Zebrafish Gastrulation: Cell Movements, Signals, and Mechanisms

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Gastrulation is a morphogenetic process that results in the formation of the embryonic germ layers. Here we detail the major cell movements that occur during zebrafish gastrulation: epiboly, internalization, and convergent extension. Although gastrulation is known to be regulated by signaling pathways such as the Wnt/planar cell polarity pathway, many questions remain about the underlying molecular and cellular mechanisms. Key factors that may play a role in gastrulation cell movements are cell adhesion and cytoskeletal rearrangement. In addition, some of the driving force for gastrulation may derive from tissue interactions such as those described between the enveloping layer and the yolk syncytial layer. Future exploration of gastrulation mechanisms relies on the development of sensitive and quantitative techniques to characterize embryonic germ-layer properties.

KEY WORDS: Zebrafish, Gastrulation, Wnt, Enveloping layer (EVL), Yolk syncytial layer (YSL), Differential adhesion.

I. Introduction

Gastrulation is the first large-scale morphogenetic process to occur during zebrafish development and results in the formation and spatial separation of the embryonic germ layers: ectoderm, mesoderm, and endoderm. This dramatic cellular rearrangement has three major features: epiboly, the spreading and thinning of cell layers; internalization of mesoderm and endoderm progenitors; and convergent extension, the narrowing and extension of the body axis (Warga and Kimmel, 1990). Conservation of these cell movements during vertebrate gastrulation has been extensively reviewed (Solnica-Krezel, 2005).

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Here, we describe in detail the different cell movements observed in the zebrafish gastrula. These movements are regulated via a number of known signaling pathways, as mentioned in Section III, but little is understood about the underlying molecular and cellular mechanisms. We explore possible effector mechanisms and discuss evidence that modulation of cell adhesion and/or the cytoskeleton could be crucial. Most interestingly, we give a special focus to the role of tissue interactions, such as that between the enveloping layer (EVL) and the yolk syncytial layer (YSL).

II. Gastrulation Cell Movements in Zebrafish

The zebrafish embryo is initially a single blastomere connected to a large yolk cell. Prior to morphogenesis, successive cell divisions form a large blastoderm that remains situated on top of the yolk cell (Fig. 1.1A). An epithelial monolayer, the EVL, becomes apparent as the outermost cell layer during the mid-blastula transition (3 hpf; Fig. 1.1B), as it undergoes morphological changes and lengthened cell cycle (Kane *et al.*, 1992). The EVL completely covers the underlying blastomeres, serving as a protective outer surface, and likely anchors to the yolk cell as has been described in the teleost *Fundulus* (Betchaku and Trinkaus, 1978; Koppen *et al.*, 2006). EVL cells are initially able to undergo divisions that give rise to both an EVL cell and a blastomere; however, at later blastula stages, cell division is restricted to the plane of the epithelium (Kimmel and Warga, 1987; Kimmel *et al.*, 1990). Another defined structure to form at the time of the mid-blastula transition is the multinucleated YSL, which is located cortically within the yolk cell, adjacent to the blastoderm (Fig. 1.1B). The YSL forms as the result of a complete fusion of the marginal layer of blastomeres to the yolk cell (Kimmel and Law, 1985) and plays an important role in specifying cell fate in the overlying blastomeres (Koos and Ho, 1998). During gastrulation, morphological changes occur simultaneously in all three of these embryonic regions: the blastoderm, EVL, and YSL.

A. Epiboly

At the onset of epiboly, the blastoderm begins to thin and take on a concave appearance as the underlying yolk cell bulges upward (Fig. 1.2A). Eventually, the thinning blastoderm spreads vegetally, past the equator of the embryo, and proceeds until the entire yolk cell has been engulfed (Fig. 1.4A). A well-described cell movement, radial intercalation, occurs within the blastoderm and is thought to play a role in driving epiboly (Fig. 1.2B). During radial

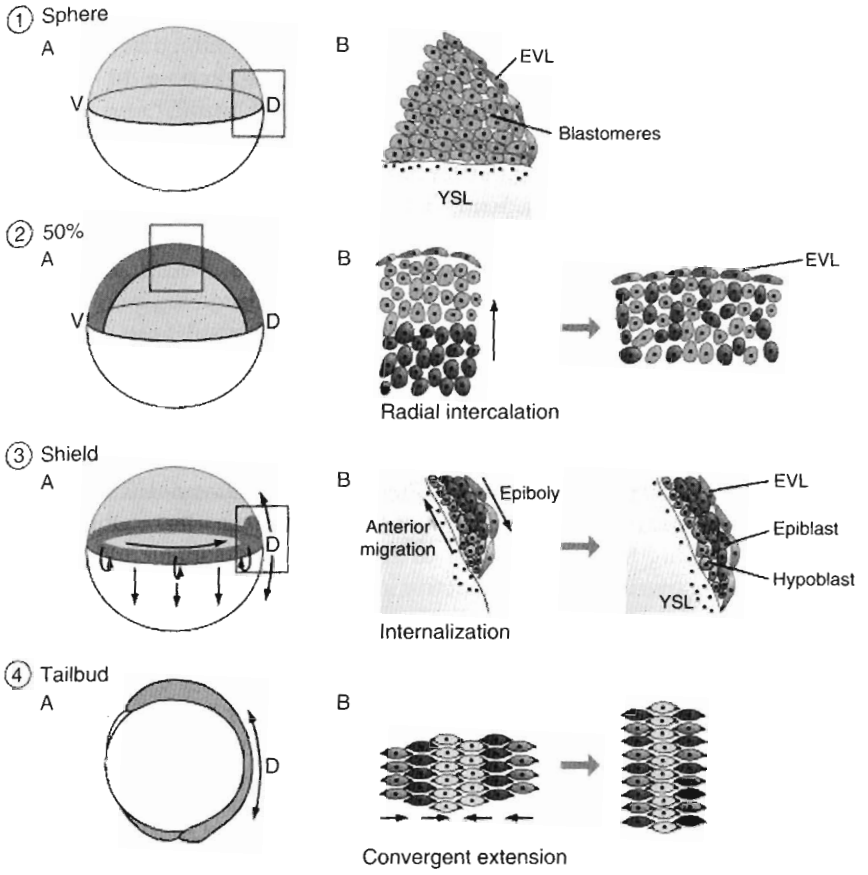

FIG. 1 Diagrams of gastrulation stage embryos (1–4A). Boxed regions shown at closer view to illustrate the different embryonic tissue layers and gastrulation cell movements (1–3B). Convergent extension movements represented in 4B. (See also color insert.)

intercalation, interior cells within the blastoderm move to occupy more superficial positions, thus intercalating with the external blastomeres, but never mixing with the EVL cells (Warga and Kimmel, 1990). Close observation of radial intercalation within the ectodermal layer at late stages of gastrulation has revealed that radially aligned internal cells intercalate into the overlying region where they assume a characteristic flattened shape (Kane *et al.*, 2005). Thus, intercalation as well as subsequent cell shape change could play an active role in epiboly.

The YSL and EVL, which link at the margin, move in concert toward the vegetal pole during epiboly. As the YSL spreads along the yolk cell cortex, its nuclei also shift, exhibiting movements similar to the overlying

blastomeres (D'Amico and Cooper, 2001). Epiboly presents a unique challenge to the multicellular EVL as this layer, which initially covers only half the embryo's surface, must expand to cover the entire surface while maintaining epithelial integrity. Cell division within the EVL is unlikely to account entirely for its expansion, given that after 50% epiboly the cell cycle dramatically slows (Kane *et al.*, 1992). EVL cells noticeably change shape over the course of epiboly, both dramatically increasing in surface area and thinning (LAR and CPH, unpublished observation). It is possible this increase in surface area is not only a passive response to EVL stretching, but also an active component of epiboly. Experiments in *Fundulus* showing an increase in apical membrane turnover in EVL cells under tension support this idea (Fink and Cooper, 1996). Cell-labeling experiments have shown that EVL cells do not undergo large-scale rearrangements during epiboly (Kimmel and Warga, 1987; Kimmel *et al.*, 1990). Cells at the leading edge, however, have been observed to narrow and align, often becoming "squeezed-out" of the leading edge, similar to *Fundulus* (Keller and Trinkaus, 1987; Koppen *et al.*, 2006). This remodeling results in a shortening of the leading edge that could create a downward pulling force on the rest of the EVL. Whether EVL cell shape changes and movements play an active role in spreading the layer during epiboly remains to be experimentally determined.

