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development**

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Annelids shed light on the evolution of spiralian development

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Title: Annelids shed light on the evolution of spiralian development

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Abstract

Spiralian development is characterized by stereotypic cell geometry and spindle orientation in early cleavage-stage embryos, and of ultimate fates of descendent clones. Diverse taxa such as molluscs, annelids, flatworms and nemerteans exhibit spiralian development, but it is a mystery how such a conserved developmental program gives rise to such diverse body plans. This review highlights examples of variation during early development among spiralian, emphasizing recent experimental studies in the annelid *Capitella teleta* (Blake, 2009). Intracellular fate mapping studies in *C. teleta* reveal that many of its cells' fates are shared among spiralian, but it also has a previously undescribed novel origin for trunk mesoderm (3c and 3d micromeres). Studies have identified an inductive signal in spiralian that has 'organizing activity', and influences cell fates in the surrounding embryo. *C. teleta* also has an organizing activity, however, surprisingly, it is localized to a different cell, it signals at a different developmental stage, and likely utilizes a distinct molecular signaling pathway compared with that in molluscs. A model is presented to provide a mechanistic explanation of evolutionary changes in the cellular identity of the organizer. Detailed experimental investigations in spiralian embryos demonstrate variation in developmental features that may influence the evolution of novel forms.

Keywords (5): *Capitella teleta*, spiralian, embryogenesis, annelid, organizer

Spiralian Development

The process of embryogenesis translates the genotype of an animal into its phenotype. Thus, genetic mutations resulting in morphological changes are often first manifest during the developmental program. During the earliest stages of embryogenesis, a period of rapid cell divisions increases the number of cells in the embryo, and the details of cell cleavage patterns are characteristic for specific animal clades. The Spiralia is a large clade of diverse animals, and a number of taxa within the Spiralia share a distinctive program of stereotypic cell divisions during early embryogenesis called spiralian development (Henry and Martindale 1999). Spiralian development is exhibited by annelids (including echiurans), molluscs, sipunculans, nemerteans, and platyhelminthes. Within metazoans, this pattern of early development is unique and occurs only in members of this clade. Although spiralian development has been lost several times (e. g. in cephalopod molluscs, bryozoans, phoronids, brachiopods, gastrotrichs, and rotifers)(reviewed in Hejnol, 2010), spiralian development is considered an ancestral feature of the Spiralia (Edgecombe et al. 2011).

In spiralian development, the pattern of cell divisions following fertilization is characterized by a series of holoblastic cleavages, and individual cells or blastomeres can be identified on the basis of position within the embryo, blastomere size and orientation of the spindle plane (Figure 1) (Henry and Martindale 1999). There is a standard nomenclature to name each cell, and this nomenclature allows for comparisons of homologous cells across species. Descendants of each of the four blastomeres at the 4-cell stage, the A, B, C and D blastomeres, roughly correspond to quadrants of the body (Figure 1A). In the transition between the four and eight-cell

stage, four typically larger cells become positioned at the vegetal side of the embryo and are called 1A, 1B, 1C and 1D macromeres, and each daughter cell is born towards the animal side of the embryo, called 1a, 1b, 1c, and 1d micromeres (Figure 1B). The letter associated with each blastomere name refers to its quadrant of origin. One hallmark of spiralian development - reflecting its name - is the orientation of the mitotic spindles with respect to the animal-vegetal axis of the embryo, which positions the birth of the animal daughter cells. At the transition from the four to the eight-cell stage, the mitotic spindles orient at a 45-degree angle relative to the animal-vegetal axis, and asymmetric divisions produce the first set of micromeres (Figure 1B). As both the micromeres and macromeres subsequently divide, the mitotic spindles shift 90 degrees relative to their previous orientation, oscillating between leiotrophic and dextrotrophic orientations (compare Figure 1B and 1C). Micromeres are named with the letter corresponding to the quadrant of their birth in lower case letters (macromeres with uppercase letters), along with a number referring to the order of their birth. Typically four rounds of micromeres are generated from each quadrant.

