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Zebrafish gastrulation movements: bridging cell and developmental biology

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During vertebrate gastrulation, large cellular rearrangements lead to the formation of the three germ layers, ectoderm, mesoderm and endoderm. Zebrafish offer many genetic and experimental advantages for studying vertebrate gastrulation movements. For instance, several mutants, including silberblick, knypek and trilobite, exhibit defects in morphogenesis during gastrulation. The identification of the genes mutated in these lines together with the analysis of the mutant phenotypes has provided new insights into the molecular and cellular mechanisms that underlie vertebrate gastrulation movements.

Key words: gastrulation / convergent extension / cell polarity / zebrafish

Introduction

> It is not birth, marriage, or death, but gastrulation, which is truly the most important time in your life.[1]

Gastrulation defines the process by which the three germ layers, ectoderm, mesoderm and endoderm are formed. Although substantial information into the mechanisms that establish cell fates during gastrulation has been gathered, much less has been learned about the molecular and cellular mechanisms underlying gastrulation movements. In vertebrates, cells undergo various types of movements during gastrulation. Gastrulation usually starts with the involution/ingression of prospective mesendodermal cells. This is followed by convergence and extension movements that lead to an accumulation of cells at the dorsal side of the gastrula and an anterior-posterior extension of the emerging body axis.

Much of the pioneering work describing vertebrate gastrulation movements has been done in the amphibian *Xenopus*.[2] Here, gastrulation is triggered by the involution of prospective mesendodermal cells at the blastopore region. Convergence of cells towards the embryonic midline and anterior-posterior elongation of the body axis is achieved by cellular rearrangements commonly termed 'convergent extension' (CE). During CE, cells move towards the embryonic midline and undergo medio-lateral cell intercalations, which leads to a medio-lateral narrowing and an anterior-posterior extension of the forming embryonic axis. A prerequisite for medial-lateral cell intercalation is the elongation of cells along the medial-lateral axis.[3]

The zebrafish has emerged as an ideal model organism to study both experimental and genetic aspects of vertebrate gastrulation movements. Zebrafish embryos develop in large numbers *ex-utero*, are transparent and accessible to various experimental manipulations such as cell- and tissue-transplantation/ablation and fate mapping. Imaging of embryos at a single-cell resolution can be achieved without additional labeling through simple DIC recordings. Finally, gene function during zebrafish gastrulation can be assessed by the identification of mutants through forward genetic screening and reverse 'knock-down' of gene function through injection of *morpholino* antisense oligonucleotides.[4]

The first part of this review describes the cellular mechanisms underlying gastrulation movements in zebrafish. In the second part, we discuss more recent advances in our understanding of the molecular control of zebrafish gastrulation movements taking into account related work in *Xenopus*.

Cellular mechanisms

Zebrafish embryos undergo meroblastic cleavage, leading to the formation of a blastoderm 'cap' on top

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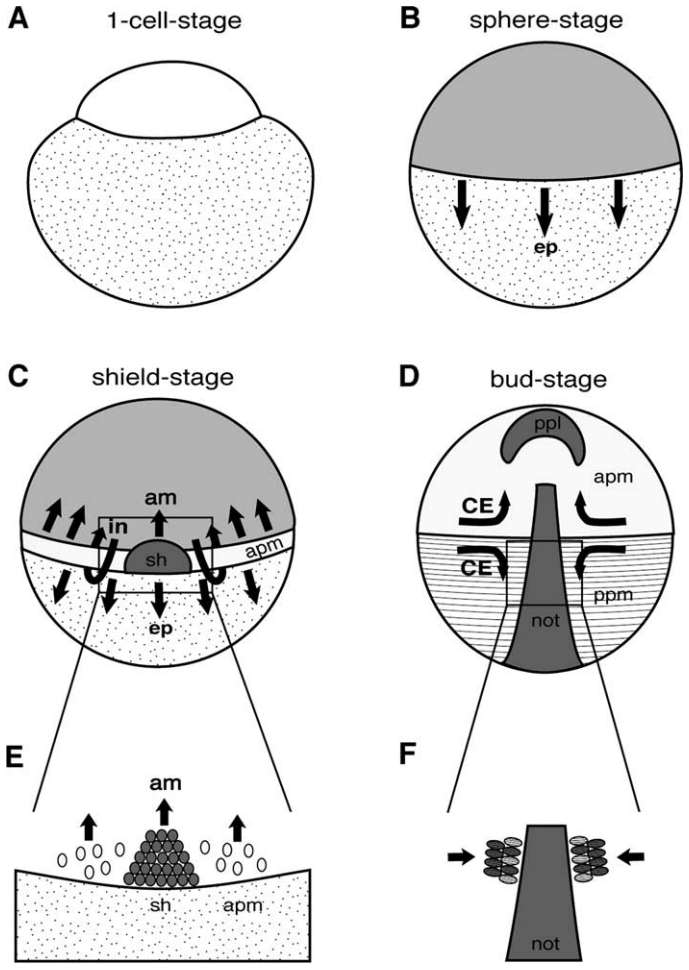

