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Ins and outs of Spiralian gastrulation

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ABSTRACT Gastrulation is a critical stage of metazoan development during which endodermal and mesodermal tissues are internalized, and morphogenesis transforms the early embryo into each animal's unique body-plan. While gastrulation has been studied extensively in classic model systems such as flies, worms, and vertebrates, less is known about gastrulation at a mechanistic level in other taxa. Surprisingly, one particularly neglected group constitutes a major branch of animals: the Spiralia. A unique feature of spiralian development is that taxa with diverse adult body-plans, such as annelids, molluscs, nemerteans and platyhelminths all share a highly stereotyped suite of characters during embryogenesis called spiral cleavage. The spiral cleavage program makes it possible to compare distantly related embryos using not only morphological features, and gene expression patterns, but also homologous cell lineages. Having all three criteria available for comparison is especially critical for understanding the evolution of a complex process like gastrulation. Thus studying gastrulation in spiralians is likely to lead to novel insights about the evolution of body-plans, and the evolution of morphogenesis itself. Here we review relevant literature about gastrulation in spiralians and frame questions for future studies. We describe the internalization of the endoderm, endomesoderm and ectomesoderm; where known, we review data on the cellular and molecular control of those processes. We also discuss several morphogenetic events that are tied to gastrulation including: axial elongation, origins of the mouth and anus, and the fate of the blastopore. Since spiral cleavage is ancestral for a major branch of bilaterians, understanding gastrulation in spiralians will contribute more broadly to ongoing debates about animal body-plan divergence, such as: the origin of the through-gut, the emergence of indirect versus direct development, and the evolution of gene-regulatory networks that specify endomesoderm. We emphasize the fact that spiralian gastrulation provides the unique opportunity to connect well-defined embryonic cell lineages to variation in cell fate and cell behavior, making it an exceptional case study for evo-devo.

KEY WORDS: *spiralia, endomesoderm, ectomesoderm, blastopore, axial elongation, epiboly, invagination*

"How do we know what is the primitive type of gastrulation? The present state of embryology certainly does not enable us to give any positive answer to this question. Whether the primary form is the epibolic or the embolic gastrula, the plakula, the unipolar or multipolar delaminate planula, or a still different type, remains to be seen; and the very fact that the differentiation of the layers is effected in such a diversity of ways proves conclusively that these early stages of development are as susceptible to secondary modification as the later."

-E. B. Wilson, 1892

Gastrulation is a critical embryonic event during which presumptive endodermal and mesodermal cells are internalized (Stern, 2004). Gastrulation is closely tied to the development of key axial properties, and to the patterning of certain organ systems, such as the digestive tract; the openings of the mouth and/or anus often arise at or close to the site of gastrulation, called the blastopore (see Technau and Scholz, 2003; Hejnol and Martindale, 2009). Gastrulation also holds a pivotal role in evolutionary theories about the emergence and divergence of bilaterian body-plans (Arendt and Nübler-Jung,1997; Nielsen, 2001; Martindale and Hejnol, 2009). Thus, understanding the phylogenetic history of this event

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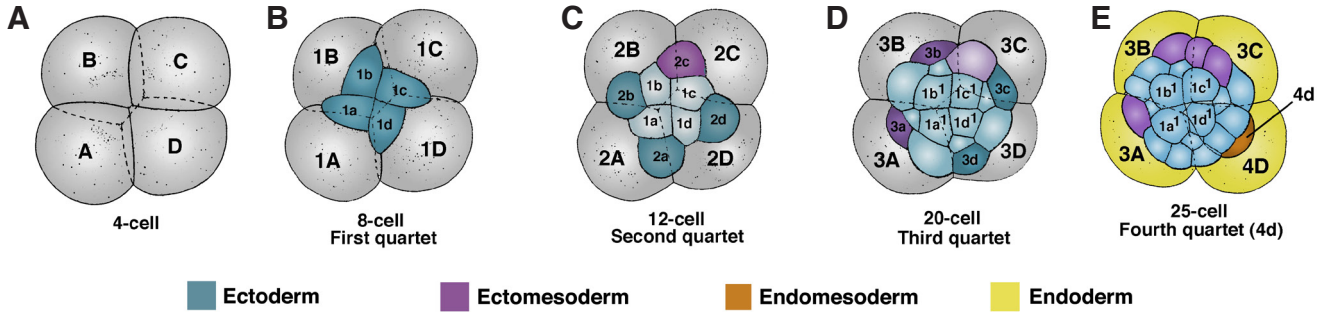


Fig. 1. Overview of early spiralian cleavage patterns. *Diagram of early cleavages from a representative spiralian, the snail* Crepidula fornicata, *viewed from the animal pole.* **(A)** *The first two cleavages establish 4 quadrants, A-D.* **(B-D)** *Micromere quartets 1a-1d to 3a-3d are born and divide, making a micromere cap on top of the yolky 3rd quartet macromeres 3A-3D.* **(E)** *When 3D divides ahead the other macromeres, it gives rise to the 4d endomesodermal precursor at the 25-cell stage. Modified from Henry and Perry (2008).*

in different metazoan lineages remains an important question for evolutionary developmental biology.

As evo-devo questions go, tracing the evolutionary history of gastrulation is particularly difficult, because the complex morphogenetic and fate specification events involved make gastrulation an emergent property of development. Gastrulation has undoubtedly undergone significant modification in extant species relative to the metazoan common ancestor; gastrulation is highly influenced by life history, and trophic, strategies (Anderson, 1973; Arendt and Nübler-Jung, 1999; Martín-Durán and Egger, 2012; Arenas-Mena, 2014, this volume). Adaptation played a role in generating the wide range of gastrulation modes found between extant taxa (Byrum and Martindale, 2004; Gerberding and Patel, 2004; Chea *et al.*, 2005; Hejnol and Martindale, 2009). For these reasons, no living species' gastrulation process can serve as a proxy for that of a distant common ancestor's. To make meaningful evolutionary inferences, it is necessary to understand the phylogenetic context of gastrulation within each group, and, from there, draw careful comparisons at broader evolutionary distances. Perhaps even more importantly, it is necessary to make comparisons at multiple levels of organization, from how gene regulatory networks (GRNs) specify individual cells, to how cells and tissues undergo complex cell rearrangements, to how various modes of gastrulation are part of developmental strategies adapted to specific environments. Spiralians are an ideal group of animals for such an approach, due to their morphological diversity, shared cleavage program, and an emerging knowledge of the molecular and cellular underpinnings of gastrulation.

The Spiralia (including the Lophotrochozoa, Halanych *et al.*, 1995) encompasses many taxonomic groups: molluscs, annelids (echiurans, sipunculans and myzostomids), nemerteans, platyhelminths, phoronids, brachiopods, entoprocts, cycliophorans, bryozoans, gnathostomulids, rotifers, mesozoans and gastrotrichs (Giribet *et al.*, 2009; Hejnol, 2010; Struck *et al.,* 2014). Many of these taxa (e.g., molluscs, annelids, nemerteans and platyhelminths) share a highly conserved suite of developmental characters collectively called spiral cleavage (Hejnol, 2010; Lambert, 2010; Henry, 2014, this issue). Spiralians (which here we mean those taxa that exhibit spiral cleavage) show a fantastic diversity of adult and larval forms, from segmented tube-dwelling marine polychaetes, to land snails, to freshwater flatworms; each of which exhibits different forms of indirect or direct development. The highly conserved spiral cleavage program allows comparison of homologous cells and tissues at single-cell resolution, across hundreds of millions of years of evolution (Wilson, 1898; Henry and Martindale, 1999; Lambert, 2010). At the same time, finer-grain cell lineage variation does exist between species, making it possible to pose hypotheses about the evolution of cleavage patterns and associated fate specification, which are the roots of morphological diversity. The unique bodyplan of each taxa begins to emerge during gastrulation stages, and so gastrulation is a critical process to study for understanding how different morphologies arise in development.

Gastrulation mechanisms vary widely amongst spiralians including: ingression/epithelial-mesenchymal transition (EMT), invagination, emboly and epiboly. (Arendt, 2004; van den Biggelaar and Dictus, 2004). These various behaviors can be compared between distantly related spiralian species because of their conserved basic fate map. Likewise, this fate map framework allows one to identify differences in both the cellular and molecular (GRN) basis of specific gastrulation behaviors. Because gastrulation often results in the establishment of the alimentary canal, we can correlate variation in gastrulation types with life history modes, such as direct vs. indirect development, or planktotrophic vs. lecithotrophic larval forms (Kato, 1968; Anderson, 1973; Singley, 1977; Arenas-Mena, 2010; Martín-Durán and Egger, 2012; Arenas-Mena, 2014, this issue). Answers to these questions will provide the necessary information about gastrulation diversity in spiralians needed for making comparisons with other metazoans.

Here we review relevant literature about gastrulation in the major spiralian groups and frame questions for future studies. Part one covers the origin of the ectodermal, endodermal, and mesodermal germ layers. Part two reviews different modes of gastrulation, including what is known about the cellular and molecular mechanisms that control them. Part three discusses morphogenetic events that follow gastrulation: mouth and anus formation, fate of the blastopore, and axial elongation. Compared to other aspects of spiralian development, gastrulation has been understudied; our main goal is to provide a framework to encourage future research on this important event.

