The MAPK cascade in equally cleaving spiralian embryos

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Abstract
Spiralian development is shared by several protostome phyla and characterized by regularities in early cleavage, fate map, and larva. Experimental evidence from multiple spiralian species implicates cells in the D quadrant lineage as the organizer of future axial development of the embryo. However, the mechanisms by which the D quadrant is specified differ between species with equal and unequal spiral cleavage. Equally cleaving mollusc embryos establish the D quadrant via cell–cell interactions between the micromeres and macromeres at the 24- to 36-cell stage. In unequally cleaving embryos, the D quadrant is established at the 4-cell stage via asymmetries in the first 2 cell divisions. We have begun to explore the molecular mechanisms of D quadrant patterning in spiralian. Previously, we showed that, in the unequally cleaving embryo of the mollusc Ilyanassa obsoleta, the MAPK pathway is activated and functionally required in 3D and also in the micromeres known to require a signal from 3D. Here, we examine the role of MAPK signaling in 4 spiralians with equal cleavage. In 3 equally cleaving molluscs, the chiton Chaetopleura, the limpet Tectura, and the snail Lymnaea, the MAPK pathway is activated in the 3D cell but not in the overlying micromeres. In the equally cleaving embryo of the polychaete annelid Hydroides, MAPK activation was not detected in the 3D macromere but was observed in one of its daughter cells, 4d. In addition, inhibiting Tectura MAPK activation disrupts differentiation of 3D and cells induced by it, supporting a functional role for MAPK in axis specification in equally cleaving spiralian. Thus, MAPK signaling may have a conserved role in the D quadrant organizer cell 3D in molluscs. However, there have been at least 2 evolutionary changes in the activation of the MAPK pathway during spiralian evolution. MAPK function in the Ilyanassa micromeres is a recent cooption and, since the divergence of annelids and molluscs, there has been a shift in onset of MAPK activation between 3D and 4d. We propose that this latter shift correlates with a change in the timing of specification of the secondary embryonic axis.

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Introduction
Several protostome phyla, including molluscs and annelids, share a conserved type of early embryogenesis known as spiralian development. Spiralian embryos have a distinctive pattern of early cell division, called spiral cleavage, which is characterized by regularities in the direction and proportion of early cleavages. Moreover, these divisions produce similar patterns of cell fates and similar larvae. The spiralian development in certain groups has been recognized as homologous for some time (Wilson, 1899). However, the mechanisms that create these patterns are not known, and among spiralian groups, copious variation exists in cleavage pattern, cell fates, and larval structure. Thus, despite the overt similarities in cleavage patterns and cell fates in spiralian embryos, it is unclear how similar the underlying patterning mechanisms are across these groups.

The regularities in the proportion and direction of early cleavages in spiralian embryos have engendered a nomenclature for describing these cell divisions. The cells of the four-cell stage are known as macromeres. At each successive cleavage cycle, these cells divide toward the animal pole to produce a set of four smaller cells that are known as micromeres. The four micromeres produced during a cleavage cycle are known as a quartet. The four divisions that produce a quartet are at the same oblique angle to the animal–vegetal axis. In successive cleavage cycles, the ori-
entation of these divisions is alternately clockwise and counterclockwise with respect to the animal pole. This produces the distinctive spiralian blastula, where the cells are arranged in a pattern that is quadrilaterally symmetrical and vaguely spiral (Wilson, 1892).

The similarities in cleavage patterns of spiralian embryos are accompanied by similarities in the fate maps of these embryos (Wilson, 1899). Most notably, one of the four macromeres in spiralian embryos is specified to become the D macromere, founder of the D quadrant. In Ilyanassa, the D macromere induces the correct pattern of cell fates among the micromeres, probably after the production of the third quartet, when contact with the micromeres specifies one cell to be the D macromere (3D; gray shading). After 3D is specified, it is thought to signal to the micromeres to specify the correct cell type. In an unequal cleaver like Ilyanassa, the first two cleavages are accompanied by the constriction of a lobe of cytoplasm at the vegetal pole, called the polar lobe. This lobe material is inherited by one of the cells at the four-cell stage, which is thereby specified to be the D macromere, founder of the D quadrant. In Ilyanassa, the D macromere induces the correct pattern of cell fates among the micromeres, probably after the production of the third quartet, when the cell is known as 3D (gray shading; red arrows represent signaling from 3D to micromeres). The chiton Chaetopleura is an equally cleaving polychaete annelid; the remainder are molluscs. The chiton Chaetopleura, the limpet Tectura, and the pulmonate snail Lymnaea have equal cleavage, while the snail Ilyanassa has unequal cleavage. Phylogenetic relationships in the molluscs are based on Ponder and Lindberg (1997) and Taylor (1996). The hatch marks on the tree indicate evolutionary transitions inferred from our survey. The red hatch mark indicates the position of the shift of MAPK activation between the anleld 4d and the mollusc 3D (or vice versa). The blue hatch mark indicates the origin of MAPK activation in the micromeres on the lineage leading to Ilyanassa (see text for details).

