeneity of this family which comprises 18 different laminin combinations. Analysis of mRNA transcripts shows that the two most highly expressed trimers are laminin α5β1γ1 and laminin α1β1γ1 (Fig. 3.2 and 3.3, red highlights), in agreement with previous studies in our lab (Copp et al. 2011). \textit{Lamb2} seems also to be expressed. This gene encodes the β2 subunit which can assemble with the corresponding α and γ subunits to give rise to the laminin isoforms α5β2γ1 and α1β2γ1.

In addition to the laminins and collagen type IV, which are the main network-forming components, nidogens (entactins) are also present: nidogen 1 (Nid1) and nidogen 2 (Nid2) (Fig. 3.2). The presence of these two glycoproteins is essential for the establishment of non-covalent interactions between the collagen type IV and laminin networks, ensuring an overall integrity of the BM (Yurchenco 2011). Also agrin and perlecan (heparan sulfate proteoglycan 2) are significantly expressed at this stage (Fig. 3.2) and they are essential for stabilising the binding of the laminin network with the interacting cell surface receptors (Yurchenco 2011).

Other minor components expressed which contribute to the heterogeneity of the BM are: fibulin 1 (Fbln1) and 2 (Fbln2), netrin 1 (Nt1) which regulates axonal guidance during spinal cord development (Kennedy et al. 1994), nephronec tin which contributes to development of the ureteric bud epithelium (Brandenberger et al. 2001) and collagen type XVIII, which was very highly expressed in the RNAseq (Fig. 3.2) and acts as a link between the stroma and BM interface (Yurchenco 2011).

The most highly expressed component of the core matrisome is fibronectin (Fig. 3.2), with an average RPKM of 7.3 (Fig. 3.3, green highlight), which makes it one of the most highly expressed genes in the entire genome. Fibronectin is encoded by a single gene (\textit{Fn1}) as an initial precursor which undergoes proteolytic cleavage to give rise to the mature dimeric protein which populates the stromal ECM and the basement membranes. Therefore, mRNA and protein analysis was needed to both define its tissue source of production and to test its contribution to the BMs surrounding the neural tube.
**Figure 3.3.** Table of normalised reads of matrisome RNA-Seq analysis. Quantification and normalisation of raw counts by Reads Per Kilobase of transcripts per Million mapped reads (RPKM). Reads for each of the three replicates are reported together with averaged values.
3.2.2 Tissue source of expression and localisation of the basement membrane constituents

The *Fn1* gene codes for 7 different transcript variants due to alternative splicing of the longest transcript, named isoform a. Therefore, an *in situ* probe was specifically designed to amplify a conserved region and to detect all the 7 different variants (see methods). Analysis of mRNA expression by *in situ* hybridisation showed that the main site of expression of *Fn1* is the very caudal region of the developing embryo at all stages, from the initiation (E 8.5) to the end of primary neurulation (E 10) (Fig. 3.4 Ai - Ci, whole mount). At this site, the paraxial mesoderm flanking the open PNP, and the recently formed somites, transcribe the *Fn1* gene and contribute to most of the mRNA transcripts which were detected by RNA-Seq. It is interesting to notice that, while the surface ectoderm and neural tube appear largely negative for *Fn1* mRNA, the most dorsal cells at SE/NT interface show a significant up-regulation of *Fn1* at the site of neural fold fusion (Fig. 3.4 B vi and C iii). This was observed in embryos with more than 16-17 somites (n=4/4 embryos analysed), but not at earlier stages of neurulation (n=3 embryos analysed).

Consistent with its mRNA expression pattern, fibronectin protein localises in stromal ECM within the paraxial mesoderm flanking the neural tube (Fig. 3.4 H-L) and eventually accumulates at the boundary zone between the somites (Fig 3.4 E and G). However, despite its main source of mRNA expression being the mesoderm, fibronectin protein is also present within a dense fibrillar BM at the interface between the surface ectoderm and neural folds (Fig 3.4 I-i). As soon as the surface ectoderm comes in contact with the dorsal neuroepithelium, a BM highly enriched in fibronectin forms and, after closure, appears to wrap around the entire dorsal neural tube. This is evident also from whole mount immunostaining of the PNP, where a dense fibronectin network accumulates in the recently closed dorsal neural tube (Fig 3.4 G, arrows). This eventually forms a series of thick fibrils oriented parallel to each other along the medio-lateral axis (Fig 3.4 G, arrowheads).
Figure 3.4

Fibronectin gives rise to a dense fibrillar BM at the dorsal interface between surface ectoderm and neuroepithelium.
In contrast to fibronectin, laminins appear to be down-regulated in the dorsal region of the closing neural tube as shown by whole mount (Fig. 3.5 B and C, arrows) and section analysis (Fig. 3.5 F arrows, G zoom in). Laminins are present in two main BM: one surrounding the surface ectoderm and another one lining the basal neuroepithelium. In the surface ectoderm the laminin-rich basement membrane does not exclusively reside on the basal side of the flat squamous epithelium but also appears to surround the lateral and apical surfaces of the cells (Fig. 3.5 E and G). The morphology of this ECM resembles a peri-cellular structure more than a basal localised BM. This is consistent with the expression pattern of the major laminin-interacting receptors integrin α3 and α-dystroglycan, which are located on both the basal and apical surfaces of the surface ectoderm cells. On the contrary, laminins form a defined BM on the basal side of the neuroepithelium (Fig. 3.5 E and F). This appears not to be fully assembled in the most caudal region of flat neural plate (Fig. 3.5 D) and instead becomes a mature structure only upon establishment of a defined epithelium, when the neural folds start to elevate.

Collagen type IV appears also to be a major component of the BMs underlying the surface ectoderm and the neuroepithelium at this stage, consistent with the distribution of laminins and fibronectin. In contrast to its presence in a mature BM on the basal surface ectoderm, collagen type IV is just beginning to assemble as part of the BM on the basal side of the flat neural plate (Fig. 3.6 A and E, arrows) and only forms a mature BM at this location upon neural fold fusion (Fig. 3.6 C, D and F). Consistent with laminins, collagen type IV seems to be down-regulated at the most dorsal region of the surface ectoderm-neural tube interface, where fibronectin fibrils are highly abundant. This has been confirmed both in early stages of neurulation (Fig. 3.6 D, closure 1) and later in development at E 9.5 and at E 10 (Fig. 3.6 C and F). Collagen type IV also localises in the mesenchymal tissue of the paraxial mesoderm flanking the neural tube, as also observed for fibronectin, since they both contribute to the interstitial ECM in addition to the BMs.
Figure 3.5. Laminins within embryonic BMs. (A-C) Whole-mount immunofluorescence analysis of laminin expression by apotome 3D reconstruction shows that laminins are downregulated in the dorsal region of the neural tube - surface ectoderm interface (C, arrows). (D-K) Immunofluorescence analysis on cryosections by laser scanning confocal microscopy shows that laminins give rise to a pericellular matrix structure in the surface ectoderm but form a defined BM on the basal side of the neuroepithelium.
Collagen type IV distribution. Collagen type IV localises in the BMs underlying the surface ectoderm and the neural tube. The latter is disorganised at the level of flat neural plate (A and E) and matures into a defined BM upon neural fold fusion (C and F). Collagen type IV is down-regulated at dorsal interface of the surface ectoderm and neuroepithelium (C, D and F). Images acquired by laser scanning confocal microscopy and processed by single z-plane projection. Scale bars: 50 μm.
Perlecan (heparan sulphate proteoglycan; HSPG II) (Sarrazin et al. 2011) and nidogens (Ho et al. 2008) are integral constituents of the BMs, but in contrast to laminins, collagen type IV and fibronectin which polymerise into networks, their main function is to regulate the structural integrity and stability of the BM by acting as bridge between the network-forming components (Yurchenco 2011). Immunofluorescence analysis shows that perlecan and nidogen-1 localise in the BMs of the neuroepithelium and surface ectoderm, showing co-expression with the main network-forming components (Fig. 3.7). Their presence can be seen as a marker of the degree of matrix assembly since their key role is in the final stabilisation of the overall architecture of the BM. Consistent with this, perlecan and nidogen-1 show continuous staining in the fully mature BM underlying the surface ectoderm, whereas they show patchy staining in the matrix complex on the basal side of the flat neural plate (Fig. 3.7 A and E), which achieves its structural maturity only after elevation and fusion of the neural folds (Fig. 3.7 C, D and F). Furthermore, while perlecan precisely outlines the BMs on the basal side at the interface between the surface ectoderm and neural tube (Fig. 3.7 D), nidogen expression highlights the peri-cellular morphology of the ECM in contact with the surface ectoderm (Fig. 3.7 G-H), as previously observed for laminins. In fact, nidogens are primarily involved in the stabilisation of the BM by establishment of non-covalent interactions between the collagen type IV and laminin networks. This supports the idea that the surface ectoderm, in addition to the fibronectin and collagen type IV basement membrane located basally, establishes contacts with an apically distributed ECM rich in laminins which envelop the entire surface of each ectoderm cell. This is also reinforced by the pericellular distribution of the laminin-interacting receptors integrin α3 (Fig. 3.11 C-D) and alpha dystroglycan (Fig. 3.12 D-G).
Figure 3.7. Perlecan (HSPG II) and Nidogen-1 immunofluorescence analysis. Perlecan and nidogen-1 localise in the BMs underlying the neural tube and the surface ectoderm. Their expression marks the state of assembly of the BMs showing that the matrix underlying the neuroepithelium is still immature at the level of flat neural plate (A) whereas it becomes fully assembled upon elevation and fusion of the folds (D and F). Nidogen-1 expression outlines the peri-cellular morphology of the ECM in contact with the surface ectoderm cells (H), like laminins. Images acquired by laser scanning confocal microscopy: perlecan (HSPG II) in red (A-D) and nidogen-1 in green (E-H). Images processed by single z-plane. Scale bars: 50 µm.
3.2.3 RNA-seq analysis of integrin receptors

The ability of cells to interact with the BM components is primarily mediated by the integrin family of transmembrane receptors. Integrins are heterodimeric complexes composed of one α and one β subunit which associate together by non-covalent bonds to give rise to a functional heterodimer. At least 24 different receptors have been described so far in vertebrates which originate from the combination of 18 α subunits with 8 different β subunits, creating a large heterogeneity both in ligand specificity and cellular function (Barczyk et al. 2010; Campbell and Humphries 2011b; Lowell and Mayadas 2012b; Takada et al. 2007). Here, the mRNA transcript levels of the α and β subunits were quantified by RNA-Seq to identify the major integrin subunits and as a result to infer the combination of functional heterodimers expressed at this stage of neurulation (Fig. 3.8).

The most highly expressed subunit is integrin β1, with an average RPKM of 6.07 (Fig. 3.8 A and E). This subunit is a central component of the family of integrin receptors due to its ability to pair with 12 different α subunits, and therefore to contribute to the assembly of half of the total 24 integrin possible combinations. Because of the high expression level of this β subunit, the amount of corresponding α subunit produced by the same cell will represent the limiting factor to determine the specific dimeric combination of the final integrin receptor which will be assembled intracellularly and then transported on the surface of the cell. RNA-seq analysis revealed that two main subfamilies of receptor combinations expressed during neurulation are the integrins interacting with the RGD motif and those interacting with laminins, while no collagen interacting receptors were expressed at this stage (Fig. 3.8 B). Among the former, the dimeric combination α5β1 was the most highly expressed which represents the major integrin which recognise the RGD motif present in fibronectin. A moderate level of integrin αvβ1 was also detected, which also interacts with fibronectin (Fig. 3.8 E). In addition to the β1 subunit, significant high levels of integrin β5 were also detected. This subunit pairs exclusively with αv, which in contrast is poorly expressed at this stage, to give rise to integrin αvβ5 which is the major receptor for the ECM protein vitronectin (Fig. 3.8 C). Nevertheless, RNA-Seq analysis of the core matrisome showed that vitronectin is not present at this stage since its transcripts levels were significantly below the threshold levels of expression.
Among the family of laminin-interacting receptors, integrin α6β1 and integrin α3β1 are the two major receptors expressed at this stage (Fig. 3.8 A and E). Finally, one of the integrin receptors expressed by leukocytes, integrin α9β1, appears also to be present alongside the major RGD- and laminin-binding integrin families. Low but still supra-threshold levels of integrin β1 binding protein 1 were also detected (Fig. 3.8 A). This is an important regulator of
integrin β1 activation which competes with talin for binding to the cytoplasmic tail of integrin β1, thus suppressing its activation and preventing clustering of the receptor within the focal adhesion complex (Bouvard et al. 2003). It is also modulates signalling downstream of integrin β1 by regulating the Rho family GTPases: Cdc42, Rac1 and RhoA (Degani et al. 2002).

In summary the two major subfamilies of integrins expressed at the stage of neural tube closure are: (1) the RGD-interacting receptors integrin α5β1 and αvβ1 which bind fibronectin, and αvβ5 which binds vitronectin, although this ligand is not expressed, and (2) the laminin receptors integrin α3β1 and α6β1 (Fig. 3.8 B) which interact with laminins α5β1γ1 and α1β1γ1, which are the most highly expressed trimeric combinations at this stage (Fig. 3.2).

3.2.4 Tissue source of expression and localisation of integrin receptors

To determine the tissue source of expression, the mRNA of each integrin subunit was examined by in situ hybridisation and the localisation of the protein was studied by immunofluorescence. Itgb1 is the most highly transcribed integrin subunit and is ubiquitously expressed from early stages to the end of primary neurulation. It shows significant expression in the open caudal region of the developing embryo (Fig 3.9 A-C). Cross section analysis of the PNP confirmed the ubiquitous pattern of expression of Itgb1 in both the neuroepithelium, in the paraxial mesoderm and with low levels in the surface ectoderm. Interestingly, Itgb1 expression in the neuroepithelium follows a ventro-dorsal gradient (Fig 3.9 A-iv & B-iii) which is observed also for the α subunit, integrin α6 (Fig. 3.11 E-G) and integrin αv (Fig. 3.10 E-F). Analysis at the protein level shows that the integrin β1 subunit eventually localises on the basal side of the neuroepithelium at the site of contact with the BM (Fig 3.9 E-F). Despite the evident mRNA gradient, the protein seems to be expressed similarly both in the ventral and dorsal parts of the neuroepithelium. Integrin β1 is also strongly expressed by the surface ectoderm cells as shown by 3D whole mount analysis of the PNP region (Fig 3.9 G). Strikingly, a significant up-regulation of integrin β1 has been observed at the site of zippering where the tips of neural folds and the most dorsal cells of the surface ectoderm come into contact (n=7/7 embryos analysed by whole mount confocal microscopy) (Fig 3.9 G). This pattern of expression was also detected at the mRNA level by in situ analysis of Itgb1 gene (Fig. 3.14 A) and has been similarly observed for the major α integrin subunits: α5, αv and α6 (Fig. 3.14). This would suggest a potential role of integrins at the site of fusion where adhesion to the dorsal fibronectin-rich basement membrane could be pivotal for the propagation of caudal zippering.
Figure 3.9. Integrin β1 is upregulated at the site of dorsal zippering. (A-C) In situ hybridisation analysis shows the ubiquitous pattern of expression of *Itgb1* gene from E 8.5 to E 10 in the neural tube, mesoderm and surface ectoderm. *Itgb1* mRNA follows a ventral to dorsal gradient with intense expression in the ventral neural tube compared to the dorsal part (A-v & B-iii). (D) Sense probe hybridisation. (E-G) Immunofluorescence analysis shows that integrin β1 localises in the surface ectoderm (G) and on the basal side of neuroepithelium (E-F). 3D reconstruction of the open PNP: integrin β1 is significantly upregulated at the site of dorsal zippering (G). Images acquired by laser scanning confocal microscopy and processed by maximal projection of z-stack planes.
Among the group of receptors interacting with the RGD motif, integrin α5 appeared to be the most highly expressed subunit. *Itga5* is largely transcribed by the paraxial mesoderm flanking the neural tube (Fig 3.10 A-C), similarly to the expression of the *Fn1* gene which codes for its main ligand (Fig. 3.4 A-C). Therefore, integrin α5 pairs with integrin β1 to give rise to the main fibronectin-interacting receptor at the site of the mesoderm, as supported also by the mesodermal defects and caudal truncation observed in the original knockout mutants of both integrin α5 (Yang et al. 1993b) and fibronectin (George et al. 1993). Similar to the β1 subunit, itga5 is also significantly upregulated at the site of zippering (Fig 3.10 C-v) and at the dorsal tips of the fusing neural folds (Fig 3.10 B & C, iv), which is evident even from whole mount analysis (Fig 3.10 B-C, ii). While this localised upregulation is marked in most of the embryos with more than 18 somites (n=4/5 embryos analysed), it is not evident at earlier stages of neurulation, below the 15 somite stage (n=0/3 embryos analysed).

In contrast, *Itgav* (integrin αv) is mainly expressed by the neuroepithelium (Fig 3.10 E-F) and follows closely the ventral to dorsal gradient which was observed for *Itgb1*. In addition to β1, integrin αv dimerises also with the β5 subunit to form the vitronectin-interacting receptor integrin αvβ5. While the expression of the two subunits co-localises at the level of the neuroepithelium in the open PNP, their pattern differs significantly in other tissues such as surface ectoderm and mesoderm where *Itgb5* is highly expressed in contrast to the low levels of *itgav* (Fig 3.10 E-F vs H-I). Despite this discrepancy, the β5 subunit is known so far to pair exclusively with the αv subunit. Therefore their dimerization would be predicted to occur only at the site of co-expression, thus in the neuroepithelial cells. *Itgb5* is also significantly upregulated at the zippering point of the fusing hindbrain neuropore (Fig 3.10 H-ii whole mount, H-iv section), suggesting a potential role of this receptor in cranial zippering.
Figure 3.10. Expression analysis of the RGD-interacting integrin receptors. (A-C) *Itga5* is mainly expressed by the paraxial mesoderm and pairs with the β1 subunit to form the main fibronectin receptor in the mesoderm. *Itga5*, together with *Itgb1*, is significantly upregulated in the dorsal apposing folds and at the site of zippering. (D) Sense probe control for *itga5*. (E-F) *Itgav* is expressed by the neuroepithelium as a ventral to dorsal gradient. It pairs with β1 and β5 subunits to form the main fibronectin (integrin αvβ1), and vitronectin receptor (integrin αvβ5). (G) Sense probe control for *itgav*. (H-I) *Itgb5* is co-expressed with the pairing subunit α5 in the neuroepithelium. It is also significantly upregulated at the dorsal tips of the fusing cranial folds at the level of hindbrain neuropore. (J) Sense probe control for *itgb5*. 
Among the family of laminin-interacting receptors, integrin α6 and integrin α3 are the two major subunits identified by RNA-seq analysis, which pair with β1 to form the functional heterodimers α6β1 and α3β1 (Fig 3.8). The Itga3 gene (integrin α3) is strongly expressed by the surface ectoderm as shown by in situ hybridisation (Fig 3.11 A-B). Consistent with the mRNA, the protein localises to the surface ectoderm (Fig 3.11 C-D). However, its expression is not confined to the basal side of the epithelium, as seen for the receptor integrin α6, but instead it appears to be expressed on the entire surface of the cells both at the apical and basal sides. This is consistent with the peri-cellular appearance of the laminin rich ECM which surrounds the surface ectoderm cells (Fig 3.5 E) and highlights the potential role of integrin α3 in the assembly and anchorage of the cells to this specialised type of peri-cellular ECM.

The main site of expression of Itga6 gene (integrin α6) is the neuroepithelium, even though low levels of expression both at mRNA and protein level could be detected in the surface ectoderm (Fig 3.11 E-G). Consistent with the β1 subunit, Itga6 follows a marked ventral to dorsal pattern of expression which also translates into a ventral to dorsal gradient of protein localisation on the basal side of the neuroepithelium at the site of contact with the laminin network (Fig 3.11 I).