The interplay among EVL, YSL, and blastoderm during epiboly is of great interest. What forces do they exert on each other? To what degree do their movements remain autonomous? A major force in epiboly appears to derive from microtubule and actin cytoskeletal structures harbored within the YSL and yolk cell. As discussed in more detail later, forces generated by these structures could act via physical interactions between the YSL, EVL, and blastoderm (Koppen *et al.*, 2006). Consistent with an important role for the cytoskeleton, global disruption of microtubules or actin within the gastrulating embryo results in epiboly defects in all three tissues (Cheng *et al.*, 2004; Solnica-Krezel and Driever, 1994; Strahle and Jesuthasan, 1993; Zalik *et al.*, 1999). Microtubules in the yolk have been detected in two main orientations: one set of arrays is directed along the animal–vegetal axis, running from the YSL through the entire yolk cell, and the second set is formed from a meshwork of microtubules associated with the YSL nuclei (Solnica-Krezel and Driever, 1994; Strahle and Jesuthasan, 1993). At the onset of epiboly, the YSL is observed to contract and as epiboly continues, the animal–vegetal microtubules become shorter (Solnica-Krezel and Driever, 1994). Also apparent at the onset of epiboly is an accumulation of actin in a ringlike band at the equator of the yolk cell (Cheng *et al.*, 2004; Koppen *et al.*, 2006; Zalik *et al.*, 1999). Prior to 50% epiboly, this region of actin is diffuse; however, once the leading EVL cells reach the equator, it becomes concentrated immediately below these cells in the YSL (Cheng *et al.*, 2004; Koppen *et al.*, 2006). As this actin-rich band in the YSL colocalizes with myosin 2,

it is possible that this region is capable of actin–myosin-based contraction (Koppen *et al.*, 2006). Importantly, YSL-specific disruption of this "actin-ring" not only affects YSL epiboly, but also impairs epiboly of both the EVL and blastoderm. The characteristic narrowing and alignment of the leading-edge EVL cells is disrupted as well (Koppen *et al.*, 2006). These results suggest that a tension generated by contraction of the actin ring in the YSL normally acts upon the EVL cells, stretching and aligning them as they are pulled toward the vegetal pole.

Experimental evidence indicates that YSL epiboly can occur independently of the EVL and blastoderm. Experiments in *Fundulus* have shown that the YSL is able to epibolize when the overlying blastoderm and EVL are removed (Betchaku and Trinkaus, 1978). Consistent with epiboly occurring independently of the blastoderm, the EVL and YSL epibolize in the zebrafish E-cadherin mutant, *weg*, in which the blastoderm does not undergo proper radial intercalation and epiboly (Kane *et al.*, 1996a, 2005). Thus, although the force provided within the blastoderm by radial intercalation may make a significant contribution to its epiboly, it is not required to spread the other tissues.

B. Internalization

After epiboly covers 50% of the yolk cell (50% epiboly), the next morphological process to initiate is the internalization of cells that will form the mesoderm and endoderm (mesendoderm). In anticipation of this process, cells accumulate at the marginal zone and stream downward toward the yolk cell, creating a thickened in-folding referred to as the "germ ring" (Fig. 1.3A) (Montero and Heisenberg, 2004; Warga and Kimmel, 1990). Cells first internalize dorsally and then at all points around the margin. Once internalized, mesendodermal cells migrate toward the animal pole, forming an internal layer termed the "hypoblast," while the overlaying, noninvoluting cells constitute an ectodermal layer, the "epiblast," which continues to epibolize (Fig. 1.3B). The separation of the epiblast and hypoblast layers is maintained throughout gastrulation (Warga and Kimmel, 1990).

How do mesendodermal cells internalize? Suggestions have included involution, whereby cells internalize as part of a coherent cell sheet, and ingression, the movement of individual cells (Carmany-Rampey and Schier, 2001; D'Amico and Cooper, 1997, 2001; Feldman *et al.*, 2000; Montero and Heisenberg, 2004; Shih and Fraser, 1995). Although the synchronous internalization of cells resembles involution (D'Amico and Cooper, 1997), several observations suggest cells most likely internalize individually via ingression movements. Transplantation experiments have shown single cells are able to internalize autonomously (Carmany-Rampey and Schier, 2001; David and

Rosa, 2001; Shih and Fraser, 1995). Additionally, Montero *et al.* (2005) visualized individual mesendodermal cells ingressing within the shield region of a live embryo using multiphoton imaging. Whether such ingression involves an epithelial to mesenchymal transition in zebrafish is not clear. Although it is true internalized cells are mesenchymal in nature, the epiblast and germ ring lack epithelial characteristics such as cell-cell junctions and apical-basal polarity (Shook and Keller, 2003). The EVL, which is an epithelium, undergoes no internalization, remaining on the outside of the embryo, in contact with the YSL (Warga and Kimmel, 1990).

C. Convergent Extension

The cell movements of convergent extension (CE) begin simultaneously with those of internalization, at 50% epiboly. Convergence is the movement of hypoblast and epiblast cells toward the future dorsal side of the embryo, resulting in a medio-lateral narrowing of the axis. Extension refers to the elongation of the anterior–posterior axis seen at the dorsal side (Figs. 1.3–1.4). The only structure in the embryo that does not take part in CE movements is the EVL, which remains equally distributed over the embryonic surface and later becomes the periderm (Kimmel and Warga, 1987; Kimmel *et al.*, 1990). The onset of CE is marked by the compaction of cells on the dorsal side of the embryo that give rise to the shield, the embryonic organizer of the zebrafish (Warga and Nusslein-Volhard, 1998). Mesodermal cells located in the shield form axial structures such as the prechordal plate and notochord, whereas paraxial and lateral mesoderm give rise to such structures as the somites and lateral plate, respectively (Kimmel *et al.*, 1990). As gastrulation progresses, CE movements become restricted such that cells of these different mesodermal subtypes do not mix (Glickman *et al.*, 2003). In addition, differing degrees of CE movement behaviors can be observed for cells at different positions along the dorsal–ventral axis. In general, CE behaviors are more pronounced in mesoderm cells that are in more dorsal positions (Myers *et al.*, 2002b). Ventral-most mesoderm, in contrast to the dorsal and lateral mesoderm, does not move dorsally or extend toward the animal pole; instead it moves toward the vegetal pole where it contributes to the tailbud (Myers *et al.*, 2002a).

Axial mesodermal cells undergo rapid extension due in part to medio-lateral elongation and intercalation behaviors (MIB) similar to those described in *Xenopus* (Glickman *et al.*, 2003; Warga and Kimmel, 1990). During MIB, cells elongate along the medio–lateral axis and use oriented bipolar or monopolar protrusions to drive intercalation between their immediate neighbors (Fig. 1.4B). MIB thus requires the efficient coupling of convergence and extension movements to result in a narrowed and elongated axis. In *Xenopus,*

MIB is considered the only motive force for CE (Wallingford *et al.*, 2002a); however, in zebrafish, MIB is not the only process contributing to axis extension. As demonstrated in the zebrafish *notail* mutant, notochord extension does not rely on convergence movements, contrary to MIB being the sole driving force (Glickman *et al.*, 2003). An additional contributing factor appears to be directed cell migration. For example, prechordal plate progenitors, once internalized, undergo directed migration as a group to the animal pole, preferentially extending cell protrusions in this movement direction (D'Amico and Cooper, 2001; Ulrich *et al.*, 2003, 2005). MIB are not observed in this particular axial mesoderm group (FU and CPH, unpublished observations). Interestingly, these cells seem to use the overlying epiblast as a surface on which to migrate, meaning that they travel animally on a substrate that is moving vegetally (Montero *et al.*, 2005). At present, deciphering the mechanisms driving this dynamic prechordal plate migration and interaction with the epiblast is an active area of research.

