

# Secondary embryonic axis formation by transplantation of D quadrant micromeres in an oligochaete annelid

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## SUMMARY

Among spiral cleaving embryos (e.g. mollusks and annelids), it has long been known that one blastomere at the four-cell stage, the D cell, and its direct descendants play an important role in axial pattern formation. Various studies have suggested that the D quadrant acts as the organizer of the embryonic axes in annelids, although this has never been demonstrated directly. Here we show that D quadrant micromeres (2d and 4d) of the oligochaete annelid *Tubifex tubifex* are essential for embryonic axis formation. When 2d and 4d were ablated the embryo developed into a rounded cell mass covered with an epithelial cell sheet. To examine whether 2d and 4d are sufficient for axis formation they were transplanted to an ectopic position in an otherwise intact embryo. The reconstituted embryo formed a secondary embryonic axis with a duplicated head and/or tail. Cell lineage analyses showed that neuroectoderm and mesoderm along the secondary axis were derived from the transplanted D quadrant micromeres and not from the host embryo. However, endodermal tissue along the secondary axis originated from the host embryo. Interestingly, when either 2d or 4d was transplanted separately to host embryos, the reconstituted embryos failed to form a secondary axis, suggesting that both 2d and 4d are required for secondary axis formation. Thus, the *Tubifex* D quadrant micromeres have the ability to organize axis formation, but they lack the ability to induce neuroectodermal tissues, a characteristic common to chordate primary embryonic organizers.

**KEY WORDS:** Spiral cleavage, D quadrant, Embryonic axis, Cell transplantation, Annelid, *Tubifex tubifex*

## INTRODUCTION

In metazoan development, a specific region in the early embryo called the organizer has a remarkable potential to instruct neighboring cells to assume proper cell fates, as well as to specify a particular embryonic axis. The characteristics of embryonic organizers have been best studied in chordate embryos. The primary embryonic organizer in chordates has two defining properties: it signals to the ectoderm to differentiate as neural tissue and it can induce a secondary axis when transplanted to an ectopic position of the host embryo (De Robertis et al., 2000; Harland and Gerhart, 1997; Spemann and Mangold, 1924; Tung et al., 1962). Embryonic organizers have also been identified in various other metazoans, including cnidarians (Broun and Bode, 2002; Kraus et al., 2007), mollusks (Clement, 1962; Damen and Dictus, 1996; Henry et al., 2006; Martindale, 1986; Rabinowitz et al., 2008; van den Biggelaar, 1977), arthropods (Holm, 1952) and echinoderms (Ransick and Davidson, 1993). These non-chordate embryonic organizers share the ability to establish embryonic axes and to induce proper cell fates, but an important difference between chordate and non-chordate embryonic organizers is that the induced tissue(s) in non-chordate embryos need not be restricted to neural tissues (see Ransick and Davidson, 1993). Recently, both chordate and non-chordate embryonic organizers have collectively been referred to as 'axial organizers' (Gonzales et al., 2007; Kraus et al., 2007). At present, it

is unclear whether axial organizers are homologous throughout the Bilateria, or how much variation exists in how they function to establish the axial organization of the embryo.

In this study, we provide an additional example of an axial organizer in the annelid *Tubifex tubifex*. We show that specific micromeres (called D quadrant micromeres) of the *Tubifex* embryo have the ability to form a secondary axis when transplanted to an ectopic position of the host embryo. Cell lineage analyses show that the D quadrant micromeres lack the ability to induce neural tissue; however, they induce secondary gut formation. These results show that the *Tubifex* D quadrant functions as the axial organizer. In addition, the present study provides the first direct evidence that annelid D quadrant cells have the ability to organize the embryonic axis (see below).

In annelids, various studies have suggested that one blastomere at the four-cell stage, the D cell, and its derivatives have the ability to organize the embryonic axis (Freeman and Lundelius, 1992; Lambert, 2008; Lambert, 2010). Cell isolation experiments in the polychaete *Chaetopterus* have shown that only embryos containing the D quadrant develop ectodermal tissues such as eyes and lateral hooked bristles (Henry, 1986). Also, it is known that equalized first cleavage gives rise to so-called double embryos in *Chaetopterus*, *Nereis*, *Platynereis* and *Tubifex* (Henry and Martindale, 1987; Penners, 1924b; Tyler, 1930), suggesting that equalization of first cleavage and twinning embryos are strongly correlated phenomena. Using MAPK activation as a molecular marker, Lambert and Nagy have suggested that the fourth micromere descendant of the D macromere, 4d, functions as the embryonic axis organizer in the polychaete *Hydroides* (Lambert and Nagy, 2003). In other polychaetes, such as *Arctonoe vittata* and *Serpula columbiana*, pharmacological analyses have suggested that the D quadrant

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functions as an axial organizer (Gonzales et al., 2007). However, the crucial transplantation test of whether the presumed axial organizer in annelids can induce host tissue to form a secondary axis has not been performed.