Therefore, integrin α6β1 and integrin αvβ1 are the two major integrins of the neuroepithelium, expressed as ventral to dorsal gradients, which mediate interactions of the neuroepithelial cells with the laminin and fibronectin basement membranes, respectively. Moreover, integrin α6 is specifically upregulated at the site of zippering where the dorsal folds come into contact (Fig 3.11 K) in agreement with the previous observation for integrin β1.
Figure 3.11
In situ and immunofluorescence analysis of the laminin-interacting integrin receptors.
Alongside the integrin family, another major receptor which mediates binding to laminins is dystroglycan. Dystroglycan is an integral component of a complex which connects the external laminin BM (through the extracellular α-dystroglycan subunit) to the F-actin cytoskeleton, via dystrophin interactions (Michele and Campbell 2003; Yurchenco 2011). The analysis of this receptor has been included alongside integrins to allow a comprehensive analysis of the laminin-interacting receptors. From early stages of neurulation, Dag1 mRNA is expressed at two main sites: the neuroepithelium and the surface ectoderm (Fig. 3.12 A,B). Later, Dag1 is expressed strongly in the dermomyotome (Fig. 3.12 C-iv), which will give rise to paraxial muscles, consistent with its role in muscle development (Hidalgo et al. 2009; Barresi and Campbell 2006). The dystroglycan receptor protein localises peri-cellularly on the surface ectoderm cells, and is also detected as less intense staining on the basal surface of the neuroepithelium (Fig. 3.12 D-F). Therefore, in addition to integrin α3 and integrin α6, cells of the surface ectoderm and of the neuroepithelium adhere to and interact with the laminin network via dystroglycan receptor.
Figure 3.12. Dystroglycan mediates interaction with laminins during neurulation, in addition to the main integrin receptors. (A-C) *Dag1*, the gene coding for α-dystroglycan subunit, is expressed by the surface ectoderm and neuroepithelium throughout neurulation. (D-G) The α-dystroglycan protein is expressed at the site of interactions with the laminin BM, both peri-cellularly on the surface ectoderm and, less intensely, on the basal surface of the neuroepithelium. Images of in situ acquired by widefield differential interference contrast. Images of immuno acquired by laser scanning confocal microscopy and processed by maximal projection of z-stack.
3.3 Discussion

The heterogeneity of the molecular constituents which sculpt the BMs, and the complexity of the dimeric integrin combinations which give rise to the great diversity of functional receptors, have both hindered the in vivo study of the structural and functional roles of the ECM during embryo morphogenesis. The present study aimed to provide a comprehensive analysis of the matrisome and ECM receptors during spinal neural tube closure. The combination of RNA-seq and in situ hybridisation analysis, together with confocal 3D imaging of protein localisation, allowed the expression of specific ECM and integrin isoforms to be identified. This defined which of the many multimeric combinations of collagens, laminins and integrins are likely to be functional during mouse spinal neurulation. Importantly, it also allowed the tissue source of each ECM component to be inferred, as well as the tissue of expression of each integrin type. Overall, the study has determined the molecular composition, as well as the structural morphology, of the basement membranes and their interacting receptors during closure of the mouse spinal neural tube.

The present data revealed not only a difference in composition, but also in the physical architecture and status of assembly among the BMs present during neurulation. While the BM underlying the surface ectoderm is structurally mature upon initiation of neurulation, on the contrary the BM underlying the flat neuroepithelium in the caudal region of the embryo is still disorganised and begins to assemble into a defined structure only upon elevation and fusion of the neural folds. This is evident by the disrupted and discontinuous pattern of expression of the main network-forming components (laminins, fibronectin and collagen type IV) on the basal side of the flat neuroepithelium, which have not yet formed a structured, continuous layer of basement membrane. It would be interesting, in future work, to compare the appearance of these two BMs at the ultrastructural level, for example by Transmission Electron Microscopy, to see if there is also a difference in the basal lamina at each location.

Not only the state of assembly, but also the overall architecture of the ECM of the surface ectoderm cells appeared to be significantly different from that underlying the neuroepithelium. The surface ectoderm has a typical BM located basally, rich in fibronectin and collagen IV, but the cells also establish contact with an apically distributed ECM rich in laminins which envelops the entire surface of each ectoderm cell, as also evident by the pericellular distribution of nidogen. Consistent with this, the expression of the main laminin-interacting receptor integrin α3 also appears not to be confined exclusively to the basal side of the surface ectoderm, but to distribute along the entire surface of the cells. A similar
appearance is seen with α-dystroglycan. This reinforces the idea of the existence of pericellular ECM rich in laminins surrounding the surface ectoderm. This is consistent with the lateral and apical localisation of the drosophila specific βPS-integrin reported both in the follicular epithelium of Drosophila egg chamber and in the amnioserosa and the epidermal cells during Drosophila dorsal closure (Bahri et al. 2010).

In addition to the state of assembly and structural architecture, the BMs of the spinal neurulation region also show a significant difference in their molecular composition. In the dorsal region, at the surface ectoderm-neural tube interface, the BM is highly enriched for fibronectin, which is the most strongly expressed constituent of the matrisome at this stage. In contrast, the network-forming components laminins and collagen type IV are downregulated in the dorsal region. Interestingly, fibronectin is mainly expressed and synthesised by the paraxial mesoderm but eventually polymerises into a fibrillar network dorsally on the basal side of the surface ectoderm. This suggests a potential mechanism of paracrine assembly of fibronectin ECM where one tissue, the mesoderm, secretes the soluble fibronectin dimer and another tissue, either the surface ectoderm or the dorsal neural tube, would mediate its fibrillogenesis. Similarly, the ability of ECM molecules to diffuse and polymerise away from the tissue of secretion has been recently observed in studies of both fibronectin (de Almeida et al. 2016) and laminins during embryo development (Copp et al. 2011). But how does the fibronectin rich BM accumulate at the dorsal interface between neural tube and surface ectoderm? One possible explanation is that fibronectin is secreted by the mesoderm, assembled upon contacting the surface ectoderm and then transported by the surface ectoderm over the dorsal neural folds as the surface ectoderm converges on the dorsal midline. Another potential explanation, not mutually exclusive, is that the dorsal rich fibronectin network is also synthesised locally as shown by Fn1 mRNA upregulation observed at the dorsal fusion point.

In summary, as the neural plate elevates and contacts the surface ectoderm dorsally, its cells become exposed to a different BM which arises from the surface ectoderm epithelium, is structurally mature, and is enriched for fibronectin but poor in laminin and collagen type IV components. This contrasts with the neuroepithelial cells that remain adjacent to the paraxial mesoderm, where the underlying BM is structurally immature and has a quite different molecular composition.
Figure 3.13. Summary of the integrin subunit combinations interacting with either laminin or fibronectin networks. (A-G) The two main laminin receptors: integrin α3β1 in the surface ectoderm (C-D) and integrin α6β1 in the neuroepithelium (E-F). (G-L) The three main fibronectin receptors: integrin α5β1 in the mesoderm, integrin αvβ1 in the neuroepithelium and αvβ5 which co-localise only at the level of the neuroepithelium. (G) β1 is the central shared subunit most highly expressed during neurulation, which pairs with α chains to give rise either to the laminin or fibronectin receptors. (M) Summary diagram of the main BM components surrounding the neural tube (yellow), underlying the surface ectoderm epithelium (blue) and present in the paraxial mesoderm flaking the neural folds. (N) Summary diagram of the main integrin receptors expressed on the basal side of the neural tube as a ventro-dorsal gradient (integrin α6 and αv), surrounding the surface ectoderm cells (integrin α3) and focally localised at the tip of the apposing neural folds (integrin α5).
The ability to regulate BM assembly and cell-ECM anchorage is primarily mediated by the family of integrin receptors. Out of the 24 potential combinations, 5 have been identified as the central integrin receptors involved in the process of spinal neural tube morphogenesis (Fig. 3.13): integrins α3β1 and α6β1 are the two receptors which primarily mediate interactions with the laminin BM, in the surface ectoderm (Fig. 3.13 C-D) and in the neuroepithelium respectively (Fig. 3.13 E-F); and integrins α5β1 and αvβ1 are the main fibronectin receptors, in the mesoderm (Fig. 3.13 J) and neuroepithelium (Fig. 3.13 K) respectively. On the other hand, for the integrin combination αvβ5, the β5 subunit is very highly expressed compared to the low levels of the αv partner. Moreover, their patterns of expression differ significantly and do not appear to co-localise in the same tissues, suggesting that integrin αvβ5 is not a major ECM receptor during neurulation. In summary, the stage of neural tube closure appears to be dominated by integrin β1, which is the most highly expressed subunit and which pairs differentially with specific α subunits to mediate interactions either with laminins or fibronectin in the different basement membranes (Fig. 3.13 G).

The significant up-regulation of α/β1 integrins at the site of dorsal fusion, which has been observed both at the mRNA (Fig. 3.14, A-D arrows) and protein level (Fig. 3.9 G and 3.11 K), would suggest a potential role of cell-ECM interactions at the site of zippering in the open PNP. A similar pattern of integrin up-regulation has been previously reported both in Drosophila and in mouse. In the former system, high levels of the receptor βPS-integrin have been observed at the leading edge surface of the most-dorsal epithelial cells migrating over the amnioserosa during the process of dorsal closure (Bahri et al. 2010). Similarly in the mouse, integrin α5 and fibronectin were shown to be up-regulated locally in the eyelid front cells as they move over the cornea during eyelid closure (Heller et al. 2014). Interestingly, this peculiar pattern of integrin expression seems to be confined to late stages of neural tube closure since the upregulation of α/β1 integrin receptors is only evident in embryos with more than 18 somites. The dorsal neural fold region has also been shown to be highly enriched for fibronectin. This would suggest a potential model where integrins α/β1 could mediate interactions and anchorage to the fibronectin BM interposed at the interface between the dorsal neural folds and the surface ectoderm at the site of dorsal fusion (Fig. 3.14 E). This cell-matrix interaction could provide the first ‘adhesions’ between the paired neural fold tips, as they become apposed. In the following chapter the potential role of fibronectin-integrin interactions at the site of zippering will be tested by employing a conditional genetic approach to investigate in vivo the function of these interactions for closure of the neural tube.
Figure 3.14. Model: possible role of integrins α/β1 at the site of zipper in the open PNP. (A-D) Integrin β1 together with the pairing alpha subunits α6, α3 and αv are highly upregulated at the site of fusion in the open PNP, where the apposing folds come into contact. Cross-sectional view. Arrows indicate the site of up-regulation: at the interface between the dorsal neural folds and overlying surface ectoderm. (E-G) Model: upregulation of integrins α/β1 (red) in the open PNP (G, dorsal view) may act as part of the mechanism of dorsal zipper by anchoring the surface ectoderm and neural folds to the fibronectin basement membrane localised at their interface, perhaps creating the first ‘adhesion’ between the paired neural fold tips (E and zoom in F, cross section).
4. Role of integrin β1-mediated adhesion during neural tube closure

4.1 Introduction

Initial insights into the role of *in vivo* integrin-ECM interactions during neural tube morphogenesis come from genetic studies in mice where open NTDs were observed in embryos deficient for specific integrin subunits or for particular BM constituents.

For example, craniorachischisis has been reported in double homozygous mutants for integrin α3 and integrin α6 (De Arcangelis et al. 1999). In this study, 43% of the mutant embryos fail to close the neural tube from the midbrain to the spinal level with deformation of the most caudal axis (kinked tail). Interestingly, the single α3 (Kreidberg et al. 1996) and α6 mutants (Georges-Labouesse et al. 1996) successfully complete closure of neural tube but they die perinatally due to kidney and lung defects and severe skin blistering, respectively, suggesting the possibility that compensatory mechanisms may be activated when a specific subunit is genetically removed. However, only occasional hindbrain exencephaly, and not the severe NTD craniorachischisis, was observed in the double α3/α6 mutants in our lab when this mouse line was imported (Carvalho and Copp, unpublished) and thus the role of these receptors in neural tube closure needs to be further elucidated.

Replacement of the most widely expressed variant of integrin β1 (isoform A) with the specific isoform D, which differs by 13 amino acids in the cytoplasmic tail, was also shown to cause defective closure in the cranial region at the hindbrain level and to affect the caudal neural tube which appeared waved and kinky in one third of the homozygous knock-in embryos (Baudoin et al. 1998). Similarly, another study reported the presence of an analogous phenotype in embryos mutated in the cytoplasmic tail of integrin β1 which develop exencephaly in 20% of homozygous mutants but successfully closed the caudal neural tube although it was waved and deformed in appearance (Hirsch et al. 2002). This directs the attention towards the potential role of the mechano-transduction signalling downstream of integrins in neural tube closure which is mediated by the integrin cytosplasmic tail.

In addition to the integrins, perturbation of specific ECM ligands was shown to prevent closure of the neural tube. For example, embryos deficient for the laminin α5 chain fail to complete cranial neural tube closure, with 60% of the mutant cases developing exencephaly.
encompassing the mid- and hindbrain regions. However, these mutants but do not show defects in the caudal neural tube (Miner et al. 1998). Analysis of the ultrastructure of the ECM in laminin α5 mutants shows that the BMs underlying the SE and beneath the NE are both structurally intact except for the region of SE in close proximity with the dorsal neural folds. The authors speculated that impairment of this BM would affect the capability of the overlying SE to generate lateral forces essential for its medial convergence and thus for neural tube closure. Recently, a study on zebrafish proposed a novel model for the role of ECM in neurulation, where laminins would act by coupling the movements of the neural tube and mesoderm during early neurulation stages (Araya et al. 2016). They in fact showed that depletion of laminin γ1 impairs neural tube morphogenesis by disrupting the coordination between these two tissues. Recent studies from the same research group also support the hypothesis that laminins may be instructive for the establishment of apico-basal polarity of the neuroepithelium as shown by the inversion of polarity phenotype observed in embryos deficient for laminins (personal communication by Dr Vineetha Vijayakumar, Prof Jon Clarke group, King’s College London).

In contrast to laminin, mouse embryos deficient for fibronectin appear to have a significantly reduced rostro-caudal axis at E8.5 due to a defect in elongation, and to display a kinky neural tube, probably as a secondary effect of the mesodermal and elongation defects (George et al. 1993). However, the early lethality of these embryos which arrest their development soon after the initiation of neurulation prevented the possibility of further investigating the in vivo role of fibronectin during neural tube morphogenesis. A recent study further investigated the mechanism of the defect by live imaging the tail bud of zebrafish embryos after perturbing the two main fibronectin interacting receptors, integrin α5 and αv (Dray et al. 2013). The authors proposed that fibronectin acts as a mechanically integrated substrate which modulates inter-tissue adhesion by regulating the viscosity between mesoderm, notochord and neural tube. Alteration of fibronectin-integrin interactions would uncouple these structures leading to elongation defects. However the underlying role of cell-fibronectin interactions during neural tube closure still remains elusive.

Overall, the above studies strongly support the possibility that integrin-ECM interactions may play a role in neurulation. However, whether these interactions are necessary for closure of the cranial and/or spinal neural tube, which specific tissue and cellular components are involved and what is the underlying molecular mechanism of cell-ECM adhesion remain to be fully determined. In the previous chapter, analysis of the ECM revealed how complex and heterogeneous is the pattern of expression during neurulation of both the ECM components
which form the BM and the integrin receptors which are expressed on the surface of epithelial cells. To investigate the functional role of cell-ECM interactions in vivo during neural tube closure two genetic approaches could be used: either to target a specific ECM component to prevent its secretion from the tissue of origin and therefore interfere with the deposition and assembly of a functional BM. Or instead to target the integrin receptors, thus blocking the ability of the cells to interact with the underlying BM. This latter approach is more likely to avoid the complication of functional redundancy between ECM ligands, and so was used in the current study.

Nevertheless, the variety of integrin receptors and the different potential combinations of alpha and beta subunits, make the integrin family a complex system to target. In the previous chapter, the main receptor combinations expressed during neurulation were identified and shown to share in common the β1 subunit. Because of its central role in the assembly of the majority of integrin receptors expressed at this stage, it was decided to target the β1 subunit (encoded by the Itgβ1 gene) using a genetic approach to prevent its pairing with the corresponding α subunits and thus to abolish in vivo the assembly of both the fibronectin-interacting receptors (integrin α5β1 and αvβ1) and the laminin-interacting receptors (integrin α6β1 and α3β1). To be able to study the role of these receptors in neural tube closure and to avoid the early embryo lethality caused by the removal of this key integrin subunit (Fassler and Meyer 1995; Stephens et al. 1995), a conditional approach based on the Cre-loxP system was employed, to confine the knock out of the gene both temporally and spatially to the stage of interest. Because of the significant up-regulation of integrins at the fusion point, as shown by in situ and immunohistochemistry analysis (Chapter 3), the genetic ablation approach was designed to target specifically the very dorsal tips of the apposing neural folds upon fusion.

The two main tissues involved at this site are the dorsal neuroepithelial cells and the dorsal surface ectoderm cells. Therefore to be able to distinguish a role of Itgβ1 within either the neuroepithelium or the surface ectoderm, two different conditional approaches were employed: a Cre driven by the Pax3 promoter was used to target mainly the dorsal neuroepithelium (Fig. 4.1 B) while a Cre driven by Grhl3 promoter was used to target mainly the surface ectoderm cells (Fig. 4.7 B).

The present study shows that integrin β1 is essential for neural tube closure since its deletion leads to the development of open neural tube defects. The occurrence of the defects was significantly higher in the Grhl3-Cre x Itgβ1-flox cross (78%) compared to the Pax3-Cre x Itgβ1-flox cross (26%), supporting a role for integrin β1 particularly in the surface ectoderm for spinal neurulation. Moreover, the abolition of the focal up-regulation of integrins at the zippering
point by targeting the surface ectoderm further confirmed this as the main tissue for critical integrin β1 function. A series of studies were then performed in an attempt to determine the likely mechanism by which focal inactivation of integrin β1 prevents spinal neural tube closure.
4.2 Results

4.2.1 Recombination of Itgβ1 in the dorsal neuroepithelium by Pax3-Cre affects cranial and caudal neural tube closure

Mice homozygous for the floxed allele of Itgβ1 gene (Potocnik, Brakebusch, and Fässler 2000) (Itgβ1<sup>f/f</sup>; gene symbol: Itgb1<sup>tm1Ref</sup>, MGI: 1926498) were crossed with the mice carrying a Cre driven by the Pax3 promoter, that were also heterozygous for the floxed Itgβ1 allele (Pax3<sup>Cre/+</sup>; Itgβ1<sup>f/+</sup>) (Fig. 4.1 A). Pax3 is a transcription factor which is expressed in the dorsal neural tube from the start of neurulation (E8.5) (Goulding et al. 1991). Pax3<sup>Cre/+</sup> is a constitutively expressed Cre line (Engleka et al. 2005) (gene symbol: Pax3<sup>tm1(Cre)Joe</sup>, MGI: 3573783) which was used here to mediate recombination of the Itgβ1 gene in the dorsal region of the neuroepithelium (Fig. 4.1 B). However, in addition to the neural tube, Pax3<sup>Cre</sup> was shown to target few sporadic surface ectoderm cells at later stages of development (20 somites) (Rolo et al. 2016), making it not entirely specific for dorsal neural tube.

The progeny generated includes four possible genotypes (Fig. 4.1 A): the mutant Pax3<sup>Cre/+</sup>; Itgβ1<sup>f/f</sup> in which both alleles of Itgβ1 gene are removed, the heterozygote Pax3<sup>Cre/+</sup>; Itgβ1<sup>f/+</sup>, and two wild type genotypes Pax3<sup>+/+</sup>; Itgβ1<sup>f/f</sup> and Pax3<sup>+/+</sup>; Itgβ1<sup>f/+</sup> in which Itgβ1 gene is fully functional due to the absence of Cre expression. The occurrence of the genotypes appears to follow closely the normal Mendelian distribution, with each genotype frequency being not far from the 25% ratio (Chi-square test, non-significant p-value = 0.2662, embryos analysed = 207) (Fig. 4.1 D). This would exclude any early lethality during development. In order to define the genotype of the progeny, a specific set of primers was designed around the site of insertion of the 5’ LoxP sequence located at the end of intron 1 of Itgβ1 gene (Fig. 1E). This allowed the floxed allele (f) (band: 480 bp), containing the LoxP sequence to be distinguished from the wild type allele (+) (band: 330 bp) (Fig. 4.1 C).

Embryos were initially collected at E 9.5 in order to assess whether removal of Itgβ1 gene could have an effect on the process of spinal and cranial neurulation. To estimate a potential defect in spinal neural tube closure the size of the PNP was quantified at different somite stages in mutants compared to wild type embryos. Two parameters were estimated: the length of the PNP along the rostro-caudal axis (Fig. 4.2 B iii), from the fusion point to the most
caudal part of the flat neural plate, and the width of the PNP along the medio-lateral axis (Fig. 4.2 B iii), representing the maximum distance between the tips of the opposing folds.