The two different mechanisms by which molluscan embryos are known to specify the D quadrant vary with early cleavage patterns (Fig. 1A; reviewed in Freeman and Lundelius, 1992). In equally cleaving molluscs (e.g., the gastropods Lymnaea sp. and Patella vulgata, and the chiton Acanthochiton crinitus), the first two cell divisions are equal, producing four equally sized and equivalent cells (see Fig. 1A). Soon after the production of the third quartet cells (24- to 32-cell stage), one of the macromeres establishes persistent contact with overlying micromeres in the animal hemisphere. Contact between the presumptive 3D and micromeres is required for 3D specification (Arnolds et al., 1983; Martindale, 1986; Martindale et al., 1985; van den Bgellaar, 1977, 1996). In unequally cleaving molluscs, the D quadrant macromere is specified earlier, by the four-cell stage. In these embryos, the first two cell divisions are unequal, producing, in most cases, a four-cell stage where one cell is larger than the other three; this cell is specified to be the D macromere (Clement, 1952; Crampton, 1896). The unequal cleavages that specify the D quadrant can be generated either by asymmetric positioning of the cleavage apparatus or by polar lobes that sequester part of the vegetal cytoplasm during division so it is inherited by one daughter cell (Fig. 1A). In some groups, both asymmetric division
and polar lobe formation operate in the same cleavage (Henry, 1986).

Fig. 3. MAPK activation in the chiton Chaetopleura apiculata. (A, B) In a vegetal pole view of the 36-cell stage, activated MAPK is detected in the third order macromere, which we identify as 3D. The antigen is enriched in the nucleus. (C, D) In a lateral view of a 36- to 40-cell embryo, the macromere with activated MAPK extends to the roof of the blastocoele cavity and contacts the micromeres, confirming that this macromere is 3D. (A) and (C) are bright-field images of embryos stained for HRP detection of MAPK activation, and (B) and (D) are the same mounts with cell boundaries and identities indicated. Scale bar is 50 μm.

Despite the difference in how the D macromere is specified, the subsequent role of this cell is similar in equally and unequally cleaving molluscs embryos. In examples of both kinds of embryos, deletion of 3D early in its life prevents the normal differentiation of tissues derived from specific micromeres, or disrupts micromere cleavage patterns (Clement, 1962; Damen and Dictus, 1996; Martindale, 1986). Micromere cleavage patterns are also affected when 3D specification is blocked, by a mutation or pharmacological inhibition of animal–vegetal contacts in equally cleaving embryos (Arnolds, 1982a,b; Arnolds et al., 1983; Martindale et al., 1985). These results indicate that, once specified, 3D signals to multiple micromeres to establish the correct pattern of cell fates. For other equally cleaving molluscs, such as the limpet and chiton examined here, signaling from 3D to micromeres has not been demonstrated experimentally. Nevertheless, the cleavage patterns in these animals also suggest that 3D specifies micromere fate. Before the specification of 3D, the cleavage patterns of the cells within a quartet are identical, whatever their quadrant of origin. However, after the specification of 3D, the cleavage patterns of some micromeres begin to differ from their quartet-mates, based on their position relative to 3D (Heath, 1899; van den Biggelaar, 1977, 1996). The transition from radially symmetrical cleavage to bilaterally symmetrical cleavage is likely due to signaling from 3D.

As in molluscs, the D quadrant lineage plays a special role in patterning the embryos of polychaete annelids (Henry, 1986, 1989; Henry and Martindale, 1987; Render, 1983). Polychaetes have classic spiralian development and include representatives with both equally and unequally cleaving embryos. In unequally cleaving polychaetes, the D quadrant is specified by inequalities in the first two cleavages. However, no experimental data address the mechanism of D quadrant specification in an equally cleaving annelid embryo—it is not known whether this event involves an interaction between the prospective D macromere and the micromeres, as it does in molluscs.

Recently, we discovered that the Erk 1/2 MAP Kinase (MAPK) pathway in Ilyanassa is activated first in 3D and later in the micromeres, in response to a signal from 3D (Lambert and Nagy, 2001). The MAPK cascade is a conserved signal transduction pathway comprised of three kinases which relay extracellular signals to targets in the responding cell (Ferrell, 1996). In order to determine whether the role of MAPK activation observed in Ilyanassa is a conserved component of spiralian patterning, we have examined the patterns of MAPK activation in other spiralian embryos: three equally cleaving molluscs and one equally cleaving annelid. The relationships of these animals are shown in Fig. 1B.