The oligochaete annelid *Tubifex tubifex* is an ideal model organism with which to address whether the D quadrant functions as an axial organizer. The cell lineage of the D quadrant has been analyzed with modern lineage tracers (Goto et al., 1999a; Goto et al., 1999b) and early embryos are amenable to cell transplantation (Kitamura and Shimizu, 2000; Nakamoto et al., 2004). To examine

the organizing properties of the *Tubifex* D quadrant, we conducted a series of cell ablation and transplantation experiments. The early development of *Tubifex* is summarized in Fig. 1. The first two cleavages are unequal and produce four macromeres denoted A, B, C and D (Fig. 1A). Each macromere undergoes a series of unequal divisions and generates small micromeres to the animal pole. For example, the D quadrant repeats unequal divisions four times, yielding four micromeres (1d, 2d, 3d and 4d) at specific positions in the embryo. The first (1d) and the third (3d) micromeres are small, whereas the second (2d) and fourth (4d) micromeres are almost as large as the macromeres (Fig. 1C,D). At the 22-cell stage, the cells  $2d^{11}$  (the critical descendant of 2d), 4d and 4D all line up on the future midline of the embryo (Fig. 1D).  $2d^{11}$  undergoes asymmetric cell division to give rise to  $2d^{111}$  and  $2d^{112}$  (see Fig. 1I). During the next cleavage,  $2d^{111}$ , 4d and 4D divide equally along the midline, generating the precursor of ectodermal stem cells (NOPQ), mesodermal stem cells (M), and endodermal cells ( $E^D$ ), respectively, in pairs (Fig. 1E). Embryos undergo gastrulation and morphogenesis during days 2-6 (see Fig. 1F-H). Embryogenesis is completed by days 7-9. Most of the tissues are differentiated at this stage (for details, see Shimizu, 1982).

Classic cell ablation experiments on clitellate (i.e. leech and oligochaete) embryos clearly showed that morphogenetic events such as body elongation and segmentation depend solely on the presence of the second (2d) and fourth (4d) micromeres of the D quadrant (Devris, 1973; Mori, 1932; Penners, 1924a; Penners, 1925; Penners, 1926). These micromeres are the main source of ectodermal and mesodermal segmental tissues (Goto et al., 1999a; Weisblat et al., 1984). When Penners eliminated both 2d and 4d by UV irradiation (using embryos within intact cocoons), these embryos developed into a ball of endodermal cells covered with an epithelial sheet of cells (Penners, 1926). This suggested that 2d and 4d, either separately or together, might function as the axial organizer.

In the present study, we re-examined Penners' experiment and confirmed that 2d and 4d are essential for axis formation. We also undertook a series of cell transplantation and cell labeling experiments and showed that these micromeres function as the axial organizer. In the light of the results obtained in this study, we discuss the similarities and differences between the *Tubifex* D quadrant and the chordate primary embryonic organizer.

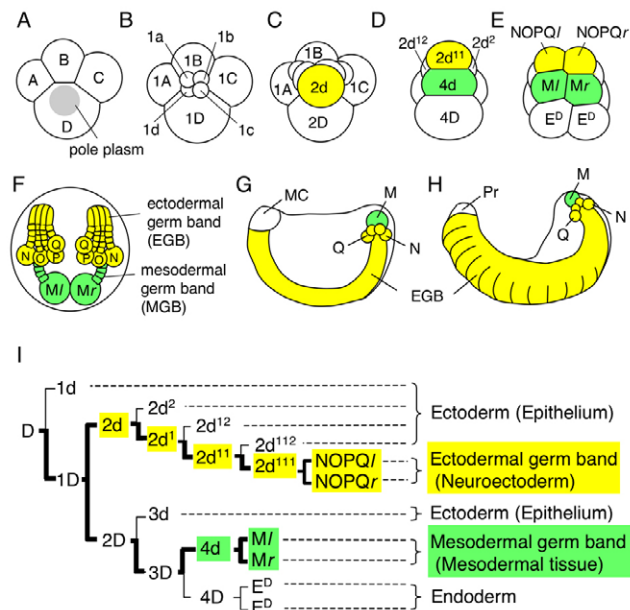
## MATERIALS AND METHODS

### Embryos

Embryos of the freshwater oligochaete *Tubifex tubifex* were obtained as previously described (Shimizu, 1982) and cultured at 22°C. Prior to experimentation, embryos were freed from their cocoons in the culture medium (Shimizu, 1982). Unless otherwise stated, all experiments were carried out at room temperature (20-22°C). The culture medium, agar, glassware and embryos were sterilized as described previously (Kitamura and Shimizu, 2000).

### Microinjection of fluorescent tracers, DAPI staining, cell ablation and transplantation

Pressure injection of 1,1'-dihexadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate (DiI; Molecular Probes) was performed as described previously (Kitamura and Shimizu, 2000). Tetramethylrhodamine dextran (Fluoro-Ruby, lysine fixable, 10,000 MW; Molecular Probes) and Oregon Green dextran (lysine fixable, 10,000 MW; Molecular Probes) were dissolved at 5 mg/ml in injection buffer (0.2 M KCl, 5 mM HEPES pH 7.2, 0.5% Fast Green). Before use, an aliquot of these solutions was filtered through a spin column (Ultrafree-MC Centrifugal Filter Unit, Millipore). Injected embryos were incubated and fixed as described previously (Kitamura and Shimizu, 2000), and stained with DAPI (I



