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Primordial germ cells in an oligochaete annelid are specified according to the birth rank order in the mesodermal teloblast lineage

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ABSTRACT

The primordial germ cells (PGCs) in the oligochaete annelid *Tubifex tubifex* are descendants of the mesodermal (M) teloblast and are located in the two midbody segments X and XI in which they serve as germline precursors forming the testicular gonad and the ovarian gonad, respectively. During embryogenesis, *vasa*-expressing cells (termed presumptive PGCs or pre-PGCs) emerge in a variable set of midbody segments including the genital segments (X and XI); at the end of embryogenesis, pre-PGCs are confined to the genital segments, where they become PGCs in the juvenile. Here, using live imaging of pre-PGCs, we have demonstrated that during *Tubifex* embryogenesis, pre-PGCs (defined by Vasa expression) stay in segments where they have emerged, suggesting that it is unlikely that pre-PGCs move intersegmentally during embryogenesis. Thus, it is apparent that pre-PGCs derived from the 10th and 11th M teloblast-derived primary mesoblast cells (designated m10 and m11) that give rise, respectively, to segments X and XI are specified in situ as PGCs and that those born in other segments become undetectable at the end of embryogenesis. To address the mechanisms for this segment-specific development of PGCs, we have performed a set of cell-transplantation experiments as well as cell-ablation experiments. When m10 and m11 that are normally located in the mid region of the embryo were placed in positions near the anterior end of the host embryo, these cells formed two consecutive segments, which exhibited Vasa-positive PGC-like cells at early juvenile stage. This suggests that in terms of PGC generation, the fates of m10 and m11 remain unchanged even if they are placed in ectopic positions along the anteroposterior axis. Nor was the fate of m10 and m11 changed even if mesodermal blast cell chains preceding or succeeding m10 and m11 were absent. In a previous study, it was shown that PGC development in segments X and XI occurs normally in the absence of the overlying ectoderm. All this strongly suggests that irrespective of their surrounding cellular environments, m10 and m11 autonomously generate PGCs. We propose that m10 and m11 are exclusively specified as precursors of PGCs at the time of their birth from the M teloblast and that the M teloblast possesses a developmental program through which the sequence of mesodermal blast cell identities is determined.

Keywords: Primordial germ cells, Oligochaete annelid, *Tubifex tubifex*, Mesodermal teloblast, Vasa, Birth rank order

Introduction

Primordial germ cells (PGCs), the precursor stem cells to the germline, are usually established early during embryonic development. The specification of PGCs that precedes the generation of germ cells is a crucial event for all sexually reproducing animals. The modes of PGC specification have been divided into two categories, preformation and epigenesis (Extavour and Akam, 2003). In the preformation mode, the localization of maternally inherited determinants is involved in PGC specification as seen in the fruit fly *Drosophila melanogaster* (Illmensee and Mahowald, 1974; Williamson and Lehmann, 1996), the nematode *Caenorhabditis elegans* (Deppe et al., 1978; Kimble and White, 1981; Strome and Wood, 1982), zebrafish *Danio rerio* (Yoon et al., 1997) and the frog *Xenopus laevis* (Tanabe and Kotani, 1974; Züst and Dixon, 1975; Ikenishi et al., 1986). In contrast, the epigenetic mode of PGC specification depends on inductive signals from surrounding tissues as seen in mice (Ying et al., 2001; Tsang et al., 2001; Saitou et al., 2002) and urodele amphibians (Nieuwkoop, 1947; Sutasurya and Nieuwkoop, 1974). Recently, however, it has been reported that small micromeres of 32-cell embryo of sea urchins, which normally contribute to the germline, are specified autonomously as precursors of PGCs even though these cells do not inherit obvious preformed germ line components (Juliano et al., 2006; Yajima and Wessel, 2011, 2012); it is apparent that some non-epigenetic mechanism for PGC specification must operate in sea urchins. This may suggest that mechanisms for PGC specification operating in metazoans are more variable than has been thought.

In this paper, we address the mechanism for PGC specification in an oligochaete annelid *Tubifex tubifex*. This animal is a hermaphrodite with a pair of testes in segment X and a pair of ovaries in segment XI (Dixon, 1915). Germ cells in these genital segments have been thought to originate from PGCs that are located therein around the time of completion of embryogenesis (Goto et al., 1999a; also see Shimizu, 1982 for review). A previous study has shown that during *Tubifex* embryogenesis, presumptive PGCs (pre-PGCs defined by *vasa* expression) emerge in a variable set of midbody segments including the genital segments (X and XI) and that nearly all of the *vasa*-expressing cells (i.e., pre-PGCs) but those in segments X and XI become undetectable by the end of embryogenesis (Oyama and Shimizu, 2007). In newborn juveniles, thus, *vasa*-expressing cells (now designated PGCs) are confined to segments X and XI.

As to the embryonic origin of *Tubifex* PGCs in juveniles, it is possible that pre-PGCs born in segments X and XI could be specified in situ as PGCs. Alternatively, it is equally possible that pre-PGCs that have migrated to these two segments from

elsewhere could become PGCs therein, since migration of PGCs in embryos and larva has often been observed in a variety of animals including a polychaete annelid (Shinomiya et al., 2000; Kobayashi et al., 2005; Rebscher et al., 2007; Yajima and Wessel, 2012). To differentiate these possibilities, it is prerequisite to have information about the behavior of pre-PGCs in living embryos, because the number and the position (along the longitudinal body axis or AP axis) of pre-PGCs are highly variable among embryos and because our previous study (Oyama and Shimizu, 2007) that was based on the observations on fixed specimens failed to provide conclusive information on this issue.

The present study was undertaken to gain an insight into the mechanism for segment-specific PGC development in *Tubifex tubifex*. The objectives of this study were (i) to determine the origin of pre-PGCs that are located in segments X and XI around the time of completion of embryogenesis and (ii) to examine whether the segment-specific PGC development depends on external cues. For this purpose, we developed a method that allows us to visualize pre-PGCs in living embryos. Furthermore, we utilized embryological techniques such as cell ablation and cell transplantation in combination with immunostaining of a germline marker protein Vasa. The results reported herein show that pre-PGCs born in segments X and XI are specified in situ as PGCs whereas those born in other segments become undetectable at the end of embryogenesis and that PGC specification in segments X and XI occurs independently of the interactions with surrounding tissues and the positional cues residing in the embryo. On the basis of these findings, we suggest that PGCs in *Tubifex tubifex* are specified according to their genealogical position in the mesodermal teloblast lineage.

Materials and methods

Embryos

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

Whole-mount immunocytochemistry

Immunocytochemical whole-mounts of fixed embryos and juveniles were prepared according to Shimizu (1993) and Shimizu and Savage (2002), with some modifications. In this study, not only juveniles but also embryos (at stages 16-18) were treated sequentially with mercaptoethanol and collagenase, prior to immunostaining (Oyama and Shimizu, 2007). A rabbit polyclonal anti-Vasa antibody raised against *Bombyx mori* (silkworm) vasa-like protein (Nakao et al., 2006) was used as a primary antibody (1:1000 diluted in PBS plus 3% BSA); a goat anti-rabbit IgG antibody conjugated to alkaline phosphatase (Sigma) was used as a secondary antibody (1:2000 diluted in PBS plus 3% BSA). Color development of alkaline phosphatase activity was carried out as described previously (Matsuo et al., 2005); unless otherwise stated, the reaction of color development was done for five minutes.

Microinjection of fluorescent lineage tracers

The fluorescent lineage tracer used in this study was Alexa Fluor 488 dextran (AF488D; Molecular Probes), which was dissolved at 50 mg/ml in injection buffer (0.2 M KCl, 5 mM HEPES pH 7.2, 0.5% Fast Green). Pressure injection was performed as described previously (Kitamura and Shimizu, 2000; Nakamoto et al., 2011). Injected embryos were cultured in the medium containing antibiotics (see below) in the darkness. Some of the injected embryos were photographed at 24-hour intervals on a Leica M165FC fluorescence stereoscope; immediately before photography, they were put into the culture medium chilled to 4°C for a few minutes, by which embryos were anesthetized. (In a preliminary experiment, we did not find any evidence for deteriorative effects on development of embryos subjected repeatedly to low temperature.) The remaining tracer-injected embryos, which had been allowed to develop through to stage 16 (see Fig. 1), were photographed on a fluorescence stereoscope and fixed with 3.5% formaldehyde in PBS. Such fixed specimens were then processed for immunostaining with Vasa antibody as described above and were finally subjected to color development for alkaline phosphatase conjugated to the secondary antibody. Stained specimens were photographed again in the bright field with incident light. Specimens were processed individually to avoid confusion of fluorescence images and bright field images.

Segment boundary experiments

M teloblasts on the right side of embryos at 30 hours after 4d cell division were injected with a lipophilic tracer DiI (5 mg/ml in safflower oil) according to Nakamoto et al. (2000). The injected embryos were cultured for three days in the

darkness and fixed with 3.5% formaldehyde in PBS for one hour. After washed in PBS, fixed embryos were treated with DAPI (1 $\mu\text{g/ml}$ in PBS) for 15 minutes. Whole-mount preparations of the experimental embryos were observed on a Leica M165FC fluorescence stereoscope.

Blastomere ablation and transplantation

Ablation and transplantation of blastomeres were carried out according to the methods described previously (Kitamura and Shimizu, 2000; Nakamoto et al., 2004; Nakamoto et al., 2011). The operated embryos were allowed to develop in the culture medium containing antibiotics (penicillin G and streptomycin, 20 units/ml each), which was renewed daily. After 7-day culture, they were fixed with 3.5% formaldehyde and processed for immunocytochemistry with Vasa antibody as described above, with some modifications. The primary antibody was diluted to 1:750 in PBS plus 3% BSA; color development of alkaline phosphatase conjugated to the secondary antibody was done for 10 minutes. These modifications resulted in the production of slight background staining, which made the contour of ventral ganglia recognizable in whole-mount preparations.