**FIGURE 4.1**

(A) **PARENTAL CROSS**

\[ \text{Pax3}^{\text{Cre}+/+} : \text{ItgB1}^{f/+} \times \text{ItgB1}^{f/+} \]

\[ \downarrow \]

**PROGENY:**

- **Pax3^{Cre}+/+ : ItgB1^{f/+}** (HETEROZYGOUS)
- **Pax3^{Cre}+/+ : ItgB1^{f/f}** (WILD TYPE)
- **Pax3^{Cre}+/+ : ItgB1^{f/+}** (MUTANT)
- **Pax3^{Cre}+/+ : ItgB1^{f/+}** (FLOXED)

(B) **DORSAL NEUROEPITHELIUM**

Pax3-cre

(C) **MENDELIAN RATIOS ANALYSIS**

(D) **E Schematic representation of the ItgB1 locus (MGI: 3573783)**: the 5’ LoxP site was introduced in intron 1, close to the start of exon 2. 3’ LoxP site is located at the end of the gene between exons 9 and 10 with the insertion of a promoterless LacZ reporter to detect cre-mediated recombination. Primers for PCR genotyping were designed to detect the presence 5’ LoxP site. Embryonic genotypes were obtained from yolk sac DNA which does not express Pax3, and hence the floxed ItgB1 alleles are not recombined.
Changes in PNP length and width over somite number were analysed by linear regression. Despite these parameters to do not follow precisely a linear pattern, both PNP length and width tend to decrease over time as somite number increases. As opposed to ANOVA analysis of the means/medians with somite number grouping, where summarised values are compared among grouped categories, the linear model takes into consideration every single measurement along variation of each somite number. Thus, it fits the best regression model to be able to predict Y (PNP length or width) from variation of X (somite number). In addition, regression equation allows to estimate the intercepts and thus to predict the somite number stage at which the PNP completes closure (equals to 0).

Mutant Pax3<sup>Cre</sup>; Itgβ1<sup>f/f</sup> embryos display significant larger PNP compared to wild type Pax3<sup>+/+</sup>; Itgβ1<sup>f</sup>/f or f/+ embryos (Fig. 4.2 A-B). Linear regression analysis of PNP width shows a significant difference between mutants and wild type (Fig. 4.2 G). \( r^2 \) estimates the degree of linear correlation between the two variables \( x \) and \( y \) and ranges from 0.0 (if no linear correlation) to 1.0 (if perfect correlation). In wild type, \( r^2 = 0.51 \) showing a strong negative linear relationship between PNP width and somite number, which predicts a decrease in PNP width over time during normal development. The calculated \( p \) value against the null hypothesis that there is no linear relationship between \( x \) and \( y \) shows statistical significance (\( p < 0.0001 \)). On the contrary, in mutant embryos, \( r^2 = 0.09 \) shows no linear relationship between PNP width and somite number, with a non-significant \( p \) value against the null hypothesis (\( p = 0.1527 \)). The linear decrease in PNP width in wild type predicts final PNP width closure at an estimated value of 35 somites (x-axis intercept), close to the observed PNP closure stage of 30 somites (Copp et al., 1982). In contrast, PNP width in the mutant does not gradually decrease. Instead the folds remain distant from each other as somite number increases, with a prediction that fusion will be achieved only at 70 somites, which is far beyond the mouse somite range. Moreover, comparison of the slopes of the two regression lines \( y = -11.93 \times + 421.1 \) (wild type) and \( y = -3.699 \times + 258.2 \) (mutant) shows a significant difference between the two models (p-value = 0.01754) (Fig. 4.2 G). Therefore, the mutant and wild type PNP width-somite stage relationships differ significantly from each other.

Interestingly, linear regression analysis of PNP width in heterozygotes shows no significant difference compared to wild type (Fig. 4.2 H). The linear regression line of the heterozygote displays a lower degree of linear correlation \( (r^2 = 0.37) \) compared to the wild type. However, fusion of the folds (x-axis intercept) is estimated to occur at 37 somites which does not differ significantly from 35 somites in wild types (p-value of the intercepts = 0.8678).
Linear regression analysis of PNP length similarly shows a significant difference between mutant and wild type (Fig. 4.2 I). Despite PNP length in mutants following a linear correlation pattern ($r^2 = 0.67$) and the slope of the linear regression line being very similar to wild type ($p$-value of the slopes = 0.74; the two lines are parallel), the intercepts differ significantly ($p < 0.0001$). In wild type, completion of PNP closure is predicted by extrapolation from the graph to occur at 35 somites (x-axis intercept when PNP length = 0), whereas in mutants closure is predicted at 40 somites. This retardation of the PNP closure is consistent with the observation of tail flexion defects (mild PNP closure delay) in some mutants, while more severe cases exhibit failure of PNP closure and development of an open spina bifida defect (Fig. 4.2 D).

In heterozygotes, PNP length follows a closely similar linear negative correlation pattern ($r^2 = 0.86$) as in wild type (non-significant $p$-value of the slopes: 0.22; $p$-value of the intercepts: 0.75) and predicts closure of the PNP at 34-35 somites (Fig. 4.2 J). Despite this close similarity, a few heterozygotes have an open PNP beyond 35 somites (Fig. 4.2 J), consistent with a small proportion of these embryos developing a spinal neural tube defect, as in the mutants.

In addition to spinal neurulation, conditional targeting of Itgβ1 gene by Pax3-Cre has an impact on cranial neurulation. Closure of the hindbrain neuropore is normally completed at 17-19 somites by progression of zippering from closure 1 and closure 2 (Copp et al. 2000). While all wild type embryos achieve full closure of the cranial neural tube at this stage (0/52 embryos with open hindbrain), many mutant and heterozygous embryos instead display an open hindbrain neuropore (Fig. 4.2 Bii). The penetrance of the defect is 28% of cases (11/39 embryos) in mutants and 10% (3/30 embryos) in heterozygotes (Fig. 4.2 E) (Fisher’s exact test: $p < 0.0001$, mutants vs wild type; $p = 0.04$, heterozygotes vs wild type).

The size of the hindbrain neuropore was assessed by measuring the distance between the fusion points along the rostro-caudal axis (Fig. 4.2 B ii) and has an estimated average value of 511 µm, ranging from a minimum of 120 µm to a maximum of 900 µm (Fig. 4.2 F). The analysis also revealed that the open hindbrain neuropore does not appear to decrease in size over time and therefore eventually leads to failure of cranial neural tube closure and development of exencephaly at later stages (Fig. 4.3 D-E).
FIGURE 4.2

Recombination of Itgβ1 in the dorsal neuroepithelium by Pax3-Cre affects spinal and cranial neural tube closure.

Open hindbrain neuropore (~18 somite stage)

G, H, I, J

slopes: \( p = 0.01754 \)

Intercepts: ns \( p = 0.1527 \)

slopes: ns \( p = 0.6377 \)

Intercepts: ns \( p = 0.8678 \)

slopes: ns \( p = 0.7423 \)

Intercepts: ns \( p = 0.7484 \)
4.2.2 Exencephaly and open spina bifida with tail flexion defects in Pax3\textsuperscript{Cre/+}; Itg\textbeta1\textsuperscript{f/f} mutants

Embryos were analysed at E14.5 to assess whether the significant increase in PNP length and width, and the presence of the open hindbrain neuropore, observed at E9.5-10 eventually lead to normal or failed neural tube closure. Neural tube defects were observed both along the cranial and caudal axes of the embryos (Fig. 4.3), either alone or in combination, with an overall penetrance of the defects of 26% within the mutant population (Fig. 4.4 B) (p = 0.0015, Fisher’s exact test, mutants (Pax3\textsuperscript{Cre/+}; Itg\textbeta1\textsuperscript{f/f}) vs wild type (Pax3\textsuperscript{f/f}; Itg\textbeta1\textsuperscript{f/f} or f/+)). The most frequent phenotype is the presence of closed tail flexion defects (37%) (Fig. 4.4 D) which range from a single, sharp flexion of the caudal axis (Fig. 4.3 B) to a more tightly curled tail appearance (Fig. 4.2 C). This is usually caused by an overall delay in PNP closure (Copp 1985), and indeed linear regression analysis showed significant delay in PNP closure in the mutant embryos (Fig 4.2). The tail flexion defect was also associated with an open spina bifida phenotype in 25% of cases (Fig. 4.4 A and D). The region of open neural tube affected was mainly at the sacral level of the embryo (Fig. 4.3 C) but in some cases it extended to the lumbar level (Fig. 4.3 D). The fact that this region is located rather caudally along the axis suggests that spinal neurulation fails at late stages as confirmed by regression analysis of PNP closure (Fig. 4.2). Embryos also failed in cranial neural tube closure in 13% cases (Fig. 4.4 A and
D) developing exencephaly in the mid-hindbrain region either alone (Fig. 4.3 E) or in association with both spina bifida and tail flexion defects (Fig. 4.3 D).

FIGURE 4.3

[Image of mutant embryos with different genotypes showing cranial and caudal open neural tube defects.]
Two cases of open spina bifida were also observed in heterozygous embryos (Pax3\textsuperscript{Cre/+}; Itgb1\textsuperscript{fl/+}) with an overall penetrance of the defect of 9% (2 out of 23 embryos) (ns, Fisher’s exact test, heterozygotes vs wild types (Pax3\textsuperscript{Cre/+}; Itgb1\textsuperscript{fl/+} and Pax3\textsuperscript{Cre/+}; Pax3\textsuperscript{fl/+})) (Fig. 4.4 A and B). One of these showed a rare situation of open spina bifida alone, not associated to any tail flexion defect, but with normal straight tail. Despite not showing a significantly different frequency from wild type embryos (Fig. 4.4 A), the presence of NTDs in the heterozygous embryos raises the question of a potential effect of heterozygosity for Pax3\textsuperscript{Cre/+}, which could have caused the defects independently of the recombination of Itgb1 gene. Pax3 encodes a transcription factor already implicated in the pathogenesis of neural tube defects, with homozygous splotch mice developing both exencephaly and spina bifida (Greene et al. 2009). In the original work (Engleka et al. 2005), the Cre knock-in allele of Pax3 in heterozygosity was reported to have only a haploinsufficient melanocyte defect which produces the typical white belly spot phenotype of these mice. NTDs were not observed in heterozygous Pax3\textsuperscript{Cre/+} embryos (103 tot embryos analysed) in contrast to the homozygous Pax3\textsuperscript{Cre/Cre} embryos which always developed NTDs (43 embryos analysed) (Engleka et al. 2005), similarly to the splotch mutants. This argues against the idea that the occurrence of spina bifida in Pax3\textsuperscript{Cre/+}; Itgb1\textsuperscript{fl/+} embryos is due to an effect of the Cre knock-in allele of Pax3.

NTDs in double heterozygotes could also result from a hypothetical genetic interaction between haploinsufficiency for Pax3 and for Itgb1. A microarray study showed that integrin β1 binding proteins Itgb1bp2 and 3 are down-regulated in splotch mutant embryos (Pax3\textsuperscript{Sod/+Sod}) together with reduced expression of several integrin α subunits (Iltgα4, Iltgα7, Iltgβ6), suggesting a potential regulation of integrins by the transcription factor Pax3 (Rolfe et al. 2014). Further evidence comes also from a recent RNAseq study of Sp\textsuperscript{fl} mice raised under different folate dietary conditions, which showed mis-regulation of several integrin subunits (Iltgα4, Iltgαv, Itgb1bp1, Iltgα6, Iltgβ5, Iltgα3) (personal communication, unpublished data, Dr Miho Ishida). Hence, a Pax3-Itgb1 gene interaction cannot be ruled out in this study.
Figure 4.4. Analysis of NTD penetrance amongst Pax3<sup>Crem</sup>/Itgβ<sub>1</sub><sup>fl</sup> embryos at E14.5-15.5. 
(A) Summary table of total embryos analysed. 6 out of 23 mutant embryos developed a defect affecting either the spinal or cranial neural tube, or both (p = 0.0015, Fisher’s exact test, mutant (Pax3<sup>Crem</sup>; Itgβ<sub>1</sub><sup>fl</sup>) vs wild type (Pax3<sup>+/+</sup>; Itgβ<sub>1</sub><sup>fl</sup> and Pax3<sup>+/+</sup>; Itgβ<sub>1</sub><sup>/+</sup>)). 2 out of 23 heterozygotes developed a similar defect but this frequency does not differ significantly from either the mutant group (p = 0.24, Fisher’s exact test) or the wild type group where no defects were observed (0/40 embryos; Fisher’s exact test, p = 0.13, heterozygote (Pax3<sup>Crem</sup>; Itgβ<sub>1</sub><sup>/+</sup>) vs wild type (Pax3<sup>+/+</sup>; Itgβ<sub>1</sub><sup>fl</sup> and Pax3<sup>+/+</sup>; Itgβ<sub>1</sub><sup>/+</sup>)). (B-C) Overall penetrance of the defects within the mutants (26%) (B) and within the heterozygotes (9%) (C). (D) Venn diagram: overall penetrance of the three main types of defects in affected cases: tail flexion defects, spina bifida and exencephaly, either alone or in combination.
4.2.3 Genetic recombination and abolition of \textit{Itgβ1} expression in the dorsal neuroepithelium

Recombination of \textit{Itgβ1} gene by Pax3-Cre was assessed by exploiting the presence of a promoterless LacZ transgene that was inserted downstream of the 3’ LoxP site of the \textit{Itgβ1} gene (Potocnik et al. 2000) (Fig. 4.1 E). Once the genomic region of \textit{Itgβ1} gene between the two LoxP sites is recombined by the Cre, the endogenous \textit{Itgβ1} promoter drives transcription of the LacZ transgene and thus expression of the β-galactosidase enzyme. Activity of the enzyme was examined \textit{in situ} by X-gal staining to confirm recombination of \textit{Itgβ1} gene in the targeted tissue at E 9.5 (Fig. 4.5 A-G) and E 10.5 (Fig. 4.5 H-K).

\textit{Itgβ1} was successfully deleted in the dorsal region of the neuroepithelium along the entire body axis of the embryo, targeting both the cranial and caudal neural tube (Fig. 4.5 A-E). In the cranial region, deletion was detected in the dorsal tips of the neural folds in both the forebrain (Fig. 4.5 G-v) and midbrain (Fig. 4.5 G-iv). However, in the hindbrain not only the tips but a large proportion of the mid-dorsal neural folds seems to be affected by deletion of the gene (Fig. 4.5 G-iii). This could explain why this region is more sensitive to failure of neural tube closure as observed by the presence of the open hindbrain neuropore at E9.5 (Fig. 4.2 B-ii) which eventually results into development of exencephaly (Fig. 4.3 D-E).

In the caudal region, deletion of the gene extends from the recently closed neural tube to the open PNP. Two regions seem to be mainly targeted by removal of \textit{Itgβ1} gene: the zippering point and the most caudal region of constriction, the so-called ‘closure 5’. While relatively few scattered dorsal neuroepithelial cells are recombined in the flat and elevating neural folds (Fig. 4.5 E and 4.5 F i-iii), strong recombination occurs at the site of neural fold fusion (Fig. 4.5 F-iv and K-ii). This is the site where the majority of integrin subunits were significantly upregulated (Fig. 3.14) and therefore represents a key site of action for integrins during neurulation. Thus targeting the β1 subunit in this region may have disrupted fusion and progression of zippering necessary to complete closure, leading eventually to failure of spinal neurulation. However, even though the Pax3-Cre mediates recombination of \textit{Itgβ1} mainly in the dorsal neuroepithelium, a few dorsal surface ectoderm cells at the fusing tips of the neural folds also appear to be affected by the deletion (Fig. 4.5 F-v and 4.5 K-ii), as previously reported (Rolo et al. 2016).

Another region which seems to be highly affected by removal of the \textit{Itgβ1} gene is closure 5 (Fig. 4.5 E-ii arrow), a constricting region at the interface of surface ectoderm and
neuroepithelium located in the most caudal tip of the developing axis where neural folds fuse and appear to zipper in a rostrally directed manner (Sakai 1989). Therefore, it is possible that the observed phenotype in the spinal neural tube may arise from a combined action of both targeting the fusion point and closure 5 together, thus altering the physiological process of PNP zippering.

Furthermore, in the region of closed neural tube, recombination was also observed in other cell derivatives of the Pax3 expression domain including the neural Crest population and the dermomyotome (Fig. 4.5 G-i), as reported previously (Engleka et al. 2005).

To confirm if Pax3-Cre mediated excision of Itgβ1 gene results eventually in loss of protein in the targeted region, expression of Itgβ1 was analysed by immunofluorescence. Itgβ1 is normally highly expressed on the basal side of the neuroepithelium along its entire perimeter (Fig. 4.6 B-C). In addition to the neural tube, Itgβ1 is also expressed on the basal side of the surface ectoderm cells and within the mesenchymal tissue flanking the neural tube. Analysis of transverse sections at the level of closed neural tube confirmed the successful deletion of Itgβ1 from the dorsal domain of the neuroepithelium in mutant embryos (Fig. 4.6 E-F, arrows) compared to wild type (Fig. 4.6 B-C).

Fluorescence intensity was quantified by measuring the mean grey values along the entire basal perimeter of the neuroepithelium, expressed as a percentage (Fig. 4.6 G), and by subtracting the average background intensity calculated within the medio-apical area of the neural tube which is negative for Itgβ1 (Fig. 4.6 H). The analysis showed that while fluorescence intensity remains constant along the entire neuroepithelial perimeter in wild type embryos (Fig. 4.6 I), in the mutants the intensity drops significantly within a region of approximately 20 % of the total perimeter of the neuroepithelium located at the mid-dorsal pole of the neural tube between 40 % and 60 % of its entire perimeter length (Fig. 4.6 J).

Whole mount immunostaining analysis confirmed the overall disruption of Itgβ1 expression from the dorsal neuroepithelium in mutants (Fig. 4.6 D, arrows) compared to wild type (Fig. 4.6 A). However, Pax3-Cre mediated excision does not appear to supress Itgβ1 focal expression at the fusion point (Fig. 4.6 D vs. A, single arrow). This would suggest that the significant upregulation of integrin subunits observed in this region does not originate from the dorsal cells of the neuroepithelium.
Figure 4.5. *Pax3*-cre mediated recombination of *Itgβ1* gene in the dorsal neuroepithelium. (A-E) Whole mount X-gal staining at E9.5 of *Pax3*cre/+; *Itgβ1*fl/fl embryos. *Itgβ1* gene is deleted in the dorsal neural tube along the entire body axis of the embryo and in the open PNP (E). Regions of intense recombination are the hindbrain (C), the caudal zippering point (arrow, E-i) and the caudal tip of the closing neural tube, so-called ‘closure S’ (arrow, E-ii). (F-G) Frozen transverse sections of the caudal (F) and trunk/cranial region (G). Intense recombination is seen in the dorsal neuroepithelium of the open PNP (F ii-iii), at the zippering point (F-iv) and in the closed neural tube roof (F-v). *Pax3* mediates recombination not only in the dorsal neuroepithelium but also in few dorsal surface ectoderm cells at the fusion point (F-iv and v). (G) Recombination in the cranial dorsal neural tube: trunk (G i-ii), hindbrain (G-iii), midbrain (G-iv) and forebrain (G-v). (H-J) Whole mount X-gal staining of E10.5 *Pax3*cre/+; *Itgβ1*fl/fl embryos. (K) Frozen transverse sections through the caudal tip of the PNP (K-i), fusion point (K-ii) and trunk region (K-iii). Both neuroepithelial cells and a few dorsal surface ectoderm cells are targeted at the point of fusion (K ii).
Figure 4.6. Targeted deletion of Itgβ1 protein in the dorsal neuroepithelium. (A-F) Whole mount (A, D) and section (B, C, E, F) immunofluorescence in wild type (A-C) and mutant embryos (D-F) (embryos analysed: n=4). Itgβ1 protein is markedly down-regulated at the basal surface of the dorsal neuroepithelium in the closed spinal neural tube of mutant embryos (E-F, arrows) whereas significant expression is still observed at the site of fusion (D, arrow). In contrast, sections through a wild type embryo show expression of Itgβ1 at the basal surface of the entire neuroepithelium, as well as on the basal surface of the surface ectoderm and within the paraxial mesenchyme (A-C). (G-J) Quantification of Itgβ1 fluorescence intensity as measured along the basal perimeter of the entire neuroepithelium (G, yellow line) (0% ventral, 50% most dorsal domain, 100% ventral clockwise) and normalised against the background intensity recorded in the medio-apical neural tube (H, yellow area). Fluorescence intensity was quantified within 3 wild type embryos, as shown by the segmented lines in blue, light blue and violet (I), and within 3 mutant embryos as represented by the segmented lines in red, yellow and pink (J). Itgβ1 fluorescence intensity drops significantly along the dorsal perimeter of the neural tube (40-60%, asterisk) in mutant embryos (J) in contrast to wild type (I) where Itgβ1 intensity remains constant along the entire neuroepithelium perimeter.
4.2.4 Deletion of Itgβ1 in the surface ectoderm by Grh3-Cre significantly affects the length and width of the PNP

To assess the role of Itgβ1 expression in the surface ectoderm, a constitutive Cre line driven by the Grh3 promoter (Camerer et al. 2010) (gene symbol: Grh3<sup>tm1(Cre)Cgh</sup>, MGI: 4430902) was used to mediate recombination of the Itgβ1 gene (Grh3<sup>Cre</sup>+/; Itgβ1<sup>f/+</sup>) in the dorsal surface ectoderm (Fig 4.7 B). Grh3 is a transcription factor that is expressed in the surface ectoderm adjacent to the neuroepithelium from E8.5 (Ting et al. 2003). Later at E9-9.5, in addition to the surface ectoderm, Grh3 also starts to be expressed in the neuroepithelium at the level of open PNP. However, at E10-10.5 its expression in the neuroepithelium ceases and it becomes predominant in the hindgut epithelium (Gustavsson et al. 2007). Therefore, despite Grh3-Cre being used to target the surface ectoderm, especially at early stages of neurulation, it is important to bear in mind that it also mediates recombination in some neuroepithelial cells and in the hindgut at later stages of development.