**Fig. 1. Early development of *Tubifex tubifex*.** (A-F) Animal pole view. (A) The first two cleavages are unequal and produce quadrants A-D of different size. The pole plasm is inherited by the D cell. (B) The third cleavage results in an 8-cell stage embryo. Quadrants A-D produce four micromeres (1a-1d) at the animal pole and four macromeres (1A-1D) at the vegetal pole. (C) A 9-cell stage embryo shortly after the formation of 2d. (D) A 22-cell stage embryo. Cells  $2d^{11}$ , 4d and 4D all come to lie at the future midline. (E) Cells  $2d^{111}$ , 4d and 4D divide bilaterally and equally.  $2d^{111}$  generates the ectoteloblast precursors NOPQ/ and NOPQr. 4d produces the mesoteloblasts M/ and Mr. 4D divides into a pair of endodermal precursors termed  $E^D$ . (F) A 2-day-old embryo. Only teloblasts and associated structures are depicted. NOPQ cells on each side of the embryo have produced ectoteloblasts N, O, P and Q. A short ectodermal germ band (EGB) extending from the teloblasts N, O, P and Q is seen on either side of the embryo. A mesodermal germ band (MGB) extending from the mesoteloblast is located under the ectodermal germ band. (G, H) Morphogenesis of the germ bands. Side view with anterior toward the left and dorsal toward the top. EGBs and MGBs on both sides of the embryo are elongated and they gradually curve around towards the ventral midline and finally coalesce with each other along the ventral midline (G). The coalescence is soon followed by dorsalwards expansion of the edge of the germ band (H). MC, micromere cap. Pr, prostomium. (I) Cell lineage diagram of the D quadrant. Pole plasm segregation is indicated by thick lines. The 2d cell undergoes unequal cell divisions and produces small micromeres  $2d^2$ ,  $2d^{12}$  and  $2d^{112}$ . Details of cell division are omitted in the portions indicated by dashed lines. Note that the timing of cell division is not reflected in this diagram. Redrawn from Penners (Penners, 1922).

$\mu\text{g/ml}$ ; Molecular Probes) to visualize nuclei. Cell ablation and transplantation were carried out as described previously (Kitamura and Shimizu, 2000; Nakamoto et al., 2004).

## RESULTS

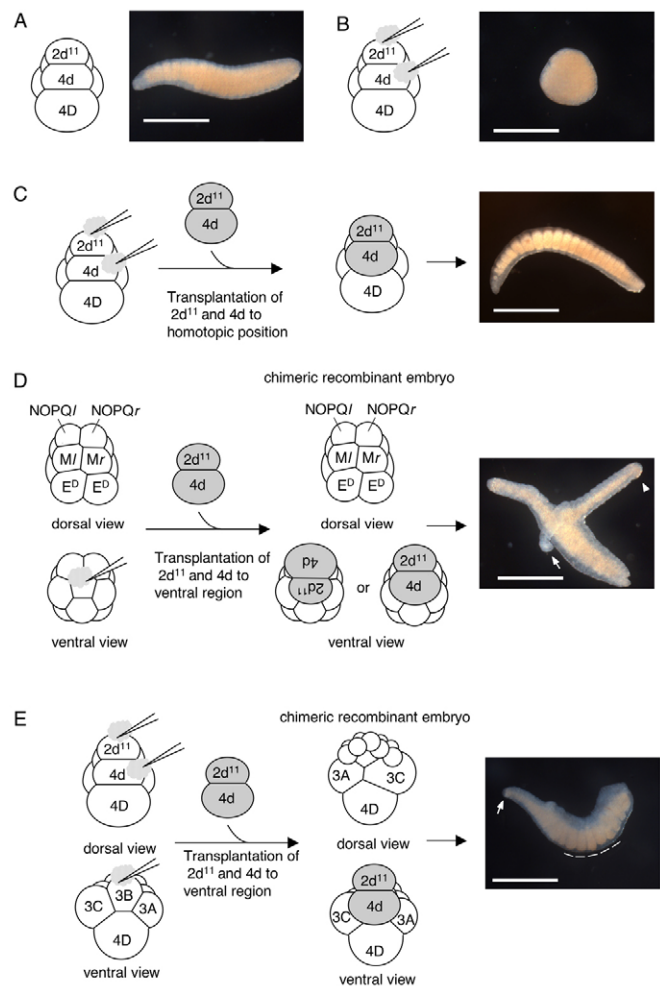
### D quadrant micromeres 2d and 4d are essential for axis formation

To re-examine the developmental role of 2d and 4d in *Tubifex* development, we ablated the two D quadrant micromeres (i.e. 2d<sup>11</sup> and 4d; see Fig. 1D) of 22-cell stage embryos by means of fine glass needles and cultured them for 9 days. All of these embryos ( $n=24$ ) developed into rounded cell masses that failed to exhibit any sign of axial development (Fig. 2B); this phenotype was essentially the same as that obtained in Penners' experiment (Penners, 1926). When the 4D cell (the largest cell of the 22-cell embryo) was ablated, the embryos developed normally (not shown), confirming that cell ablation itself does not disrupt the normal process of development. Furthermore, we also found that when 2d<sup>11</sup> and 4d (co-isolated from a donor embryo) were transplanted to the position of 2d<sup>11</sup> and 4d of 22-cell stage embryos (which had been deprived of 2d<sup>11</sup> and 4d), such reconstituted embryos 'restored' embryonic axis formation and developed into juveniles of normal morphology ( $n=8/8$ ; Fig. 2C). These results verify the notion that the D quadrant micromeres 2d<sup>11</sup> and 4d play a pivotal role in axial pattern formation (Penners, 1926).