Immunoblotting

Western blotting was performed as described previously (Shimizu, 1996). Briefly, ooplasm extracted from 50 early embryos (one-cell to 24-cell stages) was mixed with the same volume of double-strength SDS sample buffer and boiled for 5 minutes. Proteins were separated on a 12.5% acrylamide gel and transferred electrophoretically onto nitrocellulose membranes. After blocking with 3% bovine serum albumin (BSA) and 3% dry skimmed milk (DSM), nitrocellulose blots were incubated with the rabbit polyclonal anti-Vasa antibody mentioned above (1:1500 diluted in PBS plus 3% BSA and 3% DSM) for 24 hours at 4°C. A goat anti-rabbit IgG antibody conjugated to HRP (Sigma) was used as a secondary antibody (1:2000 diluted in PBS plus 3% BSA and 3% DSM) with which the blots were incubated for 6 hours. Color development of the activity of HRP was carried out according to Shimizu (1993).

Results and discussion

Summary of Tubifex development

A brief review of *Tubifex* development is presented here as a background for

the observations described below (for details, see Goto et al., 1999a, b; Shimizu, 1982). Precursors of teloblasts, which serve as embryonic stem cells, are traced back to the second (2d) and fourth (4d) micromeres of the D quadrant. At the 24-cell stage, 2d¹¹, 4d and 4D (sister cell of 4d) all come to lie in the future midline of the embryo (Fig. 1A). 4d divides equally to yield the left and right mesoteloblasts (Ml and Mr); 2d¹¹¹ (derived from 2d¹¹) divides into a bilateral pair of ectoteloblast precursors, NOPQl and NOPQr; and 4D divides equally yielding endodermal precursors E^D (Fig. 1B). Ectoteloblasts N, O, P and Q arise from an invariable sequence of divisions of cell NOPQ on both sides of the embryo (Fig. 1C; for details see Nakamoto et al., 2004). After their birth, each of the teloblasts thus produced serves as an embryonic stem cell and divides repeatedly to give rise to small cells called primary blast cells, which are arranged into a coherent column (i.e., a bandlet). Within each bandlet, primary blast cells and their descendants are arranged in the order of their birth (Fig. 1D). Bandlets from N, O, P and Q teloblasts on each side of the embryo join together to form an ectodermal germ band (GB), while the bandlet from the M teloblast becomes a mesodermal GB that underlies the ectodermal GB (Fig. 1C). The GBs are initially located on the dorsal side of the embryo (stage 14 in Fig. 1E). Along with their elongation, they gradually curve round toward the ventral midline and finally coalesce with each other along the ventral midline (stage 15 in Fig. 1E). The coalescence is soon followed by dorsalward expansion of the GBs (stage 16 in Fig. 1E). The edges of the expanding GBs on both sides of the embryo finally meet along the dorsal midline to enclose the yolky endodermal tube (stages 16-18 in Fig. 1E). Concurrently with this enclosure, the embryo becomes elongated in an anterior-to-posterior progression, and curved with the ventral convexity (stages 16-18 in Fig. 1E). Enclosed portions of the embryo begin to exhibit peristaltic movements. Embryogenesis is judged to be complete when the expanding GBs have enclosed the posterior end of the embryo, which then exhibits movement throughout its length (stage 18 in Fig. 1E).

A cross-reacting Vasa antibody stains pre-PGCs and PGCs in Tubifex embryos and juveniles

In this study, we stained *Tubifex* embryos and juveniles with anti-BmVLG antibody, which was raised to *Bombyx mori* VLG (*vasa*-like gene) protein (Nakao *et al.*, 2006). According to these authors, the amino acid sequence of the polypeptide used to raise this antibody included eight conserved DEAD-box protein motifs as well as the EARKF motif; we find that this sequence shows 52% identity to the corresponding

domain of the predicted *Tubifex* Vasa protein (data not shown). In a preliminary experiment, we found that anti-BmVLG antibody specifically stains ventrally located large cells exclusively in segments X and XI of *Tubifex* juveniles (Fig. S1B). The distribution pattern of these positive cells is very reminiscent of that of such PGCs as identified as *vasa*-expressing or Vasa-expressing cells in a previous study (Oyama and Shimizu, 2007). In immunoblotting of *Tubifex* embryos, a major band is seen at the molecular mass of approx. 65 kDa, and a minor band at 43 kDa (Fig. S1A). Given that the antibody specifically recognizes the *BmVLG* product of molecular mass ~ 66 kDa (Nakao et al., 2006), we suggest that 65 kDa polypeptides recognized by this antibody include antigen related to the *Tubifex* Vasa protein. In the following experiments, we used this antibody as a probe to detect pre-PGCs as well as PGCs; hereafter, the anti-BmVLG antibody will be referred to as Vasa antibody.

Occurrence of Vasa protein-expressing cells during Tubifex embryogenesis

As Fig. 2A-L shows, the distribution pattern of ventral Vasa protein-expressing cells (VPE cells) is highly variable among embryos (at stages 16 and 17). What is shared among embryos is that the genital segments X and XI both exhibit VPE cells without any exception. The number of and the distribution pattern of VPE cells in nongenital segments along the anteroposterior (AP) axis vary considerably, although the frequency of VPE cells in segments IX and XII is much higher than that in other nongenital segments (Fig. 2P). This may suggest that VPE cells emerge stochastically in nongenital segments.

Fig. 2M-O shows one-day-old juveniles (at stage Juv-D1) stained with Vasa antibody. In nearly all of the juveniles examined, VPE cells were found to be confined to the genital segments (Fig. 2Q). If one considers the fact that 14% of the embryos (at stages 16 and 17) examined in this study exhibited VPE cells exclusively in segments X and XI as early as stage 16 (see Fig. 2A), it is apparent that more than 80% of *Tubifex* embryos initially exhibit VPE cells not only in the genital segments but also in a few nongenital segments and that around the time of completion of embryogenesis, they undergo a certain process that leads to the “disappearance” of VPE cells from the nongenital segments.

In summary, we suggest that VPE cells emerge in a variable set of midbody segments including the genital segments (X and XI) and that at the end of embryogenesis, VPE cells are restricted to the genital segments. This distribution pattern of VPE cells is evidently comparable to that of *vasa*-expressing cells

demonstrated in our previous study (Oyama and Shimizu, 2007), where *vasa*-expressing cells seen in embryos (at stages 16-18) were referred to as presumptive PGCs (pre-PGCs), and those in juveniles as PGCs. Hereafter, VPE cells seen in embryos and juveniles will be referred to as pre-PGCs and PGCs, respectively. (It is natural to think that VPE cells are expressing *vasa* at the same time, though this point remains to be explored.)

Live imaging of pre-PGCs

During the course of previous cell lineage studies on *Tubifex* embryos (Goto et al., 1999a, b; Nakamoto et al., 2000; Shimizu and Nakamoto, 2001; Nakamoto et al., 2006), we often noticed that *Tubifex* embryos in which 4d cells or M teloblasts had been injected with cell lineage tracers (such as horseradish peroxidase, fluorescent dextran and DiI) exhibited ventrally located, tracer-labeled large cells that were distinguished from the surrounding cells and that such ventral large cells as labeled with lineage tracers showed considerably variable distribution patterns along the length of the mesodermal germ band (GB) among the embryos examined at stages 15 and 16 (unpublished observations). In a subsequent study, we found that the distribution pattern of ventral large cells labeled with lineage tracers is very reminiscent of that of ventral *vasa*-expressing cells (i.e., pre-PGCs) in embryos at stages 15 and 16 (Oyama and Shimizu, 2007). These findings prompted us to test the possibility that pre-PGCs could be visualized in living *Tubifex* embryos that are injected with fluorescent lineage tracers. In a preliminary experiment using AF488D as a lineage tracer, we confirmed that fluorescent ventral large cells derived from M teloblasts are actually visible in living embryos (at stage 16) on a fluorescence stereoscope (see Fig. 3). Furthermore, fluorescently labeled cells were viable even after they were subjected to repeated observations on a fluorescence stereoscope during a period of 7 days.

To determine whether these fluorescently-labeled cells are actually pre-PGCs, we asked if they are expressing Vasa protein. We subjected AF488D-labeled embryos ($n = 10$) to immunocytochemical staining with Vasa antibody, and found that all the large ventral cells (labeled with AF488D) present in these embryos were Vasa-positive without any exception (Fig. 3). Conversely, these embryos exhibited no Vasa-positive cells other than the large, ventral, fluorescently-labeled cells. On the basis of these results, it is safe to say that fluorescent large cells detectable in AF488D-labeled mesodermal GBs are pre-PGCs that specifically express Vasa protein.

In AF448D-labeled embryos, segments VII and VIII often appear as bright regions due to the presence of fluorescent nephridia, which are normally derived from the 7th and 8th primary mesodermal (m) blast cells (viz., m7 and m8; Kitamura and Shimizu, 2000). In this study we used these fluorescent segments as references for the distribution of ventral fluorescent large cells along the anteroposterior (AP) axis.