As in the previous genetic cross, the distribution of the four genotypes did not differ significantly from the expected frequencies of mendelian genetics (Chi-square test, non-significant p-value = 0.5282, embryos analysed: n=146) (Fig. 4.7 C), thus excluding any early lethality.

Embryos were initially collected at E9.5 to assess key parameters such as the width and length of the PNP at different somite stages. At 22 somites, PNP size of Grh3<sup>Cre</sup>+/; Itgβ1<sup>f/+</sup> mutants starts to diverge from Grh3<sup>+/+</sup>; Itgβ1<sup>f/+;f/+</sup> wild type embryos (Fig. 4.7 F length, 4.7 H width). While both wild type and heterozygous embryos complete spinal neural tube closure, mutants instead display a significant larger and wider PNP at E10.5 (Fig. 4.7 D-E, arrows open PNP). Linear regression analysis shows that the PNP length decreases linearly as somites increase in wild type embryos ($r^2 = 0.79$, p-value < 0.0001) and heterozygotes ($r^2 = 0.69$, p-value < 0.0001) (Fig. 4.7 G) (p-value of the difference in slopes = 0.38). Completion of closure of the PNP is predicted to occur at 32-34 somites (x-axis intercepts), as reported for the majority of wild type strains (A. Copp et al. 2000). In contrast, mutant embryos diverge significantly from the linear decrease in PNP length observed in wild type (p-value of the slopes < 0.0001) and show a non-linear pattern ($r^2 = 0.01$, ns p-value = 0.65) which instead predicts increase of PNP length over time and failure of PNP closure (Fig. 4.7 F).

Analogous to PNP length, PNP width of wild type embryos ($r^2 = 0.60$, p-value < 0.0001) and heterozygotes ($r^2 = 0.67$, p-value < 0.0001) also shows a negative linear relationship with
somite number (non-significant p-value of the slopes = 0.75) which predicts completion of neural folds apposition at 33 somites (Fig. 4.7 l). In contrast, linear regression analysis of mutants shows no linear relationship between somite number and PNP width ($r^2 = 0.03$, p-value = 0.49), and predicts failure of the neural folds to achieve final apposition and therefore to complete PNP closure (Fig. 4.7 H).

Cranial neural tube closure was not affected in the Grhl3$^{Cre}$/Itgβ1$^f$ cross, in contrast to the Pax3$^{Cre}$/Itgβ1$^f$ cross. Only 1 out of 18 Grhl3$^{Cre/+}$; Itgβ1$^{f/f}$ mutants collected at E 9.5 exhibited an open hindbrain neuropore, with a length of 100 µm in contrast to the average length of 511 µm found in the Pax3$^{Cre/+}$; Itgβ1$^{f/f}$ embryos.

### 4.2.5 High penetrance of open spina bifida associated with tail flexion defect in Grhl3$^{Cre/+}$; Itgβ1$^{f/f}$ mutants

The significant increase in PNP length and width in Grhl3$^{Cre/+}$; Itgβ1$^{f/f}$ mutants resulted in either retardation or failure of PNP closure leading to a tail flexion defect or open spina bifida, respectively (Fig. 4.8). Analysis at E14.5-15.5 showed an overall 78% incidence of spinal NTDs in mutant embryos (Fig. 4.9 A-B) (p-value < 0.0001, Fisher’s exact test, mutants (Grhl3$^{Cre/+}$; Itgβ1$^{f/f}$) vs wild type (Grhl3$^{+/+}$; Itgβ1$^{f/f}$ or $^{f/+}$)). The most frequent phenotype was open spina bifida affecting the lumbo-sacral level of the body axis (55% of mutants), and always associated with a shortened or flexed tail (Fig. 4.8 B, 4.9 A). In 22% of cases, mutant embryos achieved closure of the spinal neural tube and exhibited a tail flexion defect alone (Fig. 4.8 C, 4.9 A). The remaining 22% of mutant embryos had no detectable spinal abnormality at all. No cases of exencephaly or other cranial NTDs were observed, consistent with the ability of these embryos to complete closure of the hindbrain neuropore by the 17-19 somite stage.

Only 1 out of a total of 16 Grhl3$^{Cre/+}$; Itgβ1$^{f/+}$ heterozygotes was affected by a mild tail flexion defect (Fig. 4.9 C) while all other heterozygotes appeared normal. Such a low penetrance of spinal defects in heterozygotes does not differ significantly from the normal development of all 38 wild type embryos in the study (p-value = 0.29, Fisher’s exact test, heterozygotes (Grhl3$^{Cre/+}$; Itgβ1$^{f/+}$) vs wild type (Grhl3$^{+/+}$; Itgβ1$^{f/f}$ or $^{f/+}$)).
Figure 4.7. Recombination of Itgβ1 by Grhl3-Cre affects PNP length and width. (A-B) Homozygous Itgβ1<sup>f/f</sup> mice were mated with the Cre line Grhl3<sup>Cre/+</sup>; Itgβ1<sup>f/+</sup> (A) to induce recombination of Itgβ1 mainly in the dorsal surface ectoderm (B). (C) The frequency of the four filial genotypes follows closely the expected mendelian ratio distribution (B). (D-E) The frequency of the four filial genotypes follows closely the expected mendelian ratio distribution (Chi-square test, ns p-value = 0.5282). Embryos analysed total: n=146. (D-E) At E10.5, homozygous mutant embryos display an open PNP (between arrowheads in E) in contrast to the closed spinal neural tube of wild type controls. (F-G) PNP length analysis. The negative linear regression pattern of PNP length against somite number in heterozygotes does not differ significantly from the wild type pattern (p-value of the slopes = 0.3803) (G). In contrast, PNP length in mutants does not follow a linear regression pattern (r<sup>2</sup> = 0.012, ns p-value =0.6579) and differs significantly from the wild type pattern (p-value of the slopes < 0.0001) (F). (H-I) PNP width analysis. While wild type and heterozygous embryos follow an almost identical linear pattern of PNP width decrease against somite number (p-value of the slopes = 0.7461) (I), mutants show no trend towards reduced PNP width with increasing stage, and differ significantly from wild type (p-value of the slopes < 0.0001) (H).
Figure 4.8. Phenotype analysis at E14.5-15.5 (A) In Grhl3<sup>+/+</sup>; Itgβ1<sup>f/f</sup> and Grhl3<sup>+/+</sup>; Itgβ1<sup>f/+</sup> wild type embryos the neural tube is fully closed along the entire body axis and the tail is straight, without any flexion defects. (B-C) Mutant embryos Grhl3<sup>cre/+</sup>; Itgβ1<sup>f/f</sup> are affected by two main types of defects: open spina bifida in the lumbo-sacral region associated with a tail flexion defect (flexed or truncated tail) (B), or by tail flexion defect alone with fully closed neural tube (C). (D-E) Analysis at E18 shows the appearance of the lesion of open spina bifida with tail flexion defect (E) in mutants compared to fully closed neural tube in wild type (D). (F-G) Mutant embryos fail to complete closure of the eyelid at E15.5 and are affected by an open eye phenotype (G, arrow) compared to the fully closed eyelids in wild type (F ii, arrow).
At E18, the open lumbo-sacral spina bifida lesion was evident and closely resembled the condition of myelomeningocele observed in humans (Fig. 4.8 E ii, arrow). In addition to the open spina bifida and tail truncation, the mutant fetuses displayed a defect of the forelimbs which appeared swollen and not fully extended (Fig. 4.8 D vs. E). Analysis at E15.5-16 revealed also that mutant embryos failed to complete eyelid closure (Fig. 4.8 F-G, arrows) (open eyelid: 2/2 mutants Grhl3<sup>Cre/+</sup>; Itgβ1<sup>f/f</sup>, 0/5 heterozygotes Grhl3<sup>Cre/+</sup>; Itgβ1<sup>f/+</sup> and 0/5 wild type Grhl3<sup>+/+</sup>; Itgβ1<sup>f/f</sup> or <sup>f/+</sup>). This recapitulates the phenotype described in a recent study where knock-down of integrin α5, which pairs functionally with integrin β1, was shown to cause an open eye defect, due to failure to extend the eyelid epithelium over the cornea (Heller et al. 2014).

In summary, these results provide evidence of the crucial role of integrin β1 for the morphogenesis and closure of the neural tube during embryo development. The significantly higher penetrance of spinal NTDs observed in Grhl3<sup>Cre/+</sup>; Itgβ1<sup>f/f</sup> (78%) compared with Pax3<sup>Cre/+</sup>; Itgβ1<sup>f/f</sup> mutant embryos (26%) suggests that the dorsal surface ectoderm represents the main site of action of integrins and that abolition of Itgβ1 function in this tissue is the leading cause of the spinal NTDs observed in these embryos.
FIGURE 4.9

A

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<td>57</td>
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B

Penetration of defects: Grhl3^{Cre/+}; Itgβ1^{fl/fl} (MUT)

C

Penetration of defects: Grhl3^{Cre/+}; Itgβ1^{fl/+} (HET)

D

Type of NTD frequency: MUT + HET

Figure 4.9. Analysis of NTD penetrance amongst Grhl3^{Cre/+}; Itgβ1^{fl/fl} embryos at E14.5. (A) Summary table: 14 out of 18 embryos develop NTDs (p < 0.0001, Fisher’s exact test, mutants (Grhl3^{Cre/+}; Itgβ1^{fl/fl}) vs wild type (Grhl3^{+/+}; Itgβ1^{fl/fl} or fl/+)). 10 out 14 cases display an open spina bifida associated with tail flexion defects while only 4 cases out of 14 develop a tail flexion defect alone with closed neural tube. No cranial defects were observed. 1 heterozygote (Grhl3^{Cre/+}; Itgβ1^{fl/+}) had a tail flexion defect; a frequency that does not differ significantly from the wild type situation where no defects were observed (0 cases out of 38 embryos) (p = 0.29, Fisher’s exact test, heterozygotes (Grhl3^{Cre/+}; Itgβ1^{fl/+}) vs wild type (Grhl3^{+/+}; Itgβ1^{fl/fl} or fl/+)). (B-C) Penetration of the defects within the mutants (78%) (B) and within the heterozygotes (6%) (C). (D) Overall penetration of the defects (15 embryos: mutants + heterozygote): higher penetration of spina bifida associated with tail flexion (67%) compared to tail flexion defect alone (33%).
4.2.6 Genetic recombination and abolition of integrin β1 protein in the dorsal surface ectoderm

The Grhl3-Cre was used to target recombination of the Itgβ1 gene to the dorsal surface ectoderm. However, while the expression of Grhl3 at E8.5 is mainly confined to the surface ectoderm (Ting et al. 2003), later at E9.5 it is also expressed in the neuroepithelium at the level of the open PNP (Gustavsson et al. 2007). Three approaches were employed to evaluate the specificity of the Cre and to assess the tissues targeted. First the expression of Grhl3-Cre was assessed at E9 and E9.5 by exploiting the presence of the endogenous LacZ cassette inserted downstream of the Cre gene within the Grhl3 locus (Camerer et al. 2010). The nuclear pattern of endogenous LacZ confirmed that Grhl3-Cre at E9 is exclusively expressed in the dorsal/medial surface ectoderm (Fig. 4.10 C), on the tips of the neural folds in the open PNP and over the midline region of closed neural tube (Fig. 4.10 B). However, at E9.5, in addition to the surface ectoderm, a few scattered neuroepithelial cells appeared expressed Grhl3-Cre, within the dorsal neural plate and neural tube (Fig. 4.10 E-F, arrows).

A second approach, to assess the efficiency and tissue specificity of the Cre-mediated excision was to cross the Grhl3<sup>Cre/+</sup> line with the Rosa26<sup>mTmG/mTmG</sup> reporter line (Muzumdar et al. 2007). After Cre-mediated recombination, the ubiquitously expressed membrane-targeted tdTomato (mT) is converted into membrane-targeted EGFP (mG) to allow visualisation of non-recombined and recombined tissues, respectively. This confirmed efficient recombination in the dorsal surface ectoderm at the 8 somite stage both at cranial (hindbrain) and spinal levels (Fig. 4.10 M).

Thirdly, expression of the LacZ transgene induced by excision of the Itgβ1 floxed locus (Potocnik, Brakebusch, and Fässler 2000) was analysed to assess the efficiency and tissue specificity of Itgβ1 recombination. In contrast to the nuclear staining of the endogenous transgene within the Grhl3-Cre locus, the LacZ induced by recombination of Itgβ1 produces a cytoplasmic pattern. Itgβ1 was confirmed to be efficiently recombined in the surface ectoderm overlying the neural tube both in the caudal region at the level of the open PNP (Fig. 4.10 I and L, i-iii) and in the hindbrain (Fig. 4.10 I and L, iv). Interestingly, the extent of surface ectoderm recombination with this assay appeared much more extensive than with the nuclear LacZ expressed from the Grhl3-Cre locus (compare G-L with A-F). Nevertheless, and despite predominant Itgβ1 deletion in the surface ectoderm, a few scattered cells in the caudal neuroepithelium were also recombined, as previously reported from our lab (Rolo et al. 2016).
The two sites which expressed LacZ most strongly, as evidence of removal of \( \text{Itg}\beta1 \) gene, were the caudal tip of the PNP (the ventral ectoderm ridge and the site of closure 5) (Fig. 4.10 H, inset, arrow) and the zippering point (Fig. 4.10 l-iii and L-iii). In this latter region, in addition to the surface ectoderm, a few cells of the dorsal neuroepithelium appeared to be affected (Fig. 4.10 l-iii and L-iii).

Finally immunofluorescence analysis confirmed successful removal of the \( \text{Itg}\beta1 \) protein from the surface ectoderm in \( \text{Grhl}3^{\text{Cre+}}; \text{Itg}\beta1^{+/f} \) mutant embryos (Fig 4.10 N-O). This appeared to be efficient in the dorsal surface ectoderm while some residual expression of \( \text{Itg}\beta1 \) was observed in more ventral regions (Fig 4.10 O, left side). Most importantly, the focal up-regulation of \( \text{Itg}\beta1 \) at the site of zippering was completely abolished in all \( \text{Grhl}3^{\text{Cre+}}; \text{Itg}\beta1^{+/f} \) mutant cases analysed (Fig 4.10 O, arrow). This contrasted with the \( \text{Pax}3\text{-Cre} \) mediated excision which did not affect \( \text{Itg}\beta1 \) expression in this region (Fig. 4.6 D). This strongly supports the idea that \( \text{Itg}\beta1 \) up-regulation at the site of zippering is mediated by cells of the surface ectoderm. Taken together with the significantly higher penetrance of NTDs in \( \text{Grhl}3^{\text{Cre+}}; \text{Itg}\beta1^{+/f} \) mutants (78%) compared to \( \text{Pax}3^{\text{Cre+}}; \text{Itg}\beta1^{+/f} \) mutants (26%), this suggests that deletion of \( \text{Itg}\beta1 \) expression at the site of zippering, by targeting the surface ectoderm, is likely to be the main cause of spinal NTDs in these embryos. However, the potential role of integrins in the dorsal neuroepithelium cannot be discounted. Because of the overlapping neuroepithelial regions targeted by both \( \text{Grhl}3\text{-Cre} \) and \( \text{Pax}3\text{-Cre} \), it is possible that recombination of \( \text{Itg}\beta1 \) in the dorsal neuroepithelium may also have contributed to failure of closure.

In the following sections of this Chapter, several more detailed analyses were performed in an attempt to understand the embryonic mechanism(s) by which \( \text{Pax}3\text{-Cre} \) and \( \text{Grhl}3\text{-Cre} \) mediated ablation of \( \text{Itg}\beta1 \) expression leads to failure of spinal neural tube closure.
**Figure 4.10**

*Grhl3-Cre LacZ reporter: Grhl3<sup>Cre/+</sup>; lOG1<sup>+/+</sup>*

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*ltGβ1 LacZ reporter: Grhl3<sup>Cre/+</sup>; lOGβ1<sup>+/+</sup>*

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4.2.7 Loss of Itgβ1 neither perturbs integrity of the actin cytoskeleton nor prevents actomyosin contractility

A primary function of integrins is to serve as a link between the external environment (particularly the ECM) and the internal cytoskeleton which allows cells to integrate signals from the exterior and to translate them into a cellular behaviour. The majority of integrins mediate cell adhesion via signalling to the actin cytoskeleton (Geiger et al. 2009), with the exception of integrin α6β4 which is primarily involved in anchorage via the intermediate filament system (Nievers et al. 1999). Integrins do not interact directly with the actin cytoskeleton but instead recruit a large set adaptors and signalling proteins, collectively named the integrin adhesome, which mediate the linkage (Zaidel-Bar et al. 2007b). After talin promotes the initial, relatively weak, anchoring between the F-actin filaments and the integrin short cytoplasmic tails in nascent adhesion focal complexes, further components of the adhesome are recruited and several signalling cascades are initiated which regulate assembly/turnover of the actin cytoskeleton. This either reinforces binding and promotes maturation to strong focal adhesions, or induces anchorage disassembly (Hynes 2002).

Because of the key role of integrins in the regulation of anchorage to actin (Brakebusch and Fässler 2003), the possibility that deletion of Itgβ1 may have affected the integrity of the actin cytoskeleton in the mutant embryos was assessed by whole mount phalloidin staining of the PNP region. Recent studies in our lab (Galea and Nikolopoulou, unpublished data) showed the presence of a defined actin cable which appears to localise precisely at dorsal interface between the surface ectoderm and the neuroepithelium, outlining the entire perimeter of the

Figure 4.10. Genetic recombination and abolition of integrin β1 protein in the dorsal surface ectoderm. (A-F) Grhl3-Cre expression detected by the endogenous LacZ cassette inserted within the Grhl3 locus (nuclear staining). At E9, the Cre is exclusively expressed in the dorsal surface ectoderm at the tips of the PNP neural folds and dorsal to the closed neural tube (A-C). At E9.5, a few dorsal neuroepithelial cells can be seen to express the Cre (F, arrows). (G-L) Genomic recombination of Itgβ1 detected by expression of LacZ transgene inserted within the Itgβ1 locus (cytoplasmic staining). Recombination has occurred extensively throughout the surface ectoderm as well as sporadically in a few cells within the neuroepithelium. Particular sites of recombination are the zippering point (I-ii, L-iii) and the caudal end of the PNP (H, insert). (M) Lineage tracing of the reporter Rosa26<sup>tm1Stz</sup> confirmed Grhl3-Cre mediated excision in the surface ectoderm of an 8 somite stage embryo, both in the caudal (bottom) and cranial (top) regions. (N-O) Successful removal of the Itgβ1 protein from the surface ectoderm and abolition of Itgβ1 upregulation at the site of zippering (arrows) in mutant (O; n=3) compared with wild type (N; n=3) embryos.
late-stage closing PNP (Fig. 4.11 A and C). This structure appears to originate at the site of zippering and to terminate at the very caudal tip of the PNP, thus mechanically coupling the fusing neural folds with the most caudal flat neuroepithelium. Deletion of Itgβ1 either in the dorsal neuroepithelium (Pax3Cre/+; Itgβ1f/f) (Fig. 4.11 B) or in the dorsal surface ectoderm (Grhl3Cre/+; Itgβ1f/f) (Fig. 4.11 E-i and F-i) did not appear to affect assembly of the actin cable, as detected by phalloidin staining, nor to disrupt its anchorage at the site of zippering. Alongside the actin cable, the cortical actin network at the apical surface of the neuroepithelium and in the surface ectoderm overlying the neural tube, did not appear to differ significantly in mutants compared with wild type controls (Fig. 4.11 D-i and F-i).

Integrin-mediated adhesion also plays a crucial role in the regulation of actin cytoskeleton contractility and formation of stress fibres via activation of the small GTP-binding protein RhoA (Ridley and Hall 1992; Ren et al. 1999). The core FAK-Src complex which lies at the heart of the adhesome promotes formation and disassembly of focal contacts by regulating RhoA activation which induces myosin light chain phosphorylation via ROCK to induce actomyosin contractility (Huveneers and Danen 2009; Mitra et al. 2005). Recently, RhoA and ROCK signalling were shown to be required for spinal neural tube closure by modulating actin turnover and actomyosin disassembly (Escuin et al. 2015).