The basic spiralian fate map: origins of ectoderm, mesoderm and endoderm

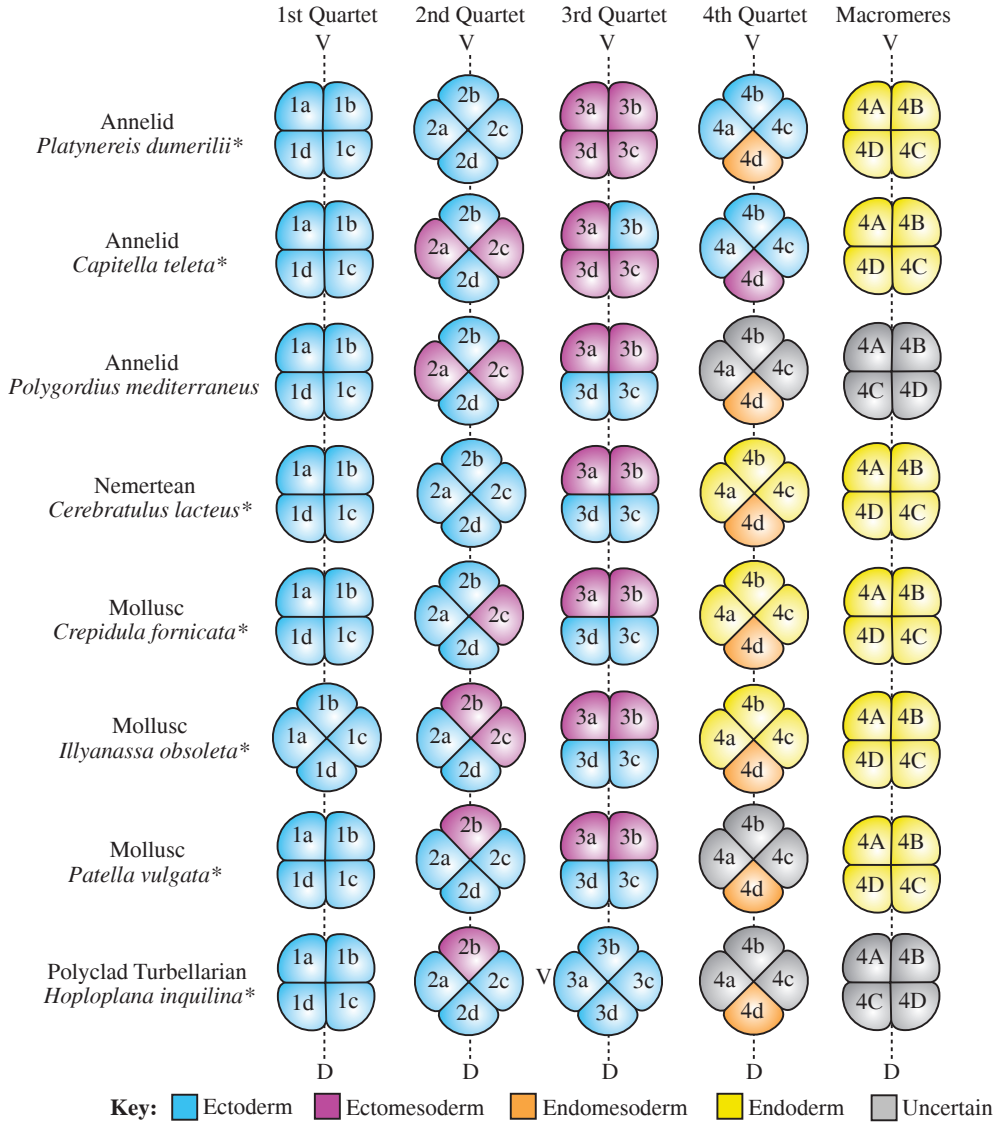
The first two cleavages of the zygote are orthogonal to one another, and parallel to the animal-vegetal axis, creating four quadrants/blastomeres called A, B, C, and D (Fig. 1A; Henry

2014, this volume). The plane of third cleavage is oblique to the animal-vegetal axis; in each quadrant, a quartet of micromeres is born at the animal pole, and situated in the furrows between the underlying macromeres. At the 8-cell stage the animal micromeres are denoted 1a, 1b, 1c, and 1d, while the corresponding vegetal macromeres are named 1A, 1B, 1C, and 1D (Fig. 1B). Depending on the species, subsequent rounds of division in the macromeres produce three to four additional micromere quartets (2a-d through 5a-5d) (Fig. 1 C-E). The fates of each quadrant, and quartet, are relatively well-conserved across distantly related taxa. In the vast majority of spiralians examined, the first quartet micromeres (1a-1d) give rise to ectodermal fates exclusively (Figs. 1,2). The first quartet makes ectoderm of the head, apical organ, brain, photoreceptors (ocelli), and anterior ciliated prototroch.

Additional ectoderm arises from cells in the second and third quartets. The second quartet also typically contributes to the nervous system, trunk ectoderm that is posterior to the prototroch, and to the posterior prototroch itself. The third quartet also contributes to the nervous system. Together, the second and third quartets contribute to the ectodermal foregut including the stomodeum (the anterior-most opening of the digestive tract) and esophagus (or foregut), and, when present, to the ectodermal proctodeum. The mesoderm comes from cells of the second, third and fourth quartets (Figs. 1,2). Second and third quartet micromeres produce so-called *ecto*mesoderm that makes larval and adult muscle cells (Boyer *et al.*, 1996; Henry and Martindale, 1999). There is considerable interspecies variation in the specific cells that generate ectomesoderm (Henry and Martindale, 1999; Hejnol *et al.*, 2007; see Fig. 2). In contrast, in most species, the 4d cell is the sole source of another type of mesoderm, the so-called *endo*mesoderm (Lambert, 2008; Hejnol, 2010; Lyons *et al.*, 2012). Finally, endoderm arises from vegetal cells, typically from macromeres that remain after the quartets of animal micromeres are born. When present, fourth and fifth quartet micromeres also contribute to the endoderm (see Fig. 2). To compare gastrulation mechanisms between spiralians, we must understand what cell lineage variation exists within this overall conserved framework. Thus next we briefly review variation within the two sources of mesoderm.

Ectomesoderm arises from diverse lineages

Ectomesoderm gives rise to muscle cells that function in the larva, and may persist into adults stages. Ectomesoderm appears to arise mainly from 3a and 3b in a variety of spiralian taxa (Table 1; Fig. 2), including the annelids, molluscs, and nemerteans (but apparently not the platyhelminthes). Thus, 3a/3b-derived ectomesoderm could represent a pleisiomorphic condition for the spira-



*Lineage analyses performed using modern cell lineage tracers.

Fig. 2. Diversity of cell fates. *Axial placement of micromere quartet derivatives and their ultimate fates in the formation of the ectoderm, mesoderm (as ecto- and endomesoderm) and endoderm (colored according to the key). Cases marked with an asterisk are based on the use of modern cell lineage tracers. For the most part (except* Ilyanassa obsoleta, *and* Hoploplana inquilina), *the final larval and adult domains of the micromere quartet are similar, and these exhibit an alternating set of relationships relative to the dorsoventral axis, as shown. Data from the studies of Anderson (1973); Henry and Martindale (1994, 1996, 1998); Damen (1994); Henry* et al., *(1995); Boyer* et al., *(1996, 1998, unpublished); Ackermann,* et al.,*2005); Hejnol* et al., *(2007); Meyer* et al., *(2010); Meyer and Seaver, (2010); Fischer and Arendt (2013); Chan and Lambert (2014). Modified from Henry and Martindale (1999).*

lians (see Hejnol *et al.*, 2007; Hejnol, 2010). In various species additional micromeres can also give rise to ectomesoderm; these might have evolved independently in different lineages (Table 1). In most species examined, micromeres of the first quartet (1a-1d) do not contribute to mesoderm (Fig. 2). There are notable exceptions: in the leech *Helobdella* the primary quartet cells a', b' and c' (= 1a, 1b, and 1c) contribute muscle fibers to the proboscis as well as epidermal and neuronal cells in the prostomium (Huang *et al.*, 2002). Furthermore, progeny of c'" (=3c) and dm' (=3d) give rise to circular muscle fibers in the proboscis in the leech. Likewise, in sipunculans, the first quartet contributes to ectomesoderm (forming the circular "apical groove"), from where the retractor muscles of the introvert appear to be derived (Torrey, 1903; Gerould, 1906; Åkesson, 1958; see Boyle and Rice, 2014). Cells derived from the D quadrant typically do not form ectomesoderm in molluscs, nemerteans or polyclads, though they may in annelids. Cells of the B quadrant do not appear to generate ectomesoderm in the annelid *Capitella* (Meyer *et al.*, 2010), though they do in all other species examined. These species differences may be due to variation in the inductive signals that specify micromeres along the animal-vegetal or dorso-ventral axes (Verdonk and van den Biggelaar, 1983). Asymmetric partitioning of cell autonomous determinants, which either promote or inhibit mesodermal specification, might also control the restriction of ectomesoderm fates to particular micromere lineages.

Not only are the various origins of the ectomesoderm a topic of interest for spiralian evolution and development, it has also been suggested that ectomesoderm as a whole might be an innovation of the Spiralia (Henry and Martindale, 1999). A better understanding of how these lineages become internalized during gastrulation in different species will shed light on their evolutionary origin. However very little is known about the cellular and molecular mechanisms that control internalization of ectomesoderm.

Endomesoderm arises from the conserved mesentoblast 4d

While ectomesoderm arises from multiple sources, endomesoderm very often arises from a single cell, the 4d micromere. This cell divides bilaterally to form left and right teloblasts that generate endodermal and mesodermal fates in a symmetrical fashion (Fig. 3K-L; Gline *et al.*, 2011; Lyons *et al.*, 2012; Fischer and Arendt, 2013). The endodermal cells generated by 4d contribute to the formation of the hindgut intestine, while the mesodermal daughters form larval, as well as most of the adult, mesodermal tissues, including: muscles, heart, components of the excretory system, additional scattered mesenchymal cells, and primordial germ cells (Gline *et al.*, 2011; Lyons *et al.*, 2012; Rebscher *et al.*, 2012; Rebscher, 2014, this issue; Chan and Lambert, 2014). There are exceptions to this generalization. In the polychaete *Capitella,* 4d behaves as an ectomesodermal progenitor that contributes to the anus, some mesoderm, and the primordial germ cells, but does not form any endoderm (Eisig, 1898; Meyer *et al.,* 2010; Seaver, 2014, this volume). In the clitellate annelid *Tubifex,* teloblasts derived from the 4d micromere generate mesodermal cells, but no endodermal cells (Goto *et al.,* 1999). In the freshwater snail, *Viviparus (Paludina*), the 4d cell appears to behave only as an endodermal precursor, forming no mesoderm (Erlanger, 1891; 1894; Tönniges, 1896; Otto and Tönniges, 1906; Dautert, 1929; Fernando, 1931; see Verdonk and van den Biggelaar,1983).

