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Establishment and activity of the D quadrant organizer in the marine gastropod *Crepidula fornicata*

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ABSTRACT

During development in metazoan embryos, the fundamental embryonic axes are established by organizing centers that influence the fates of nearby cells. Among the spiralians, a large and diverse branch of protostome metazoans, studies have shown that an organizer sets up the dorsal-ventral axis, which arises from one of the four basic cell quadrants during development (the dorsal, D quadrant). Studies in a few species have also revealed variation in terms of how and when the D quadrant and the organizer are established. In some species the D quadrant is specified conditionally, via cell-cell interactions, while in others it is specified autonomously, via asymmetric cell divisions (such as those involving the formation of polar lobes). The third quartet macromere (3D) typically serves as the spiralian organizer; however, other cells born earlier or later in the D quadrant lineage can serve as the organizer, such as the 2d micromere in the annelid *Capitella teleta* or the 4d micromere in the mollusc *Crepidula fornicata*. Here we present work carried out in the snail *C. fornicata* to show that establishment of a single D quadrant appears to rely on a combination of both autonomous (via inheritance of the polar lobe) and conditional mechanisms (involving induction via the progeny of the first quartet micromeres). Through systematic ablation of cells, we show that D quadrant identity is established between 5th and 6th cleavage stages, as it is in other spiralians that use conditional specification. Subsequently, following the next cell cycle, organizer activity takes place soon after the birth of the 4d micromere. Therefore, unlike the case in other spiralians that use conditional specification, the specification of the D quadrant and the activity of the dorso-ventral organizer are temporally and spatially uncoupled. We also present data on organizer function in naturally-occurring and experimentally-induced twin embryos, which possess multiple D quadrants. We show that supernumerary D quadrants can arise in *C. fornicata* (either spontaneously or following polar lobe removal); when multiple D quadrants are present these do not exhibit effective organizer activity. We conclude that the polar lobe is not required for D quadrant specification, though it could play a role in effective organizer activity. We also tested whether the inheritance of the small polar lobe by the D quadrant is associated with the ability to laterally inhibit neighboring quadrants by direct contact in order to normally prevent supernumerary organizers from arising. Finally, we discuss the variation of spiralian organizers in a phylogenetic context.

1. Introduction

Metazoan embryos deploy inductive organizers to set up developmental axes and to specify particular cell fates (Meinhardt, 2015; Anderson and Stern, 2016; Kraus et al., 2016). The evolutionary history of how these organizers arose and evolved is not well-understood in many groups; one of the reasons for this being the fact that it is often unclear if organizers are homologous between distantly related taxa/phyla. The Spiralia provide a test case for studying the evolution of embryonic organizers because the group exhibits substantial variation in how organizers arise and signal, but they all function within the context of an undoubtedly homologous developmental program (Henry, 2014). The Spiralia, the largest clade of metazoan phyla, includes members that exhibit spiral cleavage (e.g., annelids, molluscs and polyclad flatworms), which is believed to be an ancestral characteristic for this clade.

In animals exhibiting spiral cleavage, the dorso-ventral axis is set up by an organizer derived from the dorsal (D) cell quadrant, which induces the fates of adjacent cells. Two principle mechanisms have been described for how the D quadrant is established: autonomous cell

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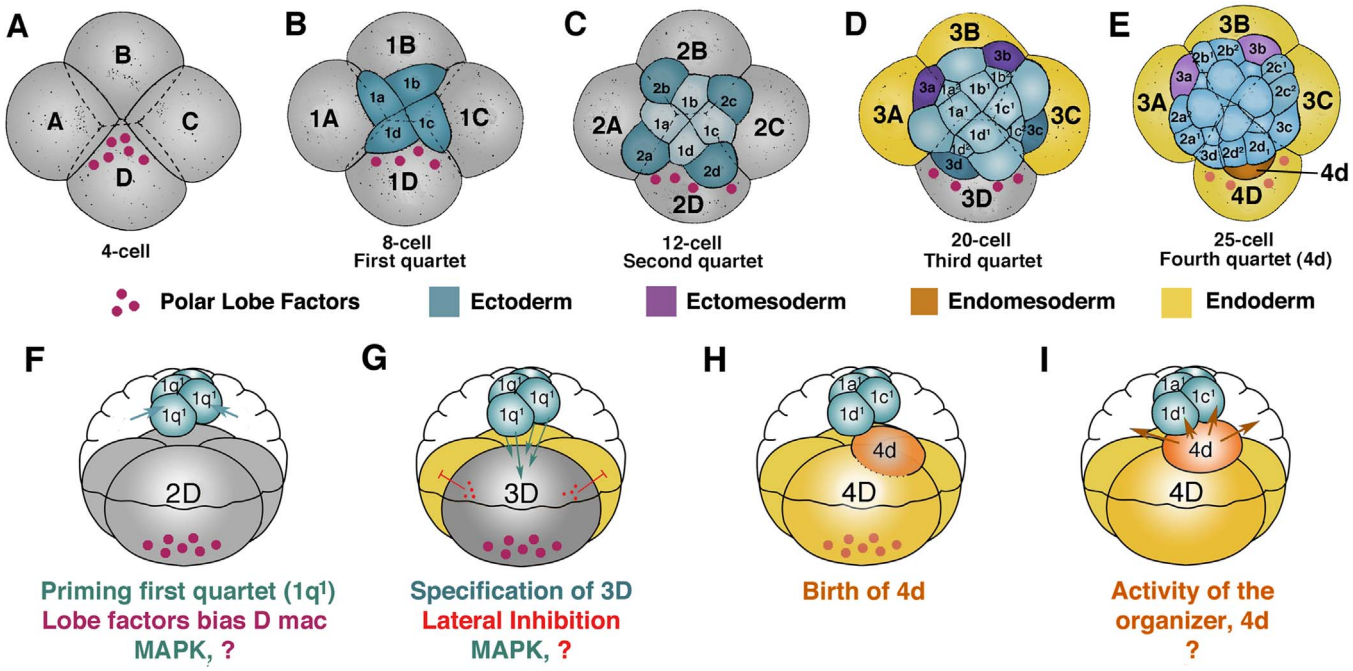

Fig. 1. Early cleavage pattern in *C. fornicata* and proposed model summarizing basic mechanisms involved in specifying the dorsal D quadrant and subsequent D quadrant organizer activity during early development. A-E). Early arrangement of blastomeres through the fifth round of cleavage divisions and the formation of the 4d mesentoblast (embryos viewed from the animal pole. Blastomere nomenclature follows that of Conklin, 1897). F-G). Hypothetical model to explain the specification of the D quadrant and organizer signaling in *C. fornicata*. In the case of equal-cleaving embryos such as *C. fornicata*, the D quadrant is established conditionally, as a result of animal-vegetal inductive interactions that involve the animal-most progeny of the first quartet micromeres ($1q^1$ cells). Typically this occurs during the interval between fifth and sixth cleavage when animal cells come into contact with one of the four vegetal macromeres and transmit an unknown signal that triggers this cell to become 3D. This series of events appears to trigger MAPK activation within the 3D macromere (Henry and Perry, 2008). F). On the basis of observations made in *C. fornicata*, there is also an earlier signal that primes the animal micromeres that also involves the activation of MAPK in those cells (Henry and Perry, 2008). G). Subsequently these $1q^1$ micromeres specify the 3D macromere, via inductive signals. Though all four macromeres are capable of becoming 3D, only one emerges, and this could potentially involve some form of lateral inhibition imparted by factors segregated to this cell via the polar lobes. Once the 3D macromere is specified, in some species it becomes the key organizer of development that sets up the dorso-ventral axis and directs the fates of adjacent cells in the other quadrants. The nature of those signals is not clear, though it appears to trigger the activation of MAPK in a subset of overlying animal micromeres. H-I). In *C. fornicata* it is the daughter cell of 3D (i.e., 4d) that serves as the organizer (Henry et al., 2006). Illustrations after Henry (2014) and Henry and Lyons (2016). Signaling interactions (factors, lines and arrows) implicated in some events are noted with corresponding colors. The molecular basis of some signaling interactions are undetermined ("?").

inheritance, or conditional cell signaling (Freeman and Lundelius, 1992; Lambert et al., 2003; Henry, 2014). Interestingly, the gastropod *C. fornicata* uses a combination of conditional and autonomous mechanisms to specify the D quadrant. This paper reveals new details about how the dorsal cell quadrant is established and when organizer signaling takes place.

In spiralian embryos, the first and second cleavage divisions form four cells, which in turn give rise to cells of the four basic embryonic quadrants, termed A, B, C and D (Figs. 1A-E, and 2A, Conklin, 1897). In some species, those first two cell divisions are unequal and these typically generate a 4-cell stage embryo in which one cell (D) is larger (Fig. 2A). That cell ultimately gives rise to the organizer that sets up the dorso-ventral axis (Freeman and Lundelius, 1992). In these cases, the unequal first and second cleavage divisions segregate unidentified factors, localized within the vegetal pole, directly into the larger D blastomere to specify this cell's fate autonomously (factors indicated by the orange color in Fig. 2A, see van den Biggelaar and Guerrier, 1983; Verdonk and Cather, 1983; Henry, 2014). The other three quadrants, particularly the two quadrants adjacent to the D quadrant (A and C) rely on inductive signaling from the D quadrant organizer to determine their fates. In some cases, cleavage asymmetries are established by the formation of vegetal polar lobes, which are transient protrusions that fuse with one of the resulting blastomeres during first and second cleavage (see Fig. 2B). Different species may form large or small polar lobes (compare Fig. 2B-C). With few exceptions (see below), experiments involving the removal of these polar lobes (or the vegetal-most region in unequal-cleavers without polar lobes) reveal that this vegetal region contains the determinants essential for specifying the D quadrant (Hatt, 1932; Novikoff, 1938; Clement, 1952, 1967; Rattenbury and Berg, 1954; Cather and Verdonk, 1974; van Dongen and Geilenkirchen, 1974, 1975; van Dongen, 1976a, 1976b; Cather et al., 1976; Dohmen, 1983; Render, 1983; Henry, 1986; Henry and Martindale, 1987; Henry et al., 2006).

Another key experiment also reveals the role of the vegetal region and the significance of its asymmetric inheritance by the larger D blastomere. In unequal-cleavers, twinning can occur if the first cleavage division is experimentally equalized and the vegetal region (or contents of the large vegetal polar lobe) is divided between both of the resulting blastomeres. The result is typically duplication of the D quadrant, the organizer, developmental axes and associated (induced) cell fates (Titlebaum, 1928; Tyler, 1930; Ghareeb, 1971; Henry and Martindale, 1987; Render, 1989). This "twinning" can be accomplished in different ways, including simply by compressing fertilized eggs under coverslips prior to first cleavage (Fig. 2D). Once the first division is equalized, and compression is relaxed, both daughter cells typically divide unequally at the next cleavage division, resulting in the formation of two larger D quadrant blastomeres and two smaller C quadrant blastomeres. The appearance of duplicated structures (e.g., ocelli), that are not derived from the duplicated D quadrant cells, reveals that each of the two D quadrants is capable of organizing a separate set of developmental axes.