To examine whether these micromeres are sufficient to restore an embryonic axis on their own, either 2d<sup>11</sup> or 4d was transplanted to the position of 2d<sup>11</sup> or 4d of a host embryo from which 2d<sup>11</sup> and 4d had been ablated. The reconstituted embryos failed to restore an embryonic axis in either transplantation (not shown), suggesting that both micromeres are required for embryonic axis formation. This result is consistent with our previous cell ablation studies (Goto et al., 1996b; Nakamoto et al., 2000). If 2d was ablated, the mesodermal germ bands (the descendants of 4d) failed to migrate to the ventral midline and they did not coalesce with each other (Goto et al., 1999b). Also, it has been shown that the mesodermal germ bands are required for the segmentation of the overlying ectoderm, which is derived from 2d<sup>11</sup> (Nakamoto et al., 2000).

### Secondary axis formation by transplantation of 2d and 4d

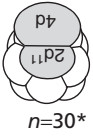



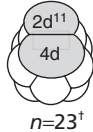
The ablation/restoration experiment shows that the D quadrant micromeres 2d<sup>11</sup> and 4d are essential for embryonic axis formation, but it does not necessarily verify a long-held view that D quadrant micromeres can function as the organizer for the embryonic axis (Gonzales et al., 2007; Henry, 1986; Henry and Martindale, 1987; Lambert and Nagy, 2003; Penners, 1924b; Tyler, 1930) because in this experiment transplanted cells were placed in their 'original' positions. The most stringent criterion for defining a cell or tissue as an organizer is to test its ability to form a secondary embryonic axis when transplanted to an ectopic position in a recipient embryo. Therefore, we transplanted 2d<sup>11</sup> and 4d (that had been co-isolated from a donor embryo) to the ventral region of a recipient embryo from which one endodermal cell had been ablated (Fig. 2D). Removal of one endodermal cell from a 22-cell stage embryo caused no developmental defects ( $n=25$ ). This allowed us to use the position of the ablated endodermal cell as the mold for the transplanted cells. We transplanted 2d<sup>11</sup> and 4d in two different orientations. In one orientation, the transplanted micromeres maintained the anteroposterior (A/P) polarity of the host, whereas in the other the A/P polarity of the transplant was reversed (Fig. 2D). The resulting chimeric recombinant embryos had pairs of NOPQ and M (the immediate progeny of 2d<sup>11</sup> and 4d) on the dorsal



**Fig. 2. Ablation and transplantation experiments with D quadrant micromeres.**

(A) Normal development of *Tubifex tubifex*. Right-hand panel shows a 9-day-old embryo which is segmented and elongated along the anteroposterior (A/P) axis. Anterior is to the left. (B) Ablation of 2d<sup>11</sup> and 4d with a fine glass needle. The embryo shown was incubated for 9 days before fixation. It developed into a rounded cell mass with no embryonic axis. (C) Homotopic transplantation of 2d<sup>11</sup> and 4d. 2d<sup>11</sup> and 4d of the host embryo were ablated and the same set of micromeres from a donor embryo were transplanted to the positions of 2d<sup>11</sup> and 4d. Right-hand panel shows a representative 9-day-old embryo with a restored embryonic axis and that developed normally. (D) Transplantation of the D quadrant micromeres to the ventral region of a host embryo. 2d<sup>11</sup> and 4d were co-isolated from a donor embryo and transplanted to the ventral region of a recipient embryo from which one endodermal cell had been ablated (see ventral view). The donor micromeres were transplanted in two different orientations. In one orientation, the transplanted micromeres maintained the A/P polarity of the host, whereas in the other the A/P polarity of the transplant was reversed. The resulting chimeric recombinant embryo was incubated for 9 days and a representative embryo is shown. The arrow and arrowhead indicate secondary head and tail, respectively. (E) Transplantation of the D quadrant micromeres to a recipient embryo from which the D quadrant micromeres had been ablated. The 3B cell of the recipient embryo had been ablated from the recipient embryo to make the mold for transplantation (see ventral view). The prospective A/P axis of the transplanted D quadrant micromeres (2d<sup>11</sup> and 4d) ran parallel to that of the host embryo. The resulting chimeric recombinant embryo was incubated for 9 days and a representative embryo is shown. Note that the endoderm is clearly segmented (dashed lines). The arrow indicates the anterior; dorsal is to the top. Scale bars: 500  $\mu\text{m}$ .

**Table 1. Results of transplantation of D quadrant micromeres to recipient embryos from which one endodermal cell had been ablated**

Orientation of transplantation	X-shape phenotype	Y-shape phenotype	No clear secondary axis	Cell mass
	 5/30 (17%)	 5/30 (17%)	 20/30 (67%)	0/30 (0%)
	0/23 (0%)	17/23 (74%)	5/23 (22%)	1/23 (4%)

\*Prospective A/P polarity of donor cells was reversed relative to that of the host embryo.