Lateral mesoderm cells initially migrate mainly toward the animal pole and exhibit less medio-lateral elongation and intercalation than the dorsally located paraxial and axial mesoderm cells. However, after 70% epiboly, these lateral mesoderm cells redirect their movement to take an overall dorsal path and increase their mediolateral elongation (Jessen *et al.*, 2002; Sepich and Solnica-Krezel, 2005). The overall slower convergence speed noted for lateral mesoderm cells versus those located more dorsally has been attributed to this late switch in migration direction (Myers *et al.*, 2002; Sepich *et al.*, 2005).

The epiblast, the outer layer of the blastoderm, which contains the ectodermal progenitors, also undergoes CE movements. According to Concha and Adams (1998), the epiblast becomes sheetlike as its cells cease independent movement and cohere at the onset of gastrulation. Epiblast cells thus move together toward the dorsal side, in contrast to the underlying mesodermal cells that take individual migration paths during convergence (Concha and Adams, 1998; Sepich and Solnica-Krezel, 2005). Epiblast cells on the ventral side do not converge dorsally and instead move toward the vegetal pole. After 70% epiboly, medio-lateral elongation and extension of protrusions by epiblast cells on the dorsal side occurs, a behavior most likely associated with the medio-lateral intercalation observed in the epiblast (Concha and Adams, 1998; D'Amico and Cooper, 2001; Warga and Kimmel, 1990).

Using Cytox green to label the nuclei in live embryos, D'Amico and Cooper (2001) followed the migration of YSL nuclei during gastrulation. By the onset of CE, the nuclei occupied positions not only at the margin of the YSL, but also in portions of the YSL that lie internally, under the blastoderm. During CE the nuclei movement within the YSL mirrored CE behaviors in the overlying blastoderm. Nuclei in paraxial and lateral positions converged dorsally, whereas axial nuclei showed the same dramatic extension along the animal–vegetal axis as the overlying notochord progenitors. In fact, the

nuclei even intercalated (D'Amico and Cooper, 2001). The mechanisms controlling nuclei movements are dependent on the cytoskeleton within the yolk cell (Solnica-Krezel and Driever, 1994); however, the significance of these nuclei movements remains to be fully explored.

III. Instructive and Permissive Cues

A. Noncanonical Wnt Signaling

1. Wnt/PCP Pathway

The Wnt family of glycoproteins is one of the most significant and actively studied groups of secreted, extracellular signaling molecules. Wnts bind 7-pass transmembrane receptors of the Frizzled (Fz) family to activate several intracellular signaling cascades that regulate cell movement and polarity, as well as other important developmental processes including fate determination and proliferation. Given this diversity of critical functions, it is not surprising that disruption of Wnt signaling has been associated with many morphogenetic defects and diseases (Logan and Nusse, 2004; Veeman *et al.*, 2003a). The importance of Wnt signaling in vertebrate gastrulation is obvious from the cell movement defects seen in zebrafish Wnt signaling mutants including *silberblick/wnt11* mutants (Heisenberg *et al.*, 1996, 2000; Ulrich *et al.*, 2003, 2005); *pipetail/wnt5* mutants (Hammerschmidt *et al.*, 1996; Kilian *et al.*, 2003; Rauch *et al.*, 1997); mutants in *knypek*, a glypican transmembrane protein that likely serves as a Wnt coreceptor (Marlow *et al.*, 1998; Ohkawara *et al.*, 2003; Solnica-Krezel *et al.*, 1996; Topczewski *et al.*, 2001); and mutants in *trilobite,* the transmembrane protein Strabismus/Van Gogh (Hammerschmidt *et al.*, 1996a; Heisenberg and Tada, 2002; Jessen *et al.*, 2002; Park and Moon, 2002; Sepich *et al.*, 2000).

Wnt signaling is categorized as either canonical or noncanonical. In the canonical, or so-called Wnt/βcatenin pathway, Wnt signaling activates the cytoplasmic protein Disheveled (Dsh), which in turn inhibits the APC-Axin-GSK3 complex from destroying βcatenin. βcatenin then accumulates and translocates into the nucleus where it forms a complex with other proteins to regulate transcription of Wnt target genes (Fig. 2A) (Moon *et al.*, 2004). Noncanonical Wnt signaling refers to a number of pathways that are independent of βcatenin.

It is now clear that vertebrate gastrulation is regulated by a Wnt-signaling pathway that acts through components similar to those found in the *Drosophila* Frizzled/Planar Cell Polarity pathway (Fz/PCP) (Fig. 2B) (Tada *et al.*, 2002). Fz/PCP signaling is responsible for establishing cell polarity

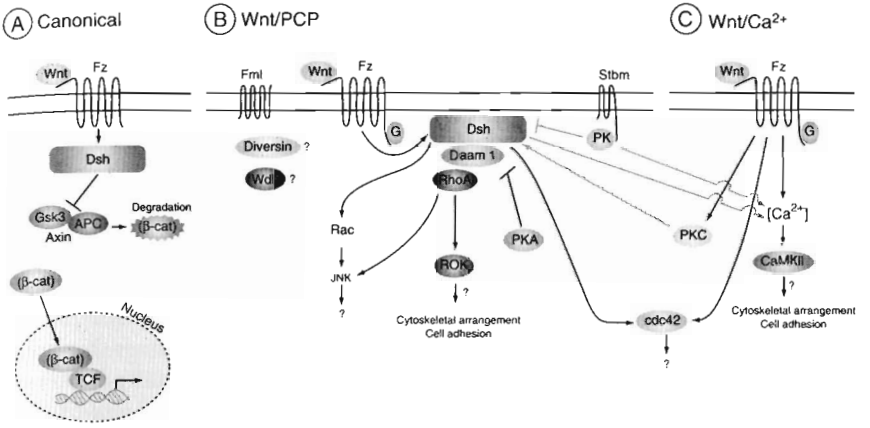

FIG. 2 Vertebrate canonical Wnt signaling pathway (A). Vertebrate noncanonical Wnt pathways, Wnt/PCP (B), and Wnt/Ca^{++}(C). (See also color insert.)

within the epithelial plane of many *Drosophila* tissues. For example, Fz/PCP regulates the actin cytoskeleton in the wing disc to produce a single wing hair from the distal end of each cell. Disruptions to Fz/PCP signaling in this case cause disorientation of hair growth (Fanto and McNeill, 2004). PCP components shared between vertebrates and *Drosophila* thus far include the transmembrane proteins, Frizzled (Fz), Strabismus, and Flamingo, as well as the intracellular proteins Dsh, Prickle, and Diego (Fanto and McNeill, 2004). Although Wnt is clearly activating the PCP pathway in vertebrates (Wnt/PCP), the identity of the polarity signal activating PCP in *Drosophila* remains unknown.

Even though work in *Drosophila* has provided insight into how PCP components interact to polarize a static epithelium, it is still unclear how they act to regulate the polarized cell movements of vertebrate gastrulation. In *Drosophila,* cell polarization within an epithelium depends on the subcellular, asymmetric accumulation of Fz and other core components. In cells of the wing disc, for example, Fz, Dsh, and Diego accumulate at the distal cell membrane, where the hair later forms, and Strabismus and Prickle accumulate on the proximal side (Strutt and Strutt, 2005). It has also been shown that these components are dependent on each other for correct asymmetric localization (reviewed in Klein and Mlodzik, 2005). Such asymmetric distribution of Fz/PCP components may be a common mechanism in establishing cell polarity. For instance, it has been reported in Caenorhabditis *(C.) elegans* that Fz is localized asymmetrically at the leading edge of migrating epithelial cells (Park *et al.*, 2004). In vertebrates, however, no obvious

asymmetry has been reported during gastrulation despite Fz and other components localizing to the cell membrane (Park and Moon, 2002).

Although Wnt/PCP signaling does play a pivotal role in polarized cell movement during vertebrate gastrulation, it may not be an instructive role. Take for example the phenotype of *silberblick/wnt11(slb)* mutant embryos. The directed cell movements of the prechordal plate progenitors toward the animal pole are defective in that they migrate slower and with less persistence, often straying from the normal path (Heisenberg *et al.*, 1996, 2000; Ulrich *et al.*, 2003, 2005). In addition, progenitors at the leading edge of the prechordal plate, which normally extend cellular pseudopod-like processes in relation to their individual movement direction, display random process orientation in *slb* embryos (Ulrich *et al.*, 2003). The progenitors, however, do maintain an overall movement direction toward the animal pole. These results suggest that Wnt11 signaling might be facilitating polarized movement by stabilizing movement direction, rather than serving as a major directional cue.