Cells originating from the D quadrant are distinct from cells originating from the other quadrants in a few ways (Lambert 2008). First, cells originating from the D quadrant make a disproportionate contribution to the body and typically generate most or all of the trunk ectoderm and mesoderm. Second, cells of the D quadrant send inductive signals to the rest of the embryo that influence cell fates and specify the dorsal-ventral and left-right axes of the body. Two different mechanisms have been described that specify the D quadrant as distinct from the other quadrants in spiralian embryos (reviewed in Freeman and Lundelius 1992). In one, cytoplasmic determinants

are shunted to one of the cells at the first and second divisions, typically resulting in the blastomere that inherits these determinants being larger than the other blastomeres. This is known as unequal cleavage. In some species, the localization of determinants to one blastomere is accomplished by asymmetric positioning of the mitotic spindle. In other species, determinants become segregated into a transient cytoplasmic protrusion at the vegetal pole of the embryo, called a polar lobe. The polar lobe becomes absorbed into only one blastomere following cytokinesis. The formation of a polar lobe during the first and second divisions has been described for several annelids that undergo unequal cleavage, including *Chaetopterus variopedatus* and *Sabellaria cementarium* (Render 1983, Henry 1986). In the annelid *Chaetopterus*, a combination of both asymmetric positioning of the mitotic spindles and polar lobe formation are utilized (Henry 1986).

In a second mechanism to specify the D quadrant, the early blastomere divisions are equal, and the D quadrant is specified by a signaling event from the first quartet of micromeres to one of the macromeres. This signaling event is contact dependent, and the vegetal macromere that makes the greatest contact with the overlying micromeres becomes the D quadrant macromere (van den Biggelaar and Guerrier 1979, Martindale et al. 1985). The inductive signal to specify the D quadrant usually occurs after birth of the third quartet of micromeres. Thus, in unequal cleaving forms, the D quadrant is specified by the four-cell stage, whereas in equal-cleaving spiralian, the D quadrant is not specified until after the 24-32-cell stage (third quartet micromere formation).

In addition to conserved cleavage patterns, many embryonic blastomeres in animals that undergo spiralian development share similar descendant cell fates and these descendants occupy similar locations within the body (Hejnal 2010). For example,

the first quartet of micromeres generates similar quadrants of head ectoderm in molluscs, annelids and some flatworms. Additionally, the blastomeres 1a and 1c generate the left and right eyes, respectively (reviewed in Henry and Martindale 1999). From this conservation, comparisons of equivalent body domains can be made across species, even though the morphology of comparable body domains can be highly variable. The highly conserved early pattern of cleavage in spiralian embryos thus presents something of a paradox with the vast diversity in larval and adult body plans seen within the clade. It is currently not known at what stage during development that species-specific differences appear. In addition, since a disproportionate fraction of the experimental studies performed in spiralian have focused on a small number of gastropod molluscs, there may be more variation among species in the spiralian developmental program, and limited sampling may have led to a somewhat limited view of spiralian development.

In spiralian development, experimental evidence supports the idea that some cell fates typically become restricted during early cleavage events (reviewed in Henry and Martindale 1999, Henry et al. 2006). This characteristic contrasts with development of embryos from other clades such as mice or sea urchins in which cell fate restrictions occur at later stages. One consequence of early cell fate restriction is that when individual blastomeres are removed from the embryo and allowed to develop into larvae or juveniles with differentiated tissues, there are missing structures that normally arise from the deleted cells. In embryos that do not undergo such fate restriction, such as in sea urchins, the embryo can often regulate for the lost tissue. Despite these differences

in early developmental modes, both types of embryos utilize embryonic organizers, or localized signaling centers, to orchestrate global patterning events in the embryo.

Fate Map of the annelid *Capitella teleta*

Recent studies of early development in the annelid *Capitella teleta* (Blake et al. 2009), formally known as *Capitella* sp. I, highlight the importance of performing additional and broad sampling to identify the extent of variation in the spiralian developmental program. Like other spiralian, *C. teleta* exhibits a spiral cleavage program, and undergoes unequal cleavages (Eisig 1899), forming a small polar lobe. Experimental evidence demonstrates that the specification of the D quadrant in *C. teleta* embryos does not rely on a signal from the first quartet micromeres. Specifically, deletion of all of the first quartet of micromeres results in formation of larvae with a normal dorsal-ventral axis, bilateral symmetry and D quadrant descendent fates, although these larvae are missing head structures that are normally derived from the first quartet cells (Yamaguchi et al. 2016). Thus, in *C. teleta*, the D quadrant is likely specified through inheritance of cytoplasmic determinants.

