Evolution of Developmental Control Mechanisms

An organizing activity is required for head patterning and cell fate specification in the polychaete annelid Capitella teleta: New insights into cell–cell signaling in Lophotrochozoa

Aldine R. Amiel a,1, Jonathan Q. Henry b, Elaine C. Seaver a,3(*)&

a Kewalo Marine Laboratory, Pacific Biosciences Research Center, University of Hawaii at Manoa, Honolulu, HI 96813, USA
b University of Illinois, Department of Cell & Developmental Biology, 601 S. Goodwin Ave, Urbana, IL 61801, USA

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Many lophotrochozoans (i.e., molluscs, annelids, nemerteans, and polyclad flatworms) display a well-conserved early developmental program called spiral cleavage that contrasts with the high diversity of adult body forms present in this group. Due to this stereotypical development, each cell can be uniquely identified and its lineage history known following intracellular injection of lineage tracers. Cell deletion experiments performed mainly in molluscs have demonstrated that one or two cells associated with the endomesodermal lineage represent an embryonic organizer of subsequent development and are causally involved in cell fate and body patterning. Utilizing the published fate map of the spiral-cleaving annelid Capitella teleta, we used infrared laser cell deletions to dissect the role of individual cells on the patterning of the larval body. Thirteen uniquely identifiable individual blastomeres and two double cell combination deletions were studied to assess larval phenotypes by scoring multiple morphological structures and cell type-specific molecular markers differentially expressed along the antero-posterior and dorso-ventral axes. Surprisingly, our results show that in C. teleta, the cellular identity of the “organizing cell” and the timing of the organizing activity are different from that of other spiralian. In C. teleta, the ectodermal primary somatoblast, 2d, is the key cell responsible for organizing activity during early embryonic development, and is necessary for bilateral symmetry and dorso-ventral axis organization of the head as well as neural, foregut and mesoderm tissue formation. Furthermore, we show that the ERK/MAPK signaling pathway does not appear to be involved in organizing activity in C. teleta. This contrasts with data from molluscs and the molecular mechanism suggested for another polychaete, Hydrodies elegans, highlighting likely molecular level variation among spiralian embryos. These results reinforce the idea that an embryonic organizing activity is present across spiralian. Our data also emphasize the developmental variation within lophotrochozoans, and may ultimately provide insight into the role of developmental processes in the evolution of diverse body forms in metazoa.

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Introduction

The developmental program must orchestrate cell fate specification, morphogenesis and axes establishment to ultimately give rise to a fully functional larva or adult. Animals of different phyla vary in their mode of development (Goldstein and Freeman, 1997; Gurdon, 1992; Prodon et al., 2004; Sardet et al., 2004), but virtually all embryos that have been investigated experimentally possess one or several signaling centers necessary to coordinate developmental events, each with its own species-specific properties (Angerer and Angerer, 2000; Bradham and McClay, 2007; De Robertis and Kuroda, 2004; Duboc et al., 2004; Lee et al., 2007; Momose et al., 2008; Momose and Houliston, 2007; Moon and Kimelman, 1998; Moussian and Roth, 2005; Nishida, 2002; Nusslein-Volhard, 1991; Riechmann and Ephrussi, 2001; Saude et al., 2000; Schier, 2001; Schier and Talbot, 2005). Determining the mechanisms of axis establishment and cell fate specification in a wide range of animals is important for identification of the developmental basis for evolution of body plan diversity across the Metazoa.

Lophotrochozoans make up a large protostome clade that includes molluscs, annelids and nemerteans with a broad range of larval and adult body forms. In contrast to the diversity in body plans, many taxa in this group display a highly conserved and
stereotypic cleavage program during early embryogenesis, called spiral cleavage, in which individual blastomeres can be uniquely identified and homologous blastomeres compared across taxa. Embryos that display this conserved mode of early development are known as spiralian embryos. During spiralian embryogenesis, the first and second mitotic divisions occur along the animal-vegetal (AV) axis of the embryo, leading to birth of four blastomeres named A, B, C and D, which correspond to the future vegetal axis and usually asymmetrically to generate a quartet of smaller cells (termed micromeres) towards the animal pole with the larger cells (termed macromeres) remaining in a vegetal position. In some species, the first quartet of micromeres are generated in a clockwise manner when viewed from the animal pole (dextral cleavage), while in others, the cells are formed in a counter-clockwise manner (sinistral cleavage). Subsequent quartets of micromeres are generated with an opposite orientation with respect to the previous quartet (e.g. clockwise vs. counter-clockwise). A standard nomenclature is used to refer to cells of the spiralian embryo (Conklin, 1897) (see Suppl. Fig. S1 for D quadrant). In this nomenclature, micromeres are identified with a small case letter of their quadrant of origin and the corresponding macromeres are identified with capital letters. For example, at the 8-cell stage, the cells born toward the animal pole are named the first quartet of micromeres (1a, 1b, 1c, 1d) and their sister cells toward the vegetal side are the first quartet macromeres (1A, 1B, 1C, 1D) (Fig. 1C). The second quartet of animal micromeres is named 2a, 2b, 2c, 2d, and the vegetal cells are the macromeres 2A, 2B, 2C, 2D, and so on (Fig. 1D, E, F).

The stereotypic nature of the spiralian cleavage program has facilitated cell lineage analyses for a number of species through the formation of the 4th quartet of micromeres (Boyer et al., 1998; Damen, 1994; Dicits and Damen, 1997; Hejnol et al., 2007; Henry and Martindale, 1998; Henry and Q, 1996, 1999). In contrast, although most spiralian embryos generate their larval eyes from two of the first quartet of micromeres, 1a and 1c, in chitons, the eyes are derived from the second quartet of micromeres, 2a and 2c (Henry et al., 2004).

Single cell deletion and blastomere isolation experiments in molluscs have shown the importance of an embryonic organizing activity in body patterning of spiralian embryos. An embryonic organizer is a cell or group of cells that has axial patterning effects on other cells of the embryo through an inductive signal, i.e., the organizing activity acts on cells that are not part of the embryonic organizer. When the cell with organizing activity is deleted, the resulting defects are greater than would be expected from loss of the tissue that normally arises from the deleted cell, demonstrating the influence that the deleted cell has on proper development of other cells in the embryo (Cather and Verdlon, 1979; Clement, 1962; Henry et al., 2006). Through such experimental manipulations, organizing activity has been shown to be restricted to one cell, 3D (e.g. Ilyanassa obsoleta, Clement, 1962) or its immediate daughter 4d (e.g. Crepidula fornicate, Henry et al., 2006), from the D quadrant. In these cases, the timing of action of organizing activity occurs prior to gastrulation and is completed when the 4th quartet of micromeres are born. If deletions are performed after organizing activity occurs, the resulting larvae are patterned normally, although there is loss of the tissue that normally arises from the deleted cell.

The molecular mechanisms that regulate embryo organization in spiralians are just beginning to be understood, and this...
information comes primarily from studies in molluscs (Henry and Perry, 2008; Koop et al., 2007; Lambert, 2010; Lambert and Nagy, 2001, 2003). In embryos of several mollusc species, the MAPK signaling pathway is activated in the cell with organizing activity, and in some cases appears to be involved in organizing activity (Henry and Perry, 2008; Koop et al., 2007; Lambert and Nagy, 2001, 2003). However, variability in the onset of MAPK pathway activation described in different species suggests variation in the molecular mechanisms controlling organizing activity (Henry and Perry, 2008; Lambert and Nagy, 2001).

Because embryonic organizing activity required for body patterning in spiralians is known largely from studies in molluscs, it is still unclear whether the organizing activity derived from the D quadrant is a conserved feature of spiralian development. Annelids, the segmented worms, are a speciose group and an important taxon for comparison with other spiralian taxa. In the direct developing oligochaete Tubifex tubifex, the only annelid investigated for organizing activity to date, grafting of cells from the D quadrant generates a second body axis (Nakamoto et al., 2010). In other annelids, the distribution of maternal determinants is necessary for specification of the D quadrant (Guerrier, 1970; Henry and Martindale, 1987; Henry et al., 2006; Tyler, 1930), and cell-cell signaling has been suggested as a mechanism of cell specification during early development in Chaetopterus variopedatus and Nereis limbata (Costello, 1945; Henry, 1986; Henry and Martindale, 1987). However, additional sampling is critical for establishing the presence of organizing activity, its cellular identity and timing of action in annelids. Furthermore, nothing is known about the molecular mechanisms that annelid embryos use to establish their main body axes.