Figure 1. Schematic drawings depicting the main cellular movements at different developmental stages during gastrulation. (A) After fertilization of the egg, cytoplasmic streaming leads to the formation of the first cell at the animal pole of the yolk sac. (B) At sphere stage, the cells of the epiblast (grey) start epibolic movements (ep) that cause a progressive spreading of the epiblast over the yolk sac. (C) At shield stage, involution (in) of the first prospective mesendodermal cells leads to the formation of axial (shield, sh) and anterior paraxial mesendodermal (apm) tissue, the cells of which migrate anteriorly (am) towards the animal pole. (D) At bud stage, convergent extension movements (CE) of both ectodermal and mesendodermal cells drive the progressive medio-lateral narrowing and anterior-posterior extension of the emerging embryonic body axis. The mesendoderm has been tentatively subdivided into anterior (apm) and posterior domains (ppm) based on their distinct cellular and molecular characteristics. (E) Close-up of the shield region shown in (C) illustrating the anterior migration (am) of axial (sh) and paraxial (apm) mesendodermal cells. (F) Close-up of the notochordal region (not) shown in (D) depicting medial-lateral cell intercalations of paraxial mesendodermal cells (ppm) that undergo convergent extension (CE) movements.

of a big yolk cell [Figure 1(A) and (B)]. Initially, this cap can be subdivided into a surface enveloping layer consisting of flattened epithelial cells and a deep layer of more loosely associated blastodermal cells. A third layer of cells, the yolk syncytial layer (YSL), is formed at late blastula stages by the fusion of marginal blastoderm cells with the yolk cell.[5] The precise function of this cell layer is not yet fully understood but it is likely that it constitutes a source of signals required for patterning of the gastrula.[6] Epiboly starts with the thinning of the blastoderm, which eventually spreads over the entire yolk cell to completely cover it at the end of gastrulation [Figure 1(B)]. Radial cell intercalations, where cells at different depths in the blastoderm intercalate between each other and trigger the characteristic thinning and spreading of the

blastoderm, appear to contribute to these epibolic movements.[7,8]

Cell internalization at the blastoderm margin, initially referred also as involution, marks the onset of gastrulation. During this process, prospective mesendodermal cells separate from the epiblast eventually leading to the formation of distinct germ layers[7] [Figure 1(C)]. The first signs of cell internalization become evident by a thickening of the blastoderm at a circumferential band at its margin, commonly called the 'germ ring'. Active movement of cells towards the blastoderm margin causes the thickening of the germ ring. Having reached the margin, these cells move internally from outer blastodermal layers towards the yolk cell surface.[7] Recent reports indicate that, although highly coordinated and restricted to the margin, the process of internalization has a cell-autonomous basis because individual cells can internalize independent of their neighbors[9,10] (Richard Adams and Miguel Concha, unpublished data). The first internalizing mesendodermal cells sharply change their direction of movement after reaching the yolk surface and actively migrate towards the animal pole. Internalized paraxial cells migrate as loosely associated cells while axial cells exhibit a tightly associated epithelial morphology and only cells at the anterior edge appear to actively migrate (Miguel Concha, Steve Wilson, Richard Adams, M. T. and C.-P. H., unpublished data [Figure 1(E)]. Anterior migration is restricted to early internalized cells whereas later internalized cells move towards the vegetal pole[7] [Figure 1(D)].