To address the possibility that Itgβ1 may be required for PNP closure via its regulation of actomyosin contractility/turnover, the distribution of the activated non muscle myosin (pMLC-2: Phospho-Myosin Light Chain 2 (Ser19)) was assessed by whole mount immunofluorescence analysis. Itgβ1 removal in the surface ectoderm did not affect the distribution of activated myosin and its co-localisation with F-actin at the level of the cable (Fig. 4.11 C-F). Further quantification of the overall length of the actomyosin cable relative to PNP length also confirmed no difference between Grhl3Cre/+; Itgβ1f/f mutants and Grhl3+/+; Itgβ1f/f or f/+ wild types (cable/PNP length ratio: 0.87 wild type, n=2; 0.92 mutant, n=2). The possibility that Itgβ1 removal may had a non-cell autonomous effect on the neural tissue was further discounted by the finding of a normal distribution of actin and pMLC-2 in the apical neuroepithelium (Fig. 4.11 A-B, C and E). Therefore, despite the dramatic change in the overall architecture of the PNP between mutant and wild type embryos, failure of neural tube closure in these embryos does not appear to result from either perturbation of the actin cytoskeleton or from disruption of the activated non muscle myosin II.
Figure 4.11. F-actin cytoskeleton and activated myosin II distribution. (A-B) F-actin staining (Phalloidin) in Pax3<sup>+/+</sup>; Itgβ1<sup>ff</sup> or f/+ wild type and Pax3<sup>cre</sup>/<sup>cre</sup>; Itgβ1<sup>ff</sup> mutant embryos. Despite the presence of a larger PNP in mutants the actin cytoskeleton does not appear to be overtly perturbed. (C-F) Distribution of F-actin and phospho-myosin light chain II (p-MLC 2) in Grhl3<sup>+/+</sup>; Itgβ1<sup>ff</sup> or f/+ wild type (C-D) and Grhl3<sup>cre</sup>/<sup>cre</sup>; Itgβ1<sup>ff</sup> mutant (E-F) embryos. Actin and activated myosin co-localisation at the level of the cable (iii) does not appear perturbed by loss of Itgβ1.
4.2.8 Loss of Itgβ1 does not impair protrusive activity at the site of zippering

Alongside RhoA signalling, integrin-mediated adhesion controls protrusive activity at the site of focal contacts during cell locomotion by regulating the action of the small GTPases Rac1 and Cdc42 to induce the formation of lamellipodia and filopodia (Nobes and Hall 1995; Price et al. 1998; Clark et al. 1998). In fact, at the leading edge of motile cells, FAK-Src complexes induce suppression of actomyosin contractility by RhoA inhibition and concomitantly stimulate lamellipodia and filopodia formation by inducing Rac1 and Cdc42 respectively (Huveneers and Danen 2009). Intense protrusive activity with a mixture of filopodia and ruffles has been observed in mouse embryos at the site of neural fold fusion in the PNP, and was recently shown to be regulated by Rac1 and Cdc42 (Rolo et al. 2016). Suppression of Rac1 in the surface ectoderm abolishes ruffle-based protrusive activity and leads to failure of spinal neural tube closure at high penetrance.

To test whether focal upregulation of Itgβ1 is required for the regulation of protrusive activity at the site of zippering, scanning electron microscopy (SEM) was performed by Dr Ana Rolo on embryos with conditional ablation of Itgβ1 expression. Deletion of Itgβ1 either in the dorsal neuroepithelium (Pax3<sup>Cre<sup>/+</sup>; Itgβ1<sup>f/f</sup>) (Fig. 4.12 A) or in the dorsal surface ectoderm (Grhl3<sup>Cre<sup>/+</sup>; Itgβ1<sup>f/f</sup>) (Fig. 4.12 D) did not disrupt or alter protrusion activity as shown by SEM analysis of the site of zippering.

Quantification analysis confirmed the presence of protrusions in both wild type and mutant embryos and showed that, at the site of zippering, protrusions consist predominantly of a mixture of ruffles and filipodia in both genotypes (Fig 4.12 C and F) (p = 1.0 for both Grhl3; Itgβ1 and Pax3; Itgβ1). This is consistent with previous observations (Rolo et al. 2016) and argues against the hypothesis that spina bifida in these mutant embryos occurs as a results of integrins regulating cell protrusive activity at the zippering site.
Figure 4.12. SEM analysis of cellular protrusions at the site of zipping. (A-C) Presence of protrusions at the site of zipping in Pax3\textsuperscript{+/+}/Itgβ1\textsuperscript{f/f} wild type (n=4) and Pax3\textsuperscript{Cre/}/Itgβ1\textsuperscript{f/f} mutant embryos (n=4). Protrusions consist mainly of a mixture of ruffles and filopodia with no significant difference between mutant and wild type (C). (D-F) Presence of protrusions both in Grhl3\textsuperscript{Cre/}/Itgβ1\textsuperscript{f/f} wild type (n=4) and Grhl3\textsuperscript{Cre/}/Itgβ1\textsuperscript{f/f} mutant embryos (n=3), with predominance of ruffles mixed with filopodia in both genotypes (F). Acquisition of SEM images performed by Dr Ana Rolo.
4.2.9 Loss of Itgβ1 does not result in an abnormal distribution of dying cells during closure

Besides the ability to regulate the cytoskeleton, and therefore cell motility, integrin-mediated adhesion also plays a crucial role in control of cell survival. In fact, detachment of epithelial cells from the underlying ECM substrate was shown to lead to a type of programmed cell death named anoikis, from the Greek meaning homelessness (Frisch and Francis 1994; Meredith et al. 1993). Several pathways have been identified for the initiation of anoikis, with a central role played by MAPK signalling, which collectively lead to the activation of apoptosis due to loss of cell adhesion (reviewed in Chiarugi & Giannoni 2008). Cell death was reported to be intimately associated with mammalian neural tube closure, correlating precisely with neural fold bending and post fusion remodelling in the surface ectoderm and dorsal neural tube (Yamaguchi et al. 2011; Weil et al. 1997). However, apoptosis was shown to be dispensable for cranial and spinal neural tube closure (Massa et al. 2009).

To test the possibility that loss of cell-ECM adhesion due to removal of Itgβ1 could have altered the extent or pattern of cell death, for example by inducing the process of anoikis, the spatiotemporal distribution of apoptosis was assessed in wild type and mutant embryos by whole mount TUNEL staining. Cell death localised precisely at the dorsal midline of the closed neural tube and in the dorsal neural folds before closure (Fig. 4.13), consistent with the pattern observed previously (Massa et al. 2009). Significant cell death could also be visualised within the somites at later stages of development (E 10) and at the limb buds extremities (Fig. 4.13 F i and H i-iii). However, no differences in the overall level of apoptosis could be detected between Pax3Cre/+; Itgβ1f/f mutant (Fig. 4.13 C-D) and Pax3Cre/+; Itgβ1f/f wild type (Fig. 4.13 A-B) embryos, either at the level of the open PNP (Fig. 4.13 A-D iii) or in the cranial region (Fig. 4.13 A-D ii). This was despite the presence of an open hindbrain neuropore in the mutants. Similarly, the level of cell death appeared to be comparable between Grhl3Cre/+; Itgβ1f/f mutant (Fig. 4.13 G-H ii-iii) and Grhl3Cre/+; Itgβ1f/f wild type (Fig. 4.13 E-F ii-iii) embryos both in the region of closed neural tube and at the level of the open PNP, which was failing to close leading to spina bifida in the mutants (Fig. 4.13 H iii). In conclusion, removal of itgβ1 either from the dorsal neuroepithelium or surface ectoderm does not alter the pattern of cell death, and in particular does not appear to induce the process of anoikis as might have been expected. Therefore the spinal and cranial defects observed in these embryos do not appear to be related to altered levels or distribution of apoptosis.
Figure 4.13. Spatiotemporal distribution of apoptosis by whole mount TUNEL staining. TUNEL-positive cells are stained in blue. (A-B) Pax3+/+; Itgβ1f/f and (E-F) Grhl3+/+; Itgβ1f/f wild type embryos show a normal distribution of apoptotic cells in the dorsal midline of the closed neural tube region and in the dorsal neural folds of the PNP. Note the presence of particularly intense apoptosis at the level of the hindbrain. (C-D) Pax3cre/++; Itgβ1f/f and (G-H) Grhl3cre/++; Itgβ1f/f mutant embryos show comparable levels and distribution of apoptotic cell as in the wild type controls. Note the presence of open hindbrain neuropore (C-D ii), enlarged PNP (G ii-iii) and spina bifida (H iii) in mutant embryos. Embryos analysed: n=3 for each genotype group.
4.2.10 Integrin β1 removal does not impair fibronectin fibrillogenesis

Along the ECM-integrin-cytoskeleton axis, binding between the integrin receptor and the ECM substrate is essential to promote deposition and assembly of BM (Yurchenco 2011). In contrast to laminins and collagen type IV which are capable of self-polymerisation in vitro, fibronectin fibrillogenesis is instead dependent on integrin binding and it is a cell-mediated process (Schwarzbauer and DeSimone 2011). Fibronectin is initially secreted as a soluble dimer with a compact conformation which prevents fibril formation. Binding to the integrin receptor induces the necessary tension to change conformation and promote dimer formation which irreversibly converts the fibres into an insoluble network.

Deletion of integrin β1 subunit either from the surface ectoderm or from the dorsal neural tube could have hindered the process of fibronectin fibrillogenesis at their dorsal interface, perhaps then affecting zippering progression in a non-cell autonomous manner. The state of the fibronectin network and the morphology of the fibrils was therefore investigated by whole mount immunofluorescence in the region of closure. Despite the presence of an open enlarged PNP at 30 somites in Grhl3Cre+/; Itgβ1f/f mutant embryos, the fibronectin BM at the interface between the surface ectoderm and dorsal neural tube appeared to be fully assembled and morphologically normal (Fig. 4.14 A, D, I and L). A closer view of the region of neural tube closure showed that orientation of the fibrils follows a precise radial pattern perpendicular to the circumference of the PNP (Fig. 4.14 D and L). High resolution imaging and deconvolution in the region of zippering confirmed the presence of radially oriented fibronectin fibrils at this site both in wild type and mutant embryos (Fig. 4.14 G-H and O-P).

Fibronectin deposition, assembly and fibril orientation also appeared to be normal in Pax3Cre+/; Itgβ1f/f mutant embryos (Fig. 4.14 S-T) compared with wild type (Fig. 4.14 Q-R). Moreover, in the region of closed neural tube, the fibronectin network had further matured to give rise to large fibrils which run parallel to each other, medio-laterally, at the interface between the surface ectoderm and dorsal neural tube (Fig. 4.14 R and T).

In conclusion, removal of integrin β1 from either the surface ectoderm or from the dorsal neural tube does not affect fibronectin fibrillogenesis or orientation of fibrils.
Figure 4.14

**Fibronectin fibrillogenesis and E-cadherin expression analysis.**
Figure 4.14. Fibronectin fibrillogenesis and E-cadherin expression analysis. Fibronectin BM in GrhI3Cre+/+; Itgβ1f/f wild type (A, D, G, H) and GrhI3Cre+/+; Itgβ1f/f mutants (I, L, O, P). Fibris are oriented radially to the open PNP (D and L) as confirmed by visualisation of individual fibronectin fibrils at the site of zippering in wild type (G-H) and mutant embryos (O-P) by high resolution imaging and post-acquisition deconvolution. Analysis of the fibronectin network in Pax3Cre+/+; Itgβ1f/f wild type (Q-R) and Pax3Cre+/+; Itgβ1f/f mutant embryos (S-T) reveals the orientation of thick fibronectin fibrils mediolaterally over the dorsal surface of the closed neural tube (R and T). Itgβ1 expression shows focal upregulation at the zippering point and at the caudal end of the PNP (closure 5) (B and E) whereas Itgβ1 gene recombination abolishes expression at both sites in GrhI3Cre+/+; Itgβ1f/f mutants (J-M). E-cadherin expression in the surface ectoderm of mutant GrhI3Cre+/+;Itgβ1f/f embryos (K, N) shows no obvious abnormalities compared with wild type (C and F), despite the enlarged PNP in the mutants (L-N).

Cadherins and integrins regulate cell-cell and cell-ECM anchorage respectively by sharing a common cytoskeletal effector. The term “adhesive crosstalk” has started to emerge to highlight the deep communication between these two systems and intricate intersection between their downstream signalling (Weber et al. 2011). Cell-ECM adhesion has been shown to regulate stability of intercellular junctions which adapts their positioning to minimise the inter- and intracellular tensile forces (Tseng et al. 2012).

Deletion of integrin β1 in the dorsal surface ectoderm did not appear to perturb E-cadherin distribution which is comparable to the wild type pattern (Fig. 4.14 C, F, K and N). This suggests that the NTDs in GrhI3Cre+/+;Itgβ1f/f mutants is unlikely to originate from loss of surface ectoderm tissue integrity due to perturbation of E-cadherin mediated cell-cell adhesion.

Analysis of integrin β1 expression at late stages of neurulation (E10-10.5), when completion of PNP closure is imminent, surprisingly revealed the presence in wild type embryos of focal upregulation not only at the site of fusion but also at the caudal end of the PNP, where protrusive activity is also observed: i.e. at ‘closure 5’ (Fig. 4.14 B and E). Expression of the β1 subunit at both sites was abolished after surface ectoderm recombination by GrhI3-Cre, in association with a dramatically enlarged PNP (Fig 4.14 J and M). This points towards a role for integrins in enabling zippering from both ends of the open PNP at late stages of neurulation, consistent with the strong recombination mediated by GrhI3-Cre as demonstrated earlier (Fig. 4.10 H, I, L). At each end of the PNP, at these late stages, neural folds come into contact and zippering propagates until the two waves of closure meet to seal the entire open region.
4.2.11 Analysis of medio-lateral mechanical stresses at the zippering point

Despite the presence of apparently normal fibronectin fibrils at the zippering point, cells deficient of integrin β1 might be not able to adhere to their underlying substrate. As a consequence, lack of cell-ECM adhesion at the zippering point could perturb the strains and stresses that are intrinsic to the closing PNP, thus hindering progression of closure biomechanically. To quantify the strength of mechanical forces at the zippering point, a 300-500 µm long region along the dorsal midline from the fusion point was laser ablated in vivo by Dr Gabriel Galea (Fig. 4.15 A and B), and the magnitude of medio-lateral forces holding the folds together was inferred by measuring the instant widening of the PNP due to their lateral displacement (‘recoil’). Change in PNP width was measured on re-sliced cross sections (Fig. 4.15 D-F, yellow), rostro caudally from the zippering point through the PNP. Upon ablation, the region just distal to the closure point (i.e. in the most rostral PNP) in wild type embryos displays an average recoil of 71.71 µm (PNP width after ablation = 77.92 ± 8.99 µm minus PNP width before ablation = 6.21 ± 0.55 µm; see points nearest to the origin in Fig. 4.15 C). However, widening of the PNP occurs not only in the region close to the fusion point but affects the mediolateral separation of the neural folds up to a distance of 200 µm caudal to the zippering point (Fig. 4.15 C).

Instant recoil was therefore measured along the rostro-caudal axis from zippering point (0 µm) as far as 225 µm caudally into the open PNP at 20-24 somite stage, which corresponds to the onset of the spinal defects in the Grhl3^{cre}\textsubscript{+}\textsubscript{-}\ Itgβ1\textsubscript{f/f} mutants (Fig 4.15 G-I). Linear regression models for wild type and mutant embryos both differed significantly from the null hypothesis (horizontal regression line through the y mean values) (p < 0.0001), indicating an inverse relationship between PNP widening and axial distance. However, there was a relatively poor fit of the data to a linear x-y relationship owing to considerable variability in the measurements (wild type r\textsuperscript{2} = 0.2459 and mutant r\textsuperscript{2} = 0.3292) (Fig. 4.15 G-H). Nevertheless, the regression models of recoil in Grhl3\textsuperscript{cre/+}; Itgβ1\textsuperscript{f/f} mutant and Grhl3\textsuperscript{f/f}; Itgβ1\textsuperscript{f/f} wild type embryos were extremely similar, as shown by the almost complete overlap between the two regression models (comparison of slopes: p = 0.9904) (Fig. 4.15 I). Instant recoils calculated at the site of closure (y-intercepts) are also very similar between the two groups with an estimated average value of 94.72 µm ± 4.996 (SEM) in Grhl3\textsuperscript{f/f}; Itgβ1\textsuperscript{f/f} wild type and 89.98 µm ± 5.193 in Grhl3\textsuperscript{cre/+}; Itgβ1\textsuperscript{f/f} mutants. These data argue that similar medio-lateral forces are holding the neural folds together in embryos deficient for integrin β1 and in normal controls. This argues against the hypothesis that lack of dorsal adhesion at the zippering point affects apposition or
medio-lateral convergence of the neural folds. However, this does not exclude the possibility that failure of closure may derive instead from an imbalance of forces oriented rostro-caudally, as opposed to medio-laterally, as the former could not be measured in this study.

**FIGURE 4.15**

**BEFORE ABLATION | AFTER ABLATION**

(A-B) A 300-500 µm long region along the dorsal midline from the fusion point (i.e. from the red dot towards the rostral end in A) was laser ablated in live embryos. Ablation caused an instantaneous lateral displacement (‘recoil’) of the neural folds (B), indicating the existence of tension at the zipper point that pulls the neural folds towards the midline. (C-E) PNP width (B and D-E, yellow lines) was measured before and after the ablation along a distance of 225 µm caudally from the fusion point along the PNP (B, white line). Measurements were performed on optically re-sliced cross sections before (D) and after ablation (E). (F) Superimposed cross sections showing the extent of lateral displacement of the neural folds upon ablation. (G-H) Linear regression analyses of the instantaneous recoil (PNP width ‘after’ minus ‘before’ ablation) in wild type (G) (n = 10) and mutant embryos (H) (n = 7) along the rostro-caudal axis from the fusion point, which is at x = 0. (I) Comparison analysis shows close overlap between wild type and mutant regression models with no significant differences between them (comparison of the slopes (p = 0.9904) and intercepts (p = 0.2029)). Mean values ± SEM in C and I. Work performed in collaboration with Dr Gabriel Galea.
4.3 Discussion

The study of the *in vivo* role of cell-ECM adhesion during neural tube morphogenesis has been hampered by the complex heterogeneity of receptors and ECM components that are expressed at this stage, and by the early lethality of some ECM or integrin gene knock-outs where embryos arrest before initiation of neurulation, preventing any analysis beyond this stage (George et al. 1993; Fassler & Meyer 1995; Stephens et al. 1995).

In the present study, conditional deletion of the integrin β1 subunit in the region of the dorsal neural folds revealed an essential requirement for this receptor for propagation of zippering and thus for neural tube closure. Genetic ablation of this subunit precludes its pairing with the corresponding alpha subunits and thus prevents *in vivo* assembly of the major integrins expressed as this stage, both the fibronectin-interacting receptors (integrin α5β1 and αvβ1) and the laminin-interacting receptors (integrin α6β1 and α3β1). The significant higher penetrance of NTDs achieved by recombination of integrin β1 in the surface ectoderm (Grhl3-Cre) as opposed to targeting the dorsal neuroepithelium (Pax3-Cre) demonstrates the key role of this receptor in the surface ectoderm for spinal neural tube closure. This is further supported by the abolition of the focal upregulation of integrin β1 at the rostro-caudal zippering point and at the extreme caudal end of the PNP, where ‘closure 5’ zippering appears to occur, by targeting the surface ectoderm in contrast to the dorsal neural tube. However, while integrin β1 in the surface ectoderm is essential for spinal neurulation, it appears instead to be dispensable for cranial neural tube closure. In fact, progression of cranial neurulation requires expression of integrin β1 in the dorsal neural tube as shown by presence of open hindbrain neuropore in some *Pax3<sup>Cms</sup>/; Itgβ1<sup>f/f</sup> mutant embryos which eventually leads to failure of cranial closure and development of extensive exencephaly encompassing the hind- and midbrain region. This highlights the different aetiology of cranial and spinal NTDs with a different requirement for integrins either in the dorsal neuroepithelium or in the surface ectoderm, respectively.

The localised pattern of up-regulation of integrins strongly directs the attention both to the fusion point and to ‘closure 5’ as the key sites of action of these receptors during neural tube closure. Integrins are primarily involved in the establishment of a structural linkage anchoring the internal cytoskeleton of the cell to the ECM and to transduce signals inside the cells which regulate almost every aspect of cell behaviour including motility, proliferation, survival and differentiation (Miranti and Brugge 2002a; Harburger and Calderwood 2009; Hynes 2002).
Regulation of actomyosin disassembly and actin turnover by RhoA was shown to be critical for spinal neural tube closure in mouse (Escuin et al. 2015; Grego-Bessa et al. 2015). Moreover, a supra-cellular actomyosin cable which lines the entire open PNP was recently identified (Nikolopoulou and Galea, unpublished data) and shown to originate at the site of zippering. However, removal of integrin β1 does not affect either the integrity of the cable or to perturb its myosin-mediated contractility (as judged by pMLC localisation) arguing against the hypothesis that integrin may be primarily required for regulation of the cellular cytoskeleton in this region. Recent evidence also demonstrated the key requirement of cellular protrusions, in particular ruffles, at the site of zippering for completion of spinal neural tube closure (Rolo et al. 2016). However, deletion of integrin β1 does not suppress protrusion activity arguing against the possibility of integrins regulating the formation of ruffles (via Rac1) or filopodia (via Cdc42) at the site of zippering.