TABLE 1

Phylum	Sources of Ectomesoderm									References
Genus, species	**2a**	**2b**	**2c**	**2d**	**3a**	**3b**	**3c**	**3d**	**4d**	
Annelida										
Capitella teleta	X		X		X		X	X	X	Meyer et al., 2010
Platynereis dumerelii					X	X	X	X		Ackerman et al., 2005
Polygordius mediteranius	X		X		X	X				Woltereck, 1904
Mollusca										
Dentalium dentale	X		X							van Dongen, 1977
Fiona marina					X	X				Casteel, 1904
Ilyanassa obsoleta		X	X		X	X				Chan and Lambert, 2014
Littorina obtusata					X	X				Deslsman, 1914
Physa fontinalis					X	X				Wierzejski, 1905
Sphaerium japonicum	X		X							Okada, 1936
Unio	X									Lillie, 1895
Crepidula fornicata			X		X	X				Hejnol et al., 2007
Patella vulgata		X			X	X				Damen and Dictus, 1994
Nemertea										
Cerebratulus lacteus					X	X				Henry and Martindale, 1998
Platyhelminthes										
Hoploplana inquilina		X								Boyer et al., 1996, 1998

Gastrulation mechanisms: formation of the archenteron, and internalization of ecto- and endomesoderm

Relatively few studies have looked at the behavior of cells during gastrulation, or studied the molecular pathways that control them, in spiralians. Below we discuss what is known about the major modes of internalization of the endoderm, ectomesoderm and endomesoderm. We highlight data from those species currently being studied, and especially in which modern intracellular lineage tracing has been carried out.

Endoderm internalization: invagination

Invagination involves a sheet of vegetal cells bending to form an inpocketing that becomes the embryonic gut, or archenteron. Gastrulation by invagination is widespread in spiralians, for example in the nemertean *Cerebratulus* (Wilson, 1900), the polychaetes *Hydroides* (Arenas-Mena, 2006: Fig. 4 A-B) and *Owenia* (Smart and von Dassow, 2009; Fig. 3 A-F), the echiuran *Urechis* (Pilger, 1997; Newby 1940), the oyster *Saccostrea* (Kakoi *et al.*, 2008), the pond snail *Lymnaea* (Morrill, 1982), and the scaphopod *Dentalium* (Wilson, 1904). In species that gastrulate by invagination, the primary quartet micromeres are typically roughly equal to, or in some cases larger than, the macromeres (Pilger, 1997). Following cleavage stages, a coeloblastula (hollow cluster of cells with a central fluid-filled lumen) is often formed (Iwata, 1985; Henry and Martindale, 1997; Pilger, 1997), and these species tend to be small and exhibit indirect development, and make planktotrophic larvae.

The details of invagination have been examined in only a few species. Prior to invagination in *Owenia,* the vegetal cells of the blastula become columnar and the vegetal plate flattens. The flattened vegetal plate consists of a few dozen cells, and then buckles to form an invagination that becomes the archenteron. The archenteron elongates and remains open via the blastopore (Smart and von Dassow, 2009; Fig. 3 A-F). A few studies have examined what mechanisms control invagination. In the scaphopod *Dentalium*, Delage (1899) and more precisely, Wilson (1904) showed via cutting experiments that specific determinants are localized to the vegetal region, which are essential for gastrula-

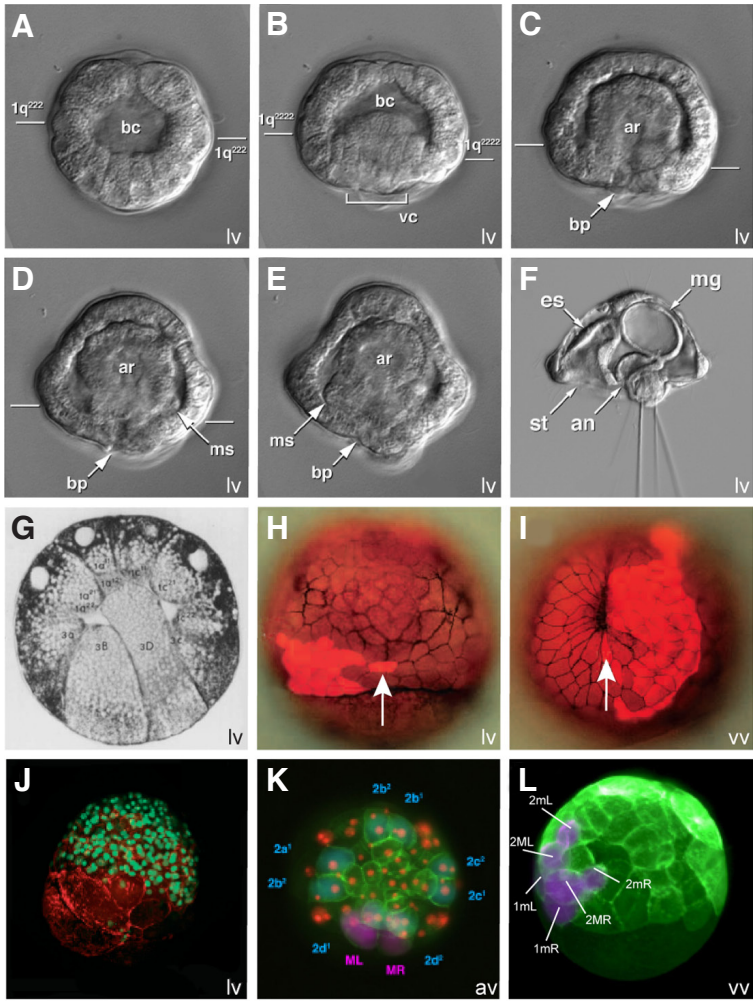


Fig. 3. Diversity of gastrulation modes. (A-F) *DIC time-lapse images of gastrulation by invagination in the polychaete* Owenia collaris. *The vegetal cells (vc) buckle into the blastocoel (bc) to form the archenteron (ar), which remains open via the blastopore (bp). Mesoderm (ms) is seen at the base of the archenteron. Later, the blastopore persists and develops into the anus (an), which makes the posterior portion of the alimentary canal, connecting to the midgut (mg), esophagus (es) and the stomodeum (st). Modified from Smart and von Dassow (2009).* **(G)** *Meridional cross-section of a 32-cell stage* Patella vulgata *embryo. The extended 3D macromere makes contact with the 1q micromeres. Modified from van den Biggelaar (1977).* **(H-I)** Helobdella robusta *gastrulation by epiboly. Modified from Smith* et al., *(1996). Silver staining of embryo in which the opq"L micromere was injected with red lineage tracer. At early stages of epiboly* **(H)** *the cells are cuboidal (arrow), while at late stages of epiboly* **(I)** *some of them have become wedged shaped where the blastopore lip is narrowing.* **(J)** *Gastrulation by epiboly in the polyclad flatworm,* Maritigrella crozieri. *Confocal images of embryos stained with sytox green (DNA, green) and phalloidin (actin, red). The micromeres make an irregular double-layered cap that spreads over the macromeres. Modified from Rawlinson (2010).* **(K-L)** *Live fluorescent images of the snail* Crepidula fornicata *during early stages of gastrulation by epiboly. (Lyons and Henry, unpublished data). Eggs were injected with mRNA for a histone-RFP fusion to visualize DNA and the actin-binding motif of utrophin, fused to GFP to visualize the actin cytoskeleton. The 2q (blue) and 4d (magenta) descendants are pseudo-colored for increased visibility. 4d daughter cells ML/MR are covered with the 2d clone* **(K)** *and as gastrulation proceeds, the micromere cap flattens and only the vegetal-most portion of the endodermal cells 1mL/R remain exposed* **(L)**. *Views in all panels are as follows: lateral view (lv); animal view (av), and vegetal view (vv).*

tion, and subsequently, normal development. Likewise, in the nemertean *Cerebratulus,* Wilson (1903), Zeleny (1904), Yatsu (1904, 1910), and Freeman (1978) showed that during the interval between the time of germinal vesicle breakdown and third cleavage (8-cell stage) determinants required for normal development, gastrulation, and the formation of animal (apical tuft) and vegetal (endodermal) tissues, are progressively restricted along the animal-vegetal axis. What these determinants are, and how exactly they control invagination, remains an important open question.

Endoderm internalization: epiboly

Epiboly involves the spreading of surface cells to form a contiguous layer that covers other cells. Epiboly is seen in the clitellate annelids *Helobdella* (Smith *et al.*, 1996; Fig. 3 H-I) and *Tubifex* (Shimizu, 1982), the polychaetes *Capitella* (Boyle and Seaver, 2008 ; Boyle *et al.*, 2014) and *Platynereis* (Fischer *et al.*, 2010), the echiuran *Bonellia* (Pilger, 1997), the spiunculan *Themiste* (Boyle and Seaver, 2010), polyclad flatworms including *Imogine* (Younossi-Hartenstein and Hartenstein, 2000) and *Maritigrella* (Rawlinson, 2010; Fig. 3J), the gastropods *Crepidula* (Conklin, 1897; Fig. 3 K-L), and *Ilyanassa* (Chan and Lambert, 2014), and cephalopods like the squid *Loligo* (Arnold, 1971; Singley, 1977). Epiboly is characteristic of spiralians with yolky eggs that form stereoblastula (a solid cluster of cells with no internal lumen). Typically in these species the macromeres are very large and the micromeres are small. The 1st-3rd quartet micromeres form an animal cap that expands by epiboly to engulf yolky macromeres (and 4th quartet micromeres).