Internalization is followed by convergence and extension movements of both mesendodermal and neuroectodermal cells. Three domains of distinct convergence and extension movements have been identified around the circumference of the gastrula.[11,12] Cells at the ventral side of the gastrula do not converge to the dorsal side but instead, move vegetally over the yolk sac to eventually contribute to the tail. In contrast, cells in lateral regions of the gastrula show increasing convergence and extension movements towards the dorsal side while cells at the dorsal side of the gastrula exhibit a high degree of extension and a low degree of convergence movements.[11,12] The movement of YSL nuclei resembles the CE movement of both ectodermal and mesendodermal cells in time and in space,[13] suggesting that cell movements are synchronized between these cell layers. In general, CE movements are achieved through medio-lateral cell intercalations of cells that stream towards the midline leading to a progressive medio-lateral narrowing and anterior-posterior extension of the forming body axis[7] [Figure 1(F)]. Cells undergoing medio-lateral cell intercalations within the posterior mesoderm and ectoderm elongate along their medio-lateral axis while ectodermal cells also show polarized protrusive activity.[14,15] In addition, preliminary studies indicate that both neuroectodermal and mesendodermal cells exhibit complex and highly dynamic morphologies with long filopodia spanning between the germ layers (Florian Ulrich and C.-P. H., unpublished data). Further analysis of cellular morphologies will be needed to uncover the full range of potential cellular interactions both within and between germ layers during CE movements.

The coordination of morphogenetic movements during gastrulation relies not only on cellular interactions within specific regions of the embryo but, equally important, between different parts/tissues of the gastrula. Thus, during the course of gastrulation, the embryo becomes subdivided into regions of distinct cellular morphology and behavior along its main axes of polarity. Most notably, the dorso-ventral axis of the forming embryo is subdivided into the three germ layers, ectoderm, mesoderm and endoderm. Signals from the underlying mesodermal germ layer regulate medio-lateral cell polarization and intercalations within the overlying neuroectoderm during *Xenopus* gastrulation suggesting that interactions between the germ layers are important for the coordination of gastrulation movements.[16] Other subdivisions of the gastrula are those along the medio-lateral axis into axial and paraxial tissues and, perhaps less obvious, along the anterior-posterior axis into tissues giving rise to head and trunk/tail structures [Figure 1(D)]. In *Xenopus*, paraxial mesodermal cells undergoing medial-lateral cell intercalations change, upon contact with the axial mesoderm, from an initial bipolar to a monopolar morphology indicating that axial–paraxial tissue interactions regulate cellular morphologies during gastrulation.[2] Interactions between other regions (anterior and posterior) might be equally important but as yet have not been studied. A detailed analysis of tissue interaction during gastrulation will be essential to understand how morphogenesis of the whole embryo is coordinated.

Molecular control

Several mutants have been identified which exhibit morphogenetic defects during gastrulation. Specification of mesendodermal precursor cells and subsequent cell internalization movements are defective

in *one-eyed-pinhead* (*oep*) mutant embryos.[9] Similarly, paraxial mesendodermal progenitor cells giving rise to the somites of the trunk are mis-specified and exhibit reduced convergence movements in *spadetail* (*spt*) mutant embryos.[17,18] Finally, mutants predominantly affecting morphogenesis and not cell fate specification are *silberblick* (*slb*), *knypek* (*kny*), *trilobite* (*tri*) and *pipetail* (*ppt*).[19–21] In *slb* mutant embryos, CE movements of both mesendodermal and neuroectodermal cells are reduced resulting in a transient shortening of the embryonic axis at the end of gastrulation and a slight fusion of the eyes at later developmental stages.[22] Although *slb* mutants show reduced medial-lateral cell intercalations in both anterior and posterior mesendodermal domains, extension of anterior regions appears to be most severely affected.[23] In contrast, *kny*, *tri* and *ppt* mutant embryos exhibit reduced CE movements in posterior mesendodermal and neuroectodermal domains while anterior extension of the embryonic body axis is less affected[19,21,24] (M. T. and C.-P. H., unpublished data).