Capitella teleta is an annelid worm (Blake et al., 2009) and an excellent model in which to study the mechanisms that control early embryonic patterning. It has a highly conserved spiral cleavage program that allows for the identification of each cell in the embryo with high accuracy prior to gastrulation (Eising, 1898; Meyer et al., 2010). The larvae of C. teleta are characterized by a number of readily identifiable morphological features positioned asymmetrically along either the anterior–posterior or dorsal–ventral axis of the body. Furthermore, C. teleta early stage embryos are amenable to accurate single cell deletions up to the birth of the fourth quartet (~64 cells) (Pernet et al., 2012). Cell lineage analysis has been performed through the formation of the 4th quartet of micromeres for C. teleta, often revealing similarities in the origins of specific tissues and body domains with other spiralians (Henry et al., 2007; Meyer et al., 2010; Nielsen, 2004). For example, the 2d somatoblast generates the trunk ectoderm and the first quartet of micromeres generates head ectoderm structures (in both C. teleta and other spiralians) (Meyer et al., 2010; Meyer and Seaver, 2010). In contrast, the mesodermal tissues of the trunk arise from the 3c and 3d cells in C. teleta, but from the 4d cell in other annelids. If there is an organizing activity in C. teleta, the variation in trunk mesodermal fates between C. teleta and molluscs raises the question of whether the cellular identity of such an organizing activity in C. teleta may also be different from what has been identified in spiralians thus far.

In the present study, we use laser cell deletions to systematically investigate the existence, cellular identity and properties of an organizing activity in C. teleta. Cell deletions, combined with the known fate map and available morphological and molecular markers, allow for detailed analysis of larval phenotypes, and for direct comparisons with data from other spiralians (e.g., molluscs). We identified an organizing activity during early development that is necessary for neural, foregut and mesodermal tissue formation, as well as for the establishment of bilateral symmetry and the dorsal–ventral (D/V) axis of anterior tissues in C. teleta. Surprisingly, this organizing signal occurs earlier and from a different cell (the 2d somatoblast) than that described in molluscan embryos (3D or the 4d mesentoblast). Furthermore, in contrast to previously published molluscan studies, MAPK signaling does not appear to be involved in the organizing activity of C. teleta.

Materials and methods

C. teleta animal care and fixation

Maintenance and handling of adults and embryos within the laboratory colony of C. teleta, were as described previously in Seaver et al. (2005).

Prior to fixation, animals were pretreated as follows: (1) for in situ hybridization, stage 1–3 embryos were incubated for 3 min at room temperature (RT) in a 1:1 mixture of fresh 1 M sucrose and 0.25 M sodium citrate, mixed immediately before addition to animals. This was followed by three 0.2 μM micro-filtered seawater (MFSW) washes. (2) for in situ hybridization plus immunohistochemistry, stage 4–9 larvae were relaxed for 5–10 min at RT in a 1:1 mixture of 0.37 M MgCl2 and MFSW. Embryos and larvae were fixed using one of two fixation methods as follows: (1) for in situ hybridization, animals were fixed in 3.7% paraformaldehyde (Electron Microscopy Sciences: EMS, Hatfield, PA) in 0.2 μM MFSW overnight (ON) at 4°C. The embryos were washed 3 times in 1× phosphate buffered saline (PBS) with 0.1% Tween-20 (PBTw), 2 times with milliQ water, dehydrated with a gradient of methanol/milliQ water (25%, 50%, 75%, 100%), and stored in 100% methanol at least ON at −20°C; (2) for immunolabeling, animals were fixed in 3.7% paraformaldehyde (Electron Microscopy Sciences: EMS, Hatfield, Pennsylvania), 0.1 M HEPES pH 6.9, 50 mM ethylene-diaminetetraacetic acid (EDTA) pH 8, 10 mM Magnesium sulfate (MgSO4), 0.4 M dextrose, 0.2% Triton X-100, ON at 4°C, or for 1 h (h) at RT. The embryos were washed 3 times in 1× PBS with 0.1% Triton X-100 (PBT) and 2 times in 1x PBS, then stored in 1× PBS for up to 3 weeks at 4°C.

Cell deletions

A 20X Hamilton Thorne XYClone laser object was used to delete individual cells. The candidate cell was ablated using brightfield illumination by delivering micro-beam pulses at 100% laser power. This approach is thoroughly described in Pernet et al. (2012) and Lyons et al. (2012). Depending on the size of the targeted cell, pulses between 270 μs and 1000 μs were used. The laser was focused on the edge of each target cell, so that a pulse would damage both the cell membrane and the egg envelope. Embryos were visually monitored for several minutes following the laser pulse to determine if the targeted cell was successfully deleted (leakage of cytoplasm from the deleted cell, and then eventual disappearance of the deleted cell). Embryos that showed signs of damage to neighboring cells were discarded. Embryos in which the candidate cell was successfully deleted were raised at 19°C to the appropriate larval stage (Stage 7, following the staging system in Seaver et al., 2005). Phenotypes resulting from the deletions were analyzed and scored on living larvae, in larvae fixed for immunolabeling, or for in situ hybridization, as described below.

Whole-mount in situ hybridization

Whole-mount in situ hybridization (WISH) was performed according to previously published protocols for C. teleta (Seaver et al., 2001; Seaver and Kaneshige, 2006). Fixed larvae were hybridized for 72 h at 65°C with a probe concentration between 0.2 and 1 ng/μl depending on the gene. Probe lengths are as follows: Capl-elat1 (NCBI acc. no. FJ830866), 820 bp; Capl-foxA (NCBI acc. no. EF651787), 929 bp; Capl-gataB3 (NCBI acc. no. EF651790), 1069 bp; Ct-piwi1 (NCBI acc. no. BK007975), 1500 bp; Capl-twist1 (NCBI acc. no. EF192048), 458 bp.
Immunolabeling

After fixation, animals were incubated in blocking solution consisting of PBT+10% heat-inactivated goat serum (Sigma, Saint Louis, MO) for 1 h at RT and then incubated with primary antibody in blocking solution ON at 4 °C. Following several PBT washes at RT, animals were incubated in secondary antibody in blocking solution for either 3 h at RT or ON at 4 °C. Animals were then washed with several exchanges of PBT for a few hours at RT. Cell membranes and larval muscles were visualized by staining with 1:200 BODIPY FL-phallacidin (Sigma, Saint Louis, MO) or Alexa Fluor 488-phalladin (Sigma, Saint Louis, MO) added during the secondary antibody incubation period. For imaging, animals were cleared by mounting them in 80% glycerol in 1x PBS plus 0.125% MgCl₂.

Primary antibodies used are as follows: 1:800 mouse anti-acetylated tubulin (6–11B–1, Sigma, Saint Louis, MO), 1:500 mouse anti-histone H1 (F152, C25,WIj, Millipore, Billerica, MA), 1:400 rabbit FRMF-amide (Antiseria, ImmunoStar, Hudson, WI), 1:400 rabbit anti-5-HT (Antiseria, ImmunoStar, Hudson, WI), and 1:200 mouse anti-diphosphorylated-Erk-1&2 (MAPK-YT, Sigma, Saint Louis, MO). Secondary antibodies used are as follows: 1:400 goat anti-mouse or anti-rabbit FITC, 1:400 goat anti-mouse or anti-rabbit rhodamine, and 1:1000 donkey anti-mouse or anti-rabbit Alexa Fluor 647 (Molecular Probes, Grand Island, NY).

U0126 treatments

U0126 is a drug that inhibits the kinase activity of MEK and thus blocks phosphorylation of the ERK/MAPK pathway. U0126 was diluted in 100% DMSO to a 10 mM stock before use. Embryos were exposed to a final concentration range of between 5 and 50 µM U0126 (Sigma, Saint Louis, MO) by direct addition of the stock solution to MFSW. Treatments representing different time windows between the 2-cell stage and larval stage 7 were performed. The drug was changed every 24 h for duration of each treatment (represented in Suppl. Fig. S4). Embryos and larvae were raised at 19 °C for the entirety of their development, and fixed at larval stage 7 for immunolabeling, as described above. In the U0126 treatment samples, DMSO was at a final concentration range of 0.05% and 0.5%. As controls, embryos were exposed to 0.1% and 0.5% DMSO in MFSW for the same length of treatment as experimental animals.

Microscopy

Both live and fixed specimens were imaged using an Axioskop 2 mot plus (Zeiss, Gottingen, Germany) with a SPOT FLEX digital camera (Diagnostic Instruments, Inc., Sterling Heights, MI). Immunolabeled animals were also imaged using an LSM 710 confocal microscope (Zeiss, Gottingen, Germany). Images were reconstructed using either the LSM 710 software or ImageJ (NIH).