The cell biological basis of epiboly has been studied best in *Helobdella* (Smith *et al.*, 1996) and *Loligo* (Singley, 1977). In *Helobdella* (Fig. 3 H-I; 5 E-F), three categories of cells are present prior to gastrulation: 1) a cap of micromeres at the animal pole, 2) bilateral germinal bands consisting of coherent columns of blast cells, generated by ten ectodermal and mesodermal teloblasts of the D quadrant beneath the cap, and 3) a syncytium formed by the fusion of the A"'-C"' macromeres (=3A-3C) beneath the germinal bands (Smith *et al.*, 1996). Expansion of the animal micromere cap towards the equator occurs in two phases: the first involves cell divisions, and the second, cell spreading. The cap then constricts towards the vegetal pole, and the cells at the leading edge become wedge-shaped (Fig. 3 H-I). Epiboly of the bilateral germinal bands occurs by teloblastic growth generated by division of the teloblasts; as the bands become longer they bend and eventually coalesce at the vegetal pole. The micromere cap and the germinal bands make epibolic movements, while staying in register, as they cover the macromere syncytium. Smith *et al.* (1996) tested which tissue generates the force that drives epiboly by experimentally reducing each separately; they laser ablated micromeres in the ectodermal cap, or chemically arrested the teloblasts by injecting them with ricin A chain (which inactivates ribosomes, thus inhibiting protein synthesis). Those experiments revealed that while each tissue *can* move independently, both are needed for proper epiboly. The primary motive force is in the germinal bands. This was demonstrated in cases where the micromere cap was experimentally reduced, in which it could be seen that

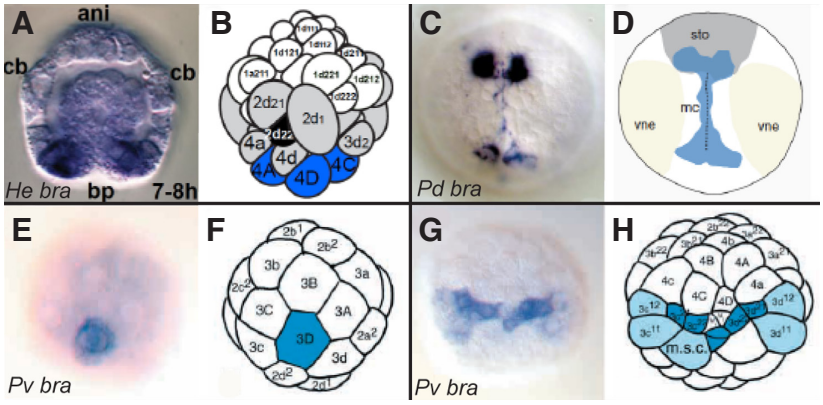


Fig. 4. *brachyury* mRNA expression during gastrulation. (A) *Lateral view of* Hydroides elegans *gastrula stained for* brachyury (Hebra), *which is expressed in cells ringing the blastopore (bp). cb, ciliary band; an, animal pole.* **(B)** *At an earlier stage, 4th quartet macromeres at the vegetal pole express* Hebra *(blue), but other vegetal cells (grey) have not turned it on yet. Modified from Arenas-Mena (2013).* **(C,D)** *Expression of in* Platynereis dumerilli brachyury *(*Pdbra*) after gastrulation has ended. It is expressed in the ventral midline posterior to the stomodeum (sto), the midline cells (mc) and a more posterior patch. vne, ventral neurectoderm. Modified from Arendt* et al., *(2001).* **(E-F)** Patella vulgata brachyury *(*Pvbra*) expressed in the 3D macromere.* **(G-H)** Pvbra *expressed in micromeres in the posterior blastopore and in the 2d-derived mesodermal stem cell (m.s.c.). Modified from Lartillot* et al., *(2002a,b).*

the movement of the germinal bands stretched the cap, despite it having fewer cells. This experiment also revealed that there is a tight connection between the micromere cap and the syncytial endoderm. After the germinal bands stretched the experimentally reduced micromere cap as far as the cap was able, a thin sheet of macromere folded back over the germinal bands, as the bands continue to move vegetally (Fig. 5E vs F).

Experiments in the squid *Loligo*, showed that the mechanisms of gastrulation are different from that in *Helobdella*. Like all cephalopods, early development in *Loligo* retains no sign of spiral cleavage. The embryonic blastomeres form a blastodisk atop a multinucleate syncytium filled with yolk, somewhat akin to that in avian embryos (Arnold, 1965). Epiboly occurs as the advancing blastodisk expands vegetally to cover the syncytial yolk cells. Singley (1977) proposed a model in which epiboly occurs by the coordinated contraction of a circumferential belt of cortical actin in the syncytium, in conjunction with active migration of the marginal cells of the blastodisk, the latter extending lamellapodia and filopodia. Thus, unlike *Helobdella*, a stable connection between the epibolizing cap/blastodisk cells and the syncytial endoderm does not occur in this species.

Since experiments in both *Helobdella* and *Loligo* suggest that the endoderm plays an active role in epiboly (and is not merely a passive substrate), one can ask if these interactions are tied with the syncytial nature of the endoderm in such extremely yolky embryos. This hypothesis could be tested by examining the process in other clitellate annelids, such as *Tubifex*, in which the germinal bands are not covered by a micromere cap, and the macromeres do not fuse to form a syncytium (Shimizu, 1982). During a recent study of gastrulation in the snail *Crepidula,* we observed that the rate at which the micromere cap advances is coordinated with divisions of the deeper macromeres, suggesting that changes in cell shape, stiffness and/or adhesion of the macromeres might regulate epiboly in this species (Lyons and Henry, unpublished observations). Experimental perturbation of gastrulation should be carried out in more species to examine what other mechanisms might exist.

Intermediate forms, emboly and other variations on gastrulation

A combination of epiboly and invagination has been reported in polychaetes (e.g. *Chaetopterus*, Malakhov, 1984), in sipunculans (Cutler 1995, Pilfer 1997), and in molluscs such as the limpet *Patella* (Lartillot *et al.*, 2002a, 2002b; Fig. 3G; 4 E-H), and the abalone *Haliotis* (Fig. 5 C-D; Koop *et al.*, 2007). Collier (1997) describes gastrulation in *Patella* as occurring by emboly, a form of invagination where the macromeres or their daughter cells extend into the blastocoel to make the archenteron. Emboly appears to be more common in equally cleaving spiralians, which require early direct contact between the macromeres and first quartet micromeres for induction of the organizer and the dorsal-ventral axis (Fig. 3G; van den Biggelaar 1977; Martindale *et al.*, 1985; Lambert and Nagy 2003). Other distinct forms of gastrulation have been described. The aplachophoran solenogaster *Neomenia* exhibits a highly modified form of gastrulation that combines invagination and ingression (Thomson, 1960; Hadfield, 1979). Uniquely, it is the invaginated cells that eventually give rise to definitive adult ectoderm (Thomson, 1960). A few described species form a flattened blastula, called a plakula, and in these cases, gastrulation proceeds through a form of invagination or cavitation, for example in the gastropod *Viviparus* (Dautert, 1929), and the polychaete *Polygordius* (Woltereck, 1904; Kato, 1968).

While gastrulation by epiboly is likely ancestral for the platyhelminths, highly divergent forms have evolved in response to increased yolk storage. For example, ectolecithal development is common, in which yolk is extraembryonic, and must be incorporated into cells later in development (Martín-Durán and Egger, 2012). In some platyhelminth species, vegetally-derived "hull" cells envelop the embryo primordium by a process of reverse epiboly (Willems *et al.*, 2009). Some species exhibit what is called anarchic cleavage, where individual blastomeres migrate through the yolk in the egg capsule, and in these cases, development is so modified that some authors have argued that no comparisons to gastrulation in other embryos can be made (discussed by Martín-Durán and Egger, 2012). However, homologs of key transcription factors associated with epithelial-to-mesenchymal transition (EMT) and gastrulation in other systems such as *twist*, *snail* and *foxA* are expressed in migratory cells of entolecithal developers. These data raise the possibility that the GRNs for morphogenetic events could be used as a basis for comparison of gastrulation between these species and other animals (Martín-Durán *et al.*, 2010).

Molecular control of endoderm internalization

We are just beginning to understand some of the pathways and regulatory factors that could control gastrulation in spiralians. For example, the beta-catenin and MAPK signaling pathways (which are known to be involved in gastrulation in other metazoans) have been inhibited in several spiralians, and in some cases, gastrulation

is perturbed. But gastrulation is not effected in all species, begging the question of whether these pathways regulate gastrulation directly (e.g., control cell shape, internalization), or indirectly (e.g. controlling cell fate along the animal-vegetal and dorsoventral axes).

For example, in *Cerebratulus*, beta-catenin protein becomes localized to the nuclei in the vegetal-most cells, which is essential not only for the specification of the vegetal (posterior) endodermal and endomesodermal progenitors, but also for invagination (Henry *et al*., 2008). In *Crepidula,* Henry (2010) showed that beta-catenin protein is broadly expressed throughout the early embryo, but later becomes restricted to the 4d lineage. At even older stages, *beta-catenin* mRNA is expressed in cells surrounding the blastopore and stomodeum. Loss-of-function experiments using injected morpholinos revealed that beta-catenin is required for the normal process of epiboly in *Crepidula*. These findings differ somewhat from those obtained for another spiralian, the polychaete annelid, *Platyneries* (Schneider and Bowerman, 2007). In that species, functional studies indicate that beta-catenin plays a role in asymmetric cell fate specification associated with cell divisions along the animal-vegetal (future anterior-posterior) axis, but epiboly, per se, was not perturbed. Future studies will need to address if beta-catenin signaling has a direct influence on morphogenetic events during gastrulation in other species.

The MAPK signaling pathway has been manipulated in several species as well. In *Crepidula*, blocking early MAPK signaling prevents the dorsal organizer from forming, resulting in a radialized embryo. Blocking MAPK signalling also prevents epiboly, and such embryos never internalize of endoderm and mesoderm (Fig. 5 A-B; Henry and Perry, 2008). In contrast, when MAPK signaling is blocked in *Ilyanassa*, the organizer is prevented from forming, but no obvious gastrulation defect was reported (Lambert and Nagy, 2001). In *Capitella*, antibody staining for anti-diphosphorylated-Erk-1&2 is seen in some cells around the blastopore during gastrula stages. However, blocking MAPK signaling in *Capitella* does not affect gastrulation, though it should be noted that in this species MAPK signaling is not involved in establishing the organizer either (Amiel *et al*., 2013). In another polychaete, *Platynereis*, dpERK signal is also seen in a small number of cells adjacent to blastopore, and in macromeres abutting these smaller cells (Pfeifer *et al.,* 2014); MAPK signaling inhibition leads to defects in gastrulation behavior of trunk mesodermal cells. In *Haliotis*, blocking MAPK signaling results in radialized embryos that often cannot complete gastrulation and have evaginated endoderm (Koop *et al*., 2008; Fig. 5 C-D). Clearly, the function of this pathway in gastrulation does not appear to be conserved, and in cases where gastrulation *is* perturbed in the absence of the organizer, it remains to be seen if this is a direct or indirect effect.