CE mutants and the Wnt/PCP pathway

Cloning of *slb* led to the identification of a member of the Wnt-family of secreted glycoproteins as a crucial regulator of CE movements. The *slb* locus encodes Wnt11, which is expressed in the entire germ ring at the shield stage and subsequently within the anterior paraxial mesoderm and lateral neuroectoderm by the end of gastrulation.[23] Cell and shield transplantation experiments showed that Slb/Wnt11 activity is required within lateral tissues of the gastrula where it regulates medio-lateral cell intercalations that underlie CE movements. The observation that *slb* embryos are predominantly affected in anterior regions of the gastrula suggests that other genes are involved in the regulation of CE movements in more posterior regions. Cloning of the *ppt* locus pointed at the intriguing possibility that another Wnt ligand might function in this territory and interact with Slb/Wnt11. Indeed, *ppt* encodes Wnt5a,[25] which during late gastrulation is expressed in the posterior paraxial mesendoderm directly adjacent to the anterior mesendodermal *wnt11* expression domain (C.-P. H., M. T. and Filipa Barbosa, unpublished data). Furthermore, in the absence of zygotic *ppt*, the *slb* homozygous phenotype is strongly enhanced (C.-P. H. and M. T., unpublished data), suggesting that Slb and Ppt exhibit partially overlapping functions in regulating CE movements in lateral domains of the gastrula.

The analysis of the *slb* and *ppt* mutant phenotypes have shown that both Wnt11 and Wnt5a are required during gastrulation for proper morphogenesis rather than cell fate specification.[20,21] This conclusion is supported by gain-of-function studies in fish and frogs, showing that unlike the canonical Wnts (for reviews see References 26, 27), Wnt5a and Wnt11 modulate CE without affecting cell fate.[28–30] One of the main functions of Wnt5a and Wnt11 is to polarize cells along their medio-lateral axis in mesodermal tissues undergoing CE movements[31] (Hannu Mansukoski, M. T. and C.-P. Heisenberg, unpublished data). This is reminiscent of the planar polarization of cells in many *Drosophila* epithelia, commonly termed *planar cell polarity* (PCP) (for reviews see References 32–34). In *Drosophila*, the PCP pathway includes the Wnt receptor Frizzled (Fz), a seven-pass transmembrane receptor, and the cytoplasmic signal transducer Dishevelled (Dsh), but not members of the canonical Wnt pathway such as Axin, GSK-3 and β-catenin. Rather, the PCP pathway signals via small GTPases (RhoA, Rac and Cdc42) and the Jun-N-terminal kinase (JNK) both of which are known to be key regulators of the cytoskeleton during cell polarization. By taking advantage of the modular structures of Dsh,[35,36] it has been shown that Slb/Wnt11 regulates CE movements through a pathway that is similar to the PCP pathway in *Drosophila*. A truncated form of Dsh, which specifically transduces the PCP pathway, is capable of rescuing the *slb* phenotype.[23] Conversely, injection of a mutant form of Dsh, which specifically blocks the PCP pathway, leads to a *slb*-like phenotype in wild-type embryos and resembles embryos that are injected with a dominant-negative version of Wnt11 in *Xenopus*.[23,37] This, together with the observation that Dsh regulates cell polarity in dorsal tissues undergoing CE in *Xenopus*,[38] presented the first evidence that the cellular and molecular processes involved in regulating vertebrate gastrulation movements and planar cell polarity in *Drosophila* might share significant similarities (for an overview see Figure 2).

The *slb/ppt* double mutant phenotype resembles the phenotype of embryos homozygous for *kny*, which encodes a member of the glypican family of heparan sulphate proteoglycans.[14] The observation that the *Drosophila* heparan sulphate proteoglycans, Dally and Dally-like, interact with the Wnt-family ligand Wingless, suggests that Kny might also function as a co-factor or co-receptor for Wnt11/Wnt5a by either facilitating the binding to Frizzled receptors or stabilizing them at the cell surface.[39–41] Consistent with these ideas, co-expression of *kny* RNA potentiates the activity of Wnt11 to rescue the *slb* CE phenotype.[14] Furthermore, *slb/kny* double mutants display a more