The second form of early cleavage found in spiralians involves equal cleavage divisions, and in these cases at the time of their birth the four cell quadrants cannot be distinguished (Fig. 2E). In equal-cleavers, the D quadrant is specified conditionally, well after the 4-cell stage. Work carried out in a few equal-cleaving species indicates that the D quadrant is specified by cell-cell inductive interactions from the progeny of the animal first quartet micromeres, by virtue of their

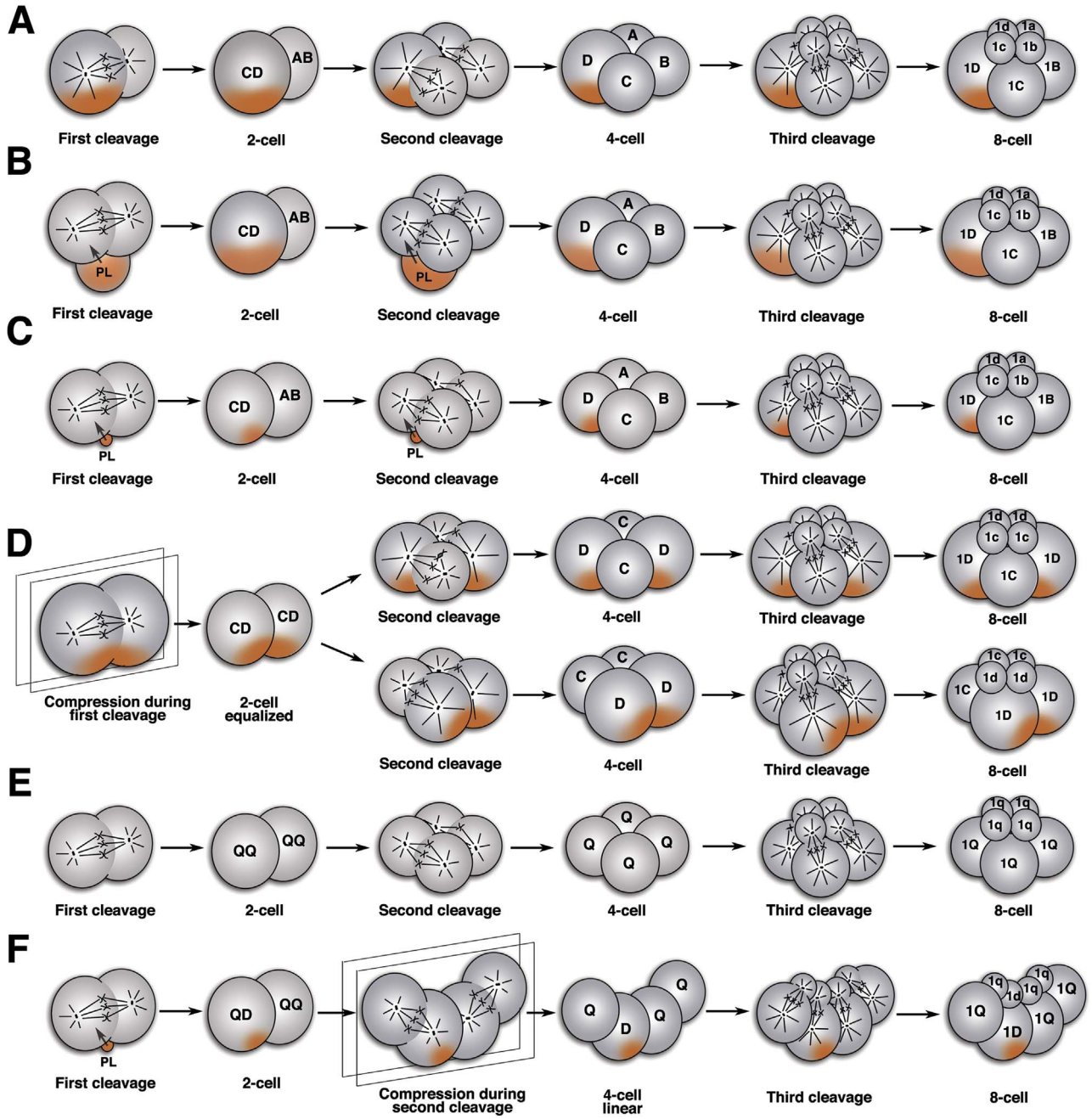

Fig. 2. Diagrams illustrating basic types of spiral cleavage leading to the 8-cell stage, and experimental manipulations to investigate mechanisms regulating specification of the dorsal D quadrant. A). Unequal spiral cleavage in species that have asymmetric first and second cleavage divisions. These asymmetries are created by shifting the cleavage spindle. The A-D blastomeres can be clearly distinguished at the 4-cell stage. The large D quadrant blastomere is specified autonomously by inheriting key vegetal components (orange color). B). Unequal spiral cleavage in species that form large polar lobes that generate the asymmetric first and second cleavage divisions. Cleavage is equal but a large enucleate vegetal lobe is protruded, which subsequently fuses with one of the daughter cells to pass key vegetal components to that cell. C). Unequal spiral cleavage in species that form small polar lobes during first and second cleavages. With a few notable exceptions (e.g., the annelid *Chaetopterus* and the mollusc *C. fornicata*) these cases exhibit asymmetric cleavages that autonomously specify the D quadrant in a similar fashion to that seen in species with large polar lobes. The two exceptions behave more like equal-cleavers, and the polar lobe does not appear to be required for development of the D quadrant (Henry, 1986, 1989; Henry et al., 2006; Henry and Perry, 2008). D). Compression of zygotes between coverslips during the first cleavage division in species that normally have unequal cleavage. This manipulation results in equalizing the first cleavage division. Once first cleavage is completed, compression is released and the embryos continue to divide as if they contain two CD cells forming a 4-cell stage embryo with two large D quadrant cells in one of two different configurations, as shown. E). Equal spiral cleavage in species that have symmetric first and second cleavage divisions. The orientation of the spindles is shown during each cleavage, as labeled. Corresponding cell stage is shown following each cleavage division. The first quartet of small animal micromeres is born at the third cleavage division. The four quadrants, (labeled as "Q") cannot be distinguished until later during development. In these cases the D quadrant is specified at a later stage of development by virtue of inductive interactions involving the progeny of the first quartet micromeres (see Fig. 1**G** and the text). F). Creation of a linear embryo in *C. fornicata* by compressing the 2-cell stage embryos through second cleavage (this study). Once second cleavage is completed compression is released and the embryos continue to divide to form micromeres at the animal pole. PL, polar lobe. Blastomeres labeled according to Conklin (1897). Diagrams modified after those of Henry (2014).

contact with one of the vegetal macromeres (the future 3D macromere) during the interval between 5th and 6th cleavage (Fig. 1D-G, van den Biggelaar and Guerrier, 1979; Arnolds et al., 1983; Martindale et al., 1985; Boring, 1986; Kühtreiber et al., 1988; Van den Biggelaar, 1996; Henry, 2002). Removal of these four micromeres (1a-1d), leads to radialized development (van den Biggelaar and Guerrier, 1979;

Arnolds et al., 1983; Martindale et al., 1985; Boring, 1986; Van den Biggelaar, 1996; Henry, 2002; Henry et al., 2006). In fact, any one of the four cell quadrants can be forced to assume the fate of the D quadrant by biasing cell contacts from the first quartet progeny towards one of the four macromeres (Arnolds et al., 1983; Boring, 1986; Henry, 2002). Once selected, typically the 3D macromere (or its daughter 4d in the case of *C. fornicata*) serves as the organizer (Fig. 1D-E, H-I).

Relatively little is known about the molecular pathways that regulate the specification of the D quadrant. However, activated MAPK, downstream of an unidentified signaling cascade, plays a role in D quadrant specification and possibly organizer activity in several spiralians (Lambert and Nagy, 2001, 2003; Lartillot et al., 2002b; Koop et al., 2007; Henry et al., 2008). Once the D quadrant is specified, activated diphosphorylated MAPK (dpMAPK) is found in the 3D macromere, and/or its progeny 4d, in both equal- and unequal-cleaving spiralians.

Embryos of the marine gastropod *C. fornicata* represent an exception to the foregoing generalizations, in that they use a combination of conditional and autonomous mechanisms to specify the D quadrant (Fig. 1A-E). These embryos appear as though they are equal-cleavers, but upon careful inspection it was found that they possess a very small polar lobe (Conklin, 1897; Henry et al., 2008). Lineage tracing experiments revealed that the polar lobe always fuses with the blastomeres that ultimately give rise to the D quadrant (Henry et al., 2006). Despite the presence of the polar lobe, *C. fornicata* utilizes a conditional, inductive mechanism to specify the D quadrant, as found in other equal-cleavers (Henry et al., 2006). We showed that, as is the case in other equal-cleavers, any one of the four quadrants can become the D quadrant in *C. fornicata*. Likewise, the first quartet micromeres are required for the specification of the D quadrant and the dorso-ventral axis in *C. fornicata*. However, unlike the case in unequal-cleavers (like *Bithynia tentaculata,* Cather and Verdonk, 1974), removal of the polar lobe does not result in the absence of the D quadrant organizer. Surprisingly, this has the opposite effect and leads to the formation of supernumerary D quadrant cells (Henry and Perry, 2008). These extra D quadrant cells were revealed in fixed samples by the presence of activated dpMAPK in multiple third quartet (3D) macromeres (14% “twins”, or more rarely, “triplets”).

As summarized in Fig. 1F-I, these observations support a multi-step model to account for the specification of the D quadrant, and for organizer activity in *C. fornicata* (Henry, 2014; Henry and Lyons, 2016). The findings suggest that the polar lobe in *C. fornicata* functions to bias D quadrant specification to insure that during normal development only a single D quadrant forms. As the *C. fornicata* polar lobe is dispensable for D quadrant formation, we hypothesize that the polar lobe may transfer factors to the future D quadrant that impart its progeny (e.g., the 3D macromere) with a contact mediated lateral inhibitor that prevents cells in the adjacent quadrant from assuming supernumerary D quadrant fates (see Fig. 1G).

Although we showed previously that the presence of the first quartet is required for the establishment of the D quadrant in *C. fornicata* (Henry et al., 2006), we did not establish the timing of these interactions, nor determine if these inductive interactions are possibly conserved. Additional experiments indicated that 4d (or else its progeny) serve as the organizer (Henry et al., 2006), but those exact cells and the window of organizer signaling was also not determined. Here we carried out sequential cell ablation experiments to define the timing of D quadrant specification and organizer signaling. While some aspects are conserved, such as the timing of D quadrant specification, we found that the specification of the D quadrant and the activity of the dorso-ventral organizer are temporally and spatially uncoupled, which differed from the situation found in other equal-cleaving spiralians.

We also report on the phenomenon of twinning in *C. fornicata,* which provides further insight into the capacity and role of the organizer in axial specification. We document the spontaneous occurrence of twins in some unperturbed sacs of embryos, which mirrors the behavior of twins generated by experimental means (via polar lobe removal). While these twins exhibited supernumerary D quadrants, they did not exhibit well defined duplicated axes (e.g., dorso-ventral axes). Therefore, organizer signaling appears to be very weak or absent in those cases. The occurrence of these twins could imply that development in this system (like that expected of equal-cleaving species) is more labile and susceptible to various perturbations. This vulnerability could have led to the evolution of autonomous mechanisms to specify the D quadrant (see also Freeman and Lundelius, 1992). In that light, and following our previously proposed model (Henry and Lyons, 2016), we tested whether the small polar lobe may play a role in regulating D quadrant specification to prevent twinning by imparting one quadrant with a contact-mediated lateral inhibitor that prevents adjacent quadrants from assuming the D quadrant fate. The results did not support this hypothesis, and suggest that the polar lobe biases D quadrant specification through a different, yet to be identified, mechanism.