Results

Early development and larval morphology of C. teleta

Early development of C. teleta has characteristic features of a spiralian unequal cleavage program (Fig. 1A–F). The D blastomere is identifiable by its larger size relative to the three other cells at the 4-cell stage (Fig. 1B). Embryos then undergo a dextral spiral cleavage to generate the first quartet of micromeres (Fig. 1C). A cell from the 2nd quartet of micromeres, 2d, called the somatoblast cell, is much larger than the other micromeres (Fig. 1D, E). The 2d somatoblast will give rise to the entire ectoderm of the trunk and the most posterior region of the larva, the pygidium (Meyer et al., 2010; Meyer and Seaver, 2010) (Suppl. Fig. S1). Following the division of 2d, the 2d₁ descendant generates the left and right ectoderm of the trunk, the left side of the ventral nerve cord and the left side of the pygidium (Meyer and Seaver, 2010) (Suppl. Fig. S1), and 2d₂ will generate the right side of the ventral nerve cord and the pygidium (Meyer and Seaver, 2010) (Fig. 1F; Suppl. Fig. S1). During gastrulation, the animal micromeres undergo epiboly around the vegetal macromeres as described for Capitella capitata (Eisig, 1898), to give rise to a metatrochophore larva (Fig. 1G–J).

C. teleta larvae possess multiple differentiated cell types and complex morphological structures positioned asymmetrically along the anterior–posterior (A/P) and the dorsal–ventral (D/V) axes, providing multiple characters that define the body axes (Fig. 1G–J). The larva has three ciliary bands: the prototroch separates the head from the anterior trunk; between the posterior trunk and the pygidium is the telotroch, and the neurotroch is positioned along the ventral midline. A ventral mouth is located immediately posterior to the prototroch, and the ventral nerve cord extends between the mouth and the telotroch. Longitudinal and transverse muscle fibers are present throughout the body (Fig. 1J). The head has an equatorial bi-lobed brain, visible as a thickening of the anterior ectoderm in live stage 7 larvae (Meyer and Seaver, 2009). Two eyes are visible by their red pigment and are located on the left and right side of the head, anterior to the prototroch. In addition, there are two cell clusters positioned along the medial-dorsal side of the brain (approximately six or seven cells total), directly or indirectly connected to the external surface of the head ectoderm by axon tracks. These cell bodies and their axon tracks are labeled with an anti-acetylated tubulin antibody (Fig. 1K, L). Their dorsal position within the brain and the presence of acetylated tubulin in their cell bodies and axon tracks support a neural identity, and they are most likely sensory cells (Fig. 1K, L). For this study, we refer to this cluster of cells as the acetylated tubulin-positive sensory cells (see fig.). In addition, the tissue-specific molecular markers Ct-piwi₁, CapI-ela₁, CapI-foxA, CapI-gataB3 and CapI-twist₁ (Boyle and Seaver, 2008; Giani et al., 2011; Meyer and Seaver, 2009) were used to score and interpret larval phenotypes resulting from blastomere deletions.

Early cell fate specification in C. teleta

To determine whether the identity of the cell with organizing activity is shared between molluscs and C. teleta (e.g. 3D in L. obsoleta and 4d in C. fornicata), the blastomere 3D or its descendant, 4d, was deleted in early stage embryos. These embryos were raised to stage 7 larvae and scored for general morphology as well as for A/P and D/V axis patterning by analyzing the presence and organization of specific larval features (Fig. 2A–F; Suppl. Tables 1 and 2). DIC observations of live larvae and immunolabeling on fixed larvae resulting from the 4d or 3D blastomere deletions, abbreviated [−4d] or [−3D], respectively, possess similar larval morphological features as control unlabaled larvae (Fig. 2A–F; Fig. 1G–J; Suppl. Tables 1 and 2). More significantly, the experimental larvae do not show any signs of mis-patterning along the A/P or D/V axis (Fig. 2A–F). Because 4d normally generates the germ line and a small number of muscle cells, which are not readily scored morphologically, we used in situ hybridization to detect the C. teleta piwi₁ transcript, a robust marker of primordial germ cells in C. teleta (Giani et al., 2011) to confirm the loss of 4d descendants in the [−3D] larvae (Suppl. Fig. S2). In undeleted controls and [−3C] larvae, as expected, Ct-piwi₁ mRNA staining is detected in primordial germ cells located in the anterior trunk on each side of the ventral midline and in the posterior growth zone of stage 7 larvae (Suppl. Fig. S2A, C) (Giani et al., 2011). No germ cells were detected in stage 7 [−3D] larvae, shown by the absence of ventro-lateral Ct-piwi₁-
cell clusters, although expression in the posterior growth zone persists (Suppl. Fig. S2B).

To determine if an organizing activity occurs prior to the birth of the 3D blastomere in *C. teleta*, we next deleted 2D, the progenitor of 3D. In additional cases, the C blastomere was deleted as a control (Fig. 2G–I; Suppl. Table 1). [−2D] or [−C] larvae show normal axial patterning and lack tissues that normally arise from the ablated cell. The left longitudinal muscle fibers arise from the 3D cell, one of the 2D daughters (Meyer et al., 2010). As expected, the [−2D] larvae lack the left longitudinal muscle fibers in the majority of cases (n=28/32) (Fig. 2H, arrow; Suppl. Table 1). The right longitudinal muscle fibers and the right eye originate from the 3c and the 1c+ cells, respectively, both of which are daughters of the C blastomere. As expected, [−C] larvae lack the right longitudinal muscle fibers in the majority of cases (n=26/29), and the right eye (n=55/72) (Fig. 2J, K, black and white arrows, respectively; Suppl. Table 1).

In the majority of [−4d], [−3D], [−2D] or [−C] larvae, the head has bilateral symmetry and shows a clear D/V axis (Fig. 2C, F, I, L), similar to unmanipulated controls (Fig. 1L). There are two well-developed bilaterally symmetric brain lobes, and the acetylated tubulin positive sensory cells (scac+) are positioned in a dorsal and medial position of the brain (white arrows in Fig. 2C, F, I, L). In some [−C] larvae, the left and right scac+ clusters are not aligned in the same antero-posterior position as they are in control larvae, making it difficult to visualize the right scac+ (compare Figs. 1 and 2L). This can be explained by the fact that daughter cells of the C blastomere (1c, 2c) give rise to the dorsal portion of the right side of the brain lobe, and in its absence, the right scac+ are displaced. However, the dorsal and medial positions of the scac+ are maintained. Thus, the bilateral symmetry and the D/V axis of the head are preserved in [−C] larvae.

In summary, following individual deletion of the blastomeres 4d, 3D, 2D and C, the resulting larvae form their normal body axes although the tissues derived from these cells are typically missing. These data reveal the early timing of fate segregation of these blastomeres during *C. teleta* embryogenesis, as well as possible blastomere regulation in some cases, but still raise the question of whether or not establishment of body axes in *C. teleta* requires organizing activity, possibly during an earlier embryonic stage than has been demonstrated for other spiralian.

The trunk mesoderm is not essential for axes establishment

A conserved feature of spiralian development is that the organizer cell (e.g., 4d in *C. fumicata*, and its mother cell 3D in *I. obsoleta*) also gives rise to most of the mesodermal tissues of the larva (Henry and Perry, 2008; Lambert and Nagy, 2001). In other words, in the molluscs studied, organizing activity is coupled with endomesodermal fates. Surprisingly in *C. teleta*, cell lineage studies have shown that the majority of the mesodermal tissue of the larval body is instead derived from the two micromeres, 3c and 3d (Meyer et al., 2010). To test whether these mesoderm-generating cells are associated with organizing activity in *C. teleta*, we analyzed the potential of 3c and 3d to establish body axes by deleting their progenitors (2c plus 2D, respectively) (Fig. 3). Since individual blastomere deletions of 2D or C (progenitor of 3c) do not result in disruption of axial patternning (Fig. 2G–I), it is possible that one cell alone is sufficient for functional organizing activity.
To determine if the combined deletion of the precursors of the mesodermal bands affects body axes establishment, we deleted 2C plus 2D (2C+2D) (Fig. 3; Suppl. Table 3). The majority of the [-2C +2D]) larvae look similar to control larvae (Fig. 3A, C, E; Suppl. Table 3), with clearly delineated A/P and D/V axes. As expected, these [-2C+2D]) larvae lack both lateral mesodermal bands (Fig. 3B, D). These larvae also have a curved body shape and narrow head relative to controls, possibly due to the significant loss of muscle fibers, which may function to support body shape.

