Investigating the Role of EphrinB1 in the Control of *Xenopus laevis* Gastrulation

by

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Abstract

During animal development, the early embryo undergoes gastrulation to bring the primary germ layers into their definitive positions. The ancestral mode of vertebrate gastrulation can be studied using the African clawed frog *Xenopus laevis*. A core function of gastrulation is the movement of prospective endoderm from the surface to the embryo interior. In *X. laevis*, endoderm internalization is achieved through vegetal rotation, a tissue morphogenetic process that has only been characterized at the tissue level. I have investigated the cell and molecular basis of endoderm morphogenesis in *X. laevis* gastrulae to connect vegetal rotation with common mechanisms of germ layer internalization in other organisms. I characterized the arrangement, shape change, and migration of cells in the endoderm, and found that movement of vegetal endoderm cells located deep to the embryo surface represents an adaptation of mechanisms of monolayer internalization (namely, invagination and cell ingression) to the multilayer structure of the vegetal cell mass. Thus, this movement was termed “deep cell ingression”. Deep cell ingression integrates an amoeboid-type motility whereby cells propel themselves forward using strategic cell shape changes to move, but with different rates, a unique mode of intercellular migration where cells move with a common direction but at different velocities with respect to neighbouring cells to drive cell rearrangement during vegetal rotation. Despite their collective
movement, endoderm cells are separated by a vast network of extracellular material. Cells are interconnected by stitch-like contacts, and cell migration requires the adhesion molecule C-cadherin, and the matrix protein fibronectin. A basic requirement for migration is that cells must detach to complete rearrangement, and in this regard, the leading cell resolves its contact with the following cell through trailing edge retraction involving ephrinB1-dependent macropinocytosis and trans-endocytosis. Beyond the endoderm, ephrinB1 is also required for the modulation of ecto- and mesoderm tissue cohesion.
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1. CHAPTER ONE: General Introduction

1.1 Xenopus laevis: A model for vertebrate gastrulation

During animal development, the embryo begins life as a relatively unstructured aggregate of cells that undergoes gastrulation, its first morphogenetic event, to pattern the germ layer primordia into primary germ layers (Beetschen, 2001). The basic metazoan body plan is established in a seemingly convoluted manner; the germ layer primordia are first specified on the surface of the embryo, followed by movement of the prospective endoderm (and mesoderm when present) to the interior of the embryo, which is then covered by the ectoderm (Gerhart and Keller, 1986; Solnica-Krezel and Sepich, 2012).

Gastrulation, named to denote the set of cell movements that form the primitive gut, is a conserved process across all animal phyla. Although gastrulation is conserved as a whole, the details of this process vary across species. In vertebrates, striking differences in egg size may account for a portion of the divergence in this process. The supposed primitive mode of vertebrate gastrulation is preserved in lampreys, lungfish, and in urodele and anuran amphibians; among the latter group, gastrulation is best known in Xenopus laevis (Collazo et al., 1994; Shook and Keller, 2008).

The African clawed frog X. laevis is prominently used as a model organism in developmental biology (Callery, 2006). Its exceptional resistance to disease, relatively short life cycle, and year-round ability to produce large quantities of eggs upon induction make X. laevis an ideal candidate for the study of vertebrate development, which requires large amounts of biological
material. Moreover, its relatively large eggs (1.3 mm in diameter) can be fertilized in vitro and give rise to large embryos suited for microsurgical manipulation such as tissue explant excision or transplantation. Using these techniques, large-scale processes such as gastrulation can be broken down into regional components for detailed analysis (Gerhart and Keller, 1986).

1.2 *X. laevis* gastrulation

The *X. laevis* gastrula can be divided into several regions based on regional gene expression (Fig. 1-1A). The gastrula consists of the ectoderm, which is animally located, it is also referred to as the blastocoel roof (BCR). The mesoderm is located along the equatorial region and forms an annulus surrounding the vegetal cell mass, which, as its name suggests, is located in the endodermal vegetal hemisphere of the embryo (Fig. 1-1A, B) (Winklbauer and Damm, 2012). At 23°C, *X. laevis* gastrulation begins 9 hours post fertilization and requires approximately 6 hours to complete (Fig. 1-1C). Gastrulation is choreographed by coordinate cell movements in each of the three germ layers. Movements include epiboly of the ectoderm, involution of the mesoderm, and vegetal rotation of the vegetal cell mass, which consists of anterior mesoderm around an endoderm core. Together, region-specific tissue movements drive internalization of the prospective mesoderm and endoderm into the gastrula (Gerhart and Keller, 1986; Bauer *et al.*, 1994).
Figure 1-1 *X. laevis* gastrulation movements.

(A) Schematic diagrams showing gastrulation movements of the three primary germ layers: ectoderm (Ecto), mesoderm (Meso) and endoderm (Endo). Colours correspond to the respective tissues. (B) Corresponding mid-sagittal gastrula fractures. (C) Gastrulation timeline relative to Nieuwkoop–Faber gastrula (10–13) stages and common range of stages referred to as early, mid, or late gastrulae. Time shown in hour:minutes; the initial reference (0:00) represents 9 hours post fertilization in standard time. Scale bar is 250 µm. Blastocoel (bc) and archenteron (arc) are indicated.

1.3 Epiboly

The ectodermal BCR can be further sub-divided into two regions based on fate maps derived from cell tracing experiments that show the fate of cells in these regions at later stages (Bauer *et al.*, 1994). The dorsal neuroectoderm region gives rise to nervous system structures, and the composite animal, lateral, and ventral regions give rise to the larval epidermis (Dale and Slack,
Both regions undergo epiboly, the morphogenetic process whereby the BCR, which initially consists of an epithelial monolayer covering 2–4 layers of deep cells, is thinned into a bilayer with a single deep layer to spread over the gastrula and cover the embryo surface (Fig. 1-2A) (Keller, 1980). Epiboly begins before gastrulation (stage 8) and ends at the onset of neurulation (stage 13), which is marked by closure of the blastopore and formation of the neural anlage, although the bulk of cell rearrangement is complete by stage 11.5 (Szabó et al., 2016). Epiboly is achieved by radial intercalation of several layers of deep cells to form a single layer beneath the superficial layer (but they do not invade the epithelium), which results in isotropic tissue expansion (Fig. 1-2B). Radial intercalation of deep cells is guided by C3a, which is a chemotactic peptide expressed by the epithelial layer to direct deep cells that express its cognate receptor C3aR (Fig. 1-2C) (Szabó et al., 2016). Concurrent with tissue expansion, the downward movement of ectoderm occurs in tandem with the inward rotation of mesoderm that begins at the dorsal marginal zone in a process called involution.
Figure 1-2 Epiboly of the ectoderm.

(A) Scanning electron micrograph of the BCR in early (left) and late (right) gastrulae. (B) Schematic diagram illustrating deep cell (left) intercalation, which leads to the thinning and spreading (right) of the ectoderm. The epithelium is omitted in this drawing. (C) Deep cell intercalation (light green) is guided by immune component C3a (red puncta) produced by the epithelium (pink). Example cell extends protrusion towards the source (dark green, left) to undergo intercalation (right). Grey circles indicate cell nuclei. Arrows indicate direction of process. Adapted from Solnica-Krezel and Sepich, 2012; Szabó et al., 2016.
1.4 Involution

Internalization of mesodermal progenitors is achieved by involution. At the beginning of gastrulation, bottle cells at the dorsal blastopore constrict their apical cell surfaces to create an inward bend in the embryo contour known as the blastopore groove. Above this groove, the blastopore lip contains mesoderm covered by a superficial layer of suprablastoporal endoderm (Essex et al., 1993). During involution, the mesoderm annulus rolls inward, beginning with the dorsal mesoderm, followed by lateral, and finally the ventral mesoderm to position the mesoderm between the ectoderm and the endodermal lining of the archenteron. It has been proposed that a “hoop stress” at the dorsal blastopore lip generated by mediolateral cell intercalation of the dorsal mesoderm acts to facilitate involution (Keller et al., 1992; Shih and Keller, 1992) by pulling the involuting marginal zone over the lip and to the interior. Such a force could work in conjunction with peak involution movements, which consists of an inward bending of dorsal mesoderm that spreads from anterior to posterior mesoderm, together with an animal–vegetal elongation and mediolateral narrowing of the dorsal lip (Evren et al., 2014) to orchestrate internalization of the dorsal mesoderm. Relative to the dorsal half, movements of the ventral blastopore remain poorly understood. However, it has been shown that Xbra-expressing cells at the blastopore lip move to the interior, and continue to move inside the embryo toward the blastocoel, suggesting that directed migration of cells contribute to mesoderm involution at the ventral blastopore (Ibrahim and Winklbauer, 2001).

Inside the gastrula, distinct mesoderm populations organize in an anterior–posterior pattern; beginning animally, these regions include the Cerberus-expressing leading edge mesendoderm (LEM), the Cerberus-Goosecoid-expressing prechordal mesoderm (PCM), and the Brachyury-
expressing chordamesoderm (CM) (Fig. 1-3A) (Damm and Winklbauer, 2011). The LEM gives rise to blood islands and ventral mesoderm in the larvae, whereas the PCM and CM produce muscles of the head and the notochord, respectively (Keller, 1976; Lane and Smith, 1999; Colas et al., 2008).

In addition to genetic differences, mesodermal regions also undergo different modes of intercellular migration. The dorsal LEM consists of spindle-like bipolar cells with multiple lamellipodia protrusions that extend toward surrounding cells. Furthermore, cells underlap one another in a shingle-like arrangement in the embryo (Fig. 1-3B) (Winklbauer and Nagel, 1991; Reintsch and Hausen, 2001). When examined on a fibronectin (FN) substrate in vitro, LEM tissue aggregates undergo isometric spreading into stationary monolayers. However, when placed on BCR pre-conditioned substrate, LEM aggregates migrate directionally toward the animal pole (Winklbauer and Nagel, 1991), which suggests that the guidance cue resides within the extracellular matrix (ECM) of the BCR. Indeed, platelet-derived growth factor alpha (PDGFA) receptor and the matrix-binding long-form lFPDGFA were found to be the guidance mechanism that directed LEM cell movement (Nagel et al., 2004).

Behind the LEM lies PCM, which internalizes ahead of the CM. PCM cells migrate at spatially graded velocities with respect to neighbouring cells. Velocity differences between leading and lagging cells expose cell gaps that are filled by the insertion of lateral cells behind the leading cell, which results in mediolateral convergence and leads to tissue elongation. (Evren et al., 2014). Moreover, from mid to late gastrulation, the PCM is transformed by thinning an initially 2.5-cell-layers-thick tissue into a monolayer. Interestingly, this radial intercalation is guided by the diffusible short-form sfPDGFA, which does not bind to ECM, but instead functions presumably as a chemotactic gradient formed by the ectoderm at Brachet’s cleft (Damm and
Winklbauer, 2011). Signaling from sfPDGFA reorients mesoderm cells towards the BCR for radial cell intercalation, and thus causes thinning and spreading of the PCM on the ectodermal surface (Fig. 1-3C).

In conjunction, the CM continues involution by radial convergence, which broadly refers to an inward movement that rotates, elongates, and thins the lip region by an unknown cellular mechanism. Inside the embryo, CM cells undergo mediolateral cell intercalation to drive convergent extension of the axial mesoderm (Fig. 1-3D). In this process, cells elongate, interdigitate, and intercalate in the mediolateral axis, which narrows the tissue to drive tissue extension in the animal–vegetal axis (Keller and Danilchik, 1988; Keller, 1984; Keller et al., 1985, Shih and Keller, 1992). Recent work has proposed that convergent extension cell movements are coordinated by septin-mediated compartmentalization of cortical actomyosin, which in turn directs junctional remodeling during cell interdigititation (Shindo and Wallingford, 2014). These two mechanisms are not mutually exclusive; in fact, it is possible that cells in this region use a combination of mediolateral intercalation together with cell-cell boundary constriction to ensure proper tissue elongation.
Figure 1-3 Mesoderm migration.

(A) Schematic of different mesoderm regions within the gastrula. The leading edge mesendoderm (LEM), prechordal mesoderm (PCM), chordamesoderm (CM), ventral mesoderm (VM), endoderm (Endo), blastocoel (BC), blastocoel roof (BCR), dorsal blastopore lip (DBPL), and archenteron (ARC) are indicated. Colours correspond to the respective tissues. (B) Schematic of LEM migration showing cells with lamellipodia (red). Select cell intercalations are shown by numbered (1–3) cells. Cell 1 remains in contact with the substrate while deeper cells interdigitate radially and insert between neighbouring cells, separating cells 2 and 3 (right). (C)
Radial intercalation of PCM cells in explants. Mesoderm cells (1–4) migrate toward the ectoderm boundary (white dashed line), interdigitating in the process (left). After 2 hour, cells had adopted a parallel configuration after interdigitating (right). Colours and corresponding cell numbers are indicated. (D) Mediolateral cell intercalation of CM cells drives convergent extension. Cells are indicated by number; select grey cells are indicated for contrast to show interdigitating cells. Arrows indicate direction of process. Adapted from Shih and Keller, 1992; Davidson et al., 2002; Damm and Winklbauer, 2011.

1.5 Vegetal Rotation

The vegetal endoderm is established by vegetal rotation. Vegetal rotation drives internalization of the endodermal vegetal cell mass in parallel to involution (Fig. 1-4) (Winklbauer and Schürfeld, 1999). The vegetal cell mass initially resembles a broadly truncated cone-shaped region delineated animal–vegetally by a narrow apex at the blastocoel floor (BCF) and a wide curved base at the vegetal epithelium, all surrounded by an annulus of mesoderm at the margin. Vegetal rotation is initiated by blastopore indentation at the vegetal edge of this mesoderm ring. The vegetal cell mass surges animally inward and turns towards the periphery near the blastocoel cavity, thus expanding the BCF while displacing the posterior mesoderm vegetally (Papan et al., 2007; Winklbauer and Damm, 2012). When examined in sagittal explants, this process appears as a rotational movement that begins at the dorsal side and then propagates to the ventral surface (Winklbauer and Schürfeld, 1999). The vegetal explant system, which uses mid-sagittal ‘slices’ of the vegetal cell mass, has been key to deconstructing how this movement is composed as a whole. Systematic dissection of explant regions has revealed that vegetal rotation is mainly
driven by endoderm, which is preprogrammed to carry out region-specific tissue-autonomous movements. Given that vegetal rotation occurs during a period of negligible cell growth or cell division (Saka and Smith, 2001; Kurth, 2005), endoderm deformation has been proposed to be driven by active cell intercalations in which cells move animally, leaving the vegetal base and inserting into the BCF. Scanning electron microscopy (SEM) of the endodermal region has revealed that endoderm cells appear elongated parallel to the BCR in the mid gastrula, which is consistent with the notion that cells undergo animally-oriented migration (Damm and Winklbauer, 2011); nevertheless, the mechanism of cell migration remains unclear.

**Figure 1-4 Schematic of vegetal rotation.**

Large-scale tissue movements of the endoderm (Endo) and peripheral mesoderm components that express Cerberus (Cer), goosecoid (Gsc), and brachyury (Xbra) are shown for stages 10, 10+, 10.5, and 11. The ectodermal BCR (Ecto) is indicated. Colours correspond to respective tissues. Dorsal is to the right. Within the tissues, single arrows indicate suggested passive movement. Double arrows indicate active movement. Inverted double arrows indicate radial intercalation. Grey arrows between stages indicate process progression. The ventral half and
suprablastoporal endoderm are omitted in the diagrams. Adapted from Winklbauer and Schürfeld, 1999.

1.6 Cell adhesion in the X. laevis gastrula

Cell rearrangement is an integral process involved in all of the morphogenetic movements described above. In this regard, proper cell migration requires spatiotemporal control of cell adhesion. In other words, cell–cell adhesion must be sufficiently strong to maintain tissue integrity, but also flexible to permit changes in cell contacts to allow cell rearrangement.

1.7 Cadherin molecules

Among adhesion molecules, classic cadherins are the best understood. Cadherins are found throughout the metazoan kingdom (Oda and Takeichi, 2011). These calcium-dependent transmembrane adhesion proteins engage in homotypic binding between adjacent cells in trans and thereby mechanically couple neighbouring cells. Cadherin molecules are composed of five tandemly repeating extracellular cadherin (EC) domains followed by a single transmembrane domain linked to a highly conserved cytoplasmic region. As implied by their name, cadherin molecules require calcium ions to function. When calcium is depleted, the extracellular domain converts from an initial rod-like structure into a globular configuration that prevents binding (Pokutta et al., 1994; Nagar et al., 1996) and intercellular adhesion.
The cadherin cytoplasmic domain can bind to β-catenin, plakoglobin, or p120-catenin (McCrea et al., 1991; Ozawa and Kemler, 1992; Aberle et al., 1996). In turn, adaptor proteins like β-catenin interact with α-catenin to link cadherins to the actin cytoskeleton. Classic vertebrate cadherins are known to mediate cell–cell adhesion in numerous embryonic systems. Pioneer experiments have shown that in the *X. laevis* gastrula, tissue cohesion is mainly mediated by cadherin-based adhesion (Heasman et al., 1994; Kuhl et al., 1996). However, only a limited number of cadherin isoforms are found at the time of gastrulation. For example, E-cadherin only becomes detectable in the late gastrula (stage 11 and beyond; Levine et al., 1994). The allelic variants EP- and C-cadherin (hereafter referred to as C-cadherin for simplicity; Levi et al., 1991) are found throughout gastrulation in conjunction with another pair of allelic variants, XB- and U-cadherin (hereafter referred to as U-cadherin; Angres et al., 1991; Herzberg et al., 1991). C- and U-cadherins are pseudoalleles generated during a tetraploidization event in *X. laevis* (Hughes and Hughes, 1993); although both are detectable in the gastrula, C-cadherin has significantly higher expression than does U-cadherin, and is therefore generally considered the main adhesion molecule of the *X. laevis* gastrula (Fig. 1-5). Seminal experiments performed using the dominant negative approach, whereby constructs were used that deleted the C-cadherin cytoplasmic domain, have shown that C-cadherin is indeed required for bulk tissue cohesion in gastrula tissues (Heasman et al., 1994; Lee and Gumbiner, 1995). However, knockdown of endogenous C-cadherin by translational blocking using morpholino oligonucleotides showed region-specific sensitivity to C-cadherin perturbation. For example, targeted knockdown of C-cadherin in the ectoderm caused cell dissociation in this tissue, whereas knockdown in the endoderm showed no discernable histological perturbations on morphogenetic movements (Ninomiya et al., 2012). These observations suggest that in addition to C-cadherin, other adhesion systems exist that mediate tissue cohesion in the gastrula.
Figure 1-5 Cadherin expression in the gastrula.

Reverse transcription polymerase chain reaction detection of XB/U-cadherin (U-Cad; 165bp) and EP/C-cadherin (C-Cad; 326bp) in stage 10 gastrula. Loading control ornithine decarboxylase (ODC; 386bp) and signal control without reverse transcriptase (-RT) are also indicated. Bands are shown at the same level for visual comparison.

1.8 Cadherin Signaling

Aside from mechanically coupling cells, cadherins are also capable of signal transduction. Pioneer experiments showed that cells in confluent cell layers exhibit cell cycle withdrawal in a phenomenon known as contact inhibition of proliferation (Eagle and Levine, 1967). For example, E-cadherin expression in a cancer cell line lacking E-cadherin showed cadherin dependent growth suppression (St. Croix et al., 1998). Moreover, it was shown that E-cadherin ligation alone was sufficient to transduce growth inhibitory signals (Perrais et al., 2007). Recently, a growing body of evidence has implicated cadherins in regulating cytoskeletal organization (Ratheesh and Yap, 2012). For example, loss of E-cadherin was shown to alter tubulin arrangement and actin density in mammary epithelial cells (Chen et al., 2014).
Overexpression of dominant negative N-cadherin deformed dendritic spine morphology by converting mushroom shaped spines into filopodia processes rich in polymerized actin (Togashi et al., 2002). In light of these findings, it is becoming increasingly clear that in addition to mechanically coupling cells together, cadherin molecules can also modulate the cytoskeleton, although the mechanism of its regulation remains to be elucidated.

1.9 Catenin

Cadherin interaction with the cytoskeleton is required for cell adhesion. To this end, the cadherin cytoplasmic domain contains two catenin-binding domains (CBD), which bind p120-catenin along with an extended region on the C-terminus that binds β-catenin (Hartsock and Nelson, 2008). Association of p120-catenin has been proposed to stabilize cadherin at the plasma membrane, whereas association with β-catenin facilitates binding to α-catenin, which links the cadherin-complex to the actin cytoskeleton. Indeed, α-catenin could bind to β-catenin, and bundle F-actin filaments in vitro (Aberle et al., 1994; Rimm et al., 1995). However, the exact configuration of the cadherin–catenin complex remains to be solved in vivo. Nevertheless, it is interesting to note that within cells of vertebrate and invertebrate organisms (such as canine and Drosophila melanogaster), α-catenin is sequestered into two distinct domains: in a monomeric membrane-bound fraction, found within the cadherin complex, and a homodimeric pool that exists within the cytosol (Drees et al., 2005; Desai et al., 2013). The membrane-bound fraction is typically associated with mediating cell–cell adhesion, but less is known about the functional role(s) of the cytosolic fraction. With respect to adhesion, it could be that the two pools of α-catenin work synergistically to promote cell-cell adhesion in two ways: 1) by acting as the
physical link between the cadherin complex and the actin cytoskeleton in the membrane-bound fraction, and 2) by regulating actin organization to support adherens junction formation in the cytosolic fraction (Yamada et al., 2005). Evidence for the latter includes observations that α-catenin dimers can regulate protrusion formation and actin ultrastructure modification in vitro (Benjamin et al., 2010).

1.10 Eph and ephrin molecules

The erythropoietin-producing hepatocellular (Eph) family of transmembrane receptors and their corresponding Eph family receptor interacting (ephrin) ligands are involved in many cell processes, including cell–cell adhesion, repulsion, migration and sorting, all of which are relevant to gastrulation (Pasquale, 2005). The Eph receptors represent the largest subset of all receptor tyrosine kinases (RTK) in the human genome (Himanen and Nikolov, 2003). Eph receptors are found in vertebrates and invertebrates; for example, Eph receptors have been found in Caenorhabditis elegans (George et al., 1998), D. melanogaster (Scully et al., 1999), and sponges (Suga et al., 1999), which suggests that they are highly conserved molecules within the animal kingdom. Eph receptors and ephrin ligands are classified into two major classes, A and B. Receptors EphA and EphB are both transmembrane RTK molecules, and are separated by amino acid sequence homologies and different ligand binding affinities (Fig. 1-6). EphA receptors preferentially bind to class ephrinA ligands, and EphB receptors to ephrinB ligands, although exceptions exist. For example, EphA4 receptors can bind to both ephrinB and ephrinA class ligands, albeit with differing affinities. In fact, promiscuous binding is a known characteristic of Eph–ephrin interactions (Himanen et al., 2007).
Structurally, Eph receptors are composed of an extracellular ligand-binding (LB) domain joined by a cysteine-rich (CR) region to two fibronectin type-III (2FNIII) repeats connected to a transmembrane domain (TM) that spans into the cytoplasmic domain components, which include two conserved tyrosine residues (2Y), a protein tyrosine kinase domain (Kinase), a sterile-α-motif (SAM), and a Post-synaptic-density-protein Drosophila-disc-large-tumor-suppressor Zonula-occludens-1-protein (PDZ) binding domain. Although the functional relevance of the different extracellular domains is not yet fully resolved, several studies have suggested that the LB and CR domains contain Sushi-epidermal growth factor (EGF) protein interface sequences that promote receptor oligomerization and clustering behaviour during signaling (Himanen et al., 2010; Seiradake et al., 2010), whereas the intracellular SAM domain is thought to stabilize the established clusters (Smalla et al., 1999; Stapleton et al., 1999).

The ephrin ligands are subdivided based on structural features. The A-class ligands have a receptor binding (RB) domain tethered onto the plasma membrane by a glycosylphosphatidylinositol (GPI) anchor. In contrast, ephrinB ligands are transmembrane molecules that consist of an RB extracellular domain with a cytoplasmic PDZ-binding motif (Pasquale, 2005). Because ephrin ligands are anchored to the plasma membrane, signal induction typically requires direct cell–cell contact. Furthermore, Eph–ephrin binding can induce signaling in the receptor-bearing cell (forward signaling), the ligand-expressing cell (reverse signaling), or both cells simultaneously (bidirectional signaling) (Fig. 1-6). The type of signaling that occurs is determined by the cell type and the signaling context of the interacting cells (Salaita and Groves, 2010).
Figure 1-6 Structural schematic of Eph receptors and ephrin ligands.

(A) Domain composition of EphA receptors and ephrinA ligands. EphA consists of an extracellular ligand-binding (LB) domain with a cysteine-rich (CR) region consisting of 20 conserved cysteine residues, two fibronectin type-III repeats (2FNIII), a transmembrane (TM) domain, two tyrosine residues (2Y), a tyrosine kinase (Kinase) domain, sterile-α-motif (SAM), and a Post-synaptic-density-protein *Drosophila*-disc-large-tumor-suppressor Zonula-occludens-1 (PDZ) binding domain. EphrinA consists of an extracellular receptor binding (RB) domain with a glycosylphosphatidylinositol (GPI) anchor to the plasma membrane. (B) EphB receptors are structurally similar to EphAs with the exception that the ligand-binding domain binds preferentially to ephrinB ligands. The ephrinB ligands have an extracellular RB domain, a TM region, and a PDZ binding domain. Double grey lines indicate the plasma membrane. Forward signaling (green arrows, left) occurs downstream of the Eph-receptor-bearing cell, and reverse
signaling (green arrows, right) occurs downstream of the ligand-bearing cell. Intra- and extracellular spaces are indicated for spatial context.

Since the initial documentation of EphA1 in human cells (Hirai et al., 1987), numerous Eph and ephrin molecules have been discovered across vertebrate species, although a common nomenclature was not established until 1997 (Table 1). Therefore, various species-specific names have been used to denote otherwise homologous molecules. This inconsistency has been reconciled, and to date, there are 14 known members of Eph receptors (EphA1–8, EphB1–6) and 8 ephrin (ephrinA1–5, ephrinB1–3) ligands (Eph Nomenclature Committee, 1997).

**Table 1 Nomenclature of Eph and ephrin molecules.**

The standardized receptor and ligand names (EphA1–8, EphB1–6, ephrinA1–5, ephrinB1–3) are shown on the left. Names previously used in the literature for mammals and birds are shown in the center. Semicolons indicate hypothetical orthologs or proposals by the Eph–ephrin naming committee to rename existing molecules. Names used in the literature for *X. laevis* molecules before the naming consolidation are shown on the right.
<table>
<thead>
<tr>
<th>Name</th>
<th>Previous name(s)</th>
<th>Xenopus</th>
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<tbody>
<tr>
<td>Mammals &amp; Birds</td>
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**Receptors**

| EphA1 | Eph, Esk          |
| EphA2 | Eck, Myk2, Sek2   |
| EphA3 | Cek4, Mek4, Hek, Tyro4; Hek4 |
| EphA4 | Sek, Sek1, Cek8, Hek8, Tyro1 |
| EphA5 | Ehk1, Bsk, Cek7, Hek7; Rek7 |
| EphA6 | Ehk2; Hek12       |
| EphA7 | Ehk3, Mdk1, Hek11, Ebk, Cek11 |
| EphA8 | Eek; Hek3         |
| EphB1 | Elk, Cek6, Net; Hek6 |
| EphB2 | Cek5, Nuk, Erk, Qek5, Tyro5, Sek5; Hek5, Drt |
| EphB3 | Cek10, Hek2, Mdk5, Tyro6, Sek4 |
| EphB4 | Htk, Myk1, Tyro11; Mdk2 |
| EphB5 | Cek9; Hek9        |
| EphB6 | Mep               |

**Ligands**

| ephrinA1 | B61; LERK1, EFL1 |
| ephrinA2 | ELF1; Cek7-L, LERK6 |
| ephrinA3 | Ehk1-L, ELF2, LERK3 |
| ephrinA4 | LERK4; EFL4       |
| ephrinA5 | AL1, RAGS; LERK7, EFL5 |
| ephrinB1 | LERK2, Elk-L, EFL3, Cek5-L; STRA1 |
| ephrinB2 | Htk-L, ELF2; LERK5, NLERK1 |
| ephrinB3 | NLERK2, Elk-L3, EFL6, ELF3; LERK8 |
1.11 Eph and ephrin signaling

As previously described, Eph–ephrin signaling could occur in a unidirectional manner whereby a ligand-induced signal is perceived by the receptor-bearing cell to transduce ‘forward’ signaling downstream of the Eph receptor. Receptors in Eph-expressing cells are dispersed throughout the plasma membrane and display minimal kinase activity (Vearing and Lackmann, 2005). In the absence of ligand, signaling is auto-inhibited due to a fold in the juxtamembrane region that prevents ATP hydrolysis and phosphorylation. Ligand binding induces a conformational change that allows activation of the kinase domain (Wybenga-Groot et al., 2001), which leads to autophosphorylation of the tyrosine residues. This activation is similar to what has been demonstrated for other receptor tyrosine kinases such as the platelet-derived growth factor receptor β (Baxter et al., 1998). Receptor–ligand interactions lead to rapid assembly of Eph–ephrin hetero-tetramers that expand laterally into higher-order clusters (Wimmer-Kleikamp et al., 2004), which have been proposed to dictate the function and strength of the biological response (Stein et al., 1998; Gauthier and Robbins, 2003). Clustering is facilitated by Eph–ephrin localization within specialized sphingolipid- and cholesterol-rich membrane rafts (Brückner et al., 1999; Simons and Toomre, 2000). Furthermore, activation of Eph–ephrin signaling has been shown to promote the formation of membrane rafts in vitro (Brückner et al., 1999), which suggests the presence of a positive feedback mechanism for multimerization.

A variety of cellular responses can be induced by Eph–ephrin signaling, typically depending on the type of receptor(s) and ligand(s) involved. The best-known example of an A-class Eph–ephrin interaction is contact-mediated repulsion, which occurs during axon guidance. Pathfinding in the visual system is dependent upon EphA receptors expressed in the retina. The receptor-
bearing neurons will project axons to their target in the region of the brain called the superior colliculus, which expresses the complimentary ephrinA ligand. Both ligand and receptor are expressed in a graded manner such that the increasing concentration of ligand will eventually induce repulsion of EphA expressing axons, and thus result in a stop signal that matches the concentration of EphA receptors on the axon (O’Leary and Wilkinson, 1999). Upon receptor activation, scaffold molecules are recruited to the cytoplasmic tail to transmit the signal. These proteins usually contain protein–protein interacting domains, such as SRC-homology 2 (SH2) and SH3, and function to facilitate the formation of protein complexes during cell signaling events (Kullander and Klein, 2002). In turn, these adaptor complexes associate with effectors such as the Eph-interacting exchange protein (Ephexin), which is a guanine nucleotide exchange factor for Rho GTPases to link Eph-ephrin signaling to functional changes in the cytoskeleton (Shamah et al., 2001). The binding of PDZ-domain-containing proteins to the carboxyl end of Eph receptors also contributes to signaling. Notable effectors that use this mechanism include Rho- and Ras-family GTPases, Dishevelled, Akt, and more (Steinle et al., 2002; Tanaka et al., 2003; Noren and Pasquale, 2004; Nakada et al., 2005; Dail et al., 2006; Kida et al., 2007).

Interestingly, despite having no intracellular domain, ephrinAs are also capable of signaling (Wang et al., 1999). A signaling event downstream of the ligand-bearing cell is considered ‘reverse’ signaling. Several downstream effectors, such as the Src-family kinases (SFK), extracellular signal-regulated kinases (ERK) 1/2, Rac, and Akt, have been shown to mediate ephrinA reverse signaling, although the mechanism of how this occurs is not yet clear (Xu and Henkemeyer, 2012). Reverse signaling is perhaps best understood in the ephrinB class of molecules because these ligands have cytoplasmic domains with known motifs for protein–protein interactions (e.g., PDZ, SH2). Signaling may be transduced by SH2-PDZ-dependent and/or SH2-PDZ-independent pathways (Daar, 2011), although the mechanism of the latter
remains unclear. A number of ephrinB-interacting partners have been identified; for example, those that use the SH2-PDZ-dependent pathway include the GTP exchange factor regulator of G-protein signaling 3 (RGS3; Lu et al., 2001), the adaptor protein growth factor receptor bound protein 4 (Grb4; Cowan and Henkemeyer, 2001), and the transcription factor signal transducer and activator of transcription 3 (STAT3; Bong et al., 2007). Those that use the SH2-PDZ-independent pathway include the planar cell polarity related partitioning-defective protein 6 (PAR-6; Lee et al., 2008), the scaffold protein Dishevelled (Dsh; Lee et al., 2006), and the gap junction protein connexin 43 (Cx43; Davy et al., 2006).