2. Materials & methods

2.1. Procuring eggs and embryos

Gravid adult *C. fornicata* were collected by the Marine Resources Center of the Marine Biological Laboratory in Woods Hole, MA. These animals were collected between the months of January and March each year and maintained in chilled, running sea water (12 °C) until needed. Spawning was stimulated by warming the animals to ambient temperatures of 18–22 °C. Embryos were raised, as previously described (Henry et al., 2006; Hejnol et al., 2007; Henry et al., 2010a, 2010b). Briefly, using sharpened number 5 watchmakers forceps, embryos were released from the egg capsules into gelatin coated petri dishes containing 0.2 μm-filtered seawater (FSW) with penicillin (100U/ml, Sigma, St Louis, MO) and streptomycin sulfate (200 μg/ml, Sigma). Embryos were subsequently reared at 20 °C.

2.2. Laser ablation

Animal micromeres (1a-1d or their progeny $1a^1$–$1d^1$ or $1a^2$–$1d^2$), or the 4d micromere (or its progeny, the bilateral teloblasts ML and MR), were ablated at specific times using two sequential 200msec pulses/cell of 1450 nm IR laser light, as previously described (Lyons et al., 2012; see also Henry et al., 2010b). To visualize these cells for laser ablation, we labeled embryos with Rhodamine B Isothiocyanate (RITC) following the protocol of Wray and McClay (1988, see Supplemental Fig. 1A-C). Briefly, embryos at the appropriate stages were placed in freshly prepared 0.4 μg/ml RITC diluted in 0.2 μm filtered sea water (FSW) for approximately 10–30 min and washed twice in FSW. As the 4d micromere forms precociously and is rather large, one does not need to label those embryos with RITC for 4d ablation. Alternatively, all nuclei were labeled with Histone H2B-mCherry by injecting synthetic mRNA into fertilized eggs (see Lyons et al., 2015). Ablation was accomplished using a Zeiss compound fluorescence microscope equipped with a XyClone laser (Hamilton-Thorne, Beverly, MA). In some cases cells were ablated by over injecting them with lineage tracer (see below). In either case one can verify that the fluorescently-labeled target cells have been removed, which occurs quickly following these treatments (see Supplemental Fig. 1D-G).

2.3. Lineage tracing

4d micromeres were pressure microinjected with fluorescent lineage tracers (Rhodamine Green Dextran, cat # D-7153, or DiIC18 (3), cat # D-282, Life Technologies, Grand Island, NY) at the 25-cell stage, as previously described (Hejnol et al., 2007; Lyons et al., 2012). In the case of twin embryos, both of the 4d micromeres were injected at the equivalent, 26-cell stage.

2.4. Removal of the polar lobe

The polar lobe was removed during first cleavage using a fine-bore pipette and suction, as previously described in Henry et al. (2006).

2.5. Generating linear embryos

2-cell stage embryos were flattened to realign the cleavage spindles and generate a more linear arrangement of blastomeres at the 4-cell stage (see Fig. 2F; similar to methods used by Titlebaum (1928), Tyler (1930), and Henry and Martindale (1987)). In contrast to normal embryos where each cell is in contact with every other cell at the 4-cell stage, linear embryos are stretched out along an axis where two middle cells are in contact with one another, and two end cells are located at opposite ends of this axis and in contact with only one of the middle cells. To accomplish this, embryos were compressed on glass slides, under the weight of a number 1 coverslip. To prevent the embryos from sticking to the glass, both the slide and the coverslip were pre-coated in a 0.1% gelatin solution and allowed to air-dry thoroughly before use (Martindale and Henry, 1997; Henry et al., 2006). The amount of compression was regulated by placing tiny bits of modeling clay (Van Aken Plastilina, Rancho Cucamonga, CA) under the four corners of the coverslip and the pressure was adjusted using forceps to push on the coverslip while observing the embryos under a dissecting scope. The amount of compression was enough to flatten the embryos and increase the cells' visible diameter by approximately 30–50%. Up to 100 embryos were placed on each slide. The slides were then transferred to a humidified chamber to prevent desiccation during compression. Compression was initiated approximately two hours after the completion of the first cleavage. Compression was released by adding additional sea water to the edge of the coverslip after these and control sibling embryos had completed the second cleavage division (embryos spent approximately one hour under compression). If necessary forceps were also used to gently lift the coverslips off of the clay feet (see results section for further details).

2.6. Fixation and immunohistochemistry

Some embryos were fixed in a 3.7% solution of ultrapure formaldehyde (made using the manufacturer's 16% stock solution, Ted Pella, Inc., Redding, CA, USA) dissolved in FSW with added Instant Ocean Aquarium Sea Salts (United Pet Group, Blacksburg, VA, USA) to adjust for reduced osmolarity (0.47 g added per 50 ml of final working volume). Embryos were fixed for one hour at room temperature and rinsed three times in 1X PBS (1.86 mM NaH_2PO_4, 8.41 mM Na_2HPO_4, 175 mM NaCl, pH 7.4), followed by three washes in 100% methanol before storage at −80 °C.

Linear embryos were fixed when sibling, uncompressed controls reached the stage at which the 3D macromere had protruded in preparation for formation of the mesentoblast (24-cell stage). Each set of embryos was immunologically stained for activated dpMAPK following the approach of Lambert and Nagy (2001, 2003); see also Henry and Perry (2008). Embryos were rehydrated with three 5–10 min washes in PBTw (PBS with 0.1% Tween-20) at room temperature (RT). Embryos were then pre-blocked in PBTw with 4% bovine serum albumin (BSA, Fraction V, Sigma, St Louis, MO) for 2 h at RT with gentle agitation, and incubated in mouse monoclonal anti-activated MAPK (anti-double phosphorylated, dpMAPK, Sigma, St Louis, MO) diluted 1:200 in PBTw with 4% BSA overnight at 4 °C. Following incubation, the samples were rinsed in six 5–10 min washes in PBTw at RT. Embryos were subsequently incubated in secondary Alexa Fluor-conjugated goat anti-mouse antibody (Alexa Fluor 594 goat anti-mouse, Molecular Probes, Eugene, OR) diluted 1:250 in PBTw with 4% BSA. The specimens were washed (six 5–10 min washes in PBTw at RT) and lastly incubated in Hoechst (diluted 1:10,000, Molecular Probes, Eugene, OR) for 20 min in order to visualize nuclei. Following two 5–10 min washes in PBTw, the embryos were mounted in PBS with 80% glycerin

In some embryos cilia were labeled using anti-acetylated tubulin (1:400, #T-7451, Sigma, St. Louis, MO) following the method described in Giani et al. (2011). These embryos were permeabilized in 0.5% Triton X-100/1X PBS and blocked in 0.5% Triton X-100/1X PBS/10% normal goat serum for 2 h at RT. Anti-acetylated tubulin antibody was added at a concentration of 1:400 and incubated overnight at 4 °C. The next day, primary antibody was removed and embryos were washed 5 times in 1X PBS/0.5% Triton X-100 over an hour. Secondary antibody (anti-mouse Alexafluor 546, 1:400) was added to the embryos and incubated at room temperature in the dark for 2 h (or alternatively overnight at 4 °C). Secondary antibody was removed and embryos were washed with 1X PBS/0.5% Triton X-100. This was followed by staining for filamentous actin in differentiated muscle cells and other tissues using Bodipy-phallacidin (2.5 μg/100 μl PBT, Molecular Probes, Eugene, OR) following the protocol of Martindale and Henry (1995) by incubating them for 24–48 h at 4 °. Stained larvae were finally washed three times for 10 min in PBT and mounted in 80% glycerin/20%PBS, as described above (see Martindale and Henry, 1995). In the case of embryos to be stained with Bodipy-phallacidin, the methanol washes were omitted following fixation and the embryos were simply stored in PBS at 4 °C.

2.7. Microscopy and image processing

Live and fixed embryos were mounted, as previously described (Lyons et al., 2015), and wide field images were captured with a Zeiss Axio Imager M2 or Axioplan microscopes (Carl Zeiss Inc., Munich, Germany). For some images, Z-stacks were prepared to make orthogonal projections with either Zen software (Carl Zeiss, Inc. Munich, Germany) or Helicon Focus image stacking software (Helicon Soft Ltd., Kharkov, Ukraine).

3. Results

3.1. Sequential ablation of 1q progeny

The D quadrant can be distinguished in several ways in *C. fornicata*. First, as is the case in many other spiralian embryos, the D quadrant exhibits a unique pattern of cell divisions, which is first apparent by the precocious formation of its fourth quarter daughter, the micromere 4d. This occurs well before the formation of the 4a-4c micromeres. In preparation for this division, the 3D macromere protrudes noticeably from the side of the embryo at the 24-cell stage (Henry et al., 2006). Furthermore, 4d represents a highly-specialized cell, which exhibits a unique pattern of bilateral cell divisions that give rise to specialized cell fates, such as a pair of autofluorescent crystal cells and a single hindgut intestine (Lyons et al., 2012). Finally, in *C. fornicata*, 4d serves as the organizer that sets up the dorso-ventral axis and specifies cell fates in the adjacent quadrants (Henry et al., 2006), which can be ascertained by the presence of morphogenesis along the bilateral and dorsoventral axes, and the differentiation of organizer dependent cell fates, such as the pigmented ocelli derived from 1a and 1c.

The fates of the $1q^1$ and $1q^2$ cells have been recently described by Lyons et al. (2017). Basically, the $1q^1$ cells form the apical organ and the brain (including the two pigmented ocelli derived from $1a^1$ and $1c^1$), anterior ciliated epithelium of the velum, and the ciliated primary trochoblast cells of the prototroch. On the other hand the $1q^2$ cells are cleavage arrested and remain as four cells that form an expansive, thin provisional ciliated epithelium located on the dorsal surface of the developing head that subsequently condenses, and is lost early during the larval stage.