Behavior of pre-PGCs during embryogenesis

It was at the beginning of stage 16 (four days after AF488D injection) that pre-PGCs become recognizable in the fluorescent mesodermal GB (data not shown). As development proceeded, pre-PGCs that appeared as fluorescent large cells became more conspicuous (Fig. 4, Day 5). This is probably because unlike non-PGC mesodermal tissues in which lineage tracer becomes diluted, pre-PGCs that undergo little to no division retain high level concentration. A previous study has shown that during the period of stages 15-17, mesodermal cells other than pre-PGCs undergo cell division several times and rearrange themselves into thin epithelial layers that wrap a resulting coelomic cavity (Goto et al., 1999b). At Day 5 (five days after AF488D injection), the distribution pattern of pre-PGCs was found to be different between embryos ($n=21$) except that segments X and XI exhibited fluorescent pre-PGCs in every labeled embryo (Fig. 4 and S2, Day 5). During the following three days, the distribution pattern seen at Day 5 appeared not to change in any of the embryos examined even when they developed to one-day-old juveniles (Fig. 4, Day 6 and Day 8; Fig. S2). This suggests that it is unlikely that pre-PGCs in *Tubifex* embryos move intersegmentally, i.e., from a segment into the adjacent segment. In some cases where multiple pre-PGCs were present in a hemisegment, they appeared to change their positions relative to each other within a hemisegment (Fig. 4A- C, Day 5 and Day 6). In juveniles, fluorescent dots were seen in nongenital segments as well as genital segments though those in nongenital segments were often smaller than fluorescent cells seen in embryos. Unlike those in genital segments which were Vasa-positive, fluorescent dots in nongenital segments did not appear to be Vasa-positive (Fig. 4, Day 8/Vasa).

In summary, we suggest that in *Tubifex* embryos, pre-PGCs that are to be specified as PGCs are placed in segments X and XI as late as the beginning of stage 16 and that they do not move intersegmentally during the period of stages 16-18. Furthermore, during this period, pre-PGCs appear not to divide.

Embryonic origin of pre-PGCs in segments X and XI

As to the origin of PGCs in segments X and XI, there is a possibility that pre-PGCs that are present in segments X and XI at the beginning of stage 16 have moved from adjacent segments prior to stage 16. Although this possibility cannot be eliminated, it seems to be unlikely. First, a previous double-labeling experiment (in which M teloblasts were injected sequentially with Texas Red dextran and fluorescein dextran at 2.5- or 5-hour intervals around 15 hours after the birth of M teloblasts) showed that mesodermal cells (i.e., descendant cells of m blast cells) appear not to move into the adjacent segments (which probably corresponded to segments V and VI; Goto et al. 1999b). In the present study, we have performed an additional, similar boundary experiment (using single tracer injection) for the boundary between segments X and XI. For this purpose, 30-hour-old M teloblasts on the right side of embryos were injected with DiI, and the resulting injected embryos were cultured for three days before fixation. A preliminary experiment showed that M teloblasts undergo cell divisions 10 times by 30 hours after their birth, suggesting that the first primary m blast cell to be labeled with DiI could be the 11th one (i.e., m11) and that the 10th primary m blast cell (i.e., m10) would not be labeled with DiI. As expected, in the embryos ($n = 3$) examined, segment XI and its succeeding segments were found to be labeled with DiI but the anterior region covering segments I to X exhibited no DiI fluorescence (Fig. S3). In any of the embryos examined, we did not find any evidence that DiI-labeled cells had migrated into segment X during stages 13-16 (Fig. S3). These observations suggest that intersegmental migration of blast cells, if any, occurs very rarely even prior to stage 16. Second, as described in the following cell-transplantation and cell-ablation experiments, PGCs are generated in segment X even if the first nine primary m blast cells are all ablated prior to the birth of m10 or none of the primary m blast cells succeeding m10 are produced. Similarly, PGCs develop in segment XI even if the primary m blast cells succeeding m11 are all removed. This suggests that PGCs in segment X (and XI) are formed without any supply of pre-PGCs from the adjacent segments. Taken together we suggest that segments X and XI are comprised of descendant cells of the 10th and 11th primary m blast cells (i.e., m10 and m11), respectively, and that pre-PGCs that are to be specified as PGCs in segments X and XI are descendants of m10 and m11, respectively. We believe that most pre-PGCs that are to be specified as PGCs, if not all, are born in segments X and XI.

Fate of pre-PGCs placed in ectopic positions

The aforementioned observation suggests that VPE cells existing in genital segments (X and XI) of young juveniles are born in segments X and XI (around stage 15), stay in their “birthplace” during embryogenesis, and are finally specified as PGCs at the end of embryogenesis. We were interested in learning how pre-PGCs that are situated in segments X and XI at the beginning of stage 16 are exclusively specified as PGCs. First, we tested the possibility that positional information along the AP axis plays a role in this PGC specification, since in intact embryos PGCs are specified solely in segments X and XI but not in others.

To address this issue, we examined whether pre-PGCs derived from m10 and m11 are specified as PGCs when placed in ectopic positions. When M teloblasts isolated from older embryos are transplanted into younger embryos, primary m blast cells (such as m10 and m11) that are derived from the transplanted M teloblasts are expected to be placed in more anterior positions than in intact embryos. To achieve such a situation in this study, M teloblasts isolated at 20 or 28 hours after 4d cell division (see Fig. 1E, stage 9) were transplanted into host embryos (at stage 8) from which 4d cells had been removed (Fig. 5). A preliminary observation on embryos injected with lineage tracers showed that M teloblasts at 20 and 28 hours have undergone cell divisions six and nine times, respectively. It was expected that if transplanted M teloblasts resume cell division in host embryos, the first cells produced by 20-hour and 28-hour M teloblasts in reconstituted embryos would correspond, respectively, to the 7th and 10th primary m blast cells in intact embryos. Reconstituted embryos were cultured for seven days before fixation, and processed for immunohistochemistry with Vasa antibody. Control embryos (i.e., those developing synchronously with donor embryos) were allowed to develop without vitelline membrane, and fixed at the same time as the reconstituted embryos. At the time of fixation, control embryos reached the juvenile stage Juv-D1 (Fig. 1E). As Fig. 6C shows, VPE cells developed in a normal fashion in control embryos.

General features of reconstituted embryos. At the end of culture, reconstituted embryos were found to have developed to fully elongated juveniles or to remain as round masses of cells. (Such experimental embryos as failed to elongate were not considered further in this study.) The endoderm of the fully elongated juveniles was seen to be segmented, suggesting that the transplanted M teloblasts continued to divide and formed m blast cell rows (and hence mesodermal GBs) in a normal fashion. It should be noted, however, that the anterior portion of these juveniles is wider than the posterior portion (compare Fig. 6A, B with Fig. 6C). When

experimental embryos (juveniles) were processed for immunostaining, Vasa-positive cells were often seen to be larger than those in intact control embryos and to be located near the posterior margin of a segment. This is probably because VPE cells located in the wide region are sandwiched tightly between the epithelial layer and the unusually thick endoderm.

Transplantation of 20-hour M teloblasts. We obtained 13 reconstituted embryos where transplantation of 20-hour-old M teloblasts initially appeared to be successful. At the end of culture, five out of the 13 reconstituted embryos were found to have developed to fully elongated juveniles. Three out of the five elongated juveniles exhibited two pairs of VPE cells in the region near the anterior end (Fig. 6A and S4A, B). Judging from the organization of ganglia visualized in stained specimens, three segments (segments 1-3 in Fig. 6A) were recognized in front of the anterior-most segment bearing VPE cells. Given that 20-hour M teloblasts that were to be isolated had undergone cell divisions six times in donor embryos, it is conceivable that these M teloblasts resumed cell division upon transplantation to form m-blast cell rows in a normal fashion. If this is the case, it is highly possible that VPE cells seen in reconstituted embryos were derived from primary m blast cells equivalent to normal m10 and m11.

The remaining two elongated juveniles exhibited VPE cells which were not distributed in a bilaterally symmetric pattern (Fig. S4C, D). Because of such disorganization in VPE cell distribution, we cannot determine the origin of these cells unambiguously. It should be noted, however, that in both of these experimental juveniles, VPE cells were detected in the anterior-most segment.

Transplantation of 28-hour M teloblasts. We obtained 16 reconstituted embryos where transplantation of 28-hour-old M teloblasts initially appeared to be successful. At the end of culture, eight out of the 16 reconstituted embryos were found to have developed to fully elongated juveniles. Three out of the eight elongated juveniles exhibited two pairs of VPE cells (Fig. 6B; Fig. S5A, B). In the juvenile shown in Fig. 6B, it is evident that VPE cells were located more anteriorly than those seen in the aforementioned embryos with transplanted 20-hour M teloblasts. Although the anterior-most VPE cells were located at some distance from the anterior end of the embryo, they were situated in the first two consecutive segments. Given that the first two primary m blast cells that were produced by transplanted 28-hour M teloblasts in host embryos correspond to m10 and m11 in intact embryos, it is evident that VPE cells seen in reconstituted embryos were derived from m blast cells corresponding to normal

m10 and m11. In the remaining two juveniles (Fig. S5A, B), VPE cells were located far from the anterior end. Judging from the organization of ganglia visualized in stained specimens, however, these VPE cells appear to be situated in the region corresponding to the first two segments (Fig. S5A, B). This result suggests that PGCs are formed even if none of the first nine m blast cells (m1-m9) are present.

Three out of the remaining five elongated juveniles exhibited aberrant distribution patterns of VPE cells (Fig. S5C-F). It should be noted that in all of the three experimental juveniles, the anterior-most segment appears as a VPE cell-bearing segment. In contrast, no VPE cells were detected in the remaining two juveniles (not shown).

In summary, we suggest that pre-PGCs derived from m10 and m11 are specified as PGCs irrespective of the positions along the AP axis they take up in the embryo. It should be noted, however, that there is a possibility that Vasa-positive cells seen in the juveniles that developed from the reconstituted embryos are derived from the germ layers (i.e., ectoderm and endoderm) of the host embryo. Although this possibility cannot be eliminated, it seems to be unlikely. During the course of the present study as well as a previous study (Oyama and Shimizu, 2007), we have never found any evidence for the ability of the ectoderm and endoderm to express *vasa* or Vasa in response to inductive signals from the mesoderm (i.e., the M teloblast lineage).