We have found that, in equally cleaving molluscs, the MAPK pathway is activated in 3D but not in the micromeres that are targets of 3D signaling. A similar observation has recently been made in the limpet Patella (Lartillot et al., 2002). Blocking MAPK activation in one equally cleaving embryo disrupts the normal pattern of cell fate specification in the micromeres. This indicates that MAPK activation in equally cleaving mollusc embryos is required for the specification or signaling activity of 3D. However, the role of MAPK in transducing the signal from 3D in the micro-

Fig. 4. MAPK activation in 3D of the limpet Tectura scutum at the 60-cell stage. (A) Bright-field image of the vegetal pole. (B) DAPI staining of nuclei in the same mount, with cell boundaries and identities indicated. As in other equally cleaving molluscs, MAPK activation in the limpet was first observed in 3D. 3ABC will divide before 3D and are in prophase here. 3D is also marked by the three smaller cells that flank it: 3c, 2d2, and 2d2. (A) is a deeper focal plane than (B), and the faint staining apparently in the position of 3B is actually from the animal end of 3D. Scale bar is 50 μm.
mers, which we have previously described for *Ilyanassa obsoleta*, does not appear to be a conserved mechanism of spiralian patterning. We also show that, in the equally cleaving embryo of a polychaete annelid, MAPK activation is not observed in the 3D cell but is observed in its daughter cell, 4d. We suggest that the role of MAPK activation in D quadrant specification in annelids is homologous with that of molluscs, but is shifted by one macromere cleavage cycle.

**Materials and methods**

**Embryo collection**

For *Lymnaea*, animal maintenance, embryo collection, staging, and prefixation processing were as described (Morrill, 1982). *Lymnaea* embryos were reared at 22–23°C. *Chaetopleura* adults were collected from the underside of rocks in the low intertidal–subtidal in the vicinity of Old Silver Beach, MA, or obtained from the Marine Resources Center (MRC) at the Marine Biological Laboratories, Woods Hole, MA. Adults were separated and allowed to spawn spontaneously, after which diluted sperm was mixed with eggs for fertilization. Embryos are surrounded by an intricate chorion, which was removed by hand with fine forceps after fixation and washing in PBTw (phosphate-buffered saline and 0.1% Tween). For *Tectura*, animals were collected in the vicinity of the Friday Harbor Laboratories or Lime Kiln, San Juan Island, WA. Maintenance and spawning were similar to *Chaetopleura* (Strathmann, 1987). Embryos were usually not dejellied prior to fixation and were raised at 14°C. *Hydroides* were obtained from the MRC and induced to spawn by chipping away the posterior portion of the calcareous tube and progressively chipping away the anterior edge of the tube until the animal spawned (Costello and Henley, 1971). *Hydroides* and *Chaetopleura* embryos were reared at 20–21°C.

**Fixation and staining**

All embryos were fixed for 30 min to 1 h in 3.7% formalin 90% 0.2-μm filtered sea water (FSW), washed two times in PBTw, and either used immediately or stored in methanol. MAPK activation was localized with the antiphosphorylated MAPK monoclonal antibody (anti-dpErk1/2; Sigma). This antibody was generated against a broadly conserved 18-amino-acid region in the kinase domain. Detection followed standard protocols (Lambert and Nagy, 2001). Briefly, embryos were rehydrated by three 10-min washes in PBTw, blocked for at least 1 h in PBTw + 2% Bovine Serum Albumin (PBTw + BSA), then incubated for 8–16 h in primary antibody diluted in PBTw + BSA. After primary incubation, embryos were washed 6 × 10 min in PBTw, then incubated with anti-mouse horseradish peroxidase secondary antibody for at least 2 h, washed as above, and detected with Pierce HRP detection kit according to the manufacturer’s instructions. For *Hydroides*, the antibody was used at a 1/20 dilution, 10 times higher than the dilution used for *Ilyanassa*, *Lymnaea*, *Chaetopleura*, and *Tectura*. DNA was stained with DAPI at 1 μg/ml in PBTw, and embryos were mounted in 80% glycerol, 1× PBS, 4% n-propyl gallate. Phalloidin staining was performed as described (Lambert and Nagy, 2001). Cell boundaries and larval diagrams were drawn from sketches made from direct observation on the microscope, or series of through-focus brightfield and DAPI images of samples.

**Inhibitor experiments**

The MEK inhibitor U0126 (Promega) was prepared at 500× final working concentration in DMSO. We found that 50 mM stock of this compound precipitated when diluted 1/1000 in FSW. Controls were treated in FSW with 1/500 dilution of DMSO. In the experiments reported here, embryos were washed 3 times with at least 10-fold dilution of fresh sea water, then cultured in FSW with penicillin (100 units/ml) and streptomycin (200 μg/ml), changed every other day. Larvae were anesthetized as described (Clement and Cather, 1957). To examine 4d differentiation, we examined larval retracting muscle rather than intestine because the *Tectura* veliger larva does not feed and lacks a developed intestinal tract. We also scored several tissues derived from the ectodermal micromeres (Dictus and Damen, 1997). Eyes disappeared during fixation and were scored in live animals. Operculum and shell were visible in fixed preparations. We could not reliably score the presence versus absence of statocysts. Larvae were scored at 200× and 400× with DIC optics and fixed specimens were DAPI stained.