**Inductive signals from the D quadrant**

To determine if any progeny from the D quadrant possess organizing activity that patterns the larval body axes, we deleted the following blastomeres: D, 1D, 2d, 2d¹, 2d², and 2d plus 2d² (2d¹+2d²). The resulting larvae were analyzed when sibling controls reached stage 7 (Fig. 4; Suppl. Tables 1–3). The majority of [-D], [-1D], [-2d] or [-2d¹+2d²] larvae show a rounded morphology (Fig. 4A, B, C; Suppl. Table 1: [-D] 97%, n=294; [-1D] 86%, n=50; Suppl. Table 2: [-2d] 93%, n=191; Suppl. Table 3: [-2d¹+2d²] 91%, n=56), which contrasts with the elongated phenotype in larvae of unablated controls (Fig. 1G–H). Because the 2d somatoblast normally gives rise to the ectoderm of the trunk and pygidium (Meyer et al., 2010), deleting the progenitors of 2d (D or 1D), 2d itself, or 2d descendants (2d¹+2d²) removes a large amount of tissue that likely results in spherically shaped larvae. As a result, all structures associated with the trunk and pygidium ectoderm are absent, such as the ventral nerve cord, neurotroch and telotroch (Suppl. Tables 1–3). In addition, the mouth cannot be detected. In a subset of these deletions, head structures were also affected. Both eyes are missing in 94% of cases in [-D] (n=294), 62% of cases in [-1D] (n=50), and 57% of cases in [-2d] (n=191) larvae (Suppl. Tables 1 and 2). In conjunction with the loss of eyes, the anterior ectoderm thickening that indicates the two brain lobes in control larvae (Fig. 1G, br) was not present or was dramatically reduced in the [-D], [-1D] and [-2d] larvae (Fig. 4A, B; Suppl. Table 1 and 2, Ant. EctoD). In contrast, two eyes and bilateral regions of anterior ectoderm thickening (Ant. EctoD in Suppl. Table 3) were observed in the majority of cases in [-2d¹+2d²] larvae (79% and 93%, respectively, n=56) similar to that found in control larvae (92%, n=344) (Suppl. Table 3; Fig. 4C).

Following 2d¹ or 2d² individual blastomere deletions, the resulting

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**Fig. 3.** Double deletions of the trunk mesoderm precursors, 2C and 2D. Diagram at the top of the figure show the identity of the deleted cell(s) (shaded in gray) in the embryo. A–E: Confocal fluorescent images of transverse sections of stage 7 larvae resulting from undeleted controls (A, B) and combined 2C plus 2D deletions (C, D, E). Larvae are in lateral view with anterior to the left in A, B, C, D, and in anterior view in E. D/V is as labeled. A, C, E: Confocal projections showing acetylated tubulin (yellow) and nuclei (cyan); white arrows show the anterior scac fibers; nt, neurotroch; pg, pygidium; pt, prototroch; tt, telotroch. Asterisk marks the mouth. Scale bar in A equals 100 μm in A, B, C, D, and 65 μm in E.

**Fig. 4.** Tissue and differentiated cell loss after D quadrant blastomere deletions. Diagrams of cleavage stage embryos at the top of the figure represent the identity of the deleted cell(s) (shaded in gray). A–E: DIC images of live larvae resulting from the following blastomere deletions: A, [-D], B, [-2d], C, [-2d¹+2d²], D, [-2d¹], E, [-2d²]. All larvae are oriented anterior to the left. Black dotted lines in C, D and E mark the contour of the anterior ectoderm thickening, which indicates the position of the brain (Ant. ectoD in Suppl. Tables 1–3). ey, eyes; nd, view not determined; pg, pygidium; pt, prototroch; tt, telotroch; ven, ventral view. Scale bar in A equals 100 μm.
larvae are similar to the controls with regard to the presence of differentiated tissues, including those associated with the trunk (Suppl. Table 2; Fig. 4D, E).

The defects observed in [-D], [-1D] and [-2d] larvae provide the first evidence of an early inducing signal from the D quadrant in C. teleta. This signal is important for eye and brain formation, tissues not derived from the D quadrant (1d generates only a small part of the brain) (Meyer et al., 2010; Meyer and Seaver, 2010). The inducing signal occurs prior to the birth of 2d1 and 2d2 in C. teleta, since both eyes and brain are present in [-2d1+2d2] larvae.

Presence of an A/P axis following successive D quadrant cell deletions

Even though some of the larvae resulting from deletion experiments possess differentiated structures such as eyes, brain lobes and cilia (Suppl. Table 1–3), the rounded shape and the reduced length of the trunk make it difficult to assess the prototroch, telotroch, neurotroch, ventral nerve cord and mouth in live larvae. Therefore, following blastomere deletions in the D quadrant, we performed immunolabelling using anti-acetylated tubulin antibodies to examine the pattern of ciliation and axon track organization, phalloidin to examine presence of actin muscle fibers, and Hoechst to label nuclei (Fig. 5; Suppl. Fig. S3B). Our results show that in [-D], [-1D], [-2d] or [-2d1+2d2]) round-shaped larvae, an asymmetrically positioned circumferential ciliary band is present ([−D] 100%, n=72; [−1D] 100%, n=22; [−2d] 100% n=26; [−2d1+2d2] 100% n=13) (Fig. 5A, E, I; Suppl. Fig. S3B). It is likely that this ciliary band is the prototroch, since the telotroch precursor cells (2d1 and 2d2) were removed in these series of deletions. Cell bodies labeled with the anti-acetylated tubulin antibody were observed in the ectodermal layer at the opposite end of the larva relative to the position of the ciliary band ([−D] 69%, n=72; [−1D] 83%, n=22; [−2d] 100%, n=26; [−2d1+2d2] 100%, n=13) (Fig. 5A, E, I; Suppl. Fig. S3B, white arrows). These cells correspond in labeling and shape to the scac+ clusters observed in control, unmanipulated embryos (Fig. 1K, L white arrows). In addition, [-D], [-1D], [-2d] or [-2d1+2d2]) spherical larvae have localized regions of a high-density of nuclei (Fig. 5B, F, J yellow arrows; Suppl. Fig. S3B). In most cases, these regions co-localize with the scac+ (Fig. 5D, H, I; Suppl. Fig. 3B). In the [−2d1+2d2]) larvae, the regions of high-density nuclei have the same number, shape and position as the brain lobes in control larvae. In control larvae, the anterior scac+ clusters are located in the dorsal part of each brain lobe. Therefore, it is possible that the dense clusters of nuclei observed in [−D], [-1D], [-2d] or [-2d1+2d2]) round-shaped larvae also possess a neural identity.

 Larvae resulting from successive deletions of the D quadrant possess differentiated muscle fibers (Fig. 5C, G, K). These muscle fibers appear disorganized compared to control larva in which there is a complex, orthogonal network of longitudinal and transverse muscle fibers observed along the body axis (Fig. 1I, J).

Fig. 5. Detailed morphological analysis following successive deletion of D quadrant blastomeres. Diagrams at the top of the figure represent the identity of the deleted cell(s) (shaded in gray) in the embryo. Each column includes images of the same embryo. A-X: Merged confocal stacks of larvae in longitudinal view following deletions indicated at the top of the figure. All larvae are oriented in lateral view with anterior to the left; A-D: [−D] larva; E-H: [−2d] larva; I-L: [−2d1+2d2] larva; M-P: [−2d1] larva; Q-T: Subset of merged longitudinal sections of [−2d1] larva; U-X: [−2d] larva. A, E, I, M, Q, U: Single channel images showing acetylated tubulin labeling in yellow; white arrows show the anterior cluster of acetylated tubulin labeled cells. B, F, J, N, R, V: Single channel projections showing nuclei (cyan); yellow arrows show clusters of high-density nuclei. C, G, K, O, S, W: Single channel images showing actin microfilaments in white; orange arrows indicate the position of the ciliary bands. D, H, L, P, T, X: Merged channel images. ac. tub., acetylated tubulin; br: brain; fg, foregut; lmf, longitudinal muscle fibers; mf, muscle fibers; nt, neurotroch; pg, pygidium; pt, prototroch; tmf, transverse muscle fibers; tt, telotroch. Asterisk marks the mouth. Scale bar in A equals 100 μm.
The complex organization of the head muscle fibers is not observed in experimental larvae (data not shown).