1.12 EphB–ephrinB interactions in tissue morphogenesis

The EphB–ephrinB signaling cascades have been implicated in numerous processes during development. In particular, EphB–ephrinB signaling is primarily involved in tissue morphogenesis; such as in the control of embryonic germ layer separation (Rohani et al., 2011), hindbrain segmentation (Xu et al., 1995, 1999; Mellitzer et al., 1999; Cooke et al., 2001), and vascular patterning (Helbling et al., 2000). Currently, as in the above examples, EphB–ephrinB signaling is best understood in complimentary interactions in which one tissue expresses the ligand while the adjacent tissue expresses the cognate receptor. In such cases, receptor–ligand interactions between tissues typically result in repulsion, which leads to the establishment or maintenance of tissue compartmentalization, and thus prevents intermingling between cells of adjacent tissues.
In comparison, relatively little is known about overlapping Eph–ephrin interactions within tissues. For example, recent work has demonstrated that ephrinB2 and its cognate receptor EphB4 are co-expressed in all tissues of the X. laevis gastrula, and yet, they function to mediate tissue separation specifically at the ecto-mesoderm boundary (Rohani et al., 2011). One plausible explanation is that EphB–ephrinB interactions within tissues must overcome a threshold to initiate tissue separation. Below this threshold, EphB–ephrinB interactions may act to modulate the level of cohesion within a tissue. It has been noted that cis-interaction between receptor and ligand on the same surface can attenuate Eph forward signaling (Böhme et al., 1996; Yin et al., 2004). However, separation of receptor and ligand into different lipid rafts on the cell surface can allow for parallel bidirectional signaling in the same cell (Marquardt et al., 2005; Kao and Kania, 2011). The study of Eph–ephrin interactions is further complicated by the fact that receptor–ligand binding can be promiscuous, meaning that receptors can potentially bind to different ligands within its class or with those of a different class. For example, EphA4 can bind to ephrinA ligands, as well as to ephrinBs (Smith et al., 1997). Likewise, EphB2 can interact with ephrinA5 (Himanen et al., 2004). Moreover, given the multitude of receptor and ligand combinations that overlap in spatial and temporal expression (Rohani et al., 2011), functional redundancies must be considered. For example, in mice, individual deletion of EphB2 or EphB3 did not induce lethality, whereas EphB2 and EphB3 double mutants died at birth (Henkemeyer et al., 1996). Furthermore, ephrinB1 controls epithelial junction integrity through the Par complex (Lee et al., 2008), whereas EphA4 is also involved in regulating cell polarity and junction assembly in the early embryo (Winning et al., 2001).
1.13 Eph–ephrin signaling in the X. laevis gastrula

A growing body of evidence in the literature implicates Eph–ephrin signaling in the control of cell movement by imposing their effects on the actin cytoskeleton, which leads to changes in cell shape, adhesion, and movement strategy through regulating Rho GTPases, which include RhoA, Rac1, and Cdc42 (Pasquale 2008, 2010). Changes in cell motility are important during early embryogenesis, particularly during morphogenetic events such as gastrulation, which occurs in X. laevis at a time of negligible cell growth or division (Saka and Smith, 2001; Kurth, 2005), when cells must migrate to specific destinations to establish patterning. Indeed, Eph–ephrin signaling has been implicated in numerous tissue morphogenetic events relevant to X. laevis gastrulation. For example, in the ectoderm, ephrinB1 is required for proper movement of cells in the anterior ectoderm into the eye field (Moore et al., 2004). In the mesoderm, EphA4 and ephrinB2 are required for mesoderm involution (Evren et al., 2014). Between the ecto- and mesoderm boundary, EphB–ephrinB signaling controls tissue separation at Brachet’s cleft via contact-induced cell detachment (Rohani et al., 2011; Rohani et al., 2014). Similarly, EphA4–ephrinA1 interactions are also involved in boundary formation (Park et al., 2011). However, the molecular regulation of these processes have yet to be fully elucidated, and further work is required to understand the detailed mechanisms that lead to these diverse cellular behaviours. Moreover, as previously stated, the X. laevis gastrula expresses all known EphB–ephrinB receptors and ligands (Rohani et al., 2011), but a full catalog of the functional roles of individual molecules in a tissue-specific context has not yet been established.

Because disease is often considered development gone awry, and given the above-described importance of Eph–ephrin interactions in gastrulation processes, it is unsurprising that aberrant
signaling has been implicated in a variety of congenital tissue disorders, such as Craniofrontonasal Syndrome (CFNS; Twigg et al., 2004; Wieland et al., 2004), and various diseases, such as cancers (Surawska et al., 2004; Ding et al., 2008; Peifer et al., 2012), which have been linked to mutation(s) in specific receptors and/or ligands. Therefore, further characterization of interactions between receptors and ligands will advance the repertoire in the literature, provide a deeper understanding of the role of Eph–ephrin interaction in developmental processes, and arm clinicians with new strategies in the fight against disease.

1.14 Thesis objectives

As mentioned above, the early embryo in most animals consists of a single layer of cells that forms a hollow sphere. This simple structure first gains complexity by organizing into multiple layers that are fated to become specialized tissues in the adult. To form the primordial gut, the endoderm must move from the surface to the interior during gastrulation. In *X. laevis*, endoderm internalization is achieved through vegetal rotation. Furthermore, the *X. laevis* gastrula expresses a full complement of ephrinB ligands; however, the functional contribution of these molecules to morphogenetic processes such as gastrulation is not fully understood. The main objective of this thesis is to evaluate the role of ephrinB1 in the control of *X. laevis* gastrulation, particularly during vegetal rotation. Additionally, ephrinB2 and ephrinB3 manipulations were performed where possible, and the results were included for a comparative analysis of ephrinB1 function.
1.14.1 CHAPTER TWO

In light of the finding that overexpression of ephrinB1 caused cell dissociation in all tissues of the X. laevis embryo (Jones et al., 1998), I systematically manipulated ephrinB1 protein levels in all three X. laevis germ layer tissues by mRNA overexpression or knockdown using morpholino oligonucleotides. I assessed the effect of ephrinB1 perturbations on tissue morphogenesis in all gastrula tissues. I used bright-field, epifluorescence, and laser scanning confocal microscopy to examine tissue morphological changes in mid-sagittally bisected embryos and region-specific tissue explants. Furthermore, I quantified the effect of ephrinB1 perturbations on tissue cohesion and tissue viscosity in gastrula tissues.

1.14.2 CHAPTER THREE

In invertebrates such as sea urchins, gastrulation entails bending the embryonic surface sheet inward at a predetermined site to generate the primordial gut. Less is known about gastrulation in vertebrates. In X. laevis, major tissue bending is not observed as the bending resulting from “bottle cell” formation is mainly local and contribute little directly to internalization, and cells do not leave the surface to move inside during gastrulation. Thus, the cellular mechanisms responsible for driving vegetal rotation were not known. Using high-resolution fluorescence microscopy in conjunction with scanning and transmission electron microscopy, I characterized cell rearrangements and assessed the requirement of fibronectin and C-cadherin during endoderm cell migration.
1.14.3 CHAPTER FOUR

The ability of cells to detach from one another is essential to cell migration. During endoderm cell detachment, cells narrow their trailing edge and retract their cell rear to complete rearrangement. However, the mechanism of cell-rear membrane remodeling during trailing edge retraction was not understood. EphB-ephrinB molecules and their corresponding interactions were shown to be crucial in regulating cell detachment (Kullander and Klein, 2002). Specifically, ephrinB1 was shown to mediate detachment by trans-endocytosis of cell–cell contacts (Marston et al., 2003; Zimmer et al., 2003). For this chapter, I used laser scanning confocal microscopy in combination with scanning electron and transmission electron microscopy to characterize endoderm cell retraction process. Furthermore, I analyzed the localization of ephrinB1 and its role in endoderm trailing edge membrane remodeling.

1.14.4 CHAPTER FIVE

Remarkably, the level of autonomous movement during endoderm differential migration is programmed to the single cell level. In contrast to classic models of single cell migration in vitro, where cells move by propelling forward using locomotory protrusions, endoderm cells lack such protrusions. Instead, endoderm cells generate movement through choreographed cell shape changes, reminiscent of cell behaviours that have been characterized for cell ingression. In this chapter, I described endoderm cell behaviours observed during endoderm internalization with
respect to other vertebrate and invertebrate systems to reconnect vegetal rotation to common mechanisms of animal gastrulation.
2. CHAPTER TWO: A role for ephrinB1 in the modulation of cell-cell adhesion in the *Xenopus* gastrula

2.1 Introduction

Intercellular migration is integral to tissue morphogenesis during gastrulation. Cell–cell adhesion must be sufficiently strong to maintain tissue cohesion, flexible to permit cell rearrangement, and selective to avoid improper mixing of tissues. In essence, the modulation of cell–cell adhesion is a fundamental requirement for proper tissue morphogenesis. A remarkable example of such a flexible adhesion system is found at the ecto–mesoderm tissue boundary known as Brachet’s cleft. Here, ecto- and mesoderm cells adhere, but despite the lack of an intact physical barrier (i.e., a basal lamina), cells do not intermix. Furthermore, when these two cell populations are mixed experimentally, they sort out *in vitro* (Krieg *et al*., 2008; Ninomiya and Winklbauer, 2008). It was revealed that contact-induced cell detachment is a core mechanism of ecto-mesoderm boundary maintenance at Brachet’s cleft. To preserve tissue separation, ecto and mesodermal cells in contact undergo repeated cycles of cell adhesion and detachment. This process allows for the establishment of cell–cell contact at the boundary for signaling, whilst segregating cells to their respective tissues, and permits the migration of mesoderm cells across an ectodermal substratum. Molecularly, this process is maintained by a quantitative balance of EphB–ephrinB signaling across the boundary (Rohani *et al*., 2011), in conjunction with Snail1/PAPC-dependent tissue separation (Luu *et al*., 2015).
Such an example also reflects the condition in which EphB–ephrinB signaling is best understood; that is, in complementary receptor–ligand interactions between two different adjoining tissues (for example, in rhombomere boundary formation, somite segmentation, and the aforementioned germ layer separation; Durbin et al., 1998; Cooke et al., 2005; Park et al., 2011; Rohani et al., 2011). Nevertheless, it is also important to consider that EphB–ephrinB interactions could occur autonomously, such as in the case where both a ligand and its cognate receptor are co-expressed in the same tissue, although the functional relevance of tissue autonomous EphB–ephrinB interactions is poorly understood. In complementary interactions between different adjoining tissues, EphB–ephrinB signaling reportedly leads to repulsive effects between tissues; therefore, it is unclear how the tissue remains cohesive when both ligand and receptor are co-expressed within the same tissue. For example, the ligand ephrinB1 and one of its reported receptors EphB4 are both expressed in the ectoderm (Rohani et al., 2011), yet the ectoderm remains cohesive throughout gastrulation. One plausible explanation is that EphB–ephrinB interactions have to overcome a threshold in order to achieve de-adhesion; however, below this threshold, EphB–ephrinB signaling could modulate the level of cohesion within the tissue. Evidence in support of this claim was reported by Jones and colleagues, who showed that overexpression of ephrinB1 induced cell dissociation in X. laevis blastulae without altering the level of C-cadherin (Jones et al., 1998). This result confirms the link between ephrinB1 interactions and tissue cohesion in X. laevis; however, the mechanism by which ephrinB1 acts to regulate tissue cohesion is not yet known.

A deeper understanding of the role of ephrinB1 within tissues is particularly important because loss-of-function mutations in the ephrinB1 gene are linked to severe tissue malformation in humans known as Craniofrontonasal Syndrome (CFNS). CFNS is a genetically rare but physically debilitating disorder that typically includes numerous tissue malformations such as
orbital hypertelorism, brachycephaly, and craniosynostosis, thoracic abnormalities, scoliosis of the spine (Twigg et al., 2004; Wieland et al., 2004; Evers et al., 2014). The gene encoding ephrinB1 is located on the X-chromosome in humans, whereas it is located on chromosome 8 in *X. laevis*. Thus, it is categorized as an X-linked disorder in humans and females are more severely affected than males. Presently, it is hypothesized that severe tissue dysmorphia occurs in females as a result of X-inactivation, which generates a heterogeneous expression of normal ephrinB1 expressing cells with cohorts of mutated ephrinB1 expressing cells that produce a truncated variant of ephrinB1 protein, thus leading to homophilic cell sorting within tissues and causing abnormal tissue compartmentalization and malformation. Given that ectopic cell sorting in tissues has been associated with the inhibition of gap junction communication, the proposed mechanism of abnormal cell sorting in CFNS has been termed “cellular interference” (Wieland et al., 2008). Although mosaic expression of ephrinB1 is apparent in heterozygous ephrinB1-mutant mice (Compagni et al., 2003), direct evidence is lacking for homophilic cell sorting *in vivo*. Moreover, mechanisms that induce cell sorting *in vitro*, such as differential expression of cadherin molecules, do not necessarily lead to cell sorting *in vivo* (Ninomiya et al., 2012). Thus, the cellular mechanism of underlying ephrinB1-induced CFNS is not fully understood.

It was recently discovered that ephrinB1 is essential for maintaining cardiac tissue cohesion in mice (Genet et al., 2012), which suggests that ephrinB1 could affect tissue morphogenesis by modulating tissue biomechanical parameters. The study of tissue mechanical properties has traditionally been difficult in model organisms such as mice due to the inaccessibility of specific tissues during early embryogenesis. In this regard, *X. laevis* offers a distinct advantage due to its external development and large, easily accessible tissues. Furthermore, it has been demonstrated that *X. laevis* embryonic tissue properties mimic those of a viscoelastic fluid (Forgacs et al., 1998). Therefore, fluid dynamics assays can be used to elucidate *X. laevis* tissue mechanical
properties. For example, when irregularly shaped tissue aggregates are excised from *X. laevis* embryos, they round up and adopt drop-shaped configurations *in vitro*. In essence, the drop-shaped outline of rounded aggregates can be interpreted as a compromise between the forces of surface tension and gravity. Tissue surface tension acts to maximize intercellular adhesion while minimizing the surface area, thus keeping the aggregate round, while gravity works in opposition to flatten the aggregate. Under the influence of these forces, the resultant tissue aggregate drop-shape is a reflection of the intensity of adhesive forces between cells; specifically, tissue surface tension is proportional to the adhesion between its constituent cells. In other words, highly cohesive tissues have strong adhesion between cells and thus would be better able to retain a more spherical drop-shape, whereas less cohesive tissues are likely to deviate from a sphere and adopt ellipsoid configurations (David *et al*., 2014). A similar condition based on intercellular behaviour can be applied to evaluate the resistance to cell rearrangement within tissues. The resistance of a fluid to shear motion can be measured as viscosity. The higher the viscosity, the more time it will take for the liquid to shear. When applied to tissue rounding, the rate of rounding is proportional to the resistance of cells within the tissue to undergo rearrangement. In other words, the more viscous a tissue is, the more time it takes for that tissue to round up.

Together, tissue surface tension and tissue viscosity provide quantitative means to assess large-scale biomechanical properties of *X. laevis* embryonic tissues (David *et al*., 2014).

Members of the EphB and ephrinB family of molecules are heterogeneously expressed in all tissues of the *X. laevis* gastrula, which thus creates a challenging landscape for comprehensive analysis of all EphB–ephrinB interactions. Nevertheless, ephrinB1 is ubiquitously expressed by all gastrula tissues (Rohani *et al*., 2011), and thus provides a reasonable starting point for analysis. For this chapter, I investigated ephrinB1 and its role in the modulation of cell–cell adhesion in the three germ layer tissues. I examined the effects of ephrinB1 manipulation on
gastrulation as a whole, on specific tissue morphology, tissue cohesion, and cell rearrangement. When possible, I included other EphB and ephrinB molecules (for example, EphB4, and ephrinB2–3) for comparative functional analysis. In light of the report that overexpression of ephrinB1 induced cell dissociation in *X. laevis* blastulae, I hypothesized that ephrinB1 functions to downregulate the level of cell adhesion within all germ layers of the gastrula such that knockdown of ephrinB1 in germ layer tissues will increase respective tissue cohesion and consequently attenuate cell rearrangement during active processes such as intercellular migration.
2.2 Results

As previously noted, members of the EphB and ephrinB families of molecules are expressed in all *X. laevis* gastrula tissues (Rohani et al., 2011); however, the functions of these molecules have not been fully elucidated. Protein structure is linked to protein function. Presently, however, structural similarities or differences between EphB and ephrinB molecules in *X. laevis* are not known. The first documented EphB receptor crystal structure was mouse EphB2 (Himanen et al., 1998), which provided valuable insight into EphB–ephrinB interactions. For instance, the structure of EphB2 revealed an unexpected “jellyroll” folding architecture in the amino terminal that shed light on ligand recognition (Himanen et al., 1998; Himanen, 2012). Since that discovery, numerous publications have focused on the crystal structures of mouse and human EphB and ephrinB molecules (Himanen et al., 2001; Toth et al., 2001; Himanen and Nikolov, 2002; Goldgur et al., 2009; Qin et al., 2010).

Presently, no such data exists for *X. laevis*; however, protein structure could be approximated if the underlying amino acid sequence is known, using the bioinformatics tool Protein Homology/analogy Recognition Engine version 2.0 (PHYRE2). During the composition of this thesis, some sequences of EphB molecules for *X. laevis* were incomplete; therefore, several molecules are notably absent. For instance, EphB2 (Accession no. AF026039.1) is missing because the amino acid sequence is incomplete. The allelic variant of EphB4-a (Accession no. AJ236867.1) is also incomplete.
2.2.1 Predicted structures of *X. laevis* EphB and ephrinB

Using PHYRE2, I assembled a catalog of predicted three-dimensional structures for *X. laevis*-specific EphB and ephrinB class molecules from existing sequences deposited in the National Center for Biotechnology Information (NCBI) database (Fig. 2-1). All EphB receptors (with the exception of EphB2, which currently contains an incomplete sequence) were predicted with a high level of accuracy with over 85% of amino acid residues modelled with 90% confidence. In the predicted models, key features of EphB receptors such as ligand-binding domains are visibly diverse among EphB isoforms (Fig. 2-1A). For example, EphB1 and EphB3 incorporate a straight “rod-like” morphology that extends throughout the entire molecule, whereas EphB4 adopts a “kinked” configuration in the extracellular domain. Unlike their cognate receptors, the ephrinB ligands were modelled with low reported confidence levels (Fig. 2-1B). Only 42–43% of ephrinB residues were modelled with 90% confidence. The presence of highly disordered structures within ligands could be linked to their low predictive scores. Indeed, an average of 41% of ligand structures were classified as disordered (meaning that they were neither α-helices nor β-strands) compared with an average of 25% disorder reported for the receptors. Nevertheless, the ligands all appear to share similar structural features, which suggests a high degree of sequence homology.
Figure 2-1 Predicted structures of EphB and ephrinB class molecules in *X. laevis*.

(A) EphB receptor structures for EphB1, -B3 and -B4-b. EphB2 structure was not available and is represented here by a boxed question mark. (B) The ephrinB ligand structures for ephrinB1, -B2-a, and -B3. Molecules are arranged such that the N-terminus is located at the top and the C-terminus at the bottom. NCBI accession numbers: EphB1 (U14164.1), EphB3 (NM_001087965.1), EphB4-b (NM_001136172.2), ephrinB1 (U31427.2), ephrinB2-a (EU334664.1), and ephrinB3 (AJ236866.1). Molecule sizes are not to scale. Predicted structures are generated and arbitrarily coloured using rainbow N→C terminus by the Protein Homology/analogy Recognition Engine algorithm (PHYRE 2.0; Kelley *et al.*, 2015).
2.2.2 Sequence of *X. laevis* EphB receptors and ephrinB ligands

I first quantified the sequence homology between EphB receptors (Fig. 2-2A-B), and then used the corresponding amino acid sequences of EphB1, EphB3, and EphB4 to reconstruct their phylogenetic tree (Fig. 2-2C). The EphB receptors had relatively variable sequences at the N-terminus, which codes for the extracellular ligand-binding domain, consistent with different ephrinB ligand affinities reported for each of the three receptors (Fig. 2-2A).

To quantify the degree of similarity between receptors, analysis of protein identity showed that EphB1 shares 54% sequence identity with EphB4 and 67% with EphB3. Moreover, EphB3 shares 56% identity with EphB4 (Fig. 2-2B). Together, these results corroborate the structural data (Fig. 2-1) which suggest that EphB1 and -B3 share a close sequence relationship; however, both receptors (B1 and B3) appear to be divergent from EphB4.
Figure 2-2 Protein coding sequence analysis of *X. laevis* EphB receptors.

(A) Amino acid sequence alignment of EphB1, EphB3, and EphB4. The N-terminus is located at the top and the C-terminus the bottom. Asterisks indicate conserved residues. Colons indicate conservation between groups with strongly similar properties. Periods indicate conservation between groups with weakly similar properties. Conservation properties were determined using the Clustal Omega sequence alignment algorithm. Hyphens within the amino acid sequence were added by the program to optimize alignment by maximizing similarities. (B) Percent amino acid
identity score matrix for EphB1, EphB3, and EphB4. (C) Phylogenetic tree comparing EphB1, EphB3, and EphB4. Phylogenetic tree was constructed using neighbour joining. Branch length is proportional to the difference between sequences; length values are shown on the right.

Next, I compared the amino acid sequences of ephrinB1–B3 (Fig. 2-3). Similar to the receptors, ephrinB ligands were most dissimilar at the N-terminus, which codes for the receptor-binding domain (Fig. 2-3A). In contrast, all ephrinB ligands share a highly conserved C-terminus. Amino acid sequence analysis revealed that ephrinB1 shares 57% identity with ephrinB2, which is a closer relationship than the 48% identity that ephrinB1 shares with ephrinB3. In addition, ephrinB2 shares 52% identity with ephrinB3 (Fig. 2-3B). Interestingly, despite the higher percentage of sequence homology between ephrinB2 and -B3 compared to ephrinB1 and -B3, phylogenetic analysis indicated a closer relationship between ephrinB1 and -B3 (Fig. 2-3C). This could be because sequence homology is scored based on local sequence alignments whereas phylogenetic analysis involves a more comprehensive approach and accounts for structural homology. In other words, homologous domains can exist outside of sequence alignment regions such that proteins with low sequence identity could still share a close common ancestry. An example for such a case was reported for Drosophila Mud, which is homologous to human NuMA despite sharing less than 27% sequence identity (Bowman et al., 2006).
Figure 2-3 Protein coding sequence analysis of *X. laevis* ephrinB ligands.

(A) Amino acid sequence alignment of ephrinB1–3. The N-terminus is located at the top and the C-terminus the bottom. Letters represent amino acid residues. Asterisks indicate conserved residues. Colons indicate conservation between groups with strongly similar properties. Periods indicate conservation between groups with weakly similar properties. Conservation properties were determined by the Clustal Omega sequence alignment algorithm. Hyphens within the amino acid sequence were added by the program to optimize alignment by maximizing similarities. (B) Percent amino acid identity score matrix for ephrinB1–3. (C) Phylogenetic tree comparing ephrinB1–3. Phylogenetic tree was constructed using neighbour joining. Branch length is proportional to the difference between sequences; length values are shown on the right.
2.2.3 Epiboly is perturbed but not abolished by ephrinB1 knockdown

EphrinB structures were predicted with a relatively high degree of confidence. All three ligands were found to be expressed in the *X. laevis* gastrula, albeit at different levels in a tissue-dependent manner (Rohani *et al.*, 2011). EphrinB1 in particular has been shown to regulate cell adhesion in *X. laevis* embryonic tissues (Jones *et al.*, 1998). Given that ephrinB1 is highly expressed in the ectoderm of *X. laevis* gastrulae, I first examined the effects of ephrinB1 manipulation on the ectoderm during epiboly (Fig. 2-4). To assess an ectodermal requirement for ephrinB1, I knocked down ephrinB1 protein levels by injection animally, at the four-cell stage of a previously characterized morpholino oligonucleotide (Rohani *et al.*, 2011).

Morphant embryos were allowed to develop in parallel with uninjected control embryos from the same clutch. I found that ephrinB1 morphant embryos initiated gastrulation at the same time as controls, as indicated by concurrent formation of the dorsal blastopore lip (not shown). To examine the ectoderm, I fractured embryos in the mid-sagittal plane at different stages of gastrulation and found that the BCR in morphant embryos was consistently thicker than in correspondingly staged controls (Fig. 2-4). Given that morphant BCR was comparatively thick at the onset of gastrulation (stage 10), this result suggests that ectodermal tissue was thickened prior to gastrulation. Mid-sagittal gastrulae fracture preparations also showed that morphant ectoderm thickness was perturbed throughout epiboly; however, gastrulation movements occurred in parallel to wildtype progression, suggesting that ectodermal knockdown of ephrinB1 did not completely abolish epiboly. Late-stage (stage 12–13) morphant gastrulae showed that regions of thicker ectoderm were adjacent to regions of overtly thin ectoderm (Fig. 2-4; inset a-
b), which suggests that tissue thinning could have contributed to restoring epiboly progression; however, this compensation mechanism has yet to be fully understood.

An absent archenteron was also noted in early–mid stage (Fig. 2-4; stage 10–11) morphant gastrulae with respect to controls. This phenotype is consistent with the report that ephrinB1 function is required for tissue separation at Brachet’s Cleft (Rohani et al., 2011); however, it was also shown that morphant animals retained the capacity for development into viable larvae. Indeed, I found that the archenteron was restored in late-stage morphant gastrulae by an unknown mechanism (Fig. 2-4; stage 12). In addition, morphant archenteron inflation occurred successfully in parallel with controls (Fig. 2-4; stage 13). Presently, how morphant gastrulae were able to restore archenteron development is not yet understood. It could involve interactions from surrounding tissues (such as the mesoderm and endoderm), which were not subjected to the ectoderm-targeted knockdown of ephrinB1. Therefore, the rescue mechanism(s) remain a subject of ongoing investigation. Nevertheless, I tentatively conclude that ectodermal knockdown of ephrinB1 perturbed ectoderm morphogenesis, but it could not inhibit the process of epiboly as a whole.
Figure 2-4 Phenotypes of wildtype and ephrinB1 knockdown gastrulae.

(A) Control gastrula phenotypes and (B) ephrinB1 ectodermal knockdown gastrula phenotypes corresponding to stages 10, 10.5, 11, 12, and 13. Magnified insets (a, b) on the right correspond to boxed regions at stage 13. Red arrow indicates an example region of irregular ectoderm thickness. Yellow arrow indicates an example region of irregularly narrow ectoderm. Animal is to the top, vegetal to the bottom. Ventral to the left, and dorsal to the right. Gastrulation stages are ordered progressively from left to right. Stage 10 marks the onset of gastrulation, whereas stage 13 marks the end of gastrulation and the concurrent beginning of neurulation.
2.2.4 EphrinB1 knockdown rescued by ephrinB1 cytoplasmic domain

The effects of ephrinB1 knockdown on ectoderm morphogenesis could result from a lack of ephrinB1 reverse signaling or ephrinB1–EphB induced forward signaling. It was shown that cytoplasmic domain of ephrinB1 is sufficient to induce cell dissociation in the *X. laevis* embryo, suggesting that at least some effects of ephrinB1 can be mediated by reverse signaling (Jones *et al.*, 1998). To test this in the ectoderm, I investigated whether co-injection of ephrinB1 cytoplasmic domain (ephrinB1CD) mRNA could rescue the effects of ephrinB1 knockdown (Fig. 2-5).

Given that the ephrinB1 morpholino was designed to target the N-terminal region of the ephrinB1 mRNA sequence, ephrinB1CD, which encodes the ephrinB1 C-terminus, is not affected by morpholino hybridization and could be co-injected in combination to enhance the accuracy of targeted reagent delivery. Within cells, the neutral charge of morpholino molecules facilitate morpholino diffusion to nearby cells (Eisen and Smith, 2008). RNA molecules, however, consist of a negatively-charged backbone that prevents their diffusion; thus, the effective range of mRNA expression is limited by cell division. Therefore, the affected region of knockdown is expected to be wider than that which could be rescued by ephrinB1CD mRNA. I found that co-injection of ephrinB1CD could partly rescue increased ectoderm thickness induced by ephrinB1 knockdown in regions nearest to the injection sites, which led to an uneven BCR appearance in the gastrulae (Fig. 2-5). Nevertheless, data here support the notion that the effects of ephrinB1 on ectoderm morphology could be mediated by ephrinB1 reverse signaling.
Figure 2-5 Rescue of ephrinB1 knockdown.

Relative to uninjected (wildtype) BCR, the ectoderm of ephrinB1 morpholino injected (ephrinB1MO; 20 ng/blastomere) BCR appeared thicker. However, injection of ephrinB1 cytoplasmic domain mRNA (ephrinB1CD; 900 pg/blastomere) which contained amino acids 215–327, rescued ephrinB1MO-induced BCR thickening.

2.2.5 Effects of ephrinB1 and -B2 perturbation on BCR morphology

Given sequence similarities shared between ephrinB1 and ephrinB2 (Fig. 2-3), it was conceivable that ephrinB1 and –B2 could perform similar functions. To investigate this, I quantified the effects of ephrinB1 and –B2 manipulation on the X. laevis ectoderm by measuring the BCR thickness of early gastrulae (Fig. 2-6). Ectodermal knockdown of ephrinB1 or ephrinB2 was achieved by targeted injection of morpholino oligonucleotides into the animal hemisphere of four-cell stage embryos.

I first confirmed that the observed changes in ectodermal thickness were not caused by morpholino-induced cytotoxicity by injecting a concentration of standard morpholino that was consistent with experimental concentrations used for ephrinB1, or ephrinB2. The standard control morpholino, which targets a human β-globin intron sequence, should not hybridize to
nucleic acid sequences in *X. laevis*. As expected, control morpholino did not alter the ectodermal thickness of *X. laevis* gastrulae. In contrast, knockdown of ephrinB1 significantly increased ectoderm thickness with respect to uninjected or control morpholino-injected gastrulae (Fig. 2-6). Interestingly, knockdown of ephrinB2 produced a similar effect in that it also increased ectodermal thickness (Fig. 2-6). The results support the hypothesis that ephrinB1 and ephrinB2 perform similar functions in the ectoderm. However, knockdown of either ephrinB1 or ephrinB2 increased ectoderm thickness, which implies that these molecules are not redundant, but could work in parallel pathways to regulate ectoderm thickness.

To complement knockdown analysis, I overexpressed full-length ephrinB1 and ephrinB2 independently, and measured their effects on ectoderm thickness. Overexpression of ephrinB1 resulted in a significant reduction of ectoderm thickness with respect to wildtype tissue (Fig. 2-6). Overexpression of ephrinB2 produced a similar effect in the reduction of ectoderm thickness (Fig. 2-6). Taken together, overexpression of either ephrinB1 or -B2 reduced ectoderm thickness by nearly one-third.
Ectodermal BCR thickness was measured (from stage 10 gastrulae) at the gastrula apex from the blastocoel to the epithelial surface. Uninjected (wildtype), control (20 ng/blastomere), ephrinB1 (20 ng/blastomere), and ephrinB2 morpholino (20 ng/blastomere) injected, ephrinB1 mRNA (900 pg/blastomere), and ephrinB2 mRNA (200 pg/blastomere) injected measurements are shown. The control morpholino used targeted Prickle1, which is not expressed by the ectoderm. Significant relationships are shown by asterisks indicating $P < 0.05$; n.s. denotes results that are not statistically significant.
2.2.6 EphrinB1 and ephrinB2 function in parallel

It has been proposed that a crucial factor in determining the outcome of ephrin signaling could be the relative abundance of ephrin molecules (Rohani et al., 2014). In this context, it is conceivable that overexpression of ephrinB1 could compensate for knockdown of ephrinB2, or vice versa. To test this possibility, I systematically injected either ephrinB1/B2 morpholino alone, mRNA alone, or morpholino in conjunction with mRNA to assess the effects of reagent combinations on ectoderm morphology (Fig. 2-7). First, I confirmed that the standard control morpholino used at a concentration comparable with ephrinB1 or -B2 morpholinos did not alter BCR morphology relative to uninjected embryos (Fig. 2-7). I then confirmed that injection of ephrinB1 morpholino alone, or of -B2 morpholino alone, induced a thick BCR phenotype, and that ephrinB1 mRNA, or -B2 mRNA, induced ectodermal thinning (Fig. 2-7). BCR thinning was associated with loose ectoderm cells located deep to the superficial epithelium, although the epithelium itself appeared unaffected by overexpression of either ephrinB1 or -B2.

Next, I tested whether ephrinB1 overexpression could compensate for ephrinB2 knockdown and rescue its effect on BCR morphology by co-injecting ephrinB2 morpholino with ephrinB1 mRNA, or vice versa. In both cases, I found that ephrinB1 mRNA could not rescue ephrinB2 knockdown, nor could ephrinB2 mRNA compensate for the effect of the loss of ephrinB1 on ectoderm morphology. Interestingly, ectoderm co-injected with ephrinB1-morpholino and ephrinB2-mRNA retained morphological characteristics of when both treatments were performed independently. In other words, the ectoderm was relatively thick, yet at the same time, cells also appeared loose. A similar BCR phenotype was produced when ephrinB2 morpholino was co-injected with ephrinB1 mRNA. Together, these results confirm that although ephrinB1
and -B2 manipulation produced similar effects on ectoderm morphology, they do so in parallel pathways, and that the loss of one pathway cannot be rescued by stimulation of the other. Alternatively, they could both be irreplaceable components of the same pathway.

Figure 2-7 Effects of ephrinB manipulation on BCR morphology.