The receptor for Wnt11 is thought to be Fz7 (Djiane *et al.*, 2000; Kilian *et al.*, 2003). Zebrafish has two Fz7 paralogues, Fz7a (El-Messaoudi and Renucci, 2001; Sumanas *et al.*, 2002) and Fz7b (Sumanas *et al.*, 2002; Ungar and Calvey, 2002), with Fz7b being expressed at the right time and place to be involved in early CE events. In *Xenopus,* Wnt11 can directly bind Fz7, and gain or loss of Fz7 function disrupts cell movements similarly to Wnt11 (Djiane *et al.*, 2000; Sumanas and Ekker, 2001). Interestingly, work in *Xenopus* has revealed that independent of Wnt11, Fz7 is also required for the separation of mesoderm from ectoderm, a process analogous to hypoblast/epiblast separation during zebrafish gastrulation (Winklbauer *et al.*, 2001).

Downstream of Fz, Dsh activity is essential for Wnt/PCP signaling (Axelrod *et al.*, 1998; Heisenberg *et al.*, 2000; Tada and Smith, 2000; Wallingford *et al.*, 2000). The role of Dsh in noncanonical signaling has noted differences from canonical signaling, including downstream targets and required Dsh protein domains (Axelrod, 2001; Axelrod *et al.*, 1998; Boutros *et al.*, 1998; Heisenberg *et al.*, 2000; Tada and Smith, 2000; Wallingford and Habas, 2005; Wallingford *et al.*, 2000). Most notably, Dsh localizes to the membrane during noncanonical signaling, whereas it remains cytoplasmic during canonical signaling (Axelrod *et al.*, 1998; Wallingford *et al.*, 2000). It has been shown in *Xenopus* that Dsh membrane localization is crucial for activation of PCP downstream targets, as well as for CE movements (Habas *et al.*, 2003; Park *et al.*, 2005). Interestingly, Fz overexpression is sufficient to recruit Dsh to the membrane in *Drosophila* as well as in zebrafish and *Xenopus*, suggesting that an increase in Fz density at the membrane may normally initiate the signaling cascade (Axelrod *et al.*, 1998; Carreira-Barbosa *et al.*, 2003; Medina and Steinbeisser, 2000; Rothbacher *et al.*, 2000; Veeman *et al.*, 2003b; Yang-Snyder *et al.*, 1996).

Similar to *Drosophila*, Fz-Dsh activity during vertebrate gastrulation appears to be modulated by Prickle, Strabismus, and the Diego-related protein, Diversin (Carreira-Barbosa *et al.*, 2003; Darken *et al.*, 2002; Hammerschmidt *et al.*, 1996; Jessen *et al.*, 2002; Park and Moon, 2002; Schwarz-Romond *et al.*, 2002; Sepich *et al.*, 2000; Takeuchi *et al.*, 2003; Veeman *et al.*, 2003b; Wallingford *et al.*, 2002). In *Drosophila*, Prickle binds Dsh and acts to inhibit it from localizing to the membrane (Tree *et al.*, 2002). This is also the case in zebrafish, as Prickle overexpression prevents Dsh from localizing to the membrane in response to Fz overexpression (Carreira-Barbosa *et al.*, 2003). Conflicting results are seen in *Xenopus* where Prickle has no effect on Dsh localization (Veeman *et al.*, 2003b; Wallingford *et al.*, 2002b). In *Drosophila,* Prickle competes with Diego, a promoter of Fz-Dsh activity, for Dsh binding (Das *et al.*, 2004; Feiguin *et al.*, 2001; Jenny *et al.*, 2005). It has been proposed that the related vertebrate protein, Diversin, also promotes Wnt/PCP signaling (Schwarz-Romond *et al.*, 2002). *Drosophila* Prickle and Dsh can both be bound by Strabismus, which recruits them to the membrane (Bastock *et al.*, 2003; Jenny *et al.*, 2003). Data from zebrafish support a genetic interaction between Prickle and Strabismus during Wnt/PCP regulation of CE (Carreira-Barbosa *et al.*, 2003).

The subcellular restriction of Fz-Dsh activity, which is obvious during polarization of *Drosophila* epithelia, may also be important for Wnt/PCP regulation of gastrulation. Research suggests the existence of distinct subcellular sites of Wnt/Fz/Dsh activity at the plasma membrane of gastrulating cells. Most importantly, these sites appear to locally enhance cell-cell adhesion (Witzel *et al.*, 2006). It could be the case that these areas of Fz/Dsh activity are reorganized throughout gastrulation to coordinate movement within groups of cells like the prechordal plate progenitors. Future experiments will tell whether this activity is restricted in a polarized fashion.

Another core component of PCP signaling is Flamingo, a 7-pass transmembrane protein that has a cadherin-like extracellular domain that can bind Flamingo molecules on neighboring cells (Chae *et al.*, 1999; Usui *et al.*, 1999). In *Drosophila,* Flamingo localizes to proximal and distal sides of wing epithelia cells where it appears to both promote and inhibit Fz-Dsh activity. In zebrafish, loss of function of multiple *flamingo*-related genes indicates that they act in combination to regulate CE (Formstone and Mason, 2005). Furthermore, mice lacking the *flamingo* homolog *celsr1* show CE-type defects during neural tube closure (Curtin *et al.*, 2003). How Flamingo is functioning during gastrulation is an open question. Interestingly, Flamingo is essential for the local enhancement of cell–cell adhesion that has been associated with the subcellular regions of Wnt/FZ/Dsh activity described previously (Witzel *et al.*, 2006).

Shared components between vertebrate and *Drosophila* PCP pathways go beyond the core components. Widerborst is a regulatory subunit of PP2A, a

regulator of both *Drosophila* PCP and vertebrate CE. In wing epithelia, Widerborst polarizes independently of core PCP components but is necessary for polarized distribution of Fz and Flamingo. Activation of PP2A via Widerborst appears to be necessary for the maintenance of a planar web of microtubules that could be required for directed vesicular transport of Fz and other components (Hannus *et al.*, 2002; Shimada *et al.*, 2006).

It is well known that Rho GTPases are key regulators of processes that are essential for cell polarization and movement, such as cytoskeletal organization, cell adhesion, and gene transcription (Hall, 2005; Montero and Heisenberg, 2004). It is little wonder then that the small GTPases, Rho, Rac, and Cdc42 are downstream components in the PCP cascade. Rho and its effectors, Rho kinase (Rok/Rock) and Diaphanous, are demonstrated mediators of both *Drosophila* and vertebrate PCP signaling, acting to regulate myosin and the actin cytoskeleton (Eaton *et al.*, 1995; Fanto *et al.*, 2000; Habas *et al.*, 2001, 2003; Kim and Han, 2005; Lai *et al.*, 2005; Marlow *et al.*, 2002; Ren *et al.*, 2006; Strutt *et al.*, 1997; Winter *et al.*, 2001; Wunnenberg-Stapleton *et al.*, 1999; Zhu *et al.*, 2006). Consistent with such a role, injection of a Rho antagonist in zebrafish embryos interferes with actin distribution within the cleavage furrow of early blastomeres (Lai *et al.*, 2005). Strikingly, overexpression of either RhoA or its effector Rok and mDiaphanous in zebrafish is able to rescue CE defects in embryos lacking Wnt11 or Wnt5 function, indicating a major role for RhoA in Wnt/PCP activity (Marlow *et al.*, 2002; Zhu *et al.*, 2006). PCP activation of RhoA has been show in *Xenopus* to require a novel formin homology protein Daam1 that can form a Wnt-induced complex with both Dsh and RhoA (Habas *et al.*, 2001). *Xenopus* Net1, a RhoA-specific guanine exchange factor that physically associates with Dsh, may also facilitate RhoA activation during PCP signaling (Miyakoshi *et al.*, 2004).