To determine the timing of D quadrant specification, we ablated the animal 1q micromeres or their animal progeny $1q^1$ at successive stages of development (8- through 24-cell stages) and looked for evidence of D

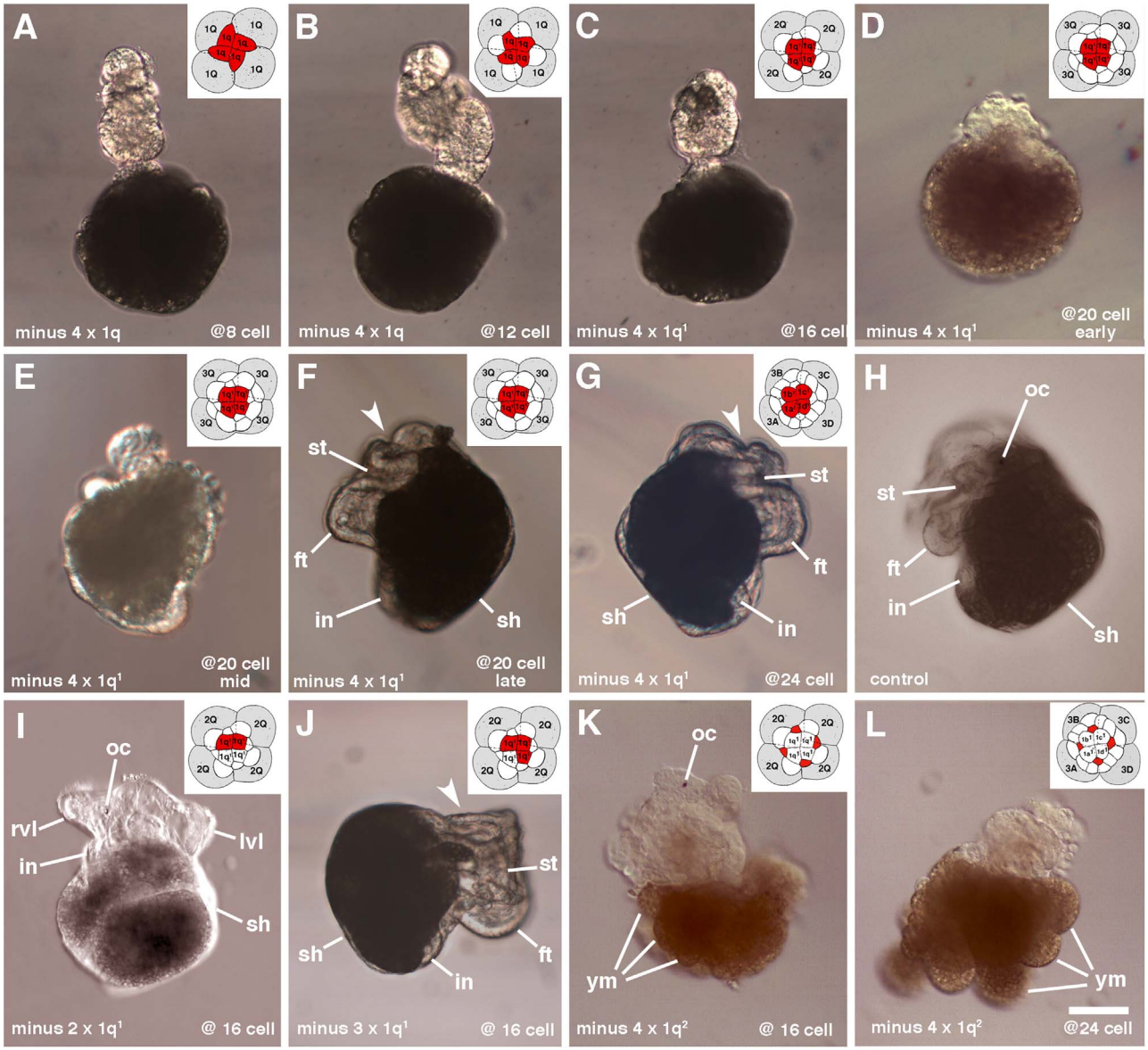

Fig. 3. Serial ablation of the animal micromeres at successive stages of development. A-F). All four of the 1q (1a-1d) or the $1q^1$ cells ($1a^1$–$1d^1$) were removed, as indicated. Ablation stages are as specified in the small inset diagrams. Arrowheads in F and G point to divots in the heads of these larvae. H). Control, unoperated embryo is shown for comparison. I-J). For the examples shown in I only two $1q^1$ cells were removed at the 16-cell stage, and three $1q^1$ cells were removed in J. Note that the post-trochal region is normal in appearance and contains the differentiated shell gland and the hindgut intestine derived from 4d. Only one ocellus has formed in I and the left velar lobe is somewhat reduced in size. The velar lobes and head are greatly diminished in J and there are no ocelli. K and L). The four $1q^2$ cells have been removed at the 16- and 24-cell stages, as indicated. These embryos lose cells and typically exhibit protruding yolky macromeres, with few overt signs of differentiation. In many cases one can observe the formation of one or two ocelli, as seen in K. Inset images show the particular cells that have been removed at those particular stages (ablated cells colored in red). The embryos shown here were reared for seven days (A-H, K-L) and 9 days (I-J). A-E and K-L are lateral views; F and H are left-lateral views; G and J are right-lateral views; I is a ventral view.; ft, foot; in, intestine; lvl, left velar lobe; oc, ocellus; rlv, right velar lobe; sh, shell; st, stomodaeum; ym, yolky macromeres. See text for further details. Scale bar in J equals 50 μm.

quadrant formation, organizer activity and the establishment of dorso-ventral and bilateral symmetry (Fig. 3). As an indicator of D quadrant formation we observed the pattern of cleavage divisions and cell shape changes (such as the protrusion the 3D macromere that leads to the precocious formation of the large 4d mesentoblast), organizer activity (e.g., formation of ocelli in the head), as well as the differentiation of specific cell fates derived from the mesentoblast (such as the auto-fluorescent kidney cells and the hindgut intestine). Embryos were first obtained at the 2- and 4-cell stages, and monitored closely for the timing of their divisions and the number of cells present in the embryo. Conveniently, embryos within a single egg capsule develop synchronously. It takes three hours for each successive set of micromeres to be born (see Hejnol et al., 2007; Henry and Perry, 2008). At least ten embryos were examined at each cell stage and the results were highly consistent amongst these cases (8-cell stage, 15 cases; 12-cell stage, 12 cases; 16-cell stage, 11 cases; 20-cell stage plus 30 min, 10 cases; 20-cell stage plus 1 h, 12 cases; 20-cell stage plus 2 h, 11 cases; 24-cell stage, 10 cases). All embryos developed in a radialized fashion when the 1q or $1q^1$ micromeres were removed at the 8-, 12-, and 16-cell stages (Fig. 3A-C). There was no indication of the formation of the D quadrant (no protrusion of the 3D macromere was observed, nor was there any indication of the precocious formation of the 4d micromere). These embryos did not complete gastrulation, and the progeny of the yolky macromeres remained uncovered (i.e., exogastrulae). When the $1q^1$ cells were removed early during the 20-cell stage (within 30 min after completion of the 5th cell division (i.e., following the birth of the third quartet of micromeres)), the embryos were abnormal and were also radialized (Fig. 3D). When these cells were removed 60 mins

following the birth of the third quartet of micromeres, they also appeared radialized but they did complete gastrulation (Fig. 3E). On the other hand, embryos more closely resembled controls when the $1q^1$ cells were removed approximately 2 h after 5th cleavage (Fig. 3F) or removed during the 24-cell stage (Fig. 3G), as compared with controls (Fig. 3H). These older embryos developed anterior-posterior, left-right and dorso-ventral axes, and posterior structures appeared to be normal including the shell, foot, intestine and a pair of autofluorescent crystal cells (Lyons et al., 2012). As expected these experimental embryos were only missing tissues derived from the $1q^1$ cells, which form parts of the head and velar lobes that were thus reduced in size. Such embryos exhibited a characteristic divot in the head, and lacked pigmented ocelli (Fig. 3E-F). These results indicate that the D quadrant (the 3D macromere) is specified during the interval between 5th and 6th cleavage (i.e., the 20-cell stage, Fig. 3D-F).

To determine if all four of the $1q^1$ cells are required to specify the D quadrant, we removed either two (14 cases) or three (12 cases) adjacent $1q^1$ micromeres at the 16-cell stage. When two $1q^1$ cells were removed, the embryos developed into veliger larvae that were missing one of the two ocelli and display one reduced velar lobe (Fig. 3I). When three $1q^1$ micromeres were removed, the embryos formed larvae that had greatly diminished velar lobes, reduced heads and were missing both of the ocelli (Fig. 3J). All of these embryos possessed anterior-posterior, left-right and dorso-ventral axes, and structures in the posterior, post-trochal region appeared to be normal, including: the shell, foot, as well as the intestine and a pair of autofluorescent crystal cells (data not shown), which are derived from 4d.

To examine the role of the $1q^2$ cells we removed all four of these at the 16- and 24-cell stages and found that the resulting embryos developed poorly and typically lost cells during later stages of gastrulation or during later stages of elongation (38, and 13 cases, respectively, Fig. 3K-L). All of these embryos formed the D quadrant, and 4d mesentoblast (data not shown). However, by 4–5 days of development, macromeres were seen to protrude from these embryos (similar to an exogastrulae phenotype, Fig. 3K-L). These phenotypes are not reminiscent of radialized embryos, but instead reflect a more general disruption of the integrity of the embryo. In fact, recently we showed that the $1q^2$ cells play a very important mechanical/structural role in the development of the head and animal-vegetal axial bending (Lyons et al., 2017). Significantly, in 15 cases (16-cell stage), and in 5 cases (24-cell stage), the embryos were found to form ocelli when raised for 7–9 days of development (an organizer dependent fate, Fig. 3K). These results indicate that, unlike the $1q^1$ cells, the $1q^2$ cells are not required for specifying the D quadrant.

3.2. Temporal ablation of 4d

In a previous paper, we ablated 4d immediately after its birth to show that 4d represents the key dorso-ventral organizer (Henry et al., 2006). However, we did not determine when organizer signaling actually takes place, nor eliminate the possibility that the progeny of 4d may serve as the organizer. To determine the timing of organizer activity we ablated the 4d micromere at successive times during the interval following its birth through the time that this cell divides to form the bilateral ML and MR teloblasts (every 10–20 min, see Supplemental Fig. 1D-G and Fig. 4). The results show that ablations carried out within 10 mins after the birth of 4d result in abnormal radialized forms of development, with no specific head structures such as the ocelli (Fig. 4A, 15 cases examined). These embryos did not complete gastrulation, appearing similar to those described above for embryos missing the 1q cells. Ablations carried out 20 (10 cases) and 30 min (17 cases) after the birth of 4d were also abnormal and failed to complete gastrulation; however, these embryos exhibited signs of anterior differentiation including ciliated velar tissue and the ocelli that normally require organizer signaling (Fig. 4B-C). Additionally, all the embryos that had the 4d cell ablated were missing all structures derived from the 4d lineage, such as the hindgut intestine and crystal cells. In contrast, ablations carried out later, after one hour (14 cases), appeared to develop normally, except they were missing all structures derived from 4d (Fig. 4D). A similar result is obtained when both the ML and MR teloblasts were removed after their birth (25 cases, Fig. 4E). These latter embryos may exhibit an elongated appearance, which is due to the presence of a straighter, elongated shell, as compared to control embryos (Fig. 4F). Furthermore, these embryos possess a well-developed head with bilateral ocelli and velar lobes, and exhibit well-developed anterior-posterior, left-right and dorso-ventral axes. These results indicate that organizer signaling begins soon after the birth of 4d and is completed within one hour.

3.3. Naturally occurring twins in C. fornicata

The earliest time that one can recognize the D quadrant is at the 24- to 25-cell stage when the D quadrant macromere(s) begins to protrude from the embryo (e.g., 3D or 4D, respectively; see Fig. 5A, which shows single protruding 3D macromere). Over the course of 12 years working with *C. fornicata* embryos, handling 100's of thousands of embryos, we occasionally encountered embryos that spontaneously formed two protruding D quadrant macromeres (see slightly older embryo in Fig. 5B, which shows two protruding 4D macromeres as well as two 4d cells). We did not maintain records for all spontaneous twins we encountered, so we cannot calculate their exact frequency of occurrence, but this is not common. When present, we observed only one or two of these embryos in a single egg capsule, which otherwise typically contained as many as 200 normal embryos. In some cases, these capsules of embryos also contained other smaller sized, uncleaved oocytes, which may suggest they arose from oocytes that were not fully mature. Usually, for a particular female, only one or two egg capsules would contain these embryos (females typically lay between 10 and 40 egg capsules), and the vast majority of egg clutches did not appear to contain any twin embryos. We did not recognize any spontaneous triplet or quadruplet embryos.