(A) BCR morphology of uninjected (wildtype) and standard control morpholino-injected (StandardMO; 20 ng/blastomere) stage 10 gastrulae. (B) Morphology of ephrinB1 (ephrinB1MO; 20 ng/blastomere), and –B2 (ephrinB2MO; 20 ng/blastomere) morpholino-injected ectoderm. (C) Morphology of full-length ephrinB1 (ephrinB1FL; 900 pg/blastomere) and –B2 (ephrinB2; 200 pg/blastomere) mRNA-injected ectoderm. (D) BCR morphology of ectoderm co-injected with ephrinB1MO and ephrinB2FL (20 ng and 200 pg/blastomere, respectively) or ephrinB2MO and ephrinB1FL (20 ng and 900 pg/blastomere, respectively).
2.2.7 Effects of ephrinB1 and ephrinB2 are independent of EphB4

Receptor EphB4 has been reported as a putative receptor for ephrinB1 and –B2, albeit with a higher binding affinity for ephrinB2 than ephrinB1. Moreover, EphB4 showed the highest level of mRNA expression relative to other EphB receptors expressed in ectoderm tissue (Rohani et al., 2011). To investigate the role of EphB4 on ectoderm morphogenesis, I manipulated EphB4 protein levels using three different strategies. First, I overexpressed full-length EphB4 in ectoderm tissue. I found that overexpression of the EphB4 could not alter ectoderm morphology (Fig 2-8). Specifically, EphB4 overexpression did not recapitulate BCR thinning or ectodermal cell dissociation induced by either ephrinB1 or ephrinB2 overexpression. Secondly, I overexpressed a dominant-negative (dnEphB4) form of EphB4, which has been reported to perturb EphB4 signaling. Overexpression of dnEphB4 to inhibit endogenous EphB4 signaling did not change BCR morphology when compared to control embryos (Fig 2-8). Finally, I knocked down endogenous EphB4 protein using a previously characterized translation blocking morpholino (EphB4MO; Rohani et al., 2011). Consistent with the dominant-negative approach, I found that injection of EphB4MO did not perturb BCR morphology (Fig 2-8). Furthermore, EphB4 knockdown could not mitigate the effect of ephrinB2 overexpression (Fig 2-8). Taken together, these results suggest that EphB4 is not the receptor responsible for mediating the effects of ephrinB1, or –B2 signaling in the ectoderm.
Figure 2-8 Effects of EphB4 manipulation.

Mid-sagittal fracture of stage 10 gastrulae showing control (wildtype), full-length EphB4 overexpression (EphB4FL; 600 pg/blastomere), EphB4 knockdown by dominant-negative EphB4 (dnEphB4; 600 pg/blastomere), and EphB4 morpholino (EphB4MO; 20 ng/blastomere), and EphB4MO co-injected with full-length ephrinB2 (ephrinB2FL; 200 pg/blastomere).

2.2.8 EphrinB-induced effects are not mediated by RhoA

EphrinB ligands undergo reverse signaling, and the small GTPase RhoA is an established downstream effector of ephrinB signaling (Daar, 2012). To explore whether RhoA could be involved in mediating the effect of ephrinB signaling during ectoderm morphogenesis, I manipulated RhoA activity in ectodermal tissue. First, I overexpressed dominant-negative RhoA (dnRhoA), which has been shown to attenuate RhoA activity. I found that dnRhoA did not affect BCR thickness with respect to control embryos (Fig 2-9A–B). Furthermore, co-injection of dnRhoA did not rescue ephrinB1 or ephrinB2 overexpression (Fig 2-9C). These results suggest that ephrinB1 and ephrinB2-induced BCR thinning is not mediated by RhoA.
In contrast, overexpression of constitutively active RhoA (caRhoA)-induced ectodermal thickening of the BCR similar to ephrinB1 or -B2 knockdown (Fig 2-9B). This outcome implies two possibilities. First, RhoA could regulate ectoderm thickness through a mechanism that is separate from ephrinB1 or -B2 signaling. Secondly, ephrinB1 or -B2 interaction(s) could attenuate RhoA activation within cells such that their respective knockdown leads to ectodermal thickening that is mimicked by overexpression of caRhoA. Presently, however, the mechanism by which caRhoA increases ectoderm thickness is not clear; future work will assess the effects of ephrinB1 and -B2 manipulation on RhoA activation.

![Figure 2-9](image)

**Figure 2-9 Effects of RhoA manipulation on BCR morphology.**

(A) Uninjected wildtype BCR is shown for comparison with (B) dominant-negative (dnRhoA; 500 pg/blastomere) or constitutively active RhoA (caRhoA; 10 pg/blastomere) injected BCRs. (C) dnRhoA co-injected with full-length ephrinB2 (ephrinB2FL; 200 pg/blastomere) mRNA, co-injected with full-length ephrinB1 (ephrinB1FL; 900 pg/blastomere) mRNA.
2.2.9 Knockdown of ephrinB1 increased ectoderm tissue cohesion

Given that knockdown of ephrinB1 induced ectodermal thickening and its overexpression induced tissue thinning, which is consistent with reports that ephrinB1 could play a role in embryonic tissue cohesion (Jones et al., 1998; Lee et al., 2008), I hypothesized that ephrinB1 could have a role in modulating ectoderm cohesion. To assess tissue cohesion, I measured ectoderm tissue surface tension using axisymmetric drop-shape analysis (ADSA), an established algorithm for determining tissue surface tension from the curvature of tissue aggregates (Luu et al., 2011; David et al., 2014).

Injection of control morpholino did not change ectoderm thickness. Correspondingly, injection of control morpholino at concentrations consistent with ephrinB1 manipulation did not alter ectoderm tissue surface tension (Fig. 2-10). It has been shown that knockdown of C-cadherin significantly reduces tissue cohesion (Ninomiya et al., 2012). I first confirmed that knockdown of C-cadherin reduced ectoderm tissue surface tension assessed by ADSA. Indeed, C-cadherin morpholino reduced ectoderm cohesion by nearly half (Fig. 2-10). Next, I asked whether knockdown of ephrinB1 would increase ectoderm tissue surface tension. I found that injection of ephrinB1 morpholino at a concentration consistent with the experimental dosage (20 ng/blastomere) required to increase ectoderm thickness correspondingly increased tissue surface tension (Fig. 2-10). Below this concentration, however, ephrinB1 morpholino (at 10 ng/blastomere) did not alter ectoderm cohesion, which suggests that a minimum effective dose (of 20 ng/blastomere) is required to elicit an effect of ephrinB1 knockdown on ectoderm cohesion.
I then investigated whether overexpression of ephrinB1 could decrease ectoderm cohesion. To define an effective concentration for ephrinB1 overexpression, I injected three different concentrations of full-length ephrinB1 mRNA (which were 300, 600, and 900 pg/blastomere). I found a dosage-dependent correlation between ephrinB1 expression and ectoderm cohesion. Specifically, ectoderm tissue surface tension decreased as ephrinB1 expression increased. Furthermore, ephrinB1 overexpression at 900 pg/blastomere (the concentration consistent with ectodermal thinning) significantly decreased ectoderm cohesion (Fig. 2-10).

It was reported that the effects of ephrinB1 were induced by reverse signaling mediated by the ephrinB1 cytoplasmic domain (Jones et al., 1998). However, the effect of the ephrinB1 cytoplasmic domain (ephrinB1CD) on ectoderm cohesion has not been examined. Similarly, the effect of ephrinB1 extracellular domain (ephrinB1ED) on ectoderm cohesion remains to be elucidated. To this end, I first examined the effect of ephrinB1ED overexpression. Interestingly, I found a trend of increasing ectoderm cohesion that was proportional to increasing concentration of ephrinB1ED expression (Fig. 2-10). Furthermore, ephrinB1ED overexpression at 900 pg/blastomere significantly increased ectoderm cohesion with respect to controls. In contrast, ectoderm cohesion decreased in response to increasing concentrations of ephrinB1CD (Fig. 2-10). Injection of ephrinB1CD at 900 pg/blastomere significantly reduced ectoderm cohesion. Taken together, the data suggest that ephrinB1 reverse signaling mediated by ephrinB1CD is involved in lowering ectoderm cohesion. Moreover, expression of ephrinB1ED could act in a dominant-negative capacity to increase ectoderm cohesion in a dosage-dependent manner.
**Figure 2-10 Ectoderm tissue surface tension assay.**

Graph indicates measured tissue surface tension from uninjected (wildtype) ectoderm, and tissue injected with standard morpholino (StandardMO), C-cadherin morpholino (CcadMO), ephrinB1 morpholino (ephrinB1MO), full-length ephrinB1 mRNA (ephrinB1FL), ephrinB1 extracellular domain (ephrinB1ED), which consists of amino acids 1–288, and ephrinB1 cytoplasmic domain (ephrinB1CD), which consists of amino acids 215–327. Boxed regions on the X-axis indicate injected concentrations. Morpholino injection concentrations are represented in ng/blastomere, whereas RNA injection concentrations are in pg/blastomere. Asterisks denote results that were significantly different from wildtype controls. Error bars indicate standard error.
2.2.10 Knockdown of ephrinB1 increased ectoderm tissue viscosity

Ectoderm cells rearrange and undergo radial cell intercalation during epiboly. The resistance of cells to movement within tissues, referred to as tissue viscosity, is expected to depend on how strongly cells are adhered to each other. Tissue viscosity can be assessed by measuring the rate of explant rounding for specific tissues (David et al., 2014). Knockdown of ephrinB1 increased ectoderm cohesion, which would imply an increased resistance to ectodermal cell rearrangement. To test this possibility, I measured tissue viscosity of ephrinB1 morpholino-injected and uninjected ectoderm tissues (Fig. 2-11A). The results confirmed that tissue viscosity was indeed higher in ephrinB1 knockdown ectoderm, which supports the notion that cell rearrangement was perturbed in ephrinB1 morphant ectoderm (Fig. 2-11B).
Figure 2-11 Knockdown of ephrinB1 increased ectoderm tissue viscosity.

(A) Time-lapse sequence of tissue aggregate rounding. Arrow indicates progression of time in hours (hr). Images show the same aggregate after isolation (0 hr), after 1 hr, and after 2 hr. An example wildtype (control) uninjected aggregate is shown at the top row, and an ephrinB1 morpholino (ephrinB1MO)-injected aggregate is shown at the bottom row. (B) Tissue viscosity measured from the rate of aggregate rounding. Error bars in this figure indicate standard deviations. Asterisk denotes statistical significance.
2.2.11 EphrinB-induced ectodermal cell intermingling

To elucidate the consequence of ephrinB1 manipulation on ectoderm cell rearrangement, I analyzed the intact BCR in mosaic embryos injected with different fluorescent cell lineage tracers to identify the left and right animal hemispheres. Ectoderm cells from the left and right animal hemispheres show some intermixing during epiboly (Fig. 2-12A).

I manipulated ephrinB1 levels in the left half of the animal hemisphere, and left the right half uninjected as an internal control. EphrinB1 knockdown ectoderm, despite being a thicker tissue, showed a pattern of intermixing that was similar to controls (Fig. 2-12A). In contrast, overexpression of ephrinB1 increased dispersion of ephrinB1-overexpressing cells into the control side (Fig. 2-12A). Moreover, ephrinB1-overexpressing cell dispersion increased in a dosage-dependent manner. Given that ephrinB1-overexpression decreased ectoderm cohesion, I examined whether ephrinB1-induced cell dispersion integrated ephrinB1-overexpressing cells into control tissues, or whether cells were dispersed on the tissue surface. To this end, I performed mid-sagittal fractures of BCR, and I found that ephrinB1-overexpressing ectoderm cells had indeed intermixed with cells in the control hemisphere (Fig. 2-12B). However, the mechanism of ephrinB1-induced cell intermingling is not yet understood.

Next, I quantified cell intermixing under different experimental conditions to examine whether the intermixing phenotype could be recapitulated by other Eph–ephrin molecules (Fig. 2-12C). Similar to ephrinB1, ephrinB2 also induced intermixing and cell dispersion into the control hemisphere. In fact, ephrinB2 overexpression showed a higher level of intermixing at a relatively lower concentration than ephrinB1 (Fig. 2-12C). Interestingly, overexpression of EphB4 did not induce morphant cell dispersion. In contrast, when EphB4 was overexpressed, control cells
appeared to have spread into the morphant tissue (Fig. 2-12C). Taken together, the results suggest that the extent of cell intermixing is a controlled process that could be modulated by EphB or ephrinB molecules, although the mechanism of this regulation is not yet understood.
**Figure 2-12 Ectoderm cell intermixing assay.**

(A) Mosaic ectoderm of embryos injected with cell lineage tracers only (left column), embryos half-injected with ephrinB1 morpholino (ephrinB1MO; 20 ng/blastomere; central column), and embryos half-injected with ephrinB1 mRNA (ephrinB1FL; 600 pg/blastomere; right column). Perspective shows the ectoderm cells located on the inner surface of the BCR; the superficial epithelium is still attached but hidden behind cells, which are shown. Cells of the left hemisphere were injected with fluorescein dextran amines (FDA, green), and cells of the right hemisphere were injected with rhodamine dextran amines (RDA, red). Label colours correspond to the co-injected tracer. Merged images are shown at the top row. FDA-only images are shown in the middle row, and RDA-only images are shown in the bottom row. (B) Mid-gastrula slices taken from fixed intact mosaic embryos. Representative images show embryos with lineage tracers only (left), and ephrinB1FL half-injected embryo (right). Image shows the BCR, blastocoel and surrounding tissue only; the vegetal hemisphere is omitted for simplicity. FDA-expressing cells intermingled deep within RDA-expressing cells are indicated (white arrowheads). The yellow colour of the endodermal region results from mixing of the green and red cell lineage tracers due to the injection of lineage tracers prior to the complete segregation of A–V blastomeres. (C) Quantification of cell intermingling. Whole animal caps were removed from mosaic embryos. A standardized area of 500 µm² was marked at the center of the animal cap and used for quantification. The numbers of red and green cells normalized to the total number of cells within the marked area were used for analysis. Averaged values from at least three independent experiments for each treatment were compiled into the combination graph. X-axis indicates the percentage of cells with respect to a particular cell tracer that were counted within the marked area. Vertical bar (grey) indicates 50%.
2.2.12 Knockdown of ephrinB1 delayed blastopore closure

In light of the importance of ephrinB1 function in the ectoderm, I investigated whether ephrinB1 could play a role during mesoderm morphogenesis. The mesoderm also expresses ephrinB1, albeit at lower level relative to the ectoderm (Rohani et al., 2011). To examine the requirement for ephrinB1 in mesoderm morphogenesis, ephrinB1 morpholino was injected into both dorsal blastomeres equatorially at the four cell stage to target prospective dorsal mesoderm. Based on standard external criteria, knockdown of ephrinB1 resulted in delayed gastrulation progression in morphant gastrulae with respect to controls (Fig. 2-13A). Morphant embryos appeared one full stage behind controls, where stage 12 morphant embryos phenotypically resembled stage 11 controls (Fig. 2-13A). To further analyze this discrepancy, I measured blastopore diameter and confirmed that the blastopore diameter of stage 12 morphants paralleled stage 11 controls (Fig. 2-13B). Presently, however, the mechanism by which knockdown of ephrinB1 delayed blastopore closure is not yet understood.
Figure 2-13 Injection of ephrinB1 morpholino to the dorsal marginal zone delayed gastrulation.

(A) Vegetal view of uninjected wildtype (control) gastrulae and ephrinB1 morpholino (ephrinB1MO) injected gastrulae at stages 11 and 12. (B) Blastopore size comparison. Average blastopore diameters from control and ephrinB1MO embryos at stages 11 and 12. Grey colour gradient corresponds to size variation.

2.2.13 Knockdown of ephrinB1 led to tissue thickening in the dorsal marginal zone

To investigate how knockdown of ephrinB1 could perturb mesoderm morphogenesis, I examined the dorsal mesoderm region in mid-sagittal fractured gastrulae. Interestingly, I found that ectodermal tissue at the dorsal marginal zone appeared thicker with respect to controls, reminiscent of ephrinB1 knockdown in the BCR (Fig. 2-14).
In comparison to control embryos at stages 11 and 12, the ectoderm of morphant embryos remained thicker than controls (Fig 2-14, compare a-b to c-d). These results confirm that ephrinB1 knockdown effects were successfully targeted to the dorsal marginal zone. However, two different populations of tissue exist in this region; the ectoderm and mesoderm. Ectoderm and mesoderm are connected within the embryo, and although knockdown of ephrinB1 in this region perturbed ectoderm thickness, it was not clear whether ephrinB1 morpholino had effect(s) on mesoderm morphogenesis.

Figure 2-14 Thickening of dorsal ectoderm induced by ephrinB1 knockdown.

(A) Gastrula phenotypes of control embryos (top row), and ephrinB1 morpholino (ephrinB1MO; 20 ng/blastomere) knockdown embryos (bottom row) at stages 11 and 12. Magnified insets (a, b) show boxed gastrula regions corresponding to control gastrulae. Magnified insets (c, d) show boxed gastrula regions corresponding to ephrinB1MO gastrulae. Yellow lines indicate ectodermal tissue thickness.
2.2.14 Knockdown of ephrinB1 perturbed chordamesoderm cell movements

Given that knockdown of ephrinB1 delayed blastopore closure, a corresponding effect on involution was expected. Mesoderm tissue within the dorsal involuting marginal zone is composed of chordamesoderm and prechordal mesoderm. Morphogenetic movements of the former tissue can be examined using tissue explants, which could autonomously undergo convergent extension movements \textit{in vitro} (Wallingford \textit{et al.}, 2000). To this end, I explanted dorsal marginal zone tissue from control and ephrinB1-morphant gastrulae (Fig. 2-15). When given an identical amount of time to develop, explants healed and were expected to elongate due to convergent extension cell movements of the chordamesoderm. Consistently, I found that control and morphant explants both elongated \textit{in vitro} (Fig. 2-15). Elongated dorsal mesoderm explants display a characteristic morphology with a bulbous region at one end of the explant that corresponds to the non-involuting marginal zone of the embryo. The bulbous non-involuting marginal zone narrows into a neck region formed by the involuting marginal zone, where the stem of the neck is mainly composed of cells from the chordamesoderm. The neck, which represents the prospective notochord, extends into a bend that resembles a hook at the other end of the explant that consists of cells from the prechordal mesoderm. Despite successful elongation in both explants, the neck region representing chordamesoderm was thicker with respect to controls. To further examine this discrepancy, I examined mid-sagittal fractures of control and morphant explants (Fig. 2-15B). I found that the chordamesoderm was in fact thicker within morphant explants with respect to controls, which suggests that chordamesoderm morphogenesis was perturbed by knockdown of ephrinB1.
Figure 2-15 Mesoderm convergent extension explant assay.

(A) Control explant elongation showing the side view of an explant (center), the mid-sagittal fracture of the explant (left) and the dorsal view of the explant (right). (B) Elongation of ephrinB1 morpholino-injected (ephrinB1MO) mesoderm explant showing the side (center), mid-sagittal (left), and dorsal view (right) of the explant. Red lines indicate mesodermal tissue thickness.

2.2.15 Knockdown of ephrinB1 inhibited prechordal mesoderm cell migration

Given that knockdown of ephrinB1 perturbed chordamesoderm morphogenesis, I asked whether ephrinB1 morpholino would have a similar effect on prechordal mesoderm migration. To this
end, I analyzed prechordal mesoderm cell rearrangement using an established cell migration assay that recapitulates the directed movement of prechordal mesoderm cells *in vitro* (Damm and Winklbauer, 2011). In this assay, the prechordal mesoderm is placed in direct contact with ectoderm from the BCR. Mesoderm cells are then directed by a secreted gradient of PDGFA, which guides mesoderm cells by a chemotactic gradient toward the BCR. In control explants, prechordal mesoderm cells from 3–5 cell layers deep to the ecto-mesoderm boundary were seen to rearrange by undergoing cell intercalation toward the ectoderm (Fig 2-16A). Control cells typically reach the ecto-mesoderm boundary within two hours. In contrast, ephrinB1 knockdown cells remained relatively stationary throughout the observation period, and the movements observed from marked cells correlated with background explant fluctuations (Fig 2-16B). Taken together, knockdown of ephrinB1 attenuated prechordal mesoderm cell migration and perturbed chordamesoderm morphogenesis.
Precordial mesoderm (PCM) cell migration is perturbed by ephrinB1 knockdown.

(A) Control PCM cells intercalate towards the ectodermal BCR. Panels show cell migration starting from 45 minutes after explant isolation. An initial PCM cell (green) is joined by two more deep cells (blue and red) at the 60 minute mark, and the three cells migrate towards the BCR. During intercalation movement, the green and red cells advance in front of the blue cell to reach the ectoderm (yellow arrows). (B) Cell migration in ephrinB1 morpholino (ephrinB1MO; 20 ng/blastomere) PCM cells. Relative to control explants, morphant cells showed oscillatory motion consistent with explant tissue fluctuations; however, morphant cells did not intercalate toward the BCR. The position of three (red, green, and blue) stalled morphant cells are shown (white arrow). The tissue interface between the PCM and BCR is indicated by a dashed white line.
2.2.16 Mosaic ephrinB1 knockdown larvae showed craniofacial abnormalities, axial, and somite defects

Given that knockdown of ephrinB1 perturbed chordamesoderm morphogenesis and prechordal mesoderm cell migration, I asked whether ephrinB1 morphant animals could develop beyond gastrulation. Surprisingly, morphant animals developed to the larval stage; however, numerous tissue abnormalities were notable (Fig. 2-17). To characterize the effect of mesodermal ephrinB1 knockdown, I generated mosaic morphants that had ephrinB1 signaling attenuated by morpholino injection on the right side, whereas the left side was injected with standard morpholino as an internal control. Relative to similarly staged wildtype larvae, morphant larvae showed deformed axial structures; in particular, severe scoliosis was notably pronounced at the tail (Fig. 2-17A). Relative to the control side, ephrinB1 morpholino injection abolished somite segmentation (Fig 2-17A). Furthermore, numerous craniofacial abnormalities, including strabismus, increased inter-ocular distance, and facial asymmetry, were observed (Fig 2-17B). Interestingly, the observed spinal and facial deformations in *X. laevis* larvae appear similar to ephrinB1-linked CFNS in humans, which suggests that *X. laevis* could provide an experimental model for unravelling the cellular mechanisms behind CFNS. Presently, how loss of ephrinB1 affects somite segmentation is not understood. Future work will focus on characterizing the effect of ephrinB1 knockdown on somite morphogenesis.
Figure 2-17 Knockdown of ephrinB1 induced larval somite and craniofacial defects.

(A) A comparison of morphant (top) and control larvae (bottom). Images show a front view of facial features (left), a right side view (center), and a left side view (right). Mosaic embryos were generated by equatorially injecting the left dorsal blastomere with standard morpholino (StandardMO; 20 ng/blastomere) and the right dorsal blastomere with ephrinB1 morpholino (ephrinB1MO; 20 ng/blastomere) at the 4-cell stage. Control larvae were uninjected. Panels show the left and right halves of the same larvae. Experiment and control larvae were obtained from the same clutch of eggs and fixed in parallel at identical developmental stages. (B) Schematic of facial structure abnormalities in mosaic embryos (left) relative to wildtype controls (right). Anatomic positions for eyes, nasal pits, mouth, and cement gland are indicated for reference. Left-right facial asymmetry is highlighted by dashed line.
2.2.17 Mosaic ephrinB2 knockdown larvae showed somite segmentation defects

In light of the functional similarities between ephrinB1, and -B2, I asked whether knockdown of ephrinB2 could recapitulate the larval phenotype induced by knockdown of ephrinB1. To this end, I generated ephrinB2 mosaic morphants, which were injected with ephrinB2 morpholino on the right side. In parallel, standard morpholino was injected using an identical concentration on the left side (Fig. 2-18). I found that in contrast to ephrinB1 morphants, ephrinB2 morphants developed intersomitic boundaries (Fig. 2-18A). However, the width of somites appeared expanded relative to controls (Fig. 2-18B). Measurements revealed that only a fraction (approximately one-third) of the full complement of somites were present in the morphant half, and morphant somites were on average 1.8-fold wider than controls, which suggested that somite segmentation was perturbed but not abolished. Furthermore, ephrinB2 morphants also developed scoliosis. Taken together, the results showed that although ephrinB1 and -B2 morphant larvae manifested similar defects, tissue malformations were phenotypically different between respective morphants, which suggests that ephrinB1 and -B2 signal through different pathways and produce different phenotypes.
Figure 2-18 Knockdown of ephrinB2 induced larval somite defects.

(A) Mosaic morphant larvae. Mosaic embryos were generated by equatorially injecting the left dorsal blastomere with standard morpholino (StandardMO; 20 ng/blastomere) and the right dorsal blastomere with ephrinB2 morpholino (ephrinB2MO; 20 ng/blastomere) at the 4-cell stage. (B) Control larvae were uninjected. Example left and right image panels represent two halves of the same larvae. Experiment and control larvae were obtained from the same clutch of eggs and fixed in parallel at identical developmental stages.

2.2.18 Perturbations of ephrinB1 during endoderm internalization

Given the role of ephrinB1 in ectoderm and mesoderm morphogenesis, I then asked whether ephrinB1 could play a role in endoderm internalization. To begin with, I knocked down endogenous ephrinB1 levels using morpholino oligonucleotides. Analysis of mid-sagittally bisected gastrulae revealed that ephrinB1 perturbed endoderm internalization (Fig. 2-19A).
When compared to late-stage control gastrulae, it could be inferred from established features of endoderm morphogenesis that morphant gastrulae did not complete vegetal rotation. First, the BCF in morphant gastrulae retained a flat morphology, reminiscent of early gastrulae at the onset of gastrulation, in contrast to the curved bicuspid BCF surface that had developed in control late-stage gastrulae. Secondly, the dorsal archenteron in morphant gastrulae was short in comparison to control gastrulae (Fig. 2-19A). To complement ephrinB1 knockdown, I then overexpressed full-length ephrinB1 in the *X. laevis* endoderm. Analysis of bisected ephrinB1-overexpressing gastrulae revealed that overexpression of ephrinB1 abolished endoderm cohesion of the vegetal cell mass, consistent with a report of ephrinB1-induced cell dissociation in the early embryo (Jones *et al.*, 1998). Endoderm cells in ephrinB1-overexpressing gastrulae appeared detached from one another. Interestingly, detached endoderm cells did not collapse; instead, they were suspended by an extracellular material that was retained after sample fixation (Fig. 2-19A). The constituents of this interstitial material are not yet known, and work is currently underway to examine its composition.

To investigate the cellular consequence of ephrinB1 manipulation on endoderm cells, I next analyzed the effect of ephrinB1 knockdown in vegetal slice explants. Membrane-labelled explants were used to visualize endoderm cell morphology. At the cellular level, ephrinB1-morphant cells appeared elongated in a manner similar to controls. However, a majority of morphant cells were arranged in linear rows, in contrast to control cells whose arrangement resembled oblique tiles, which suggests that knockdown of ephrinB1 perturbed endoderm cell rearrangement (Fig. 2-19B). Conversely, ephrinB1-overexpressing cells were rounded and found with copious amounts of interstitial space between cells. (Fig. 2-19B). Furthermore, a consistent pattern in cell arrangement was not observed as cells appeared loose and unattached. Taken together, ephrinB1 manipulation perturbed endoderm internalization in two ways. First,
knockdown of ephrinB1 halted endoderm morphogenesis, potentially by perturbing cell rearrangement. Secondly, overexpression of ephrinB1 perturbed endoderm morphogenesis by reducing endoderm cohesion, inducing cell rounding and cell detachment.

**Figure 2-19 Manipulation of ephrinB1 protein levels perturbed vegetal rotation.**

(A) Mid-sagittal fractures of stage 12 wildtype (left), eB1MO-injected, and eB1FL-injected gastrulae. BCF length (red line) and dissociated cells (red arrows) are indicated. (B) Morphology of wildtype (left), eB1MO-injected (center), and eB1FL-injected (right) labelled (mRFP) endoderm cells. Animal is to the top, vegetal to the bottom, ventral to the left, and dorsal to the right.
2.2.19 Effects of ephrinB1 on endoderm tissue cohesion

To quantify the effects of ephrinB1 manipulation on endoderm tissue cohesion, I measured endoderm tissue surface tension of various treatments (Fig. 2-20). To reduce batch-to-batch variation, surface tension measurements under different experimental conditions were normalized to respective wildtype control values. To confirm that morpholino used at the experimental concentration did not alter endoderm cohesion, I first evaluated the effect of standard control morpholino on endoderm tissue surface tension. The normalized ratio of tissue surface tension for standard morpholino-injected endoderm was nearly one; therefore, standard morpholino did not change tissue surface tension with respect to wildtype endoderm and was subsequently used as experimental control. In contrast, knockdown of ephrinB1 significantly increased endoderm tissue surface tension (Fig. 2-20). To complement knockdown, I evaluated the effects of ephrinB1 overexpression for three different concentrations (300, 600, and 900 pg/blastomere). I found that overexpression of ephrinB1 at 900 pg/blastomere significantly decreased endoderm surface tension (Fig. 2-20), similar to that observed for the ectoderm. In light of this result, I analyzed the effect of ephrinB1CD overexpression, and consistent with results for full-length ephrinB1, overexpression of ephrinB1CD significantly reduced endoderm cohesion at 900 pg/blastomere (Fig. 2-20), which suggests that the effects of ephrinB1 overexpression in the endoderm are mediated by the ephrinB1 cytoplasmic domain. Furthermore, I quantified the effect of ephrinB1ED overexpression, and I found that ephrinB1ED increased ectoderm cohesion in a dosage-dependent manner, and significantly increased ectoderm tissue surface tension at 900 pg/blastomere (Fig. 2-20). Taken together, the results suggest ephrinB1 modulates endoderm tissue cohesion in a similar manner to its effect on ectoderm cohesion, and suggest that ephrinB1 could have a conserved role in the modulation of tissue cohesion in all
tissues of the *X. laevis* gastrula. I next tested whether the effect of ephrinB1 knockdown on endoderm cohesion could be rescued by overexpression of ephrinB1. I found that when ephrinB1CD was expressed at the effective dosage of 900 pg/blastomere, it was able to rescue the effect of ephrinB1 knockdown (Fig. 2-20), thus suggesting that ephrinB1 modulates endoderm cohesion by reverse signaling.
Figure 2-20 Endoderm tissue cohesion assay.

Graph indicates tissue surface tension measurements normalized to wildtype controls for standard (20 ng/blastomere) and ephrinB1 morpholino (20 ng/blastomere), ephrinB1 full-length (eB1FL), ephrinB1 cytoplasmic domain (eB1CD), and ephrinB1 extracellular domain (eB1ED) constructs injected at 300, 600, and 900 pg/blastomere, and rescue of ephrinB1 morpholino (20
ng/blastomere) knockdown by eB1CD injected at 900 pg/blastomere. Error bars indicate standard error.

2.2.20 Knockdown of ephrinB1 increased endoderm tissue viscosity

As knockdown of ephrinB1 increased endoderm tissue surface tension, a corresponding decrease in endoderm cell rearrangement was expected. To investigate this, I performed tissue viscosity analysis on ephrinB1 morpholino-injected tissues (Fig. 2-21). I found that ephrinB1 morphant tissue viscosity was in fact significantly higher than wildtype endoderm tissue viscosity (Fig. 2-21A). To support the claim that the observed increase in endoderm viscosity was specific to ephrinB1 knockdown, I analyzed the effect of ephrinB3 knockdown in endoderm tissue. The ligand ephrinB3 is highly expressed in the ectoderm but not expressed in the endoderm (Rohani et al., 2011) and can therefore function as a control for morpholino off-target effects in endoderm tissue. Accordingly, I found that ephrinB3 morpholino did not alter endoderm viscosity.

Tissue viscosities were estimated using the rate of explant rounding. Endoderm cells were visible and cell positions could be tracked during explant deformation. To take advantage of their relatively large cell diameter, I monitored endoderm cell rearrangement in explants from time-lapse recordings (Fig. 2-21B). As expected, I found that endoderm cells exchanged neighbours during rounding in wildtype explants. In contrast, however, cells within ephrinB1 morphant explants appeared relatively immobile, and cell rearrangements were rarely observed (Fig. 2-
Taken together, tissue viscosity analysis revealed that knockdown of ephrinB1 inhibited endoderm cell rearrangement.

**Figure 2-21 Endoderm cell rearrangement assay.**

(A) Tissue viscosity of wildtype (control), ephrinB1 morpholino (eB1MO10), and ephrinB3 morpholino (eB3MO10)-injected endoderm. Injection of eB3MO was used as a control. Error bars indicate standard error (B) Cell rearrangement during explant rounding showing wildtype (top row) and eB1MO-injected (bottom row) endoderm. In wildtype, cell–cell boundaries are reduced (yellow line), and cells (yellow) detach, whereas new borders (blue line) form between initially separate cells (blue). In contrast, eB1MO-injected cells (red, green) do not rearrange, and no boundaries (brown lines) are exchanged.
2.3 Discussion

2.3.1 Eph–ephrin binding and biology

It was previously proposed that Eph–ephrin interaction specificities are rigorously separated by different binding affinities (Gale et al., 1996; Flanagan and Vanderhaeghen, 1998). However, recent evidence has shown that receptor–ligand interactions are promiscuous, meaning that within a particular class of molecules, the ligands can bind to and activate signaling in multiple receptors, or vice versa in the case of bidirectional signaling. Among receptors, a notable example is EphB2, which can bind to and be activated by ephrinB1 (Riedl et al., 2005; Luxey et al., 2013; Jiang et al., 2015), ephrinB2 (Dravis et al., 2004; Zhou et al., 2015), or ephrinB3 (McClelland, 2010). The same promiscuity holds true for ligands; for example, like other ephrinBs, ephrinB2 can be activated by EphB2 (Essmann et al., 2008) or EphB4 (Bouzioukh et al., 2007; Wang et al., 2010). To complicate the matter, Eph and ephrin molecules of different classes can cross-react to induce signaling. For example, EphA4 can bind to and be activated by ephrinB2 (Blits-Huizinga et al., 2004; Qin et al., 2010; Evren et al., 2014; Poitz et al., 2015). However, it is necessary to consider that physical interactions in vitro do not strictly imply physiological relevance in vivo.