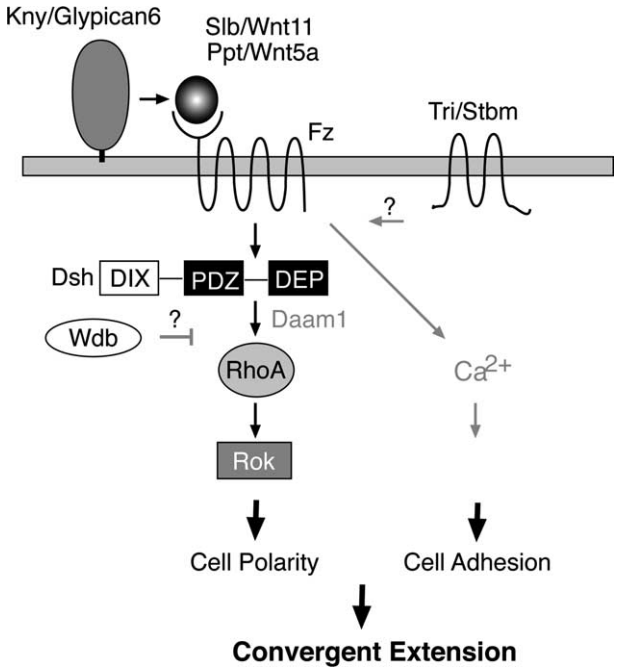

Figure 2. A model for the Wnt/PCP pathway regulating convergent extension during zebrafish gastrulation. The ligands, Slb/Wnt11 and Ppt/Wnt5a, signal through their potential receptors, Fz7 andFz2, to the intracellular transducer Dsh. Kny/Glypican6 presumably facilitates Wnt activity extracellularly. The PDZ and DEP domains of Dsh are responsible for the activation of RhoA, and thereby its effector Rok, which directly regulates the actin cytoskeleton. Fz7might be involved in the separation of hypoblast from epiblast by regulating cell adhesion through a Wnt/Ca^{2+} pathway. Tri/Stbm participates in the Wnt/PCP pathway via an unknown mechanism, while another PCP gene, Wdb, is involved in the regulation of convergent extension. Possible regulators anticipated from studies in other species are shaded. The Wnt/Ca^{2+} pathway is not shown (for review see Reference 65).

severe phenotype than either mutant alone, indicating that Kny acts as a positive regulator of Wnt11 similar to which has been shown for LDL-receptor related proteins (LRPs) in the canonical Wnt signaling pathway.[14,42–45]

Additional evidence for a connection between the *Drosophila* PCP pathway and the Wnt signaling pathway regulating CE movements has come from the functional analysis of vertebrate homologues of the *Drosophila* PCP gene *strabismus/van gogh* (*stbm/vang*),[46,49,51] which encodes a unique four-pass membrane protein.[47,48] Positional cloning of the *tri* locus, shown to be required for CE movements, revealed that *tri* encodes the zebrafish *stbm/vang* homologue.[46] Similarly, 'knock-down' of *stbm/vang* gene function through *morpholino* antisense oligonucleotide injections in zebrafish and *Xenopus* leads to CE defects during gastrulation.[49–51] The observation that Tri/Stbm directly binds to Dsh and activates JNK[49] together with the finding that *tri* genetically interacts with *slb* and *kny* in the regulation of CE movements[22,24] further supports the notion that Tri/Stbm acts within the Wnt/PCP pathway. In addition to its function during gastrulation, Tri/Stbm also regulates migration of branchiomotor neurons.[46] This later function, however, appears to be independent of the Wnt/PCP pathway,[46] which is in agreement with observations in *Drosophila* showing that Stbm is not a simple linear component of the Fz/PCP pathway.[47]

Function of the Wnt/PCP pathway in regulating gastrulation movements

How do cells acquire polarity within the plane of the mesodermal/ectodermal tissue during gastrulation? An initial step in the establishment of planar cell polarity in the *Drosophila* wing epithelium is the recruitment Dsh onto the plasma membrane in response to Fz signaling, followed by the establishment of an asymmetric localization of the Fz–Dsh signaling complex to the distal edge of the cells.[52–57] Similarly, Dsh accumulates at the membrane of cells undergoing CE in *Xenopus*.[38] However, it has not been reported that Dsh or other components of the functional signaling complex are preferentially localized to the medio-lateral edges of cells undergoing CE movements.