PGC specification in truncated mesodermal germ bands

Next we examined the possibility that segment-specific development of PGCs depends on their surrounding cellular environments. In a previous study, it was shown that ablation of 2d¹¹¹ cell, precursor of ectoderm, did not exert any significant effect on PGC development (Oyama and Shimizu, 2007), suggesting that PGC specification in segments X and XI does not depend on the overlying ectoderm. In this study, we examined another possibility that m blast cells (or mesodermal segments) that precede or succeed the genital segments (X and XI) play a role in specification of PGCs in segments X and XI. In the aforementioned 28-hour M teloblast transplantation experiment, we found that pre-PGCs derived from m10 and m11 are specified as PGCs even in the absence of entire m blast cell row that precedes the genital segments (Fig. 6B). This apparently suggests that PGC specification in segments X and XI does not require the presence of the preceding mesodermal segments ranging from I to IX.

To test the possibility that PGC specification in segments X and XI depends on the presence of mesodermal segments (or m blast cell row) succeeding the genital

segments, M teloblasts were ablated from embryos at 24, 28, 30, 33 and 36 hours after 4d cell division, and operated embryos were cultured for eight days before fixation and processed for immunostaining. In this study 25-30 embryos were operated on in each series of ablation experiments. A preliminary experiment with embryos injected with lineage tracers showed that M teloblasts produce seven, nine, 10, 11 and 12 primary m blast cells, respectively, by 24, 28 30, 33 and 36 hours after 4d cell division (Fig. 7A).

Irrespective of the timing of M teloblast ablation, primary m blast cells that had been produced before teloblast ablation were found to be organized in normal appearing germ bands, which subsequently underwent mesodermal segment formation in a normal fashion. It should be noted, however, that in contrast to the normal appearing anterior portion of an operated embryo, the posterior portion was wide and has blunt end (as indicated by square brackets in Fig. 7B-F); posterior-most regions were referred to as posterior tips (PT). The length of operated embryos appears to be proportional to the number of primary m blast cells that were present at the time of teloblast ablation. VPE cells were detected in embryos from which 30-, 33- or 36-hour M teloblasts were ablated (Fig. 7D-F). These cells were present exclusively in the segment X after 30-hour M teloblast ablation (Fig. 7D and 8B) and in the segments X and XI after 33- and 36-hour M teloblast ablation (Fig. 7E, F and 8C, D). In some of the operated embryos, segments other than X and IX occasionally exhibited Vasa-positive tiny spots (Fig. 7D, E and 8B-D). In embryos from which 28-hour M teloblasts were ablated, however, no trace of Vasa-positive (large) cells was detected (Figs. 7C and 8A). Embryos ($n = 25$) which were deprived of 24-hour M teloblasts exhibited neither Vasa-positive cells nor tiny spots (Fig. 7B; data not shown). These results suggest that PGC specification in segments X and XI does not depend on the presence of the succeeding m blast cell row and that development of PGCs in these segments occurs without any supply of pre-PGCs from the primary m blast cells succeeding m11.

In summary, we suggest that it is unlikely that PGC specification in segments X and XI occurs in response to inductive signals from the m blast cell rows either preceding or succeeding the genital segments. It remains to be explored, however, whether pre-PGCs derived from m10 and m11 are specified as PGCs in the absence of the entire mesoderm but descendants of m10 and m11.

PGC specification according to the birth-rank in the M teloblast lineage

As described earlier, pre-PGCs that are to be specified as PGCs are derived from the 10th and 11th primary m blast cells (i.e., m10 and m11), which serve as founder cells of segments X and XI, respectively. In contrast, pre-PGCs that originate from m blast cells other than m10 and m11 are destined to “disappear” around the time of completion of embryogenesis (Fig. 9). The aim of this study was to examine whether PGC specification in segments X and XI depends on external cues. The results of the present cell transplantation experiments suggest that it is unlikely that PGCs are specified in response to the positional cues residing in the embryo. Nor is it likely that specific regions of ectoderm and endoderm exert inductive signals for PGC specification (Oyama and Shimizu, 2007). The results of the present cell ablation experiments suggest that PGC specification in segments X and XI does not require the presence of m blast cell chains that precede or succeed m10 and m11. Nor is it likely that PGC specification depends on the presence of a contralateral m blast cell chain (or mesodermal GB), since in embryos where one of the two M teloblasts was ablated, PGC specification occurred normally in the mesodermal GB (unpublished observation).

Taken together, it is unlikely that PGC specification in segments X and XI is achieved through inductive signals from the neighboring germ layers. Rather, it appears that PGC specification in these two segments is brought about through mechanisms intrinsic to m10 and m11 (or segments X and XI). That is to say, irrespective of their surrounding cellular environments, m10 and m11 autonomously and exclusively generate VPE cells that are destined to become PGCs. In contrast, other m blast cells seem to lack such properties as lead to PGC generation, though they stochastically produce VPE cells themselves. Thus, we suggest that in terms of generation of PGCs themselves, m10 and m11 are intrinsically different from other m blast cells in the mesodermal GB (Fig. 10). In other words, m10 and m11 are exclusively specified as initial precursors of PGCs.

Then, how are the fates of m10 and m11 specified? It is possible that their fates are specified according to their physical position in the m blast cell chain (i.e., the mesodermal GB). Alternatively, specification of m blast cells may result from the genealogical position in the M teloblast lineage, i.e., the rank order of their birth. In the present cell transplantation experiments, we observed that in the absence of preceding m blast cells, PGCs were detected in the region near the anterior end of the reconstituted embryos; PGC-bearing segments were the fourth and fifth segments in the former case and the first and second segments in the latter case (Fig. 6A, B). Evidently, these results are consistent with the birth-rank hypothesis, but they do not favor the

former regional specification mechanism. In a previous study, specification of primary m blast cells according to their genealogical positions in the M teloblast lineage has already been suggested for the seventh and eighth primary m blast cells (m7 and m8), which are specified to form a nephridium (Kitamura and Shimizu, 2000). Thus, we suggest that the commitment of m7 and m8 to nephridium-generating fates and of m10 and m11 to PGC-generating fates, is brought about through a developmental program intrinsic to the M teloblast. We envisage that the M teloblast of the *Tubifex* embryo counts cell division cycles by a certain mechanism to segregate specific fates to primary m blast cells at specific rounds of cell division. As to the segment-specific development of PGCs, we propose that at the time of their birth from the M teloblast, m10 and m11 are endowed with some factors (indicated by blue shading in Fig. 10) that give these cells a PGC-generating fate. At present, we cannot rule out the possibility that the specification of m10 and m11 as initial precursors of PGCs occurs through inductive signals emanating from their mother cell, M teloblast, during or shortly after their birth.

Whether inheritable factors or inductive signals are involved in primary m blast cell specification, it is evident that some kind of determination must occur in these blast cells at the time of their birth from the M teloblast. So far, in *Tubifex*, there has been no evidence for the presence of obvious preformed germ line components either in m blast cells or M teloblasts, suggesting that it is unlikely that the putative determination of m10 and m11 is brought about by inherited cytoplasmic determinants from maternal stores. As discussed above, however, m10 and m11 are specified autonomously as precursors of PGCs, even though they do not inherit preformed cytoplasmic determinants for PGC specification. This situation is reminiscent of that in sea urchins, where small micromeres of 32-cell embryo are specified autonomously as precursors of PGCs even though there is no evidence for the inheritance of obvious preformed cytoplasmic determinants (Juliano et al., 2006; Yajima and Wessel, 2011, 2012). It appears that some non-epigenetic mechanism for PGC specification must operate in primary m blast cells and/or M teloblasts of *Tubifex* embryos and in small micromeres and/or their precursor micromeres of sea urchin embryos. In this regard, it is intriguing to note that M teloblasts in *Tubifex* embryos appear to be specified autonomously as mesodermal stem cells at the time of their birth (see Goto et al., 1999b; Kitamura and Shimizu, 2000; Nakamoto et al., 2011). It is probably at this time when M teloblasts are endowed with a developmental program that is responsible for temporal control of primary m blast cell specification.

At present it is not known whether the aforementioned cell-intrinsic mechanisms for PGC specification is unique to *Tubifex* or widespread among clitellate annelids. Recently Kang et al. (2002) have shown that in the leech *Helobdella robusta*, 11 paired sets of pre-PGCs (defined by *nanos* expression), which arise in a segmentally iterated manner from the M teloblast lineage, appear in mid-body segments (M8-M18) during embryogenesis and that only a subset of these pre-PGCs (four to six paired sets) participate in gonadogenesis to form testisacs. This suggests that as in *T. tubifex*, 'supernumerary' pre-PGCs that are not to be specified as PGCs are formed in embryos of *H. robusta*. Whether cell-intrinsic or position-dependent cues are responsible for this selection remains to be determined. In this regard, it is interesting to note that in another leech, *Theromyzon rude*, m blast cells derived from the M teloblast give rise to segment-specific structures according to their birth rank rather than to their actual segmental position (Gleizer and Stent, 1993). It remains to be explored whether the fates of pre-PGCs in leech embryos are specified according to the birth rank order in the M teloblast lineage.