Within tissues, multiple combinations of Ephs and ephrins can be expressed in complementary patterns between adjacent tissues, or be co-expressed in complex patterns where numerous cross-regulatory pathways could potentially interfere. An example of trans-interaction is found at the
ecto–mesoderm tissue boundary of the *X. laevis* gastrula, known as Brachet’s cleft. Here, EphB–ephrinB interactions are involved in maintaining tissue separation by modulating repeating cycles of cell attachment/detachment across the boundary (Rohani *et al*., 2011). Rohani and colleagues showed that during tissue separation, the final outcome of whether separation occurred was largely based on the selectivity of receptor–ligand interactions and their relative abundance (Rohani *et al*., 2014). In this way, individual Eph–ephrin interactions were not absolute signaling determinants, but interacting pairs contributed to a network that collectively determined the physiological response. Moreover, Rohani and colleagues claim that components within this network are semi-selective, meaning that individual receptors or ligands could have multiple partners (as noted above, ephrinB2 could bind to EphB1, EphB2, or EphA4). This implies that interactions in this network have the capacity for compensation, such that individual molecules should be interchangeable to some extent. For example, ephrinB1 and ephrinB3 can bind to different cognate receptors; however, ephrinB3 overexpression could partly compensate for loss of ephrinB1 in restoring tissue separation at Brachet’s cleft (Rohani *et al*., 2014).

Conversely, relatively little is known about the biological consequences of Eph–ephrin *cis*-interactions. Reports of EphA–ephrinA binding within the same membrane domain have shown that *cis*-interactions are predominantly inhibitory (Yin *et al*., 2004). For instance, EphA3–ephrinA5 *cis*-binding in chick retinal axons prevent tyrosine phosphorylation and activation of EphA3, which results in a loss of sensitivity to trans-ephrinA signaling (Hornberger *et al*., 1999; Carvalho *et al*., 2006). The mechanism of how *cis*-interactions could interfere with signal activation is not clear. What is known, however, is that *cis*-attenuation depends on the second FN-III domain of Eph receptors (Carvalho *et al*., 2006), which is required for signal transduction (Xu *et al*., 2013). Similarly, *cis*-attenuation has been reported for EphB–ephrinB interactions. Antion and colleagues found that EphB2–ephrinB3 co-expression inhibited EphB2
phosphorylation of the NR2B subunit within NMDA receptors (Antion et al., 2010).

Interestingly, ephrin can also undergo cis-interaction with other cell surface molecules. For example, ephrinA3 and ephrinB3 can bind to heparan sulfate proteoglycans using their respective extracellular domains (Irie et al., 2008; Holen et al., 2011). EphrinB1 can interact with Claudin1 and Claudin4 in cis on the surface of Madin-Darby Canine Kidney Epithelial (MDCK) cells (Tanaka et al., 2005). EphrinB1 can also cis-interact with the receptor tyrosine-protein kinase ErbB2 in cancer cells (Vermeer et al., 2012). EphrinB1 is a known regulator of tissue morphogenesis. Global loss of ephrinB1 in mice is embryonically lethal due to multiple tissue malformations (Compagni et al., 2003). In heterozygous ephrinB1 mutants, it was found that loss of ephrinB1 restricts cell movement, although the mechanism by which cell migration became impeded was not understood (Compagni et al., 2003). EphrinB1 transcripts are significantly elevated during X. laevis gastrulation (Jones et al., 1997). Moreover, ephrinB1 is ubiquitously expressed in all tissues of the X. laevis gastrula (Rohani et al., 2011); thus, ephrinB1 is implicated as a key regulator of gastrulation movements in the X. laevis embryo.

2.3.2 EphrinB1 modulates tissue cohesion in all three germ layer tissues

To disentangle the role of ephrinB1 in tissue morphogenesis, I systematically examined ephrinB1 function in all three germ layers of the X. laevis gastrula. It has been shown that the same ephrin isoform could have different functional roles in cell adhesion or de-adhesion in a tissue-dependent context. For example, Jellinghaus and colleagues showed that ephrinA1 increased the adhesion of human monocytes to the underlying endothelium (Jellinghaus et al., 2013). In contrast, Yang and colleagues showed that ephrinA1 decreased the adhesion of human
fallopian tube epithelial cells to the substratum (Yang et al., 2012). Moreover, Fujii and colleagues showed that ephrinA1 induced dissociation of human endometrial epithelial cells (Fujii et al., 2011). In contrast to these reports, a key finding from my experiments indicates that the function of ephrinB1 on tissue cohesion is conserved among all gastrula tissues. Overexpression of ephrinB1 induced dissociation of all germ layer tissues, consistent with the report that ephrinB1 induced cell dissociation in the *X. laevis* blastula (Jones et al., 1998). In complementary experiments, when endogenous ephrinB1 levels were knocked down using morpholino oligonucleotides, tissue cohesion increased in all tissues with respect to controls. Tissue viscosity analysis confirmed that ephrinB1-knockdown tissues are indeed more viscous and are therefore less able to undergo cell rearrangement in vitro. Together, these results support the hypothesis that ephrinB1 modulates the level of cohesion within gastrula tissues. Specifically, ephrinB1 acts to lower the level of cohesion within tissues in order to facilitate proper cell rearrangement during tissue morphogenesis.

Tissue cohesion is a reflection of constituent cell–cell adhesion. Consistent with this notion, I showed that knockdown of ephrinB1 increased cohesion and decreased cell motility, which suggests that proper cell de-adhesion is required for cell rearrangement. It is conceivable that ephrinB1 could modulate adhesion by regulating the level of C-cadherin. For example, it was reported that in cancer cells, EphB receptors, E-cadherin, and the metalloproteinase ADAM10 interact at the membrane. Upon ephrinB1 binding, EphB–ephrinB1 interactions activate ADAM10 locally to induce enzymatic cleavage of E-cadherin, and thus lower E-cadherin levels (Solanas et al., 2011). Moreover, ephrinB1-upregulation during epidermal wound healing in mice coincided with a strong downregulation of E-cadherin and Claudin1, which have been speculated to facilitate cell migration (Nunan et al., 2015). However, Jones and colleagues noted that in *X. laevis* embryonic cells, ephrinB1 induced cell de-adhesion without directly affecting
levels of C-cadherin, or its ability to bind β-catenin (Jones et al., 1998). Alternatively, it could be that ephrinB1 affects adhesion by modulating local cadherin distribution and cluster density, such as in the case reported for the notochord–somite boundary. It was shown that Eph–ephrin signaling across the notochord–somite boundary triggers an increase in local cell contractility, which causes cell detachment and prevents cadherin clustering at this surface to decrease cell adhesion and results in tissue separation (Fagotto et al., 2013). Furthermore, ephrinB1 could regulate cell adhesion by inducing endocytosis of cell–cell membrane contacts to terminate cell adhesion. For instance, when fibroblasts engage in mutual contact, EphB–ephrinB interactions form complexes that are rapidly trans-endocytosed within contacting cells to promote detachment (Zimmer et al., 2003; Marston et al., 2003).

2.3.3 Functional similarity between ephrinB1 and ephrinB2

Different ephrin molecules have been shown to perform similar functions. For example, ephrinB1 and ephrinB2 are both involved in regulating tissue separation in X. laevis embryonic tissues (Rohani et al., 2011). EphrinB1 and -B2 were upregulated and implicated in regulating epidermal wound repair in mice (Nunan et al., 2015). Likewise, ephrinB1 and -B2 both regulate contact-dependent cell repulsion in mouse embryonic fibroblasts (Zimmer et al., 2003; Marston et al., 2003). Together, these results suggest that different ephrinB1 and -B2 co-expression within the same tissue could be functionally redundant. However, my results show that ephrinB1 could not rescue the loss of ephrinB2, or vice versa, in the X. laevis ectoderm. In fact, when knockdown of one molecule was combined with overexpression of the other, both malformation phenotypes manifested simultaneously, which suggests that ephrinB1 and -B2 transduce their
signal through different pathways that can operate in parallel to modulate tissue cohesion. Consistent with this finding, McClelland and colleagues reported that ephrinB1 and -B2 can function independently in regulating the formation of synaptic contacts; however, they act in a similar capacity in recruiting syntenin1 to mediate presynaptic development (McClelland et al., 2009). Analogously, X. laevis ephrinB1 and -B2 are functionally similar but not redundant.

2.3.4 EphrinB1 and ephrinB2 induce ectodermal cell intermingling

EphrinB1 and -B2 are frequently co-expressed or expressed in alternating patterns in developing tissues. For example, in rhombomeres (R) of the developing zebrafish hindbrain, ephrinB1 is expressed in R2 and R4, whereas ephrinB2 is expressed in R1–4 and R6 (Bergemann et al., 1995; Flenniken et al., 1996; Gale et al., 1996; Xu et al., 2000). Moreover, in chick somites, ephrinB1 is highly expressed in the ventral quadrant of epithelial somites, whereas ephrinB2 is highly expressed in the dorsal dermomyotome (Baker and Antin, 2003). Consistent with segmental expression patterns, ephrinB1 and -B2 are known regulators of tissue segmentation in the zebrafish hindbrain (Xu et al., 2000). When the balance of Eph and ephrin interactions was experimentally manipulated by overexpression of ephrinB2, cells from R3 and R5 intermixed into R2, R4, and R6, which suggests that over-activation of ephrinB2 reverse signaling induced cell intermingling. In support of this notion, truncated EphA4, which could bind to and activate ephrinB2 reverse signaling but cannot itself undergo forward signaling, was able to recapitulate the ephrinB2 overexpression phenotype (Xu et al., 1995, 1999, 2000; Mellitzer et al., 1999). Thus, shifting Eph–ephrin interactions toward unidirectional signaling induces a bias toward cell intermingling of different cell populations. In contrast, Mellitzer and colleagues used cells from
the zebrafish animal cap to show that when bidirectional signaling is augmented in the same cell population, which is normally capable of some intermixing, cell intermingling becomes restricted by overexpression of EphB2 in cells juxtaposed to neighbouring cells that overexpress ephrinB2 (Mellitzer et al., 1999).

Cell intermixing can be induced in the X. laevis animal cap. Here, ephrinB1 or -B2 overexpressing cells intermingle into the control side of mosaic animal cap explants. It is not yet known whether cell intermingling in X. laevis ectoderm is induced by forward or reverse signaling. However, overexpression of EphB4, which has been reported as a cognate receptor of ephrinB1 and -B2 (Gerety et al., 1999; Nikolov et al., 2005), could not recapitulate cell intermixing. This suggests that ephrinB1, or -B2 could signal through different EphB receptors other than EphB4; for example, ephrinB1 can bind EphB1–4 but not EphB6 (Nikolov et al., 2005), whereas ephrinB2 can bind to all EphB-class receptors including EphB6 (Munthe et al., 2000). Interestingly, expression of the ephrinB1 extracellular domain alone could not induce intermixing in the X. laevis ectoderm (not shown), which suggests that ephrinB1 may potentially regulate intermingling by reverse signaling. Presently, the mechanisms of ephrinB-reverse signaling in X. laevis have not yet been fully elucidated; however, with respect to other isoforms within the ephrinB family, reverse signaling is best understood in the context of ephrinB1.

2.3.5 EphrinB1 reverse signaling activation

Given that the ephrinB1 cytoplasmic domain was sufficient to reduce tissue cohesion (this thesis) and induce cell dissociation (Jones et al., 1998) in X. laevis embryonic tissues, these results
suggest that ephrinB1 mediated cell de-adhesion is a bonafide result of ephrinB1 reverse signaling. Nevertheless, how ephrinB1 signaling becomes activated in *X. laevis* germ layer tissues is not yet understood. As Eph and ephrin molecules are membrane-bound, signaling activation presumably requires cell–cell contact. Ligands ephrinB1 and -B2 bind EphB receptors with different binding affinities (Blits-Huizinga *et al.*, 2004). Messenger RNA analysis of *X. laevis* gastrula tissues revealed that EphB1–4 are present in all germ layer tissues (Rohani *et al.*, 2011). Of the four candidate receptors, EphB2 and EphB4 are frequently reported as prototypical examples of bidirectional signaling, and were thus likely candidates for activating ephrinB1 and -B2 reverse signaling (Holland *et al.*, 1996; Brückner *et al.*, 1997; Cowan and Henkemeyer, 2001). As the full sequence for EphB2 is not yet available, I analyzed EphB4. EphB4 manipulation did not recapitulate nor rescue tissue phenotypes induced by ephrinB1 or -B2 in the ectoderm, consistent with results from cell intermingling, which suggests that other Eph receptors (potentially EphB2), but not EphB4, are responsible for activating ephrinB1 and -B2 signaling in *X. laevis* germ layer tissues.

Reverse signaling can also occur via contact-independent mechanisms. In fact, ephrinB1 and –B2 can signal in a cell-autonomous manner (Mellitzer *et al.*, 1999; Xu *et al.*, 1999; Davy *et al.*, 1999); however, the mechanism by which this feature could be achieved is not yet fully understood. Recently, it was reported that cell–cell contact-independent signaling could be generated through interaction with exosomes. Exosomes are specialized vesicles secreted by other cells. Gong and colleagues showed that Eph and ephrin molecules are present on the membrane surface of exosomes secreted by many different cell types. Eph and ephrin molecules present on exosome membranes are functional and able to interact with their cognate binding partners; in particular, they bind to cells expressing ephrinB1 to induce reverse signaling (Gong *et al.*, 2016). Furthermore, autocrine-type signaling could be achieved by autonomous membrane
undulations. For example, during ruffle formation, the cell membrane could extend outward and fold back on itself at distal margins to form contacts that could potentially trigger receptor–ligand signaling within the same cell. Such an event has been observed and documented during large-scale membrane remodeling processes such as macropinocytosis (Welliver et al., 2011).

In the aforementioned scenarios, ephrinB signal transduction requires receptor interaction. It is necessary to note that a third option of receptor-independent signaling is also possible for ephrinB ligands. For example, in human endothelial cells, overexpression of ephrinB1 or ephrinB2 induced repeated cycles of cell body contraction followed by extension of lamellae and membrane ruffling (Bochenek et al., 2010). Expression of truncated ephrinBs, which lacked the cytoplasmic domain, could not reproduce cell contractions, which suggests that ephrinB reverse signaling is required for this process. Interestingly, when Bochenek and colleagues investigated the mechanism of reverse signaling activation, they found that ephrinB-induced cell contractions occurred independently of Eph receptor binding. Expression of mutant forms of ephrinB2 in which crucial sites for Eph receptor binding were abolished could not impair cell contractions. The authors supplemented their analysis by treating ephrinB-expressing cells with peptide antagonists that block EphB–ephrinB interactions and found that cell contractions persisted, further supporting the notion that ephrinBs functioned in a receptor-independent manner.

Similarly in human cervical cancer cells, Cho and colleagues found that ephrinB1 interacts with connector enhancer of KSR1 (CNK1) in a receptor-independent manner (Cho et al., 2014). Taken together, ephrinB signaling could be transduced through cell–cell contact, through contact-independent mechanisms that involve receptor–ligand interactions, or via receptor-independent mechanisms.
2.3.6 A role for ephrinB1 in cytoskeleton remodeling

EphrinB1 is a known regulator of the actin cytoskeleton. The adaptor protein Grb4 (Cowan and Henkemeyer, 2001) transduces ephrinB1 reverse signaling to activate downstream effectors such as p21 protein-activated kinase 1 (Pak1) (Cowan and Henkemeyer, 2001), the Wiskott–Aldrich Syndrome protein (WASP)-binding protein WIRE (Irie and Yamaguchi, 2004), and the PDZ-domain containing regulator of G protein-signaling (PDZ-RGS) (Lu et al., 2001) to regulate actin architecture through actin-related proteins (ARP) such as ARP2/3 (Irie and Yamaguchi, 2004), and the small GTPase Ras homolog gene family member A (RhoA) (Tanaka et al., 2003). Recent work has shown that ephrinB1 can activate RhoA in several ways. In human embryonic kidney cells and human cervical cancer cells, ephrinB1 interacts with connector enhancer of kinase suppressor of Ras1 (CNK1) to activate RhoA (Cho et al., 2014). In X. laevis, ephrinB1 interacts with the scaffolding protein Dishevelled (Dsh) through the Dsh, Egl-10, pleckstrin (DEP)-domain and protein kinase C-delta (PKCδ) to activate RhoA (Tanaka et al., 2003; Lee et al., 2006). Activated RhoA at the level of the cell cortex induces actin polymerization and regulates actin architecture by increasing cortical F-actin density (Hall, 1998; Bishop and Hall, 2000). Given that RhoA is frequently reported as a downstream effector of ephrinB1, it was surprising to find that active RhoA does not rescue loss of ephrinB1 in the X. laevis ectoderm. In contrast, expression of active RhoA alone induced a similar phenotype to ephrinB1 knockdown which suggests two possibilities: 1) within ectoderm cells, ephrinB1 may act to attenuate RhoA activation, or 2) ephrinB1 and RhoA could regulate ectoderm thickness through different pathways.
Aside from RhoA, ephrinB1 can also modulate the cytoskeleton through Rac1. For example, Rac1 mediates ephrinB1-induced membrane ruffling in human endothelial cells (Nagashima et al., 2002). Activated ephrinB1 interacts with the adaptor protein Crk through its SH2 binding domain, and in turn, Crk binds to DOCK180 on its SH3 domain to activate Rac1 (Nagashima et al., 2002). Furthermore, it was shown that ephrinB1 promotes formation of F-actin filled filapodia-like protrusions in zebrafish hepatic endoderm cells through the PDZ-binding domain; however, the exact molecular mechanism underlying protrusion formation is not yet known (Tomita et al., 2006; Cayuso et al., 2016). Nevertheless, a growing body of evidence implicates ephrinB1 as a crucial regulator of the actin cytoskeleton. Since knockdown of ephrinB1 leads to an increase of tissue surface tension across all tissues of the X. laevis gastrula, it is conceivable that this result could be because ephrinB1 modulates a core parameter of tissue cohesion that is common to all germ layer tissues: the actin cytoskeleton.

It has long been considered that cell–cell adhesion is mainly mediated by adhesion molecules such as C-cadherin. However, recent studies have shown that the binding energy released upon trans-cadherin binding is low, and insufficient to overcome cortical tension such that cells remain rounded and unable to spread on one another (Maître et al., 2012; Youssef et al., 2011). Cell contact spreading is required to establish cell–cell adhesion. To accomplish this, cells must lower their cortical tension at the contact interface. Cortical tension is predominantly regulated by cortical actin density (Krieg et al., 2008; Yamada and Nelson, 2007; Hidalgo-Carcedo et al., 2011; Chugh et al., 2017). In turn, cortical tension reflects constituent cell–cell adhesion (David et al., 2014). It is tempting to speculate that in the X. laevis ectoderm, overexpression of ephrinB1 leads to overt Rac1 activation and actin polymerization at the cortex, which leads to increased cortical tension, and consequently to the loss of cell–cell adhesion; however, work is required to support this hypothesis.
2.3.7 EphrinB1 in development and disease

The gene coding ephrinB1 is located on the X-chromosome in humans and mice. Loss-of-function mutations of ephrinB1 lead to tissue patterning disorders in the adult, collectively referred to as CFNS (Twigg et al., 2004). In females, the gene that codes for ephrinB1 is subjected to random X-inactivation. Therefore, two different populations of ephrinB1-expressing cells would be present in females who are heterozygous for the ephrinB1 mutation; a portion of cells would express wildtype ephrinB1 and another portion would express a truncated ephrinB1. Within tissues, these two populations sort out from one another, which leads to the mosaic segregation of normal and mutant cells and gives rise to ectopic tissue boundaries that contribute global tissue malformations, supposedly by ectopic cell sorting and perturbed cell migration (Twigg et al., 2004; Wieland et al., 2004). Thus, the phenotypic manifestations of CFNS are particularly severe in affected females.

Hallmark characteristics of CFNS patients include facial asymmetry (for example strabismus), abnormal thorax, and scoliosis (Wieacker and Wieland, 2005). In preliminary results, I show that knockdown of ephrinB1 in half of the embryo to mimic X-inactivation results in a *X. laevis* larval phenotype that manifested prominent aspects of CFNS pathology. Larvae developed craniofacial asymmetry, abnormal somite patterning and fusion, and scoliosis of the spine. Although it has been proposed that the abnormal compartmentalization of cells, referred to as ‘cellular interference’, could be responsible for CFNS (Twigg et al., 2013; Evers et al., 2014), the precise molecular mechanism, or the cellular events that ultimately lead to tissue malformations, are not fully known. The study of CFNS disease progression in mice has largely
been hindered by the inaccessibility of live tissues during early embryogenesis. Here, I propose that *X. laevis* represents a novel and powerful model system for studying the cellular basis of CFNS due to its external development, large cells, and well-established explants for experimental investigation of cellular processes.
3. CHAPTER THREE: The cellular basis of endoderm morphogenesis

3.1 Introduction

The basic body plan of metazoans is established by gastrulation, and at the core of this process is the movement of endoderm and mesoderm from the surface to the interior of the embryo. Among invertebrates, a common mechanism of germ layer internalization is invagination, the localized, coordinated inward bending of a single layered epithelium. A classical paradigm of gastrulation by invagination is the sea urchin embryo (Kominami and Takata, 2004), and a thoroughly studied example is the invagination of the mesoderm during D. melanogaster gastrulation (Rauzi et al., 2013). An alternative mechanism closely related to invagination is ingression, where individual cells leave the epithelial layer to move interiorly (Katow and Solursh, 1980).

Germ layer internalization by ingression also occurs among vertebrates, such as at the avian or mammalian primitive streak (Arendt and Nübler-Jung, 1999). However, gastrulation in these groups is highly derived (Stower and Bertocchini, 2017). In vertebrates in general, the development of a blastula with a multilayered wall and a compact, endodermal vegetal cell mass appears to be the primitive condition, and gastrulation is modified to adapt to these features. In particular, the multilayered vegetal cell mass that harbours most of the endoderm precludes invagination. Because massive ingestion of superficial vegetal cells is also not observed (Keller, 1978), the question arises as to how the vegetal endoderm is internalized during the ancestral
mode of vertebrate gastrulation, which is conserved in lampreys, lungfish, and amphibians (Collazo et al., 1994; Shook and Keller, 2008).

Surprisingly, although endoderm internalization is an essential feature of gastrulation, it has barely been studied in these vertebrate groups, not even in X. laevis, where gastrulation is best understood at the cellular and molecular level. In this species, the inward movement of the endoderm has been analyzed only at the tissue level (Winklbauer and Schürfeld, 1999; Papan et al., 2007). At the onset of gastrulation, the vegetal cell mass surges animally into the embryo, expands at the BCF, and displaces the posterior mesoderm at its periphery in the vegetal direction. The whole process appears in sagittal view as a rotational movement, and was accordingly termed vegetal rotation. As shown by respective vegetal mass explants, this process is based on active, region-specific, autonomous tissue deformations, although the precise cellular mechanisms that drive vegetal rotation are not known.

Here, I analyzed the cellular basis of vegetal rotation. I show that endoderm cells elongate and align at the onset of gastrulation. Cells move by differential migration; spatially graded differences in velocity lead to cell rearrangements that narrow the vegetal-most part of the tissue and expand the animal part, leading to the inward surge of the vegetal mass. During migration, endoderm cells are separated by wide interstitial gaps, which are bridged by dynamic filiform protrusions. Despite the gaps, C-cadherin is required to maintain tissue cohesion and to facilitate cell migration. Interaction with the extracellular matrix (ECM) protein fibronectin (FN) is also necessary for vegetal rotation. Cells move using amoeboid behaviours, which include cell elongation, leading edge expansion, and trailing edge retraction. They reproduce this behavior in vitro on an appropriate FN-containing substratum.
3.2 Results

3.2.1 Vegetal rotation is driven by endoderm cell migration

The vegetal slice explant, which consists of an approximately five-cell-layer-thick mid-sagittal tissue slice of the vegetal hemisphere, has played an instrumental role in deciphering the cellular basis of vegetal rotation (Winklbauer and Schürfeld, 1999). The vegetal explant contains two cell types, located in distinct regions. Namely, a central column of endoderm is bilaterally flanked by mesoderm positioned at the explant periphery. I first confirmed that vegetal rotation movements were driven by endoderm cells within the vegetal cell mass (Fig. 3-1). To test this notion, I removed the mesoderm components located at the explant periphery (Fig. 3-1A). I found that isolated mesoderm fragments became round and remained relatively stationary (not shown). In contrast, explanted endoderm alone could broadly recapitulate vegetal movements from the intact embryo in vitro (Fig. 3-1B), which supports the notion that vegetal rotation is an endoderm-driven phenomenon. The endoderm, which initially resembled a rectangular column, changed shape and progressively adopted a truncated cone shape after one hour (Fig. 3-1B). This geometric transformation is reminiscent of tissue deformation that the endoderm undergoes in vivo, due to expansion of the BCF in conjunction with tissue narrowing at the vegetal base (Fig. 3-1C). A small population of primordial germ cells (PGCs) reside within the endoderm. During gastrulation, there are approximately 8.7 ± 3.0 PGCs in the endoderm (Whittington and Dixon, 1975), whereas the early gastrula consists of approximately 20,000 cells (Gerhart and Keller, 1986); thus, PGCs represent less than 1% of all cells within the endoderm. Therefore, the
contribution of PGCs to vegetal rotation is likely negligible, and the following sections are focused on endodermal contributions to morphogenesis.

![Diagram of tissue-autonomous movement](image)

**Figure 3-1 Tissue-autonomous movement in central endoderm explants.**

(A) Vegetal slice explant was subdivided to isolate the central column of endoderm. Peripheral mesoderm was excluded from the explant. (B) Endoderm explant deformation from an initial rectangular shape to a truncated-cone shape after 2 hours. Actual explant (top) is shown in conjunction with a two-dimensional illustration of tissue geometric changes (bottom). (C) Tissue deformation of the early gastrula to the mid gastrula. Mid-sagittal gastrula fractures are shown.
The central column of endoderm is highlighted (yellow), and geometric changes are indicated (dashed lines, top). Endoderm region is estimated using cell position tracking reported by Bauer et al., 1994. Corresponding three-dimensional illustrations of tissue geometric changes are noted (bottom).

3.2.2 BCF expands by cell rearrangement and oriented cell shape changes

To shed light on the cellular basis of explant deformation, I first quantified tissue shape changes in vegetal slice explants. Explants were isolated from stage 10 gastrulae and tissue movements were monitored in parallel with intact embryos from the same clutch. Beginning at gastrulation the upper region of explants expanded, and the BCF widened by 1.8-fold over two hours before reaching a plateau (Fig. 3-2A). Simultaneously, the equatorial waist of explants narrowed by 0.8-fold (Fig. 3-2A). In the absence of significant cell division or cell growth during gastrulation (Saka and Smith, 2001; Kurth, 2005), this tissue deformation must be due to cell rearrangement, cell shape change, cell orientation, or any combination of these factors.

Next, I quantified the number of cells along predetermined landmarks in explants immediately after explant excision and 90 minutes following explant deformation. Landmarks for the BCF were determined based on the position of opposing endoderm cells located at the explant margins. The equatorial waist was determined from the explant center, which aligned with an indentation created by the dorsal blastopore. I found that the average cell number increased from
11.7 to 17.3 at the BCF and remained unchanged along the A–V axis, but decreased from 21.3 to 14.3 along the waist of explants (Fig. 3-2B). Thus, cells rearranged to expand the upper part of the explant at the expense of the lower, narrowing part. Cells also elongated during rearrangement. The average cell length–width ratio (LWR) was 1.4 at 30 minutes after explantation, and reached a plateau of 1.6 after the first hour in explants (Fig. 3-2C). Correspondingly, the cell LWR increased from 1.3 at stage 10 to 1.5 in stage 10.5 gastrulae (Fig. 3-2C).

![Figure 3-2 Cell number and cell shape changes during explant deformation.](image)

(A) Endoderm explant deformation. BCF lengths (red lines) from cell tracking (red dots). Equatorial waists (dashed white lines), and blastopores (red arrowheads) are indicated. Animal (An) is to the top, vegetal (Vg) to the bottom, ventral (V) to the left, and dorsal (D) to the right.
(B) Number of cells at the BCF and equator. Error bars indicate standard deviations. Asterisks denote statistical significance where $P < 0.05$. (C) Cell LWR in explants (left) and embryos (right). Averages are 1.41 (30 min), 1.57 (60 min), and 1.57 (90 min) in explants; 1.27 (S10), 1.53 (S10.5), and 1.46 (S11) in embryos. Box plots show the median, interquartile range, maximum and minimum.

Cell layer measurements were taken at the midline position of the vegetal cell mass. Measurements revealed that the vegetal tissue was approximately 12-cell layers thick at the onset of gastrulation. To uncover regional contributions to large-scale tissue deformation during vegetal rotation, video recordings of vegetal slice explants were evenly subdivided into the top, mid, and bottom regions according to their respective cell layers for analysis. Interestingly, I found that cell elongation coincided with a pronounced cell re-orientation event. In the initial 30 minutes after explant excision, endoderm cells appeared obliquely oriented in all regions of explants (Fig. 3-3A). During the next half hour, cells from the top layers oriented progressively toward the mediolateral axis, whereas cells from the middle layers became aligned to the A–V axis (Fig. 3-3A). In contrast, cells at the bottom remained obliquely oriented. Thus, cells at the top became perpendicularly oriented with respect to cells in the center. Regional cell orientations appeared similar in the embryo at comparable stages (Fig. 3-3A). In particular, cells of the BCF appeared flattened in parallel to the tissue surface. Cells from the BCF were also oriented perpendicular to cells located farther vegetally (Fig. 3-3B). These data support the notion that a combination of cell rearrangement and oriented cell elongation is associated with the distinct shape change of vegetal explants. In fact, these two factors work together to advance tissue morphological changes. For instance, an increase in cell number in conjunction with cell
reorientation at the top layers, whereby cells near the BCF lie with their long axes in parallel to the surface, contribute to rapid expansion of the BCF. Moreover, a decrease in cell number and cell alignment along the A–V axis in the mid region, synergistically constrict the vegetal region.

Figure 3-3 Endoderm cell orientation in explants and in embryos.

(A) Rose diagrams indicating the number of cells in explants (left) and in embryos (right) oriented at 0–90° angles relative to the A–V axis in the top, middle, and bottom cell layers. At stage 10, the endoderm has an average of twelve cell layers, evenly divided into three regional layers. Bars represent angle ranges, and lengths indicate numbers of occurrence in 5° bins. (B) SEM images of endoderm in mid-sagittally fractured embryos.
### 3.2.3 Endoderm cells rearrange by differential migration

To examine cell rearrangement during tissue deformation, I recorded the movement of membrane-labelled endoderm cells in wildtype vegetal explants. In groups of contiguous cells, A–V neighbours typically separated due to the higher velocity of the more animally positioned cell, whereas laterally situated neighbouring cells converged toward the trailing end of the advancing cell, thus filling the space behind them (Fig. 3-4A). To achieve tissue deformation in this way, cell velocity must increase in a vegetal to animal pattern. Indeed, I discovered such a pattern when I analyzed the kinetics of cell movement in explants (Fig. 3-4B). Relative to the vegetal epithelial surface, cells moved faster the farther animally they were situated, up to a point near the BCF. This increase amounted to a 4-fold difference between the extremes of the velocity gradient (Fig. 3-4C, D). Moreover, movement also became faster toward the center of the explant relative to the periphery (Fig. 3-4D). Individual cell movements appeared discontinuous, and the fits and starts of cell movement were apparent from corresponding cell velocities recorded for the same cell at different time points (Fig. 3-4C). Discontinuous movement is consistent with the notion that cells temporarily use adjacent cells as substratum while engaged in a burst of locomotion, to serve in turn as the substratum for neighbouring cells. Nevertheless, the average velocity increases gradually in the direction of movement to promote cell rearrangement by differential migration.
In isolation, endoderm cell elongation coincided with single cell translocation. The intended migration pathway indicated by the long axis of migrating cells and its actual movement trajectory were aligned (examined below). For cell cohorts, however, such as the case of endoderm cells in explants, I noted that cell displacement and cell body elongation did not always align with the direction of cell migration (Fig. 3-5A). Thus, net movement of cells was the result of both active movement and passive influence by neighbouring cells (Fig. 3-5A, B). I then asked whether cell alignment could have an impact on cell migration. To address this question, I quantified cell alignment by measuring the angular deviation of cell displacement relative to the long axis of the cell. I found a significant correlation between pathway alignment and movement velocity (Fig. 3-5C). In other words, cells that had their actual movement
trajectory matched to their intended path as projected by cell elongation also tended to move faster.

Figure 3-5 Cell body elongation and directional alignment during cell movement.

(A) Longitudinal cell axis is indicated (blue line, top panels) with corresponding cell displacement (bottom panels) from origin (red centroid) to target (green centroid). Migration path is shown (red line). (B) Trajectory alignment of cell in A. Deviation of the resultant path (red line) from the intended path (blue line) is represented by alignment $\theta$. (C) Inverse relationship of alignment $\theta$ to cell velocity in explants. Fitted curve (red line) is indicated in the scatter plot.

As previously noted, cells moved faster the farther animaly they were situated, although how this velocity gradient became established is not yet understood. To investigate this, I examined endodermal tissue architecture, and strikingly, I found a latticework of interstitial spaces (gaps) that permeated the endoderm in the intact embryo (Fig. 3-6A). A pattern of different gap widths could be seen where gaps appeared narrow near the vegetal base and the BCF, whereas they widened toward the middle (Fig. 3-6B). To characterize the pattern of gaps, I measured the
intercellular distance produced by gaps along the transverse (cell columns) and vertical (cell rows) axes of mid-sagittally fractured embryos (Fig. 3-6C). In both directions, width correlated with movement velocity, and cell gaps were widest near the tissue center relative to narrow peripheries. In addition, gaps also widened in the A–V direction, where gaps were wider the more animally they were situated, until they became narrow again at the BCF (Fig. 3-6C).

Figure 3-6 Interstitial gaps between endoderm cells.
(A) Gaps between cells in the embryo. TEM image of endoderm (left). Negative of TEM image (right). Different gap widths between the top (yellow arrow), middle (orange arrow), and bottom cell layers (red arrow). (B) A vertical series of gaps shows gap widths widening in the vegetal to animal direction and narrowing again at the BCF. (C) Gap widths in cell columns (left) and rows (right) in gastrulae. Error bars indicate standard error.