**Results**

**MAPK activation in the snail *Lymnaea palustris***

To characterize the activation of the MAPK pathway during *Lymnaea palustris* embryogenesis, we used a monoclonal antibody that specifically recognizes the diphostorylated, activated form of MAPK (Gabay et al., 1997; Yung et al., 1997). If the MAPK pathway is activated by a cell–cell signaling event, then this antibody will detect the activated protein in the cell that receives the signal. As in *Ilyanassa*, we did not detect MAPK activation before the production of the third quartet of micromeres, nor did we detect MAPK activation 60 min after the production of the third quartet (hereafter 3q + 60). However, soon afterward (3q + 90), MAPK was activated in one of the macromeres (Fig. 2). This is the stage when 3D is specified by contact with the micromeres (Martindale et al., 1985; van den Biggelaar, 1976). Two lines of evidence indicate that the cell with MAPK activation is 3D. First, the cross-furrow
macromeres are the two cells that are in contact with each other at the vegetal pole. It is essentially always one of the cross-furrow macromeres that is selected to be 3D (van den Biggelaar, 1976), and we always detected MAPK in a cross-furrow cell. Second, 3D is the only macromere that establishes lasting contacts with the animal micromeres. In animal views, the stained cell was observed to be in contact with all four 1q cells, the most animal micromeres (not shown). MAPK activation is still observed in 3D more than an hour later (3q + 180), just prior to its division. After 3D divides, MAPK activation is no longer observed. Throughout the period of activation in 3D, and for 3 h after the division, we did not detect MAPK activation in the micromeres. This is in contrast with Ilyanassa, where MAPK activation is initially observed in 3D but is then observed in micromeres as they respond to signaling from 3D. These results suggest that, in Lymnaea, MAPK activation in 3D may be involved in specification of this cell, but that the MAPK pathway does not transduce the subsequent signal from 3D to the micromeres.

**MAPK activation in the chiton Chaetopleura apiculata**

We examined the pattern of MAPK activation during cleavage and D quadrant specification in the chiton Chaetopleura apiculata. In chitons, the first indication of which macromere is 3D comes at the 36-cell stage, when the presumptive 3D macromere makes contacts with the micromeres on the roof of the blastocoel cavity (van den Biggelaar, 1996). We first see activation of MAPK in one macromere at this stage. Through-focus lateral images reveal that this macromere is in contact with the micromeres, demonstrating that MAPK activation is first observed in 3D in the chiton embryo (Fig. 3). We examined embryos fixed at several points after the division of 3D and found no MAPK activation in the macromeres. This shows that, in Chaetopleura, like Lymnaea, the MAPK cascade does not transduce the signal from 3D to the micromeres, but is likely involved in specification or function of 3D.

**Expression and function of MAPK in the limpet Tectura scutum**

We examined the role of MAPK in 3D of the equal-cleaving mollusc Tectura scutum. Tectura is a limpet, and its embryology is very similar to its well-characterized relative Patella vulgata (van den Biggelaar, 1977; Eric Edsinger-Gonzales, personal communication). We examined the pattern of MAPK activation in Tectura. About 30 min after the production of the third quartet, at the 32-cell stage, MAPK was detected in the 3D macromere (Fig. 4, and data not shown). Thus, the timing of MAPK activation in Tectura is similar to the other equally cleaving molluscs examined and indicates that MAPK activation is involved in 3D specification or function.

To examine the role of MAPK in 3D, we used the MEK inhibitor U0126. This agent specifically inhibits the kinase that is directly upstream of Erk MAPK, and thus inhibits signal transduction through the pathway (Favata et al., 1998; Gould and Stephano, 1999; Lambert and Nagy, 2001). We treated embryos from about 30 min before the production of the third quartet until about 30 min after the birth of 4d. This period spans the observed activation of MAPK in 3D. We then reared embryos for 6–7 days. Since the cell lineage of the major larval organs is known, our approach was to treat embryos with the inhibitor when 3D is thought to induce certain micromere fates and determine whether the organs derived from these micromeres differentiate.

We found that 50 μM U0126 prevented the normal differentiation of eyes, shell, and foot (Fig. 5): these structures derive from various cells of the first, second, and third quartets (Dictus and Damen, 1997). While the relevant deletion experiments have not been performed in a limpet, early deletion of 3D affects the differentiation of these structures in Lymnaea, suggesting that 3D induces these fates in micromeres in equally cleaving molluscs (Mant-dale, 1986). All treated larva (n = 15) lacked both shell and operculum (the biomineral plate on the posterior face of the foot). Unlike Ilyanassa, all treated larvae developed eyes, and surprisingly, half had extra eyes (Fig. 5C; n = 14; three had four eyes, four had three, and seven had two). When two eyes were present, they were usually not spaced on opposite sides of the pretrochal region as in controls, but were more variably spaced, often near each other. Treatment with 25 μM U0126 had similar results (not shown). These experiments show that the MAPK activation observed in 3D is required for the normal development of the micromeres that produce the foot and shell as well as the proper spatial pattern of specification or differentiation of the cells that produce eyes. Thus, MAPK activation is required in 3D to induce the normal pattern of cell fates in the micromeres.