Twelve spontaneous twins were followed in detail for this study. In each case the two D quadrant macromeres gave rise to 4d micromeres that underwent normal bilateral patterns of cell division. This was verified by injecting those cells with diI or rhodamine green dextran lineage tracer at the 26-cell stage. Each of these twin embryos formed duplicated V-shaped mesendodermal bands (Fig. 5C-F, see also Lyons et al., 2012). The four cell quadrants within these twin embryos assumed one of two different configurations. In some cases, the duplicated D quadrants are located opposite one another (5 cases of "CDCD" types, Fig. 5C) and in others they are adjacent to one another (7 cases of "CCDD" type, Fig. 5D, also compare a later stage shown in 5E with the control in 5F). These configurations are similar to those reported for the experimentally generated twins in Henry and Martindale (1987) for the annelid *Chaetopterus variopedatus*. These twin embryos subsequently underwent epiboly with a single blastopore. Unlike the case in normal embryos (Lyons et al., 2015), the blastopore appears to close completely at the vegetal pole in these twins, and did not appear to be displaced to the side of the embryo (e.g., they appeared to be radially symmetrical). When the twin embryos begin to undergo axial elongation after approximately 5 days of development, they assumed a radial, pear shape with a more highly constricted animal/anterior region (Fig. 5G, see descriptions of stages and ages in Lyons et al. (2015)). Subsequently, after approximately 7–8 days of development, the anterior region (derived from the animal pole) ended up forming a constricted small "head," which gave rise to more transparent anterior tissues (Fig. 5H). In some cases, a small passageway could be seen in these anterior tissues (possibly representing the stomodaeum, but its relationship to the blastopore could not be determined, Fig. 5I). The larger yolky vegetal region contributed to the posterior region of the embryo and contained the labeled progeny of the duplicated 4d mesentoblast cells. Development was abnormal

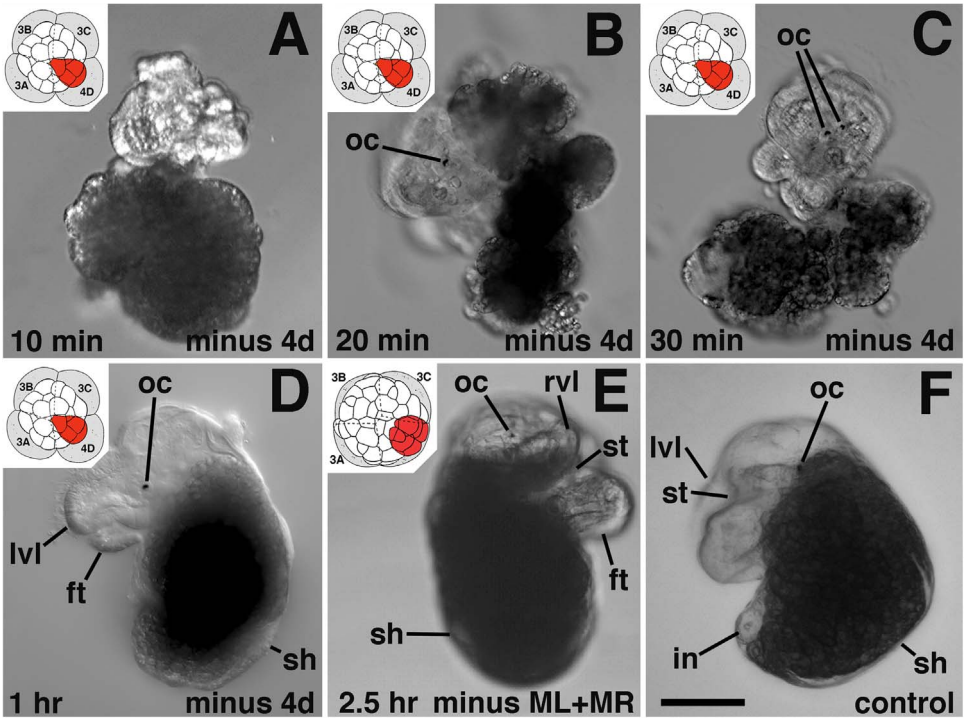

Fig. 4. Serial ablation of the 4d organizer at successive time points following its birth at the 25-cell stage. A-E). The 4d micromere or the ML and MR daughter cells (teloblasts) were removed, as indicated. Ablation stages are as specified in the small inset diagrams. F). Control, unoperated embryo is shown for comparison. ft, foot; in, intestine; lvl, left velar lobe; oc, ocellus; rvl, right velar lobe; sh, shell; st, stomodaeum. Scale bar in F equals 50 μm.

and generally the head seemed radialized, with minimal signs of differentiation. Only in one case did we observe signs of possible duplication of structures within the head (three close-set ocelli, which could have been a single fragmented ocellus, Fig. 5J). In two other cases, there were two ocelli (Fig. 5K); in four other cases there was only a single ocellus; and in the remaining six cases there were none (Fig. 5H-I). No distinct velar lobes were present, though there were some patches of ciliation. On the other hand, duplications were always observed in the posterior region and included two pairs of duplicated auto-fluorescent crystal cells in all cases (Fig. 5I, K, compare with control shown in 5L). and in six cases we observed duplicated hindgut intestines (Fig. 5H, J). These are all structures derived from the duplicated 4d mesentoblast cells. Typically, there was no foot, operculum or shell, except that two cases had a single posterior-lateral protrusion, which could be a foot, and one of those had a thin plate of birefringent material, (possibly an operculum, data not shown). These two cases appeared to exhibit duplicated statocysts, which are normally derived from 3c and 3d (data not shown, see Lyons et al., 2015).

A few cases were stained for acetylated tubulin and filamentous actin. For comparison, sibling control embryos possessed a well-developed ciliated velum and head vesicle, as well as muscle cells, including the main larval retractor muscles (Fig. 6A-C). In the case of the twins, ciliated cells were located mainly in the anterior animal regions, though a smaller number of ciliated cells also differentiated in the posterior regions (Fig. 6D-F). Some muscle fibers could also be seen in both the anterior (animal) and posterior (vegetal) regions (Fig. 6E). Eventually, these embryos fall apart when sibling controls have reached young veliger stages (10–14 days). The results indicate that each of the duplicated D quadrants formed 4d mesentoblasts cells that underwent bilateral cell divisions and differentiated 4d specific cell fates. However, inductive signaling by each one of the duplicated organizers was very weak or absent, as there were few signs of duplication in structures derived from other lineages and no clearly defined dorsoventral or bilateral axes.

3.4. Experimentally induced twins in C. fornicata: Lobeless embryos

In previous studies we removed the polar lobe, and observed early signs of twinning (multiple D quadrants), but we did not observe evidence of twinning at later stages of development (Henry et al., 2006; Henry and Perry, 2008). Here we have repeated these experiments to follow a greater number of cases. Polar lobes were removed from 206 embryos and 19 cases exhibited duplications (9%), as revealed by the formation of two protruding 3D quadrant macromeres (compare control in Fig. 5A and twin embryo in 5B). Both CDCD (8) and CCDD (11) configurations of blastomeres were observed. Development and morphology of these embryos is identical to that described for spontaneous twins above. This included the formation of duplicated 4d mesentoblast cells that gave rise to duplicated set of mesentoblast bands (Fig. 5M), and each formed a pair of autofluorescent crystal cells in all cases examined (a total of four autofluorescent crystal cells, see Fig. 5N). Two hindgut intestines were observed in six cases (data not shown). Six cases had a single ocellus that was also seen in the reduced heads, while four others had two, and the others had none. In four cases duplicated statocysts appear to be formed (data not shown). In addition, we examined 149 sibling controls and all but one of these developed normally with no signs of duplication (Fig. 5O, the one other case failed to cleave). Therefore, removal of the polar lobe increases the frequency of twinning and these cases exhibit some signs of duplications (specifically 4d derived structures) at later stages of development (see also Henry and Perry, 2008).

3.5. Development of linear embryos

In *C. fornicata* all four of the vegetal macromeres are normally in direct contact with one another during early development, and inductive interactions from the overlying animal cap of micromeres ($1a^1$–$1d^1$ cells, see above) provide inductive signals that specify one of the macromeres to become the 3D macromere (see Fig. 1). This 3D