†Prospective A/P polarity of donor cells was the same as that of the host embryo.

side and had the transplanted  $2d^{11}$  and 4d cells and their progeny on the ventral side (Fig. 2D). After 9 days in culture, they were examined for secondary axis formation (Table 1). When the A/P polarity of the transplanted cells was opposite to that of the host embryos, 33% of the reconstituted embryos ( $n=30$ ) had a secondary axis. Of these, five embryos (17%) had clearly duplicated heads and tails (designated as an ‘X-shape phenotype’); the other five (17%) exhibited a ‘Y-shape phenotype’, with either a duplicated head or tail. This result shows that the transplanted  $2d^{11}$  and 4d cells have the ability to form a secondary embryonic axis.

Interestingly, when the A/P polarity of the donor cells was equivalent to that of the host embryo ( $n=23$ ), none of the reconstituted embryos exhibited the X-shape phenotype (0%); most of them (74%) developed the Y-shape phenotype. Thus, the orientation of transplantation affected the phenotype of the reconstituted embryos. At present, it is unclear whether the transplanted cells received a signal(s) from the host embryo or developed in a cell-autonomous manner.

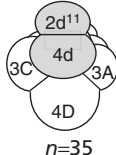

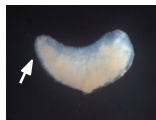
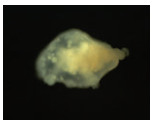
The ability of  $2d^{11}$  and 4d to form an embryonic axis was verified by observations of another form of chimeric recombinant embryo.  $2d^{11}$  and 4d (co-isolated from a donor embryo) were

transplanted to the ventral side of a recipient embryo that had been deprived of the same set of micromeres (from the dorsal side, see Fig. 2E). In this experiment, 3B of the recipient embryo was ablated to make a mold for the transplanted cells (Fig. 2E). As with other endodermal ablations, embryos developed normally in the absence of 3B (A.N. and T.S., unpublished). Eleven percent of the reconstituted embryos ( $n=35$ ) exhibited an elongated body with a distinct head and tail as well as a clearly segmented endoderm; their overall morphology was similar to that of an intact embryo (Table 2). In addition, 71% of the reconstituted embryos elongated to a significant extent and had either head or tail (Table 2). These results suggest that the embryonic axis and endodermal segmentation are partially rescued by transplanted  $2d^{11}$  and 4d.

### Neuroectoderm and mesoderm along the secondary axis are derived from the transplanted micromeres

To determine the origin of cells comprising the secondary axis, we analyzed the cell fates of transplanted D quadrant micromeres. Either the  $2d^{11}$  or 4d cell of donor embryos was labeled with fluorescent tracers (DiI or Rhodamine dextran) and then co-isolated

**Table 2. Results of transplantation of D quadrant micromeres to recipient embryos from which D quadrant micromeres had been ablated**

Orientation of transplantation	Distinct head and tail*	Either head or tail†	Cell mass
	 4/35 (11%)	 25/35 (71%)	 6/35 (17%)

Arrows indicate the anterior.

\*Embryos were very similar to intact embryos.

†Embryos were elongated to a significant extent and segmented.

and transplanted to the ventral region of a complete, normal recipient embryo. Our previous cell lineage analyses have shown that major ectodermal tissues, such as ganglia, peripheral neurons, epidermis and setal sacs, are differentiated at the 7-day stage (Goto et al., 1999a; Goto et al., 1999b). The distribution pattern of mesoderm has also been characterized at the 7-day stage. Mesoderm derived from 4d underlies the ectoderm as well as envelops the endoderm (Goto et al., 1999a; Goto et al., 1999b). In the present study, we assessed the cell fate of transplanted 2d<sup>11</sup> and 4d based on these criteria.

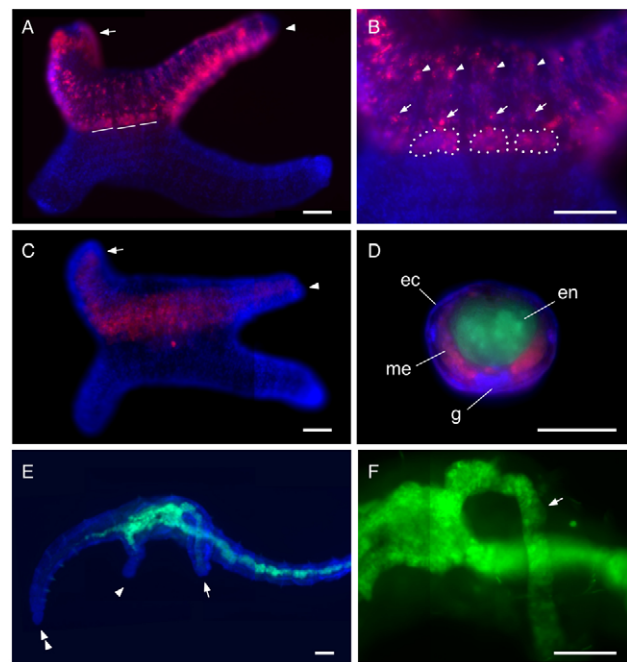
The distribution patterns of labeled cells in 7-day-old reconstituted embryos demonstrated that neuroectoderm and mesoderm along the secondary axis were derived from the transplanted 2d<sup>11</sup> and 4d cells, respectively (Fig. 3A-D). The descendants of transplanted 2d<sup>11</sup> differentiated into ganglia, peripheral neurons, setal sacs and epidermis along the secondary axis. Similarly, transplanted 4d contributed to the mesoderm of the secondary axis. These distribution patterns were comparable to those in normal development (Goto et al., 1999a; Goto et al., 1999b). To examine whether the endoderm along the secondary axis was derived from the host embryo, endodermal macromeres (E<sup>D</sup>, see Fig. 1E) of the host embryo were labeled with Oregon Green dextran. The reconstituted embryos were incubated for 7 days to examine the distribution and cell fate of the labeled cells (Fig. 3D) and for 14 days to examine gut differentiation (Fig. 3E,F). We observed that descendants of the host macromeres contributed to the endoderm and gut of the secondary axis (Fig. 3D-F). Thus, transplanted micromeres recruit endoderm from the host embryo and induce secondary gut formation. Note that the D quadrant micromeres rescued endodermal segmentation when transplanted to an ectopic position in the host embryo from which endogenous D quadrant micromeres had been ablated (see Fig. 2E). This suggests that the D quadrant micromeres regulate segmental patterning and morphogenesis of the endoderm.