Some studies have begun to ask if genes known to be necessary for normal gastrulation in other metazoans, for example the transcription factors *brachyury* and *foxA* (Kusch and Reuter, 1999; Fritzenwanker *et al*., 2004; Annunziata *et al*., 2014), could also be expressed during gastrulation stages in spiralians (Arenas-Mena, 2013; Boyle and Rice 2014). In the polychaete *Hydroides, brachyury* and *foxA* are expressed dynamically in the blastopore as cells invaginate (Arenas-Mena, 2006, 2013; Fig. 4 A-B). Additional transcription factors such as *T-brain, blimp, otx,* and *SalI/spalt* are likewise expressed transiently in the invaginating vegetal tissue (Arenas-Mena and Wong 2007; Arenas-Mena 2013, 2014, this volume). In another polychaete, *Platynereis*, *brachyury* and *otx* are not expressed in the endoderm during gastrulation, and instead come on only later after epiboly is complete (Arendt *et al*., 2001; Fig. 4 C-D). Arenas-Mena (2013) hypothesized that these differences could be explained by the different trophic strategies (feeding versus non-feeding larvae) and different cellular behaviors

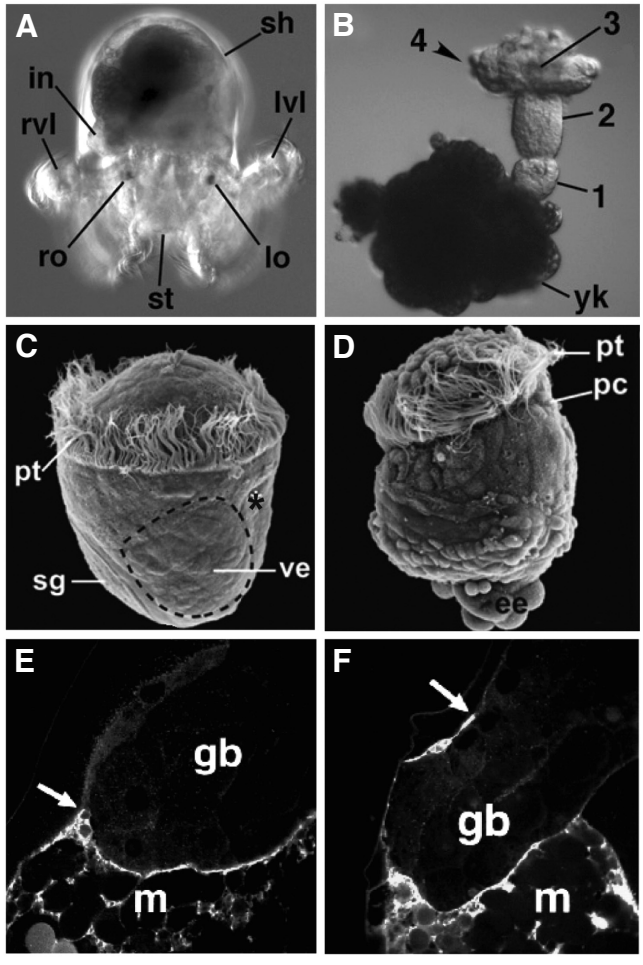


Fig. 5. Cellular and molecular control of gastrulation. (A,B) *Veliger stage* Crepidula fornicata *control* **(A)** *and U0126-treated embryo to inhibit MAPK signaling* **(B)**. *The U0126-treated embryo fails to complete epiboly, as seen by the exposed yolky cells (yk). The numbered structures are derived from the correspondingly numbered micromere quartets. Arrowhead points to radial extensions derived from the fourth quartet micromeres. in, intestine; sg, shell gland;, lvl, left velar lobe; rvl, right velar lobe; lo, left ocellus; ro, right ocellus; st, stomodeum. Modified from Henry and Perry (2008).* **(C)** *SEM of control* Haliotis asinina *larva. (*), stomodeum; pt, prototroch; sg, shell gland; ve, ventrolateral ectoderm.* **(D)** *stage-matched U0126-treated embryo showing evaginated endodermal cells (ee) at the vegetal pole; modified from Koop et al. (2007).* **(E,F)** *Thick section of the connection between the micromere cap and the macromere syncytium (m), surrounding the germinal band (gb) in* Helobdella robusta. **(E)** *Control embryo where the boundary between the micromere cap and syncytium lies at the vegetal side of the germinal band (arrow).* **(F)** *Embryo in which the micromere cap was experimentally reduced by ablation, showing that a thin fold of macromere cytoplasm and membrane extends around the germinal band, making contact with the reduced ectoderm cap on the animal side of the germinal band (arrow). Modified from Smith et. al. (1996).*

(invagination vs. epiboly) used by the two species. To test this interesting hypothesis, it will be necessary to survey expression of these and additional components of endomesodermal gene regulatory networks in a wide range of spiralians that have diverse mode of gastrulation. For example in *Tubifex* (which gastrulates via epiboly), *brachyury* is not expressed in the endoderm during gastrulation (Kitakoshi and Shimizu, 2010), similar to *Platynereis*. On the other hand, in the oyster *Saccostrea*, which gastrulates by invagination, *brachyury* is not expressed in the endoderm during invagination, in contrast to *Hydroides* (Kin *et al*., 2009). In the polychaete *Capitella,* and in the sipunculan *Themiste*, which both gastrulate by epiboly, *foxA* is expressed in the endoderm during gastrulation (Boyer and Seaver, 2008; 2010), which is at odds with the hypothesis presented by Arenas-Mena (2013). Likewise, brachyury is expressed prior to gastrulation in vegetal macromeres in Capitella (Boyle and Rice, 2014). The expression patterns of genes such as *brachyury* and *foxA* have been reported in several species that exhibit "intermediate" forms of gastrulation. In *Patella,* which gastrulates by emboly (Fig. 1G), *brachyury* is first expressed in 3D; then later in 4D/4d, prior to their internalization and it becomes expressed in the 3c/3d/2d micromeres, which reside at the posterior edge of the blastopore (Fig. 4 E-H; Lartillot *et al*., 2002a). In *Haliotis*, *brachyury* is likewise seen at the posterior edge of the blastopore, but not observed in the 3D lineage. In *Chaetopterus*, *foxA* is expressed in four vegetal cells and then expands to more cells of the vegetal plate during gastrulation. In *Patella*, *foxA* comes on in vegetal cells at the 32-cell stage, in 3A-3C, then in their macromere and micromere daughters more strongly, but notably not in the D quadrant (Lartillot *et al*., 2002b).

These results show that expression patterns for morphogenetic regulatory genes are different in each species, and that more data are necessary to assess whether such differences correlate with varying gastrulation modes per se, or are associated with particular cell fates. It will also be important to inhibit the function of these genes to test their role in gastrulation versus cell fate specification in the future.

Ectomesoderm internalization

Details describing the behavior of ectomesodermal precursors are particularly scarce. It appears that the ectomesoderm may become internalized either by directed cell divisions that ultimately position these cells deep below the surface, or by cellular delamination or ingression from the ectoderm. In polyclad flatworms, the 1st-3rd quartet micromeres form an irregular double-layered micromere cap (Fig. 3J), with the ectomesoderm already internalized prior to the beginning of epiboly (Younossi-Hartenstein and Hartenstein, 2000; Rawlinson, 2010). In *Imogine*, although the internalization of these ectomesodermal cells could not be determined definitely, mitotic figures were never seen to be perpendicular to the surface as this cap proliferated, suggesting that either the ectomesodermal cells are born to the interior very early, or they delaminate prior to, and during, epiboly.

In molluscs, some descriptions indicate that the definitive ectomesoderm ultimately appears as two deeper, bilateral masses of cells located to the sides of the blastopore (Verdonk and van den Biggelaar, 1983).

It has also been suggested that the ectomesoderm forms from the anterior edges of the blastopore lip (van den Biggelaar and Dictus, 2004), but details of how it becomes internalized are lacking. In *Ilyanassa*, ectomesoderm arises mainly from the 3a and 3b cells, which appear initially as large cells that lie lateral to the anterior blastopore and beneath the superficial ectoderm (Chan and Lambert, 2014). During gastrulation stages in *Crepidula,* ectomesodermal cells reside along the anterio-lateral lip of the blastopore, as bilateral progeny of 3a and 3b. We have observed progeny of 3a and 3b leaving the lip of the blastopore and passing deeper into the embryo where they become covered by trailing ectodermal cells at the surface (Lyons and Henry, unpublished). These mesodermal cells become mesenchymal and migrate to remote locations in the embryo. Few genes that might be involved in internalization of the ectomesoderm have been investigated, however some likely candidates are genes such as *goosecoid* and *foxA* that are expressed at the anterior/anterior lateral lip of the blastopore (Lartillot *et al*., 2002b). Likewise, the transcription factor *twist* (associated with the process of EMT in many metazoans) is expressed in ectomesoderm in the gastropod *Patella vulgata* (Nederbragt *et al*., 2002).