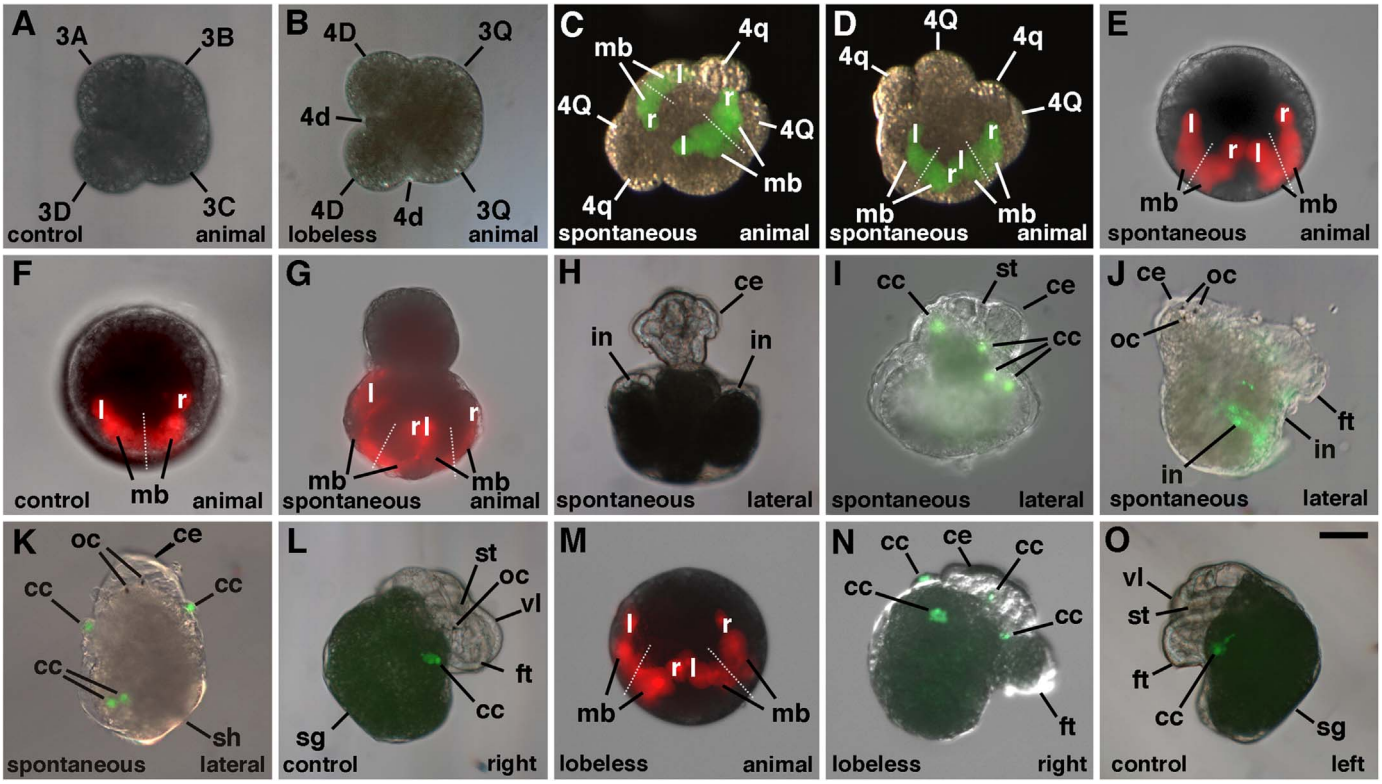

Fig. 5. Natural occurrence of twin embryos in *C. fornicata,* and development of experimentally generated twins following removal of the polar lobe. Examples of embryos with duplicated D quadrants in *C. fornicata*. A). Control embryo with a single protruding 3D macromere (24-cell stage). B). Example of an embryo with duplicated D quadrants following removal of the polar lobe during the first cleavage division, (note two protruding 4D macromeres each having given rise to duplicated 4d blastomeres of the 26-cell stage). Note that the two D quadrants lie adjacent to one another. C). A 48 h old twin embryo that arose spontaneously. In this case the two 4d blastomeres were injected with fluorescent Rhodamine Green dextran at the 26-cell stage, and allowed to undergo subsequent development. The D quadrants lie opposite of one another (CDCD type embryo). Note presence of twinned sets of mesodermal bands. D). Another example of a 48 h old spontaneous twin embryo. In this case the two D quadrants and their sets of mesodermal bands lie adjacent to one another (green fluorescently labeled cells in this CCDD type embryo). E). Another spontaneous twin embryo at a later stage of development (compacted round stage at approx. 52 h) that has duplicated pairs of mesodermal bands (red fluorescently labeled progeny of the 4d cells lie adjacent to one another, CCDD type embryo). In this example 4d cells were injected with DiI (red fluorescence) at the 26-cell stage. F). Same spontaneous twin embryo as that seen in E at a later "pear" stage (120 h) with duplicated pairs of growing mesodermal bands (red fluorescently labeled progeny of the twinned 4d cells). G). Spontaneous twin embryo after some cellular differentiation has occurred (CCDD type embryo). This embryo contains four auto-fluorescent crystal cells (green) derived from the 2 mL^1 and $2mR^1$ progeny of each duplicated 4d cell. H). Right lateral view of a control embryo, showing one of the two auto-fluorescent crystal cells (green). The other crystal cell is hidden on the opposite side of the embryo. I). Twin embryo containing two hindgut intestinal rudiments, and a single radialized head covered with ciliated epithelium (CDCD type embryo). J). Spontaneous twin with three ocelli and what appears to be a protruding foot rudiment (CDCD type embryo). K). Another spontaneous twin with only two pigmented ocelli (CCDD type embryo). Birefringent shell material is present in the posterior region. L). A compacted control embryo at approx. 52 h. In this example 4d cell was injected with DiI (red fluorescence) at the 26-cell stage. M). Compacted twin embryo following removal of the polar lobe at approx. 52 h. Duplicated pairs of mesodermal bands (red fluorescently labeled progeny of the 4d cells) lie adjacent to one another (CCDD type embryo). N). Twin embryo with two pairs of auto-fluorescent crystal cells (CDCD type embryo). O). Embryo from which a small amount of vegetal cytoplasm was removed equal to the volume of the polar lobe. Development appears to be normal. One of the two auto-fluorescent crystal cells (green) is seen, while the other one is hidden on the opposite side of the embryo. A-D are animal pole views. E, L-M are oblique/dorsal views. F-K, N-O are lateral views. Fine dotted lines in C-F and L-M separate the bilaterally symmetrical left and right bands derived from each of the labeled 4d clones. l and r label the tips of the left and right mesodermal bands, respectively. The embryos shown in G-K and N-O were reared for 7–9 days. cc, auto-fluorescent crystal cells; ce, ciliated epithelium; ft, foot; in, intestine; mb, mesodermal bands; oc, ocellus; sg, shell gland; sh, shell; st, stomodaeum; vl, velar lobe; 3Q, third quartet macromeres; 4d, 4d mesentoblast cells; 4q, fourth quartet micromeres; 4Q, fourth quartet macromeres. Scale bar in O equals 50 μm.

macromere is always derived from the quadrant that inherits the vegetal material, sequestered by the small polar lobe (Henry et al., 2006). Furthermore, removal of the polar lobe results in the formation of multiple D quadrants (Henry and Perry, 2008). To test the hypothesis that the future D quadrant macromere inherits polar lobe factors that may allow it to inhibit adjacent macromeres from assuming the D quadrant fate, we altered the arrangement of these cells by generating linear embryos.

By compressing 2-cell stage embryos, one can shift the orientation of the second cleavage spindles and generate 4-cell stage embryos with linear arrangements of the four blastomeres (Figs. 2F and 7A-B). The orientation of the embryos is random with respect to the plane of compression, so embryos with the appropriate linear morphology (Fig. 7A-B) were selected after compression was released (compare these with uncompressed controls shown in Fig. 7C-D). Other configurations of cells were also observed following compression embryos, that were not followed further in this study. However, some compressed embryos had a relatively normal 4-cell stage geometry, and these were followed along with uncompressed controls (Fig. 7E-F). The linear embryos continue to divide, and divisions proceed at a normal pace, compared to sibling controls. Micromeres form at the animal pole similar to those found in control embryos (Figs. 1F and 7A-B, G-L). It is difficult to distinguish the identities of the four quadrants in these linear embryos based simply on morphological appearance; however, one can examine the pattern of MAPK activation, as a readout for 3D macromere specification, as 3D exhibits activated dpMAPK (Henry and Perry, 2008). These linear embryos have the configuration shown in Figs. 2F and 7A-B, where two middle blastomeres or quadrants are in contact with both each other and only one of the two end blastomeres or quadrants. In contrast, the end cells are only in contact with one of the two middle cells. More frequently, the two end cells or quadrants are a little smaller than the two middle cells. As the first cell division was allowed to take place before any compression was applied, the polar lobe is segregated into one of the first two cells as it proceeds

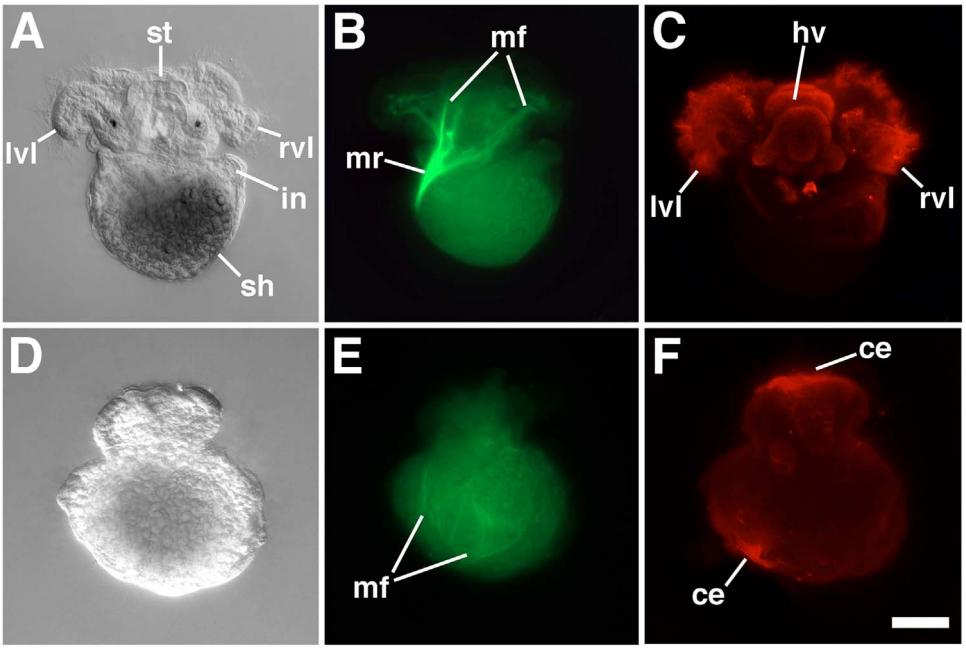

Fig. 6. A-C). Complementary DIC (A) and fluorescence micrographs of an early control veliger larvae stained with Bodipy-phallacidin (green fluorescence in B) and anti-acetylated tubulin (red fluorescence in C). D-F). Complementary DIC (D) and fluorescence micrographs of a spontaneous twin larva stained with Bodipy-phallacidin (green fluorescence in E) and anti-acetylated tubulin (red fluorescence in F). ce, ciliated epithelial cells; hv, head vesicle; in, intestine; lvl, left velar lobe; mf, muscle fibers; mr, main retractor muscle; sh, shell; st, stomodaeum. Scale bar in F equals 50 μm.

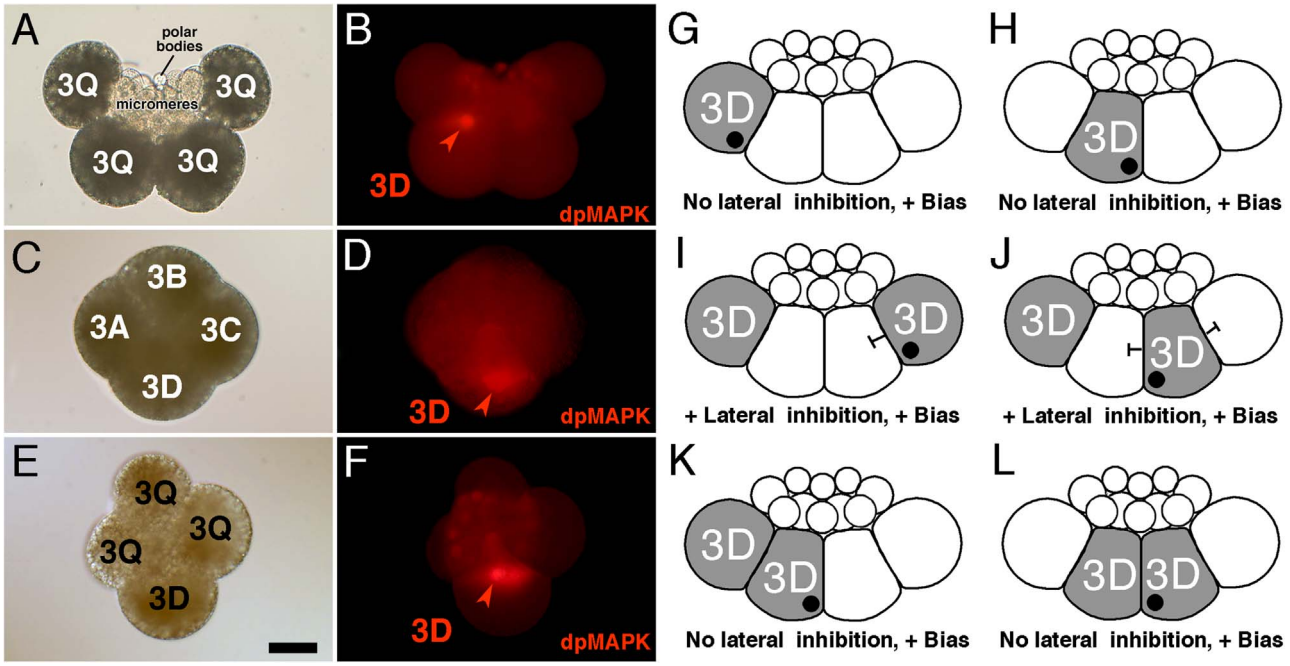

Fig. 7. Development of "linear embryos" generated by compression at the 2-cell stage and various models showing possible configurations for the activation of MAPK in resulting D quadrant(s). A-B). Corresponding light and fluorescence micrographs, respectively, showing typical result where activated dpMAPK is present in a single middle 3D macromere. Red fluorescence in B shows localization of anti-dpMAPK antibody. C-D). Corresponding light and fluorescence micrographs, respectively, showing control uncompressed embryo with activated dpMAPK in the single 3D quadrant macromere. E-F). Corresponding light and fluorescence micrographs, respectively, showing a compressed embryo that did not obtain the linear configuration of blastomeres. Activated dpMAPK is present in a single 3D quadrant macromere. G-L). Models depicting possible configurations of D quadrants in linear embryos either without or with the proposed D quadrant contact mediated lateral inhibition. G-H). Predicted results assuming no lateral inhibition and an intrinsic bias for specification of a single D quadrant via inheritance of the polar lobe. (Grey shading depicts 3D MAPK activation. Black dot signifies inheritance of the polar lobe.) The most likely scenario is that depicted in H, as the polar lobe would generally be distributed to one of the larger middle cells. Note that these outcomes could also be explained if there is long range inhibition provided via inheritance of the polar lobe (signified by the black dots). I-J). Predicted results assuming contact mediated lateral inhibition via components provided by the polar lobe. T bars indicate contact mediated lateral inhibition. The most likely scenario is that depicted in F as the polar lobe would generally be distributed to one of the larger middle cells. K-L). Two other possible results that could only be encountered in the absence of any contact mediated lateral inhibition or imposed bias for the cell that inherited the polar lobe. Some other variations are also possible, but not depicted here. A-B and G-L are all lateral views. C-E are all animal pole views. Cells are labeled according to Conklin (1897). "3Q" signifies generic third quartet macromeres. The scale bar in E equals 50 μm and applies to A-F.