### Both 2d and 4d are required for secondary axis formation

To examine whether 2d<sup>11</sup> or 4d is sufficient for secondary axis formation, we transplanted 2d<sup>11</sup> or 4d separately to the ectopic ventral position of a recipient embryo. The reconstituted embryos did not form a secondary axis in either transplantation ( $n=9$  for 2d<sup>11</sup>,  $n=8$  for 4d). This result is consistent with the homotopic transplantation of 2d<sup>11</sup> or 4d. As described above, when either 2d<sup>11</sup> or 4d was transplanted to the position of 2d<sup>11</sup> or 4d of a host embryo from which the endogenous 2d<sup>11</sup> and 4d had been ablated, the reconstituted embryos failed to restore embryonic axis formation. Taken together, these results show that both 2d and 4d are necessary to establish an embryonic axis.

### 4d is required for proper neural development

In the mollusks *Ilyanassa* and *Crepidula*, cell ablation experiments have shown that 4d functions as an organizer (Rabinowitz et al., 2008; Henry et al., 2006). In addition, it has been suggested that 4d has organizer activity in the polychaete *Hydroides* (Lambert and Nagy, 2003). These reports led us to examine whether *Tubifex* 4d also plays a role in the cell fate determination of neighboring cells, especially in the 2d<sup>11</sup> lineage. Our previous cell ablation experiments have shown that 4d and its derivatives (M teloblasts and mesodermal germ bands) are required for the early morphogenetic processes that give rise to the formation of ectodermal segments (Nakamoto et al., 2000). In the present study, we extended the observations to a more advanced developmental



**Fig. 3. The neuroectoderm and mesoderm along the secondary axis are derived from transplanted 2d<sup>11</sup> and 4d, respectively.** The 2d<sup>11</sup> or 4d cell of a donor embryo was labeled with fluorescent tracers (Dil or Rhodamine dextran) and then co-isolated and transplanted to a recipient embryo. The reconstituted embryos developed for 7 days before fixation and were stained with DAPI (blue) to visualize nuclei. **(A,B)** Cell fate of the transplanted 2d<sup>11</sup>. **(A)** The Dil-labeled descendants of transplanted 2d<sup>11</sup> are confined to the upper half (along the secondary axis) of the recombinant embryo. Arrowhead and arrow indicate secondary head and tail, respectively. Dashed lines indicate the position of consecutive segments and the boundary between the primary axis (below) and secondary axis (above). **(B)** Higher magnification view of a mid-body region. Cell clusters indicated with dotted lines are ganglia. Dil-labeled cells are also seen in peripheral neurons (out of focus), epidermis, dorsal setal sacs (arrowheads) and ventral setal sacs (arrows). **(C)** Cell fate of the transplanted 4d. The descendants of the transplanted 4d cell differentiate into segmented mesoderm of the secondary axis. Arrowhead and arrow indicate secondary head and tail, respectively. **(D)** Cross-section of the anterior region of a secondary axis. The transplanted 4d was labeled with Rhodamine dextran and endodermal macromeres of the host embryo were labeled with Oregon Green dextran. The reconstituted embryo was incubated for 7 days before fixation. The descendants of the transplanted 4d cell differentiate into the mesodermal layer (me) underlying the ectoderm (ec; labeled with DAPI). Note that the descendants of host macromeres contribute to the endoderm (en) of the secondary axis. g, ganglion. **(E,F)** Cell fate of the host endodermal macromeres. Host macromeres were labeled with Oregon Green dextran and the reconstituted embryo was incubated for 14 days before fixation. **(E)** The descendants of host macromeres contribute to the gut tissue of the secondary axis. Arrowhead and arrow indicate secondary head and tail, respectively. Double arrowheads indicate the head of the primary axis (i.e. of the host embryo). **(F)** Higher magnification view of the secondary gut (arrow). Scale bars: 100  $\mu$ m.

stage when ectodermal cells are terminally differentiated. For this purpose, 2d<sup>11</sup> (precursor of neuroectoderm) was labeled with Rhodamine dextran and 4d of the same embryo was ablated shortly after its birth. The embryos were incubated for 7 days before fixation. We found that elongation of the A/P axis was significantly