Rac appears to act in parallel to RhoA downstream of Dsh during PCP signaling and can form complexes with Dsh independently of Daam1 (Fanto *et al.*, 2000; Habas *et al.*, 2001, 2003; Tahinci and Symes, 2003). Rac activates the Jun N-terminal kinase (JNK) module, a known target of PCP signaling in both vertebrates and *Drosophila* (Boutros *et al.*, 1998; Fanto *et al.*, 2000; Habas *et al.*, 2003; Hammerschmidt *et al.*, 1996; Park and Moon, 2002; Strutt *et al.*, 1997; Weber *et al.*, 2000; Yamanaka *et al.*, 2002). Alterations in Rac or JNK activity disrupt *Xenopus* gastrulation and polarized cell movements (Habas *et al.*, 2003; Kim and Han, 2005; Ren *et al.*, 2006; Tahinci and Symes, 2003; Yamanaka *et al.*, 2002). RhoA has also been suggested to activate JNK independent of Rok activation (Boutros *et al.*, 1998; Fanto *et al.*, 2000; Kim and Han, 2005; Strutt *et al.*, 1997). In the context of *Drosophila* PCP, JNK has been proposed to regulate transcription; however, in vertebrates its function is unknown (Fanto *et al.*, 2000; Weber *et al.*, 2000).

Protein kinase-A (PKA) has been shown to interfere with Wnt/PCP signaling (Park *et al.*, 2006). Activation of PKA inhibits PCP signaling, whereas inhibition of PKA can rescue loss-of-PCP activity. PKA likely interacts with the PCP signaling pathway by inhibiting the formation of a Daam1-Dsh-RhoA protein complex required for RhoA activation. PKA also functions downstream of heterotrimeric G-proteins, critical components of the Wnt/Ca^{2+} signaling pathway (Ahumada *et al.*, 2002; Slusarski *et al.*, 1997a). This suggestion that the Wnt/PCP and Wnt/Ca^{2+} pathways interact is explored further later in this chapter.

2. Wnt/Calcium Pathway

Noncanonical Wnt signaling via calcium also occurs during embryonic development (Fig. 2C; Kuhl *et al.*, 2000). This has been demonstrated by a number of experiments in which the overexpression of Wnt and Fz molecules known to act in noncanonical signaling increases intracellular Ca^{2+} release and activates Calcium/Calmodulin-dependent kinase II (CamKII) and PKC (Kuhl *et al.*, 2000; Sheldahl *et al.*, 2003; Slusarski *et al.*, 1997a,b; Westfall *et al.*, 2003). For example, in zebrafish embryos, overexpression of *Xenopus* Wnt5a, Wnt11, or Rat Frizzled 2 (Rfz2) induces an increase in the frequency of intracellular Ca^{2+} release (Ahumada *et al.*, 2002; Sheldahl *et al.*, 1999; Slusarski and Corces, 2000; Slusarski *et al.*, 1997a,b; Westfall *et al.*, 2003). This is in contrast to Wnt and Fz molecules that are solely associated with canonical/β-catenin signaling, such as Wnt8, which fail to generate such a response (Sheldahl *et al.*, 1999; Slusarski *et al.*, 1997b).

Downstream mediators of Wnt/Ca2+ signaling include heterotrimeric G-proteins which are most likely coupled to and directly activated by Fz receptors (Wang *et al.*, 2006). Blocking the activity of heterotrimeric G-proteins or another downstream component, Phosphodiesterase, interferes with the induction of intracellular Ca^{2+} release by noncanonical Wnts (Ahumada *et al.*, 2002; Slusarski *et al.*, 1997a,b). Release of intracellular Ca^{2+} has been postulated to activate Ca^{2+}-sensitive enzymes such as PKC and CAMKII, which in turn control both cell adhesion and movement during gastrulation (Kuhl *et al.*, 2000; Sheldahl *et al.*, 1999, 2003). Evidence for PKC as a downstream mediator of Wnt/Ca^{2+} signaling during gastrulation comes from studies in *Xenopus* that show blocking PKCγ activity disrupts CE movements (Kinoshita *et al.*, 2003). Conversely, PKCα activation rescues tissue separation defects caused by loss of Fz7 function (Winklbauer *et al.*, 2001). Cdc42 is also thought to function as a downstream effector because blocking Cdc42 activity rescues CE defects caused by the overactivation of Wnt/Ca^{2+} signaling (Choi and Han, 2002; Winklbauer *et al.*, 2001).

Increasing evidence suggests noncanonical Wnt/Ca^{2+} and Wnt/PCP pathways functionally interact during gastrulation. PKCγ activated by Wnt/Ca^{2+}

signaling has been shown to interact with Dsh, leading to Dsh phosphorylation and plasma membrane translocation (Kinoshita *et al.*, 2003). This translocation, a required step in noncanonical signaling, is inhibited when PKCγ function is absent (Kinoshita *et al.*, 2003). Dsh in turn has been shown to trigger intracellular Ca^{2+} release and activate PKC and CamKII, presumably by signaling through the PCP pathway (Sheldahl *et al.*, 2003). Further evidence for a Wnt/Ca^{2+} and Wnt/PCP interaction comes from studies in *Xenopus*, showing that Prickle, a PCP component, induces intracellular Ca^{2+} release in gastrulating cells (Veeman *et al.*, 2003b).

Overall, the Wnt/Ca^{2+} pathway appears to play a critical role in regulating cell movement and adhesion during gastrulation. Future work is needed to elucidate the degree of interaction between the Wnt/Ca^{2+} pathway and other noncanonical Wnt pathways. The role played by such communication during gastrulation is bound to be interesting.

3. Downstream Effector Mechanisms of Noncanonical Wnt Signaling

The cytoskeleton is a probable target of noncanonical Wnt signaling during gastrulation (Veeman *et al.*, 2003b). Direct control of the cytoskeletal rearrangement is suggested by evidence in *Xenopus* and zebrafish that noncanonical Wnt signaling activates RhoA effector proteins and known actin modulators such as Rok and Diaphanous (Habas *et al.*, 2001; Marlow *et al.*, 2002). This notion is further supported by findings that loss of Wnt signaling results in the defective orientation of actin-rich cellular protrusions in mesenchymal and migratory cells and reduced apical localization of actin in ciliated epithelial cells (Jessen *et al.*, 2002; Park *et al.*, 2006; Ulrich *et al.*, 2003). Interestingly, this Wnt-dependent actin localization appears to be required for the proper organization of cilia microtubules. This indicates an ability of Wnt signaling to control microtubule organization via its effect on actin localization (Park *et al.*, 2006).

Studies both *in vitro* and *in vivo* have shown noncanonical Wnt signaling also plays an important role in modulating cell adhesion (Solnica-Krezel, 2006). Both gain-of-function and loss-of-function of Wnt signaling interferes with the ability of primary cultures of gastrulating cells to form coherent cell assemblies and to efficiently adhere to substrates coated with E-cadherin and the extracellular matrix component Fibronectin (Puech *et al.*, 2005; Torres *et al.*, 1996; Ulrich *et al.*, 2005). Defects in cell adhesion are also observed in response to loss of Fz7 function. In *Xenopus* lacking Fz7, for example, the germ layers fail to properly separate at the onset of gastrulation (Winklbauer *et al.*, 2001). These results in combination indicate noncanonical Wnt signaling modulates both cell-cell and cell-matrix adhesion. Although the molecular mechanisms by which noncanonical Wnts modulate cell adhesion are not

yet fully understood, observations in both zebrafish and *Drosophila* indicate Wnts control cell adhesion by regulating the subcellular localization and/or turnover of Cadherin molecules (Classen *et al.*, 2005; Ulrich *et al.*, 2005; Wodarz *et al.*, 2006). Future studies will have to identify downstream effector molecules of noncanonical Wnt signaling in this process.

B. Other Signaling Pathways

1. PDGF-PI3K Pathway

Localized activation of Phosphoinositide 3-kinase (PI3K) at the leading edge of single cells such as *Dictyostelium* and leukocytes is crucial to directional sensing and cell polarization during chemotaxis (Merlot and Firtel, 2003). It also appears to be important for vertebrate gastrulation cell movements (Ataliotis *et al.*, 1995; Ghil and Chung, 1999; Montero *et al.*, 2003; Nagel *et al.*, 2004; Symes and Mercola, 1996). PI3K can regulate movement direction by activating small GTPases and catalyzing the conversion of phosphoinositide-4,5-diphosphate (PI(4,5)P2) to phosphoinositide-(3,4,5)-triphosphate (PIP3), which can then bind Pleckstrin Homology (PH)-domain–containing proteins such as Protein kinase B (PKB). The local activation of GTPases and PH-domain proteins at the leading edge is thought to subsequently regulate cytoskeletal dynamics that contribute to the formation of polarized protrusions (Merlot and Firtel, 2003; Wymann *et al.*, 2003).