In these deletions, the ciliary prototroch and populations of high-density nuclei associated with the scac+ are consistently positioned at opposite ends of the larva along a main axis. To further investigate the A/P axis in the set of successive D quadrant deletions, we examined expression of the following markers by in situ hybridization: elav (CapI-ela1), an early neural identity marker that labels the brain and ventral nerve cord (Meyer and Seaver, 2009), and CapI-foxA, a foregut marker (Boyle and Seaver, 2008). Since the brain is located anterior to the prototroch, and the foregut is located posterior to the prototroch, CapI-elav and CapI-foxA serve as anterior and posterior markers, respectively, for experimental larvae that are missing the trunk (Fig. 6). In the majority of cases in [−2d] and [−(2d1+2d2)] larvae (Fig. 6C, D, G, H, K, L, O, P, S, T), although CapI-foxA mRNA is not detectable in [−D] larvae, the presence of CapI-elav mRNA on the opposite side of the larva to the ciliary band suggests the presence of an A/P axis. Taken together, these data reveal that larvae form an A/P axis following deletion of D, 2d, or 2d1+2d2 cells.

Larvae resulting from single deletions of 2d1 or 2d2 were also analyzed using the same set of markers (Figs. 5M–X, 6Q–T). In [−2d1] larvae, the majority of the trunk ectoderm is missing (Fig. 5M–T). In the anterior part of the head, the brain and scac+ are visible (Fig. 5M, N, Q, R, white arrows). In the trunk (derived from the 2d2 cell), the mouth and a short ciliary band (likely a partial neurotroch originating from the 2d2 cell) are positioned ventrally, and the pygidium is present and posterior to the telotroch (Fig. 5M, N, Q, R). The orientation of the prototroch and telotroch (orange arrows in Fig. 5M and S) are variable in their angle relative to one another instead of being parallel as is the case in control larvae (Fig. 1I). The [−2d2] larvae are nearly normal, but frequently have a smaller pygidium (Fig. 5U–X). Although [−2d1] larvae look misshapen due to lack of trunk ectoderm, there is a clear A/P axis for both [−2d1] or [−2d2] larvae. In both cases, the head is anterior to the trunk and the trunk is anterior to the
pygidium, and the mouth and neurotroch are present at their correct locations (Fig. 5Q, U). Moreover, \textit{Capl-ela}1 and \textit{Capl-fox}A are expressed in [−2d] larvae in the anterior part of the head and surrounding the mouth respectively, in similar positions as observed in controls (Fig. 6Q–T).

Organizing activity from the D quadrant is essential for D/V axis formation of the head

Most previous studies that examined the organization of the larval body in spiralians examined coiled veliger larvae of molluscs, using anterior larval morphological markers such as the eyes, the stomodeum, and bilateral organization of the velar lobes to interpret phenotypes resulting from blastomere deletions or drug treatment experiments (Clement, 1962; Henry and Perry, 2008; Lambert and Nagy, 2001). In these studies, the absence of anterior markers was interpreted as a radialization of the larval body. Only one study, performed in the mollusc \textit{Halocles asinina}, shows a clear radialization of the larval head by changes in the pattern of the neural marker serotonin (Koop et al., 2007). As we show in our study, anterior larval markers such as the eyes, brain lobes, ciliated sensory cells and \textit{Capl-ela}1 expression are affected to varying degrees following successive D quadrant deletions in \textit{C. teleta}. To investigate whether radialization of the anterior part of the larval body occurs after deletion of D quadrant blastomeres, we analyzed head organization. \textit{Capl-ela}1 and \textit{Capl-fox}A were used as markers of brain and foregut respectively, to orient the resulting round-shaped larvae, and allow for examination of bilateral symmetry and D/V axis establishment of the head (Fig. 7). In contrast to no deletion controls (Fig. 7M), organization of head tissues is disrupted in [−D], [−1D], and [−2d] larvae (Fig. 7A–F; Suppl. Fig. S3A). In addition to loss of eyes (Fig. 4A–B; Tables 1 and 2), the remaining territories of neural tissue are reduced in size and disorganized relative to controls (Fig. 7A, D; Suppl. Fig. S3A). In these deletions, the number of neural domains (\textit{Capl-ela}1 expression, sc\textsuperscript{++} and domains with high-density of nuclei) in the anterior-most part of the larva is typically three, but varies from one to six clusters, consistent with a loss of bilateral symmetry in the head (Fig. 7A, D, C, F white arrows). Bilateral symmetry was disrupted in all cases for this set of deletions and no signs of a D/V axis were detected ([−D] (n=50), [−1D] (n=18), or [−2d] (n=26)) (Fig. 7A–F; Suppl. Fig. S3A white numbers and orange numbers, respectively).

In contrast, bilateral symmetry of the brain was observed in the majority of cases in [−(2d\textsuperscript{1}+2d\textsuperscript{2})], [−2d\textsuperscript{1}] or [−2d\textsuperscript{2}] larvae (Fig. 7G, J; Suppl. Fig. S3C white numbers). The normal medial and dorsal position of the sc\textsuperscript{++} in each brain lobe was observed in 17 of 42 cases for [−(2d\textsuperscript{1}+2d\textsuperscript{2})], in 12 of 23 cases for [−2d\textsuperscript{1}] and in all cases for [−2d\textsuperscript{2}] larvae (n=11) (Fig. 7G, I, J, L; Suppl. Fig. S3C orange numbers). Because head organization is disrupted in larvae resulting from 2d deletions, but restored when the same amount of tissue is removed one division later by deletion of both daughter cells (2d\textsuperscript{1}−2d\textsuperscript{2}), the axial organization of the head is established by the time 2d\textsuperscript{1} and 2d\textsuperscript{2} are born.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{fig7.png}
\caption{Loss of bilateral symmetry and the dorso-ventral (D/V) axis in the head following deletions of D quadrant progeny. Cleavage stage embryo diagrams at the top of the figure represent the identity of the deleted cell(s) (shaded in gray) in the embryo. Each column shows images of the same specimen; all larvae are head anterior view. A–C: [−D] larvae; D–F: [−2d] larvae; G–I: [−(2d\textsuperscript{1}+2d\textsuperscript{2})] larvae; J–L: [−2d\textsuperscript{1}] larvae; M–O: undeleted control larvae. A, D, G, J, M: Confocal stacks of transverse sections of the head; white arrows indicate the sc\textsuperscript{++}, acetylated tubulin protein is labeled in yellow and nuclei in cyan. The white numbers in the lower left of each panel are the number of cases with bilateral symmetry in the head (Fig. 7A, D, C, F white arrows). Bilateral symmetry was disrupted in all cases for this set of deletions and no signs of a D/V axis were detected ([−D] (n=50), [−1D] (n=18), or [−2d] (n=26)) (Fig. 7A–F; Suppl. Fig. S3A white numbers and orange numbers, respectively).}
\end{figure}
2d is required for trunk mesoderm formation

As we show in this study, deleting the 2d somatoblast leads to the loss of bilateral symmetry and disorganization of the D/V axis of the head ectoderm, including neural tissues (1st quartet descendants). We also investigated the influence of 2d signals on cell fates of other micromeres, in particular the descendants of the 3rd quartet, 3c and 3d, which generate the majority of the trunk mesoderm. Previous studies in other animals show that the mesoderm plays an important role in specification and organization of different tissues (Halpern, 1997; Harland, 2008; Harland and Gerhart, 1997). We therefore investigated mesoderm formation in the absence of the organizing activity (2d somatoblast deletion) using the mesodermal markers, gataB3 (CapI-gataB3), and twist1 (CapI-twist1) (Boyle and Seaver, 2008) (Fig. 8).