Implications for germ cell sex determination

As described earlier, the body segments X and XI of *Tubifex tubifex* are genital segments bearing a pair of testes and ovaries, respectively (Dixon, 1915). It is conceivable that germ cells comprising these gonads are derived from descendants of PGCs residing in the segments X and XI at the time of completion of embryogenesis. More specifically, PGCs in juveniles proliferate as spermatogonia in segment X and oogonia in segment XI to form gonads. In this hermaphrodite animal, gonads become morphologically recognizable within 40 days after the completion of embryogenesis under laboratory culture conditions, and testes and ovaries that are functionally mature are formed simultaneously in individual organisms (I. Shiomi, unpublished observation). At present, however, nothing is known about how gametes of different sexes develop in the two consecutive segments. If one considers that PGC-generating fates are segregated exclusively to the 10th and 11th primary m blast cells at the time of their birth from the M teloblast, it is tempting to speculate that whether PGCs become spermatogonia or oogonia would be determined as early as the time of their emergence as pre-PGCs (i.e., VPE cells) in the mesodermal GB. Alternatively, it is equally possible that germ cell sex determination depends on external cues, such as surrounding germ layers, positional information along the AP axis and physical positions in the mesodermal GB. It is also plausible that sexes of germ cells are specified in response

to “extrinsic” cues residing in the segments in which PGCs exist. One of the best ways to differentiate these possibilities would undoubtedly be to utilize such classic embryological techniques as introduced in this study. In the present study we have shown that pre-PGCs (i.e., VPE cells) are visualized and can be followed in living embryos by simple injection of fluorescent lineage tracers into M teloblasts. This apparently allows us to manipulate pre-PGCs in living embryos. Furthermore, laser ablation of pre-PGCs (labeled with fluorescent tracers) would be plausible as well. Studies using such fluorescent, living pre-PGCs in *Tubifex* embryos are currently under way.

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Figure legends

Fig. 1. Summary of *Tubifex* development. (A) A stage 8 embryo comprised of 24 cells, dorsoposterior view. Cells 2d¹¹, 4d and 4D (sister cell of 4d) all come to lie in the future midline of the embryo. (B) A stage 11 embryo shortly after formation of mesodermal teloblasts Ml and Mr (yellow), ectodermal teloblast precursors NOPQl and NOPQr (green), and endodermal precursors E^D (brown). (C) A stage 12 embryo undergoing germ band (GB) formation, dorsal view with anterior to the top. (D)

Longitudinal section showing the relative positions of the endoderm and bandlets extending from teloblasts M and O. Anterior is to the left and posterior is to the right. In both teloblasts, metaphase mitotic spindles are depicted to indicate the direction and inequality of their divisions. Asterisks indicate the presence of a single primary blast cell in each block of the bandlet; the remaining blocks individually represent a cell cluster, which is derived from a single primary blast cell. (E) Time course of *Tubifex* development (stages 9 to Juv-D1 or one-day-old juvenile, at 22°C). A stage 9 embryo completes 4d cell division into a pair of teloblasts Ml and Mr. Embryos at stages 14-18 are viewed from side with anterior to the left and dorsal to the top. The GBs (hatched) are initially located on the dorsal side of the embryo (stage 14). Along with their elongation, they gradually curve round toward the ventral midline and finally coalesce with each other along the ventral midline (stage 15). The coalescence is soon followed by dorsalward expansion of the GBs (stage 16). The edges of the expanding GBs on both sides of the embryo finally meet along the dorsal midline to enclose the yolky endodermal tube (stages 16-18). Concurrently with this enclosure, the embryo becomes elongated in an anterior-to-posterior progression, and curved with the ventral convexity (stages 16-18). Enclosed portions of the embryo begin to exhibit peristaltic movements. Embryogenesis is judged to be complete when the expanding GBs have enclosed the posterior end of the embryo (stage 18), which then exhibits movement throughout its length.

Fig. 2. Occurrence of Vasa-expressing cells in *Tubifex* embryos and juveniles. (A-O) Ventral views with anterior to the top are shown. A-I, stage 16; J-L, stage 17; M-O, Juv-D1 (one-day-old juvenile). A pair of asterisks in each panel indicate the boundary between segments IX and X. Arrowheads in K and L indicate Vasa-positive cells in segment VII. An arrowhead in O indicates the boundary between segments XII and XIII, where a Vasa-positive cell is seen. Scale bar: 200 μm (for A-O). (P, Q) Frequency of Vasa-positive cell occurrence with reference to body segments in stages 16-17 embryos (P) and one-day-old (Juv-D1) juveniles (Q). Abscissa: frequency (in percentage) of cell occurrence. Data from the left side and the right side of the embryo are presented separately. Ordinate: position of segments (VI to XIII).

Fig. 3. Live imaging of pre-PGCs in *Tubifex* embryo. Embryos whose 4d cells had been injected with a fluorescent tracer AF488D at stage 8 were allowed to develop up to stage 16 (for four days), photographed on a fluorescence stereoscope, and fixed for

immunostaining with Vasa antibody. Each panel is composed of two images of the same embryo, AF488D fluorescence image (left) and Vasa bright field image (right). Ventral views with anterior to the top are shown. Asterisks indicate positions of ventral fluorescent large cells. Note that every fluorescent large cell is Vasa-positive. Vertical lines in A and C indicate the position of segments VII and VIII, where some fluorescence originating from a developing nephridium (mesodermal origin) is detectable (for details see Kitamura and Shimizu, 2000). Scale bar: 200 μ m (for A-C).

Fig. 4. Behavior of pre-PGCs in living *Tubifex* embryos. Embryos whose 4d cells had been injected with AF488D at stage 8 were photographed on a fluorescence stereoscope at five, six, and eight days after tracer injection. Immediately after acquiring fluorescence images at Day 8, they were fixed for immunostaining with Vasa antibody. Each panel is composed of five images of the same embryo, upper three AF488D fluorescence images (Day 5, 6 and 8) and lower two Vasa bright field images (Day 8, Vasa). Upper four images are at the same magnification; the lowest image is an enlargement of the fourth image (Vasa). Ventral views with anterior to the left are shown. Asterisks indicate positions of ventral fluorescent large cells. Horizontal lines indicate the positions of segments VII and VIII. Note that pre-PGCs were changing their positions relative to each other in segment X (left and right) of Embryo 1, segment X (right) of Embryo 2, and segment X (left) and XI (right) of Embryo 3. Arrowheads in bright field images (Day 8, Vasa) indicate Vasa-positive cells; a double arrowhead in A points to the site where a Vasa-positive cell exists though it is not visible here due to its location deep inside. Note that Vasa-positive cells are restricted to segments X and XI at Day 8. Scale bar: 200 μ m (for A-C).

Fig. 5. Teloblast-transplantation procedure. A bilateral pair of M teloblasts that have undergone cell divisions nine times (at 28 hours after 4d cell division) are transferred to a 22-cell stage (stage 8) host embryo from which the 4d cell has been removed. The donor pair of M teloblasts are placed in the dorsoposterior region of the host embryo and they are oriented so that their anterior end is associated with the 2d¹¹ cell of the host embryo. Red asterisks indicate the posterior end of M teloblasts. A, anterior; P, posterior.

Fig. 6. Development of PGCs in ectopic positions. (A, B) M teloblasts to be transplanted were isolated from donor embryos at 20 (A) or 28 (B) hours after 4d cell

division and transplanted to host embryos from which 4d cell had been ablated (see Fig. 5). The experimentally reconstituted embryos were cultured for seven days before fixation. Fixed specimens were processed for immunostaining with Vasa antibody. Whole-mount preparations of the experimental embryos are shown. Ventral views with anterior to the left. Horizontal lines with numerals indicate anterior segments that have been established under the control of m blast cells derived from the transplanted M teloblasts. Square brackets indicate the regions which are devoid of ganglion-like cell clusters. (A) Following the transplantation of the M teloblasts that produced six primary m blast cells, Vasa-positive cells were detected in segments 4 and 5 of the reconstituted embryo. In segments 3 and 6, Vasa-positive tiny spots are seen. (B) Following the transplantation of the M teloblasts that produced nine primary m blast cells, Vasa-positive cells were detected in segments 1-3 of the reconstituted embryo. Note that these cells are located in the posterior half of each segment. (C) An intact control embryo exhibiting Vasa-positive cells in segments X and XI. Scale bar: 200 μm (for A-C).

Fig. 7. Development of PGCs in truncated mesodermal germ bands. (A) Schematic representation of the primary m blast cell production from the M teloblast indicating the number of primary m blast cells that have been produced by 24 (B), 28 (C), 30 (D), 33 (E) and 36 (F) hours after the birth of M teloblast (i.e., 4d cell division; see Fig. 1E). (B-F) M teloblasts were ablated at 24 (B), 28 (C), 30 (D), 33 (E) or 36 (F) hours after 4d cell division and the resulting operated embryos were cultured for eight days before fixation and processed for immunocytochemistry with Vasa antibody. Square brackets indicate posterior tips. (B) Side view with anterior to the left. No Vasa-positive cells were detected. (C) Ventral views with anterior to the left. No Vasa-positive cells were detected. (D-F) Ventral Vasa-positive cells were restricted to segment X (D) or segments X and XI (E, F). Vasa-positive cells seen on the right in D and on the left in E are out of focus here. Arrowheads in D and E indicate Vasa-positive tiny spots. (E) An intact control juvenile that developed synchronously with that shown in E. Vasa-positive cells are seen in segments X and XI. Scale bar: 200 μm (for B-E).

Fig. 8. Frequency of Vasa-positive cell occurrence with reference to body segments in embryos with truncated mesodermal germ bands. (A-D) M teloblasts were ablated at 28 (A), 30 (B), 33 (C) or 36 (D) hours after 4d cell division and the resulting operated embryos were cultured for eight days before fixation and processed for

immunocytochemistry with Vasa antibody. (E) Unoperated control embryos were cultured without vitelline membrane for nine days after 4d cell division and fixed for immunostaining. Abscissa: frequency (in percentage) of cell occurrence. Data from the left side and the right side of the embryo are presented separately. Ordinate: position of segments (VI to XIII). PT, posterior tip. Asterisks in A-E indicate Vasa-positive tiny spots (see Fig. 7D, E).