PI3K activity during gastrulation is regulated by Platelet Derived Growth Factor (PDGF) (Ataliotis *et al.*, 1995; Ghil and Chung, 1999; Montero *et al.*, 2003; Nagel *et al.*, 2004; Symes and Mercola, 1996). PDGF is a secreted signaling molecule known to regulate many different processes such as cell proliferation, migration, and tissue remodeling (Hoch and Soriano, 2003). For example, during gastrulation in *Xenopus,* mesodermal cells expressing PDGF receptors (PDGFR) undergo directed migration across the blastocoele roof which expresses PDGFA (Ataliotis *et al.*, 1995). Loss of PDGFA function in the blastocoele roof results in disoriented mesodermal migration and reduced cellular protrusions (Nagel *et al.*, 2004). Likewise, use of dominant negative PDGFR to affect signaling also disrupts mesodermal migration (Ataliotis *et al.*, 1995; Nagel *et al.*, 2004). Thus PDGF appears to serve as an instructive cue for directed migration during *Xenopus* gastrulation.

PDGF has also been shown to play a role during the anterior-ward migration of prechordal plate cells in the zebrafish. It this situation, however, it is unclear whether PDGF is acting as a guidance cue like it is in the *Xenopus*. Montero *et al.* (2003) has shown that PDGF-PI3K activity is required for the formation of polarized cell processes in prechordal plate

progenitors and localization of PKB at the leading edge of these cells. Despite the obvious loss of cell polarization and reduced speed however, the overall movement direction remains correct. Zebrafish PDGF and PDGFR are expressed ubiquitously in the embryo (Liu *et al.*, 2002a,b), and it remains to be seen whether the protein is localized to a particular region or tissue.

2. Eph-Ephrin Signaling

Signaling through Eph transmembrane receptor tyrosine kinases plays a role during many morphogenetic events such as axonal guidance, cell migration, boundary formation, and angiogenesis. Ephs are activated by binding Ephrins, extracellular proteins tethered to the cell membrane through a GPI anchor (EphrinAs) or a transmembrane domain (EphrinsBs). Eph activation therefore depends on cell–cell contact (Poliakov *et al.*, 2004). After binding, the Eph-Ephrin complexes can cluster, undergo tyrosine phosphorylation, and interact with cytoplasmic proteins (Holder and Klein, 1999; Pasquale, 2005).

Proper cell movement during gastrulation relies on Eph-Ephrin signaling (Chan *et al.*, 2001; Jones *et al.*, 1998; Oates *et al.*, 1999a). In zebrafish, soluble Eph-A3 and Ephrin-A5, which act as dominant negative inhibitors of signaling, result in somite, brain, and notochord defects consistent with disruption of convergence and extension movements (Oates *et al.*, 1999a). A similar approach has also shown that disruption of Eph-Ephrin B signaling in zebrafish impairs gastrulation movements without affecting cell fate specification (Chan *et al.*, 2001). The mechanisms by which Eph-Ephrin signaling regulates gastrulation movements are unclear. Considering its role in other events, it is possible this signaling regulates cell adhesion or cytoskeletal dynamics in the gastrula to mediate repulsion or attraction between cells or with the extracellular matrix (ECM) (Murai and Pasquale, 2005; Pasquale, 2005).

3. Jak/Stat Signaling

Jak/Stat (Janus kinase/signal transducer and activator of transcription) signaling has been implicated in a variety of processes including cell polarization, cell motility, proliferation, and cell fate specification (Hou *et al.*, 2002). Jaks associate with receptors for various cytokine and growth factors. As a consequence of the receptors binding ligand and multimerizing, the associated Jaks are activated via transphosphorylation. Jaks then phosphorylate targets such as Stats, which dimerize and enter the nucleus where they act as transcription factors to regulate gene expression (Rawlings *et al.*, 2004).

Jak/Stat signaling appears to play a role in regulating cell movements during gastrulation (Conway *et al.*, 1997; Miyagi *et al.*, 2004; Yamashita

et al., 2002, 2004). Potential Jaks that may activate Stat3 include Jak1 and Jak2b which are expressed at the right time during zebrafish gastrulation (Conway *et al.*, 1997; Oates *et al.*, 1999c). Dominant-negative Jak1 kinase expressed in zebrafish embryos results in slowed epiboly and a shortened, broad axis characteristic of gastrulation defects (Conway *et al.*, 1997). Activation of Stat3 likewise appears to be required during zebrafish gastrulation as Stat3 loss-of-function disrupts CE, as well as anterior migration of the prechordal plate mesoderm (Yamashita *et al.*, 2002). Despite the ubiquitous expression of Stat3 during gastrulation (Oates *et al.*, 1999b), phosphorylation and subsequent localization of Stat3 to the nucleus occurs only within dorsal mesoderm, including the prechordal plate progenitors. This activation, which can be detected prior to the onset of gastrulation, is dependent on Wnt/β-catenin signaling. Transplantation experiments have shown that the anterior migration of prechordal plate progenitors cell-autonomously requires functional Stat3 (Yamashita *et al.*, 2002). Although it remains unclear how Stat3 regulates migration in these cells, LIV1, a ZIP zinc transporter, may be an important downstream target. LIV1 has been speculated to function downstream of Stat3 by regulating the nuclear localization of Snail, a known regulator of epithelial to mesodermal transition (Yamashita *et al.*, 2004). Stat3 might therefore affect prechordal plate migration by influencing the mesenchymal character of the cells.

Stat3 activity in the prechordal plate also has a cell-nonautonomous role in regulating the convergence of the paraxial mesoderm (Yamashita *et al.*, 2002). It is possible Stat3 is upstream of a signal that produces a gradient sensed by the paraxial mesoderm. Interestingly, Wnt/PCP signaling has been proposed to function downstream of Stat3 in this role. Dsh overexpression can rescue the elongation of paraxial cells in Stat3 loss-of-function embryos (Miyagi *et al.*, 2004). However, the orientation of these cells remains disrupted, suggesting the directional component is not supplied through Wnt/PCP. Future studies are needed to identity the directional signals regulated by Stat3.

4. Slit-Robo Signaling

In zebrafish, the secreted Slit molecule and its receptor Roundabout (Robo) are involved in regulating convergence extension movements (Yeo *et al.*, 2001). Slit and Robo homologs in vertebrates and *Drosophila* are best known for their part in axon guidance. Slits expressed at the midline of the nervous system serve to repel axons of *robo*-expressing cells, preventing them from crossing the midline (Hammond *et al.*, 2005; Kidd *et al.*, 1998a,b; Mambetisaeva *et al.*, 2005). Other cell migration events also rely on Slit and Robo. For example in chick, Slit expression in the dermomyotome is suggested to guide the migration of *robo*-expressing neural crest cells

(Jia *et al.*, 2005). Additionally, Slit and Robo appear to regulate cell polarity and coordinate migration during *Drosophila* heart morphogenesis (Macmullin and Jacobs, 2006; Qian *et al.*, 2005).

The expression pattern of *slit* and *robo* in the gastrulating zebrafish suggests that Slit-Robo interactions are important for organizing domains of cell movement. *slit* expression is restricted to the axial mesoderm during gastrulation, whereas *robos* are expressed in the entire embryo (Challa *et al.*, 2001; Lee *et al.*, 2001). Although evidence is still lacking, Slits released at the midline may control the migration or protrusiveness of *robo*-expressing cells to prevent them from crossing the midline region. In accordance with such a role, global overexpression of *slit2* in zebrafish embryos results in impaired prechordal plate migration and convergence extension defects (Yeo *et al.*, 2001). In the future it will be interesting to determine targets of Slit-Robo signaling during gastrulation, as well as the particular cell-cell interactions affected.