We also tested whether the differentiation of 4d was perturbed after U0126 treatment. This cell, the daughter cell of the 3D macromere, gives rise to the primary mesoderm and some endoderm, such as intestine. The most obvious 4d derivative in Tectura is the larval retractor muscle. However, this muscle is difficult to score in animals treated with 50 μM U0126, because they lack structures—like shell—that serve as landmarks for identifying the muscle bands. Fortunately, we found that the larval retractor muscle was perturbed at concentrations of inhibitor low enough that other ectodermal organs differentiated normally. Treatment with U0126 at 10 μM, from 30 min before the third quartet to 30 min after 4d formation in controls, allowed largely normal differentiation of ectodermal organs like shell and eyes (development of the foot was variable after this treatment). We stained treated and posttorsional control larvae with phalloidin to visualize the larval musculature (Fig. 6). In controls, the main larval retractor was observed connecting the visceral mass to an insertion on the
posterior-right of the interior of the shell. In 10 μM U0126-treated larvae, this muscle was either absent or much smaller (Fig. 6). We have not determined whether this treatment blocks production of the 4d micromere itself, or whether 4d is born normally but does not differentiate properly. However, these results suggest that inhibiting MAPK activation in 3D disrupts the birth or differentiation of a daughter cell of 3D, the 4d micromere.

Fig. 5. The effects of blocking MAPK activation in the limpet Tectura. (A) Control 7-day-old veliger larva, anterior view. (B) Seven-day-old larva after 10 μM U0126 treatment. The head is rotated 90 degrees from the normal relation with the shell. No operculum was present in this animal. (C) Seven-day-old larva after 50 μM U0126 treatment. A ciliated velar band is evident, as are three eyes. Extra eyes ranged from normal sized to small as in this animal. No operculum or shell is present. Scale bar is 50 μm.

posterior-right of the interior of the shell. In 10 μM U0126-treated larvae, this muscle was either absent or much smaller (Fig. 6). We have not determined whether this treatment blocks production of the 4d micromere itself, or whether 4d is born normally but does not differentiate properly. However, these results suggest that inhibiting MAPK activation in 3D disrupts the birth or differentiation of a daughter cell of 3D, the 4d micromere.

We noticed that all of these larva were incompletely torted—the head–foot mass was at a 90-degree angle to the shell compared with controls (Fig. 5). The normal relationship of the head and foot to the shell is established during the process of torsion, where the body is twisted 180 degrees between the head–foot mass and the shell. Therefore, the configuration observed after 10 μM treatment suggested that torsion was incomplete. This could be a result of the lack of a larval retractor muscle or foot structures; both of these structures have been proposed to drive torsion (Page, 1997).

MAPK in the polychaete Hydroides hexagonus

To assay whether the MAPK pathway is used in a similar way in embryos of other spiralian groups, we examined MAPK activation in the embryo of the polychaete annelid Hydroides hexagonus. This embryo has equal cleavage in the sense that the four macromeres are the same size, but it is not known whether these four cells are equipotent as in molluscan equal cleavers. We did not detect MAPK activation before the birth of the third quartet, nor did we detect MAPK activation at several time points during the life of 3D. Surprisingly, MAPK activation was detectable in one of the daughter cells of 3D, the 4d micromere. Activation was not detected immediately after the birth of 4d, but was strong at a later time point, after the division of the third quartet cells and during the division of 2d2 (Fig. 7). This division represents the shift from radial to bilateral cleavage, and thus signals the specification of the secondary axis. Specifically, 2d2 is smaller than 2abc2 (our personal observations), as in some other equally cleaving polychaetes (Treadwell, 1897, 1901). Since the equivalent cells in all four quadrants behave the same until this point, the cleavage of 2d2 is the earliest manifestation of dorsal–ventral axis specification. Thus, MAPK activation in 4d coincides with overt dorsal–ventral axis specification in Hydroides.

Fig. 6. MAPK inhibition impairs development of the main larval retractor muscle in Patella. (A) Dark-field image of a right-lateral view of a control larva. (B) Phalloidin-AlexaFluor 488 staining of muscle in the same mount. The main larval retractor is visible at the posterior connecting the visceral mass to a point on the right–posterior inside surface of the shell. After 10 μM treatment with the MAPK inhibitor U0126 (C, D), the main larval retractor is greatly reduced or absent. The arrowhead in (D) indicates a muscle that may be the remnant of the main larval retractor. In other treated larvae, the muscle was gone entirely. All of these larvae were incompletely torted. Scale bar is 50 μm.
Discussion

In Ilyanassa, there are two phases of MAPK activation during early cleavage: activation is first observed in the 3D macromere and subsequently in a specific subset of micromeres (Lambert and Nagy, 2001). Since the signal from 3D to the micromeres has been inferred to take place in equally cleaving mollusc embryos, we expected that the role for the MAPK pathway demonstrated in Ilyanassa would be conserved in these embryos and possibly those of other spiralian phyla. We did find evidence that MAPK is involved in the specification or function of the D quadrant cells in various spiralian embryos with equal cleavage. However, we also documented two changes in the spatiotemporal pattern of MAPK activation. One change was the observed shift in the initial onset of activated MAPK expression between 3D (molluscs) and 4d (annelids). We propose that the MAPK activation in the molluscan 3D and annelid 4d reflect a conserved role for MAPK in activating these cells as the organizers of future embryonic dorsal–ventral polarity. We argue that this change in MAPK activation corresponds with a shift in the timing of the establishment of D quadrant specification in the two groups. The second change involves the acquisition of a new function for MAPK in the micromeres of Ilyanassa, which was not observed in the other spiralian tested.