Fig. 9. Summary of PGC specification in *Tubifex*. (A-C) Mesodermal germ bands elongating from M teloblasts are shown at stages 13 (A), 14 (B), 15 (C), 16 (D) and 18 (E). Anterior is to the left. Each block in germ bands represents one segment. For brevity, mesodermal cells comprising each segment are not depicted except for pre-PGCs and PGCs (filled circles). Open circles in E indicate pre-PGCs that have not been specified as PGCs. (A-C) Emergence of pre-PGCs in the mesodermal germ band during stage 15. Red shading indicates *vasa* mRNA (see Oyama and Shimizu, 2007). (D, E) Development of PGCs in segments X and XI but not in other segments. Red shading indicates Vasa protein.

Fig. 10. Model for specification of primary m blast cells m10 and m11 as initial precursor of PGCs. Early mesodermal germ bands with M teloblasts on the right are shown shortly after formation of m9 (A), m10 (B), m11 (C), m12 (D) and m13 (E). Anterior is to the left. m10 and m11 are exclusively endowed with factors (blue shade) that give the cells a PGC-generating fate.

Fig. S1. Anti-BmVLG antibody recognizes a 65 kDa polypeptide in *Tubifex* embryos. (A) Immunoblot of ooplasmic extracts from *Tubifex* embryos probed with anti-BmVLG antibody. Molecular mass markers (kDa) are shown on the left. A major band is seen at the molecular mass of approx. 65 kDa, and a minor band at 43 kDa. (B) Whole-mount immunocytochemistry of a one-day-old juvenile. Ventral view with anterior to the top. Immunoreactive staining is present in cells (arrowheads) located in genital segments X and XI. Scale bar: 200 μ m.

Fig. S2. Live imaging of pre-PGCs in *Tubifex* embryos undergoing body elongation. Embryos whose 4d cells had been injected with AF488D at stage 8 were photographed on a fluorescence stereoscope at five (Day 5) and six (Day 6) days after tracer injection. Ventral views with anterior to the left are shown. Asterisks indicate positions of

ventral fluorescent large cells. Horizontal lines indicate the positions of segments VII and VIII. Scale bar: 200 μm (for A-D).

Fig. S3. Descendant cells of m11 (the 11th primary m blast cell) appear not to move into segment X. (A-C) Right M teloblasts in embryos at 30 hours after 4d cell division were injected with DiI (5 mg/ml in safflower oil), and the injected embryos were cultured for three days before fixation. After stained with DAPI (1 $\mu\text{g/ml}$ in PBS), a whole-mount preparation of the experimental embryo was viewed on a fluorescence stereoscope. Right side view with anterior to the left and ventral to the top. (A) DiI fluorescence. (B) DAPI fluorescence. Ventral ganglia are visible. (C) Merge. The positions of segments X and XI are indicated by short horizontal lines. Scale bar: 200 μm (for A-C).

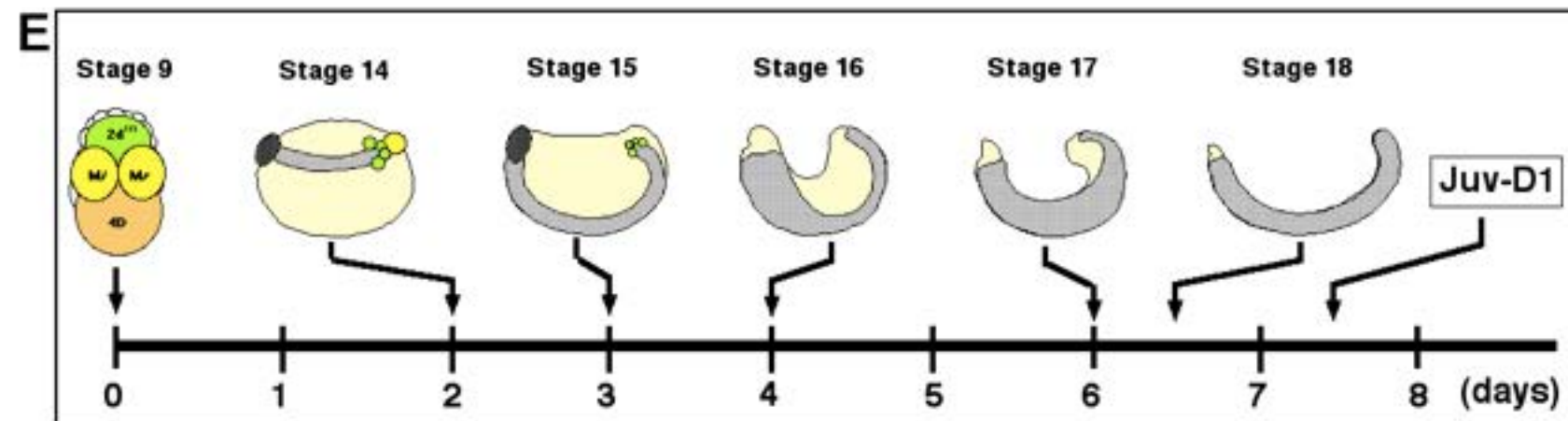
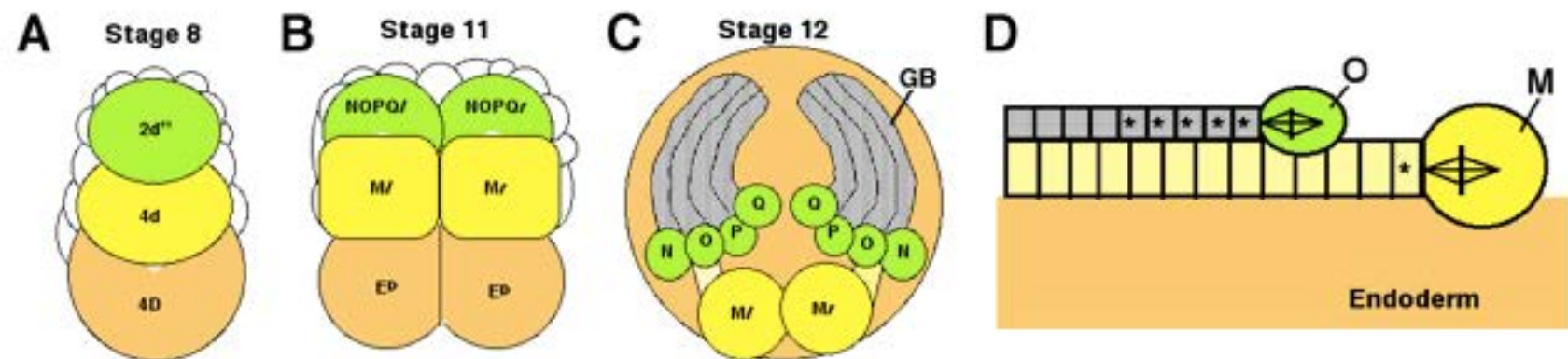
Fig. S4. Development of PGCs in ectopic positions. (A-D) M teloblasts to be transplanted were isolated from donor embryos at 20 hours after 4d cell division and transplanted to host embryos from which 4d cell had been ablated (see Fig. 5). The experimentally reconstituted embryos were cultured for seven days before fixation. Fixed specimens were processed for immunostaining with Vasa antibody. Whole-mount preparations of the experimental embryos are shown. Ventral views with anterior to the left. Short horizontal lines indicate the positions of anterior segments that have been established under the control of m blast cells derived from the transplanted M teloblasts; asterisks indicate segment 1 (i.e., anterior-most segment). A square brackets indicate the regions which are devoid of ganglion-like cell clusters. (A, B) Vasa-positive cells are seen in segments 4 and 5 of the reconstituted embryo. A square bracket in B indicates segment 5 on the left side; note that this segment is shorter than those on the right side and appears to be shifted anteriorly relative to the right side. (C, D) Vasa-positive cells on the right side are located in segments 4 and 5. By contrast, Vasa-positive cells (arrowheads) on the left side appear to be present in segments 1 and 5. The regions indicated by square brackets appear to be devoid of mesodermal and endodermal tissues. Scale bar: 200 μm (for A-D).