Consistent with the overall integrity of the actin cytoskeleton, the degree of medio-lateral neural fold recoil, quantified by the response to laser ablation, was almost identical between Grhl3+/-; Itgβ1f/f wild type and Grhl3Cre/+; Itgβ1f/f mutant embryos. This is in contrast to the significant higher recoil observed in mutant Zic2Ku/Ku embryos than their wild type littermates, which suggested significant biomechanical abnormalities in the neuroepithelium that opposes closure in these mutants, correlating with their subsequent development of severe spina bifida (Galea, unpublished data). However, ablation at the zippering point limited the analysis to quantification of changes in PNP width as a readout of stresses along the medio-lateral axis. Therefore, it cannot be excluded that perturbation of integrin β1 at the zippering point may instead have an effect on mechanical forces oriented along the rostro-caudal axis that might affect PNP closure. Moreover, while the genetic defect in Grhl3-Cre embryos is mainly localised to the surface ectoderm, the displacement quantified by laser ablation may largely reflect recoil of the neuroepithelium. Hence, this approach does not allow assessment of changes in mechanical forces originating from the surface ectoderm alone. Therefore, it will be necessary to design a surface ectoderm specific ablation to be able to distinguish between the forces generated by the neuroepithelium or by the overlying surface ectoderm.

Integrins are able to regulate cell survival by activating the apoptotic pathway upon loss of adhesion to the ECM (anoikis) (reviewed in Chiarugi & Giannoni 2008). However, removing integrin β1 in the dorsal neural folds did not appear to induce anoikis, as no additional TUNEL-positive cells were present in mutants compared with wild type. Integrins are also key modulators of cell cycle progression via activation of the Ras/Raf/Mek/Erk signalling pathway (Hynes 2002; Miranti and Brugge 2002b) and regulators of orientation of the plane of cleavage.
by defining the spindle position (Streuli 2009). It will therefore be important in future to investigate the potential effect of integrin β1 removal on the frequency and orientation of cell division among the cells of the surface ectoderm which are normally aligned preferentially along the medio-lateral and rostro-caudal axes (Galea, unpublished data).

Deletion of the integrin β1 subunit does not prevent fibronectin fibrillogenesis suggesting that the defect does not arise from perturbation of BM structure. However, loss of cell adhesion to a structurally normal fibronectin network is a possible consequence of integrin ablation, and this might be particularly marked at sites of zippering. Adhesion to fibronectin at these sites could serve as primary anchorage to couple mechanically the dorsal neuroepithelium to the overlying surface ectoderm. Fibronectin was shown to regulate tissue mechanics and inter-tissue adhesion during vertebrate trunk elongation in zebrafish (Dray et al. 2013). In fact, both the dorsal cells of the neuroepithelium and dorsal cells of the surface ectoderm adhere basally to a common fibronectin-rich network interposed at their interface. This could act to mechanically couple the two epithelia at the site of neural fold fusion before continuity between cells on the opposing neural folds become established. This mechanism is pursued further in the General Discussion chapter (Fig. 6.1).
5. Cell dynamics of the neuroepithelium

5.1 Introduction

Focal bending of the neuroepithelium is an essential morphogenetic event of spinal neurulation. Initially at E8.5, the flat neuroepithelium bends at the midline (MHP, median hinge point) enabling the neural folds to elevate. Later at E9.5, as neurulation progresses further caudally, the elevating neural folds undergo additional bending dorsolaterally (DLHPs, dorsolateral hinge points) enabling neural fold apposition at the dorsal midline. While midline bending in mouse and chick appears to be dispensable, as shown by successful closure of the neural tube when MHP is inhibited either genetically (Ang and Rossant 1994; Chiang et al. 1996) or surgically (Smith & Schoenwolf 1989; Ybot-Gonzalez et al. 2002), dorsolateral bending is instead essential and its suppression severely affects spinal neural tube closure. The critical requirement of dorsolateral bending is in fact demonstrated by the Zic2-Kumba loss of function mutant where the elevated neural folds lack dorsolateral bending leading to failure of neural tube closure and development of an extensive spina bifida (Ybot-Gonzalez, Gaston-Massuet, et al. 2007).

However, little is known about the cellular mechanisms underlying DLHP formation in mouse embryos. Early in 1947, Lewis proposed the first physical model of epithelial bending based on local increase in apical contractile tension which could drive invagination of the epithelium and therefore bending (Lewis 1947). Later the observation that actin microfilaments accumulate at the apical surfaces of the neuroepithelial cells together with non-muscle myosin II led to the idea that acto-myosin contractility could be the motor which drives bending of the neuroepithelium by inducing apical constriction. While actomyosin contractility seems to be essential for neural tube closure in lower vertebrates such as chick (Kinoshita et al. 2008) and Xenopus (Rolo et al. 2009), it does not seem to be required for spinal neurulation in mouse. Disassembly of F-actin by cytochalasin D and also inhibition of actomyosin contractility by blebbistatin lead to failure of mouse neural tube closure only at the cranial level without halting spinal closure or affecting DLHP formation (Austin et al. 1982; Wiley 1980; Escuin et al. 2015; Ybot-Gonzalez and Copp 1999).

Cells shape changes and nuclear localisation has been proposed as alternative potential mechanism to explain focal bending. Each non-mitotic cell of the neural plate extends to both basal and apical surfaces of the pseudostratified neuroepithelium and undergoes interkinetic nuclear migration along the apico-basal axis as the cell cycle progresses, causing cells to
change their shape depending on nuclear position. In fact both in chick (Schoenwolf and Franks 1984) and mouse (McShane et al. 2015), the MHP appears to be highly enriched in wedge shaped cells with basally localised nuclei as a consequence of a prolonged cell cycle. Nuclear localisation is predicted to cause basal expansion with consequent adoption of a wedge shape by the cells in the MHP, causing focal bending.

In contrast to the MHP, analysis of dorsolateral bending showed that these sites display asynchronous cell cycle progression and, similarly to non-bending regions, contain a mixture of cells of different shapes: wedge (basal nucleus), spindle (intermediate nucleus) and inverted wedge (apical nucleus), arguing for a different mechanism of bending in the DLHPs from that seen in the MHP. Interestingly, the transition from midline to dorsolateral bending is characterised by a significant increase in cell number in the dorsal neural plate, where it is in contact with the surface ectoderm, whereas the ventral neural plate that contacts the paraxial mesoderm increases in cell number much less. Concomitantlally, the dorsal region appears to be affected by a decrease in cell width and an increase in cell density, supporting the idea that cells become increasingly crowded dorsally as the neuroepithelium elevates and bends dorsolaterally. Analysis of the cell cycle confirmed that dorsal neuroepithelium proliferates more rapidly than the ventral region and this could potentially explain the increase in cell number observed dorsally. However, there were more cells in the dorsal neural plate than the expected number calculated on cell cycle analysis and on the contrary there were fewer cells than expected in the ventral neural plate (McShane et al. 2015).

This discrepancy led to the hypothesis that cells from the ventral neuroepithelium may relocate dorsally during DLHP formation, contributing to the large increase in cell number observed in this region. To test this hypothesis, vital cell labelling was performed in embryo culture to trace the fate of ventrally labelled neuroepithelial cells over the time course of neural fold elevation and dorsolateral bending. The analysis showed that after a time period of 18 h, cells from the ventral cell population relocate into the most dorsal region of the neuroepithelium supporting a model of dorsally directed translocation. Further analysis of the relationship between the ventral and dorsal neuroepithelial cells showed that cells from the ventral neuroepithelium actually reach and intermingle with the dorsally localised cell population, therefore contributing to the significant increase in cell number observed in this region. Finally, live imaging of the developing neuroepithelium at the level of the PNP was attempted and provided initial insights into the migratory trajectories and behaviour of the cells during morphogenesis of the neuroepithelium. Part of the results presented in this chapter were published in McShane et al (2015) with myself as joint first author.
5.2 Results

5.2.1 Cells translocate from ventral to dorsal within the plane of the neuroepithelium

To investigate the possibility of a dorsally directed movement in the neural plate, cells of the mid-ventral neuroepithelium were labelled by injection of the carbocyanine DiI and traced over 18 h of whole embryo culture. DiI is a lipophilic red dye which displays strong fluorescence and high photostability when incorporated into the cell membrane. Most importantly, due to the low toxicity at elevated concentrations, it allows long-term visualisation of living cells. Embryos were initially recovered at late E8.5, within a somite range of 7-9 somites, as the neural folds are bending locally at the MHP but have not yet developed focal DLHPs. The injection was performed half-way along the rostro-caudal axis of the open PNP (Fig. 5.1 D-E) by trans-neural fold injection of the dye. Despite the focal application, this method is not suitable to precisely label single cells but only to target group of cells in a specified area. Nevertheless, the dye remains confined to the targeted cells and is not transferred between adjacent cell membranes. This method, in fact, labelled the entire thickness of the mid-ventral neuroepithelium (Fig. 5.1 B-C), in a region that was on average between 26% (ventral border) and 47% (dorsal border) of the entire ventro-dorsal distance along the neuroepithelium (Fig. 5.2 C, time 0). In addition to the neuroepithelium, a defined track of DiI was also visible in the flanking mesoderm (Fig. 5.1 C).

Precise quantification of cells along the neuroepithelium ventro-dorsal axis can only be performed after fixation and histological processing of the samples, therefore limiting the possibility to directly assess the localisation of cells at the initial time of labelling (time 0) and the end of the culture (time 18 h) within the same embryo. Therefore, to be able to estimate the above parameters, upon injection half of the labelled embryos were blindly assigned to the time 0 group and thus immediately fixed and processed to confirm the ventral localisation of the initial site of injection. The remaining half of the embryos were instead cultured over a period of 18 h to track the fate of the ventrally labelled cells over the period of neural tube closure (Fig 5.2 A-C).
Strikingly, at the end of the 18 h culture period, the ventrally labelled cell population appeared to have translocated as a whole into the dorsal neuroepithelium. The presence of dorsal Dil positive cells was confirmed at the level of both the recently closed (Fig. 5.1 J-K) and the open neural tube (Fig. 5.1 L-M). To quantify the degree of dorsal translocation, the ventral and dorsal borders of each Dil positive cell cluster was calculated as a percentage of the total ventro-dorsal distance along the neuroepithelium (Fig. 5.2 A) at time=0 compared to time=18 (Fig. 5.2 B). Both the ventral and dorsal borders of the Dil positive cells showed a significant shift from the initial site of injection in the ventral neural folds at time= 0 (26% and 47% respectively) towards the most dorsal region of the neuroepithelium at time=18 (62% and 97% respectively) (Fig. 5.2 C) (Mann-Whitney test: ventral borders t = 0 vs t = 18, p = 0.0028; dorsal borders t = 0 vs t = 18 p = <0.0001). This supports the idea that the ventral neuroepithelium at the level of the open PNP acts to supply cells to the dorsal neural plate and therefore contributes to the significant increase in cell number observed in this region during DLHP formation. This finding also provides an explanation for the discrepancy between the observed and predicted cell number (based on cell cycle length) along the ventro-dorsal axis of the neuroepithelium. That is, more cells accumulate in the dorsal neuroepithelium than expected, as this population is generated not only by mitotic division but also by relocation of ventral neuroepithelial cells to the dorsal region.
Figure 5.1

Fluorescent cell labelling reveals a ventral to dorsal translocation of neuroepithelial cells during dorsolateral bending.
Interestingly, although the paraxial mesoderm and neuroepithelium were initially labelled at precisely the same axial level, labelling in the two tissues was no longer in frame along the rostro-caudal axis at the end of 18 h culture. While mesodermal Dil-labelled cells were found rostrally within the somite near the initial site of injection (Fig. 5.1 I), the neuroepithelial Dil-labelled cells appeared to have relocated more caudally compared to the mesodermal track (Fig. 5 G-H). Dil-labelled cells were visible both at the level of the open and closed neural tube (Figure supplementary 5.2, video, 3D reconstruction at time 18 h). This would suggest a more complex pattern of movement of the cells within the neuroepithelium than in the mesoderm. Neuroepithelial cells appear to migrate not only along the ventro-dorsal axis but also along the rostro-caudal axis of the embryo.

Figure 5.1. Fluorescent cell labelling reveals a ventral to dorsal translocation of neuroepithelial cells during dorsolateral bending. (A) Schematic representation of the embryo at the time of injection (8 somites). (B) Injection at the site of the open PNP by insertion of a fine surgical glass micropipette and then release of Dil as the pipette is withdrawn, in order to label the entire thickness of the mid-ventral neural fold. (C) Dil-labelling at time=0 with visible track in the mid-ventral neuroepithelium and adjacent mesoderm. Dil in red, anti-fibronectin immunohistochemistry in green. The focal Dil injection was performed by Prof A. J. Copp. Lateral (D) and dorsal views (E) of the embryo with focal site of injection in red. (F) Appearance of an embryo that was injected while preserving the yolk sac and amniotic membrane for long term culture. (G-H) Embryo at the end of 18 h culture period with enlargement of the region positive for Dil (H). (I-M) Serial sections through a labelled embryo after 18 h culture, with enlargements to show the labelled cells (ii and iii). Mesodermal Dil-labelled cells are visible rostrally within the somite, marking the original injection site (I) whereas Dil-labelled neuroepithelial cells are located more dorsally and more caudally at the levels of the closed (J-K), closing (L) and open PNP (M). No of embryos analysed: 38.
Figure 5.2. Quantification of cell displacement along the ventro-dorsal axis of the neuroepithelium. (A-B) The ventral (blue) and dorsal (red) borders containing the Dil-positive cells were calculated as a percentage of the total ventro-dorsal distance along the neuroepithelium (yellow): at time 0 h (A) (group of embryos fixed upon labelling) and at time 18 h (B) (group of embryos cultured for 18 hours). (C) Statistical analysis comparing the ventral border of the embryos at time 0 vs the embryos at time 18 h, and the dorsal border at time 0 vs time 18 shows significant dorsal shift of the borders along the neuroepithelium axis, with the dorsal border reaching the most dorsal extremity of the neural folds. Statistical test: non-parametric Mann-Whitney test. Mean values ± SEM: comparison of the ventral borders t = 0 V = 26.21 ± 7.43 vs. t = 18 V = 62.37 ± 3.97 (** p = 0.0028). Comparison of the dorsal borders t = 0 D = 47.39 ± 7.49 vs. t = 18 D = 97.36 ± 1.65 (**** p < 0.0001). 9 embryos analysed for each time point.
5.2.2 Dil ventral neuroepithelial cells reach the DiO dorsal cells

To further investigate the relationship between the ventral and dorsal cells, the neuroepithelium was co-labelled simultaneously with two carbocyanine dyes: Dil (red) in the mid-ventral region (Ventral (V) - Dorsal border (D) = 19-46%) and DiO (green) in the most dorsal neural folds (V-D = 74-93%), at the same rostro-caudal axial level (Fig. 5.3 A-D). After 20 h culture, no change was observed for the DiO-positive cells which retained a dorsal position while the Dil-positive ventral cells translocated dorsally reaching the DiO population (Fig. 5.3 E-F). However, a more detailed analysis of the pattern of displacement along the rostro-caudal axis of the embryo revealed that in the most caudal regions, where the neural tube was open, the Dil-positive cells maintained a significant ventral localisation (Fig. 5.3 H-I). In contrast, in the rostral closed neural tube the Dil-positive cells had reached the most dorsal region of the neural tube and intermingled with the population of dorsal DiO-positive cells (Fig. 5.3 J-L) (Fig. 5.2 supplementary video).

A similar quantification of the degree of translocation along the ventro-dorsal axis was performed for both the Dil and DiO-labelled cells (Fig. 5.4). However, in this case, the analysis was performed separately at different axial levels: from the very rostral level where the neural tube is already closed to the most caudal region where the neuroepithelium is still flat (Fig. 5.3 G). No significant difference was observed for both the ventral and dorsal borders containing the DiO-positive cells between time 0 and time 20 (Fig 5.4 A, green). These cells were initially labelled in the dorsal neuroepithelium (V-D = 74-93%) and maintained a dorsal localisation after 20 h culture along the entire rostro-caudal axis of the embryo (V-D = 66-100% at very rostral, 69-100% at rostral, 68-99% at fusion, 62-100% at elevated, 57-92% at caudal) (Kruskal-Wallis test, dorsal and ventral borders DiO, t = 0 vs t = 20: non-significant at very rostral, rostral, fusion, elevated and caudal level). In contrast, the Dil-positive cells, which were initially labelled in the ventral region (V-D = 19-46%) of the neuroepithelium, after 20 h showed a significantly more dorsal position at the most rostral (38-89%) and rostral levels (28-83%) of the closed neural tube (Fig. 5.4 A) (Kruskal-Wallis test, dorsal borders Dil, t = 0 vs t = 20: *** p = 0.0009 at very rostral, ** p = 0.0019 at rostral). Conversely, in the caudal axial levels, the Dil-positive cells retained a ventral position (D = 71% at fusion, 56% at elevated, 49% at caudal) which did not significantly differ from the initial ventral localisation at the initial time of injection (D = 46%) (Fig. 5.4 A) (Kruskal-Wallis test, dorsal borders Dil, t = 0 vs t = 20: ns. at fusion level, ns. at elevated, ns. at caudal).
Figure 5.3. Double labelling of the neuroepithelium. (A & C) Schematic representation of the two sites of labelling (A) and cross section of the PNP at time 0 (C): Dil (red) was injected in the mid-ventral region, while DiO (green) was injected in the dorsal neuroepithelium. (B & D) Ventral view of whole embryo showing the two focal sites of labelling in the ventral (Dil) and dorsal neuroepithelium (DiO). Stage of injection at 6 somites due to the flat appearance of the PNP which facilitates targeting of the dorsal vs. ventral neuroepithelium. (E-G) Distribution of cells labelled with the two dyes at the end of 20 h culture period. Note that Dil-labelled cells (red) have reached the region occupied by dorsal DiO-labelled cells (green). This dorsal displacement occurs both along the ventro-dorsal and rostro-caudal axes as shown by the rostro-caudal spreading of the two dyes and by the rostral localisation of the mesoderm track (star in G), indicative of the initial site of labelling. (H-L) Analysis at different levels along the rostro-caudal axis. Dil translocates and merges dorsally with DiO in the closed neural tube (very rostral, rostral and fusion levels). Dil remains ventral and separate from the dorsal DiO in the open PNP (elevated and caudal levels).
To further quantify the proximity and the degree of overlap between the Dil- and DiO-positive cell populations at each axial level, the ventral border of Dil was compared against the ventral border of DiO, while the dorsal borders of Dil and DiO were compared (Fig. 5.4 B). The analysis showed that the ventral borders of the two dyes remained fully separated from each other along the entire rostro-caudal axis (two-way ANOVA, ventral border Dil vs. DiO: *** p=0.0005 at very rostral, **** p < 0.0001 at rostral, elevated, fusion, caudal). In contrast, the lack of statistical significance between the dorsal border of Dil and the dorsal border of DiO at the level of closed neural tube (Fig. 5.4 B, very rostral) demonstrated that the Dil-positive cells came close and intermingled with the DiO-positive cells dorsally (two-way ANOVA, dorsal border Dil vs. DiO: ns. very rostral), supporting the model of cell crowding in the dorsal neuroepithelium (Fig. 5.4 B, region of overlap: dark grid). However, at more caudal levels, the two populations remain significantly separated from each other with ventral located Dil-cells and dorsal located DiO-cells (Fig. 5.4 B) (two-way ANOVA, dorsal border Dil vs. DiO: * p = 0.02 at rostral, *** p = 0.0001 at fusion, **** p < 0.0001 both at elevated and caudal), reminiscent of the initial time of injection (two-way ANOVA, dorsal border Dil vs. DiO, time 0: **** p < 0.0001)