Following embryogenesis, *C. teleta* passes through a short pelagic larval stage prior to undergoing metamorphosis into a burrowing juvenile worm. *C. teleta* can be cultured in the laboratory, and has several favorable characteristics for studies of early development, including the ability to raise all stages of embryonic and larval development in a dish of sea water (Seaver 2016). Individual identifiable blastomeres can be microinjected using a variety of different fluorescent lineage tracers or mRNA, and a comprehensive fate map of the embryonic origin of larval tissues has been

performed (Meyer et al. 2010). Like in other spiralian, the fates of individual blastomeres in *C. teleta* are stereotypic, and each blastomere gives rise to a unique clone of descendents. The fate map of *C. teleta* shows many features conserved with other spiralian. For example, first quartet micromeres generate the ectodermal structures of the head, the larval eyes arise from the micromeres 1a and 1c, the prototroch originates from the vegetal daughters of the first quartet (1q²), 2d generates the trunk ectoderm, macromeres from all four quadrants contribute to the midgut, and second and third quartet micromeres contribute to the foregut and the tissue surrounding the mouth.

In contrast, the embryonic origin of trunk mesoderm in *C. teleta* demonstrates a surprising difference from that described for other spiralian. In *Capitella*, the trunk mesoderm originates from two cells from two different quadrants, the 3c and 3d micromeres. Each of these cells generates a mirror image descendant clone across the midline of the body. To date, this is the only report of trunk mesoderm originating from 3c and 3d in any spiralian. A highly conserved hallmark of the spiralian developmental program is the origin of the trunk mesoderm, which originates from a single cell, the mesentoblast 4d (reviewed in (Lambert 2008)). In the few annelids for which there is fate map data using intracellular lineage tracers, the mesodermal bands also arise from 4d. Such studies include *Platynereis* (Ackermann et al. 2005), the leech *Helobdella* (Weisblat et al. 1984), and the oligochaete *Tubifex* (Goto et al. 1999). It will be important to examine the origin of the mesodermal bands in other annelids, including close relatives of *C. teleta* to determine if this change in developmental program is a relatively recent evolutionary event or if a trunk mesoderm from 3c + 3d can be found more

broadly in annelids. Recent phylogenomic analyses of annelids support echiurans as sister taxa with *C. teleta* (Struck et al. 2011, Weigert et al. 2014). In a historical study by Newby (Newby 1940), trunk mesoderm was reported to arise from 4d in the echiuran *Urechis caupo*. Although this observational study needs to be confirmed using intracellular tracers, if this result holds true, it would indicate that the trunk mesoderm origin from 3d and 3d in *C. teleta* represents a derived trait within annelids. It is noteworthy that in many spiralian, including in *C. teleta*, the 4d micromere also generates the germ line and the hindgut, indicating that some characteristic descendant cell types of 4d have been maintained in *C. teleta*.

Embryonic organizers

Embryonic organizers are signaling centers that are critical for establishing the body axes and inducing fates of nearby cells. Embryonic organizers induce the surrounding cells to undergo changes in their cell fate and can direct formation of an extra body with all three axes. Removal of an embryonic organizer can lead to animals with missing structures due to loss of cell fates, and often have global defects such as loss of dorsal-ventral or left-right body axes. In contrast, transplantation of embryonic organizers to an ectopic location is often sufficient to induce a secondary axis, and this has been shown in a number of organisms. One well known embryonic organizer was discovered by Mangold and Spemann in the amphibian embryo (Spemann and Mangold 1924). The amphibian organizer is a population of cells located along the lip of the blastopore of the gastrulating embryo, and when it is transplanted to an ectopic location, it influences the surrounding cells to adopt a dorsal fate, and induces a second axis.

Other examples of embryonic organizers include the vegetal micromeres in sea urchin embryos, which were first transplanted by Horstadius (Horstadius 1939), the blastopore lip in the gastrula of the cnidarian *Nematostella vectensis* (Kraus et al. 2007) and the 3D macromere in the mud snail *Ilyanassa obsoleta* (Clement 1962).

Spiralian organizers are characterized by being localized to an individual cell, and having the signaling event occur within a relatively short developmental time frame at early cleavage stages when the embryo has fewer than 100 cells. For example, in the gastropod mollusc *Ilyanassa obsoleta*, embryonic organizing activity is localized to the blastomere 3D at the 32-cell stage. Organizing activity in *Ilyanassa* was first demonstrated by Clement by deletion of individual blastomeres at successive stages of early development (Clement 1962). The phenotype of the resulting larvae demonstrated that a number of cells remaining in the embryo did not adopt their normal fates, leading to larvae with missing structures and overall disorganization of the structures that did form. It is important to note that some of the missing structures were not derived from the deleted blastomere. Subsequently, organizing activity was identified in the molluscs *Dentilium* (Cather and Verdonk 1979), *Lymnaea stagnalis* (Martindale 1986) and *Crepidula fornicata* (Henry et al. 2006). Organizing activity is localized to the blastomeres 3D and 4d in *Lymnaea* and in the slipper snail *Crepidula fornicata* respectively, suggesting that the cellular identity of organizing activity is relatively conserved in molluscs, and is localized to either 3D or its daughter cell 4d. The nature and timing of organizing activity in molluscs contrasts with that in amphibians and zebrafish, which occurs at gastrulation when the embryo is composed of over 1000 cells, and organizing activity comes from a population of cells.