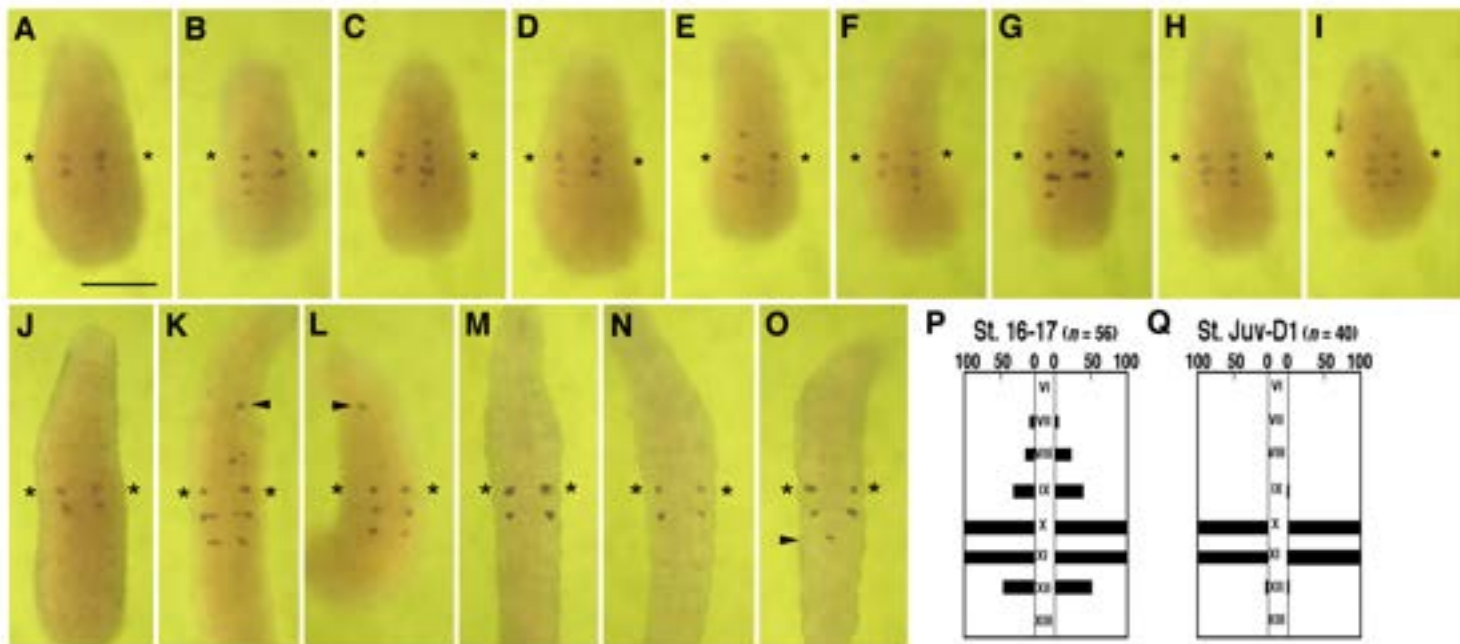
Fig. S5. Development of PGCs in ectopic positions. (A-E) M teloblasts to be transplanted were isolated from donor embryos at 28 hours after 4d cell division and transplanted to host embryos from which 4d cell had been ablated (see Fig. 5). The experimentally reconstituted embryos were cultured for seven days before fixation.

Fixed specimens were processed for immunostaining with Vasa antibody.

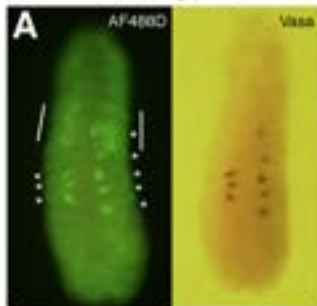
Whole-mount preparations of the experimental embryos are shown. (A-C, E) Ventral views with anterior to the left; (D) left side view with anterior to the left and dorsal to the top. Short horizontal lines indicate the positions of anterior segments that have been established under the control of m blast cells derived from the transplanted M teloblasts; asterisks indicate segment 1 (i.e., anterior-most segment). Square brackets indicate the regions where ganglion-like cell clusters are not seen. (A, B)

Vasa-positive cells were detected in segments 1 and 2. Note that Vasa-positive cells seen on the right side in B appear to be thin. This is probably because these cells are associated with the septum. (C-E) Vasa-positive cells show 'aberrant' distribution patterns in these experimental juveniles. Note that in all of the three juveniles, the anterior-most segment appears as a Vasa-positive cell-bearing segment. Scale bar: 200 μm (for A-E).