These results show that cells do not translocate uniformly towards the dorsal neuroepithelium but instead acquire different ventro-dorsal positions at different levels along the rostro-caudal axis. While the mesodermal track remains confined focally at the axial site of the initial injection, the neuroepithelial labelled cells instead populate the entire length of the embryonic axis, from the region of closed neural tube to the most caudal flat neural plate. This suggests a relatively complex pattern of distribution of neuroepithelial cells and their progeny during neural tube morphogenesis. The apparent discrepancy of dorsal Dil-labelled cells rostrally and ventral Dil-labelled cells caudally, reminiscent of the initial situation at time 0, would suggest two potential models of cell and tissue dynamics (Fig. 5.5). The first predicts that cells from the initial site of labelling move dorsally and caudally, as the body axis elongates (Fig. 5.5 B). However, this would not explain the presence of labelled ventral cells in more caudal regions. The second model would predict instead that the initial site of labelling is retained at the most caudal region and thus may act as source of cells for the developing axis which are initially supplied ventrally to ultimately relocate dorsally, as the neural folds elevate and bend (Fig. 5.5 C). This latter model seems most likely to be correct as it not only explains the distribution of labelled neuroepithelial cells along the body axis, but also can explain the finding of ventrally located Dil-positive cells at caudal levels and dorsally located labelled cells at more rostral levels, in the region of closed neural tube.
Figure 5.4. Quantification of the degree of dorsal translocation of labelled cells along the rostro-caudal axis of the neural tube. (A) The ventral and dorsal borders containing the Dil-positive cells (red) and DiO-positive cells (green) were calculated at time 0 and at time 20 h at different axial levels. Statistical analysis compared each border at time 0 vs time 20. DiO cells maintained a dorsal localisation from the initial labelling to the end of culture period in every axial level (Kruskal-Wallis Test, ns.). Dil cells translocate significantly dorsally in the closed neural tube (Kruskal-Wallis Test, Dil dorsal border, *** p = 0.0009 at very rostral, ** p = 0.0019 at rostral). In more caudal axial levels, Dil cells maintain a significant ventral localisation which does not differ from the initial ventral site of labelling (Kruskal-Wallis Test, Dil dorsal borders, ns. at fusion, elevated, caudal). (B) Statistical analysis on the relationship between the two populations by comparing the ventral borders of the two dyes and the dorsal borders. Dil cells translocate and reach dorsally located DiO cells (black hatching: region of overlap between the two dyes) in the closed neural tube (Two-way ANOVA, Dil dorsal vs DiO dorsal, ns. at very rostral). More caudally Dil cells remains ventral and fully separated from the dorsal DiO cells (Two-way ANOVA, Dil dorsal vs DiO dorsal, *** p = 0.0001 at fusion, **** p < 0.0001 at elevated and caudal). Borders: mean values as a percentage of the total neuroepithelium length ± SEM. n = number of embryos analysed at each time point and at each axial level.
Figure 5.5. Hypothetical models of cell dynamics of the neuroepithelium during PNP morphogenesis. (A) Schematic representation of the axial levels analysed. (B) Model 1 which predicts a caudally directed displacement of neuroepithelial cells from the initial ventral site of labelling (marked by the mesodermal track) towards caudal and dorsal regions of the developing axis. (C) Model 2 which predicts that the labelled pool of neuroepithelial cells is retained caudally and acts as a supply of cells from the ventral-caudal neuroepithelium to the dorsal regions of the elevating and closing neural tube.
5.2.3 Live imaging of the neuroepithelium reveals two main streams of cell translocation:
ciaudal directed and rostro-dorsal directed

to test the validity of the two models (Fig. 5.5) and to further investigate the in vivo kinematics of the neuroepithelial cells during neural tube morphogenesis, live imaging of the open PNP was performed in static culture conditions on an inverted scanning confocal microscope, using a similar mounting protocol as developed by the Lee Niswan lab (Massarwa and Niswander 2013). To visualise the dynamics of neuroepithelial cells over time, the cytoplasmic ROSA26-EYFP reporter (Srinivas et al. 2001) was recombined genetically by the Nkx1.2creER^{T2} line (Rodrigo Albors, Halley, and Storey 2016) using low tamoxifen concentrations to generate mosaic labelling of both the neuronal and mesodermal lineages in the most caudal region of the embryo. The Nkx1.2creER^{T2} driver allows full visualisation of the caudal neuroepithelium beneath the surface ectoderm, which is negative, even though the precise site of zippering cannot be easily distinguished. In addition, to allow full penetration of the lasers and visualisation of the neural tube, a small aperture in the yolk sac was created to expose the region of open PNP while the amniotic membrane was kept fully intact (Fig. 5.7 A).

A total time period of 5h 57 min imaging was successfully recorded by acquisition of a z-stack at 7 minute intervals (Fig. 5.6) (Fig. 5.4 supplementary, video). Despite the short period of imaging, extensive tissue dynamics of the neuroepithelial cells at the level of the open PNP can be observed. To investigate the trajectories of the neuroepithelial cells during PNP morphogenesis, automatic tracking was performed in 2D (x-y axes) by Icy software (Fig. 5.7 E) after rotational and translational registration of the time lapse frames, which accounts for any displacement of the embryo during the time acquisition. The analysis showed that the PNP acts as a biphasic structure with a caudally directed stream of cells in the posterior region where the neuroepithelium is flat, and a rostro-dorsally directed stream at the level of neural fold elevation and fusion (Fig. 5.7 D and F, separation of the two regions by dotted lines). Neuroepithelial cells at the very caudal tip of the PNP, where the neural plate is relatively flat, appear to collectively migrate caudally, contributing probably to assembly and elongation of the new body axis (Fig 5.7 E and F). In net contrast, cells in the region of neuroepithelium which is undergoing neural fold elevation and fusion appear instead to move collectively in the opposite direction (rostral) and perpendicular to the plane of the epithelium (dorsally) (Fig. 5.7 E-G), supplying further evidence for the process of dorsally directed translocation observed by vital cell labelling. The presence of the two opposed streams could be further visualised by analysis of the averaged vector displacement (Fig. 5.7 D) which highlights both the
directionality (direction of the arrows) and the magnitude of displacement (size of the arrow), showing a net separation of tissue directionality between the caudal region (caudally directed) compared to rostral neural tube (rostro-dorsally directed).

**FIGURE 5.6**

Figure 5.6. Live imaging of the closing mouse spinal neural tube by laser scanning confocal microscopy. Caudal is to top, and rostral to bottom of each image. (A-I i) Time lapse frames showing the dynamics of the neuroepithelial and mesodermal cells flanking the neural tube over a period of 5 h 57 min. (A-I ii) Binarised time lapse frames and examples of manual cell tracking in 2D by Icy software (red cells). (J) Comparison between start (cyan, time 0 min) and the end of the time lapse (magenta, time 5 h 57 min). Note expansion and growth of the neuroepithelium in the very caudal tip of the developing axis. Stage at the end of culture: 16 somites.
Figure 5.7. 2D cell track analysis. (A) Aperture through the yolk sac (arrows) to image the caudal neural tube by laser scanning confocal microscopy. The amnion is left fully intact but, due to its thin transparent nature, does not impair imaging. (B-C) Time lapse at the start (B) and the end of the culture (C). (D) Averaged vector displacement analysis, comparing the transitions between start and end of imaging, shows the two streams of tissue displacement in the caudal flat neuroepithelium (caudally directed) and elevating/closing neural folds (rostro-dorsally directed). Directionality is illustrated by the direction of the arrows and the magnitude of movement by the size of the vectors. Note the transition zone (dotted lines) between the two regions where the cell movement is strongly reduced in magnitude and changing directionality. (E-G) 2D automatic cell track analysis showing the trajectories of the cells from the start (green) to the end of the time lapse (red). Note the caudally directed displacement of the cells in the flat neuroepithelium (F, above dotted line) and rostrally and dorsally directed displacement in the region of the elevating and closing neural folds (F, below dotted line). The analysis was done in collaboration with Dr Gabriel Galea.
Further optimisation of live imaging led to the successful recording of a second time lapse movie, although shorter in time (1 h 21 min), by using two-photon technology instead of confocal microscopy (Fig. 5.8 A-B, supplementary 5.5 video). The main advantages of using this technology are the significant reduction in photo-toxicity during acquisition, and deeper penetration through the tissue. In fact, in contrast to the first time lapse, the neural tube here was successfully imaged through the intact yolk sac, therefore preserving the optimal conditions for the embryo growth in static culture. Another major improvement has been the use of the ROSA26-mT/mG reporter which allows detailed detection of cell membranes by converting the ubiquitous Tomato fluorescence into EGFP upon cre excision (Muzumdar et al. 2007). The major improvement in z-resolution in this time lapse allowed a 3D analysis of the patterns of cell movements along the x, y and z axes by using Imaris software. Initially, the centroid of each cell was identified by automatic spot detection (Fig. 5.8 E, red spheres) and the tracks were generated on a colour coded scale (Fig. 5.8 F). In agreement with the previous 2D analysis, the neuroepithelial cells in the mid-posterior regions of PNP (above dotted line) show a caudally directed displacement, as shown by directionality of the vectors (Fig. 5.8 G) and by the end points of the tracks, in red, pointing towards the caudal axis (Fig. 5.8 F). In contrast, cells in the elevating neural folds and in the recently closed neural tube (region below dotted line), translocate following a rostro-dorsal pattern as shown by analysis of both tracks and displacement vectors (Fig. 5.8 F & G). A further aspect of the biphasic architecture of the neuroepithelium is the striking difference in velocity between the two oppositely migrating streams: caudal displacement in the flat neuroepithelium of the mid-posterior region is very rapid compared to rostro-dorsal displacement in the elevating and fusing neural folds which is comparatively slow (Fig. 5.8 H). This reflects the propensity of the cells to migrate over longer distances in the former region (longer tracks and vectors) compared to the short trajectories observed in this latter.
Figure 5.8. Live imaging of the closing spinal neural tube by multiphoton microscopy and 3D cell track analysis. Caudal is to top, and rostral to bottom. (A-D) Only the first (time 0 min) and last frame (time 1 h 21 min) of the time lapse are included. Enlargements (C-D) show two tracked cells (red and blue) in the opposing neural folds getting closer as the folds converge medially and zipperung progresses further caudally. (E) Cell centroid identification (red spheres) by Imaris spot detection filtered on quality of the signal and length of the tracking. (F) Cell tracks colour coded with blue indicating the beginning of the track and red the end point. (G) Vector displacement plot shows directionality (direction of the arrow) and magnitude of displacement (size of the arrow). (H) Tracks colour coded by mean velocity. Stage end of culture: 22 som. Time lapse acquisition and analysis was done in collaboration with Dr Gabriel Galea.
5.3 Discussion

The present study provides novel insights into the pattern of cell movements which occur within the neuroepithelium during spinal neural tube morphogenesis. In contrast to a static view of the neural plate, where cells maintain their specific ventro-dorsal and rostro-caudal positions during fold elevation and fusion, cells instead appear to change their relative positions within the neuroepithelium and to translocate over long distances as the embryonic body axis develops. Cells residing in the mid-ventral regions of the flat neural plate translocate and migrate collectively towards the dorsal neuroepithelium as the neural folds elevate, bend and fuse dorsally. As this dorsally directed translocation progresses, the pool of initially ventrally-located cells reaches and intermingles with the dorsally resident cell population, as shown by the region of overlap between DiO- and Dil-positive cells in the dorsal neuroepithelium. Interestingly, a similar dorsal-ward displacement of cells has been recently reported in a fate map study of the *Xenopus* neural tube (Edlund et al. 2013). Here, cells labelled in the ventral superficial layer of the neuroepithelium were shown to be significantly displaced dorsally during the process of radial intercalation, which converts the double layered neural tube into a single layered epithelium. Once in this new position, cells started expressing the dorsal neural marker Pax3, despite their origin from the ventral region of the neuroepithelium. It will be important in future to investigate to what extent this process of dorsally directed neuroepithelial cell movement is conserved among different species.

Previous analysis in our lab showed that cells in the dorsal neural folds increase significantly in number during dorsolateral bending. Despite the dorsal neuroepithelium proliferating more rapidly than the ventral compartment, the increase in cell number observed in this region exceeds the number of cells predicted by cell cycle analysis; conversely in the ventral region the observed number is significantly lower than the predicted number, based on proliferation alone (McShane et al. 2015). The evidence that cells are able to translocate provides an explanation for the discrepancy in cell number observed between the ventral and dorsal compartments, showing that the significant increase in cell number in this region occurs as a result of a net cell translocation dorsally. The resulting cell crowding in the dorsal neuroepithelium has been proposed as a potential driving mechanism of focal bending. The physical discontinuity in cell number between the less populated mid-ventral neuroepithelium compared to a highly cell dense dorsal neuroepithelium would be predicted to cause an inward buckling at the site of transition and therefore inducing DLHP formation (McShane et
al. 2015). The dorsally directed cell translocation itself, together with the medial convergence of the surface ectoderm over the elevating neural folds, could also potentially contribute further to the inward deformation of the dorsally dense neuroepithelium and therefore DLHP formation (Morita et al. 2012; Alvarez and Schoenwolf 1992). Ultimately, the fine balance and cooperation between several external and internal forces within the neuroepithelium would be predicted to act together to eventually achieve focal bending dorsolaterally.

A more detailed analysis revealed that the process of dorsal translocation involves also a strong rostro-caudal displacement, as the body axis elongates. Moreover, the rostral region of the closing/newly closed neural tube where cells relocate dorsally differs from the caudal flat neural plate where cells retain a significant ventral localisation, reminiscent of the initial site of dye injection. In vivo analysis of cell position dynamics by live imaging is able to reconcile this apparent discrepancy by revealing the presence of two opposite streams of cell displacement at the level of the open PNP. A caudally directed stream coincides with formation of new body axis while a rostro-dorsally directed stream coincides with the dorsal translocation of cells at the level of neural fold elevation and apposition. Thus, cells initially labelled in the ventral neural plate are moved further caudally as new body axis is formed, retaining their ventral localisation. Concomitantly, this region is predicted to act as a source of new cells rostrally for the developing neuroepithelium, giving rise to the rostro-dorsal stream, in which cells initially supplied ventrally relocate dorsally as the folds elevate. The presence of these two opposite streams thus explains the difference in ventro-dorsal position along the body axis of the Dil-positive cells which retain their original ventral localisation caudally while translocating into the dorsal neural folds rostrally.

The model proposed here could imply a difference in potency between the caudal flat neural plate which acts as a reservoir of new cells compared to the growing neuroepithelium in the region of neural fold elevation and fusion. There is in fact increasing evidence for the existence of a bi-potential cell population, named neuromesodermal progenitors (NMPs), in the ventral-posterior region of the embryo (reviewed in Wilson et al. 2009; Henrique et al. 2015). This pool of proliferating progenitors is characterised by the co-expression of the neuronal marker Sox2 and mesodermal marker T (Brachyury) (Henrique et al. 2015; Wymeersch et al. 2016). Fate mapping studies have identified the location of this population at E8.5 in the border between the caudal node and rostral primitive streak (node-streak border: NSB) and in the caudal lateral epiblast (CLE), lateral to the primitive streak which, later at E10, gives rise to the chordo-neural hinge (CNH) in the tail bud region (Cambray and Wilson 2007; Cambray and Wilson 2002). The extending body axis is thought to be supplied from this pool of proliferating
cells which contribute both to the neuronal and mesodermal lineages as shown by retrospective clonal analysis (Tzouanacou et al. 2009). It is intriguing therefore to consider the possibility that the initial ventrally labelled cells at the site of the open PNP in the above experiments may belong to the pool of NMPs. In support to this hypothesis, Dil positive cells can still be detected at the end of the culture in the NMP region at the very caudal end of the embryo, in addition to other labelled cells contributing rostrally to the closed neural tube. This is consistent with the published fate map of the NMPs (Cambray and Wilson 2002; Wymeersch et al. 2016), and suggests the possibility that descendants of the NMPs may eventually relocate dorsally as new cells of the neuronal lineage are supplied to the growing neuroepithelial axis, as initially observed for the Dil-labelled cells. To test specifically the hypothesis that cells deriving from the NMP pool may be involved in the process of dorsal translocation, the NSB region was labelled with Dil and similarly the degree of dorsal displacement was calculated along the body axis (work conducted by PhD rotation student Dorothee Mugele, as part of a 3-months’ project under my supervision). Consistent with the previous results, descendants of the NMPs colonised the dorsal neural tube rostrally, while other labelled cells were retained within the NMP pool within the tail bud (data not shown).

These results, together with the analysis from live imaging, suggest a model of cellular dynamics where the proliferative pool of NMPs is retained caudally as new body axis is formed (caudally directed stream), while supplying new cells to the developing neuroepithelium which translocate dorsally during neural fold elevation and apposition (rostro-dorsal stream) (Fig. 5.9). Upon mitosis, one daughter cell is predicted to retain potency and thus to remain within the NMPs pool. The second daughter cell of the mitotic pair instead to differentiate towards the neuroepithelial lineage, leaving the progenitor pool and joining the rostro-dorsal stream.
Figure 5.9. Model of cell translocation during body axis extension and formation of the caudal neural tube. (A-B) Cross section views of the neural tube. Cells labelled at E8.5 in the mid-ventral neuroepithelium (A) relocate dorsally at E9.5 upon neural fold elevation and fusion (B). Cell crowding in the dorsal neuroepithelium causes an inward buckling and thus DLHP formation. (C-D) Dorsal (C) and lateral view of the PNP region (D). Neuromesodermal progenitors residing in the NSB (square) and in CLE (brown) (C) are retained caudally (caudal stream) as new body axis is deposited. NMPs descendants of the neuronal lineage exit the progenitor pool and become deposited ventrally to the developing neuroepithelium to eventually relocate dorsally (rostro-dorsal stream) during neural folds elevation and apposition. Neuroepithelium in yellow, surface ectoderm in light blue, cells labelled by Dil in red.
5.4 Supplementary figures

FIGURE S5.1
(related to figure 5.2)

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Figure S5.1 supplementary. Quantification measurements of Dil positive cells along the ventro-dorsal axis at time 0 and time 18 h, calculated as a percentage of the total length of the neuroepithelium. Analysis embryos t = 0: n = 9. Analysis embryos t = 18: n = 9. Number of experiments performed = 5.

Figure S5.2 supplementary (video). 3D reconstruction by confocal microscopy of the Dil and DiO tracks at the end of 20 h culture period. Note the dorsal localisation of DiO-labelled cells (green). Note the stream of Dil-labelled cells (red) which become located dorsally in the closed NT but maintain a significant ventral position in the caudal region of the open PPN. Bright-field: refraction to visualise the entire caudal axis.
**Figure S5.3** supplementary. Quantification measurements of DiI and DiO positive cells along the ventro-dorsal axis at time 0 and time 20 h, calculated as a percentage of the total length of the neuroepithelium. Measurements were taken at each level of the body axis: very rostral, rostral, fusion, elevated and caudal.

**Figure S5.4** supplementary (video). Related to Figure 5.6. Live imaging of the neural tube by laser scanning confocal microscopy (total duration 5 h 57 min). On the left: YFP cells induced by Nkx1.2 cre which labels the neuronal and mesodermal lineages in the open PNP. On the right: binarised image of the time lapse by Icy. Stage at the end of culture: 16 som.

**Figure S5.5** supplementary (video). Related to Figure 5.8. Live imaging of the neural tube by multiphoton microscopy (total duration 1 h 27 min). mEGFP cells are induced by Nkx1.2 cre. Stage at end of culture: 22 som.
6. General Discussion

The present study provides novel insights into the in vivo role of integrin-mediated adhesion during mouse neural tube morphogenesis. During neurulation, the most highly expressed receptor is integrin β1 which pairs functionally with 4 specific α subunits to form the main fibronectin-interacting receptors (integrin α5β1 and αvβ1) and the laminin-interacting receptors (integrin α6β1 and α3β1). At late stages of neurulation (above 18 somites), integrins become focally localised and significantly upregulated at the site of zippering where the apposing folds come into contact and fuse. Genetic ablation of integrins by targeting the dorsal cells of the surface ectoderm abolishes the localised upregulation of the receptors at the site of fusion leading to failure of neural tube closure and development of open spina bifida. This may result in cells losing their ability to interact basally with the structurally normal network of fibronectin fibrils, which sits at the interface between the dorsal neuroepithelium and the dorsal surface ectoderm cells. This could impede forward progression of the two waves of zippering which propagate caudally from the fusion point and rostrally from closure 5, leading eventually to failure to seal the open PNP.

6.1 The role of cell-ECM adhesion during epithelial gap closure

Epithelial gap closure is a fundamental process during embryogenesis characterised by the progression of zippering and the collective convergence of an overlying epithelium towards the midline to eventually establish or re-establish tissue connectivity and integrity between apposing epithelia, thus sealing a gap (Begnaud et al. 2016). Examples of mechanisms of epithelial gap closure in vivo are the process of dorsal closure in Drosophila melanogaster (Jacinto et al. 2002), embryonic wound healing (Redd et al. 2004; Martin and Parkhurst 2004) and eyelid closure (Heller et al. 2014). Strong similarities exist between the above systems and mouse neural tube closure. In fact during spinal neurulation, the open region of the PNP becomes eventually sealed dorsally to establish epithelial continuity of the surface ectoderm and neuroepithelium between the tips of the apposing neural folds.