What are the other components mediating the function of the Wnt/PCP signaling pathway regulating CE movements during gastrulation? Several lines of evidence support the notion that Frizzled 7 (Fz7) can function as a receptor for Slb/Wnt11. First, Fz7 can directly bind to Wnt11 and is expressed in domains similar to those of *wnt11* in late gastrula stage embryos.[58,59] Second, both gain- and loss-of-function of Fz7 disturbs CE movements in *Xenopus*.[60–63] Finally, the blocking of Fz7 function through *morpholino* oligonucleotide injections leads to reduced CE movements that are preceded by a defect in cell adhesion and tissue separation between ectoderm and mesoderm during involution of presumptive mesodermal cells.[63] Similar to Fz7, Frizzled 2 (Fz2) might act as a receptor for Wnt5a considering the fact that injection of *fz2 morpholinos* into wild-type embryos phenocopies the *ppt* mutant phenotype[64] (Hannu Mansukoski and C.-P. H., unpublished data).

In addition to their proposed function within the Wnt/PCP pathway, both Fz2 and Fz7 can signal through an alternative pathway that involves activation of protein kinase C (PKC) in a G-protein dependent manner and the mobilization of intracellular calcium (Ca^{2+}) (for review see Reference 65). The activity of Fz7 in tissue separation during gastrulation (as mentioned above) is mediated by PKC.[63] Similarly, Fz2, together with Wnt5a, can activate its effectors including PKC through the mobilization of intracellular Ca^{2+}.[66,67] This indicates that at least a part of the morphogenetic function of Fz2 and Fz7 during gastrulation is mediated through a Wnt/PCP independent pathway involving intracellular Ca^{2+} and PKC. How the Wnt/PCP pathway interacts with this alternative pathway has still to be clarified.

Several more molecules, which are shared between the *Drosophila* PCP pathway and the Wnt signaling cascade regulating polarized cell behaviors during CE have been uncovered in recent studies in zebrafish and *Xenopus*. A homologue of Rho kinase (Rok), a downstream effector of RhoA and part of the Fz signaling cascade in *Drosophila*,[68] acts downstream of Slb/Wnt11 to regulate cell polarity during gastrulation.[31] In zebrafish, over-expression of a dominant-negative form of *Rho kinase 2 (Rok2)* leads to an inhibition of CE movements, while over-expression of wild-type *Rok2* can partly rescue the *slb* mutant phenotype.[31] Serving as a crucial linker molecule between Dsh and RhoA/Rok, Daam1 has been identified, a formin-like molecule, which appears to participate in the Wnt/PCP pathway during *Xenopus* gastrulation.[69] Finally, zebrafish homologues of the *Drosophila widerborst* (*wdb*) gene, which encodes a B regulatory subunit of protein phosphatase 2A (PP2A) involved in regulating planar cell polarity in the wing disc, are also required for correct CE movements during zebrafish gastrulation.[70] This effect on CE movements is only observed when zebrafish Wdb activity is partially suppressed while a more complete 'knock-down' of Wdb function leads to severe defects in dorso-ventral patterning of the gastrula.[70] This indicates that Wdb regulates CE as well as dorso-ventral patterning, but that CE is more sensitive to the dose of Wdb.

Other signaling pathways regulating CE movements

In the absence of Wnt/PCP signaling CE movements are strongly reduced but not completely abolished. Similarly, Fz/PCP signaling in *Drosophila* is responsible for the coordination of epithelial cell polarities but not for the overall establishment of cell polarity.[33] This raises the question to what extent the Wnt/PCP signaling pathway is required for CE movements and what is the contribution of other signaling pathways to this process (for an overview see Table 1).