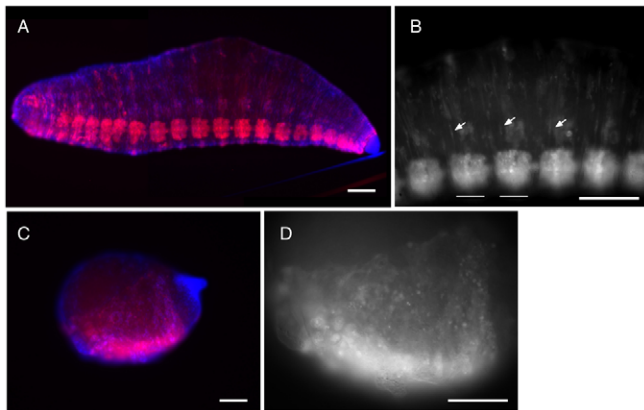
reduced and 2d<sup>11</sup> failed to develop differentiated ganglia and peripheral neurons, which are typically detectable using lineage tracers at this stage of development (Fig. 4) (Arai et al., 2001; Goto et al., 1999a). This result indicates that 4d or the mesodermal germ band provides a signal(s) to the overlying ectoderm to differentiate into the proper neural tissues.

## DISCUSSION

We have conducted a series of ablation and transplantation experiments of the D quadrant in the oligochaete *Tubifex tubifex*. We showed that the D quadrant micromeres (2d<sup>11</sup> and 4d) are necessary and sufficient for embryonic axis formation. Cell lineage analyses revealed that the neuroectoderm and mesoderm along the secondary axis were derived from the transplanted micromeres and that host macromeres contributed to the endodermal tissue (gut) of the secondary axis. These results provide the first direct evidence that the D quadrant in an annelid has the ability to organize the formation of the embryonic axis.

### Secondary axis formation by D quadrant transplantation

Based on the results of transplantation and cell labeling experiments, the process of secondary axis organization is envisaged as follows. Transplanted 2d<sup>11</sup> and 4d undergo a series of asymmetric cell divisions to produce five ‘bilateral’ pairs of teloblasts on the ventral side of the reconstituted embryo. These teloblasts generate ectodermal and mesodermal germ bands in a normal fashion, which subsequently elongate and undergo morphogenetic movements to envelop endodermal cells derived from host macromeres. Importantly, these processes should proceed independently of the host teloblasts and germ bands, both of which are located on the dorsal side of the reconstituted embryo. In the middle region of the reconstituted embryo, however, germ bands that originated from the transplanted cells coalesce with host germ bands (see Fig. 3B). This is likely to be because the host germ bands curve around towards the ventral midline (see Fig. 1G),



**Fig. 4. 4d is required for proper neural development and elongation of the anteroposterior axis.** (A–D) To examine whether 4d has a role in the differentiation of neural tissues it was ablated from embryos that possessed a 2d<sup>11</sup> cell that had been labeled with Rhodamine dextran. In the intact embryo (A), descendants of the 2d<sup>11</sup> cell differentiate into neural tissues such as ganglia (horizontal lines in B) and peripheral neurons (arrows in B). By contrast, when 4d is ablated, elongation of the A/P axis is significantly reduced (C) and descendants of 2d<sup>11</sup> do not show any sign of segmental organization or differentiated neural tissues (D). Scale bars: 100  $\mu$ m.

whereas donor germ bands curve around towards the dorsal midline. As a result, host and donor germ bands coalesce together in the middle region. In the anterior and posterior regions, however, donor and host germ bands are able to envelop the endodermal cells by themselves owing to the smaller size of the underlying endodermal cell mass in these regions.

These processes are reminiscent of egg fusion or embryonic parabiosis. When eggs or embryos are fused/joined, the reconstituted embryos/parabionts develop to form conjoined twins with duplicated heads and tails (Micciarelli and Colombo, 1972; Tompkins, 1977; Yamaha and Yamazaki, 1993). However, axial pattern formation and cell differentiation proceed independently in each embryo/parabiont (Micciarelli and Colombo, 1972; Tompkins, 1977; Yamaha and Yamazaki, 1993). In the *Tubifex* D quadrant transplantation, donor micromeres recruit the endoderm from the host embryo to induce secondary gut tube formation (Fig. 3E,F). Therefore, D quadrant transplantation is different from egg fusion or parabiosis in that inductive cell-cell interaction occurs between the host embryo and donor cells.

Interestingly, the orientation of the transplantation affected the phenotype of the reconstituted embryo. When the A/P polarity of the transplant was reversed relative to that of the host embryo, 17% of the reconstituted embryos exhibited the X-shape phenotype (Table 1). However, when the transplanted micromeres maintained the A/P polarity of the host, none of them developed an X-shape phenotype (Table 1). At present, it is unclear whether the transplanted D quadrant receives signal(s) from the host or whether it has already established polarity and develops autonomously. In the former case, the signal might come from the host endoderm. A gradient of positional information throughout the host embryo could affect the development of the transplanted micromeres. Consistent with the existence of polarizing signaling or graded positional information in *Tubifex* embryos, our previous cell transplantation study showed that the dorsoventral (D/V) polarity of the NOPQ cell (the critical daughter cell of 2d) is determined by an interaction with neighboring cells (Nakamoto et al., 2004). However, transplanted D quadrant micromeres might have already established A/P and D/V polarity and undergo morphogenesis autonomously. The differences in gastrulation movements between the host and donor might affect secondary head and/or tail formation. Our attempts at cell isolation experiments undertaken to determine whether 2d and 4d are polarized at their birth have been unsuccessful, as the isolated 2d and 4d cells underwent aberrant cell divisions or ceased dividing (our unpublished observations).