3.2.4 Endoderm cell reorientation near the BCF

As previously noted, during cell rearrangement, A–V neighbours typically separated due to the higher velocity of the more animally positioned cell. Therefore, an A–V cell velocity gradient was expected, and such a gradient was indeed found (Fig. 3-4). However, at the anticipated maximum of the velocity gradient, which corresponded to cells positioned near the BCF and where cells were expected to migrate fastest, the velocity gradient was reversed and became diminished instead. To investigate this discrepancy, I analyzed the movement of cells near the BCF in explants. I found that cells at or near the BCF moved rapidly; however, they underwent reorientation during movement (Fig. 3-7). Consequently, the A–V component of their movement velocity became diminished as they approach the BCF (Fig. 3-7A), which appeared as a reversal in the velocity gradient. As the vertical component of cell movement slowed down near the BCR, this reversal of the velocity gradient allowed for a directional change from mediolateral to A–V cell intercalation. Reorientation was achieved by rotation of the entire cell body (Fig. 3-7B).
To investigate whether cell movement patterns observed in the explant apex could occur in the embryo, I examined cell morphology of the intact BCF. I found cells in oblique (Fig. 3-7C) and parallel (Fig. 3-7D) orientations with respect to the BCF surface, consistent with cell reorientation in explants. Together, these data suggest that at the BCF, elongated cells move laterally, and that cells from deeper layers insert themselves into opened gaps, thus expanding the BCF by intercalation as well as by cell reorientation.
Figure 3-7 Endoderm cell reorientation.

(A) Original velocity is maintained while its vertical component is reduced at the BCF. Cell movement is shown (top row). Cohort (top-mid row) shows the leading cell (green) advancing relative to the lagging cell (orange); the gap that opens is filled by an inserting deep cell (purple). A cell near the surface (red) is inserted, and remains parallel to the BCF. A deep cell (blue) that
was initially perpendicular to the surface is re-oriented and becomes parallel to the BCF. Cell re-orientation is shown (bottom-mid row). Re-orienting cell vectors (bottom) showing the vertical component (black arrow) relative to the original (grey arrow) vector. (B) Cell re-orientation; movement is indicated (orange arrow). (C) Cell morphology and arrangement in the embryo is consistent with cell insertion at the BCF. SEM image of cells at the BCF (left). Morphology is highlighted (orange, right) with the long axis of the cell (black arrow), and blastocoel (bc) indicated. (D) Intact BCF showing endoderm (endo) cells.

### 3.2.5 Endoderm cells are coupled by stitch contacts

Given the presence of interstitial space between cells, it was not clear how cells attached to one another during intercellular migration. To investigate this question, I visualized cell–cell contacts using SEM (Fig. 3-8A). Consistent with mid-sagittal plane semi-thin sections of the endoderm (Fig. 3-6A), cells were surrounded on all sides by copious amounts of interstitial space. Interestingly, cells extended thin processes to contact neighbouring cells through the gaps (Fig. 3-8A). Under transmission electron microscopy (TEM), interstitial spaces appeared even more prominently and included irregular gaps at three or four-cell junctions (Fig. 3-8B). Similar to the SEM observations, thin cytoplasmic protrusions were observed under TEM. Protrusions were distributed throughout the cell surface and formed stitch-like contacts between cells (Fig. 3-8B). Additionally, cells appeared connected through short stretches of closely apposing cell membranes, where intermembrane distances were approximately 30 nm (Fig. 3-8C), consistent with the intercellular binding distance of classic cadherins.
To confirm that gaps were not artefacts induced by sample fixation, I examined intercellular dynamics in live tissues. To visualize cell gaps, I incubated vegetal explants in a medium containing AvidinFITC (Fig. 3-8D). Cells were separated by gaps 1 µm wide on average, similar to those observed under SEM (Fig. 3-8E). Furthermore, using an ECM stain on TEM samples prepared from whole embryos, I found numerous globules of electron-dense bodies on the surfaces of cell membranes. These darkly-stained amorphous residues were reminiscent of dehydrated heteroglycans found on the surfaces of cell membranes (Komazaki and Hirakow, 1982), consistent with the notion that cell gaps are filled with ECM in the intact embryo (Fig. 3-8F). Interestingly, cells in live explants were interconnected by long (1–10 µm) and thin (0.5–1.0 µm) F-actin filled protrusions, which were also observed under TEM (Fig. 3-8B, G). The filiform protrusions extended and retracted rapidly (Fig. 3-8G). For example, a 5-µm-long protrusion could undergo a complete cycle of extension, attachment and retraction in under 3 minutes. Thus, the protrusions provide highly dynamic cell contacts, potentially well-suited for cell rearrangement.
Figure 3-8 Endoderm cells are linked by stitch contacts.

(A) SEM images of endoderm (left) in embryos. Magnification (center) depicts interstitial space (red arrows). Cells are linked by stitch contacts (right; yellow arrows). (B) TEM images of endoderm (left) in embryos. Cell gaps (red arrows) and cell contacts (yellow arrows) are indicated. Magnification of neighbouring cells (center) shows contacts (green arrows) interspersed between gaps (red arrows). Base of stitch contacts appear raised (white arrows), indicating tethers are taut (right). (C) Cell–cell contact distances (green arrow) are compatible with cadherin-based adhesion (~30 nm). (D) Interstitial gaps in explants. Labelled (mRFP) explants (left) in medium with AvidinFITC to visualize gaps (red arrows). Stitch contacts (yellow arrows) extend between cells (center). Magnified view of contacts (right). (E) Quantification of gap widths in explants, whole embryo SEM and TEM images. Measurements were taken from cells located in the central, mid-endodermal region. (F) Interstitial gaps are filled with ECM. Heteroglycans stained using Alcian blue appear as black cell surface residues under TEM (left). Dehydrated matrices (blue arrows) were also found linking cells together (yellow arrow). (G) Cells actively form dynamic intercellular contacts. Membrane label (mGFP; top) and Lifeact–Ruby (middle) co-expressing cells show contacts are rich with polymerized actin (merged; bottom). Time-lapse sequences (four right panels) of a region of interest (box) showing that protrusions extend (yellow arrows) and retract (green arrows).
3.2.6 Amoeboid migration of endoderm cells

To elucidate the mechanism of endoderm cell migration, I examined endoderm cell movement in membrane labelled vegetal explants (Fig. 3-9). I found that endoderm cells exhibit an amoeboid-type movement. Cells undergo cycles of body elongation in the direction of movement, expansion of the cell front, narrowing of the cell rear, and retraction of the trailing edge (Fig. 3-9A). Endoderm cells are rich with yolk platelets, which are stabilized in the cytoplasm by a microtubule basket that surrounds the cell body (Selchow and Winklbauer, 1997). Thus, yolk platelets may be considered markers of cytoplasmic positioning. To confirm that motile cell behaviours correspond to cell displacement, I tracked yolk platelet displacement within moving cells and between neighbouring cells. In the example shown, yolk platelets near the leading edge within the same cell maintain their relative positions during migration (Fig. 3-9A), which indicates that the cytoplasm advanced forward behind the leading edge. In contrast, yolk platelets of different cells separated from one another during migration, which confirmed that cell displacement occurred in parallel with cyclic amoeboid cell behaviours (Fig. 3-9A).

Amoeboid-type cell migration is characterized by specific cell morphological features associated with distinct steps in the migration cycle. Thus, cell morphology may be used to identify cells that undergo amoeboid migration in vivo. I assessed whether endoderm cells undergo amoeboid migration in the intact embryo by analyzing endoderm cell morphology in mid-sagittally fractured gastrulae (Fig. 3-9B). I found that endoderm cells from mid-stage gastrulae showed numerous morphological features consistent with amoeboid migration. Moreover, distinctive cell
shapes of different steps within the cell migration cycle were found between neighbouring cells, consistent with differential migration (Fig. 3-9B, C).

Importantly, endoderm cells lacked locomotory protrusions, such as lamellipodia, at the cell front both in time-lapse recordings (Fig. 3-9A), and in the embryo, where the leading edge of cells appeared blunt (Fig. 3-9C). Taken together, these results support the notion that endoderm cell translocation is achieved by coordinate cell behaviours that correspond to steps of a cell migration cycle consistent with amoeboid-type cell migration.
Figure 3-9 Morphological changes associated with endoderm cell migration.

(A) Amoeboid behaviour of endoderm cells. Labelled cells are shown (top row), with major cell body changes indicated (dashed arrows). Corresponding yolk platelets are also shown (bottom row). A selected cell (pink dashed lines) is highlighted with respect to neighbouring cells (grey dashed lines). Yolk platelets (pink solid lines) within the selected cell and a neighbouring cell (blue and orange platelets) are indicated. Platelets (yellow, blue) of different cells move with respect to cell rearrangement; platelet movements are indicated by displacement length ($\Delta d$, white double arrows). (B) Endoderm cell morphology in the embryo. SEM image of endoderm cells reveals morphology consistent with amoeboid movement. Morphologies consistent with 1)
cell elongation, 2) leading edge expansion, 3) trailing edge recession, and 4) trailing edge retraction are indicated. (C) Endoderm cell topography. Panels show an elongated cell (left), a cell undergoing leading edge expansion in parallel with trailing edge recession (center), and a cell undergoing trailing edge retraction (right). The cell front (yellow arrows) and cell rear (blue arrows) are indicated.

To further characterize cell rearrangement, I analyzed the spatiotemporal relationship between cell shape changes and the relative displacement of neighbouring cells. Cells appeared elongated during movement; however, cell body LWR typically did not extend beyond an average of approximately 1.5, which suggests a dynamic balance between extension of the cell front and retraction of the cell rear. I found that leading edge advancement and trailing edge retraction occurred simultaneously, and thus propelled the cell body forward with respect to neighbouring cells while preserving the cell LWR (Fig. 3-10). With respect to its neighbours, the leading edge of the moving cell remained in contact with the cell ahead of it, with both cells apparently moving in tandem. On other occasions, cells were observed to invade the space evacuated by the retraction of a leading cell. At the rear, tail retraction was accompanied by reduction of contact with the lagging cell (Fig. 3-9A). Together, synchronized contact formation at the cell front, in conjunction with cell–cell contact resolution at the cell rear, contributed to the apparent smooth creeping locomotion of endoderm cells during cell rearrangement.
Figure 3-10 Coordinate behaviours drive cell locomotion.

Elongated cells poised for migration maintain stable lateral borders (yellow arrowed lines) throughout migration (parallel dashed white lines). To advance forward, the leading edge is extended (green arrows) relative to its initial length (top grey lines), while the trailing edge is retracted (red arrows) relative to its initial length (bottom grey lines). Extracellular debris anchored to the leading cell (blue arrows) and lagging cell (white arrows) are indicated to show displacement. Enlargement of the trailing edge shows contact reduction (mRFP panels) between cells. AvidinFITC puncta are also visible (white arrows) at sites of membrane undulation. An illustrated interpretation of trailing edge retraction is depicted in the bottom row.

3.2.7 FN interaction is required for endoderm cell migration

It has been shown that the matrix-related glycoprotein FN accumulates on the surface of endoderm cells (Winklbauer, 1998). However, it was reported that endoderm cells do not migrate on FN-adsorbed tissue culture plastic (Wacker et al., 1998). To confirm this finding, I analyzed
the morphology of endoderm cells seeded on FN-plastic substrate. Cells appeared multipolar and extended numerous lamellipodia and filopodia outward from the cell body (Fig. 3-11A). In contrast, when cells were seeded on a substratum of FN-adsorbed to gelatin, cells adopted morphological and behavioural features reminiscent of the situation in vivo (Fig. 3-11A, B). While on gelatin, cells elongated and showed discernable cell polarity. A presumptive cell front could be distinguished based on its characteristically broad edge that tapered into a narrow cell rear. However, as in tissues in vivo (Fig. 3-11B), no leading lamellipodia structures were observed in cells in vitro (Fig. 3-11A).

I then examined endoderm cell migration on different in vitro substrates (Fig. 3-11C, D). When seeded on FN-plastic, endoderm cells spread onto the plastic surface, which resulted in non-migratory cells that flattened over time (Fig. 3-11C). This non-migratory multipolar morphology appeared to be an artefact of substrate incompatibility. When seeded on FN-gelatin, cells translocated using an amoeboid-like migration cycle that mimicked the behaviour of cells in tissues (Fig. 3-11D). I quantified cell movement velocities in vitro and found that on FN-gelatin, single cells translocated with an average velocity of 4 µm/min, which was approximately 2.5-fold faster than that within tissues (Fig. 3-11E). These data support the notion that cell movement velocity within tissues is attenuated by the influence of neighbouring cells (Fig. 3-5C).
Figure 3-11 Endoderm cell movement on FN in vitro.

(A) Morphology on different substrates. Cells on plastic coated with FN (left) appear multipolar (red arrows). Cells on gelatin coated with FN (right) appear unipolar with distinct front (yellow arrow) and rear (blue arrow). (B) Morphology in vivo. Example cell within the endoderm with front (yellow arrow) and rear (blue arrow) polarity. Animal (An) is up, vegetal (Vg) is down. (C) Spreading on FN. Cell spreading morphology is noted in A (top row). Morphological changes are outlined (bottom row). Time after seeding is indicated. (D) Locomotion on gelatin–FN in vitro. Cell movement on gelatin–FN (top row). Morphological changes (black arrows) from the previous time points are outlined (black lines) with respect to the next time point (grey lines). Cell behaviours (bottom row) with respect to time after seeding. (E) Cell migration velocity in vitro. Velocities of cells in explants with respect to the epithelium, and of single cells with respect to the substrate.
To assess the requirement for FN during cell migration, I blocked cell–FN interactions using a well-characterized synthetic peptide that contained an Arg–Gly–Asp (RGD) amino acid sequence, which inhibits FN-binding by competitive inhibition. I first injected either RGD-, or the corresponding control RGE-peptide into the blastocoel cavity of blastula-stage embryos. I then followed the developmental progression of injected and uninjected embryos to the late-gastrula stage. I found that the endoderm of RGD-injected embryos was morphologically perturbed. Rather than a cohesive tissue, numerous round cells were found scattered in the position where endodermal tissue was expected, which suggested that endoderm cells had undergone cell dissociation. In contrast, however, control RGE-injected embryos appeared indistinguishable from wildtype gastrulae (Fig. 3-12A).

Given that RGD-peptide perturbed endoderm cohesion, I expected that this inhibition of FN-binding would perturb endoderm cell migration. To test this prediction, I incubated vegetal slice explants in media containing either RGD- or RGE-peptides. Consistent with whole embryo manipulation, exposure of vegetal slice explants to RGD-containing media perturbed endoderm cell morphology (Fig. 3-12B). Relative to cells that were exposed to control RGE-peptide, RGD-treated cells became significantly more rotund (Fig. 3-12C). However, some elongated cells were still present in RGD-treated explants. Cell elongation and axial orientation of cells could be used to infer effective cell migration (Fig. 3-5). By measuring alignment congruity between the expected migratory pathway (in parallel to the A–V axis) and the observed long-axis of endoderm cells, I found that cell elongation congruity was significantly lower in RGD-treated cells, which indicated that even when elongated cells appeared under RGD-treatment, they were less aligned with the direction of cell migration (Fig. 3-12D). Consistent with this observation, I
measured cell movement velocity in explants and found that the velocity was significantly decreased, and the majority of RGD-treated cells became non-migratory (Fig. 3-12E). In contrast, control RGE-treated cells showed movement velocities comparable to those of wildtype cells in explants (Fig. 3-11E; 3-12E). Taken together, these results suggest that the presence of FN was required for cell translocation.

Figure 3-12 Endoderm cell movement on FN in vivo.

(A) Inhibition of FN binding in embryos. Embryos were injected with a bolus of either RGD (left) or RGE peptides (center), or were left uninjected (right) at stage 8, and then grown until stage 11. RGD treatment perturbed endoderm morphology relative to controls (yellow arrows).

(B) Inhibition of FN binding in explants. Explants in media containing either Arg–Gly–Asp (RGD; left) or Arg–Gly–Glu (RGE) peptides (right). RGD-treated cells appear rotund (pink outline). RGE-treated cells remained elongated (yellow outline). Schematic showing the region
of interest (red box) is indicated in the top-right corner. (C) Cell LWRs in explants incubated in RGE (left) and RGD peptides (right). (D) Cell elongation congruity in explants incubated in RGE (left) and RGD peptides (right). (E) Cell migration velocity in explants incubated in RGE (left) and RGD peptides (right).

3.2.8 Cell body oscillation during single cell translocation

To my knowledge, this work is the first to demonstrate that X. laevis endoderm single cell migration can be studied in vitro. Although I have shown that endoderm cells migrate using amoeboid-type locomotion, the mechanism by which cells translocate without locomotory protrusions such as lamellipodia is not yet clear. To further investigate cell membrane dynamics during single cell translocation, I monitored membrane behaviours using time-lapse microscopy. Interestingly, I found that endoderm cells undergo membrane undulations that propagated along the lateral sides of the cell body. I quantified membrane undulations by measuring the corresponding changes in cell width (Fig. 3-13). I found that cell width oscillations resembled pulsatile contractions. Moreover, contractions were biphasic; rapid cell body oscillations occurred over approximately two minutes, and were then followed by a refractory period of approximately one minute (Fig. 3-13).
Dissociated cells were seeded and given 15 minutes to recover before measurements were taken. Cell body widths from the cell center were measured from successive frames in a time-lapse recording of elongated motile endoderm cells.

In *Cynops pyrrhogaster*, it was found that pulsating cell body movements were directional and capable of moving small particles attached to the cell body in a polarized manner from the cell front toward the cell rear. Oscillations were wave-like and correlated with membrane undulations (Kubota, 1981). In light of this observation, I asked whether endodermal cell body oscillations were also capable of moving extracellular material. I monitored the position of a yolk platelet that fell close to the lateral cell border of an abutting endoderm cell. I found that the yolk platelet that was adjoined to the endoderm cell underwent displacement over time, in contrast to an
unattached yolk platelet that remained stationary throughout the observational period (Fig. 3-14). As noted previously, endoderm cell polarity could be distinguished in vitro based on a broad presumptive cell front and a narrow cell rear (Fig. 3-11A). Interestingly, the movement trajectory of the attached yolk platelet suggests that the cell displaced the yolk platelet toward the cell rear. This finding implies that an external flow could be generated from cell surface membrane contractions of endoderm cells, and that this flow was capable of moving extracellular components directionally, although the implications of such movements in *X. laevis* are not yet understood. Work is ongoing to further characterize the kinetics of cell body oscillations.

**Figure 3-14 Displacement of cell debris attached to the surface of an endoderm cell.**

A yolk platelet that fell within the proximity of an elongated endoderm cell was moved posteriorly down the cell body. Successive frames show the movement of an external yolk platelet (yellow) attached to the endoderm cell relative to another yolk platelet (red) that is unattached. The displacement is shown (Δd). Cell polarity was determined based on cell morphology. Time is shown at the bottom left in hours:minutes.
Given that endoderm cells undergo single cell movement \textit{in vitro}, as well as the infrequent nature of cell–cell contacts \textit{in vivo}, I asked whether C-cadherin is necessary for endoderm cell migration in tissues. C-cadherin is the main adhesion molecule in the early gastrula (Kühl and Wedlich, 1996). To determine the requirement of C-cadherin, I knocked down endodermal C-cadherin protein levels using a previously characterized C-cadherin morpholino antisense oligonucleotide (CcadMO; Ninomiya \textit{et al.}, 2012).

I first examined endoderm cell morphology in CcadMO-injected morphant tissue explants. Compared to wildtype controls (Fig. 3-14A), morphant cells extended fewer lateral protrusions between cells (Fig. 3-14B) and became significantly more rounded (Fig. 3-14C). To evaluate whether cells were elongated in parallel to the axis of migration, I assessed the congruence between cell elongation and the direction of cell migration. I found that knockdown of C-cadherin significantly lowered cell elongation congruity, which indicated that CcadMO-injected cells were poorly aligned with respect to the axis of cell migration (Fig. 3-14D). Given that knockdown of C-cadherin significantly reduced the number of protrusions projected by morphant cells (Fig. 3-14E), I asked whether this phenomenon was due to a decrease in protrusion formation or a loss of protrusion stability. To address this question, I monitored changes in the number of protrusions between successive frames in time-lapse recordings. I found that protrusive activity was in fact reduced upon C-cadherin knockdown, but the few processes that did form in morphant cells retained comparable stability to controls (Fig. 3-14F, G). This finding suggests that C-cadherin is not only responsible for the attachment of protrusions, but is also required to initiate protrusion formation during cell migration.
Nevertheless, CcadMO-injection diminished endoderm tissue cohesion, which is essential for cell migration (Fig. 3-14H). Consistently, I found that CcadMO-injected cells were nearly non-migratory relative to controls (Fig. 3-14I–K).
Figure 3-15 C-cadherin is required for endoderm cohesion.

(A) Protrusions engage in cell–cell adhesion. Labelled (mRFP) endoderm cells appear elongated (yellow outline) in explants (left). Cells project protrusions (yellow arrows) onto neighbouring cells (center). Protrusions are enriched with α-catenin at sites of cell–cell contact (right). (B) C-
cadherin knockdown altered cell morphology and reduced cell protrusions. C-cadherin morpholino (CcadMO)-injected cells (pink outline) appear rotund (left). Few protrusions were found in morphant cells (center), although α-catenin was still present within protrusions (right). Schematic showing the region of interest (red box) is provided in the top-right corner of select panels. (C) Cell LWRs in explants comparing wildtype and CcadMO-injected cells. (D) Cell elongation congruity in explants comparing wildtype and CcadMO-injected cells. (E) Average number of protrusions per cell projected by wildtype or CcadMO-injected cells. For all histograms, error bars indicate standard error (F) Protrusion dynamics of wildtype (control) cells. (G) Protrusion dynamics of morphant (CcadMO-injected) cells in explants. Zero indicates no change (dashed grey line); net gain (positive axis) and net loss (negative axis) of protrusions over time are shown. Colours represent individual cells. (H) Quantification of tissue cohesion. Tissue surface tension measured from wildtype and CcadMO-injected endoderm. (I) Cell migration velocity in explants comparing wildtype and CcadMO-injected cells. (J) Movement trajectories of cells in wildtype explant. (K) Trajectories of morphant cells in CcadMO-injected explant after 60 minutes. Colours represent individual cells.

The protein α-catenin is essential for cadherin function in cell adhesion. Within the cell, β-catenin is constitutively linked to the cadherin cytoplasmic domain, whereas α-catenin associates with the cadherin–catenin complex at sites of cell–cell adhesion in the *X. laevis* gastrula (Kurth *et al.*, 1999). To characterize α-catenin dynamics in endoderm cell contacts, I expressed α-catenin–GFP at levels where no overexpression effects were apparent. I found that α-catenin was enriched at the base of protrusions in wildtype and CcadMO morphant cells (Fig. 3-15A, B). Surprisingly, α-catenin–GFP enrichment persisted well beyond sites of cell–cell contact (e.g., at
the tip of protrusions). Expression of α-catenin filled the entire length of cytoplasm within projected protrusions. Moreover, α-catenin extended beyond the base of protrusions and into the lateral cortical cell membrane domains that were devoid of cell–cell contacts, which suggested that α-catenin may have a role in endoderm cells beyond cell adhesion (Fig. 3-16A). Overall, α-catenin was enriched at the lateral cell cortex in the presence of C-cadherin.

Interestingly, α-catenin density fluctuated with contact behaviour. For instance, contact formation induced a local increase in α-catenin density (Fig. 3-16B), whereas contact detachment was correlated with a decrease in local α-catenin density (Fig. 3-16C). Beyond sites of contact, α-catenin was also concentrated at the base of nascent protrusions (Fig. 3-16B, C). In CcadMO-injected morphant cells, the distribution profile of α-catenin appeared similar, although, α-catenin levels were lower overall, in agreement with the low frequency of protrusions (Fig. 3-15A, B). Thus, a dynamic interplay between contact formation and detachment was associated with α-catenin fluctuations at the cell cortex.
Figure 3-16 Localization of α-catenin.

(A) Localization of α-catenin–GFP at sites of cell–cell contact (white arrows). (B) Protrusion extension is correlated with α-catenin accumulation (top row, white arrows). The α-catenin enrichment fills protrusions from the tip to the base (bottom row). (C) Protrusion retraction is correlated with α-catenin reduction (top row, white arrows). After complete retraction, α-catenin is diminished in the cortex (bottom row).
3.3 Discussion

3.3.1 Endoderm cell orientation during gastrulation

For this chapter, I investigated endoderm morphogenesis by characterizing the cellular behaviours and molecular components responsible for driving cell migration during vegetal rotation. I monitored endoderm cell translocation in live tissue explants in parallel with fixed samples from correspondingly staged gastrulae to correlate events observed in vitro to processes that occur in vivo. The vegetal hemisphere represents nearly two-thirds of the early gastrula. To investigate regional contributions to large-scale tissue deformation, the endodermal vegetal cell mass was subdivided into top, middle, and bottom regions for further analysis.

At the onset of gastrulation, prior to vigorous cell migration, endoderm cells of all regions appeared elongated. This observation is consistent with reports that subtle vegetal cell movements occur prior to gastrulation (Papan et al., 2007; Winklbauer and Damm, 2012). During pre-gastrulation emboly, the vegetal tissue is moved inward toward the embryo center. Consistent with this movement, cell orientation of the vegetal cell mass is aligned oblique to the A–V axis at the start of gastrulation. Despite initial uniformity, however, shortly after the start of gastrulation, different region-specific cell orientations become apparent. Cells of the top layers progressively orient perpendicular to the A–V axis, whereas cells in the middle layers align along the A–V axis. Thus, endoderm cells near the BCF become nearly perpendicular to cells positioned near the vegetal center. Concurrently, cell reorientation events occur in parallel with
regional changes in cell migration behaviour. For example, cells in the middle layers surge animally, while cells positioned near the top rotate and then migrate toward the periphery. Region-specific tissue deformation may be linked to differences in regional cell orientation. In support of this notion, Schürfeld and Winklbauer reported that when the vegetal explant is subdivided into fragments representing discrete zones, each fragment autonomously reproduces region-specific movements in isolation. Moreover, regional movements can be used to reconstruct and interpret large-scale tissue movements of the vegetal cell mass as a whole (Schürfeld and Winklbauer, 1999). In light of the fact that some cell movements begin prior to gastrulation, how the endoderm may be organized prior to gastrulation is not understood. Pre-gastrulation cell arrangements suggest that during cleavage divisions, which generate the multilayer structure of the vegetal cell mass, the initial apical–basal polarity of vegetal blastomeres is retained by deep cells of the endoderm by an unknown mechanism. Taken together, cell shape change and cell orientation contribute to endoderm morphogenesis. In the absence of cell growth, however, endoderm deformation is largely expected to be the result of cell rearrangement.

3.3.2 Tissue deformation by cell rearrangement

Although cell rearrangements in monolayered epithelia have been extensively studied (Blankenship et al., 2006; Zallen and Blankenship, 2008; Simões et al., 2010), relatively few studies have investigated the mechanisms of cell movement in multilayered tissues. In an epithelium, cells are interconnected by adherens junction proteins, which link the actin cytoskeletons of adjacent cells to facilitate cell adhesion and maintain tissue integrity. To
rearrange, cells must coordinate cell boundary behaviours with neighbouring cells. In the well-studied example of the *D. melanogaster* germband, cells undergo intercalation to achieve rearrangement through processes known as T1-transitions (Blankenship *et al*., 2006). In foams, T1-processes are defined by the scenario in which two vertices joined by a short edge are merged into a single vertex, which then resolves into two new vertices such that the network topography is changed (Fletcher *et al*., 2014). Such scenarios are also encountered in tissues. In confluent cell layers such as epithelia, vertices are created by shared cell–cell interfaces such as tri-cellular junctions. During germband extension, the anterior–posterior boundaries between neighbouring cells contract, which brings vertices shared with dorsal–ventral cell neighbours together, and shrinks cell contacts into four-cell vertices. Subsequently, a new boundary is formed in an orthogonal plane between dorsal–ventral neighbouring cells to complete cell rearrangement and elongate the tissue along the anterior–posterior axis (Guillot and Lecuit, 2013).

### 3.3.3 Endoderm cells undergo differential migration

Differential migration is potentially an archetypical mode of cell rearrangement in multilayer mesenchyme. For instance, mesoderm involution (Evren *et al*., 2014), radial intercalation of prechordal mesoderm (Damm and Winklbauer, 2011), and mediolateral cell intercalation during neural convergence (Keller *et al*., 2000) are some examples of this mechanism in the frog embryo. Moreover, the dorsal migration of lateral mesoderm in zebrafish involves differential migration (Roszko *et al*., 2009; Yin *et al*., 2009), and limb/fin bud morphogenesis in chick, fish, and mouse all depend on three-dimensional patterns of cell migration to alter tissue geometry (Ede *et al*., 1974; Wyngaarden *et al*., 2010; Mao *et al*., 2015).
In explants, cell velocity increases along the A–V axis, from the vegetal base toward the BCF. Movement velocity increases progressively toward the tissue center; however, the vertical component of this velocity is reversed and becomes diminished near the BCF surface. This particular shift in movement velocity leads to narrowing of the explants’ lower part by mediolateral cell intercalation, and expansion of the upper part by A–V cell intercalation. In addition, cell movements are not restricted to the endoderm periphery as previously suggested (Winklbauer and Schürfeld, 1999). The timing of explant excision may account for this discrepancy, as vegetal rotation initiates and continues from the dorsal side to the center of the vegetal cell mass. Instead, cell movements were mainly observed at the core of the vegetal cell mass where the endodermal cell population resides. In intact gastrulae, endoderm cell movements visualized by time-lapse X-ray microtomography showed a similar pattern of cell migration velocity (Moosmann et al., 2013). Specifically, a reversal of the velocity gradient near the BCF was also observed in live embryos, which corroborates results observed in explants.

Mechanistically, differential migration relies on velocity differences between adjacent cells or cell groups. If the leading cells are slow, encroaching cells from behind would intercalate into the cohort of slower moving cells ahead. This situation is encountered near the BCF. However, in the case where leading cells move faster than follower cells, cell intercalation would require additional mechanisms. For instance, a way this could be achieved is by intermittent (or discontinuous) cell migration, whereby cell movements are punctuated by fits and starts. To move forward as a whole, some cells of the cohort slow down momentarily to serve as substratum for advancing cells; thereafter, these cells translocate again at a velocity higher than that of their neighbours, which in turn have decelerated. This asynchronous pattern of cell motility between neighbouring cells is facilitated by cyclical cell migration behaviours found in endoderm cells (chapter four). Moreover, maximal and minimal velocities along with the timing
of burst movements can be modulated such that a spatial gradient of the average velocity is maintained and tissue deformation as a whole appears smooth and continuous.

Presently, the mechanism responsible for generating the cell velocity gradient is not known. I found that intercellular distances narrow and widen in parallel with the magnitude of cell movement velocity. Thus, it is conceivable that the close intercellular distance in the vegetal region compared to the animal region constitutes a possible mechanism for decreasing cell movement velocity. Moreover, regional variance in intercellular distance may translate into corresponding differences in cell–cell adhesiveness. For example, close intercellular distances may facilitate cadherin clustering which increases cell adhesion strength (Yap et al., 1997). In support of this notion, I observed that in calcium-free medium, explants do indeed dissociate faster in the animal region compared to the vegetal half (not shown). Endoderm cells show a range of movement velocities \textit{in vitro} (Wacker et al., 1998). Therefore, cell movement velocities may depend on regional differences such that cells near the top autonomously move faster than more vegetally-located cells. Furthermore, it is also possible that different adhesive components within the interstitial space may generate regional differences in cell adhesion (Taubenberger et al., 2016). However, the molecular composition of interstitial gaps is not yet known. Work is currently underway to characterize the interstitial space and its functional significance during tissue morphogenesis.
3.3.4 Amoeboid cell movement during endoderm migration

Amoeboid migration is associated with low, non-specific substratum adhesion, a rounded cell shape, and cell movement in confined spaces where traction is generated by repeated cell shape changes, in the absence of locomotor protrusions such as lamellipodia (Paluch et al., 2016; te Boekhorst et al., 2016). Amoeboid cell behaviours have been demonstrated in single cells moving through three-dimensional ECM or between stationary cells. Well-studied examples include human leukocytes, zebrafish primordial germ cells, and numerous cancer cells such as melanoma and colon carcinoma cells (Mandeville et al., 1997; Sahai and Marshall, 2003; Wolf et al., 2003; Blaser et al., 2006).

Amoeboid cell behaviours are observed in the *X. laevis* endoderm during cell rearrangement by differential migration. C-cadherin is involved in endoderm migration; however, its function extends beyond cell–cell adhesion (explained below). To generate locomotion, endoderm cells undergo cycles of elongation and recoil from an initially round shape as they knead forward during migration. A principal condition for amoeboid migration, low adhesion, is met by the endoderm, which represents the least cohesive tissue in the *X. laevis* gastrula (David et al., 2014). Nevertheless, the endoderm is formed from a coherent cell mass, which exhibits tight confinement such that cells must squeeze between neighbouring cells during movement.

A striking feature of the endoderm is that cells are separated by wide interstitial gaps. Presently, the constituents and mechanical properties of these gaps are not known. Heteroglycan staining confirmed that ECM material is present and abundant between cells (Fig. 3-8F). At the tissue level, however, a large *trans*-cellular ECM scaffold is unlikely to be present in the endoderm for several reasons. First, when tissues are fixed for TEM, matrices collapse into densely stained
globules on the surfaces of cell membranes. Secondly, when the endoderm is dissociated into single cells using calcium-free medium, no conspicuous, covalently linked ECM scaffold remains. Moreover, vegetal explants deform rapidly beyond their initial morphological contours, which is an observation that is difficult to reconcile with a stable pre-formed matrix scaffold. Furthermore, molecular components that form covalently cross-linked ECM fibrils such as collagens, laminin or fibrillin are not expressed during gastrulation (Winklbauer and Ettensohn, 2004). Finally, the consistency of similar gaps found in between X. laevis ectoderm and mesoderm are fluid-like (Barua et al., 2017).