C. Extracellular Matrix and Fibronectin

The Extracellular Matrix (ECM) plays an important role in the regulation of a wide range of cell behaviors such as adhesion, proliferation, differentiation, and migration. It does this through direct cell-ECM interactions, as well as by harboring growth and differentiation factors (Rosso *et al.*, 2004) A major component of the ECM is Fibronectin (FN), which is assembled into a fibrillar network in response to interactions with adjacent cells. Fibrils form when FN binds to Integrin receptors, frequently Integrin $\alpha5\beta1$, causing receptor clustering at the cell membrane and increased FN-FN interactions. The subsequent contraction of the cellular actin cytoskeleton, which is anchored to Integrin, along with conformational changes in FN, also contributes to fibril assembly (Mao and Schwarzbauer, 2005).

In *Xenopus*, FN and Integrin $\alpha5\beta1$ are involved in mesoderm migration and CE during gastrulation (Davidson *et al.*, 2002, 2006, Goto *et al.*, 2005; Howard *et al.*, 1992; Marsden and DeSimone, 2001, 2003; Winklbauer and Keller, 1996). Throughout early *Xenopus* development, FN-ECM is deposited at tissue boundaries and is remodeled in a dynamic fashion (Davidson *et al.*, 2004; Nakatsuji *et al.*, 1985). The FN matrix across the blastocoele roof is particularly essential for the migration of mesodermal cells. Disruption of this FN matrix results in the loss of polarized cell protrusions and mesodermal-spreading defects (Davidson *et al.*, 2006; Winklbauer and Keller, 1996). Similarly, when Integrin-FN interactions are blocked, mesodermal cells exhibit disoriented protrusions, reduced radial cell intercalation, and aberrant CE (Davidson *et al.*, 2006; Marsden and DeSimone, 2001, 2003).

Wnt/PCP signaling has been proposed to be involved with FN-ECM interaction in mesodermal cells. Proper binding of Integrins to FN at early stages of *Xenopus* gastrulation is needed for the localization of Dsh to the plasma membrane, suggesting that Wnt/PCP signaling depends on an Integrin-FN interaction (Marsden and DeSimone, 2001). Conversely, perturbing the expression of the Wnt/PCP signaling components Strabismus, Fz, and Prickle during *Xenopus* gastrulation affects polarized FN fibril assembly and, in the case of Fz or Strabismus, the ability of mesodermal cells to move in a polarized way on FN substrates (Goto *et al.*, 2005).

Integrin-ECM interaction has also been suggested to modulate cadherin-mediated cell-cell adhesion. Prevention of Integrin-FN binding can perturb C-cadherin–mediated mesodermal cell sorting as well as medio-lateral cell intercalation and axial extension (Marsden and DeSimone, 2003). Although this suggests that an Integrin-ECM interaction modulates C-cadherin activity in the gastrula, the underlying molecular mechanisms wait to be elucidated.

In zebrafish, mutants in *fibronectin-1 (natter)* display defects in epithelial organization and myocardial progenitor cell migration (Trinh and Stainier, 2004). A role for FN in the directed migration and polarization of these progenitors is likely and is suggested to be regulated by signals from the underlying YSL (Sakaguchi *et al.*, 2006; Trinh and Stainier, 2004). Moreover, maternal-zygotic *natter* mutants exhibit severe gastrulation defects, suggesting FN is also important early in development (Trinh and Stainier, 2004).

In sum, the evidence so far shows the importance of polarized deposition of ECM, including FN, for cell polarization and directed migration in the *Xenopus* and zebrafish gastrula. Future studies are needed to determine upstream regulators and downstream effectors of ECM function during gastrulation.

IV. Tissue Interactions

The precise coordination of cellular rearrangements and tissue interactions is central to the control of morphogenetic events. An important means of achieving this is through the physical linkage of different tissues via adhesion and extracellular matrix molecules. During zebrafish gastrulation, it is clear that the forming tissue layers physically interact, but how these interactions are regulated and to what degree they control morphogenesis remain open questions.

Mesendodermal progenitors (hypoblast) in the zebrafish physically interact with the overlying ectodermal layer (epiblast) as they ingress and undergo

CE movements (Montero *et al.*, 2005). Intriguingly, as the epiblast cells epibolize toward the vegetal pole of the gastrula, hypoblast cells migrate toward the animal pole. These tissues thus move on top of each other in opposite directions. Ultrastructural analysis of the interface between epiblast and hypoblast indicates that cells from both layers are not separated by basal lamina (Montero *et al.*, 2005). This direct cellular contact at the interface is likely to be very dynamic as the cells must rapidly adhere and deadhere to migrate. How this dynamic interaction is regulated at a molecular level is as yet unknown. It is conceivable, given the reorganization of cell adhesion that likely occurs, that intracellular adhesion molecule trafficking plays an important role. Consistent with this, blocking early endocytosis in mesendodermal progenitor cells reduces migratory activity during gastrulation (Ulrich *et al.*, 2005).

Despite clear indications that dynamic epiblast–hypoblast interactions occur, they have not yet been proven essential for morphogenesis. Evidence suggests that epiblast and hypoblast layers can move independently. For instance, in maternal-zygotic *one-eyed pinhead (mz-oep)* mutant embryos, which lack hypoblast cells, epiblast cells still epibolize properly (Hammerschmidt *et al.*, 1996). This reveals that interaction with the underlying hypoblast is dispensable for this particular epiblast cell movement. Conversely, in *weg* mutant embryos, which have severely impaired epiblast epiboly, CE of hypoblast cells is only mildly affected, indicating that epiblast epiboly is not essential (Kane *et al.*, 1996). In general, detailed study is necessary to determine the particular aspects of epiblast and hypoblast morphogenesis that depend on interactions between the tissues.

One tissue–tissue interaction that appears to be important for the coordination of zebrafish epiboly is the linkage of the EVL to the YSL (Fig. 3). Cells at the leading edge of the EVL appear to establish tight junctions with the YSL. At the same time, Actin accumulates within the YSL at the contact site (Cheng *et al.*, 2004; Koppen *et al.*, 2006; Zalik *et al.*, 1999). As mentioned in the introduction, this accumulation of Actin along the gastrula equator may serve as a "purse string." As the purse string tightens, it could presumably pull both the YSL and the EVL toward the vegetal pole (Fig. 3). This potential coordination of EVL-YSL morphogenesis by an Actin purse string requires at least two conditions: (1) the Actin must assemble after the EVL has reached the equator of the underlying yolk cell, otherwise a purse string-like contraction would drive closure toward the animal pole instead of the vegetal pole; and (2) the EVL must be tightly linked to the Actin purse string so that the contractile force within the YSL can be directly transmitted. Although these conditions are fulfilled during gastrulation, direct molecular and biophysical evidence for a purse-string mechanism remains, for the moment, elusive. Experiments have demonstrated that a YSL-specific loss of the Ste20-like kinase *misshapen,* impairs Actin and Myosin recruitment

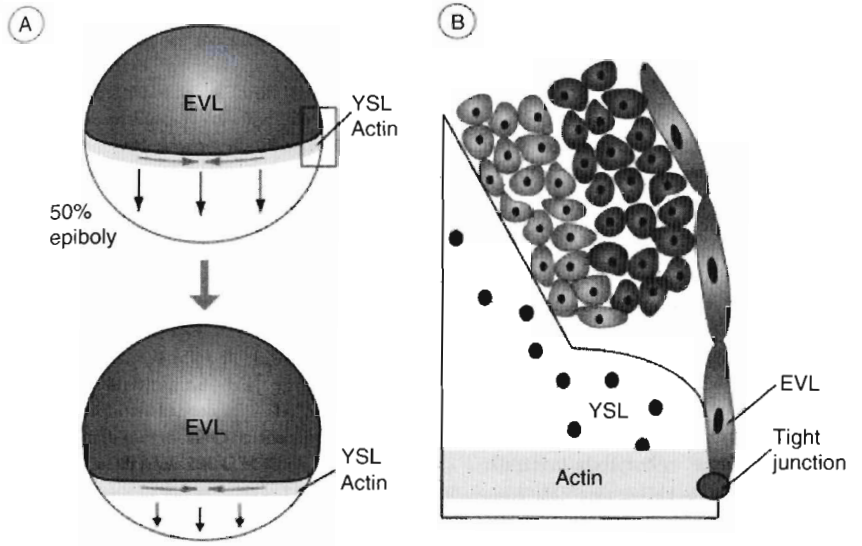

FIG. 3 Model of Actin purse string-driven EVL/YSL epiboly. Contraction of the Actin purse string drives EVL/YSL epiboly toward the vegetal pole (A). EVL anchored to YSL via tight junctions to the YSL (B). (See also color insert.)

within the YSL, and results in both disrupted EVL cell constriction and defective YSL and EVL epiboly (Koppen *et al.*, 2006). Importantly, this reveals that the force-generating process controlling EVL epiboly likely resides within the YSL. Additional contributions to YSL coordination of EVL constriction and epiboly, such as endocytosis of YSL plasma membrane at the EVL–YSL border, are equally possible (Cheng *et al.*, 2004).