Identification of organizing activity in annelids

Another key hallmark of the spiralian developmental program is the ability of 4d (or its mother cell 3D) to act as an embryonic organizer (Lambert 2008). Because the cell fate of 4d in *C. teleta* is different from that in other spiralian, it was important to ask whether the cellular identity of organizing activity also changed. Specifically, does 4d, or its precursor blastomere 3D, have organizer function in *C. teleta*? To answer this question, an infrared laser microbeam was used to delete individual blastomeres, and phenotypes of larvae that develop from these manipulated embryos were analyzed for presence of their three body axes (Amiel et al. 2013). Following deletion of either 4d or 3D, resulting larvae form all three body axes. Typically, spiralian embryos do not regulate following deletion of a blastomere, and such embryos form partial larvae (Reverberi 1971). Larvae resulting from deletion of 3D appear relatively normal, but lack *vasa* expression in the location in which germ line cells usually reside, demonstrating that one of the cell types was missing that normally arise from 3D and 4d. These blastomere deletion experiments demonstrate that the 3D and 4d blastomeres in *C. teleta* do not possess organizing activity as they do in molluscs.

However, a systematic investigation that included deletion of individual blastomeres at successive cleavage stages up to the formation of the third quartet of micromeres identified an organizing activity in *C. teleta* embryos. The organizing activity is localized to the primary somatoblast, 2d, and it is critical for patterning the dorsal-ventral and the left-right axes of the head in *Capitella* (Amiel et al. 2013). Although 2d generates a large proportion of the ectoderm posterior of the prototroch (Meyer and

Seaver 2010), it is important to note that the affected tissues do not arise from 2d, demonstrating that 2d generates an inductive signal that influences patterning of other cells in the embryo. Larvae resulting from 2d-deleted embryos are circular and lack their typical elongated, barrel shape (Figure 2A, B). The overall morphology of the larvae resulting from deletion of 2d is not unexpected since 2d forms the trunk and pygidium ectoderm, and loss of this tissue results in a non-elongated larva, in which the majority of the tissue present is head tissue. Larvae resulting from 2d-deleted embryos have an anterior-posterior axis, demonstrating that organizing activity is not required for formation of the anterior-posterior axis (Amiel et al. 2013). In addition, there is a substantial reduction of muscle fibers, although it is unclear whether the reduction of mesoderm derivatives is due to lack of organizing signal or an indirect effect of deleting 2d. By performing carefully timed blastomere deletions, it was determined the signal is no longer required after 2d divides. When both daughter cells of 2d were deleted (called 2d¹ and 2d²), resulting larvae showed clear bilateral symmetry through the presence of left and right larval eye spots and bilateral brain lobes, and the presence of a subpopulation of neurons positioned along the dorsal edge of the brain to support the presence of a dorsal-ventral axis (Figure 2C). In summary, *C. teleta* has organizer activity, it is localized to the cell 2d, and although the cellular identity of organizing activity is different from what has been reported for molluscs, in both *C. teleta* and in molluscs, the cell with organizing activity originates from a D quadrant.

The blastomere 2d is the largest cell of the second quartet of micromeres in *C. teleta*, and its size approaches that of the macromeres (Meyer and Seaver 2010). The relative large size for 2d is characteristic of embryos in which there is early specification

of the D quadrant. In *C. teleta*, 2d extends deep into the center of the embryo, and makes direct contact with the first quartet micromeres, which are target cells of the inductive signal from 2d (Amiel et al. 2013). Thus, it is possible that the organizing signal may be transmitted via direct cell-cell contacts.