The ECM likely mediates weak adhesion between cells. At the ecto-mesoderm boundary, adhesive cell–cell contacts, or so called “intermediate contacts”, are 100–300 nm wide and filled with ECM (Luu et al., 2015). These intermediate distances are typically absent in the endoderm; instead, cells are separated by 10-fold wider distances. Consistent with a significant role for cell–ECM interaction during migration, the adhesive ECM glycoprotein FN and receptors like α5β1 integrin are expressed in gastrula endoderm (Winklbauer and Ettensohn, 2004). It has been shown that FN is deposited on the endodermal cell surface (Winklbauer, 1998), and that endoderm cells strongly adhere to FN in vitro (Winklbauer, 1988).

Another highly hydrated, space-filling constituent of ECM is hyaluronic acid, which is abundant in the X. laevis gastrula (Müllegger and Lepperdinger, 2002). Hyaluronic acid is a large glycosaminoglycan molecule that is well-distributed in the interstitial space of many tissues, including epithelial and endodermal tissues. A low concentration of hyaluronic acid could facilitate cell–ECM adhesion, although, counterintuitively, a high abundance of hyaluronic acid could act to inhibit adhesion (Zimmerman et al., 2002). Thus, the presence of hyaluronic acid between cells could contribute to cell confinement while maintaining low adhesion. When
exerting pressure against one another using an incompressible, hyaluronate-based ECM, cells could generate sufficient friction through cell–ECM interaction to develop the traction necessary for locomotion. In support of this notion, I found that when single cells are seeded onto FN adsorbed gelatin-coated dishes and influenced solely by their own weight, cells adhere weakly to the substratum and translocate using amoeboid-like behaviours reminiscent of the situation in vivo. This finding contrasts with previous reports, which have claimed that endoderm cells appear multipolar but remain non-migratory in vitro (Wacker et al., 1998; Luu et al., 2008). In their work, however, Wacker and colleagues used FN-coated polystyrene as the substratum, which proved to be unsuitable for endoderm cell migration, perhaps due to incompatibility of substrate stiffness. In this chapter, I have shown that endoderm cells are capable of amoeboid cell migration in vitro when seeded on a soft-substratum. The onset of motile behaviour occurs within (10–15) minutes after seeding, which indicates that changes are unlikely the result of changes in gene expression, but rather a direct shift in the mode of cell behaviours. Migration mode ‘plasticity’ is a known phenomenon in other cell types, and has been shown to be regulated by changes in substratum properties (Ladoux et al., 2010; Paluch et al., 2016; te Boekhorst et al., 2016).

3.3.5 Endoderm cell body elongation and oscillation

Endoderm cells can be distinguished based on their elongated kite shape in vivo. When isolated in culture, single endoderm cells undergo autonomous elongation during cell translocation in vitro. In parallel with migration, endoderm cells extend and recoil their cell bodies during locomotion. In contrast to bottle cells, which can extend their initial body length by over four-
fold during gastrulation, endoderm cells maintain a relatively stable LWR around 1.5–1.6, which suggests that cell length is actively regulated by an unknown mechanism. It was reported for Madin–Darby canine kidney (MDCK) epithelial cells that mechanical stretching of cells beyond 1.4-fold the initial cell length induced a corresponding five-fold increase in cell division (Gudipaty et al., 2017). In terms of cell diameter, *X. laevis* endoderm cells are four-fold larger than the average MDCK cell; however, given that minimal cell division occurs during *X. laevis* gastrulation, it may be that endoderm cells analogously restrict elongation to avoid excess cell division.

Conversely, cell elongation coincides with cell migration; therefore, it is tempting to speculate that this behaviour could contribute to cell migration. It has been shown that cells actively contract their cell body in response elongation induced by mechanical stretching (Micoulet et al., 2005). Guevorkian and colleagues found that when tissues are stretched by external forces – for example, when tissue aggregates are subjected to micropipette aspiration – they undergo repetitive shiver-like contractions. Two types of contractions are observed and classified based on magnitude. Large contractions, which result from multicellular responses, occur over a significant portion of the aggregate and have a typical amplitude of 3 μm. In contrast, small contractions represent single-cell behaviours and have a typical amplitude of 1 μm or less (Guevorkian et al., 2011). Given the small diameter of mouse cells used in that study (8–10 μm), the cell contractions correspond to a 10–30% fluctuation in diameter. Shivering behaviour is also observed in elongated *X. laevis* endoderm cells *in vitro*, where cell width varies by approximately 10% during the oscillation phase. In contrast, shivering behaviour is absent in round, stationary endoderm cells, consistent with the notion that oscillations are linked to cell elongation (not shown).
How pulsatile cell body oscillations contribute to cell migration is currently not known. Interestingly, when extracellular debris was incidentally attached to the membrane of an isolated endoderm cell in culture, the debris was moved ‘downward’ relative to the cell body, from near the cell front toward the cell rear. A similar phenomenon was observed in *C. pyrrhogaster*; when external carbon particles were intentionally placed on the cell body, the particle was shifted down the cell body, which suggests that the cell surface contains a continuous flow directed from the cell front to the cell rear (Kubota, 1981). Similarly, it was shown in mouse embryonic fibroblasts that cells can actively move the substrate during locomotion. By embedding fluorescent beads into the collagen substrate on which fibroblasts moved, Pelham and Wang showed a radial distribution of force at the cell–substrate interface, which moved the substrate content from the cell perimeter to the cell rear (Pelham and Wang, 1999).

Presently, the molecular mechanisms responsible for endoderm cell elongation are not known. It was reported that disruption of microtubule stability during early stages of gastrulation had negligible effects on gastrulation movements as a whole. For example, when early gastrulae were treated with the tubulin destabilizing agent nocodazole for 2 hours and then removed, embryogenesis proceeded as normal (Lane and Keller, 1997). Similarly, it was shown that nocodazole inhibited apical constriction in bottle cells without affecting cell elongation (Lee and Harland, 2007). Furthermore, the microtubule polymerization inhibitor colchicine, which competitively binds to tubulin, could not disrupt gastrulation movements despite being directly injected into the blastocoel. In contrast, the competitive actin-binding inhibitor cytochalasin B strongly perturbed gastrulation movements (Nakatsuji, 1979), which suggests that actin polymerization could contribute to endoderm cell elongation. To date, however, the cytoskeletal architecture of endoderm cells has not been fully elucidated.
3.3.6 A role for C-cadherin beyond adhesion

Although endoderm cells are separated by wide interstitial gaps, C-cadherin is nevertheless required for proper cell migration in tissues. Cadherins are widely known for their role in mechanically coupling cells together. Rather than playing a strictly adhesive role, however, Cadherins are also bonafide signaling molecules responsible for controlling numerous aspects of the actin cytoskeleton (Priya and Yap, 2015; Padmanabhan et al., 2017). In tissues, cells can move one after another following in the same path laid out by their predecessor in a process known as ‘multicellular streaming’ (Kulesa and Gammill, 2010). However, unlike streaming, where cells are typically bound in a chain-like fashion, cell rearrangement is prevalent during differential migration in the endoderm. Follower cells do indeed converge into the path of leading cells. It has been shown that cadherin-rich protrusion structures, so-called ‘cadherin-fingers’, are extended at the trailing end of the leading cell and engulfed by the following cell as a mechanism of guidance during collective cell migration (Hayer et al., 2016). Indeed, knockdown of C-cadherin decreased endoderm cell polarity and congruence of cell elongation toward the direction of migration, consistent with a loss in cell polarity. Taken together, these findings suggest that C-cadherin may have a key role in pathway guidance during endoderm cell migration in tissues.
4. CHAPTER FOUR: EphrinB1 is required for endoderm cell-rear retraction during differential migration

4.1 Introduction

Tissue cohesion is essential for tissue morphogenesis. Thus, it is not surprising to find that cell–cell adhesion is an intensely studied topic in developmental biology, with over 7,000 peer-reviewed articles published on this topic in 2016 alone. Cell rearrangement is a key process of numerous tissue morphogenetic events. To complete rearrangement, cells must detach and move away from one another; therefore, cell de-adhesion (or detachment) is an unequivocally important aspect of tissue morphogenesis. Yet, only 490 articles were published in 2016 that focused on cell de-adhesion (or detachment). Thus, cell de-adhesion is a vital, but relatively under-studied aspect of cell rearrangement.

The best studied example of cell rearrangement is exemplified by D. melanogaster germband extension (Bertet et al., 2004). During epithelial extension, cell–cell boundaries contract and resolve while new contacts form in a pattern that leads to controlled cell neighbour exchange (Blankenship et al., 2006). In non-epithelial tissues, cells must also move apart to complete rearrangement; however, in this circumstance, the mechanism by which cells de-adhere (or detach) is poorly understood. Pioneering studies of cultured cells have shown that trans-membrane endocytosis and enzymatic cleavage of cadherin molecules are basic mechanisms of cell detachment (Marston et al., 2003; Solanas et al., 2011; Hayer et al., 2016). In both
processes, plasma membrane remodeling plays a key role in regulating de-adhesion; although, how this occurs for cells within tissues is not yet fully understood.

The *X. laevis* gastrula provides an excellent system for studying vertebrate cell rearrangement due to its external development, the use of tissue explants, and well characterized region-specific tissue movements. In particular, the endoderm offers an ideal platform for the study of cell–cell behaviours during rearrangement due to the large size of its cells and the capability of endodermal explants for subdivision into region-specific parts where the movement of distinct tissue fragments could be reconstructed to interpret large-scale tissue deformation as a whole (Winklbauer and Schürfeld, 1999). However, the process by which endoderm cells detach from one another during cell migration is not yet understood.

In gastrula tissues, the mesoderm detaches from the ectoderm using alternating cycles of attachment and detachment to maintain tissue separation, a process that requires EphB–ephrinB signaling (Rohani et al., 2011). In fact, EphB–ephrinB signaling has been implicated in regulating cell de-adhesion (Zimmer et al., 2003; Füller et al., 2003). The protein ephrinB1 in particular has been shown to induce cell de-adhesion in *X. laevis* embryonic tissues (Jones et al., 1998). Indeed, ephrinB1 is expressed in all tissues of the *X. laevis* gastrula (Rohani et al., 2011).

Here, I found that within the endoderm, ephrinB1 expression is present and surrounds the cell membrane; specifically, ephrinB1 is enriched at the rear membrane, which suggests that it may have a role in modulating cell-rear detachment during cell migration. Additionally, endoderm cells undergo trailing edge retraction by first narrowing their trailing edge in order to detach from following cells. Trailing edge retraction is accompanied by active plasma membrane undulations at the cell rear and by the internalization of membrane-bound vesicles via macropinocytosis, which is involved in the ingestion of large membrane vesicles. Furthermore,
ephrinB1 is enriched at the rear of migrating endoderm cells. To investigate the role of ephrinB1 in trailing edge retraction, I knocked down ephrinB1 levels using morpholino oligonucleotides and found that ephrinB1 is required for trailing edge narrowing. Mechanistically, ephrinB1 is a membrane constituent of internalized endosomes, and knockdown of ephrinB1 reduced endosome internalization. Correspondingly, overexpression of ephrinB1 increased endosome internalization, which suggests that ephrinB1 is required for endosome biogenesis and trailing edge membrane remodeling during cell detachment.
4.2 Results

4.2.1 Endoderm cells undergo trailing edge retraction

Within tissues, cells must detach from one another to undergo rearrangement by differential migration. Presently, the mechanism underlying endoderm cell detachment is not understood. When the endoderm was examined using SEM, cell polarity could be distinguished based on a characteristically broad cell front that tapers into a narrow cell rear (Fig. 4-1). Endoderm cells showed approximate bilateral symmetry about the long axis of the cell (Fig. 4-1A), and this morphology was maintained during the retraction process (Fig. 4-1B), which suggests that a mechanism must exist to regulate trailing edge tapering and cell detachment.

To investigate this notion, I monitored endoderm cell shape changes in vegetal slice explants. Consistent with SEM data, cells in explants gradually narrowed and retracted their trailing edges during cell translocation (Fig. 4-1B). Cell rear membrane undulations and dynamic protrusions were found to accompany the retraction process. Interestingly, numerous small vesicle clusters were visible at the trailing end, which appeared in correlation with edge retraction (Fig. 4-1B).

During retraction, cells extended and retracted numerous protrusions in and out of the rear-lateral cell membrane; thus, I speculated that cytoskeletal remodeling could play a role in trailing edge retraction. To this end, I examined cortical actin dynamics during tail retraction using Lifeact, an in vivo probe for polymerized actin, in conjunction with a membrane label (Fig. 4-1C). Indeed, I
found that cortical actin was enriched at the trailing edge. Actin enrichment was particularly apparent at the tip of the trailing edge. Numerous actin puncta were also visible near membrane fluctuations. Interestingly, actin puncta, which formed during membrane undulations, resembled ring-like structures and were co-localized with membrane-bound vesicles in the trailing edge cytoplasm (Fig. 4-1C). Taken together, these findings suggest that trailing edge narrowing is an actively controlled process that involved cortical actin and membrane remodeling.
**Figure 4-1 Cell rear detachment is associated with trailing edge narrowing.**

(A) Scanning electron micrograph of endoderm cells within the vegetal cell mass (left). Schematic illustration of endoderm cells (right). An example cell with front and rear polarity is shown in the center. Cell front (yellow), lateral (orange), and rear (red) borders are indicated. Animal is to the top, vegetal to the bottom. (B) Time-lapse recording of a membrane-labelled endoderm cell moving animally within the endoderm (top). Inset shows the trailing edge. Magnification of the trailing edge (bottom) shows notable vesicle clusters (yellow arrows). (C) Enrichment of cortical actin at the narrowing trailing edge. Panels are separated into merged (top row), membrane-only (middle row), and Lifeact-only images (bottom row). Co-localization of Lifeact puncta with membrane vesicles (red arrows) were notable in several instances. Numerous membrane vesicles (yellow circle) could also be seen. Lifeact was enriched near the trailing edge membrane cortex (yellow arrow).

### 4.2.2 Trailing edge retraction is associated with vesicle internalization

To confirm that vesicles were not artefacts of membrane labelling or Lifeact mRNA injection, I examined the trailing edge of wildtype uninjected cells using SEM (Fig. 4-2A). As expected, I found numerous vesicular clusters in the trailing edge cytoplasm of wildtype cells. Next, I examined endoderm cells using TEM. Ultrastructural analysis revealed that within the endoderm cell body, vesicle clusters were predominantly localized to the trailing edge of endoderm cells (Fig. 4-2B).
I found that many differently sized vesicle were present in clusters at the trailing edge. To quantify vesicle size heterogeneity during gastrulation, I measured the diameters of vesicles at different gastrula stages (Fig. 4-2C). In stage 10 endoderm cells, vesicle diameters were relatively narrow, and averaged around 0.15 μm, compared to stage 10.5 cells where vesicle diameters were notably dispersed and averaged about 0.49 μm. After stage 10.5, however, average vesicle diameters were once again reduced and averaged between 0.34 and 0.30 μm in stages 11 and 12, respectively. Furthermore, vesicle size variation was highest in stage 10.5 gastrulae, which coincides with the developmental time point of vigorous cell rearrangement during vegetal rotation. Nevertheless, it was noted that regardless of developmental stage, measured vesicle sizes ranged from 0.05 to 3 μm, which greatly exceeded diameters known for clathrin-mediated endocytosis (McMahon and Boucrot, 2011).
Figure 4-2 Trailing edge vesicles.

(A) Scanning electron micrograph of the trailing edge. A segment of the cell rear with the membrane partly broken off reveals numerous heterogeneous vesicles (arrows) within the trailing edge cytoplasm (left). Size comparison of internalized vesicles (arrow) within the trailing edge and a yolk platelet (Y). (B) Transmission electron micrograph of an endoderm cell within the vegetal cell mass (left). Inset (i) indicates a magnified region of the trailing edge (right). Numerous vesicle clusters were found within the trailing edge cytoplasm (arrows). (C) TEM
image showing vesicle size heterogeneity (left). Quantification of trailing edge vesicle size variation from different gastrula stage endoderm cells.

4.2.3 Endosomal internalization by macropinocytosis

Although vesicles in the trailing edge cytoplasm were abundant, it was not clear whether these vesicles were internalized by endocytosis or formed to be secreted via exocytosis. However, the relatively large and inhomogeneous vesicle sizes were inconsistent with typical vesicle diameters associated with exocytosis. For example, in neuromuscular junctions of D. melanogaster larval body wall muscles, exocytosis vesicle diameters were uniform, typically ~40 nm (Zhang et al., 1998). To investigate further, I first examined trailing edge plasma membrane morphology. Ultrastructure analysis under TEM revealed membrane deformations consistent with endocytosis, namely, endocytic pit formation, bicuspid membrane invagination, and membrane scission leading to vesicle internalization (Fig. 4-3A). Pit-stage membrane indentations ranged from 150 nm to ~3 μm in diameter and were consistent with vesicle parameters known for macropinocytosis. To confirm that extracellular contents were internalized, membrane-labelled explants were incubated in media supplemented with fluorescein-conjugated dextran to mark the extracellular fluid. I found that dextran was taken up in vesicles that initiated from membrane pits (Fig. 4-3B).

Furthermore, Rab5 is known to be a key regulator of endosome biogenesis and a marker of early endosomes (Lanzetti et al., 2004). To examine whether endoderm vesicle internalization involved endocytic machinery, I examined the expression of fluorophore-tagged Rab5.
Remarkably, I found flares of Rab5–CFP puncta accumulated and resolved on the scale of minutes alongside membrane clusters, which entered the cytoplasm, particularly at the trailing edge (Fig. 4-3C). The spatiotemporal accumulation of Rab5 alongside endosomes suggested that Rab5 could play a role in the formation or trafficking of these large, heterogeneously sized vesicles.

Taken together, the association of Rab5 with high protrusive activity, membrane ruffling, and the internalization of large vesicles was consistent with hallmark features of macropinocytosis. However, the exact mechanism, by which Rab5 acts to regulate macropinocytosis is not yet clear. Work is currently ongoing to investigate potential cross-talk between the endocytic machinery and the mechanism of macropinocytosis.
**Figure 4-3 Vesicle internalization by macropinocytosis.**

(A) TEM images of trailing edge membrane showing progressive endocytic stages. From left to right, the stages indicated (arrows) are pit formation, pit invagination, and vesicle scission. (B) Engulfment of extracellular fluid marked with fluorescein dextran. Merged images showing sequence (yellow arrows) of engulfment into vesicle (top row). A yolk platelet (Y) is indicated for relative size comparison. Individual labels for membrane and dextran are shown for clarity (middle rows). An illustrated interpretation of the process is shown (bottom row). (C) Localization of Rab5c–CFP within migrating endoderm cells. Rab5 is preferentially enriched at the trailing end. Insets (i–iii) show magnification of individual regions for clarification. Rab5 was co-localized to regions of membrane internalization (yellow arrows).

### 4.2.4 Endosome internalization and narrowing of the trailing edge

Macropinocytosis involves the internalization of relatively large vesicles, which could be measured using TEM. Taking advantage of this feature, I investigated whether membrane surface area internalized by vesicles could correspond to surface area lost at the rear membrane during trailing edge retraction. Using confocal fluorescent microscopy, I first determined the three-dimensional parameters of the trailing edge, which approximately resembled a rectangular prism (Fig. 4-4). By defining relative initial and final time points (within a timeframe of 10 minutes) in time-lapse videos, I was able to estimate that the trailing edge membrane surface area was reduced by approximately 30 μm² per minute. An assumed average vesicle diameter of ~0.5 μm (typical for stage 10.5 endoderm cells) would correspond to the expected generation of about
40 vesicles per minute. I found that the trailing edge contained an average of 22 vesicles. Therefore, the order of magnitude for observed vesicle numbers was well-matched to expected values, which suggested that the rate of vesicle internalization could be correlated with trailing edge retraction such that vesicle internalization could be the rate-limiting process during rear-membrane remodeling. However, information is lacking regarding the net transit rate and movement kinetics of vesicles inside the cell. For example, it is not known whether internalized vesicles coalesce, are recycled, or become degraded. Thus, future work will focus on investigating vesicular dynamics within the cell.

**Figure 4-4 Trailing edge narrowing.**

The rate of change in membrane surface area (ΔSA) after 10 minutes (\(t_0 \rightarrow t_{10}\)) was determined by the following equation: \(2[d_{t0}(h_{t0} + w_{t0}) + h_{t0}w_{t0}] - 2[d_{t10}(h_{t10} + w_{t10}) + h_{t10}w_{t10}]\), where if at \(t_0\): the average depth (d), height (h), and width (w) were 3.1, 25.0, and 8.5 μm, respectively, and at \(t_{10}\), the corresponding d, h, and w were 2.1, 20, and 5.5 μm, then ΔSA would be \(\approx 30 \mu m^2/min\) (or 500,000 nm²/sec). Assuming an average vesicle diameter of 0.5 μm (or 500 nm), it would take approximately 40 vesicles to account for the change in surface area per minute.
4.2.5 EphrinB1 is expressed at the membrane and enriched near the cell rear

The molecular components involved in regulating trailing edge retraction are not yet fully known. Given that Eph–ephrin molecules are well-known for their role in mediating cell detachment, they were ideal candidates for this investigation. Particularly, ephrinB1 mRNA is highly expressed in the endoderm of the early gastrula, which suggested that it could play a role in modulating endoderm tissue cohesion (Rohani et al., 2011). Indeed, I had previously noted that manipulation of ephrinB1 perturbed endoderm cohesion and vegetal rotation (chapter two). To investigate how ephrinB1 could modulate adhesion at the cellular level, I first examined the localization of ephrinB1 in endoderm cells using immunofluorescent labelling of ephrinB1 in vegetal explants (Fig. 4-5). EphrinB1 was detected at the plasma membrane in all cells of the vegetal cell mass; however, expression levels were most prominent near the explant center, which is an active zone of vigorous cell rearrangement (Fig. 4-5A). High-resolution confocal microscopy revealed that ephrinB1 was detected in the cytoplasm and cell membrane (Fig. 4-5B). In both cases, ephrinB1 was enriched near the posterior end of the cell, particularly in the trailing edge membrane (Fig. 4-5B), consistent with a role for ephrinB1 in trailing edge retraction.
Figure 4-5 Detection of ephrinB1 localization by immunostaining.

(A) Immunostaining of vegetal explant. A surface view of the entire explant is shown. Presence of ephrinB1 was detected at the endoderm cell plasma membrane. Signal was occasionally detected within the nucleus; however, this could be a potential artefact of staining because the nuclear signal was absent when staining duration was reduced, whereas membrane staining remained (not shown in panel). (B) High-resolution micrograph showing ephrinB1 staining of endoderm cells within a vegetal explant. Image represents an optical section taken 0.5 µm above the substrate surface. Two overlapping cells (yellow and white) are shown by outlines. Although ephrinB1 staining is diffuse around the plasma membrane, it is abundant within the posterior half of the cell, and particularly enriched near the trailing edge membrane (arrow). Animal (An) is up, vegetal (Vg) is down.

4.2.6 EphrinB1 is enriched at the trailing edge
To investigate the functional contribution of ephrinB1 to trailing edge retraction, I monitored the localization of ephrinB1 in migrating endoderm cells of live tissue explants. I expressed fluorophore-tagged ephrinB1 along with a membrane label to visualize ephrinB1 distribution in endoderm cells. I found that consistent with immuno-staining results, ephrinB1 expression was dispersed around the cell in the plasma membrane; however, ephrinB1 was notably enriched at the trailing edge (Fig. 4-6A). Interestingly, ephrinB1 puncta were also observed within the cytoplasm at the tip of the trailing edge, in a pattern that was reminiscent of vesicle cluster distribution (Fig. 4-1).

Next, I investigated the spatiotemporal kinetics of ephrinB1 localization in endoderm cells from newly explanted tissues. I found that immediately following explant excision, endoderm cell morphology appeared irregular. Cells extended multiple bleb-like protrusions and were relatively rotund in comparison to the characterized kite shape of cells from intact endoderm tissue (Fig. 4-6B). In this condition, ephrinB1 expression appeared evenly expressed around the membrane. In time, cells progressively recovered their elongated morphology. After approximately 5 minutes, ephrinB1 enrichment was notably localized to the presumptive cell rear (Fig. 4-6B). On average, cell morphology was completely restored approximately 15 minutes after explantation. Upon recovery, robust endoderm cell rearrangement was observed. In parallel with cell shape recovery, after 15 minutes, ephrinB1 enrichment was prominently localized to the cell rear (Fig. 4-6B). Quantification of regional membrane fluorescence intensity verified that ephrinB1 was indeed enriched at the posterior end relative to the leading or lateral membrane domains in migrating cells (Fig. 4-6C).

Given that ephrinB1 is a membrane-associated protein, the mechanism of ephrinB1 internalization into the trailing edge cytoplasm was not clear. Cytoplasmic ephrinB1 was
detected in fixed samples processed for immunohistochemistry (Fig. 4-5) and in live samples undergoing active cell migration (Fig. 4-6A, B). In both cases ephrinB1 puncta were found in proximity to vesicles at the trailing edge. Thus, to investigate this relationship, I examined ephrinB1 localization with respect to membrane-label expression at the trailing edge. Remarkably, I found that ephrinB1 was included as a membrane component of internalized endosomes (Fig. 4-6D), which suggested that ephrinB1 could have a role in endosome biogenesis.

Figure 4-6 Enrichment of ephrinB1 at the trailing edge.
(A) EphrinB1 is dispersed around the membrane and enriched at the cell rear. Membrane–GFP and ephrinB1 co-expression revealed ephrinB1 enrichment at the cell rear (yellow arrows), particularly at the tip of the trailing edge (blue arrows). (B) EphrinB1 localization kinetics. Image sequence captured immediately after explant isolation. EphrinB1 was initially evenly dispersed (1 min); however, expression became enriched at the cell rear. Strong enrichment was noted as soon as 5 min after explantation (white arrows). (C) Quantification of ephrinB1 fluorescence intensity after subtracting membrane–GFP intensity. (D) EphrinB1 was found as a membrane constituent of internalized endosomes. Magnified insets (i–iii) show corresponding channels of ephrinB1 (white arrows) and membrane–GFP, along with merged images (top two rows). Magnification of vesicle shows that ephrinB1 is part of the endosome membrane (bottom row).

4.2.7 Single- and double-membraned endosomes

To further examine ephrinB1 in relation to internalized endosomes, I visualized ephrinB1-mCherry and membrane-labelled endosomes in the trailing edge cytoplasm using high-resolution fluorescence confocal microscopy. To track ephrinB1 localization within cells, the monomeric fluorophore mCherry was fused onto the cytoplasmic domain of ephrinB1. The mCherry molecule offers high photo-stability and tolerance to pH fluctuations, which are an advantageous characteristics for monitoring molecular tracking in vivo (Shaner et al., 2008; Doherty et al., 2010). A disadvantage of mCherry is the slow maturation time of this fluorophore, which has been reported as 155 mins at approximately 20°C (Macdonald et al., 2012). However, this potential setback is compensated for by the fact that embryos were injected with ephrinB1-
mCherry mRNA at the four-cell stage and analyzed at gastrulation stages (>420 mins later at 23°C), which provided ephrinB1-mCherry with sufficient time for protein expression. Within vesicles, mCherry was expected to be situated on the endosome exterior. In line with this expectation, vesicles with exteriorly-placed mCherry were observed (Fig. 4-7A). Surprisingly, I found that in a subset of vesicles within multi-vesicular clusters, mCherry fluorescence was present on both sides of the same endosome (Fig. 4-7B). This subset of vesicles also possessed endosomal membranes that were approximately 2-fold higher in relative intensity. Together, the findings suggested that these particular endosomes could contain two membranes in close proximity of one another. Presumably, one of the two membranes could have been contributed by the leading cell while the other was contributed by the following cell. Therefore, double-membraned endosomes could have been formed due to trans-endocytosis, which is a reported phenomenon associated with ephrinB1 (Marston et al., 2003; Zimmer et al., 2003; Lauterbach and Klein, 2006; Gaitanos et al., 2016). To confirm that double-membraned endosomes were not artefacts produced by experimental conditions, I examined the trailing edge of wildtype uninjected endoderm cells using TEM (Fig. 4-7C). Consistent with results obtained using fluorescence confocal microscopy, I found double-membraned vesicles in the trailing edge of wildtype cells (Fig. 4-7C). Furthermore, cytoplasm-like contents were found within the lumen of double-membraned vesicles, consistent with the notion that these vesicles could be internalized by trans-endocytosis (Fig. 4-7C).
Figure 4-7 Endosomal membrane morphology.

(A) Membrane labelled (mGFP) single-membraned vesicle within the cytoplasm. (B) mGFP labelled double-membraned endosomes. Panels show a composite image of mGFP and ephrinB1–mCherry (eB1–mCh), along with respective channels independently for visual clarity. An illustrated interpretation of molecular localization is shown (right). (C) TEM images of trailing edge cytoplasm. (i) Cell–cell contact. A double-membraned endosome that contains
cytoplasm is indicated (arrow). (ii) Double-membraned vesicle in proximity to mitochondrion (arrow). (iii) Numerous double-membraned vesicles within the trailing edge cytoplasm (arrows).

4.2.8 EphrinB1 is required for trailing edge retraction

Given that ephrinB1 is enriched at the cell rear, and is a membrane component of internalized endosomes, I hypothesized that ephrinB1 could play a role in membrane remodeling during trailing edge retraction. To assess the requirement for ephrinB1 during trailing edge retraction, I knocked down ephrinB1 protein levels using a previously characterized morpholino oligonucleotide (Rohani et al., 2011). To avoid variance between embryos, I used an internal control by generating mosaic embryos in which ephrinB1 was knocked down in half of the gastrulae, whereas the other half maintained endogenous expression (Fig. 4-8A). Endoderm cells in the control half of the embryo rearranged, and trailing edge retractions were observed during cell translocations. In contrast, morphant endoderm cells seldom rearranged during observation. In fact, morphant cells showed blunted cell rears (Fig. 4-8A), consistent with failure in trailing edge retraction, which could have led to failure in cell detachment and rearrangement.

Quantification of trailing edge width showed that on average, control cells narrowed their trailing edge widths by half in approximately 10 minutes, whereas morphant rear widths remained nearly unchanged after 40 minutes (Fig. 4-8B, C).

Given that knockdown of ephrinB1 perturbed cell detachment, I next examined ephrinB1 overexpression. I found that ephrinB1 overexpression induced endoderm cell dissociation (Fig. 4-8D). In correlation with dissociation, ephrinB1-overexpressing cells showed overt and ectopic
endosome accumulation around the cortical membrane (Fig. 4-8D). Furthermore, quantification of endosomes after ephrinB1 knockdown or overexpression corresponded to a significant decrease or increase, respectively, in endosome number relative to control cells within the same tissue (Fig. 4-8E). Thus, the results suggested that ephrinB1 modulates cell detachment by regulating endosome biogenesis during trailing edge retraction.

Figure 4-8 EphrinB1 is required for trailing edge retraction.

(A) Wildtype cells undergo trailing end retraction during rearrangement (top row), whereas ephrinB1-morpholino-injected cells did not rearrange, and subsequently did not undergo rear retraction (bottom row). (B) Quantification of trailing edge widths in wildtype cells during retraction. (C) Quantification of rear edge widths in ephrinB1 morphant cells. Colours represent individual cells. Black line indicates average. (D) Overexpression of ephrinB1-induced cell
dissociation. Sequence shows cell dissociation and rounding. Furthermore, ephrinB1-overexpressing cells showed overt and ectopic vesicle formation (white arrows). (E) Quantification of endosomes within the cytoplasm of wildtype (control), ephrinB1-morpholino-injected (eB1MO), and ephrinB1-overexpressing (eB1FL) cells.

4.2.9 Endosomal coalescence and heterogeneity

Endosomal clusters showed vesicle size heterogeneity. Vesicle diameters ranged from 0.05 to 3 μm. Such vesicle size heterogeneity is a hallmark feature of macropinocytosis. Interestingly, many differently sized vesicles were often found in close proximity to each other within cytoplasmic clusters. Confocal microscopy revealed that vesicles within clusters were often in direct contact with each other (Fig. 4-9A). Furthermore, vesicles that clustered together tended to stay together, which suggested that vesicles within a cohort could interact with one another. To investigate this behaviour, I tracked movement of endosomes within the trailing edge cytoplasm. Surprisingly, I found that relatively small vesicles coalesced with one another to form larger vesicles (Fig. 4-9A). Furthermore, large vesicles were remarkably stable; for instance, I found that large vesicles (diameter > 1 μm) persisted within the cytoplasm beyond one hour. To verify that endosomal coalescence was not an artefact induced by membrane labeling or ephrinB1 expression, I examined unlabeled wildtype endoderm cells from the embryo using TEM (Fig. 4-9B). In agreement with fluorescence microscopy results, TEM ultrastructure analysis revealed numerous vesicles of different sizes that appeared to share a lumen, which supported the notion that vesicles could undergo fusion (Fig. 4-9B). Thus, vesicular coalescence could be a contributing factor for generating larger vesicles within endoderm cells.
Figure 4-9 Endosome coalescence.