We suggest that the second (2d) and fourth (4d) micromeres of the D quadrant in the *Tubifex* embryo not only serve as exclusive sources of segmental ectoderm and mesoderm, respectively, but also organize the formation of A/P and D/V axes through the ability of their immediate descendants (i.e. teloblasts) to produce and elongate germ bands, which then induce the underlying endoderm to form a proper gut.

### Comparison with other spiralian

Our results are surprising given the highly conserved cell lineages of spiralian embryos. It is known that 3D and/or 4d function as axial organizers in mollusks such as *Ilyanassa* (Clement, 1962; Lambert and Nagy, 2001; Rabinowitz et al., 2008), *Crepidula* (Henry and Perry, 2008; Henry et al., 2006), *Lymnaea* (Martindale, 1986), *Tectura* (Lambert and Nagy, 2003) and *Patella* (Lartillot et al., 2002; van den Biggelaar, 1977). Similarly, it has been suggested that 4d or the D quadrant macromere has organizer activity in the annelids *Hydroides*, *Arctonoe* and *Serpula* (Lambert

and Nagy, 2003; Gonzales et al., 2007). In *Tubifex*, we have shown that 2d<sup>11</sup> and 4d, but not the D quadrant macromere, function as the axial organizer. This suggests that the evolution of the spiralian organizer is more dynamic than previously thought (Lambert and Nagy, 2003; Lambert, 2008).

At present, the cell fate and function of 1d and 3d have not been demonstrated in *Tubifex*. Penner's classic cell lineage analysis suggested that 1d and 3d contribute to epithelial ectoderm (Fig. 1I) (Penners, 1922); however, this has not been confirmed with modern lineage tracers. In addition, cell ablation of 1d or 3d has not been performed in *Tubifex* embryos. In future studies, it will be interesting to compare the cell fates and organizer activity of D quadrant micromeres with those of other annelids, such as the leech *Helobdella* and the polychaete *Capitella teleta*.

Our cell lineage analysis has shown that transplanted *Tubifex* D quadrant micromeres have the ability to induce secondary gut formation. Inductive interactions between the D quadrant and the endoderm have been described previously in the embryos of *Helobdella*. In the leech, the D lineage macromere (D', which corresponds to the precursor of 2d<sup>11</sup> and 4d in *Tubifex*) induces the cell-cell fusion of endodermal macromeres (Isaksen et al., 1999). In addition, ablation of segmental mesoderm (which is derived from the D quadrant) disrupts endodermal segmentation and gut tube morphogenesis, suggesting that mesoderm provides a short-range signal(s) to the endodermal cell layer (Wedeen and Shankland, 1997). Therefore, it is likely that the regulation of endodermal development by the D quadrant is conserved in clitellates.

### Comparison with other metazoans

The present study shows that precursors of neuroectoderm and mesoderm (2d and 4d) of the *Tubifex* embryo have the ability to establish body axes and to induce endodermal differentiation. These properties fit the definition of an axial organizer: it can establish embryonic axes as well as induce proper cell fates (see Gonzales et al., 2007; Kraus et al., 2007). Although axial organizers have been described in both chordate and non-chordate embryos (Gonzales et al., 2007; Kraus et al., 2007), some non-chordate axial organizers differ in their inductive properties from the chordate axial organizer (historically referred to as the primary embryonic organizer). For example, micromeres of the sea urchin embryo induce a secondary gut and oral/aboral axis instead of neural tissues and the A/P and D/V axes (Ransick and Davidson, 1993). We note that the chordate primary embryonic organizer consists of mesoderm and endoderm precursors [i.e. mesendoderm (see Lambert, 2008; Rodaway and Patient, 2001)] and is characterized by an ability to instruct ectodermal cells to differentiate into neural tissues (De Robertis et al., 2000; Harland and Gerhart, 1997; Spemann and Mangold, 1924; Tung et al., 1962). By contrast, in *Tubifex*, precursors of mesoderm (4d) and neuroectoderm (2d) function as the axial organizer and we did not observe induction of the host cells toward a neuroectoderm fate. Cell transplantation data or the precise lineage of the cell or cells that constitute the signaling center or the responding tissues are not available in mollusks (Clement, 1962; Damen and Dictus, 1996; Henry et al., 2006; Martindale, 1986; Rabinowitz et al., 2008; van den Biggelaar, 1977) and arthropods (Holm, 1952), respectively, so the degree to which these axial organizers share functional properties is not known. Future studies to identify the functional properties of axial organizers will provide important clues to understanding the evolution of axial patterning in metazoan embryos.

### Acknowledgements

We thank David A. Weisblat for invaluable discussions during the initial course of this study and for adult specimens of *T. tubifex*; and Maey Gharbiah and Julia Bowsher for their helpful comments on the manuscript. A.N. was supported by Uehara Memorial Foundation. This work was partially supported by a grant (0820564) from N.S.F. to L.M.N.

### Competing interests statement

The authors declare no competing financial interests.

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