Endomesoderm internalization

Among some spiralians that gastrulate by epiboly, the 4d cell becomes internalized by the advancing cap of micromere cells. In others, the 4d cell is born beneath, or partially covered by, the ectoderm. For example, in *Crepidula,* the 4d cell is tear-dropped shaped, and only the most peripheral side of the cell is exposed to the surface near the equator (Fig. 3K; 6 A-B,E). The rest of this cell lies under the micromere cap (Conklin, 1897; Lyons *et al*., 2012). When the 4d cell starts dividing, all of the mesodermal sublineages (along with some endodermal progeny) are born beneath this cap, while the progeny that remain exposed to the surface are exclusively endodermal (Fig. 3K; 6 A-B,E; Lyons *et al*., 2012). These exposed endodermal progeny (as well as 4a-4c) move along with the advancing lip of the blastopore (Fig. 6E), but eventually, ectoderm associated with the blastopore lip covers those cells as well (Fig. 6 C-D,F). In the leech *Helobdella,* the 4d cell (DM") becomes enveloped at its birth by a lamellapodial extension from the macromeres (Isaksen *et al*., 1999).

Less is known about the internalization of 4d in species that gastrulate by invagination, though it is likely internalized with the vegetal plate. In some reports, mesoderm is said to ingress around the same time as invagination, taking residence near the base of the archenteron (*Owenia*; Fig. 3 D-E Smart and von Dassow, 2009), or off the tip of the archenteron (*Saccostrea*, Kakoi *et al*., 2008). In other reports it is the 4d lineage itself that initiates invagination: in the mussel *Septifer,* for example, the 1ML/R and 1mL/R cells initiate invagination (Kurita *et al.,* 2009). Thus, the 4d lineage can become internalized in different ways and may occur when the clone contains one to several cells.

Morphogenetic events following gastrulation: formation of the mouth and anus, fate of the blastopore, and axial elongation

Technically, the process of gastrulation ends when endoderm and mesoderm are fully internalized. Yet, several significant morphogenetic events continue beyond this point that bear on the establishment of the through-gut in spiralians. Most extant animals derive their mouth and/or anus from the blastopore, a transient embryonic structure, broadly defined as the site of gastrulation (Technau, 2001;

Hejnol and Martindale, 2009). Echinoderms, hemichordates and chordates are classically defined by deuterostomy, a condition in which their anus forms in association with the posterior blastopore, while the mouth forms as a separate invagination in anterior, ventral ectoderm. Deuterostomy is also seen in some ecdysozoans and spiralians (Smart and von Dassow, 2009; Hejnol and Martindale, 2009; Martín-Durán *et al.*, 2012). More typically, however, species that exhibit spiral cleavage have been referred to as exhibiting protostomy, where the blastopore gives rise to the mouth, and the anus forms as a secondary posterio opening (van den Biggelaar *et al.*, 2002; Arendt, 2004; Hejnol and Marindale, 2009). In reality, the relationship between the blastopore lip/blastopore and the mouth/anus is not very well understood, and varies between spiralian species (Hejnol and Martindale, 2009). What mode is ancestral for the spiralians (or bilaterians for that matter), and how did deuterostomy and protostomy evolve? The spiralians are a particularly interesting group for asking these questions, because the fate of the blastopore, and the origin of the mouth and anus, can be studied in the context of a homologous cleavage program.

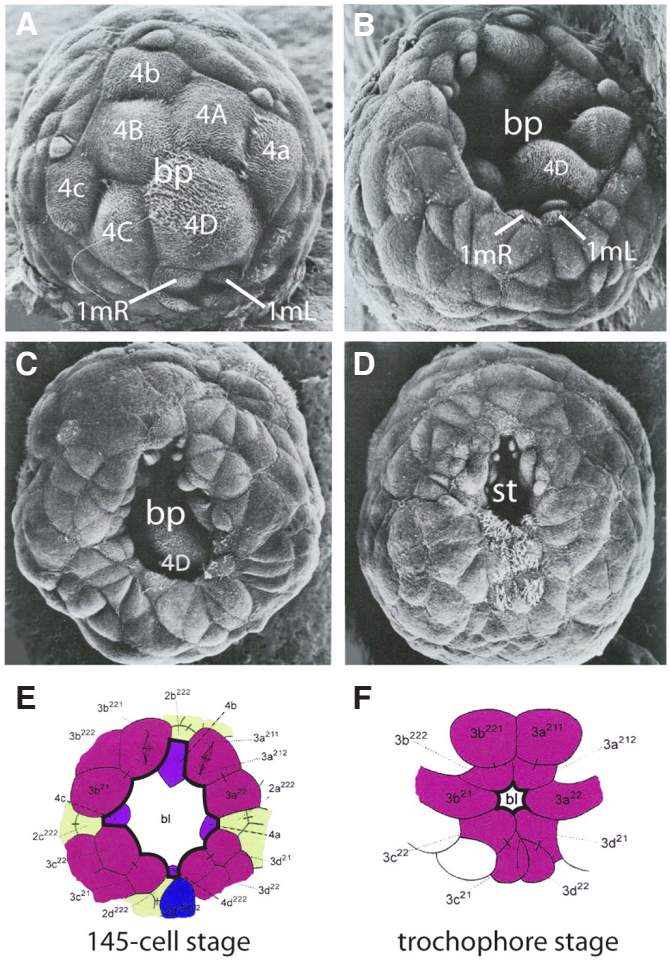


Fig. 6. Behavior of cells at the lip of the blastopore. (A-D) *SEMs of* Crepidula fornicata*, showing vegetal views of internalization of the endoderm (derived from 4D-4A, 4a-4d [including 1mL/1mR]) during epiboly. Also seen is the narrowing of the blastopore (bp), which in this species matures into the opening of the stomodeum (st) or mouth. Modified from Verdonk and van den Biggelaar (1983).* **(E-F)** *Cell-rearrangement of micromere lineages at the blastopore lip (thick black line) during epiboly in* Trochus magus*. Purple, 4th quartet micromeres; magenta, 3rd quartet micromeres; yellow, 2nd quartet micromeres; blue, 2d1222. Modified from van den Biggellar and Dictus (2004).*

How do we define the spiralian blastopore?

In metazoans, in general, the blastopore is typically defined as the site of internalization of gastrulating cells (which includes presumptive endoderm and mesoderm), or as the cells surrounding the opening into the archenteron during gastrulation. The composition of cells at the blastopore is often transient. For example, during sea urchin and frog invagination, cells destined to be endoderm and mesoderm initially reside at the surface of the embryo and become internalized by moving over the blastopore lip as the archenteron grows (Stern, 2004). At the end of gastrulation in those embryos, the site of the transient blastopore persists as the boundary between the internalized endoderm and the adjacent external ectoderm (Technau and Scholz, 2003). In spiralians that gastrulate by invagination (e.g. *Hydroides* Fig. 4 A-B and *Owenia* Fig. 3 A-D), the blastopore can be defined in a similar fashion.

However, defining the blastopore in species that gastrulate via epiboly is more subjective, because no actual "pore" forms until very late in the process. We define the blastopore and blastopore lip as follows. The endodermal macromeres and fourth quartet micromeres lie exposed within the "opening" of the blastopore itself. We define the "lip" of the blastopore in such species as the leading edge of the ectodermal micromere cap, which is made of 2nd and 3rd quartet micromeres and their progeny (Fig. 6 E,F). As epiboly proceeds, the circumference of the blastopore lip decreases, and the arrangement and composition of micromere descendants located at the lip changes (Fig. 6 E,F). In some species, the constricted blastopore remains open, while in others it closes completely, and thus ceases to exist; in still other species, the cells meet along the left and right lateral edges of the blastopore lip, leaving persistent openings only at the anterior and/or posterior ends (Hejnol and Martindale, 2009). This variability has lead to considerable debate about the exact relationships between the blastopore lip/blastopore and the origin of the mouth and the anus (van den Biggelaar *et al.,* 2002; Arendt, 2004; Hejnol and Martindale, 2009). Only careful examination of defined cell lineages during gastrulation can speak to such debates. In the next sections we review what data already exist and point out remaining lacunae.

Is the mouth derived from the blastopore?

The mouth, and the esophagus, are ectodermal inpocketings that make the foregut, and connect to deeper parts of the alimentary canal, which come from endoderm (see discussion in Meyer *et al.*, 2010). Cells that give rise to the mouth are often called "stomatoblasts" (Wilson, 1892). Classical descriptions of development reported, and modern lineage tracing confirmed, that the stomatoblasts are often derived from the vegetal daughter cells of 2nd quartet micromeres (2q2); cells from the 3rd quartet can also contribute to the mouth. The deeper esophageal tissues arise from 2nd and 3rd quartet micromeres, and the relative contribution of 2q vs. 3q to the mouth vs. the esophagus varies between species (Meyer *et al.*, 2010; Chan and Lambert, 2014). The 2nd and 3rd quartet micromeres, including the stomatoblasts, generally lie at the blastopore lip (Wilson, 1892; Lartillot *et al.*, 2002a,b; Lambert and Chan, 2014). For example, in the polychaete *Nereis* the

stomatoblasts were reported to come from 2a22-2c22 and these cells reside at the right (2a22), anterior (2b22) and left (2c22) edges of the blastopore lip at late epiboly stages (Wilson, 1892). At the anterior edge of the blastopore, between 2a22 and 2b22, and 2b22 and 2c22, respectively, are derivatives of 3a and 3b micromeres. The 3d and 3c progeny reside at the posterior edge of the blastopore. Similarly, stomatoblasts are arranged at the blastopore lip in molluscs such as *Ilyanassa* (2a22, 2b22, 2c22; Chan and Lambert, 2014), *Crepidula* (2a2, 2b2, and 2c2; Conklin 1897) and *Physa* (2a22, 2b22, 2c22; Wierzejski, 1905). Modern lineage tracing in *Capitella* revealed that 2a-2c and 3a-3d contribute to the mouth (Meyer *et al*., 2010), but the contribution to the early blastopore lip itself is not known. In the nemertean *Cerebratulus* 2a, 2c, 2d and 3a-3d all contribute to the perimeter of the mouth and the esophagus, while 2b seems to be located deeper inside the esophagus and does not extend to the external opening of the mouth (Henry and Martindale, 1998). It is not known where these cells are relative to the blastopore in this species.