under normal circumstances. However, no visible polar lobe forms in these embryos while they are under compression; hence, we could not follow the final path of those second polar lobes. Given the slightly asymmetric orientation of the cleavage spindles at the next division, the contents of the small polar lobe most likely end up being inherited by the larger, middle quadrant blastomeres (black dots shown in Fig. 7H, J-L), though it could have ended up in the smaller cell, and these other possible scenarios are recognized in Fig. 7G-I. If the polar lobe conveys a contact mediated, lateral inhibitor to the blastomere that finally inherits it at the 4-cell stage, one can predict several different outcomes, as shown in Fig. 7G-L**.** For example, if lateral inhibition ensures that adjacent quadrants do not assume the D quadrant fate, some outcomes are possible in which cells will not be in direct contact with the cell that inherited the polar lobe (e.g., Fig. 7I-J). In those cases, we might expect to see some embryos that exhibit MAPK activation in more than one macromere, as predicted in Fig. 7I-L (gray shading). Other arrangements are possible depending on where the contents of the polar lobe end up, and whether or not there is indeed lateral inhibition (e.g., Fig. 7I-J vs. K-L). In contrast, if the polar lobe simply confers a direct bias to the cell that inherits it (with no lateral inhibition), then the predicted outcomes shown in Fig. 7G-H are possible.

We prepared these linear embryos and fixed them at the same time that sibling control embryos began to protrude the 3D macromere (as they prepared to form the 4d mesentoblast), to examine the patterns of MAPK activation, which are indicative of D quadrant specification (Henry and Perry, 2008). We examined 76 linear embryos (Fig. 7A-B) along with 63 sibling uncompressed controls (Fig. 7C-D) and 8 compressed embryos that ended up with a more normal arrangement of the four blastomeres (Fig. 7E-F). Of these, 17 linear embryos did not exhibit any activated MAPK. In the remaining 59 linear embryos, all but one exhibited MAPK activation in a single 3D quadrant macromere (Fig. 7A-B), and this was always found to be in one of the two middle cells, like the configuration shown in Fig. 7H. In the one other linear embryo we saw MAPK activation in all four quadrants, although there was also activation of MAPK in the micromeres, as well (data not shown). The 63 control embryos (Fig. 7C-D), and 8 non-linear compressed embryos (Fig. 7E-F), all exhibited activated MAPK in only a single 3D macromere. 24 additional linear embryos were allowed to develop to later stages but these embryos lost cells and did not form clearly differentiated structures. These results do not support the model of contact-mediated, lateral inhibition, as illustrated in Fig. 1G.

4. Discussion

4.1. Specification of the D quadrant occurs during the interval between fifth and sixth cleavage in gastropod molluscs

Experimental studies revealed that the first quartet cells are required to specify the D quadrant in equal-cleaving molluscs. This includes the archaeogastropod *Patella vulgata* (van den Biggelaar and Guerrier, 1979), the pulmonate gastropods *Lymnaea stagnalis* (Arnolds et al., 1983; see also Freeman and Lundelius, 1992) and *L. palustris* (Martindale et al., 1985), the opisthobranch gastropod *Hamineoa callidegenita* (Boring, 1986), and the polyplachophoran *Accanthochiton crinitus* (Van den Biggelaar, 1996). Observations reported by those investigators, as well as others (van den Biggelaar, 1971, 1976, 1977, 1993; van den Biggelaar and Guerrier, 1979), indicates that contacts between the animal-most progeny of the first quartet micromeres (the $1q^1$ cells), and one of the four vegetal macromeres (presumably the future 3D macromere) take place during the interval between fifth and sixth cleavage, shortly after the birth of the third quartet micromeres. However, only one functional study has directly probed the timing of these inductive events, by using cytochalasin B to prevent these contacts from forming over specific time intervals (for the pulmonate gastropod *L. palustris*, see Martindale et al., 1985). That study confirmed the morphological observations by showing that induction does occur during the interval between fifth and sixth cleavage.

Ours is the first study to systematically remove the first quartet micromeres at successive stages of development in any spiralian. Through a series of ablations, we show that D quadrant specification involves the $1q^1$ micromeres and that this also takes place between fifth and sixth cleavage in the caenogastropod *C. fornicata*. This is consistent with the pattern of MAPK activation appearing in a single macromere (3D) at the 20-cell stage. Therefore, it appears that this mechanism of D quadrant specification is highly conserved among the few molluscs that have been examined. Yet, it is not yet clear if a similar series of events takes place in other equal-cleaving spiralians. Equal cleavage is represented widely amongst the Spiralia, and is likely the ancestral condition for this clade (including members of the phyla Annelida, Nemertea, and the Platyhelminthes, see Freeman and Lundelius, 1992; Henry, 2002, 2014; but see Dohle (1999) for an alternative argument). However, only two other studies outside of the molluscs demonstrated that the first quartet is required for establishing the D quadrant and dorso-ventral axis. One of these studies was carried out using the nemertean *Cerebratulus lacteus*, but the timing of those inductive events was not specifically examined (Henry, 2002). The other study was conducted by Boyer (1989, 1995), who examined the role of the first quartet in the flatworm *Hoploplana inquilina*. She concluded that the first quartet is required for establishing the D quadrant and dorsal-ventral polarity, but these inductive interactions must occur at a different time, as the third quartet macromeres ("3Q") never come into contact with the animal pole micromeres (see also Van den Biggelaar, 1996).

4.2. Organizer activity is completed soon after the birth of 4d

The dorsal organizer arises from the D quadrant in Spiralians; however, the exact D quadrant progeny that serve as the organizer varies depending on the species examined. Hence, organizer signaling can occur over different time intervals. In many cases the organizer is 3D (*Titria* (formerly *Ilyanassa)*, *Dentalium, Patella*, and *Lymnae*a, (Clement, 1962, 1976; Cather and Verdonk, 1979; van den Biggelaar and Guerrier, 1979; Martindale, 1986), and organizer signaling appears to occur during the interval between fifth and sixth cleavage, over the same cleavage interval in which the D quadrant is initially specified (Clement, 1962, 1976; Cather and Verdonk, 1979; Martindale, 1986). On the other hand, 4d serves as the organizer in *C. fornicata* (Henry et al., 2006), and some evidence suggests that both 3D and 4d provide organizer activity in the mollusc *T. obsoleta*, (Lambert, 2009). In the polychaete annelid *Capitella teleta,* the 2d micromere serves this role (Amiel et al., 2013); in the leech *Helobdella robusta*, both 2d (DNOPQ) and 2D (DM) exhibit inductive activity (Isaksen et al., 1999), and in another annelid, the oligochaete *Tubifex*, it is thought that both $2d^{11}$ and 4d serve as organizers (Shimizu and Nakamoto, 2014). Here we show in *C. fornicata* that 4d organizer signaling is completed within one hour following the birth of 4d. Therefore, D quadrant specification and organizer activity are uncoupled in *C. fornicata* in both a temporal and spatial context. Taken together, these reports indicate that there is a great deal of variation in terms of the timing and precise cells involved in dorsal organizer signaling in spiralians.

4.3. Twinning in Crepidula fornicata: Insights into organizer activity

Normally a single set of body axes emerges during the course of development, and robust developmental mechanisms help ensure that supernumerary body axes do not arise to cause abnormal forms of development (e.g., twins). However, development of these axial systems involves a complex set of cell and molecular processes.

Spontaneous examples of twinning in nature are rather rare, especially among invertebrates (Penners, 1924a; Eyster, 1995). Most reported cases have been generated experimentally, and have only been reported for unequal-cleaving spiralians (Heymans, 1893 in Conklin (1897); Conklin, 1897, 1917; Penners, 1922; Newman, 1923; Penners, 1924b; Morgan, 1927; Titlebaum, 1928; Tyler, 1930; Ghareeb, 1971; Sander, 1976; Guerrier et al., 1978; Itow and Sekiguchi, 1979; Itow et al., 1991; Eyster, 1995; Henry and Martindale, 1987; Render, 1989; Duboc et al., 2005; Lapraz et al., 2015). Such cases provide valuable insight into fundamental developmental mechanisms, in particular, the role of inductive organizers that set up the main body axes.

Here we document spontaneously occurring and experimentally generated twin embryos that possess duplicated D quadrants in *C. fornicata*, an equal-cleaver. Spontaneous twins arise at a very low frequency. A previously published study (Eyster, 1995) reported on the occurrence of twin, triplet, and even quadruplet larvae in *C. fornicata* at a low frequency: less than 0.5% of the cases observed. Those cases were only discovered later during larval stages and had not been followed through early development. In fact, Eyster (1995) was careful to state that she did not know how these multiples arose, and suggested that they could have resulted from the fusion of embryos inside the egg capsules. Indeed, the embryos contained with each egg capsule do not possess vitelline envelopes, and it is conceivable that they may fuse during the weeks preceding hatching. Many representative cases shown in her figures are actually much larger, compared to normal control larvae. In addition, the wide range of reported axial orientations within these multiples suggests they may have arisen from fused embryos. Conklin (1897, 1917) also reported on the occurrence of such multiple embryos of *C. fornicata* and suggested they likely arose through fusion. Other examples of fusion have been reported, such as for the opisthobranch *Umbrella mediterranea* (Heymans, 1893 in Conklin, 1897), and de Lacaze-Duthiers (1875) observed side-by-side, foot-to-foot and posterior to posterior fusion in embryos of the opisthobranch *Philine aperta*.

Unlike many of the multiples reported by Eyster (1995), which survived to metamorphosis and exhibited more normal morphological features with duplicated heads, all of the twin embryos recovered in this study exhibited grossly abnormal forms of development and did not survive longer than two weeks. The twin embryos studied here did produce duplicated D quadrants, each forming 4d sub-lineages that underwent differentiation to form larval kidney complexes (e.g., crystal cells) and intestines, but these embryos exhibited very poor development of anterior structures. Only one case appeared to organize supernumerary head structures (i.e., ocelli) and a few cases appeared to have duplicated statocycsts. Furthermore, though we did find twins, we did not observe spontaneously occurring triplets or quadruplets. These results indicate that supernumerary D quadrants can form spontaneously and the progeny of the duplicated 4d micromeres can undergo differentiation to form structures normally derived from the mesentoblast. However, D quadrant organizer activity was very weak or absent in these twins (see further discussion below).