In control larvae, gataB3 mRNA is localized to the mesodermal bands in the trunk and mesoderm on each side of the foregut (Fig. 8A). In [−D], [−2d] or [−(2d¹+2d²)] larvae, the CapI-gataB3 mRNA expression domains are considerably reduced in the majority of cases and, when detected, is localized to a small domain on the posterior-most side of the larva (Fig. 8E, I, M). The expression domain detected is larger in [−(2d¹+2d²)] larvae compared to [−D] or [−2d] larvae. Twist1 mRNA is also localized to the mesodermal bands in the trunk of control larvae, in cells surrounding the foregut, and in mesodermal cells at the anterior-most region of the larval head (Fig. 8C). The twist1 expression domain in the head arises from the 3a blastomere in C. teleta (Meyer et al., 2010), and is adjacent to the scac⁺ (Fig. 8C, D, white arrows). In [−D] and [−2d] round-shaped larvae, CapI-twist1 mRNA expression domains are considerably reduced in the majority of cases and, if detected, are localized to small patches on the anterior-most side of the spherical larvae adjacent to the scac⁺ (Fig. 8G, K, H, L). In [−(2d¹+2d²)] larvae, CapI-twist1 expression is also localized on the anterior-most side of the round-shaped larva adjacent to the scac⁺ (Fig. 8O black arrow). In addition, in 13 out of 44 cases, CapI-twist1 expression is observed around the mouth, on the opposite side of the anterior expression domain (Fig. 8O, P white arrow). Interestingly, the posterior expression domain of the two mesodermal markers in the [−D], [−2d] or [−(2d¹+2d²)] larvae is not expressed in a bilateral pattern as they are in control larvae. Although it is possible that the trunk ectoderm is necessary for the bilateral organization of CapI-gataB3 and CapI-twist1 expressing cells surrounding the mouth, it is likely that the lack of trunk tissue may interfere with the

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**Fig. 8.** 2d is important for trunk mesoderm formation. Diagrams at the top of the figure represent the identity of the deleted cell(s) (shaded in gray) in the embryo. All larvae are oriented with anterior to the left. A-D: Undeleted control larvae; E-H: [−D] larvae; I-L: [−2d] larvae; M-P: [−(2d¹+2d²)] larvae. A, E, I, M: DIC images of CapI-gataB3 gene expression; black arrows show posterior staining in E, I, M, B, F, J, N are corresponding fluorescent images of the same specimens of the panels above showing acetylated tubulin staining. C, G, K, D: DIC images showing CapI-twist1 gene expression; black arrows indicate head expression, which is localized near the scac⁺ cluster (white arrows in D, H, I). The white arrow in O indicates posterior staining and the yellow arrow in P shows the neuropyle of the brain. Numbers in bottom left of each panel are the number of cases for each represented phenotype. ac. tub., acetylated tubulin; dor, dorsal; nd, not determined; pt, prototroch; tt, telotroch; ven, ventral. Asterisk marks the mouth. Scale bar in A equals 100 µm.
ability of these mesodermal cells to properly organize in discrete positions. These data plus our observations of a reduction in muscle fibers resulting from the same set of deletions (Fig. 5) support the idea that the organizing activity from the 2d cell is also required for correct mesoderm formation in *C. teleta*.

**Cell–cell contacts of the 2d micromere**

Together, our data show that a signal crucial for normal development of the embryo emanates from the 2d somatoblast and influences development of descendants of the 1st, and 3rd quartet. The 2d cell [Fig. 9A–F] shows that it is in contact with the 1st and 3rd quartets and the macromeres. The 2d cell is a mesodermal trunk precursor, and it is known to be involved in the formation of muscle fibers [Fig. 5].

**Fig. 9.** Cell–cell contacts between 2d and other blastomeres of the embryo. A–F: Confocal fluorescent images of embryos following the birth of the 2nd quartet. Cell membranes and nuclei are labeled in white (phalloidin staining) and cyan (Hoechst staining) respectively. Pseudo-colored images are shown in A–B, where yellow indicates 2d; pink represents the position of the 2nd quartet. A: Subset of stacks to show the morphology of 2d during interphase. 16-cell stage embryo, the first quartet has divided to generate 1q1 and 1q2. White dotted line represents the contour of the 1st quartet. B–F are from the same embryo, showing different depths along the animal–vegetal axis. 18-cell stage, the first quartet has divided to generate 1q1 and 1q2, and the third quartet cells 3c and 3d are born. At this stage, 2d is undergoing mitosis and assumes a round shape. Cell identities are indicated. B: Maximum projection; C: Subset of stacks showing the animal-most cells; D: Subset of stacks beneath the animal surface showing 2d contacts with a subset of 1st quartet micromeres; E: Subset of stacks showing contacts between 2d and the two mesodermal trunk precursors, 3c and 3d; F: Subset of stacks showing 2d contacts with the vegetal macromeres. emb1: embryo1; emb2: embryo2. Scale bar in B equals 50 μm.
quartet micromeres. To determine whether the 2d cell is in direct contact with these and other cells of the embryo, we observed the morphology and position of the 2d cell (Fig. 9). 2d is a large cell relative to the other micromeres, and during its interphase, 2d extends deep inside the embryo and makes direct contacts with the four macromeres and all four 1st quartet micromeres (n = 14) (Fig. 9A; dotted line). The 2d somatoblast also contacts the other 2nd quartet micromeres (pink shaded domain), although in none of the embryos observed did 2d have direct cell–cell contacts with 2a, 2b and 2c all at the same time. Later during the cell cycle, as 2d becomes round prior to dividing (Fig. 9B, F), direct contacts persist with the 1st quartet micromeres (n = 20) (Fig. 9B, C) and with the macromeres 2A, 2B, 3C, 3D (n = 20) (Fig. 9F). In some embryos, the 3rd quartet is born just prior to the division of 2d, and in these cases 2d makes contact with the 3c and 3d mesentoblasts (Fig. 9E). During the mitotic phase of 2d, contacts with the 2nd quartet micromeres are no longer detected (Fig. 9B), and possibly prevented by the position of the 1st quartet micromere progeny in the embryo (1a1, 1b1, 1c1, 1d1) (Fig. 9C). These observations suggest that the 2d somatoblast in C. teleta has the expected morphology of a “contact-mediated” organizing cell. Such characteristic morphology has been described in detail for other spiralians such as Lymnaea palustris and Haminoea callidegenita for 3D, and in Crepidula fornicata for 4D (Boring, 1989; Henry and Perry, 2008; Martindale et al., 1985). Thus, our observations document direct cell contact between 2d and cells that show a disruption of fate when the organizer of C. teleta, the 2d somatoblast, is deleted.

**ERK/MAPK activation does not appear to be involved in axial patterning**

Molluscs represent the only group of spiralians in which specification of the D quadrant and its role during early embryonic development have been studied at a molecular level. In these animals, the ERK/MAPK pathway appears to be important in specification of the D quadrant (Henry and Perry, 2008; Lambert and Nagy, 2001). There is evidence to suggest that ERK/MAPK signaling also has a role in patterning the dorsal–ventral body axis of molluscs (Koop et al., 2007; Lambert and Nagy, 2001). In annelids, ERK/MAPK activation has been reported for the equal-cleaving Hydrodides elegans, and is restricted to the 4d cell; however, its role during development remains unknown (Lambert and Nagy, 2003). In the leech Helobdella, inhibition of ERK/MAPK has been suggested to not affect axes formation (Gonsalves and Weisblat, 2007).

To determine if the ERK/MAPK pathway is activated in early stage embryos of C. teleta, we used an anti-phospho-ERK-2 antibody. During early development, activated MAPK is not detected in any cells, including in 3D or 4D, even 30 min after the birth of the 4d cell, although signal was detected in at least one of the two polar bodies (Fig. 10A–H; data not shown). The earliest detectable ERK/MAPK activation is during epiboly (gastrulation) in cells positioned around the blastopore lip (Fig. 10I–L). In early larval stages (stage 4–5), ERK/MAPK activation is barely detectable in a few cells in the head region, in the posterior hindgut, trunk ectomesoblasts, and in cells surrounding the mouth opening (data not shown).

To gain further insight into the role of this pathway during C. teleta development, embryos were exposed to the drug UO126 to inhibit the kinase activity of MEK, and thus block phosphorylation of ERK/MAPK (Fig. 10M–R; Suppl. Fig. S4). We performed inhibitory drug treatments at different concentrations and during different time intervals (Suppl. Fig. S4, bottom panel). UO126 prevents ERK/MAPK activation in C. teleta embryos at the lowest UO126 concentration used (5 μM), verified by using the MAPK/ERK2 antibody following UO126 treatment (Fig. 10M, O). We found that larvae resulting from treatment with the UO126 inhibitor do not show any disruption of axial patterning (Fig. 10Q, R; Suppl. Fig. S4). This is true for all concentrations and time windows tested. Although larvae resulting from the UO126 treatments have all the differentiated tissues and normal axes organization present in control larvae (Fig. 10Q, R), most have a slightly compact shape, smaller eyes, a shorter head, reduced foregut, a constriction in the position of the posterior growth zone, a decreased number of muscle fibers and disorganization of axon tracts, regardless of the condition tested (Fig. 10Q; Suppl. Fig. S4). These observations suggest that ERK/MAPK activation is not required for D quadrant specification, organizing activity or establishment of the larval body axes in C. teleta, and contrasts with previous data from molluscs.