Thus, some of data exist about the position of specific micromere lineages relative to the blastopore. However, in most species, a detailed description of their behavior *over time* is missing. Where it has been examined, the composition of cells that reside at the blastopore lip changes over time (e.g., Wilson, 1898; Child, 1900; van den Biggelaar and Dictus, 2004; Chan and Lambert 2014; Fig. 6). These studies show that cells can divide parallel and/or perpendicular to the circumference of the blastopore, can exchange neighbors, or may leave the blastopore lip entirely (Heath 1899; van den Biggelaar and Dictus, 2004). For example, in the gastropod *Trochus*, the blastopore lip at the 145-cell stage embryo initially includes cells derjved from 2a22-2c22, 3a2-3d2, and at the very posterior edge, cells from the 2d lineage: 2d222 and 2d12222 (Robert,1902; van den Biggelaar and Dictus, 2004; Fig. 6E). As epiboly proceeds, only the 3rd quartet cells remain at the lip of the blastopore; the 2nd quartet cells are excluded, becoming more centrifugal (i.e., more peripheral, if the vegetal pole is the central point of reference; Fig. 6F). Eventually, the 2nd quartet cells do make the mouth (stomatoblasts=2a2-2d2), even though they are excluded from the lip during epiboly. We can presume that after the 3rd quartet cells extend into the embryo to form the esophagus, the more peripheral second quartet cells come together to form the mouth. Thus, although there is species-specific variation, cells within the blastopore contribute to the mouth and anterior gut in several of the best-studied spiralians (see discussion by Chan and Lambert, 2014), and in such cases, they could be considered "protostomes".

On the other hand, an alternative interpretation is possible. If one favors the definition of the blastopore as defined strictly by the boundary between the internalized endoderm, and the adjacent ectodermal 2nd and 3rd quartet micromeres, it could be argued that this ectoderm/endoderm interface becomes internalized as the archenteron grows; those cells that give rise to the mouth then come from a separate subset of the 2nd and 3rd quartet micromeres that are more peripheral to this interface (e.g. in *Trochus*, Fig. 6 E-F). In other words, only ectodermal lineages in direct contact with the endoderm can be called the blastopore lip; those that have lost contact can no longer be considered part of the blastopore, or the blastopore lip. In this interpretation, the mouth does not actually form from the lip of the blastopore, given that the stomatoblasts are not in contact with the endoderm.

Semantics aside, the only way to know whether the mouth comes from the blastopore in any given species is to follow the dynamic behavior of defined cell lineages throughout gastrulation, to avoid building scenarios on misleading "snap shots".

Is the anus derived from the blastopore?

Most spiralians possess an endodermal hindgut connected to an ectodermal anus formed form an ectodermal invagination (i.e., proctodeum). The relative contribution of ectoderm versus endoderm in the terminal parts of the alimentary canal varies between species (see discussion by Meyer *et al*., 2010). The anus is typically formed late in development, and so the exact origin is often not known.

Early reports describe the anus as being derived from specific "anal cells" or "terminal cells" that lie at the posterior end of the larva (e.g., *Crepidula, Littorina, Physa, Patella, Ilyanassa, Umbrella* and *Lymnaea*, see Verdonk and van den Biggelaar, 1983). In *Patella,* the anus is hypothesized to be formed by "terminal" cells, which derive from 3c and 3d, and are located in the posterior end of the larva (Lartillot *et al.,* 2002a). Although he does not indicate which specific lineages form them, Conklin (1897) states that the posterior end of the *Crepidula* embryo is marked by a pair of large ciliated "anal" cells. Using lineage tracers, we have observed that two large posterior cells are formed from progeny of 3c and 3d, which appear to represent these same cells Conklin observed (Lyons and Henry, unpublished data). These cells do not form the anus, however. We have observed that the termination of the hindgut intestine is actually located under a patch of ectoderm derived from 2d, while the two cells Conklin called "anal cells" are located just adjacent to this point, never contributing to the anus (Lyons and Henry, unpublished data). Chan and Lambert (2014) provide a different account in *Ilyanassa,* where lineage tracing showed that two cells derived from 3c appear to form what have historically been termed "anal cells" near the termination of the hindgut. They also mention, however, that there are 1-2 other cells in a similar location derived from 3d. It is unclear, therefore, whether these, or yet other cells, actually form the anus in *Ilyanassa.* A very different arrangement from *Ilyanassa* or *Crepidula* is found for the annelid *Capitella*, where the ectodermal anus (proctodeum) arises from 4d (Meyer *et al*., 2010). Surrounding these 4d-derived presumptive anal cells are cells derived from 2d, 3c and 3d. These results underscore the need for modern lineage tracing, and suggest that earlier classical descriptions should be regarded with caution.

It is also possible that there are no pre-fated cells that give rise to the proctodeum/anus. Rather, it may be that the anus is induced to form by the presence of the underlying hindgut endoderm (e.g., intestine). In fact, this was suggested in the study by Lyons *et al.,* (2012) in *Crepidula*, where it appeared that the anus might not form when specific progenitors of the endodermal hindgut were ablated. Induction of anal cell fate might explain interspecies variation; any one of several cell lineages situated over the hindgut at the time it sends its inducing signal could then form the anus, and the arrangement of particular lineages in this posterior region could vary between species.

A key question remains, are the cells that make the anus ever part of the blastopore lip? Some spiralians are described as deuterostomes, where the opening of the anus forms directly from the blastopore. These species include the polychaete *Owenia* (Smart and von Dassow, 2009; Fig. 3A-F) and the gastropod *Viviparus*

(*Paludina)* (Blochmann, 1883; Erlanger, 1891; Tönniges, 1896; Otto and Tönniges, 1906; Dautert; 1929 and Fernando, 1931). The classical literature generally describes the posterior portion of the blastopore to be occupied by 4d (endoderm/endomesoderm) and more posterior/lateral to it, 3c, 3d, and 2d, which make the posterior blastopore lip (e.g. Wilson, 1898, Child, 1900; van den Biggelaar and Dictus, 2004, Fig. 6E). In *Aplysia* (Blochmann, 1883) and *Umbrella* (*Umbraculum*, Heymons, 1893), the anal cells are reported to lie at the posterior margin of the blastopore, but the specific clonal origins were not determined. In *Crepidula* 2d-derived cells (which likely make the anus) lie at the posterior edge of the blastopore lip transiently during early development (Lyons and Henry, unpublished data). The 2d cells quickly become displaced from the lip by 3c and 3d-derived cells. Furthermore, while some progeny of 3c and 3d also occupy positions along the posterior lip of the blastopore in *Crepidula*, the two large 3c- and 3d-derived cells, which Conklin claimed to be anal cells, are ultimately located far posterior of the blastopore lip. The situation appears to be similar in *Ilyanassa* (Chan and Lambert, 2014). In *Patella*, the progeny of the 3c/3d cells are reported to give rise to the anus and they are born away from (more peripheral to) the blastopore. Hence it is important to underscore that at early stages of epiboly, specific 2nd and 3rd quartet micromeres may initially contribute to the leading edge of the micromere cap, but as the blastopore constricts and the clones proliferate, particular cells that make the anus may be born away from, or very quickly become displaced from, the lip of the blastopore.

The dynamic behavior of cells at the blastopore lip likely explains why so much variation has been documented in the literature about the ultimate fate of these cells and the blastopore itself. The blastopore may remain open in some cases, such as in *Crepidula, Dentalium*, *Littorina*, *Viviparus*, *Ischnochiton*, *Aplysia* and *Limas*; in other species it closes, as in *Anodonata, Sphaerium, Lymnaea, Dreissensia, Ilyanassa*, and *Nereis* (Wilson 1898; Verdonk and van den Biggelaar, 1983). In some cases the blastopore closes at its lateral edges and only the anterior side is described as remaining open (e.g. *Hydroides*, Arenas-Mena, 2013). Note also that the 2d lineage cleavage pattern is highly variable between species (see for example Child, 1900, and Seaver 2014, this issue). If the anus typically comes from the 2d lineage, detailed cell lineage studies are necessary to definitely assess how these and other cells may make up parts of the blastopore lip and how transient their association with the blastopore lip is in different species.