Specification of 3D in equally cleaving molluscs

We examined the embryos of three molluscs with equal cleavage: the pulmonate gastropod Lymnaea, the limpet Tectura, and the chiton Chaetopleura. In all of these embryos, MAPK is activated in 3D during the period when this cell is specified but not in dorsal and lateral micromeres after activation in 3D. This is consistent with a recent report of MAPK activation exclusively in the 3D of Patella (Lartillot et al., 2002). In equally cleaving molluscs, 3D specification requires contact between the presumptive 3D macromere and the micromeres. This suggests that the micromeres signal to this macromere to specify it as 3D. In the equally cleaving molluscs surveyed here, MAPK activation is observed in the 3D macromere around the time that it makes contact with the overlying micromeres. Thus, the simplest model is that the MAPK pathway transduces a signal from the micromeres to the presumptive 3D. However, the role of MAPK in 3D specification may be more complex, since the available data are also consistent with MAPK activation mediating interactions between macromeres. In equally cleaving molluscs where cell contacts have been carefully examined at this stage, micromeres contact multiple macromeres before 3D is selected, and there is a period of more transient contact between micromeres and 3D before this cell is centralized (van den Biggelaar, 1976, 1977). It is thus possible that the signal from the micromeres may not have sufficient spatial resolution to select a single macromere, and that interactions between macromeres might be required to refine a general spatial cue from the micromeres to the selection of a single cell as 3D. For instance, there could be lateral inhibition of neighboring macromeres by the presumptive 3D. MAPK activation that we observe may be downstream of such macromere–macromere signals, or could integrate micromere and macromere signals to select 3D.

Specification of 3D in equally cleaving polychaete annelid Hydroides. Vegetal view of an embryo fixed about 40 min after the birth of 4d. (A) Bright-field image. Strong MAPK activation is observed in the nucleus of 4d, a daughter cell of 3D. (B) Same mount with cell boundaries and identities. Scale bar is 10 μm.

Fig. 7. MAPK activation in the equally cleaving polychaete annelid Hydroides. Vegetal view of an embryo fixed about 40 min after the birth of 4d. (A) Bright-field image. Strong MAPK activation is observed in the nucleus of 4d, a daughter cell of 3D. (B) Same mount with cell boundaries and identities. Scale bar is 10 μm.

Fig. 8. Heterochrony in cleavage landmarks and axis specification in equally cleaving spiralians. Schematic shows the temporal progression of development in four different spiralians (horizontal black lines). Key landmarks in development and axis specification are shown (colored bars), as is the onset of MAPK activation in a macromere (red ovals). Despite the overt similarities between spiralian embryos, there is considerable variation in the sequence of events in cleavage and axis specification. In equally cleaving spiralians, the onset of MAPK activation coincides with the first indication of bilateral symmetry (red ovals). Both the onset of bilateral symmetry in cleavage pattern and the onset of MAPK activation are before 4d in equally cleaving molluscs and after 4d in equally cleaving annelids.
MAPK appears to be required for 3D specification. Lartillot et al. (2002) found that, in *Patella*, a close relative of *Tectura*, the characteristic division pattern of 3D was abolished by U0126 treatment. Our data also indicate that MAPK is required for 3D specification since inhibiting MAPK activation in *Tectura* affected two specific behaviors of 3D. Low concentrations of inhibitor prevented differentiation of main larval muscles that are produced by the 4d micromere, a daughter cell of 3D. This argues that MAPK is required for proper cell fate specification in the asymmetric division of 3D. Higher concentrations of inhibitor prevented the normal specification of micromere-derived ectodermal structures, such as operculum and external shell. Since 3D appears to be required to signal to micromeres to specify particular fates (Martindale, 1986), the absence of these structures suggests that signaling from 3D requires MAPK activation. Lartillot et al. (2002) found that, in *Patella*, a close relative of *Tectura*, even high concentrations of U0126 (50 μM) did not completely radialize the larvae. This is in contrast to their finding that these doses usually radialized the cleavage pattern, and to our own finding that the same concentration of the inhibitor reproducibly produced radialized larvae, i.e., larvae that lack differentiation of a secondary or dorsal–ventral axis. This could be due to differences in delivery of the drug: in our hands, U1026 precipitates when diluted as described by Lartillot et al. (2002), so it is possible that their phenotypes represent a partial MAPK inhibition. The discrepancy could also be caused by differences in scoring (we scored our larvae later in development), or by real differences between these two species of limpets in the requirement for MAPK or the sensitivity of this pathway to the inhibitor.