**Discussion**

In this study, we identified the 2d somatoblast as the key cell responsible for organizing activity during early embryonic development in the marine polychaete annelid C. teleta. By using a number of morphological characters, and tissue and cell type-specific markers that are differentially expressed along the A/P and D/V axes (Ct-elav1, Ct-forkhead, Ct-gataB3, Ct-twist1, Ct-piw1, acetylated tubulin), we analyzed tissue differentiation and patterning of the larval body. The known cell lineage and the availability of previously characterized tissue-specific makers greatly facilitated the interpretation of body organization in larvae resulting from blastomere deletions, which frequently involved an extensive loss of tissues, making analysis of the remaining tissues more difficult. Our results show that the C. teleta organizing activity is essential for nervous system, foregut and mesoderm formation, as well as for establishment of bilateral symmetry and the D/V axis of the head. The radialization of neural markers (sc acn and Copelav1) in the larval head of C. teleta following 2d blastomere deletions are similar to the observed radialization of the neural marker serotonin in the larval head resulting from ERK/MAPK inhibition in Haliothis asinina (Koop et al., 2007). The timing of C. teleta organizing activity occurs prior to completion of the birth of the 2d daughters (2d1 and 2d2), and differs from the timing and cellular identity of organizing activity in other spiralians (Suppl. Table 4). Another difference between C. teleta and other spiralians is in the molecular pathway involved in organizing activity. In C. teleta, ERK/MAPK signaling does not appear to be necessary for either the establishment of the organizer or its activity. This contrasts with the importance of the ERK/MAPK pathway for establishment of the D quadrant and organizing activity in molluscs studied thus far (Henry and Perry, 2008; Koop et al., 2007; Lambert and Nagy, 2001), and highlights likely molecular level variation among spiralians. Our identification of an embryonic organizing activity in the annelid C. teleta further strengthens the idea of a conserved embryonic organizing activity in spiralians.

**Consequences of variation in cellular identity of the spiralian organizer**

The present study shows that in C. teleta, an organizing signal is necessary to orchestrate cell fate, axis formation and cell behavior during early steps of embryogenesis, earlier than in molluscs or in T. tubifex. The organizing activity necessary for head formation has occurred by the time that 2d1 and 2d2 are born, approximately two cell divisions earlier than in other spiralians studied. In the molluscs, L. obsoleta and C. fornicata, organizing activity is completed shortly after the 4th quartet (4d cell) is born, or prior to the birth of its daughters (ML and MR), respectively. In another annelid, the oligochaete T. tubifex, organizing activity appears to
occur during the lifetime of the 4th quartet (involving both 2d11 and 4d cells). Although these embryological experiments show clear species-specific differences, one cannot rule out earlier action of a D quadrant organizing activity, which allows for temporal overlap in signaling among species.

An intriguing consequence of differences in the timing and identity of the cell necessary for the organizing activity is that in C. teleta, the organizing activity is associated with a cell that does not have either a mesodermal and/or endodermal fate as has been found in molluscs and in T. tubifex, respectively (Henry and Perry, 2008; Lambert and Nagy, 2001; Meyer et al., 2010; Nakamoto et al., 2010). In C. teleta, the organizing activity is associated with a cell that has restricted ectodermal fates; the 2d somatoblast gives rise to the entire trunk and posterior ectoderm (Meyer et al., 2010). These data reveal that the germ layer precursors associated with organizing activity may differ among species, and reveals more variation in spiralian embryogenesis than had previously been appreciated. In addition, because the identity of the mesodermal precursors is different from the cell with organizing activity in C. teleta, the potential role of the organizing activity on mesoderm formation can be addressed for the first time in spirilians using directed cell deletions. We demonstrate that deletion of 2d leads to substantial reduction of mesodermal precursors in resulting larvae.

Studies in vertebrates have demonstrated the importance of mesodermal precursors in specification and organization of other tissues during early development (Halpern, 1997; Harland, 2008; Harland and Gerhart, 1997). It is possible therefore that 2d has an indirect influence on head patterning through activity on mesodermal precursors. For example, if 2d has a role in mesoderm specification and/or maintenance as our results suggest (Fig. 8), the mesoderm may have an organizing activity that functions subsequently to signaling from 2d. However, since double deletion of trunk mesoderm precursor cells (2C plus 2D) result in larvae with normal head patterning, it is clear that the trunk mesoderm precursors are not essential for head patterning. In C. teleta there are additional embryonic sources of mesoderm, and the 3a blastomere generates anterior head mesodermal tissues (Meyer et al., 2010). Therefore, not all mesoderm is removed after deletion of both 2C and 2D. Deletion of the precursor of 3a at the 4-cell stage (A cell) gives rise to larvae with head patterning similar to control larvae; nevertheless, it is possible that the presence of any

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**Fig. 10.** Activation of the ERK/MAPK pathway during early development in C. teleta. A–L: Pattern of ERK/MAPK activation. Labeling is indicated to the left of the figure. A–D: Same embryo following birth of the fourth quartet micromeres. E–H: Same embryo 30 min after the birth of 4d. ERK/MAPK activation is not detected at the third quartet stage, during birth of the fourth quartet, or 30 min after the birth of 4d. I–L: MAPK activation is first detected in gastrula stage embryos in a few cells surrounding the blastopore lip (white arrows in I). The blastopore lip is delineated as a discontinuous line in I. M–R: Inhibition of ERK/MAPK activation via treatment with U0126. DMSO and U0126 treatments do not interfere with axes establishment in larvae. M–N: Control embryo at gastrula stage treated with 0.1% DMSO. O–P: Experimental embryo at gastrula stage exposed to 10 μM U0126. The blastopore lip is delineated as a discontinuous line in M and O. Q: Control larva exposed to 0.1% DMSO treatment. R: Experimental larva exposed to 10 μM U0126. Q–R: Multichannel confocal projections showing actin filaments in white, nuclei in blue and acetylated tubulin in yellow; white arrows show the scac+ on the dorsal side of the brain. Both panels are lateral views with anterior to the left. A, E, I, M, O: Fluorescent images of Erk-1&2 diphosphorylated kinase in red; B, F, J, N, P: Fluorescent images of actin filament staining in green; C, G, K: Fluorescent images of nuclei in blue. L: Multichannel projection of the channels shown in I, J, K, D and H. Diagrams of the embryos shown in A–C and E–G, respectively, to illustrate cell outlines. br, brain; M, underlying macromeres; mf, muscle fibers; nt, neurotroch; pg, pygidium; pt, prototroch; tt, telotroch. Asterisk marks the mouth.
one of these mesoderm precursors (3a, 3c or 3d) may be sufficient to orchestrate bilateral symmetry and the dorsal–ventral axis of the head. Our results contrast with previous experiments performed in the mollusc I. obsoleta, in which larvae resulting from deletion of the trunk mesoderm (2D) have disorganized axial patterning (Clement, 1962; Lambert and Nagy, 2001).

Possible mechanisms underlying changes in spiralian organizer identity

Our data reveal differences in the timing of the organizing activity during spiralian development. This shift in timing may be a consequence of differences in the distribution of cytoplasmic contents along the animal–vegetal (A/V) axis or differences in the control of mitotic apparatus formation and positioning. Either during egg maturation or after fertilization, variation in the distribution of cytoplasmic contents along the A/V axis can result in differential segregation of maternal determinants among blastomeres during early mitotic cleavages (Costello, 1948; Goldstein and Freeman, 1997; Raven, 1938). This may have significant consequences on subsequent cell fates, such as has been documented in diverse animal phyla, including in molluscs and annelids (Roegers et al., 1999; Yoneda et al., 1982). It has been proposed that cortical contractions along the A/V axis of the egg, combined with attachment of molecules to the egg cortex lead to heterogeneous spatial reorganization of egg contents, resulting in bipolar cytoplasmic organization in cleitellate eggs (T. tubifex) and uninipolar cytoplasmic organization in polychaete eggs (N. virens and P. dumerili) (Shimizu, 1999).