The development of twin embryos in *C. fornicata* contrasts with other examples of twinning seen for other species (e.g., *Chaetopterus*), which exhibit more complete forms of duplication, both in the body and the head. In those other cases, twins will arise by experimentally equalizing the distribution of vegetal determinants (or teloplasm in the case of leeches) in species that exhibit unequal cleavage (the molluscs *Cummingia tellinoides, Pholas dactylus, Spisula subtruncata, T. obsoleta,* and *Dentalium dentale*; Tyler, 1930; Guerrier, 1970b; Ghareeb, 1971; Render, 1989; Guerrier et al., 1978) and the annelids (*Chaetopterus variopedatus, Platynereis dumerilii, Nereis limbata, Tubifex rivulorum*, and *Helobdella triserialis*; Astrow et al., 1987; Nelson and Weisblat, 1992; Dorresteijn et al., 1987; Titlebaum, 1928; Penners, 1922, 1924; Tyler, 1930; Henry and Martindale, 1987). In most of those cases it is clear that twinned D quadrants are able to organize two discrete dorso-ventral embryonic axes.

Given the occurrence of multiple D quadrants following the removal of the polar lobe in *C. fornicata*, we suggest that spontaneous twins may arise through failed distribution of the polar lobe factors during development. In other spiralians that form polar lobes, removal of the polar lobe typically results in radialized development due to the failure of D quadrant specification, and subsequent lack of organizer activity (Guerrier et al., 1978; Verdonk and Cather, 1983; Henry and Martindale, 1987; Henry, 2014). In contrast, we have shown that removal of the polar lobe in *C. fornicata* does not prevent the specification of the D quadrant(s) (Henry et al., 2006; Henry and Perry, 2008 and the present study). In an earlier study (Henry and Perry, 2008), we observed the activation of dpMAPK in multiple 3D macromeres following removal of the polar lobe (14% of 73 cases examined); however, our preliminary analyses did not detect signs of later duplications. Here we have followed these embryos more thoroughly, and detected duplications, particularly in terms of the fates derived from the 4d mesentoblast. Different explanations could account for missing these duplications in the earlier study: 1) smaller sample sizes were examined in those earlier studies and we missed some instances of later duplications in those lobeless embryos, 2) perhaps some lobeless embryos were able to undergo regulation during later stages of development so that only one D quadrant emerged that organized a well-developed dorso-ventral axis, 3) our ability to completely remove the contents of the polar lobe may have varied between embryos, or critical vegetal factors may not have been entirely restricted to the polar lobe.

Additional studies have revealed different functions for polar lobes present in other species. For instance, removal of the first polar lobe in the annelid *Chaetopterus variopedatus* does not prevent the formation of the D quadrant (Henry, 1986, 1989). Such lobeless embryos only exhibit one defect: a loss of the ability to produce bioluminescence. Removal of the second polar lobe that forms during second cleavage in *Bithynia tentaculata* also does not affect development, presumably because this lobe has lost the specialized vesicular body present in the first polar lobe, which contains the key determinants required for D quadrant specification (Cather and Verdonk, 1974). These findings indicate that polar lobes may play very different roles in different spiralians, and this is consistent with arguments that these structures likely arose independently over the course of evolution (Fioroni, 1979).

4.4. Does the polar lobe in C. fornicata contain factors required for organizer activity?

The extent of axial duplications varies depending on the species. For instance, nearly complete symmetrical forms of axial duplications were observed for the annelids *Chaetopterus variopedatus*, and *Nereis limbata* (Titlebaum, 1928; Tyler, 1930; Henry and Martindale, 1987) and *Sabellaria cementarium*, (Novikoff, 1940; Guerrier, 1970a) and the mollusc *Spisula solidisima* (Guerrier, 1970b). On the other hand, less extensive forms of axial duplication have been reported for the molluscs *T. obsoleta* (Styron, 1967; Crampton, 1896; Tyler, 1930; Ghareeb, 1971; Render, 1989), and *Cumingia tellinoides*, (Tyler, 1930), and the annelids *Tubifex*, Lehmann (1948, 1956); however, see Penners (1924) and *Platynereis* (Dorresteijn et al., 1987). However, even in these latter cases certain forms of duplications were observed for specific structures, particularly those generated by D quadrant progeny. These results suggest that the level of organizer signaling may be weaker in some cases. A point to be made is that duplications of the D quadrant are not necessarily tied with duplications of organizer activity and the dorsoventral axes. In the case of *C. fornicata*, the small polar lobe does not appear to be required to form the D quadrant, but it is unclear if it contains factors needed to regulate later organizer activity.

Previous investigators have proposed that inhibitory inductive interactions could be emanating from the D quadrant. Cather (1967) claimed that the presence of the polar lobe and the D quadrant

prevents shell secretion by the other three quadrants (the shell gland was thought to be derived from the 2d micromere). Henry and Martindale (1987) found fewer duplications and poorer overall development and absence of duplications in CCDD twins, when compared to CDCD twins resulting from the equalization of the first cleavage in the annelid *Chaetopterus*. They argued that hypothetical D quadrant inhibitory interactions could be reinforced when these quadrants were adjacent to one another, such that they negate each others' activity to set up dorsal-ventral axes. In the cases reported here for *C. fornicata*, the most incomplete forms of duplications and abnormal forms of development were observed in CCDD embryos. On the other hand, Render (1989) refutes such an explanation, as she did not observe differences in the frequency of duplicated structures between CDCD and CCDD embryos in the mollusc *T. obsoleta*. However, she did find an increase in the number of duplications for certain structures when the two CD blastomeres were separated from one another, following equalization of first cleavage division, which might suggest that there were some mutual inhibitory influences. In another study, Render (1983) showed that a factor supplied by the second polar lobe in the annelid *Sabellaria*, which is inherited by the D blastomere during second cleavage, contains an inhibitor that prevents progeny of the D quadrant from forming apical tuft cilia (presumably regulated by lobe factors passed to the 1d micromere). Likewise, Sweet (1998) showed that the 1d micromere inherits an inhibitor, transferred via the second polar lobe, that prevents 1d from responding to D quadrant inductive interactions required for the formation of left and right ocelli (normally formed by 1a and 1c, respectively).

4.5. The polar lobe biases the specification of the D quadrant

Our observations showed that the polar lobe is always distributed to the future D quadrant (Henry et al., 2006). Furthermore, the occurrence of multiple D quadrants following removal of the lobe shows that this structure is dispensable for D quadrant formation in *C. fornicata*. In a previous study on *C. fornicata* (Henry and Perry, 2008), we suggested that the polar lobe may regulate D quadrant specification by providing that macromere with a lateral inhibitor. Here we sought to test this model of lateral inhibition by changing the arrangement of the four cell quadrants in the intact embryo (Figs. 2F and 7). The results of the linear embryo experiments, however, do not support a model in which the small polar lobe in *C. fornicata* conveys a contact mediated inhibitor to the presumptive D quadrant macromere, to block adjacent quadrants from assuming the D quadrant fate. Therefore, the findings leave us with an open question as to the exact function of the polar lobe. An alternative explanation might be that there are inhibitory interactions which operate over a longer range. Some signaling interactions operate over short range involving direct cell contacts. Delta-Notch signaling represents one such contact-mediated signaling pathway that is known to regulate lateral inhibition (Bray, 2006); however, treatments with the Notch inhibitor DAPT do not result in multiple axes in *C. fornicata* (data not shown). Other signaling pathways operate over long ranges such as the BMP signaling (Duboc, 2005). Another possible complication could be that D quadrant specification requires interactions with a certain number of $1q^1$ micromeres. As such, the end cells in the linear embryos might be in contact with a smaller number of those cells. This seems unlikely, however, as D quadrant specification can still take place when two or even three of the four $1q^1$ micromeres have been removed (Fig. 3I-J).

4.6. The evolution of unequal cleavage in the Spiralia

Freeman and Lundelius (1992) showed that one can bias D quadrant specification in an equal-cleaver by experimentally biasing contacts between the macromere and the first quartet micromere progeny. They accomplished this by changing the size of the blastomeres. The larger macromere is favored to become the D quadrant by presenting better contact with the $1q^1$ progeny, and they proposed that such an evolutionary transition could have supported the eventual asymmetric distribution of materials that influence D quadrant specification. The polar lobe in *C. fornicata* is only about 20 μm in diameter and represents far less than 1% of the embryo's volume. It seems rather unlikely that this could have an appreciable effect on the volume of the D quadrant macromeres to favor $1q^1$ inductive interactions. However, factors contained in the polar lobe could cause a change in the shape and/or arrangement of the blastomeres that favors contacts between the animal $1q^1$ cells and the macromere that inherits the polar lobe. In fact, we reported a change in the opacity of the future D quadrant macromeres beginning at the 8-cell stage (1D) and later part of presumptive 3D quadrant macromere does extend deeper into the center of the embryo, beneath the $1q^1$ cells at the time that these inductive interactions are now known to take place (Henry et al., 2006). Furthermore, these morphological changes were not observed following removal of the polar lobe (see Henry et al., 2006).

Further observations and experimental evidence clearly show that macromere position and contact dictate the choice of which macromere ultimately becomes 3D in equal-cleaving spiralians. For example, in the case of *Lymnaea*, it is typically one of the two, more central, cross-furrow macromeres that is specified to become the 3D macromere. This occurs by virtue of their central geometric locations (Martindale et al. (1984) and references therein, see also Van den Biggelaar (1996) for the Chiton *Acanthochiton crinitus*). Furthermore, Boring (1986) reported for the opisthobranch *Haminoea* that the animal cap of micromeres normally becomes asymmetrically displaced to one side, over one of the vegetal cross-furrow macromeres at the 24-cell stage when D quadrant specification occurs. In addition, Boring (1986 in *Haminoea*) and Henry (1986 in *Cerebratulus*) showed experimentally that one can shift these inductive contacts, by ablating specific 1q micromeres at the 8-cell stage, to bias the later specification of the D quadrant.

Based on a phylogenetic survey, Freeman and Lundelius (1992) argued that equal cleavage is ancestral in the Spiralia. They suggested that subsequent asymmetries in the early cleavage divisions may have permitted asymmetric distribution of localized factors that could be used to regulate D quadrant specification. They argue that this transition may have facilitated evolutionary transitions towards more rapid and direct modes of development. Dohle (1999) proposes that asymmetric cleavage is ancestral in the clitellate annelids and describes its phylogenetic deviations, though he has a different set of hypotheses than those proposed by Freeman and Lundelius (1992), as to how these variations have arisen. As described above, equal and unequal-cleaving spiralians use different mechanisms to specify the D quadrant. Here we find that *C. fornicata* behaves more like an intermediate form proposed by Freeman and Lundelius (1992) in which both conditional (i.e., equal cleavage) and autonomous (i.e., unequal cleavage) mechanisms are used to specify a single D quadrant. Interestingly, evidence suggests that both autonomous and conditional mechanisms may also be operating in the mollusc *T. obsoleta* (Nagy et al., 2009). Clearly, further experiments need to be carried out using additional spiralian representatives to examine the relative contributions of these two mechanisms of D quadrant specification.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.ydbio.2017.09.003.

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