There is very limited experimental evidence demonstrating presence of organizing activity in annelids, and in some animals it is likely that inheritance of determinants is sufficient for specification of the body axes. Besides *C. teleta*, there is currently a report for only one other annelid species, the oligochaete *Tubifex tubifex* (Nakamoto et al. 2011). In *Tubifex*, transplantation of two cells from the D quadrant, 2d¹¹ plus 4d (which generate the majority of the body ectoderm and mesoderm, respectively), to an ectopic location were reported to be sufficient to form a body duplication. In these experiments, it is difficult to assess whether these results argue strongly for an organizing activity in *Tubifex* or whether the transplanted cells simply execute their own internal program in a new location, since very little surrounding tissue is affected by the presence of these cells. *Tubifex* and leeches are in the same clade within annelids, the clitellates, and they share similar modifications of the spiralian cleavage patterns that generate the trunk tissues. In leech embryos, inheritance of determinants has an important role in specifying aspects of the cleavage patterns that generate the trunk of the body (Astrow et al. 1987, Lyons and Weisblat 2009), whereas local signaling events are critical for specification of the identity of other trunk precursor cells (Kuo and Weisblat 2011). The importance of inheritance of cytoplasmic determinants is also important for establishment of the D quadrant and for patterning in embryos of the polychaetes *Chaetopterus* and *Sabellaria*. Equalization of the cleavage

from the one to the two-cell stage in *Chaetopterus* embryos leads to twinning in the resulting larva, presumably because cytoplasmic determinants that are normally segregated into the CD cell and are critical for induction of the body axes become distributed to both blastomeres during the first cleavage event (Henry and Martindale 1987, Tyler 1930). In *Sabellaria*, cytoplasmic determinants are shunted to the D quadrant via formation of a polar lobe, and removal of the first polar lobe affects the formation of head structures (Render 1983). Even from these few examples, it is clear that there is considerable variability among annelids in how body axes are established during embryogenesis, and there is not yet a reported example in annelids in which 3D is the organizer. It will be necessary to sample species from other parts of the annelid tree to try to reconstruct the ancestral condition for annelids with respect to how the body axes are established.

Towards a molecular understanding of organizing activity in spiralian

There is limited information on molecular nature of the signal from the organizing cell in spiralian. In *Ilyanassa*, knockdown of the gene *Dpp* by antisense morpholino injection results in loss of clear dorsal-ventral patterning, while exposure of early stage embryos to exogenous human BMP protein can induce the formation of ectopic eyes, implicating BMP signaling in global patterning (Lambert et al. 2016). In the annelid *Helobdella*, functional studies have demonstrated that some patterning of the trunk ectoderm along the dorsal-ventral axis requires BMP signaling (Kuo and Weisblat 2011). In this context, a local signaling event is critical for specifying the fate of two ectodermal lineages that initially develop as an equivalence group (O and P lineages),

and later form a portion of the trunk ectoderm. In another set of studies to characterize the molecular signals involved in specification of the D quadrant and in organizing activity, the pattern of activation of ERK/MAPK signaling during early cleavage stages in *Ilyanassa* as well as in a number of equal-cleaving molluscs was characterized (Lambert and Nagy 2001, 2003). ERK/MAPK is a signal transducer and can be used to identify cells that are receiving external molecular signals. In the unequal-cleaving spiralian *Ilyanassa*, activation of MAPK is observed in the target cells of the organizing signal as well as the organizing signal itself (3D). In equal-cleaving molluscs, MAPK is activated in the blastomere 3D. Furthermore, exposure of *Ilyanassa* embryos to a chemical inhibitor of the MAPK/ERK pathway, U0126, results in larvae with a similar phenotype as those in which the D quadrant is deleted (organizer is deleted) (Lambert and Nagy 2001) or in which there is a knockdown of *Dpp* signaling (Lambert et al. 2016). These results suggest that the organizer signal is transduced via activation of MAPK. In annelids, activation of MAPK in *Hydroides* is detected in a single cell during cleavage stages, in the blastomere 4d. In contrast, ERK/MAPK activation does not appear to have a role in axial patterning in the annelids *Capitella* or *Platynereis*, since activated MAPK is not detected in early cleavage stage embryos (Amiel et al. 2013, Pfeifer et al. 2014), and exposure to U0126 results in larvae with normal body axes in both species. Organizing activity has not yet been reported for *Platynereis*. In some spiralian, such as in the mollusc *Crepidula*, specification of the D quadrant and the organizing activity are tightly coupled in time, potentially during the same cell cycle, posing a challenge for distinguishing the molecular signals responsible for these two events (Henry et al. 2006). In contrast, *C. teleta* embryos have a unique advantage for

investigations of the molecular character of the organizing activity since the timing of specification of the D quadrant is uncoupled from the timing of organizing activity; the D quadrant is specified by the third cleavage and the organizing signal occurs during the fourth cleavage (Amiel et al. 2013, Yamaguchi et al. 2016). The observed variation in patterns of activation of MAPK within molluscs and annelids and between molluscs and annelids strongly suggests differences in the molecular mechanisms utilized for transducing organizing signal within spiralian.