Two major mechanisms driving epithelial gap closure have been proposed: the actomyosin purse-string mechanism and the cell crawling model (Begnaud et al. 2016). The former is characterised by accumulation of actin and myosin II at the circumference of the gap,
assembling a supra-cellular contractile cable which links neighbouring cells and propels collective centripetal movement of the epithelium to close the gap (Martin and Lewis 1992). Similarly, a supracellular actomyosin cable is visible at the surface ectoderm/dorsal neuroepithelium interface which runs caudally along the open PNP (Galea, unpublished). At early PNP closure stages, the cable extends only about 3/4 of the length of the PNP, and so cannot mediate simple purse-string closure throughout most of spinal neurulation. Towards the end of spinal neurulation, the cable extends further to surround the entire circumference of the open PNP, and at this late stage it could exert a purse-string closure influence. However, in contrast to a uniform circumferential contraction of an actomyosin purse string, as for example happens in embryonic wound healing, PNP closure appears to occur specifically from the rostral zippering point, and at the end of neurulation also from the new (closure 5) causal zippering point. This is similar to zippering progression during neurulation in Ciona intestinalis which was shown to be driven by sequential contractions localised at individual cell junctions at the zippering site (Hashimoto et al. 2015).

The cell crawling model is based instead on forward movement over the gap driven by basal lamellipodial or filopodial protrusions emanating from the leading edges of the advancing epithelium (Theveneau and Mayor 2013). Indeed, rosettes of protrusions are present at intervals along the entire perimeter of the PNP with intense accumulation at the sites of zippering (Rolo et al. 2016). However, unlike systems such as wound healing, there is no physical substrate between the closing neural folds (i.e. it is a fluid filled space) on which ‘crawling’ surface ectoderm cells could exert traction to mediate closure. Hence, this mechanism appears unlikely to apply to neural tube closure.

Loss of integrin β1 in the surface ectoderm cells does not disrupt either contractility of the supracellular actomyosin cable (as judged by expression of pMLC) or abolish protrusions formation at the site of zippering, even though progression of closure is halted. Instead of perturbation of the cytoskeleton, failure of closure could result from loss of adhesion to the underlying ECM substrate which may uncouple the surface ectoderm from the dorsal neuroepithelium. Perturbation of cell-ECM adhesion, either by removing the receptors or by perturbing BM assembly, was shown to severely affect the ability to close the epithelial gap in all the above systems leading to development of congenital open defects.

In Drosophila, for example, the myospheroid mutant which results from deletion of the integrin βPS subunit, orthologous to integrin β1 in vertebrates, fails to complete dorsal closure leading to a hole in the dorsal epidermis (Leptin et al. 1989; Wright 1960; Brown 1994). In these embryos the leading edge epidermis was shown to eventually detach from the
underlying amnioserosa leading to failure of closure (Narasimha and Brown 2004; Peralta et al. 2007). This highlights the pivotal role of the integrin receptor in mechanical coupling of the two epithelia at their basal interface. However, precise mathematical analysis and laser ablation experiments showed that a defect of closure arises before detachment of the two epithelia due to impairment of zippering at late stages of closure due to loss of integrin mediated adhesion (Hutson et al. 2003). Similar to the two sites of zippering in the PNP (fusion point and closure 5), in Drosophila dorsal closure the eye-shaped opening develops two sites of zippering progression, the anterior and posterior canthus. Significant reduction in the rate of zippering in the myospheroid mutant (80% decrease compared to wild type) was predicted to affect the tension of the actomyosin cable along the direction of motion (Hutson et al. 2003). Despite compensatory forces coming from the amnioserosa and the epidermis allowing further progression, zippering from the two canthi eventually stops and the lateral edges of the migrating epidermis fail to meet at the dorsal midline to re-establish epithelial continuity. The decreased rate of zippering in myospheroid mutants however is not associated with lack of protrusive activity or activation of apoptosis via anoikis (Gorfinkel et al. 2009). This is consistent with the normal appearance of ruffle-filopodia rich protrusions observed at the site of fusion in Grhl3 cre/+ Itgβ1 f/f mutants and by normal level of apoptotic cells compared to the wild type controls.

Cell-ECM interactions appear also to be essential for mouse embryonic eyelid closure. Loss of either fibronectin or integrin α5 causes failure to close the eyelid at E16.5 leading to an eye-open-at-birth defect (Heller et al. 2014). In particular, integrin α5/fibronectin interactions were shown to regulate intercalation of the cells at the eyelid front. The forces generated by cell intercalation in turn tow the skin epidermis over the cornea to eventually seal the open eyelid. Interestingly, perturbation of the integrin-ECM axis did not affect cell-cell junction or the architecture of the F-actin fibres in the eyelid front. Instead knock-down embryos displayed a significant reduction in cell elongation as well as in the speed of intercalation which eventually led to an overall reduction of the speed of eyelid closure. In summary, this study highlights the pivotal role of cell-ECM adhesion in the cells of the eyelid front for towing the epidermis over the cornea. In agreement with the above study, removal of the β1 subunit, which functionally interacts with α5, led to failure of closure of the eyelid, similar to failure of closure of the PNP. This strong similarity between the two systems highlights the importance of fibronectin-integrin interaction for the collective moment and medial convergence of an epithelium to achieve epithelial closure.
Recently, gap closure of stromal tissue was also shown to be dependent on cell-ECM interactions at the edge of the wound (Sakar et al. 2016). By using a combination of live cell imaging and traction force microscopy, the authors found that closure of the wound in 3D follows an initial phase dependent on RhoA mediated contractility and a second late phase dependent on interactions with a newly deposited fibronectin scaffold. This is consistent with the process of dorsal closure in *Drosophila* which is initially dominated by cell contractility followed by the onset of zippering where myospheroid mutant are deficient (Hutson et al. 2003). During the fibronectin dependent phase, fibroblast at the edge of the wound engage into a tangential process of migration along the circumference of the wound which drags cells at the back towards the centre of the wound. Live imaging revealed that while towing, fibroblasts extend existing fibronectin fibrils into the wound which act as substrate for cells to migrate centripetally. Inhibition of either integrin α5 or of the downstream target FAK abolishes closure in the second fibronectin dependent phase without affecting the rate of closure during the initial contraction dependent phase. The late onset of the closure defect due to integrin deficiency seen in both wound healing and dorsal closure closely resembles the timing of failure in PNP closure which occurs at the final stages of spinal neural tube closure.

Preliminary analysis of live imaged surface ectoderm cells conducted in collaboration with Dr Gabriel Galea also showed that cells at the closing edge migrate tangentially and converge medially at the site of zippering as it propagates forward (data not shown). Together with the enrichment of fibronectin in this region, this could suggest a similar mechanism of surface ectoderm mediated zippering due to medial convergence of apposing cells into the gap area by remodelling the basal fibronectin substrate which is used as a scaffold for collective migration.

The role of collective movement of surface ectoderm cells towards the dorsal midline was recently demonstrated for closure of the neural tube in *Xenopus* (Morita et al. 2012). The deep layer of the surface ectoderm was shown to be the driving force for collective migration of both the superficial and deep surface ectoderm layers towards the dorsal midline. A strong similarity with the above study is the identification of the surface ectoderm as the key tissue of action of integrins. The authors reported as well that deletion of integrin β1 subunit in the surface ectoderm leads to failure of neural tube closure. Moreover, loss of the receptor was shown to impair the ability of the surface ectoderm to migrate collectively dorsalwards; migration still progresses but with a significant reduction in velocity. This strongly reinforces the role that integrin-substrate adhesion may have for collective medial convergence of the surface ectoderm towards the dorsal midline to allow progression of zippering. The precise spatio-temporal orientation of the fibronectin fibrils observed in the current study would give further support to the above scenario. In fact, fibrils are oriented radially to the opening of the
PNP which may in fact act as a track for collective medial convergence of the surface ectoderm centripetally. In addition, in the recently closed neural tube thick fibronectin fibrils are specifically aligned along the dorso ventral axis further supporting a potential role of cell-ECM interaction for dorsal-ward collective movement.

In conclusion, cell-ECM adhesion is essential during the final stage of neurulation (above 22-24 somites) for closure of the open PNP. Surface ectoderm cells upregulate integrins at both sites of zippering: at the fusion point in the rostral region of the PNP and caudally at the level of closure 5 (Fig. 6.1 A-B). Deletion of integrin β1 halts forward progression of zippering at these two sites leading to failure to seal the dorsal midline of the neural folds. The defect arises from the perturbation of zippering progression and not from impairment of neural fold apposition as shown by the similarity in medio-lateral recoil in both wild type and integrin β1 deficient embryos. In fact, the forces governing neural fold elevation and apposition are likely to be intrinsic to the neuroepithelium and not to derive from the thin layer of the overlying surface ectoderm cells. Rapid elevation and fold apposition (within 33 min) can be observed in live imaging studies of the surface ectoderm independently of zippering progression (collaboration with Dr Galea). Instead, constriction of the more caudal, flat PNP region has been proposed as generating force to induce neural fold midline apposition, via the actin cable that mechanically couples both regions (Galea, unpublished data). It would be predicted that neural fold apposition would be permissive for forward progression of zippering by medial convergence of the surface ectoderm, which is the first tissue to come into contact during fusion (Rolo et al. 2016).

I would like to propose here a model where integrin-fibronectin adhesion would act by mechanically coupling the surface ectoderm with the dorsal neuroepithelium allowing forward progression of zippering by the collective medial convergence of the surface ectoderm toward the dorsal midline (Fig. 6.1). Surface ectoderm cells are predicted to migrate collectively along the direction of the radially distributed fibronectin fibrils which will predict a centripetal displacement of the epithelium into the gap (Fig. 6.1 A-B, C), similarly to the towing mechanisms reported in wound healing (Sakar et al. 2016) and eyelid closure (Heller et al. 2014). At the sites of zippering, medial convergence of the surface ectoderm via the fibronectin substrate re-establishes continuity of the surface ectoderm epithelium between apposing neural folds before the tips of neuroepithelium come into contact. Therefore, cell-ECM adhesion at this site of transition will be essential to both govern medial convergence of the surface ectoderm cells (Fig. 6.1 C) and to provide a transitory robust inter-epithelial
adhesion (between dorsal neuroepithelial and surface ectoderm cells) before cell-cell adhesion is eventually re-established at fusion (Fig. 6.1 D-E).

It will therefore be essential in the future to optimise further the live imaging set-up in order to quantify the morphogenetic movements of the surface ectoderm which accompanies PNP closure and zippering progression. This, together with the introduction of a reporter into the integrin β1 line would allow visualisation in vivo of the behaviour of cells when adhesion to the ECM substrate is abolished to eventually provide insights into the role of adhesion during zippering. Finally quantification of forces by a surface ectoderm targeted laser ablation and by using 3D force traction microscopy could potentially unravel the mechano-biology of the defect which arises from lack of integrin adhesion.
Figure 6.1. Model of integrin-mediated adhesion at the sites of zippering. (A-B) Schematic (A) and confocal acquired (B) dorsal view of the open PNP at 30 somites. Integrin β1 in red, fibronectin in green. Fibronectin fibrils are oriented radially to the open PNP and aligned medio-laterally in parallel to each other in the rostral region of the closed neural tube. Note focal upregulation of integrin β1 at the sites of zippering emanating from closure 1 and from closure 5. (C) Model of medial convergence of the surface ectoderm cells at the site of zippering led by cells that strongly express integrin β1. (D-E) Cross section view of the site of zippering showing neural fold apposition. Localised up-regulation of integrin β1 by two surface ectoderm cells at the site of fusion which strongly adhere to the underlying network of fibronectin interposed between the dorsal neuroepithelium and the basal surface ectoderm. Mechanical coupling between the two tissues ensures adhesion before cell-cell cadherin-mediated adhesion is re-established between opposing neural folds.
6.2 Role of cell-ECM adhesion in human neural tube closure

The essential requirement for integrin β1-mediated adhesion in the surface ectoderm may serve as a novel mouse model for the study of the mechanisms of spinal neural tube closure. The open lesion evident at the level of the lumbo-sacral axis in these fetuses closely resembles the condition of myelomeningocele observed in humans. Therefore, this model may be of particular significance to investigate the aetiology of open spina bifida, and in particular myelomeningocele, observed in humans. In fact, only a few mouse genetic models of spina bifida exist while the majority of genetic factors of NTDs identified so far mainly affect the process of cranial neural tube closure (Harris and Juriloff 2010). Mouse NTD mutants affected by exencephaly alone are in fact four times more frequent than those affected by open spina bifida alone or associated with exencephaly.

The requirement for integrin-mediated adhesion for neural tube closure in humans has also started to emerge, through studies of genome sequencing of cases affected by myelomeningocele. Recently, whole exome sequencing of a cohort of 132 cases of myelomeningocele among Mexican Americans and Caucasian Americans, who have the highest prevalence of open spina bifida in the USA, identified deleterious mutations in Itga6 gene, coding for integrin α6 subunit, as a significant risk factor for the development of NTDs (conference communication, Kit Sing Au from Dr Hope Northrup lab). In mouse, deletion of Itga6 alone does not cause failure of neural tube closure (Georges-Labouesse et al. 1996), but only when associated with simultaneous removal of Itga3 (De Arcangelis et al. 1999). However, both are obligatory partners of the central receptor integrin β1.

Moreover, a recent multicentre study of the genetic risk factors associated with myelomeningocele focused on the analysis of the Mexican Mestizo population due to the very high prevalence of the defect. Association analysis of 1023 families compared to 500 healthy controls found significant differences between the two groups for 4 top candidate genes which included Lama5, coding for laminin α5 chain (conference communication, Osvaldo Mutchinick from Beatriz Sanchez lab). This further reinforces the likely role of cell-ECM in human neural tube closure.

The localised pattern of up-regulation of integrins strongly directs attention towards the two focal sites of zippering at the opposite ends of the open PNP: at the main fusion point, deriving from progression of closure 1, and at closure 5 which is located at most caudal end of the late-stage open PNP. Closure 5 was first proposed in mouse as the latest site where neural fold
fusion initiates, located at the very caudal end of the neural plate at the level of the 33rd somite (sacro-coccygeal region) (Sakai 1989). Histological analyses led Sakai to propose the idea that completion of PNP closure at the very end of spinal primary neurulation occurred by the joining of the caudally directed wave of zippering propagating from progression of closure 1 with the rostrally directed wave coming from closure 5. A few years later, evidence for the existence of closure 5 in humans emerged from an analysis of clinical NTD cases at late fetal stages. Exposure to the teratogen valproic acid was noted to cause NTD lesions at a very low level of the body axis, and this was interpreted to have resulted from a ‘closure 5 defect’ (Van Allen et al. 1993). Further evidence came from the analysis of human fetuses with open spina bifida that were either restricted to the sacral region alone, or else affected the thoracic-lumbar region whereas the sacral level had closed normally (Seller 1995). This argued in favour of a different zippering process for the sacral region (closure 5) distinct from the one involved in closure of the thoracic-lumbar region (progression from closure 1). The late occurrence of the defect and the localisation of the open spina bifida in the low lumbo-sacral axis in Grhl3 cre/+ Itgβ1 f/f mutants strongly suggests the possibility that zippering emanating from closure 5 may have failed to further progress rostrally and join with the caudal directed zippering emanating from closure 1.

In future, it will be important to quantify stresses and strains acting at this site by using a similar approach based on laser ablation of the recently closed neural tube roof and the site of closure 5. Eventually, live imaging of the open PNP at the final stages of neurulation should ultimately be able to distinguish between defects arising from failure of progression of closure 1 or closure 5.

6.3 Molecular mechanisms driving neuroepithelial cell translocation

Cells of the neuroepithelium were shown to translocate rostro-dorsally during elevation and closure of the neural folds. But a fundamental question which still remains open is the nature of the cellular and molecular mechanism that regulate and drive this process of cell movement. It is unlikely to involve single cell migration, but instead probably requires fine coordination between cells of the entire neuroepithelium which are likely to migrate collectively. This could be coordinated by the strong interconnection that exist between neuroepithelial cells by both adherens (via N-cadherin) and occluding junctions. Furthermore, the neuroepithelial cells appear to be mechanically coupled by a supracellular network of
cortical actin which spans the entire neuroepithelium and resolves into a thick supracellular actin cable at the SE-NE border, which runs from the site of fusion towards the caudal end of the PNP.

Insights into the mechanisms of collective migration of cells within an epithelium have been provided by genetic and live imaging analyses of D. melanogaster and zebrafish models. An example is the migration of border cells of the D. melanogaster oocyte (Montell 2003). In the anterior egg chamber, the polar cells which are not motile become surrounded by the border cells which engage in a process of intra-epithelial collective emigration. It has been shown in this instance that migration is not regulated by adhesion to an ECM substrate but instead by direct cell-cell adhesion of the border cells to the nurse cell epithelium (Pacquelet and Rørth 2005). Transient cell-cell contact mediated by E-cad, in fact, was shown to generate the traction necessary to drive the posteriorly directed migration. In addition, directionality was shown to be regulated by a gradient of growth factors (epidermal and platelet derived growth factors) which causes the stochastic establishment of leader cells at the leading edge of the emigrating cells (Bianco et al. 2007). Once the posterior dorsal region of the oocyte is reached, the motility ceases as a consequence of the even distribution of the growth factors (Prasad and Montell 2007).

Another example is the migration of the lateral line primordium in zebrafish: a group of 100 cells which translocates posteriorly in the zebrafish axis to deposit the neuromasts, epithelial rosette like structures which are used as mechano-receptive organs for detection of water movements by the fish (Ghysen and Dambly-Chaudière 2007). In this case, a chemokine gradient, dependent on the SDF1-CXCR4 system, is established which guides the migration (David et al. 2002).

Recently new examples of collective epithelial cell migration were reported in mouse thanks to the advances in live imaging techniques. During the process of embryonic eye-lid closure in mouse, epidermal cells in the eyelid front were shown to become elongated and undergo a process of cell intercalation which tows the epidermal tissue over the cornea via cell-ECM adhesion (Heller et al. 2014). Another recent example is the intraepithelial cell migration which is required for positioning of the organ primordium during tooth development in mouse (Prochazka et al. 2015). In this case, directionality and magnitude of movement were shown to be regulated cooperatively by the expression of Fgf8 by the migrating epithelium and by a Shh gradient produced by the region of the mandible towards which migration occurs.
The above examples highlight the presence of two fundamental components for cell migration: the existence of a molecular motor which drives the movement and the presence of a chemoattractant gradient which establishes directionality and regulates persistency. Forces which drive the collective movement of the neuroepithelial cells towards the dorsal neural folds may for example derive from the contraction of the supra-cellular cable which could generates a dorsally directed pulling force. Cell proliferation has been shown to be able to drive directed motility in many other models. However, it seems unlikely to be the driving force in this system: first because proliferation is more rapid in the dorsal region compared to the ventral neuroepithelium (McShane et al, 2015) which would be predicted to encourage cells to move in the opposite direction, dorso-ventrally. Moreover, the plane of division was shown to be mainly oriented in the rostro-caudal direction in the closed neural tube and randomly in the open PNP (unpublished data, Galea) suggesting that cell division is unlikely to be a driving force for dorsal cell translocation.

Adhesion to the basement membrane and generation of traction forces by basal interactions with the ECM substrate could also provide motility to the neuroepithelium for its dorsal displacement. One potential way in which directional migration into the dorsal neural folds may occur is by haptotaxis where cells move along a gradient of ECM molecule: cells tend to migrate from regions of low concentration of a ECM molecule towards regions of increasing concentration to eventually arrest if the adhesion becomes too strong (Rozario and DeSimone 2010). The differential composition of the BM along the ventro-dorsal axis which is poor in fibronectin ventrally at the NT-MES interface and rich dorsally at NT-SE interface would predict that cells may move from the ventral neural folds where fibronectin concentration is low and still under assembly towards dorsal regions where fibronectin is highly concentrated, thus causing cells to translocate into the dorsal neural folds. The precise orientation of the fibronectin fibrils along the medio-lateral axis further suggests that neuroepithelial cells may use the fibronectin substrate as roadway to relocate dorsally. The role of fibronectin in migration during embryogenesis has been already documented in several studies: fibronectin has been implicated in myocardial precursor migration in the formation of the heart tube (Trinh and Stainier 2004), and in several morphogenetic movements during gastrulation (epiboly, convergent extension, mesoderm migration) (Schwarzbauer and DeSimone 2011). Finally the ventro-dorsal expression gradient of the integrin subunits α6 and αv further suggests the possibility that alongside a ventro-dorsal gradient of ECM ligand, migration may be regulated by a ventro-dorsal gradient of integrin receptors expressed by the neuroepithelial cells. This model predicts that motile ventrally located cells which express high levels of
integrins will progressively downregulate integrin expression along the way towards the dorsal folds until residual expression dorsally causes them to stop.

In addition to the identification of the molecular motor, it will be important to understand eventually the significance of this movement in relation to the process of neural tube bending and closure: to investigate whether dorsal translocation would be necessary for bending and closure of the dorsal neural folds, and whether abolition of dorsal translocation would lead in turn to failure of neural tube closure.
7. Bibliography


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