(A) Sequence shows endosome fusion of the right endosome into the central endosome. Respective panels show membrane–GFP only (top row), ephrinB1 only (mid row), and their superimposed composite (bottom row). Time is indicated (hours:minutes). (B) TEM image of the cytoplasm at the trailing edge of an endoderm cell showing two differently sized vesicles (white outline) in the process of fusion between vesicle 1 and vesicle 2.
4.2.10 Dishevelled puncta kinetics in correlation with membrane protrusion dynamics

It was shown that ephrinB1 could interact with Dishevelled (Dsh), and that Dsh mediates ephrinB1 signaling in the *X. laevis* eye-field (Lee *et al.*, 2005). Thus, Dsh could mediate ephrinB1 signaling during rear retraction in endoderm cells. To assess whether Dsh is involved in trailing edge retraction, I visualized Dsh localization with respect to the cell membrane by co-expressing Dsh-GFP, together with a membrane label, or with ephrinB1-mCherry (Fig. 4-10). Co-expression also allowed for co-localization analysis of Dsh with respect to ephrinB1 (Fig. 4-10A). Under fluorescence confocal microscopy, Dsh-GFP appeared in puncta that were dispersed throughout the cytoplasm of endoderm cells. In addition to the cytoplasmic pool, however, a discrete sub population of Dsh-GFP could be identified near the cell cortex based on increased puncta density, larger puncta size, and relatively high fluorescence intensity (Fig. 4-10A). This particular sub-population (hereafter referred to as the cortical pool) appeared to fluctuate in fluorescent signal intensity. The cortical pool was primarily observed along the lateral cell membrane near the trailing edge, and co-localized with regions that were enriched with ephrinB1 (Fig. 4-10A).

Furthermore, cortical Dsh-GFP fluctuations occurred in tandem with membrane undulations at the cell membrane. Numerous membrane protrusions rapidly formed and resolved at the trailing edge. I found that cortical Dsh-GFP puncta accumulated with protrusion formation (Fig. 4-10B). In contrast, Dsh-GFP clusters dissolved in tandem with protrusion retraction (Fig. 4-10B). Together, these observations implicated Dsh as a potential regulator of protrusion dynamics at the trailing edge. To confirm this speculation, work is ongoing to assess the effect(s) of Dsh manipulation, by knockdown or overexpression, on protrusion formation and retraction.
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- Protrusion Formation
- Protrusion Resolution
- Dsh-Puncta Formation
- Dsh-Puncta Resolution
Figure 4-10 Disheveled puncta at the trailing edge.

(A) Dishevelled (Dsh)-GFP puncta are localized to the subcortical regions near the trailing edge. Dsh puncta were found throughout the cytoplasm; however, the population of Dsh puncta near the cell cortex are mainly localized to the trailing edge (arrows), which coincides with local enrichment of ephrinB1-mCherry (eB1-mCh). (B) Mobility of Dsh-GFP puncta correlates with membrane protrusion dynamics. Panels show the sequence of protrusion formation, and resolution for two protrusions (yellow and blue arrows) over 15 minutes. Panels are divided into composite (top row), membrane-RFP label (middle row), and Dsh-GFP (bottom row) for clarity. Dsh puncta were recruited to the site of membrane protrusion during formation, whereas Dsh puncta were dissolved during protrusion resolution. Protrusions formation and resolution are indicated (brackets).
4.3 Discussion

4.3.1 Endoderm cell endocytosis

Plasma membrane remodeling is a basic morphogenetic mechanism that can be achieved by active cell processes such as exo- or endocytosis. Until recently, the spatiotemporal sequence of clathrin-dependent endocytosis was not fully understood. Two distinct mechanisms for clathrin recruitment have been proposed: 1) clathrin is first recruited to the site of endocytosis, and the membrane then invaginates with a full assembly of clathrin molecules, or 2) the membrane begins to invaginate, and clathrin is then sequentially recruited to the site of membrane bending to form the clathrin lattice (Avinoam et al., 2015). Recent insight into clathrin-dependent endocytosis, based on correlative fluorescence microscopy in conjunction with electron tomography to visualize the molecular composition of clathrin-coated pits and reconstruct the three-dimensional volume of membrane vesicles, suggests that clathrin is first deposited on a flat membrane, after which the membrane bends inward toward the cytoplasm with a fully assembled clathrin lattice (Avinoam et al., 2015). Crucially, this finding implies that the temporal recruitment of clathrin is a rate-limiting step in clathrin-mediated endocytosis. It is estimated that the upper limit of a typical clathrin-coated endosome diameter is approximately 100 nm, with the exception of virally induced endosomes, which can produce diameters beyond 200 nm (McMahon and Boucrot, 2011). Given that each leg of the clathrin triskelion is approximately 48 nm long (Fotin et al., 2004), within a 100 nm diameter endosome, coating would require
approximately 60 clathrin-triskelion molecules. In turn, a conservative estimate is that it would take roughly 30 seconds to complete the assembly of such a latticework (Perrais and Merrifield, 2005; Loerke et al., 2009). Disregarding endosome recycling and assuming a constant endosome traffic-rate for rough estimation, if the endoderm membrane reduced the trailing edge surface area by 30 µm²/min, then roughly 480 endosomes and 480 endocytic pits per minute would be expected to form at the trailing edge of migrating cells. However, such vesicle densities were not found when the ultrastructure of the trailing edge was examined using TEM. Moreover, no discernable clathrin-coated structures were found near or at the site of endosome formation. Together, these data suggest that endoderm cells undergo clathrin-independent endocytosis.

4.3.2 Macropinocytosis at the trailing edge

At the trailing edge of endoderm cells, clusters of vesicles were found that appeared distinct from other forms of endocytic vesicles. The vesicles were large and heterogeneous in size, reminiscent of macropinosomes generated by macropinocytosis. Macropinocytosis involves the uptake of relatively large volumes of extracellular fluid and membrane. In contrast to phagocytosis, which is another way for cells to intake large macromolecules, macropinocytosis is non-selective, and vesicles do not form around a solid object (Bloomfield and Kay, 2016). Typical macropinosomes range from 0.01 to 5.00 µm in diameter. Macropinosomes were first discovered in mammalian cells by Warren Harmon Lewis in 1931, due to their characteristically large vesicles that were visible under light microscopy. Macropinocytosis is found in many cell types, such as in leukocytes, Dictyostelium, human embryonic kidney cells, and numerous types of cancer cells, which suggests that macropinocytosis is a highly conserved process (Hacker et al., 1997;
Buckley and King, 2017). In contrast to specialized endocytic processes such as phagocytosis, where the primary function is the targeted removal of pathogens and cell debris, macropinocytosis has diverse roles in different cell types. For example, epithelial cancer cells use macropinocytosis to transport nutrients from the environment into the cell (Comisso et al., 2013), macrophages use macropinocytosis for antigen capture and presentation to T-cells (Liu and Roche, 2015), and neurons use macropinocytosis to modulate the amount of cell surface receptors (Clayton and Cousin, 2009). Beyond cell-autonomous roles, macropinocytosis can be induced by viruses in pathological conditions to enhance viral infection. For example, the influenza virus, vaccinia virus, and cytomegalovirus can induce macropinocytosis through different host-dependent mechanisms (Mercer and Helenius, 2009; Rossman et al., 2012; Hetzenecker et al., 2016). Thus, macropinocytosis represents a general mechanism for the large-scale removal of cell membrane and extracellular fluid uptake which can be adapted to a variety of cellular processes including cell migration. Experiments on mouse fibroblasts in vitro showed that during migration, integrins from the cell front are redistributed to the cell rear where they are internalized through macropinocytosis. The internalized integrins are then recycled to repopulate newly formed focal adhesions at the cell front (Gu et al., 2011). In another report, Wen and colleagues showed that in human cancer cell lines, N-cadherin regulates the maturation of macropinocytosis through Rab5 at the leading edge (Wen et al., 2016). Here, I show that macropinocytosis is involved in trailing edge membrane remodeling in X. laevis endoderm cells during differential migration.

4.3.3 Macropinosome formation
Macropinocytosis is a multistep process that begins with membrane undulations and ruffling, which progress to protrusion formation. Protrusion extension generates a ring structure that encircles the future site of internalization. When viewed from the sagittal plane, membrane protrusions resemble a cup where bilateral cusps extend toward the interstitial space. Cup formation can be induced in vitro by growth factor stimulation or can occur spontaneously from cell surface ruffles (Swanson, 2008), and in some cells macropinocytosis is a constitutively active phenomenon (Lim and Gleeson, 2011). However, relatively little is known about its initiation in vivo. Moreover, it has recently been proposed that the site of macropinosome formation and its size are stochastically determined (Buckley and King, 2017), but further study is required to investigate these claims. However, macropinocytic cup structures are known to be rich in F-actin from the base to the tip of each protrusion. Several molecular interactions have been identified in the formation of macropinocytic cups. First, patches of active Ras and PIP3 self-assemble at the cortex, which corresponds to the inner surface of the future macropinocytic cup. Then, Ras–PIP3 recruit a ring of suppressor of cAMP receptor/WASP-family verprolin-homologous proteins (SCAR/WAVE) to the margins of the patch. At the margins, SCAR drives actin polymerization through ARP2/3 to extend protrusions outward and generate the lateral walls of the future macropinocytic cup (Veltman et al., 2014). Thus, macropinocytosis is predominantly an actin-driven phenomenon. As actin polymerization is localized to the circumferential margins of the cup, a distinctive actin-ring is visible during macropinocytic cup formation. Consistent with this notion, the trailing edges of X. laevis endoderm cells are rich in F-actin. Moreover, numerous ring-like structures are observed at the cell rear that appear and disappear in parallel with membrane undulations (Fig. 4–1C). Presently, the cue(s) responsible for sequestering Ras–PIP3 to the cortex for macropinocytic cup formation are not known. Moreover, it is not clear how SCAR becomes restricted to the cup margins. However, SCAR is
known to act downstream of Rac1. Molecular analysis places Rac1 at the sites of Ras−PIP3 patches and the inner surfaces of macropinocytic cups, which suggests that Rac1 may have a role in localizing SCAR to the rim of the cup. In addition, Rac1 is sufficient to induce macropinocytic cup formation, and is required for macropinosome closure and maturation (Fujii et al., 2013). Following cup initiation, protrusions extend outward and close at the distal tip to complete macropinosome formation. The molecular mechanism for cup-closure is not known. However, it may involve a mechanism where cell membrane would extend outward and fold back onto itself at the distal margins and activate contact-dependent signaling (chapter three), such as Eph–ephrin signaling. Consistent with characteristic behaviours of macropinocytic cup formation, the trailing edge of *X. laevis* endoderm cells undergo frequent membrane undulations. Membrane ruffles extend and retract in a pulsatile manner, along with protrusions that follow the ebb and flow of cortical fluctuations. Protrusions are filled with F-actin from the base to the distal tip, which suggests that protrusions are formed during active membrane remodeling.

### 4.3.4 EphrinB1 in cell detachment

In *X. laevis*, macropinocytosis occurs throughout the trailing edge. The trailing edge membrane is typically in contact with the ECM or a neighbouring cell from which the moving cell would have to detach to complete rearrangement. It is conceivable that macropinocytosis may narrow the trailing edge by reducing the membrane surface area at free margins via the uptake of extracellular fluid. Indeed, macropinosomes can form at the free surface, such as by receptor-independent ephrinB2 signaling in isolated human endothelial cells (Bochenek et al., 2010). Although the exact mechanism of ephrin-mediated macropinocytosis is not yet known, it has
been reported that ephrinB-reverse signaling can induce integrin clustering (Jülich et al., 2009), and upon clustering, integrins become internalized into the cytoplasm (Upla et al., 2004). In X. laevis, macropinosomes can form at the free surface of the trailing edge; this process requires ephrinB1.

Moreover, Eph–ephrin-mediated *trans*-endocytosis can also occur between cells. In cells undergoing *trans*-endocytosis, endosomes once formed, are taken up by both of the interacting cells. For example, in mouse fibroblasts expressing EphB4 and ephrinB2, cell contact induces EphB–ephrinB signaling, which leads to dynamic membrane ruffle formation and *trans*-endocytosis of cell membranes between cells, resulting in cell detachment (Marston et al., 2003). Similar results in which EphB–ephrinB bi-directional signaling induces *trans*-endocytosis to terminate cell–cell adhesion leading to cell repulsion were also reported for human cervical cancer cells expressing EphB2 and ephrinB1 (Zimmer et al., 2003). Furthermore, ephrinB1 is required for *trans*-endocytosis in Chinese hamster ovary cells (Parker et al., 2004). In X. laevis, *trans*-endocytosis occurs at points of cell–cell contact between leading and following cells at the cell rear; this process also depends on ephrinB1. In support of this notion, double-membraned vesicles are observed at the trailing edge under TEM. In these vesicles, one layer of membrane would be contributed by the leading cell, and the other layer contributed by the following cell. Double-membraned vesicles typically contain cytoplasm-like cargo, consistent with cell–cell *trans*-endocytosis. Taken together, X. laevis endoderm cells use both ephrinB1-dependent mechanisms to undergo macropinocytosis of the rear-cell membrane at the free margins and *trans*-endocytosis of cell–cell contacts at the trailing edge to facilitate retraction and cell detachment.
4.3.5 Macropinosome maturation

Presently, it is not known whether vesicles internalized at the free margin of the trailing edge through macropinocytosis or at cell–cell contacts by trans-endocytosis adopt different fates within the cell, and although advances have been made in understanding the steps involved in macropinosome maturation, the exact molecular pathway(s) involved in macropinosome trafficking are not yet fully known. What is known, however, is that macropinosomes can undergo recycling back to the plasma membrane, become broken down for protein extraction, or can coalesce into larger vesicles (Buckley and King, 2017).

Macropinocytosis involves the internalization of large membrane areas; therefore, to maintain steady-state levels of cell surface proteins, macropinosome recycling of key proteins has been observed in numerous cell types. For example, in *Dictyostelium*, The Wiskott–Aldrich and SCAR homologue (WASH) complex drives early macropinosome recycling of integrins back to the cell surface. WASH is recruited to select macropinosomes as rapidly as within one minute post-macropinosome closure (Buckley et al., 2016). WASH then recruits and interacts with the retromer sorting complex (Gomez and Billadeau, 2009) to drive recycling of early macropinosomes. In *X. laevis*, macropinosomes are predominantly localized to the trailing edge; yet, a small fraction of vesicles can be observed near the lateral border of the cell, and an even smaller number of vesicles are scattered toward the cell front. At the cell front, characteristics of macropinocytosis such as membrane ruffling and protrusions are notably absent. In this regard, it is conceivable that the small number of vesicles present at the front and to the sides could represent recycling macropinosomes in transit back to the membrane surface; however, further analysis is required to confirm this claim.
Despite many different maturation pathways, the small GTPase Rab5 is the first protein to be recruited to all macropinosomes and is required for macropinocytosis (Lanzetti et al., 2004; Kerr et al., 2006; Feliciano et al., 2011). Although the mechanism behind its initial recruitment is not yet understood, Rab5 acts to stabilize macropinosomes during their formation (Feliciano et al., 2011), and directs macropinosome traffic within the cell (Schnatwinkel et al., 2004). Exogenous expression of Rab5 leads to a two-fold increase in macropinocytosis, whereas knockdown of Rab5 prevents fluid uptake through macropinocytosis (Schnatwinkel et al., 2004). The persistence of Rab5 expression after internalization depends on macropinosome diameter. For example, Rab5 expression can be detected on macropinosomes with ~2 µm diameters for approximately 10 minutes, and on ~7 µm diameter vesicles for nearly 30 minutes (Feliciano et al., 2011). In X. laevis endoderm cells, Rab5 is enriched at the trailing edge of migrating cells. Specifically, Rab5 is present on nascent macropinosomes at the cell cortex and on some presumably mature macropinosomes in the trailing edge cytoplasm. However, Rab5 is also absent on some macropinosomes, which suggests that Rab5 is only involved in particular part(s) of the maturation pathway. Correspondingly, in human epidermal cancer cells, macropinosomes lose Rab5 and adopt Rab7 expression shortly before they are broken down by lysosomal degradation (Racoosin and Swanson, 1993; Hewlett et al., 1994). Macropinosomes fated for degradation undergo size reduction by membrane tubulation and fission where initially round macropinosomes become elongated and small vesicles bud off from the main tubule structure to form successively smaller vesicles. Secondary macropinosomes then merge with tubular lysosomes for subsequent degradation (Racoosin and Swanson, 1993; Bright et al., 2005). The exchange of Rab5 for Rab7 requires the Rab7-associated GEF Mon1–Ccz1 (Yasuda et al., 2016), although the mechanism of how Rab5 is exchanged for Rab7 is not fully known. What is known, however, is that after the adoption of Rab7, the filamentous GTPases Septins associates with
Rab7-expressing macropinosomes and regulates macropinosomal traffic and fusion to the lysosome compartment (Dolat and Spiliotis, 2016).

In addition to fission, macropinosomes can also undergo fusion. Fusion requires the early endosomal autoantigen 1 (EEA1), consistent with the notion that macropinosomes converge upon existing endosomal pathways within the cell to mediate their maturation (Hamasaki et al., 2004). For example in human epidermal cancer cells, small vesicles are seen to coalesce with other vesicles to form large macropinosomes in a process that requires EEA1 (Araki et al., 2006); however, the functional relevance of these relatively large macropinosomes is not known and may be cell type dependent. For example, in human cervical cancer cells, endosome fusion plays a role in the endosomal recycling pathway (Puri et al., 2013). Vesicle fusion also plays a role in the formation of the excretory canal in C. elegans (Buechner et al., 1999). In X. laevis, macropinosomes also undergo fusion, and vesicular coalescence contributes to the enlargement of macropinosomes at the trailing edge. Interestingly, large (~3 µm) macropinosomes do not appear to be degraded and can persist within endoderm cells for upwards of an hour. It has been proposed that internalized endosomes retained within the cell (neither recycled nor degraded) can persist as signaling hubs within cellular compartments and continue to signal after internalization (Marston et al., 2003; Pitulescu and Adams, 2010). Support for such a phenomenon was discovered when studies of interacting fibroblasts examined cell–cell contact repulsion between cells that expressed EphB2 and ephrinB1. Cell–cell contacts were found to induce bi-directional EphB–ephrinB signaling, which resulted in membrane trans-endocytosis of the contacting region and led to cell repulsion (Zimmer et al., 2003). Notably, intact EphB2–ephrinB1 complexes were found within endocytosed vesicles. Moreover, the presence of phosphorylated EphB2 within vesicles supports the notion that signaling may persist even after vesicle internalization. In fact, persistent signaling has been documented for activated receptor tyrosine kinases internalized by
endocytosis, and signaling continues from intracellular compartments until deactivated by dephosphorylation or recycling back to the membrane (Goh and Sorkin, 2013), such as in epidermal growth factor receptor signaling in human endothelial cells (Burke et al., 2001). Similarly, nerve growth factor-induced activation of Tropomyosin receptor kinase-A was detected in endosomes during neurite attraction and growth, which led to sustained activation of Rap1, a small monomeric GTP-binding protein of the Ras family, in rat adrenal gland phaeochromocytoma cells (Wu et al., 2001). Thus, it is conceivable that persistent macropinosomes may act as signaling centers within endoderm cells; however, further analysis is required to investigate this claim.

**4.3.6 Dsh in protrusion formation and retraction at the trailing edge**

Macropinosome formation is associated with protrusion formation. The ligand ephrinB1 is enriched at the trailing edge and is a membrane constituent of internalized macropinosomes. Given its role in modulating the cytoskeleton, it is possible that ephrinB1 may regulate protrusion dynamics. Support for this claim is found in the scaffold protein Dsh, which is a known downstream effector of ephrinB1 signaling in *X. laevis* (Lee et al., 2006, 2009). Here, I found that at the endoderm cell cortex, Dsh was co-localized to regions of ephrinB1 enrichment at the trailing edge. Moreover, the kinetics of Dsh puncta behaviour correlated with protrusion formation and resolution, consistent with a presumptive role for Dsh in modulating actin dynamics at the membrane. Furthermore, Dsh has been implicated in modulating endocytosis by interacting with the disheveled-associated activator of morphogenesis (Daam1) protein. Dsh was shown to form a complex with Daam1 and EphB receptors. This complex is incorporated into
endocytic vesicles, which leads to the removal of EphB from the cell surface to alter cell adhesion and cytoskeletal remodeling (Kida et al., 2007). Other notable effectors of ephrinB1 include the SH2/S3 adaptor protein Grb4, which has been shown to transduce ephrinB-reverse signaling (Cowan and Henkemeyer, 2001). The SH3 domain of Grb4 is known to bind to other proteins that are known effectors of cytoskeletal remodeling, such as p21-activated kinase (Pak1). Interestingly, Pak1 has been shown to regulate E-cadherin levels at adherens junctions and basolateral membranes within cells of the D. melanogaster embryonic salivary gland by interacting with Rab5 to regulate E-cadherin endocytosis (Pirraglia et al., 2010). Taken together, a growing body of evidence implicates ephrinB1 and several of its known effectors in specialized endocytic mechanisms.
5. CHAPTER FIVE: A Relative Perspective on Endoderm Cell Migration

5.1 Endoderm internalization in *X. laevis* gastrulation

In metazoan development, the early embryo typically consists of a group of relatively unspecialized cells arranged in a simple hollow monolayered sphere. A key event begins when progenitor cells of the prospective endoderm (in diploblastic organisms) or mesoderm and endoderm (in triploblastic organisms) becomes specified on the embryo surface at pre-localized sites and then move to the embryo interior by tissue-specific morphogenetic movements to generate a complex multilayered structure with separate germ layers through the process of gastrulation (Beetschen, 2001).

As gastrulation occurs in both diploblastic organisms such as cnidarians and triploblastic organisms such as *X. laevis*, the internalization of endoderm represents a primary function of gastrulation. Indeed, when endoderm internalization is prevented, animal development is halted. For example, when amphibian embryos are stripped of their vitelline membrane and cultured in a high salinity medium, the endoderm is moved outward rather than inward toward the blastocoel (Holtfreter, 1933). The resultant process, termed “exogastrulation”, produces embryos that form only epidermis without neural tissue, and further development is aborted.

In triploblastic embryos, gastrulation movements organize the stratification of three primary germ layers that surround the blastocoel such that by the end of gastrulation, the endoderm becomes situated as the innermost layer and is enveloped by the mesoderm, which in turn is
surrounded by ectoderm. Additionally, in X. laevis, the whole embryo is covered by the superficial epithelium (Chan and Etkin, 2001; Keller et al., 2003).

Classic studies of triploblast gastrulation have largely focused on echinoderms such as sea urchins to take advantage of their relatively large embryos and simple culturing conditions. Thus, sea urchin gastrulation has come to be regarded as the “textbook” definition of animal gastrulation (Kominami and Takata, 2004; Lyons et al., 2012). General gastrulation-related movement strategies include tissue invagination, involution, and cell ingression. These processes are not exclusive of one another; in fact, multiple mechanisms are typically used by the same organism in a region-specific manner. For example, sea urchin embryos undergo cell ingression and tissue invagination during gastrulation (Kominami and Takata, 2004).

In sea urchin blastulae, presumptive mesoderm cells specified at the vegetal pole are internalized by ingestion (McClay et al., 1992). During ingestion, single cells initially situated on the surface of the vegetal pole progressively lose cell adhesion at their apical surface, and ingress by constricting their apical surface and expanding their basal surface to “pinch-off” and migrate toward the blastocoel to enter the embryo interior and become primary mesenchymal cells (Katow and Solursh, 1980). The process of pinching-off can be interpreted as a series of cell morphological changes that begin with initially columnar cells transitioning into a wedge shape (with a narrow apical surface and wide basal surface) that develops into kite/flask/bottle shaped cells, which have an apical stalk that extends into an Erlenmeyer Flask-like cell body. Finally, cells retract their apical stalk and round up to complete the process. Similar behaviours are also observed during amoeboid-type cell migration in vitro (Friedl and Wolf, 2009). Morphological changes are regulated by cytoskeletal remodeling, such as by disassembling the microtubule framework of initially columnar cells (Tilney and Gibbins, 1969). However, the precise order of
molecular interactions responsible for spatiotemporal control of morphogenetic changes are not fully known.

During sea urchin gastrulation, the entire vegetal pole undergoes invagination, which bends cells at the vegetal plate inward toward the interior to form the archenteron. To drive invagination, presumptive endoderm cells elongate along the A–V axis, which significantly increases the thickness of the vegetal plate with respect to the animal hemisphere to generate the distinctive asymmetric appearance of sea urchin blastulae right before gastrulation. At the start of gastrulation the presumptive endoderm cells constrict their apical-exterior cell surface and expand their basal-inner cell surface to initiate an inward bend of the entire vegetal plate region which proceeds to invaginate and internalize the endoderm (Kominami and Takata, 2004).

The cephalochordates and tunicates, subphyla of Chordata, also initiate gastrulation originally as a single-layered epithelium. In ascidians like *Ciona intestinalis*, for example, the vegetal cells are relatively large with respect to other cells in the single-layered embryo, which confer ascidian blastulae with a distinct asymmetric appearance (Fig. 5-1A) (Sherrard *et al.*, 2010). However, in contrast to sea urchins, ingestion is not observed in these animals, and germ layers are internalized by invagination (Shook and Keller, 2008). A more complex embryo structure is found in the third chordate group, the vertebrates, for which the process of gastrulation is best characterized in *X. laevis*. Here, a dramatic increase in egg size immediately set vertebrate embryos apart. Moreover, pre-gastrulation, blastulae form multiple cell layers that surround the blastocoel and thus can be structurally considered a multilayered epithelium (Fig. 5-1B). Despite the drastic change into a multilayered embryo, the asymmetric appearance of blastulae where the vegetal hemisphere is thicker with respect to the animal hemisphere is conserved in *X. laevis* (Fig. 5-1B). Instead of columnar epithelial cells, however, the vegetal part of the *X. laevis*
blastula is occupied by the multilayered vegetal cell mass, which consists of the presumptive endoderm (Bauer et al., 1994).

In *X. laevis*, a landmark event marking the initiation of gastrulation occurs by the apical constriction of bottle cells, which leads to the formation of the dorsal lip. This initial event generates an inward bend at the site of bottle cell formation that is reminiscent of invagination; however, bottle cells themselves are not required for gastrulation movements (Keller, 1981). Instead, the dorsal mesoderm is internalized by involution, an active tissue autonomous movement that rolls the tissue inward to ultimately place it between the ectoderm and the inner endodermal lining of the archenteron (Evren et al., 2014). The endoderm is internalization by vegetal rotation (Winklbauer and Schürfeld, 1999). Thus, invagination is not a major tissue morphogenetic movement during *X. laevis* gastrulation. Furthermore, the round morphology of embryos is retained throughout gastrulation.

Gastrulation progression can be tracked by monitoring external morphological criteria such as blastopore closure and epiboly. However, movements continue despite complete removal of the BCR, which suggests that blastopore closure is not induced by pushing from the ectoderm but is driven by active cell movements in the vegetal hemisphere (Keller and Jansa, 1992). Monitoring by time-lapse cinemicrography confirms that cells do not leave the vegetal epithelium during gastrulation (Keller, 1978). Given that vegetal rotation in *X. laevis* appears deprived of classic cell or tissue internalization strategies such as ingress or invagination, respectively, it is not surprising that vegetal rotation has been ascribed to a novel tissue movement unique to amphibians. However, this thesis presents evidence indicating that vegetal rotation represents a process that has adopted monolayered morphogenetic mechanisms for use in a multicellular context as it contains cell behaviours reminiscent of traditional ingress and invagination, thus
bringing vegetal rotation back into the fold as a novel use of ancient cell behaviours during amphibian gastrulation.

5.2 Shrinking on the outside and expanding on the inside

The *X. laevis* endoderm occupies almost the entire vegetal hemisphere. At the onset of gastrulation, the endoderm represents nearly a 12-cell-layer tissue in contrast to the three-cell-layer BCR, which gives rise to a characteristic asymmetry in the *X. laevis* gastrula. When observed from the mid-sagittal plane of bisected gastrulae, the endoderm initially resembles a truncated cone with a narrow flat surface at the BCF and a wide vegetal epithelial surface. At early cleavage stages, the initial shape of prospective endoderm in *X. laevis* resembles the morphology of endodermal vegetal plate cells in monolayered embryos such as the ascidian blastulae (compare Fig. 5-1A and 5-1B).

In other initially monolayered embryos, such as the sea urchin blastulae, the vegetal plate thickens by presumptive endodermal cell elongation to adopt a columnar cell morphology (Sawyer *et al*., 2010). An analogous scenario is encountered in *X. laevis*, where endoderm cells are elongated prior to gastrulation, which suggests that cell elongation is initiated at the late blastula stage. Cell elongation along the A–V axis leads to thickening in the vegetal hemisphere that is apparent in vegetal explants. In the embryo, vegetal thickening is represented as an upward rise in the endoderm (roughly a 6% rise at the apex of the BCF from stage 10 to stage 10+) that forms a convex dome shaped BCF surface, as seen in nuclear magnetic resonance microscopy images of intact *X. laevis* gastrulae (Papan *et al*., 2007). Thus, cell elongation in the
*X. laevis* vegetal cell mass thickens the vegetal hemisphere in a manner reminiscent of single cell morphological changes in monolayered embryos.

In *X. laevis*, endoderm cell elongation occurs concomitantly with differential cell migration, which generates tissue deformation in the entire vegetal cell mass. Moreover, as BCF expansion is constrained by the spherical geometry of the embryo, advancement of the leading edge mesendoderm in the animal direction reverts the initially convex BCF into a concave surface by the mid-gastrula stage (as the apex of the BCF falls by nearly 11% from stage 10+ to stage 10.5 when measured in mid-sagittally fractured gastrulae). In the present work, morphometric analysis of tissue-geometric changes in the vegetal hemisphere revealed that BCF expansion occurs at the expense of narrowing in the more vegetally located region by differential cell migration. Thus, endoderm cell rearrangement leads to tissue-autonomous narrowing such that by the late-gastrula stage, only a small “yolk plug” remains of the original blastopore. Interior surface expansion in parallel with narrowing of the exterior-facing apical epithelial surface is also seen in monolayer gastrulation. In ascidian gastrulation, endoderm cells undergo apical constriction to shrink their external cell surfaces and basal expansion to increase their internal surfaces (Rhee *et al.*, 2005; Sherrard *et al.*, 2010). Thus, it is conceivable that tissue morphogenetic changes of the endoderm during *X. laevis* vegetal rotation as a whole functionally represent a multicellular adaptation to endodermal cell shape changes that occur in preparation for monolayer invagination; however, invagination itself does not occur during *X. laevis* gastrulation. Furthermore, endoderm cells are elongated prior to gastrulation, and pre-gastrulation emboly movements are observed in the vegetal cell mass (Winklbauer and Damm, 2012), which suggest that endoderm cell-autonomous behaviours are preprogrammed prior to the onset of gastrulation; however, the cue(s) responsible for this programming are not known.
5.3 Endoderm cell-autonomous behaviours during migration

Both diploblastic and triploblastic animals undergo gastrulation; however diploblastic animals such as cnidarians do so without the mesoderm, which has been taken to imply that of the three germ layers, mesoderm was the last to evolve (Martindale et al., 2004; Ryan et al., 2013). Recent analysis of embryonic gene expression in *C. elegans* revealed that endodermal genes are phylogenetically older and are activated earlier than ectoderm genes. Hashimshony and colleagues (2015) identified and grouped genes that are uniquely expressed by each germ layer. Then, by mapping germ-layer-specific gene groups to their induction times with respect to the known developmental timeline, they showed that the endoderm program is first to activate in *C. elegans*. Additionally, they extended their analysis to orthologous gene groups in other animals, including *Xenopus tropicalis* (a species closely related to *X. laevis*) and found similar results across all investigated organisms. Furthermore, they used phylostratigraphic analysis (Domazet-Lošo and Tautz, 2010), by which gene-age is inferred from the phylogenetic reach of its orthologs (for example, polymerase is expressed in all cells and is thus older relative to histones for which expression is restricted to eukaryotic cells) to show that the endodermal gene program contains the oldest genes among the three germ layers. Together, these findings imply that the endoderm evolved first, ectoderm second, and mesoderm last (Hashimshony et al., 2015). Furthermore, this places the endoderm cell program back toward the origin of multicellularity, which suggests that single-cell migration may be an ancestral program retained by endoderm cells in *X. laevis*. Similar phenomena have been observed in endoderm cells of other amphibian species. For instance, LeBlanc and colleague noted that elongated “sausage-like” cells could be
isolated from *Rana pipiens* endoderm (LeBlanc and Brick, 1981). Holtfreter noted that *Ambystoma* endoderm cells appear “sausage-shape” when isolated and engage in an “obscure gliding movement without need of support” (Holtfreter, 1944). Moreover, it was observed in *C. pyrrhogaster* that elongated endoderm cells were capable of generating “creeping locomotion” when placed on an *in vitro* substrate of soft agarose (Kubota, 1981). Together, these data suggest that amoeboid migration is a conserved program of amphibian endoderm cells that is adapted for use in a multicellular context for differential migration.

In the multilayered tissue environment, cells are confined by the presence of other cells such that movement of a contiguous cell layer, as occurs in epithelia, would not be feasible and is not observed in *X. laevis* endoderm. Instead, endoderm cells move by differential migration, wherein cells move at spatially graded velocities with respect to neighbouring cells. Cell movement is discontinuous such that cell displacement is punctuated by fits and starts, which correlate with discrete cell body behaviours that propel the cell forward. In other words, the moving cell is typically bordered by stationary neighbours or slower moving cells that the cell uses as substrate for movement. Endoderm cells undergo repeated cycles of cell body elongation, expansion of the cell front in tandem with narrowing of the cell rear, and finally retraction of the cell rear to complete a rearrangement step. Remarkably, cell translocation occurs without the extension of lamellipodial, filopodial, or bleb-like protrusions, consistent with an amoeboid-type movement associated with low substratum adhesion where cells translocate by modulating cell shape changes to squeeze through the ECM, which is typical behaviour for cells undergoing ingression (Friedl and Wolf, 2010). In the sea urchin embryo, mesoderm cells do not have or use locomotory protrusions to move during ingression (Katow and Solursh, 1980); instead, cells use cell shape changes to generate traction for locomotion. To achieve translocation, mesoderm cells knead their cell bodies to squeeze through the basal ECM, presumably with the help of several
matrix metalloproteases (Quigley et al., 1993) to enter the blastocoel cavity (Wu et al., 2007; Lyons et al., 2012).