V. Differential Adhesion

Differential cell adhesion has long been suggested to drive various cell populations to rearrange relative to each other (Steinberg, 1996). This differential adhesion hypothesis, introduced by M. Steinberg over 40 years ago, proposes that differences in intercellular adhesions create *tissue interfacial free energies* that trigger the sorting out of heterotypic cell mixtures. Upon sorting out, it is postulated that tissues with low intercellular adhesion spread over tissue with higher intercellular adhesion. Ultimately, these cellular rearrangements determine the configuration of tissues.

Increasing evidence indicates that the different germ layer progenitors in the zebrafish gastrula display distinct adhesive properties that might drive

separation and morphogenesis. Adhesion molecules known to regulate cell–cell adhesion, such as E-cadherin, show different expression levels in the ectoderm and mesendoderm (Montero *et al.*, 2005). Importantly, mixtures of ectodermal and mesendodermal progenitors in culture efficiently sort into distinct clusters, with the mesendoderm eventually surrounding the ectoderm (Montero *et al.*, 2005; Y. Arboleda, M. Krieg, and C. P. Heisenberg, unpublished observations). This tissue configuration suggests that ectodermal intercellular adhesion is higher than that within mesentoderm. Consistent with this, quantitative measurements of single-cell adhesion using atomic force microscopy show that the deadhesion forces needed to separate ectodermal progenitors is significantly higher than that needed to separate mesentodermal progenitors (Puech *et al.*, 2005; M. Krieg and C. P. Heisenberg, unpublished observations).

How relevant are these *in vitro* observations to the actual cellular rearrangements in the zebrafish gastrula? One set of cell behaviors that could be driven by germ-layer specific changes in adhesion is the internalization and subsequent migration of mesendodermal progenitors. This idea is supported by work in mutants that lack mesendodermal cell specification. In these embryos, cells still ingress at the germ ring, but fail to deadhere from surrounding cells and are unable to move toward the animal pole, indicating the need for the proper regulation of adhesive properties (Carmany-Rampey and Schier, 2001). Additionally, when mesendodermal cell migration is impaired by inhibition of PI3K activity, ingression and movement toward the animal pole still occurs. Combined, these studies suggest migratory activity alone is not responsible for all mesendodermal rearrangement (Montero *et al.*, 2003).

Although the previous observations highlight a potential role for differential adhesion in germ-layer formation and morphogenesis, direct experimental evidence is missing. One crucial future experiment will be to systematically identify and characterize adhesion molecules that create differential adhesion in the gastrula. This may be a challenge as the inhibition of individual adhesion molecules appears insufficient to disturb germ-layer morphogenesis. For example, despite the elevated E-cadherin levels found in internalized mesendodermal cells, E-cadherin is not critical for germ-layer separation and formation (Kane *et al.*, 1996a, 2005; Montero *et al.*, 2005). Similarly, mutants of other adhesion molecules expressed at gastrulation, such as N-cadherin *(parachute)* and Fibronectin-1 *(natter)*, do not exhibit obvious defects (Jiang *et al.*, 1996; Lele *et al.*, 2002; Trinh and Stainier, 2004). The potential functional redundancy between adhesion components must therefore be overcome to directly test the role of differential adhesion during germ-layer formation and morphogenesis.

What is controlling the differential expression and activity of adhesion molecules in the germ layers? Multiple possibilities exist, and all may play a role. First, within a specific germ layer, adhesion molecules might be

upregulated or downregulated at the transcriptional or translational level. Second, the subcellular localization, trafficking, and degradation of adhesion molecules might be differentially controlled. Lastly, differences in cytoskeletal architecture might impact the anchoring and activity of adhesion molecules. Although evidence implicating any of these processes in differential germ-layer adhesion is sparse, there are some hints. Noncanonical Wnt signaling has been proposed to regulate mesendodermal cell cohesion by controlling E-cadherin trafficking (Ulrich *et al.*, 2005). However, it remains unclear whether this effect is restricted to mesendodermal progenitors, as would be expected in the case of differential adhesion. Finally, TGFβ-like Nodal signals, which regulate mesoderm specification and formation (Schier, 2003), appear to control the cytoskeleton by phosphorylating Ezrin2, a molecule that anchors Actin to the plasma membrane (Link *et al.*, 2006). Whether any of these mechanisms are crucial to the establishment of differential germ-layer adhesion remains to be shown.

Differential adhesion is only one of multiple processes that determine germ-layer separation and morphogenesis. There are indeed cellular rearrangements that cannot be solely explained by differential adhesion mechanisms. For example, internalized mesendodermal progenitors in lateral regions of the embryo move only for a short time toward the animal pole, then suddenly make a 90-degree turn toward dorsal (Myers *et al.*, 2002b; Sepich *et al.*, 2005). Differential adhesion-induced spreading of mesendodermal tissue could explain mesendoderm movement away from the margin, but not the rapid change in direction. To determine the contribution of differential adhesion, the intercellular adhesion of specific tissues must be measured and placed into theoretical models that predict cellular rearrangements resulting from the *tissue interfacial free energies.* Comparing the actual cellular rearrangements in the gastrula to those predicted by differential adhesion-based models should aid in revealing the role of differential adhesion.

VI. Concluding Remarks

Studying gastrulation movements in zebrafish has become increasingly popular as it offers the unique possibility to analyze cell movements in a simple cellular context *in vivo.* Although the major cell movements of zebrafish gastrulation have been heavily investigated, there is little known about the underlying molecular and cellular mechanisms. A picture of the signaling pathways required during gastrulation has emerged, most prominently so for noncanonical Wnt signaling. However, insight into the effector mechanisms by which these pathways control cell movement is largely missing.

To address the downstream effector mechanisms, one needs to examine basic cellular processes controlling cell adhesion and cytoskeletal rearrangement. These are two key factors in embryonic morphogenesis. The main difficulty

with such an investigation is that the inactivation of molecules tied to the regulation of cell adhesion and the cytoskeleton could have widespread consequences. The resulting pleiotropic phenotypes would preclude the analysis of specific morphogenetic function. Different methods and tools must be developed to circumvent this problem. First, assay systems must be developed with the capability to quantify specific parameters of cell/tissue shape, movement, and adhesion. Second, cell adhesion assays, both *in vitro* and *in vivo,* are needed to determine the specific adhesive properties of different gastrula cell types. Third, image analysis and quantification software is required to determine three-dimensional cell shape and movement over time. Finally, and most importantly, imaging tools such as two-photon confocal microscopy need to be enhanced to visualize single cells at subcellular resolution *in vivo.*

Besides assay development, methods to modulate gene or molecule activity in a temporally and spatially restricted manner are essential. Single cell or tissue transplantations are very useful in this respect, but less invasive methods are needed to determine the endogenous requirements of particular genes. The generation of transgenic lines expressing genes of interest under the control of specific promotors is one very effective way to dissect differences in gene requirements. Also, screens identifying small molecules and drugs that interfere with the activity of specific molecules and cellular processes will be helpful.

Last but not least, both forward and reverse genetic screens will continue to be an indispensable tool to identify genes with essential functions in morphogenesis during zebrafish gastrulation. However, the success of such screens in the future will depend on the development of highly sensitive screening assays (e.g., screens that can detect changes in cell migration and/or adhesion). Only screening assays that are sensitive, reliable, and feasible for high-throughput screening are likely to reveal new genes with important functions in morphogenesis.

In summary, the development and improvement of genetic and biophysical tools to identify and characterize gene function during morphogenesis is essential to obtain new insight into the mechanisms underlying gastrulation movements in zebrafish.

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