Model of the evolution of the cellular identity of the spiralian organizing activity

Although there is variation in the identity of cells with organizing activity amongst different spiralian embryos, in all cases that have been examined, the cell with organizing activity is a descendent of the D quadrant. This feature allows one to propose a model to mechanistically explain how the identity of the cell with organizing activity can change during evolution (Figure 3). In this model, the cellular identity of the organizer could change as the result of both heterochronic and heterotopic shifts in the segregation of determinants that confer organizing properties to different blastomeres derived from the D quadrant in distinct evolutionary lineages. Equal cleavage is proposed to represent the ancestral condition in the Spiralia (Freeman and Lundelius 1992). In equal-cleaving forms, induction of the D quadrant and subsequent specification of the cell with organizing activity are closely linked, and occur after the birth of the third or fourth quartet of micromeres (Figure 3A). In this case, cytoplasmic determinants that confer potential to become an organizer are distributed to all four quadrants in the vegetal macromeres. One macromere is induced to become the D

quadrant (3D) by contact with overlying first quartet micromeres, and would become the organizer (Figure 3A, e. g. *Lymnea stagnalis*). In the case in which the identity of the organizer changed from 3D in an equal cleaving form to 4d in an equal cleaving form, one of the third quartet macromeres would be induced to become the D quadrant macromere, and the requirement for organizing activity would be delayed by one cell cycle by determinants segregated to the 4d micromere during its birth (Figure 3A, e. g. *Crepidula fornicata*). In unequal cleaving forms, the D quadrant is specified precociously at the four-cell stage by asymmetric localization of cytoplasmic determinants to the D quadrant macromere at the four-cell stage (Figure 3B). In these embryos, the cellular identity of the organizer could change as the result of heterochronic or heterotopic shifts in the segregation of determinants that confer organizing properties to different D quadrant blastomeres. In the scenario in which 3D is the organizer, determinants that confer potential to become an organizer would be retained by the vegetal macromere during the sequential divisions that produce the first, second and third quartet of micromeres. In the case of *C. teleta* in which the identity of the organizer shifts to 2d, there is precocious segregation of developmental determinants into the D quadrant micromere, and the requirement for organizing activity ends one cell division earlier. A unique feature of this case is that determinants become segregated towards a more animal position in the embryo, away from vegetal pole. It is clear that there have been a number of evolutionary changes between equal and unequal cleaving forms within the Spiralia. Therefore, taken together with the inadequate available data due to limited sampling, it is not currently possible to determine whether the proximal ancestor of *C. teleta* was an equal or unequal cleaving form or what was the cell identity of organizing

activity. The differences in timing and cellular identity of organizing activity among species provide a nice example of heterochronic changes that have occurred during the evolution of spiralian embryos.

Even though the nature of the determinants confer organizing properties is unknown, transient localization of *Eve* and *Dpp* transcripts to the centrosome during cell division in *Ilyanassa* provide a cellular mechanism to explain asymmetric inheritance in spiralian embryos (Lambert and Nagy 2002). In addition, an expression screen in *Ilyanassa* embryos has identified numerous mRNAs that differentially segregate to one of two daughter blastomeres via centrosome localization during the birth of the micromeres (Kingsley et al. 2007). These data along with a large body of experimental evidence highlight the importance of inheritance of cytoplasmic determinants to specify cell fate in spiralian embryos, and these embryos provide an exciting opportunity to determine the molecular regulation of cell fate determination.