5.4 Endoderm deep cell ingestion

Mesoderm cell ingression has been extensively studied in the sea urchin where embryos are large and translucent; thus, cell lineages, cell positions, and cell behaviours can be accurately monitored throughout gastrulation. Within the monolayer, presumptive mesoderm cells are packed in tight columnar cell arrays where each cell is flanked on its lateral cell borders by other blastomeres and sandwiched between two ECM layers located on their external-facing apical and interior-facing basal surfaces. Presumptive mesoderm cells are attached to neighbouring cells using classic cadherins (Miller and McClay, 1997) and adhere to the apical and basal surfaces through integrins (Hertzler and McClay, 1999). Prior to ingression, presumptive mesoderm cells are morphologically indistinguishable from their epithelial neighbours (Wu et al., 2007).

Ingression is a cell-autonomous process, and mesoderm cells will undergo ingression even if transplanted into ectopic sites in the embryo (McClay et al., 1992; Peterson and McClay, 2003). Ingression begins with a loss of both cell–cell and cell–ECM adhesion in the apical half of the mesoderm cell; in contrast, adhesion is quantitatively increased in the basal half of the cell to the basal lamina (Fink and McClay, 1985; Hertzler and McClay, 1999). In parallel with adhesive changes, mesoderm cell shape also changes from cuboidal to an elongated kite shaped morphology with a broad basal surface and narrow apical surface that tapers into a stalk. Analogously, X. laevis endoderm cells adopt similar morphological features at the start of
differential migration. In vegetal explants, endoderm cells widen their cell front to increase cell–cell contact with the leading cell, while simultaneously narrowing the cell rear to de-adhere from the following cell.

During apical constriction and membrane resorption of the apical stalk in sea urchin mesoderm cells, β-catenin–cadherin complexes are internalized through endocytic mechanisms, and numerous large vesicles (> 1 µm) can be seen in the apical half of ingressing cells (Miller and McClay, 1997). Apical surface remodeling through endocytosis appears to be a conserved process of ingressing cells (Fig. 5-1C). For example, cnidarian cell ingression during Clytia gregaria gastrulation showed large (> 1 µm) heterogeneous vesicle clusters at the trailing edge of ingressing cells (Byrum, 2001). Similarly large vesicle clusters were found at the trailing edge of ingressing cells in the sea urchin Lytechinus pictus (Katow and Solursh, 1980; Anstrom and Raff, 1988), in the frog Rana pipiens (Johnson, 1978), in the apical surface of ingressing mesoderm cells of the white rabbit Oryctolagus cuniculus (Viebahn et al., 1995), and in the primitive streak of the mouse (Batten and Haar, 1979). Furthermore, large vesicles are also found in the apical cytoplasm of bottle cells in the newt Triturus (Perry and Waddington, 1966). In these examples, vesicle sizes well exceed those typically known for clathrin-mediated endocytosis, which suggests that these vesicles are formed from macropinocytosis. Macropinocytosis is also encountered in X. laevis; macropinosomes can be seen inside the apical cytoplasm of bottle cells during apical constriction (Lee and Harland, 2010), and in endoderm cells during differential migration (Fig. 5-1D). Here, macropinocytosis is required for endoderm cell-rear retraction to rapidly resorb the trailing edge membrane during differential migration.

To leave the epithelium during sea urchin gastrulation or to complete rearrangement during X. laevis endoderm migration, cells must detach from one another. In L. pictus, cells progressively
lose cell–cell adhesion by losing cadherin on the apical half of ingressing cells (Miller and McClay, 1997). However, TEM reveals that desmosomes are still present at the tip of the apical stock in ingressing cells (Katow and Solursh, 1980). Thus, cadherins are still expressed and engaged in cell–cell binding at the apical membrane; however, cell–cell membrane contacts and adhesion complexes are removed by specialized endocytosis, conceivably by trans-endocytosis. Evidence in support of trans-endocytosis is found in electron micrographs showing that double membrane vesicles filled with cytoplasmic content are found in the cytoplasm of ingressing cells. Indeed, “multi-lamellar” vesicles with cytoplasm-like cargo have been observed at the apical surface of ingressing cells of numerous animals, including the sea urchin (Katow and Solursh, 1980; Anstrom and Raff, 1988), the chick (Harrisson et al., 1991), the rabbit (Viebahn et al., 1995), and cnidarians (Byrum, 2001), which suggests that trans-endocytosis constitutes a common mechanism of detachment employed by ingressing cells. In double-membraned vesicles, one layer of membrane presumably belongs to the ingressing cell, whereas the other layer belongs to an adjoining cell. As the cell–cell contact region is endocytosed by the ingressing cell or epithelial cell, it takes a portion of the neighbouring cell’s cytoplasm into the lumen of the double-membraned vesicles. In X. laevis, double-membraned vesicles are found primarily in the trailing edge cytoplasm of endoderm cells, consistent with the notion that endoderm cells undergo trans-endocytosis to detach from neighbouring cells during differential migration.

At the molecular level, termination of cell–cell adhesion by trans-endocytosis is mediated by EphB–ephrinB interactions in multiple cell types, including mouse fibroblasts, mouse primary neurons, human cervical cancer cells (Zimmer et al., 2003), human umbilical vein endothelial cells (Marston et al., 2003), human neuroblastoma cells, and human embryonic kidney cells (Gaitanos et al., 2016). In particular, ephrinB1 signaling is involved in mediating cell–cell trans-
endocytosis (Parker et al., 2004; Gaitanos et al., 2016), potentially to direct endosome traffic into the advancing cell. A recent study showed that the direction of endosome internalization is regulated by the configuration of EphB–ephrinB1 interactions such that in an ephrinB1 mutant lacking the cytoplasmic domain, the endosome formed is internalized into the EphB-expressing cell. Conversely, in an EphB mutant lacking the cytoplasmic domain, the endosome formed is internalized into the ephrinB1-expressing cell. In the condition where EphB and ephrinB1 are both intact, endosomes preferentially enter the ephrinB1-expressing cell (Zimmer et al., 2003).

In X. laevis, ephrinB1 is expressed in the cell membrane, and it becomes preferentially enriched at the trailing edge during differential migration. Furthermore, ephrinB1 is required for vesicle biogenesis at the trailing edge, consistent with a role for ephrinB1 in regulating endoderm trans-endocytosis.

5.5 Post-ingression migration

It is necessary to note that although cells within a large area may all undergo ingestion at some point, they do not ingress all at once; instead, cells ingress in order at localized regions, presumably to maintain epithelial integrity (Shook and Keller, 2003). In its course, as the ingressing cell pinches-off toward the embryo interior, its laterally neighbouring cells converge, creating a new cell–cell contact interface that is facilitated by narrowing the apical surface of the ingressing cell. In multilayered systems such as the primitive streak of rat or chick, internalized mesoderm cells directly transition from ingestion to migration. The binding of presumptive mesoderm cells to FN is required for their ability to migrate away from the streak and move laterally to form the mesoderm (Harrisson et al., 1993). During post-ingression migration, cells
alter their morphology and transition away from their bottle shapes to adopt a more mesenchymal architecture where cells appear more elongated and flattened, and extend numerous cell processes onto neighbouring cells (Solursh and Revel, 1978). In addition, internalized cells also produce and accumulate hyaluronic acid on the cell surface and generate interstitial space (Sanders, 1979; Sanders and Prasad, 1986; Solursh et al., 1979).

Tracking of cell movement trajectories in the chick shows that cells move asynchronously during ingression-migration (Chuai et al., 2006). Asynchronous cell movements are characteristic of differential migration in X. laevis (Fig. 5-1E). Here, differences in cell velocity allow advancing cells to use temporarily stalled adjoining cells as substrate for movement. In addition to the intermittent nature of intercellular rearrangements, endoderm cells move using a graded movement velocity with respect to laterally neighbouring cells. Identification of an A–V cell velocity gradient in the vegetal cell mass emerged from quantification of endoderm cell translocation in explants (this thesis), and the monitoring of vegetal cell movement trajectories in live embryos using X-ray microtomography (Moosmann et al., 2013). However, the mechanism responsible for generating this pattern is not yet understood.

Endoderm cells deep to the superficial epithelium possess mesenchyme-like morphology, and cells extend thin cell processes that interconnect neighbouring cells across channels of interstitial space (Fig. 5-1F). Instead of protrusion-based propulsion, cells migrate using vigorous cell shape changes in amoeboid migration to drive locomotion (Fig. 5-1G). However, adhesive cell–cell contacts are still engaged between endoderm cells in vivo, perhaps to mediate directional persistence during differential migration (Fig. 5-1H) (Petrie et al., 2009). Amoeboid migration behaviours have been shown for numerous cell types, including CD8+ T-cells, dendritic cells, monocytes, and in organisms such as Dictyostelium on experimental substrates containing
mixtures of collagen, hyaluronic acid, and chondroitin sulfate \textit{in vitro} (Devreotes and Zigmond, 1988; Friedl \textit{et al}., 2001; Wolf \textit{et al}., 2003). In \textit{X. laevis}, gene expression analysis of the early gastrula using \textit{in situ} hybridization reveals that hyaluronic acid synthase (HAS), an enzyme responsible for hyaluronic acid production, increases in a A–V direction in the vegetal cell mass; HAS expression is low on the vegetal side, and expression increases toward the animal direction (Taverner \textit{et al}., 2005). Interestingly, this A–V gradient of HAS expression overlaps spatiotemporally with gap size and with the A–V gradient of endoderm cell movement velocity, which suggests a possible link between these two factors; however, more work is required to confirm this relationship.

Endocytic remodeling at the trailing edge, combined with amoeboid-type translocation of endoderm cells, suggests that differential migration may be derived from an invagination- or ingression-type process, which are in fact closely related processes. For example, the genes folded gastrulation (fog) and t48 are expressed and required for mesoderm invagination in \textit{D. melanogaster} (Sweeton \textit{et al}., 1991; Kölsch \textit{et al}., 2007); however, these genes are not expressed in the midge \textit{Chironomus riparius}, which undergoes mesoderm ingression. Experimental expression of fog and t48 in \textit{C. riparius} induced mesoderm invagination similar to \textit{D. melanogaster} (Urbansky \textit{et al}., 2016), which supports the notion that these two processes are mechanistically linked. Given the similarities between endoderm cell rearrangement and cell ingression in other organisms, I propose that in the deep cells within \textit{X. laevis} endoderm, differential migration represents an adaptation of ingression to a multilayer tissue context. In other words, within the vegetal cell mass, endoderm cells undergo ingression-type cell migration during vegetal rotation.
Figure 5-1 Significant findings that place vegetal rotation in the context of animal gastrulation.

(A) Endodermal cells (yellow) invaginate in ascidians. Schematic of *Ciona intestinalis* embryos at 64- and 112-cell stages. (B) Vegetal rotation in amphibians. Schematic of *X. laevis* embryos at 32-cell, late blastula, and mid-gastrula stages. Vegetal endoderm cells are shown in yellow. Inset indicates cells shown in (D). (C) Epithelial cell ingression. Schematic shows ingressing cells (yellow) next to non-ingressing cells (grey). Internalized membrane vesicles are shown at the trailing edges. (D) Deep cell ingression. Schematic shows endoderm cells undergoing differential amoeboid migration in the vegetal cell mass. (E) Schematic of cell migration velocity gradient
within the endoderm explant. Colours and sizes of arrows represent the magnitude of speed shown on a scale to the right. (F) Large interstitial spaces between cells within the slice explant, similar to the situation \textit{in vivo}. (G) Endoderm cells rearrange by cycling through a series of amoeboid migration behaviours (indicated by dashed lines), including cell body elongation, cell front expansion, and, in tandem, cell rear narrowing, which is required for trailing edge retraction. (H) Despite large gaps between cells, endoderm cells are coupled by filiform protrusions, and require the adhesion molecule C-cadherin. Protrusions are thin but taut, and the molecule \(\alpha\)-catenin is enriched within the bodies and bases of protrusions.
6. Materials and Methods

Embryos

Lab-bred African clawed frogs (*X. laevis*) were housed in aquaria, water temperature 19–20°C. Research animals were used in accordance with guidelines approved by the University Animal Care Committee (Protocol no. 20011765, University of Toronto, Canada).

Egg collection

To induce egg laying, female frogs were first primed with pregnant mare serum gonadotropin (PMSG; 50 U; Sigma–Aldrich, Missouri, USA) one week before use. One day prior to egg collection, PMSG-primed females were injected with human chorionic gonadotropin (400 U; Sigma–Aldrich, Missouri, USA) to induce ovulation. Eggs were harvested from female frogs by gently squeezing the abdominal region near the cloaca. Eggs were placed in a petri dish incubated with 1X Modified Barth’s Saline (MBS; 88 mM NaCl, 1 mM KCl, 2.4 mM NaHCO₃, 0.82 mM MgSO₄, 0.33 mM Ca(NO₃)₂, 0.41 mM CaCl₂, 10 mM Hepes (+NaOH), 1% Streptomycin, 1% Penicillin, pH 7.4) and used immediately after collection.
**Harvesting testes**

To harvest testes, male frogs were first anesthetized by submerging male frogs for 1 hour in Ethyl 3-aminobenzoate methanesulfonate solution buffered with sodium bicarbonate to approximately pH 7.0 (1 g/L, MS-222; Sigma–Aldrich, Missouri, USA). After anaesthesia, animal responsiveness was checked to ensure that frogs were fully sedated prior to surgery. A vertical incision was made in the middle of the belly followed by horizontal cuts to the lateral sides to create an opening in the abdominal wall to extract the testes without damaging them. Testes were used immediately following excision.

**In vitro fertilization**

For *in vitro* fertilization, eggs were fertilized with macerated testes in 1X MBS for 5 minutes. Then, 1X MBS was removed, and embryos were incubated in 0.1X MBS for 1 hour until removal of the jelly coat. To remove the jelly coat, embryos were incubated in a solution of 2% cysteine (w/v, pH 8.0) in 0.1X MBS for 5 minutes and then cultured in 0.1X MBS until the desired stage for experimentation. Embryos were staged using external criteria outlined by Nieuwkoop and Faber (1967).

**Reagents**

*Constructs and mRNA synthesis*

Full-length *X. laevis* ephrinB1 in pSP64A was a gift from Dr. I.O. Daar (National Cancer Institute Center for Cancer Research, Maryland, USA; Jones *et al.*, 1998). Fluorophore tag was
added by sub-cloning eB1FL into pCS2+8CmCherry (Addgene, Massachusetts, USA). Membrane–RFP in pCS2+ was a gift from Dr. N. Kinoshita (National Institute for Basic Biology, Okazaki, JPN; Iioka et al., 2004). Membrane–GFP in pCS2+ was a gift from Dr. R.M. Harland (UC Berkeley, California, USA). Lifeact–GFP was a gift from Dr. C.P. Heisenberg (Institute of Science and Technology Austria, Klosterneuburg, AUT; Riedl et al., 2008). Lifeact–Ruby was a gift from Dr. M. Tada (University College London, London, UK). GFP-fused α-catenin was a gift from Dr. W.J. Nelson (Stanford University, California, USA; Schepis et al., 2012). Rab5c–CFP was a gift from Dr. M. Brand (Center for Regenerative Therapies, Dresden, GER; Yu et al., 2009). Capped messenger RNA was synthesized from linearized constructs using mMessage mMMachine (Ambion, Massachusetts, USA) as per manufacturer instructions. To assist with RNA isolation, GlycoBlue (50 µg/mL; Ambion, Massachusetts, USA) was added to the reaction during isopropanol precipitation. RNA was prepared in Gurdon’s Buffer (15 mM Tris, 88 mM NaCl, 1 mM KCl, pH 7.5) for microinjection.

Morpholinos

Morpholino oligonucleotides (MOs; GeneTools, Oregon, USA) were reconstituted in autoclaved double-distilled water (ddH2O) and microinjected at the concentrations indicated. The following MOs were used for knockdown: efnB1-morpholino (eB1MO): 5’-
GGAGCCCTTCCATCCGACACGGTGG-3’; efnB2-morpholino (eB2MO): 5’-
ACACCGAGTCCCCGCTCAGTGCCAT-3’; and efnB3-morpholino (eB3MO): 5’-
CGGGAAAACATGCTGATTAAAGGGC-3’. StandardMO, 5’-
CCTCTTACCTCAGTTACAATTTATA-3’, which targets a human β-globin intron mutation was used as negative control. MOs were designed using the following NCBI reference.
sequences: ephrinB1 (BC169964.1), ephrinB2 (NM_001086485.1/ NM_001122597.1), and ephrinB3 (NM_001085842.1).

Fluid-phase markers

To monitor the uptake and internalization of vesicles, explants were incubated in media supplemented with fluorescein-conjugated dextrans (FDA; 10 kDa, Molecular Probes, Massachusetts, USA) in 1X MBS at 5 mg/mL. To mark the interstitial space, fluorescein isothiocyanate-conjugated avidin (AvidinFITC; Sigma–Aldrich, Missouri, USA) was diluted 1:500 in 1X MBS.

Chemical Inhibitors

Dynamin inhibitor (Dynasore; Sigma–Aldrich, Missouri, USA) was reconstituted to 40 µM in 1X MBS. To block dynamin activity, 50 µL of inhibitor was injected into the blastocoel of stage 8/9 blastulae; explants were then excised from stage 10 gastrulae and transferred into 1X MBS supplemented with 40 µM of inhibitor.

Microinjections

Injection needles were crafted by heating and pulling glass capillaries (Drummond, Pennsylvania, USA) using a glass capillary puller (Narishige, New York, USA). The distal tips of pulled needles were sharpened to a 20° angle using a microgrinder (Narishige, New York,
USA). Desired injection volumes were delivered using the Nanoject II injector (Drummond, Pennsylvania, USA). Embryos were microinjected in a 3% Ficoll (w/v; Sigma–Aldrich, Missouri, USA) solution in 1X MBS on plasticine-coated injection dishes. All embryos were seeded into Ficoll-dishes 15 minutes before injection and allowed to heal in Ficoll for 1 hour after injection. To target ectoderm, mesoderm and endoderm, reagent injections were targeted to the animal, dorsal-equatorial and vegetal regions, respectively, on the blastomeres of four-cell stage embryos.

**Microsurgery**

Before dissection, the vitelline membrane was removed from de-jellied embryos with forceps. Microsurgical manipulations were carried out on sterilized plasticine-coated petri dishes in 1X MBS. Specific tissue regions of interest were excised with eyebrow-knife and hair-loop tools and then transferred onto glass-bottom dishes (MatTek, Massachusetts, USA) or tissue-culture dishes (Cellstar, Missouri, USA) rendered non-adhesive by pre-coating with 1% bovine serum albumin (w/v, BSA) solution in 1X MBS or poly-2-hydroxyethyl methacrylate (Poly-HEMA; Sigma–Aldrich, Missouri, USA). Micro-dissections were carried out under a Zeiss Stemi SV 11 microscope and observed with a Zeiss AxioCam MRc digital camera using AxioVision 4.8 software.
Polymerase Chain Reaction

For PCR, RNA was extracted using TRIzol (Life Technologies, California, USA), and cDNA was synthesized using Superscript III reverse transcriptase (Life Technologies, California, USA) kit as per manufacturer instructions. The following primers were used in PCR reactions:

- **housekeeping gene ornithine decarboxylase (ODC; 386bp)** forward (FWD): 5′-GTCAATGATGGAGTGTATGGATC-3′, reverse (REV): 5′-TCCATTCCGCTCTCCTGAGCAC-3′;
- **XB/U-cadherin (165bp)** FWD: 5′-GCTTGGCAAAGTGAACTTTGTT-3′, REV: 5′-CGTGCATCCACGTACTGATTG-3′;
- **EP/C-cadherin (326bp)** FWD: 5′-GGGGGCACCAGGCTTAGAAACG-3′, REV: 5′-CGTGCATCCACGTACTGATTG-3′. PCR reactions were carried out using platinum Taq polymerase (Life Technologies, California, USA) as per manufacturer instructions. PCR was performed for 30 cycles at an annealing temperature of 58°C.

Tissue Explants

Vegetal slice

All explants were prepared and observed in 1X MBS unless otherwise specified. Tissue was excised from NF stage 10 gastrulae. A mid-sagittal vegetal tissue slice (without the BCR) about five-cell-layers thick was secured onto a glass-bottom dish with a cover glass, together held in place by silicon grease as described by Winklbauer and Schürfeld (1998).
Microscopy

All images were captured in an ambient temperature of 21–23°C using brightfield, epi-fluorescence, confocal laser scanning fluorescence microscopy, scanning electron microscopy (SEM), and transmission electron microscopy (TEM).

Fluorescence microscopy

Brightfield and epi-fluorescence time-lapse videos were recorded on a Zeiss Axiovert 200M inverted microscope with a Zeiss AxioCam MRm digital camera using AxioVision 4.8 software. High-resolution images were captured with a Leica TCS SP8 confocal laser scanning microscope equipped with HCX-PL-APO-CS 10x/NA0.40, HC-PL-APO-CS 20x/NA0.75, 40x/NA1.30, 63x/NA1.20, HC-PL-APO-CS2 100x/NA1.4 oil-immersion objectives, HCX-IRAPO-L 25x/NA0.95, HC-PL-APO-CS2 63x/NA1.20 water-immersion objectives, and a resonant scanning system using Leica LAS AF 3.2 software.

Scanning Electron Microscopy

Embryos were fixed in 2.5% glutaraldehyde and 2% paraformaldehyde in 50 mM HEPES buffer (pH 7.4) overnight at 4°C. Embryos were rinsed in 0.1 M sodium cacodylate (pH 7.0) and bisected on the mid-sagittal plane using a microsurgical knife. Post-fixation was performed by incubating embryos with 1% osmium tetroxide in 0.1 M sodium cacodylate overnight at 4°C. Samples were then dehydrated through a graded ethanol series (30%, 50%, 70%, 2X 100%),
dried overnight, and sputter-coated with gold-palladium. Images were obtained using the Hitachi S2500 Scanning Electron Microscope.

Transmission Electron Microscopy

*X. laevis* gastrulae with the vitelline membrane removed were gently punctured in the BCR with a tungsten needle to facilitate stain infusion. Perforated gastrulae were fixed for one week at 4°C in 3% glutaraldehyde (Fisher Scientific, Massachusetts, USA), 2% paraformaldehyde (Fisher Scientific, Massachusetts, USA), and 1% Alcian blue (Sigma–Aldrich, Missouri, USA) in 0.05 M sodium cacodylate (pH 7.0; Sigma–Aldrich, Missouri, USA). Embryos were rinsed in 0.1 M sodium cacodylate (pH 7.0), bisected mid-sagittally, embedded in 3% low-melting-point agarose (Sigma–Aldrich, Missouri, USA), and fixed overnight at 4°C in 1% osmium tetroxide (Electron Microscopy Sciences, Pennsylvania, USA) and 1% lanthanum nitrate (Sigma–Aldrich, Missouri, USA) in 0.1 M sodium cacodylate (pH 7.0). Embryos were then rinsed in 0.1 M sodium cacodylate (pH 7.0), dehydrated in a graded series of ethanol solutions (30%, 50%, 70%, 90%, 2X 100%), and gradually infiltrated with Spurr’s resin overnight at room temperature. Embryos were then cured at 65°C overnight. Semi-thin (1–1.5 µm) sections were made using a Leica RM2235 microtome, and ultrathin (90–100 nm) sections were prepared using a Leica EM UC6 microtome. Semi-thin sections were stained with 1% toluidine blue for sample inspection under brightfield microscopy, whereas ultrathin sections were stained using 3% uranyl acetate and Reynolds’ lead citrate to provide contrast for imaging using the Hitachi HT7700 transmission electron microscope operated at 80 kV.
**Tissue mechanical properties**

*Tissue surface tension*

Tissue aggregates round up into drop shapes *in vitro*. The drop shape of an aggregate at equilibrium represents a balance of forces between surface tension (which acts to minimize the surface area and compact the tissue into a sphere) and gravity (which acts to flatten the tissue). Because the force of gravity is known, tissue surface tension can be deduced with the Laplace equation using the radii of the aggregate curvature, which reflect the pressure difference over an interface between two static fluids (David *et al.*, 2009). The Laplace equation of capillarity defines the equilibrium of a liquid surface as the following:

**Equation 1 Young–Laplace equation**

\[
\Delta P + \Delta \rho g z = \gamma \left( \frac{1}{R_1} + \frac{1}{R_2} \right);
\]

from left to right, \(\Delta P\) is the difference in pressure across the interface between the tissue and the immersion medium (MBS), \(\Delta \rho\) is the difference in density between the tissue aggregate and MBS, \(g\) represents gravitational acceleration, \(z\) is the vertical distance from the aggregate apex, \(\gamma\) is the tissue surface tension, and \((R_1, R_2)\) are the radii of the aggregate profile. The specific numerical values of \(\Delta \rho\) for gastrula tissues (ecto-, meso-, and endoderm) were obtained from prior measurements (David *et al.*, 2009).

Tissue excised from the early gastrula (at NF stage 10 unless otherwise specified) were placed in a well for 30 minutes to facilitate rounding. Tissue aggregates were then transferred onto BSA-coated tissue culture dishes to equilibrate for 2 hours before aggregate profiles were imaged.
using an inclined mirror calibrated at a 45° angle to the substrate surface. Tissue surface tension was quantified using a modified ADSA algorithm (del Río and Neumann, 1997) adapted for use with tissue explants by introducing more robust methods for image analysis to accommodate the irregular curvatures of tissue aggregates (David et al., 2009; Luu et al., 2011; David et al., 2014). The curvature outline was identified using an edge detection algorithm (Canny) in MATLAB. All processed images were visually inspected to ensure that Canny detections generated accurate depictions of the tissue aggregate. ADSA is a program written for use in MATLAB that numerically integrates the Laplace equation to generate theoretical drop shapes for hypothetical surface tensions, which are optimally fitted onto experimental profiles obtained from tissue aggregates. The drop shape that best fits the aggregate is used to derive the specific tissue surface tension for that particular aggregate. Surface tension is numerically equivalent to surface energy, which is the energy required to expand the surface of a droplet by a unit of area, and is also equal to half the energy required to split a droplet into two equal parts (i.e., creating two new surfaces); therefore, tissue surface tension is an effective measure of tissue cohesion.
Figure 6-1 Tissue aggregate drop shape analysis.

Aggregate curvature detected by Canny in MATLAB (left) is shown in comparison to the reference tissue aggregate (right) visualized by microscopy. A Laplacian curve (red outline) corresponding to calculated value of surface tension is fitted onto the tissue aggregate outline (blue).

Tissue viscosity

Elongated tissue fragments tend to round up in vitro due to surface tension. In other words, roughly ellipsoidal aggregates transition toward becoming nearly spherical aggregates. The viscosity of the fluid (in this case, the tissue) resists surface tension-driven rounding. Therefore, tissue viscosity can be mathematically derived from the rate of explant rounding over time as defined by the following equation (Gordon et al., 1972; David et al., 2014):
Equation 2 Tissue viscosity equation

\[
\frac{dA}{dt} = \frac{3}{8t_c(A^2 - 1)} \left( \frac{4\pi A^3}{3} \right)^{\frac{1}{3}} \left[ -2 - A^2 + \frac{A^2(4 - A^2)}{\sqrt{A^2 - 1}} \sin^{-1} \left( \frac{\sqrt{A^2 - 1}}{A} \right) \right].
\]

where \(A\) represents the explant aspect ratio, and the time constant \(t_c\) is defined as \(t_c = V^\frac{1}{3} \eta/\gamma\), using the volume \(V\) of the aggregate and the surface tension \(\gamma\) of the tissue determined from separate experiments (described above). By rearrangement of terms, viscosity \(\eta = t_c V/\gamma^\frac{4}{3}\) can be determined using measured values of \(V\) from images in time-lapse recordings of aggregate rounding. The aspect ratios for tissue fragments at different time points in time-lapse videos were defined using Canny in MATLAB. All aspect ratios were visually inspected prior to further processing to ensure that computer-generated ratios were accurate representations of the tissue. The aggregate aspect ratio was used to estimate \(V\), and the radius of each ellipsoid aggregate perpendicular to the aggregate’s long axis was assumed to equal the smaller of the two detected radii.

Because cell rearrangement is associated with tissue rounding (David et al., 2014), the rate of surface tension-driven tissue rounding was used to infer the rate of cell rearrangement within tissues. For example, tissues with high tissue viscosity are more resistant to tissue deformation, which reflects a lower rate of cell rearrangement (\(\uparrow\eta \Leftrightarrow \downarrow\) cell rearrangement, and vice versa). To quantify tissue viscosity, ellipsoid tissue explants were excised from the region of interest and transferred onto a BSA-coated tissue culture dish for imaging. For gastrula tissues, it was empirically determined that the rounding process is completed in approximately 60 minutes.
Explant rounding was recorded by time-lapse video microscopy and analyzed using a custom routine in MATLAB.

Figure 6-2 Workflow schematic of experimental procedure for tissue surface tension and tissue viscosity measurements.

(A) Surface tension was measured from (A1) tissue aggregates that were allowed to (A2) round up in a pitted dish for 30 minutes, and were then placed in (A3) a BSA pre-coated dish for monitoring. Tissues in the dish were undisturbed for 2 hours, and (A4) images of the aggregate
profile in the sagittal plane were then captured and analyzed using (A5) ADSA. (B) The video capture dish consists of (B1) an upright dissecting microscope, which housed (B2) the sample dish containing (B3) 1X MBS buffer. To capture the sagittal profile, the microscope objective is focused on (B4) an inclined mirror, which reflects the image of (B5) the tissue aggregate rested on top of (B6) a platform pre-coated with BSA to prevent non-specific substrate adhesion. The captured images were systematically analyzed in laboratory computers using MATLAB software (right). (C) Tissue viscosity was measured using (C1) cylindrical tissue fragments that were placed horizontally (C2) on a dish pre-coated with BSA and (C3) imaged immediately after excision. The rate of tissue rounding was analyzed using (C4) a custom MATLAB algorithm designed to monitor changes in aspect ratio.

**Single-cell migration assay**

Endoderm cells were isolated using tissue dissected from a central column of the vegetal cell mass between the vegetal pole and the BCF, and incubated in cell dissociation buffer (88 mM NaCl, 1 mM KCl, 2.4 mM NaHCO₃, 10 mM Hepes (+NaOH), pH 7.4) to separate cells. Culture dishes were coated with 10% (w/v) fish skin gelatin (Sigma–Aldrich, Missouri, USA) overnight and surface-coated with bovine plasma fibronectin (FN, 5 µg/cm²; Sigma–Aldrich, Missouri, USA) for 2 hours at room-temperature prior to seeding. Cells were then seeded onto FN–gelatin dishes and incubated in modified Holtfreter’s Solution (59 mM NaCl, 0.67 mM KCl, and 2.4 mM NaHCO₃) for imaging.
Image and statistical analysis

The central columns of endoderm cells within the vegetal cell mass were used for morphometric analysis.

Morphological metrics

The LWR provides a measure of cell elongation parallel with the long axis of the cell by dividing the length of the cell by its width perpendicular to the long axis. Endoderm cell body LWRs were measured based on cell outlines captured from time-lapse videos of membrane-labelleled explants and scanning electron micrographs of mid-sagittally fractured embryos in various gastrulation stages as indicated.

Cell body orientation was measured by determining the angle of the long axis of cells relative to the vertical axis. To identify the long axis of individual endoderm cells, best-fit ellipses were matched to traced cell outlines (Blanchard et al., 2009). Individual fittings were manually checked to contain the same area, orientation and centroid as the original cell. The long axis of the ellipse was then used to represent the long axis of cells.

Cell movement trajectories were tracked using the MtrackJ (Meijering et al., 2012) plug-in for ImageJ. Cell displacement and velocity measurements were made by connecting the centroids of individual cells from lapsed time-points. Cell path alignment was measured by determining the angle of the cell displacement path relative to the long axis of cells.
Figure 6-3 Schematic of morphometric analyses.

To characterize cell morphology, I analyzed time-lapse recordings of membrane-labelled cells in explants and in the embryo. For computer-assisted analysis, I first used cell segmentation to identify cell outlines. Segmentation accuracy was manually verified. To find the long axis of cells, I used the best-fit ellipse to identify the major (a) and minor (b) axes of cells. The cell aspect ratio (a/b) was used to measure the cell body LWR. The long (major) axis of cells were used to determine cell orientation (α) with respect to the animal (An)–vegetal (Vg) axis. To measure the congruence (y/x) of cell elongation relative to the direction of cell migration (along the A–V axis), I measured the corresponding horizontal (x) and vertical (y) cell axes.

Statistical Analysis

All experiments were replicated at least three times; representative images are shown. Statistical testing was conducted using two-tailed unpaired Student’s t-tests to compare different populations.

Significant findings (P-values < 0.05) are indicated by asterisks. Numbers of asterisks indicate different P-values (* P ≤ 0.05; ** P ≤ 0.01; *** P ≤ 0.001; **** P ≤ 0.0001). For visualization, figures were cropped and presented after intensity adjustment using Photoshop (Adobe Systems,
California, USA). All adjustments within experiments were performed equally. Figures were composed using Illustrator (Adobe Systems, California, USA).
References