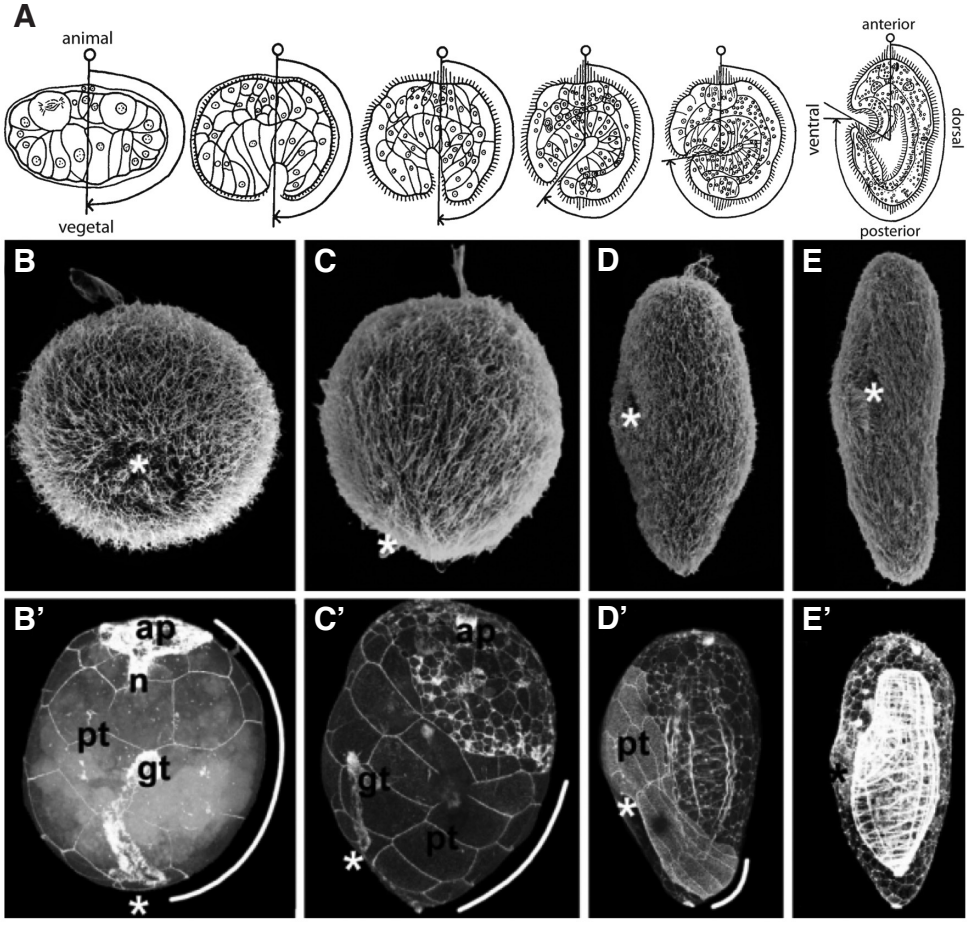


Fig. 7. Distortion of the animal-vegetal axis and axial elongation. (A) *Diagram of the curvature of the animal-vegetal axis by expansion of the dorsal side in the nemertean* Procephalothnx stimulus. *Modified from Iwata (1985).* **(B-E)** *SEM of the nemertean* Carinoma tremaphoros *showing the position of the stomodeum (*) during axial elongation.* **(B'-E')** *Stage matched embryos showing confocal imaging of phalloidin staining to reveal cell outlines beneath the ciliated surface. ap, apical plate; n, neural invaginations; pt, prototroch; gt, gut; (*) stomodeum. In this species the dorsal side of the prototroch is bent by proliferation of 1d-derived cells in the pre-trochal ectoderm. Modified from Maslakova* et al., *(2004b).*

Some spiralians have been described as exhibiting "amphistomy", where the anterior lip of the blastopore remains open and gives rise to the mouth, and the posterior lip remains open to become the anus, as has been argued for the polychaete *Polygordius* (Arendt and Nübler-Jung, 1997, Arendt, 2004). But the inference of amphistomy from Woltereck's (1904) original description of *Polygordius* has sparked debate. While some authors interpret Woltereck's description to support amphistomy (Arendt and Nübler-Jung, 1997), other authors argue that the posterior portion of the blastopore closes and the anus derives from even more posterior 2d-derived cells (van den Biggelaar *et al.*, 2002; Hejnol and Martindale 2009). We are not aware of any study that clearly documents amphistomy via modern, intracellular lineage tracing. Much of the debate is likely rooted in semantics about the definition of the "blastopore" and "blastopore lip." One must consider how the transient association between the anal cells and the posterior blastopore lip may impinge to these arguments.

Regardless of whether or not the mouth and the anus both derive from the blastopore in the strictest sense, the cellular progenitors that make these structures are indeed initially near one another at the vegetal pole at the end of gastrulation (van den Biggelaar and Dictus, 2004).

Subsequent morphogenetic events eventually separate the mouth and anus anlagen, as discussed in the following section.

Axial elongation and anterior displacement of the mouth

In most animals, the mouth forms in the animal hemisphere (Martindale and Hejnol, 2009). In spiralians, regardless of the mode of gastrulation, or the ultimate fate of the blastopore, the definitive mouth forms just posterior to the first quartet-derived cells in the animal hemisphere (Wilson, 1898, Child, 1900). The spiralian stomatoblasts derive from cells that are born in the animal hemisphere. But in species that gastrulate by epiboly, gastrulation takes place such that the constricting blastopore relocates these lineages to the vegetal pole, transiently. Thus, cells that make the mouth must later relocate to the animal pole. This process is poorly understood.

In most spiralian species the anterior-posterior axis (which at this time point is typically still coincident with the original animal-vegetal axis) is roughly perpendicular to the future dorsal-ventral axis in the more or less spherical embryo throughout gastrulation (Fig. 7). Through proliferation and cell rearrangement, the embryo begins to lengthen along the anterior-posterior axis. Coincidently, the vegetal pole and future stomatoblasts become relocated anteriorly along the ventral side and the animal-vegetal axis becomes curved by approximately 90 degrees (Iwata, 1985; van den Biggelaar and Dictus, 2004 Fig. 7). Through these processes, the cells that will make the mouth are separated from posterior cells that will ultimately generate the anus, and collectively undergo what is called axial elongation.

The cellular basis of axial elongation is not well understood, and might vary between species. Some classical studies describe the displacement of the mouth, and axial elongation, as the result of cell proliferation on the dorsal side, within the 2d clone (Child, 1900). In *Patella,* 2d2 has been hypothesized to act as a midline stem cell, dividing repeatedly as the embryo elongates along the anterior-posterior axis (Lartillot *et al.,* 2002a). Flanking 2d2, the 3c1 and 3d1 cells presumably divide in an anterior-posterior direction to give rise to an anterior stomatoblast (3c12/3d12 presumptive mouth contributor) and a posterior "terminal cell" (3c11/3d11, possible presumptive anus). During elongation, stomatoblasts and terminal cells become separated from one another. Whether this is due to intercalation/convergent extension with other lineages, or the result of stem cell like divisions of 2d2 lineage, remains to be demonstrated.

In most spiralians, the prototrochal cells (which separate pretrochal ectoderm from post-trochal ectoderm) remain in an arc perpendicular to the anterior-posterior axis during axial elongation, while the proliferation/movement that drives the mouth anteriorly/ventrally occurs within the post-trochal 2d-derived ectoderm. In contrast, in the palaeonemertean *Carinoma tremaphoros,* the dorsal portion of the prototroch is driven posteriorly during axial elongation by proliferation of 1d-derived *pre*trochal ectoderm (Fig. 7B-E; Maslakova *et al.,* 2004a; 2004b), which so far has only been reported in this species.

We have some clues about the genes that may be involved in regulating axial elongation. In *Patella*, *Platynereis*, and *Haliotis*, *brachyury* mRNA is expressed by cells in the vicinity of the posterior blastopore lip and in the ventral midline during elongation (Arendt *et al.*, 2001; Lartillot *et al.*, 2002a; Koop *et al.*, 2007). However, *Brachyury* protein function has not been inhibited in these species (or any spiralian), to test if it is necessary for axial elongation. When MAPK signaling is blocked in *Haliotis*, the mouth forms at the vegetal pole, and the embryo does not elongate (Fig. 5C), suggesting that this pathway could play a role in the anterior movement of the mouth; however, the expression pattern of *brachyury* in MAPK-inhibited embryos was not drastically different from that in controls (Koop *et al.,* 2007). In *Platynereis,* homologs of Jnk pathway members are expressed in the ventral territory during elongation, and inhibiting Jnk signaling perturbed elongation (Steinmetz *et al.,* 2007). Thus, the role of *brachyury*, MAPK and Jnk signaling in axial elongation should be explored further.

Future directions

With the data at hand, can we infer the ancestral mode of spiralian gastrulation? Yolk content and life history strategy, more than any other factors, seem to dictate the mode of gastrulation in extant species. Yolk-poor eggs tend to make hollow blastulae that gastrulate by invagination and give rise to planktotrophic feeding larvae; yolk-rich eggs tend to make stereoblastulae that gastrulate by epiboly and develop directly or make lecithotrophic non-feeding larvae (Pilger, 1997; Arenas-Mena, 2010). Both extremes are seen in two major groups of spiralians, the annelids and molluscs. Gastrulation by epiboly is probably ancestral for platyhelminths, and gastrulation via invagination is likely ancestral for nemerteans (Wada, 1985; Rawlinson, 2010). Thus, a key question might be: is direct, or indirect, development ancestral for the spiralians, and bilaterians at large? Debate remains, but if we consider the scenario that indirect development is ancestral for the spiralians, then the spiralian ancestor may have gastrulated by invagination, and epiboly might have evolved several times independently, as a response to increased yolk content associated with lecithotrophy (Nielsen, 2001; Dohle, 1999; Freeman and Lundelius, 2005). This might explain why there is so much cell behavior diversity as the blastopore narrows in species that gastrulate by epiboly. On the other hand, if direct development is ancestral, then the spiralian ancestor might have gastrulated by epiboly or emboly. Most of the published lineage tracing data comes from species that gastrulate by epiboly, likely because their eggs are large, develop slowly, and are easy to inject. It would be useful to generate more lineage data, and descriptions of gastrulation, from indirect developing species, or species that are facultative planktotrophs (Allen and Pernet, 2007). It will also be necessary to study gastrulation mechanisms in the lesser-known spiralian groups, and non-spiralian lophotrochozoans.

Another topic for future research is the evolution of ectomesoderm. Classically the diverse origins of ectomesoderm have been tied to the wide variety of larval forms; it was reasoned that ectomesoderm gives rise largely to larval mesoderm, while 4d-derived endoderm gives rise to adult mesoderm (Henry and Martindale, 1999). This dichotomy is probably an over-simplification, as modern lineage tracing has demonstrated that ectomesoderm and endomesoderm can both give rise to larval and adult structures (Lyons *et al.*, 2012 and references therein). It is interesting that ectomesoderm is derived mainly from the 3a and 3b lineages in most species examined, and that these cells appear to become internalized at the anterior/lateral lip of the blastopore during epiboly. It will also be important to ask if other sources of ectomesoderm are likewise internalized at the blastopore. Furthermore, are particular genes associated with all ectomesoderm internalization?

Unlike endomesoderm, which has an evolutionarily conserved gene regulatory network (e.g., Röttinger *et al.,* 2012 , Boyle and Rice, 2014), the factors regulating ectomesoderm remain to be uncovered, and might be very different between species.

Lastly, future studies would benefit from analyses that follow the dynamic morphogenetic events of gastrulation by time-lapse microscopy. Spiralian gastrulation takes place when the embryo has a relatively small number of cells, making it easy to follow their behavior. Careful examination in this diverse group might uncover novel mechanisms of gastrulation that can shed light on the evolution of morphogenesis. Since the techniques for live-imaging using conventional lineage tracers, membrane dyes, and fluorescently-tagged proteins already exist in several species (e.g., Steinmetz *et al*., 2007; Henry *et al*., 2010; Gline *et al*., 2011), we can expect to learn much more about spiralian gastrulation in the near future.

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