In addition to differential localization of egg contents, control of the dynamics and positioning of the mitotic apparatus are necessary for correct segregation of determinants involved in cell fate specification, asymmetric cell division and axis establishment during development (Velarde et al., 2007; Wallenfang and Seydoux, 2000). In spiralians, asymmetric cell division and stereotyped cell positioning in the early stage embryo are well-conserved characteristics, suggesting precise control of mitotic spindle formation and positioning. Species-specific timing differences in the organizing activity among spiralians may be explained by differences in the control of mitotic spindle dynamics and positioning that result in variation in both the amount of cytoplasm and the nature of maternal determinants inherited by each blastomere. A morphologically observable example of species-specific variation in inheritance of cytoplasmic contents is illustrated by size differences of the 2d somatoblast. In some spiralians, the 2d cell is equal in size to other cells from the 2nd quartet (e.g. C. fornicata and I. obsoleta) (Freeman and Lundelius, 1992), and in others, as in C. teleta, the 2d cell is bigger than other 2nd quartet cells (e.g. T. tubifex, P. dumerili, Unio, Freeman and Lundelius, 1992). In our current knowledge of the organizing activity, when the 2d cell is equal in size to other cells from the 2nd quartet, 4d or its progenitor 3D, is the organizing cell (mesendodermal fate) (Freeman and Lundelius, 1992; Clement, 1962; Henry and Perry, 2008; Lambert and Nagy, 2001, 2003). When the 2d cell is bigger than other 2nd quartet cells, the organizing activity is associated with the 2d ectodermal lineage (Nakamoto et al., 2010). To further investigate whether the size of 2d may indicate the identity of the organizing cell as well as to determine if there is a more extended link between the ectodermal lineage (2d) and the organizing activity in annelids, it will be of interest to determine the cellular identity of the organizing activity in the annelid P. dumerili or in the bivalve mollusc Unio, in which 2d is bigger than the other cells of the 2nd quartet (Ackermann et al., 2005; Freeman and Lundelius, 1992; Lillie, 1895; Schneider et al., 1992).

Variation in the distribution of cytoplasmic contents along the A/V axis of the egg, plus control of mitotic apparatus formation and positioning, may generate differences in the identity of the organizing cell, and thus in the timing of action of organizing activity. For example, the molecules involved in organizing activity may be inherited by the 3D and 4d mesentoblast (endomesoderm lineage) or passed earlier into the 2d cell (ectoderm lineage), depending on the species. As a consequence, the organizing activity may occur in different embryonic contexts for both the cell that is signaling and the targeted cells in different species. Shifts in cell fate have been observed in spiralians; a well-documented example is a change in the competence to generate an eye from the 1st quartet to the 2nd quartet of micromeres in chitons relative to other molluscs (Clement, 1967; Henry et al., 2004). Such shifts may be the result of differences in segregation of determinants and positioning of the mitotic spindle, and this variation may play an important role in generating the diversity of form found across Lophotrochozoa.

Organizing activity in annelids

The only other annelid for which there is functional data related to organizer activity is in the oligochaete T. tubifex. In this oligochaete, two cells are necessary for organizing activity, 2d11 (a 2d descendant, ectodermal fate) and 4d (mesodermal fate) (Nakamoto et al., 2010). Our current data for C. teleta associate the organizing activity to a single cell with ectodermal fate (2d). Although both annelids require a precursor from the 2d lineage for organizing activity, the signal occurs at a later developmental stage in T. tubifex relative to C. teleta, and involves an additional mesendodermal lineage (4d).

In T. tubifex, a heterochronic heterotopic graft of 2d11 plus 4d into the empty “bed” of a depleted endodermal precursor in an older host embryo leads to double axis formation in the resulting animal. The grafted cells give rise to the tissues they normally generate: 2d11 gives rise to epidermis and neural tissues, and 4d makes its normal mesodermal tissues (Nakamoto et al., 2010). Since cell fate specification occurs during the first few embryonic cleavages in T. tubifex (Penners, 1926), the grafted cells may have already been specified prior to transplantation and they may simply be executing their intrinsic developmental program. In addition, the graft does not appear to change the fate of the host cells. The subsequent elongation of the ectoderm and mesoderm in the secondary axis derived from the transplant may influence morphogenesis of host tissue endodermal precursors. Therefore, one has to question whether these transplanted cells can actually organize a second axis in T. tubifex. Since our data in C. teleta clearly show that the organizing activity from the 2d cell emanates earlier, and is necessary for both patterning and specification of multiple cell fates, it will be of great interest to perform a homochronic heterotopic graft of 2d in T. tubifex. This experiment will allow for a more direct comparison between these two annelids and determine whether the 2d somatoblast produces an inductive signal necessary for both cell fate specification and axes establishment in T. tubifex, such as is the case in C. teleta. Combining our results from C. teleta with previous results from the T. tubifex, we may also hypothesize that in annelids, the 2d cell is necessary for head (anterior) patterning, and the descendants of 2d plus mesoderm progenitors are involved in trunk (posterior) patterning.

Blastomere deletions were performed in T. tubifex after the birth of both ectodermal (2d descendants) and mesodermal precursors (4d cell) at the 22-cell stage. When 2d descendants are deleted in T. tubifex, the authors observed that “the mesoderm” GBs [germbands] lengthen and curve round toward the ventral side of the embryo, in a very similar manner to that in

intact embryos” and “the [mesoderm] GBs on either side are located at a distance from the ventral midline; they are separated from each other furthest at their anterior ends” (Goto et al., 1999b), indicating that both D/V (ventral) and A/P (anterior) axes can be identified in the resulting embryo. These data suggest a role of the descendants of 2d cell in orchestrating mesodermal precursor behavior in T. tubifex embryos, but not a role in axis establishment, per se. If an organizing activity is important for axial patterning in T. tubifex, it likely occurs prior to the birth of the 2d descendants. In addition, combined 2d11 (a 2d descendant) plus 4d blastomere deletions (at the 22-cell stage) result in “rounded cells that failed to exhibit any sign of axial development” (Nakamoto et al., 2010). This phenotype appears to be superficially similar to the larval phenotype resulting from individual 2d blastomere deletions in C. teleta, although the phenotypes in T. tubifex were analyzed in limited detail. It is possible that these two sets of experiments (2d11 plus 4d cell deletions in T. tubifex or 2d blastomere deletions in C. teleta) give rise to a similar phenotype because, as our results suggest, removal of 2d in C. teleta results in larvae with considerably reduced mesodermal domains (Fig. 9). For a better understanding of possible similarities or differences between these two species, it will be of interest to analyze head patterning in detail in T. tubifex using tissue-specific markers following deletion of 2d, 2d descendants or 2d11 plus 4d blastomeres.

Trunk patterning

In C. teleta, deletion of 2d precursors (D or 1D), 2d itself, or its daughter cells (2d1 plus 2d2) removes the entire trunk ectoderm, and therefore analyses of these cell deletion experiments are limited to investigating effects of organizing activity on the remaining anterior head tissues and endodermal precursors normally derived from the four macromeres (4A, 4B, 4C, 4D). In control larvae, the endoderm becomes internalized at gastrulation and is positioned posterior to the prototroch in the trunk. Interestingly, in deletions of trunk ectoderm (D, 1D, 2d or 2d1 plus 2d2 deletions), the endodermal precursors (4A, 4B, 4C plus 4D only in 2d or 2d1 plus 2d2 cell deletions) are still internalized, but become localized anterior of the prototroch. These observations suggest that the endoderm requires either organizing activity or presence of the trunk ectodermal tissue to become correctly positioned (Fig. 4A–C). In addition, because not all muscle fibers are absent from larvae resulting from D quadrant deletions, we cannot exclude the possibility that the mesoderm or the combination of both mesoderm and ectoderm controls endoderm cell behavior as previously suggested for T. tubifex (e.g., the influence of 2d11 plus 4d on the elongation of endodermal tissue) (Nakamoto et al., 2010). These data suggest that the organizing activity in C. teleta may also influence, directly or indirectly, morphogenetic movements in the developing embryo.

Organizing activity and the evolution of animal complexity

Spiralian embryos represent an advantageous system for understanding the emergence of diversity of body forms because this phylum exhibits a high diversity of body shapes yet shares a stereotypic cleavage program. Coupled with the fact that the spiralian embryonic organizer is represented by one or two identifiable cells allows for a detailed characterization of the timing and role of organizing activity in generating these body plans. From a comparative standpoint, if organizing activity occurs much earlier during development when embryos only have a few cells, as opposed to embryos with many more cells, the consequences may be radically different in terms of the potential number of distinct tissues and cell types that can be generated in the animal. The signaling mechanisms utilized to effect these changes may also need to be different (i.e., direct cell–cell contact versus long-range signaling). With regard to the latter, long-range signals involving many cells may permit gradients that can be interpreted to create more numerous intermediate states, which have the potential to define additional cell fates and lead to the emergence of novel cell types and tissues. Comparing the effects of an organizing activity that occurs in an embryo with a limited number of cells, as in spirarians, to organizing activity in an embryo with thousands of cells, as in vertebrates, may give valuable insight into the evolution of complexity and specific properties of different organizing activities.

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Appendix A. Supporting information

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References