We found that inhibiting MAPK activity in 3D often produced an excess of eyes, which appeared to be positioned around the dorsal–ventral axis of the head. This is strikingly different from *Ilyanassa*, where blocking MAPK activation prevented the differentiation of eyes. The simplest explanation of this difference is that, in *Tectura*, signaling from 3D has a negative effect on eye differentiation, perhaps serving to block eye specification in some first quartet cells. In experiments where 3D specification was blocked in *Lymnaea*, cleavage patterns indicated that more than the normal two lateral quadrants were sometimes produced, consistent with the production of more than two eyes (Martindale et al., 1985). However, the effect of these cleavage pattern alterations on eye development was not determined in this case, and the frequency of embryos with supernumerary lateral quadrants was lower than the frequency of extra eyes in our experiments.

*The evolution of MAPK function in molluscs*

We unexpectedly found that MAPK is not activated in the micromeres of equally cleaving molluscs. Together with current understanding of the phylogenetic relationships of the molluscs, our results suggest that the role of MAPK observed in equally cleaving gastropods (only in 3D) is ancestral and that the biphasic pattern of MAPK activation observed in *Ilyanassa* (3D then micromeres) evolved later. Equal cleavage is believed to be the ancestral character state for the gastropods (Ponder and Lindberg, 1997). We found the same pattern of MAPK activation in the two equally cleaving gastropods we surveyed. One of these (*Tectura*) is a member of the basally branching patellogastropod clade along with *Patella*, in which the pattern of MAPK activation is the same. This provides strong evidence that the ancestral role for MAPK in the gastropods was similar to *Tectura* and *Lymnaea*. Since *Ilyanassa* shares a more recent common ancestor with *Lymnaea* than with *Tectura*, this implies that the second phase of MAPK activation observed in *Ilyanassa* evolved in this lineage since the last common ancestor with *Lymnaea* (see Fig. 1). The polar lobe-dependent patterning found in *Ilyanassa* appears to have evolved near the base of the higher gastropod clade Caenogastropoda (Ponder and Lindberg, 1997). One possibility is that the origin of the second phase of MAPK activation observed in *Ilyanassa* was associated with the origin of the polar lobe in early Caenogastropods.

The role of MAPK in equally cleaving embryos likely represents the ancestral character state, not only for gastropods but also for the whole phylum. Although the class level phylogeny of the molluscs remains unclear, the Polyplacophora, or chitons, are generally considered to be one of the most basally branching extant clades in the phylum (see references in Taylor, 1996). Chitons exhibit equal cleavage, and in these embryos, 3D is specified by contact with the micromeres (Heath, 1899; van den Biggelaar, 1996). The role that we have demonstrated for MAPK in equally cleaving molluscs, including the chiton *Chaetopleura*, may therefore have been in place in the common ancestor of all extant molluscs. It will be interesting to see whether MAPK plays a role in early patterning in representatives of other extant molluscan classes—like the cephalopods, bivalves, and aplacophorans.

*MAPK in a polychaete*

Molluscs and polychaete annelids embryos have similar cleavage patterns, fate maps, and roles of the D quadrant in embryonic organization, suggesting that the underlying patterning mechanisms would also be conserved. The role of MAPK in the equally cleaving polychaete *Hydroides* was markedly different from the molluscs we examined. In *Hydroides*, MAPK is not activated in 3D, but it is specifically...
activated in its daughter cell, 4d. We propose that the activation of MAPK in 4d of Hydroides is homologous to activation in 3D of molluscs, and similarly downstream of a signal that specifies the D quadrant and axis of bilateral symmetry. This implies that the timing of this event in development has shifted between molluscs and annelids (see Fig. 1). This hypothesis is based on comparisons of cleavage patterns between representatives of the two groups, the function of MAPK in molluscs, and the patterns of MAPK activation we have described here.

If the role of MAPK in the annelid 4d is indeed homologous to the role in the molluscan 3D, then this pathway has undergone a heterochronic shift, i.e., a temporal change in the role of MAPK in patterning the D quadrant lineage relative to the landmarks in the cleavage program. Indeed, comparison of the cleavage patterns of equally cleaving annelids to that of molluscs suggests a shift in D quadrant patterning events relative to cleavage cycle. In equally cleaving spiralians, there is an initial period of radial—or more properly, quadrilateral—symmetry in the arrangement of cells and the pattern of early cleavages. At some point during cleavage, this symmetry is broken with the onset of bilateral symmetry, when cells of one quadrant behave differently from the corresponding cells in the other quadrants. The first indication of bilateral symmetry can be the movement of the 3D macromere to the center of the blastula, or micromere divisions that differ in the timing, direction, or proportion from the divisions in other quadrants. Wherever it occurs, the emergent bilateral symmetry is the first overt indication of the secondary or dorsal–ventral axis in these equally cleaving embryos. Specification of this axis is assumed to occur at or shortly before the onset of bilateral symmetry. In equally cleaving molluscs, the onset of bilateral cleavage in the micromeres depends on specification of the D quadrant macromere 3D.