The canonical Wnt pathway has been associated with the initiation of CE movements[26] although it cannot control Dsh-mediated cell polarity directly.[38] This has been most clearly demonstrated by the analysis of the morphogenetic activities of several downstream targets of the canonical Wnt signaling pathway such as the signal transducer and transcriptional activator *stat3* which is known to be activated by cytokines.[71] In *Drosophila*, JAK/STAT signaling in equatorial regions of the eye disc can cell-non-autonomously influence planar cell polarity via an unknown secondary signal, which appears to be downstream of Fz.[34] Similarly, Stat3 in zebrafish cell-autonomously regulates cell movements in medial tissues and displays a cell-non-autonomous function in lateral tissues during gastrulation.[71] It is therefore conceivable that Stat3 induces a chemotrophic signal in medial cells that attracts lateral cells to converge medially although the existence and molecular nature of such a signal has still to be determined.

Another regulatory pathway that is thought to act in medial tissues and can influence CE movements

Table 1. Genes—outside of Wnt/PCP pathway—involved in the regulation of convergent extension movements during gastrulation

Gene	Gene product	Molecular role	Function	Reference
papc	Protocadherin	Cell adhesion	LOF (DN): defective CE	78, 79
stat3	Transcription factor	Mediator of chemokines	LOF (MO): defective movement of axial tissues	71
sprouty	Intracellular molecule	FGF inhibitor	GOF: defective CE	77
slit2	Secreted ligand	Repulsive cue	GOF: defective CE	74

Abbreviations: LOF: loss-of-function; GOF: gain-of-function; DN: dominant-negative; MO: morpholinos; CE: convergent extension.

is the Slit-Robo signaling cascade. In *Drosophila*, the large extracellular matrix protein Slit and its receptor Robo are required in medial cells of the embryo to inhibit the crossing of neuronal axons.[72,73] In zebrafish gastrula embryos, *slit2* is expressed in medial tissues and mis-expression of *slit2* inhibits CE movements.[74] It is tempting to speculate that a repulsive signal mediated by the Slit-Robo complex at the midline of the gastrula might influence CE movements by converting the initial bipolar protrusive activity of paraxial mesodermal cells into a monopolar cell morphology as soon as these cells reach the midline (see also 'cellular mechanisms').

Evidence that the fibroblast growth factor (FGF) signaling pathway is also involved in regulating vertebrate CE movements comes from studies showing that genes involved in regulating zebrafish gastrulation movements such as *slb* and *spt* are directly and/or indirectly activated by FGF signals.[37,75,76] The notion that FGF signaling can influence cell movements without affecting cell specification is also supported by the observation that in *Xenopus*, over-expression of *sprouty*, an FGF inducible FGF antagonist, can inhibit CE without changing cell fate.[77] *Sprouty* functions in this process by interfering with a Ca^{2+} dependent signal,[77] pointing at a potential link between the FGF and the Wnt/Ca^{2+} signaling pathways (see also above).

The coordination of cellular movements during gastrulation crucially depends on cell–cell signaling within and between tissues. The regulation of cell adhesion is an important factor that allows large population of cells to communicate with each other. In the *Drosophila* eye, the graded distribution of protocadherin family members such as *fat* (*ft*) and *dachsous* (*ds*) determine, via an unknown secondary signal, the asymmetric localization of the Fz/PCP signaling complex.[56] One possible candidate for a protocadherin family member that regulates cell polarity and movements during zebrafish gastrulation is *paraxial protocadherin* (*papc*). *Papc* is expressed within the paraxial mesoderm during gastrulation and is required for proper CE movements in zebrafish and *Xenopus*.[78,79] By analogy to the function of *ft* and *ds* in *Drosophila*, *papc* might influence CE movements by establishing a graded activity of the Wnt/PCP signaling required for proper cell polarity and movement during gastrulation.

Perspective

In recent years, a steadily increasing number of molecular pathways have been implicated in the regulation of zebrafish gastrulation movements; however, their precise function on a cellular basis has, in most cases, not yet been addressed. To understand how cellular rearrangements during gastrulation are achieved, it will be important to explore the precise contribution of these molecules to cell biological processes that are central to tissue morphogenesis during gastrulation such as cell-shape changes, cell migration and cell–cell/extracellular matrix interaction. The combination of genetic and molecular studies with cellular analyses, for which zebrafish constitutes an ideal experimental model organism, will help to uncover the developmental mechanisms underlying morphogenesis during vertebrate gastrulation.

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