Summary and Future Directions

Much of what is known about spiralian development comes from studies in gastropod molluscs, and by extending these studies to include other taxa such as annelids, there is an emerging picture of variation in several aspects of early spiralian development. This is perhaps not surprising since there is incredible diversity in larval and adult forms in the Spiralia, which includes almost half of the number of animal phyla. However, it is possible that differences among developmental programs across species only become apparent at later stages in development, and clade-specific differences may not be detectable during early stages. The gradual accumulation of

documented examples of exceptions to what have been considered to be highly conserved hallmarks of spiralian development suggest that there is underappreciated variation in spiralian early development (Lambert 2008). For example, the highly conserved embryonic origin of trunk mesoderm from 4d has an exception in *C. teleta* development in which the trunk mesoderm instead arises from 3c and 3d, while 4d retains the ability to generate the germline and contribute to the posterior gut. Another example comes from chitons, in which larval eyes are generated from the second quartet micromeres, 2a and 2c, instead of the first quartet cells 1a and 1c, another highly conserved feature (Henry et al. 2004). The finding that *C. teleta* has organizing activity localized to 2d represents an example of variation in the timing and cellular identity of global patterning. The limited information currently available suggests that the molecular nature of the organizing signal may also vary across spirilians.

One question that emerges from studies in *C. teleta* is whether the cellular identity of the organizing activity in other annelids is 2d or whether *C. teleta* represents a unique case. Thus, it will be important to determine if other annelids pattern their axes through an organizing signal, and if so, to determine the cellular identity of the source of the signal. Most annelids are members of one of two large clades, the Errantia and Sedentaria (Struck et al. 2011, Weigert et al. 2014). *C. teleta* is a member of the Sedentaria. To begin to reconstruct the ancestral state of the organizing activity for annelids, it will be particularly important to sample in the Errantia, and among the annelid groups that branch sister to these large clades. It will also be important to sample among equal cleaving annelids, since organizing activity has not been demonstrated for any equal cleaving annelid to date, and equal cleavage has been

argued to be the ancestral condition in spiralian (Freeman and Lundelius 1992). Studies of other spiralian such as the equal cleaving nemerteans (Henry 2002) will be important to generate a broader view of spiralian development. There are fate maps available for a wide range of spiralian, and this strong framework can be utilized to build a broad sampling of inductive signals that influence global patterning. Additional sampling is necessary to build an understanding of the evolutionary history of global patterning in the spiralian lineage. A future challenge will be to determine whether some of this early variation can be linked to phenotypic differences in the larval or adult forms.

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Figure Legends

Figure 1: Cleavage pattern during spiralian development. Each column shows schematics of lateral (top) and animal (bottom) views of cleavage-stage embryos. The stage is listed below each column, and cells are labeled using standard nomenclature for spiralian embryos. Gray shading indicates D quadrant blastomeres. Short straight lines between cells connect sister cells. The example shown is an unequal cleaving embryo. Note the 90 degree shift in the position of the birth of subsequent micromere daughters between the 8- and 16 cell stage (1d vs. 2d). In lateral view schematics, animal is up and vegetal is down. Polar bodies are indicated as a pair of small circles. An, animal view; lat, lateral view.

Figure 2: Single cell ablations reveal precise timing of organizing signal that

patterns head structures. A. B. C. Filled blastomeres indicate deleted cell in each

experiment, and name of deleted cell is indicated above embryo. Arrows indicate

images of larvae resulting from blastomere deletion. Images in left hand column are of

live specimens in latera/ventral (C) and anterior views. Right column shows maximal

projections of confocal images of larvae labeled with Hoechst (blue) and anti-acetylated

tubulin (yellow) Cleavage stage embryo schematics are oriented in an animal view. Br,

brain lobes; ey, eye pigment; sc^{ac+} , acetylated tubulin-positive sensory neurons.

Figure 3: Model to explain evolutionary changes in the cellular identity of

organizing activity in spiralian. Schematics of cleavage stage spiralian embryos

viewed from the animal pole. Each column represents a different cleavage stage. The

four-cell stage (2nd cleavage) is in the left-most column, with subsequent cleavages

moving from left to right. Cleavage stages are denoted at the bottom of the figure.

Asterisks denote cytoplasmic determinants that have the potential to confer identity of

the cell with organizing activity. Cell with organizing activity is shaded and the cell name

is indicated beneath each embryo schematic. Unfilled embryos signify the stage at

which organizing signal is no longer necessary. Changes in the identity of the cell with

organizing activity occur by heterochronic and heterotopic changes in asymmetric

segregation of cytoplasmic determinants to distinct daughter micromeres of the D

quadrant. Model assumes that inheritance of cytoplasmic determinants confers

organizing properties. A. In equal-cleaving spiralian, all four quadrants have the

potential to adapt a D quadrant fate and subsequently serve an organizing function. In