Freeman and Lundelius (1992) have pointed out that the first appearance of bilateral symmetry has shifted in relation to cleavage cycle between equally cleaving molluscs and equally cleaving annelids. In equally cleaving molluscs, the first indications of bilateral organization—the centralization of the 3D macromere—always occur before the birth of 4d (Fig. 8; Heath, 1899; van den Biggelaar, 1977; Wierzejski, 1905). Moreover, in equally cleaving molluscs, there are often departures from the quadrilateral symmetry of the cleavage pattern before the birth of 4d (Heath, 1899; van den Biggelaar, 1977). In contrast, in all equally cleaving polychaetes examined, all divisions leading up to and including the birth of the fourth quartet cells are radially symmetrical. The onset of bilateral symmetry does not appear until some point after the birth of the fourth quartet (Groepeler, 1986; Mead, 1897; Treadwell, 1901). When the onset has been identified—as in Podarke—it is marked by the asymmetric and early division of 2d2 but not 2abc2. This may be generally true for equally cleaving polychaetes: Treadwell reported 2d2-specific asymmetric division in several species where it had apparently not been noted earlier (Mead, 1897; Treadwell, 1897). The later onset of bilateral symmetry in polychaetes indicates that the specification of the D quadrant may be accelerated in molluscs versus polychaetes relative to the birth of the fourth quartet (or the converse).

Our results add a new level of support to this idea. We have found that, in both molluscs and an annelid, the MAPK pathway is first activated at or before the onset of bilateral cleavages. However, the cell where activation first observed in the polychaete (4d) is one cell cycle later than the onset of MAPK activation in molluscs (in 3D). Thus, both the onset of MAPK activation and the onset of bilateral cleavages are shifted between these two groups, by one cleavage cycle. Since the MAPK pathway is often activated in response to extracellular signals, our observation of MAPK activation in 4d raises the possibility that inductive interactions may determine the dorsal–ventral axis by specifying 4d, rather than 3D as in molluscs. No experimental evidence is yet available that directly addresses the mechanism of D quadrant specification in equally cleaving polychaetes, although Treadwell reported extensions between animal and vegetal cells in Podarke which could indicate inductive interactions between these two populations (Treadwell, 1901). In Ilyanassa, MAPK activation in 3D is required for 4d to be born at the normal time and for differentiation of tissues derived from this cell (Lambert and Nagy, 2001). We show here that the latter is true in Tectura as well. This suggests that MAPK is important for 4d specification in molluscs and supports the homology between the molluscan 3D activation and the polychaete 4d activation.

Our argument for the homology of MAPK function in the polychaete 4d and mollusc 3D is stronger if equal spiral cleavage was in place in the most recent common ancestor of these groups, rather than independently derived in each group. Both the molluscs and the polychaetes contain some representatives that display equal spiral cleavage and some that have unequal cleavage. In both groups, equal spiral cleavage has been inferred to be ancestral (Freeman and Lundelius, 1992; van den Biggelaar et al., 1997). This was based on available phylogenies, a proposed mechanism for the transition from equal to unequal cleavage, and a putative selective pressure for this transition. Recent phylogenetic analyses of the gastropods are consistent with equal cleavage being ancestral for this group (Ponder and Lindberg, 1997). On the other hand, Dohle (1999) has argued that unequal cleavage is ancestral for annelids, based on the assumption of monophyly of the Polychaeta, as well as the highly similar cleavage patterns of 2d between polychaetes and clitellates (leeches and oligochaetes). Resolution of this question will improve with advances in annelid phylogenetics and more data on developmental mechanisms in polychaetes. However, we note that the homology of 2d cleavage patterns proposed by Dohle is perfectly compatible with equal cleavage being ancestral for the annelids, as long as the clitellates are derived from within the polychaetes.
This topology is usually recovered in molecular analyses of these groups (see McHugh, 2000).

We note that the MAPK pathway is used repeatedly in different contexts during development of diverse metazoans. This suggests that it has frequently been coopted to perform different functions, and by extension, that it need not indicate homologous developmental events. Our assessment of homology could be further strengthened by examination of additional equally cleaving polychaete embryos, as well as functional analyses of both the role of MAPK in the polychaete 4d and the role of 4d in polychaete micromere patterning. Our hypothesis predicts that ablation of 4d in Hydroidea would mimic 3D ablation in an equally cleaving mollusc.

The evolution of spiralian embryogenesis

The developmental programs of many phyla have been described as spiralian (see Costello and Henley, 1976). In some cases, the similarity of cleavage patterns and cell fates provides strong evidence that the mechanisms of development are homologous. These include the molluscs, the annelids, the polyclad flatworms, the sipunculids, the echiurans, and the nemerteans. Other protostome taxa have been described as spiralian, but the evidence for homologous spiralian development is either equivocal or lacking; these taxa include some lophophorates, acoel flatworms, and the arthropod crustaceans. Examination of the role of the MAPK pathway in other protostome taxa should provide an important new class of evidence supporting the existence of homologous patterning mechanisms among protostome embryos. As such evidence accumulates, comparative analysis of the MAPK pathway will provide the first glimpse into the evolutionary changes in molecular patterning that have occurred during the long history of spiralian embryogenesis. As we have already observed in the case of MAPK activation in 4d of Hydroidea and in the micromeres of Ilyanassa, molecular markers of patterning events can reveal evolutionary transitions that have remained hidden beneath the highly conservative spiralian cleavage pattern.

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