one scenario (e. g. *Lymnea*), both the specification of the D quadrant and organizing activity occurs after the birth of the third quartet. In the case for *Crepidula*, specification of the D quadrant occurs (3D) one cell division before organizing activity is no longer required (4d), and developmental determinants are asymmetrically segregated to the 4d micromere upon the birth of the fourth quartet. Note, although *Crepidula* has a small polar lobe, experimental evidence demonstrates that it functions as an equal cleaving form in that the D quadrant is specified through interactions with the first quartet of micromeres. B. In unequal cleaving spiralian, cytoplasmic determinants are localized to the vegetal pole of the D quadrant, and as micromeres are born, determinants remain in the D quadrant macromere. Changes in timing of organizing activity and of its cellular identity are achieved by changes in segregation of cytoplasmic determinants. In the case for *Capitella* (top row), determinants are segregated to the micromere when the second quartet of macromeres is born instead of remaining in the macromere. The bottom row represents the case for *Ilyanassa*, in which determinants remain in the macromere through the birth of the third quartet, and organizing activity remains in the third quartet macromere (3D).

Figure 1: Cleavage pattern during spiralian development. Each column shows schematics of lateral (top) and animal (bottom) views of cleavage-stage embryos. The stage is listed below each column, and cells are labeled using standard nomenclature for spiralian embryos. Gray shading indicates D quadrant blastomeres. Short straight lines between cells connect sister cells. The example shown is an unequal cleaving embryo. Note the 90 degree shift in the position of the birth of subsequent micromere daughters between the 8- and 16 cell stage (1d vs. 2d). In lateral view schematics, animal is up and vegetal is down. Polar bodies are indicated as a pair of small circles. An, animal view; lat, lateral view.

153x189mm (300 x 300 DPI)

Figure 2: Single cell ablations reveal precise timing of organizing signal that patterns head structures. A. B. C. Filled blastomeres indicate deleted cell in each experiment, and name of deleted cell is indicated above embryo. Arrows indicate images of larvae resulting from blastomere deletion. Images in left hand column are of live specimens in latera/ventral (C) and anterior views. Right column shows maximal projections of confocal images of larvae labeled with Hoechst (blue) and anti-acetylated tubulin (yellow) Cleavage stage embryo schematics are oriented in an animal view. Br, brain lobes; ey, eye pigment; sc^{ac+} , acetylated tubulin-positive sensory neurons.

175x147mm (300 x 300 DPI)

A. Equal cleaving spiralian

B. Unequal cleaving spiralian

Figure 3: Model to explain evolutionary changes in the cellular identity of organizing activity in spiralian. Schematics of cleavage stage spiralian embryos viewed from the animal pole. Each column represents a different cleavage stage. The four-cell stage (2nd cleavage) is in the left-most column, with subsequent cleavages moving from left to right. Cleavage stages are denoted at the bottom of the figure. Asterisks denote cytoplasmic determinants that have the potential to confer identity of the cell with organizing activity. Cell with organizing activity is shaded and the cell name is indicated beneath each embryo schematic. Unfilled embryos signify the stage at which organizing signal is no longer necessary. Changes in the identity of the cell with organizing activity occur by heterochronic and heterotopic changes in asymmetric segregation of cytoplasmic determinants to distinct daughter micromeres of the D quadrant. Model assumes that inheritance of cytoplasmic determinants confers organizing properties. A. In equal-cleaving spiralian, all four quadrants have the potential to adapt a D quadrant fate and subsequently serve an organizing function. In one scenario (e. g. *Lymnea*), both the specification of the D quadrant and organizing activity occurs after the birth of the third quartet. In the case for *Crepidula*, specification of the D quadrant occurs (3D) one cell division before organizing activity is no longer required (4d), and developmental determinants are asymmetrically segregated to the 4d micromere upon the birth of the fourth quartet. Note, although *Crepidula* has a small polar lobe, experimental evidence demonstrates that it functions as an equal cleaving form in that the D quadrant is specified through interactions with the first quartet of micromeres. B. In unequal cleaving spiralian, cytoplasmic determinants are localized to the vegetal pole of the D quadrant, and as micromeres are born, determinants remain in the D quadrant macromere. Changes in timing of organizing activity and of its cellular identity are achieved by changes in segregation of cytoplasmic determinants. In the case for *Capitella* (top row), determinants are segregated to the micromere when the second quartet of macromeres is born instead of remaining in the macromere. The bottom row represents the case for *Ilyanassa*, in which determinants remain in the macromere through the birth of the third quartet, and organizing activity remains in the third quartet macromere (3D).

230x196mm (300 x 300 DPI)

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