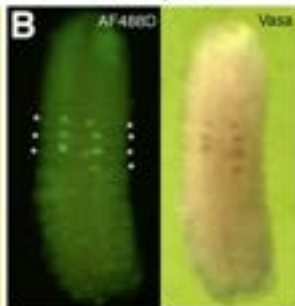




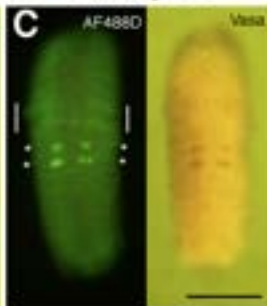
Embryo 1



Embryo 2



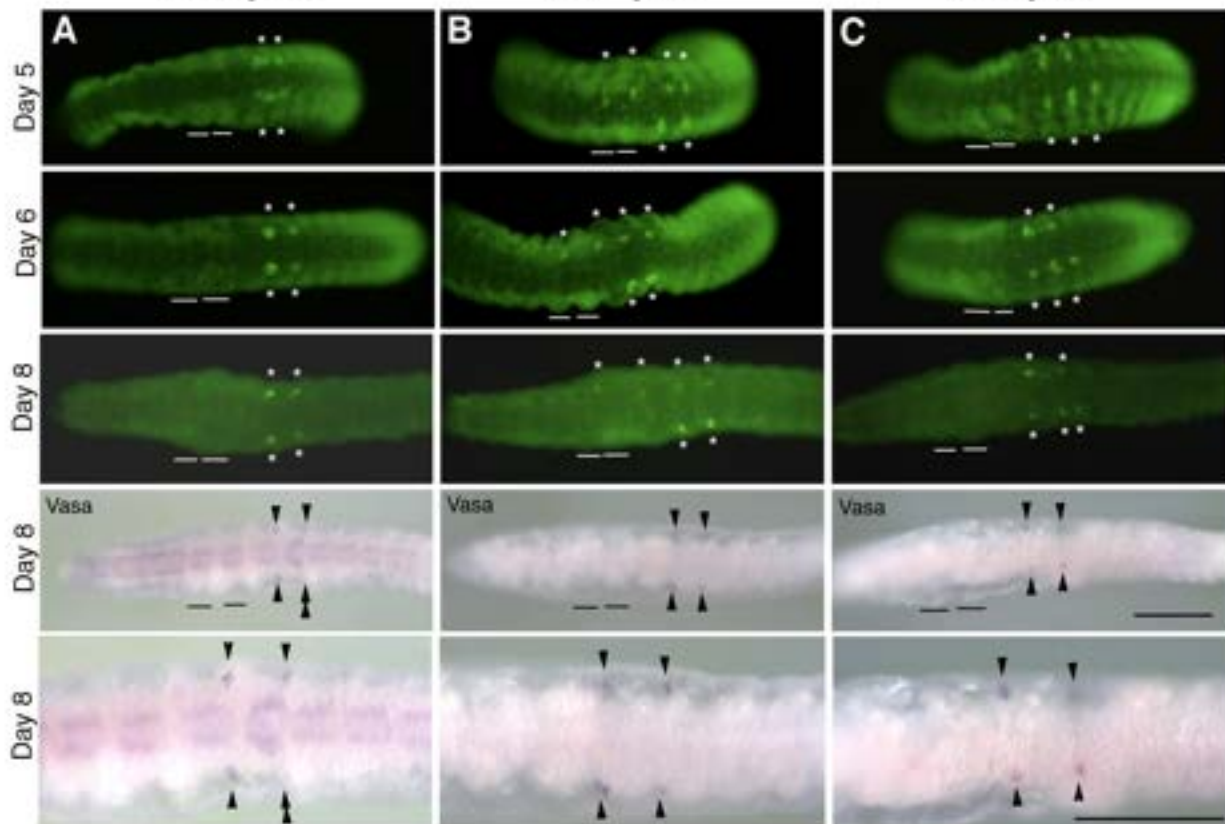
Embryo 3

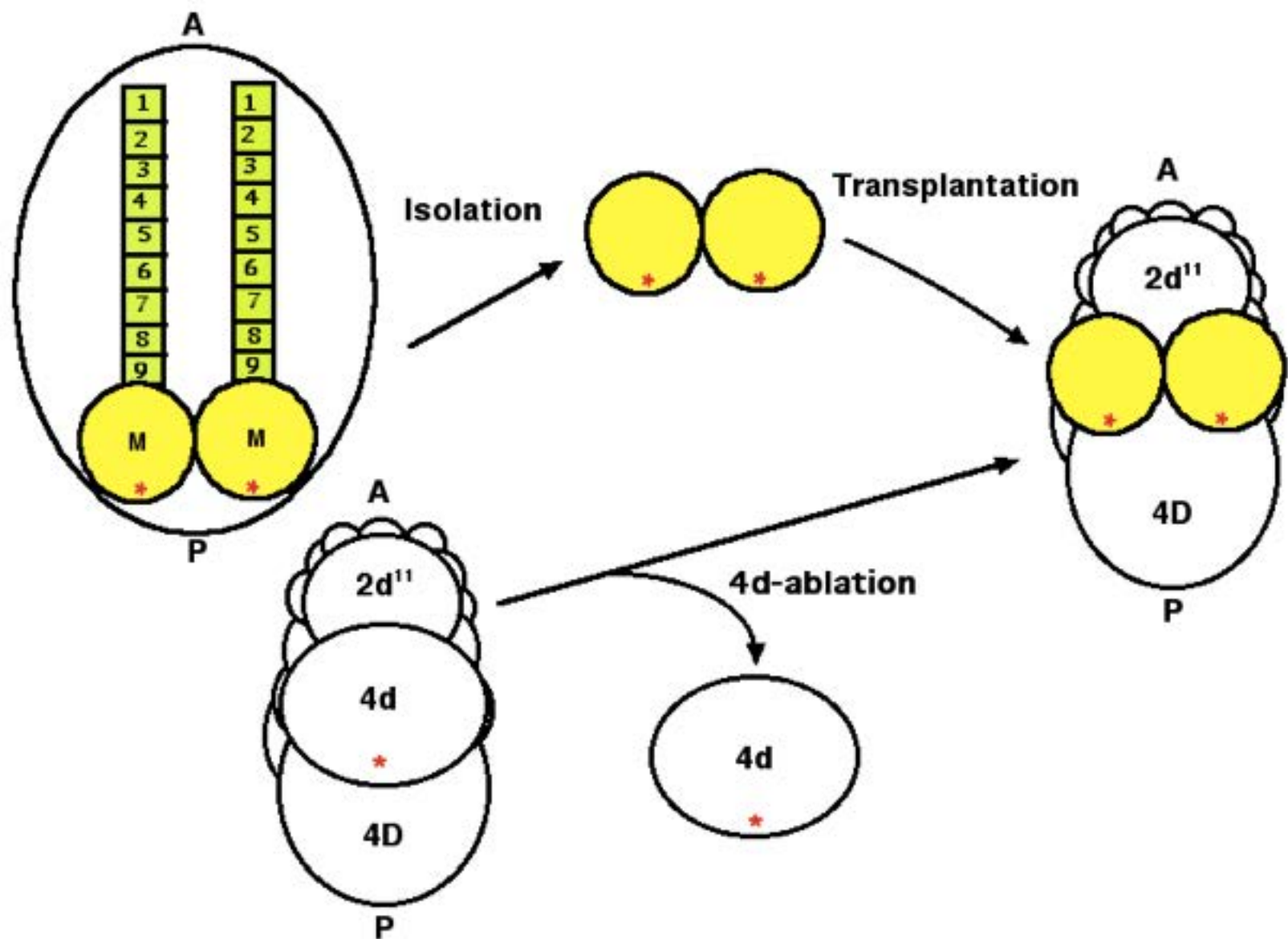


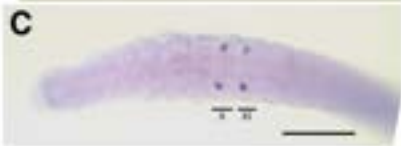
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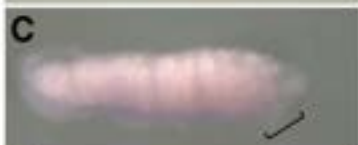
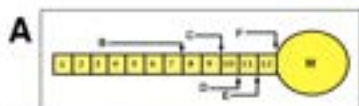
Embryo 2

Embryo 3



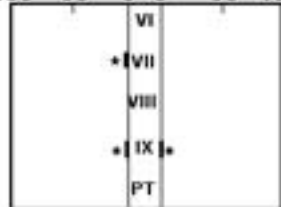




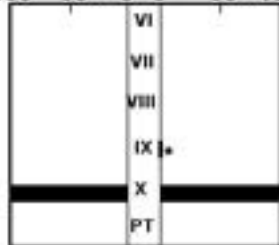


A**28 hours** ($n = 25$)

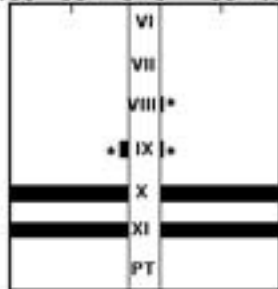
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**B****30 hours** ($n = 25$)

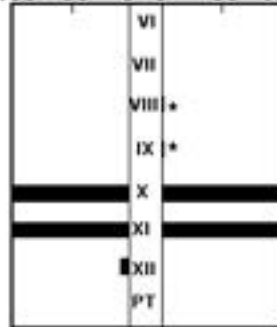
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**C****33 hours** ($n = 30$)

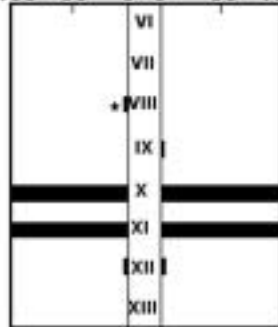
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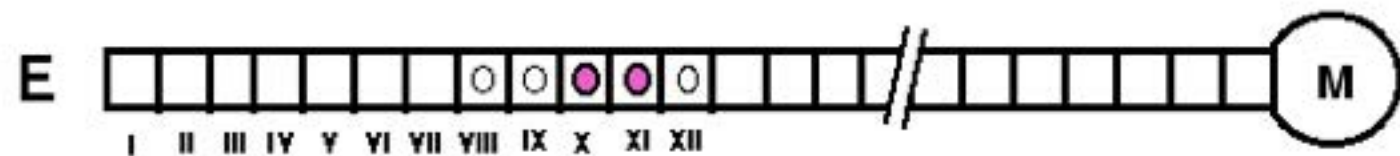
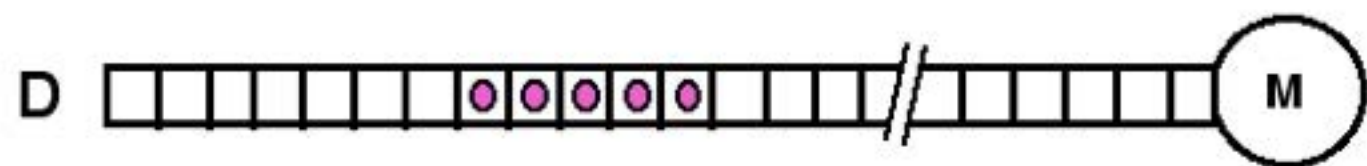
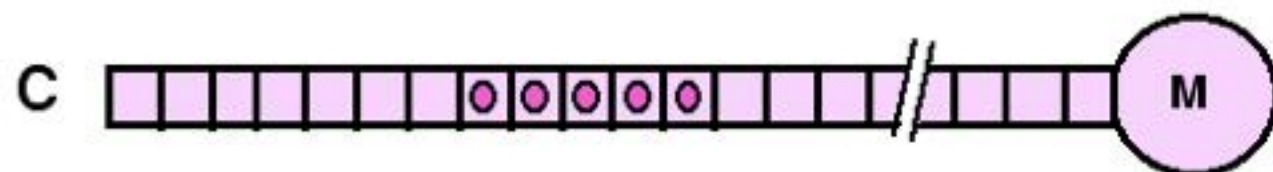
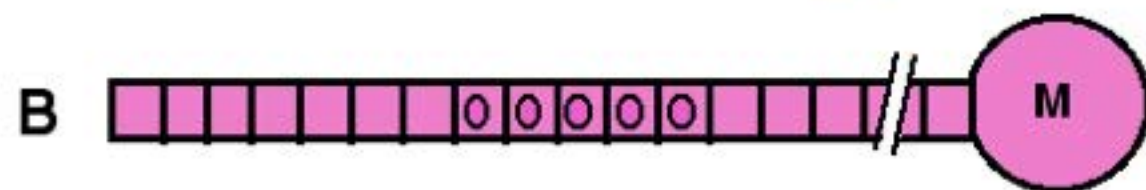
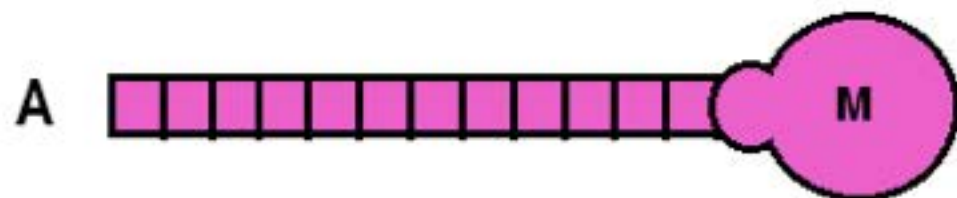
**D****36 hours** ($n = 26$)

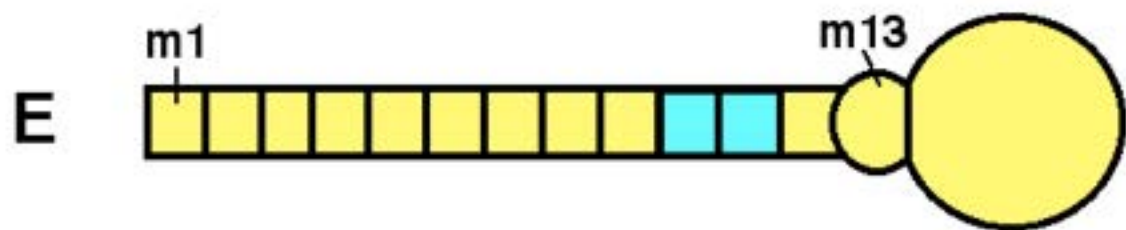
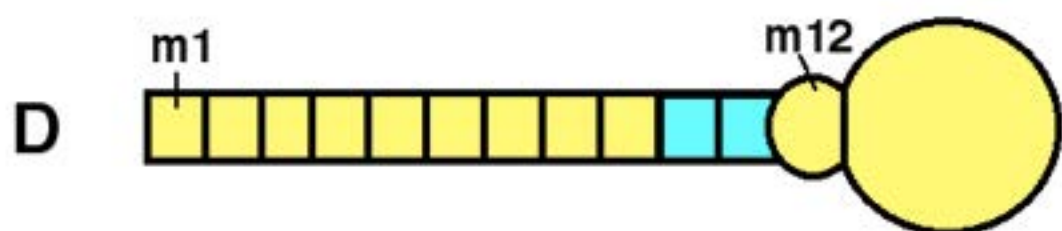
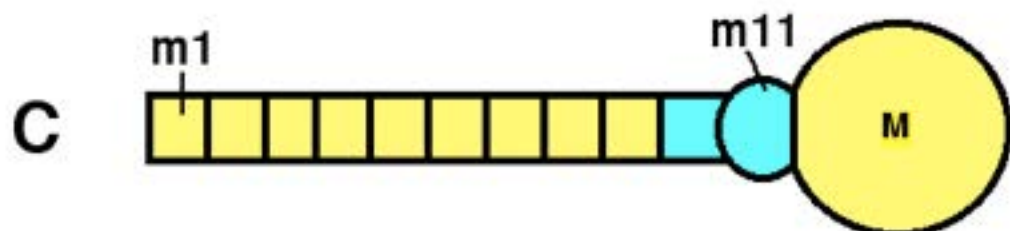
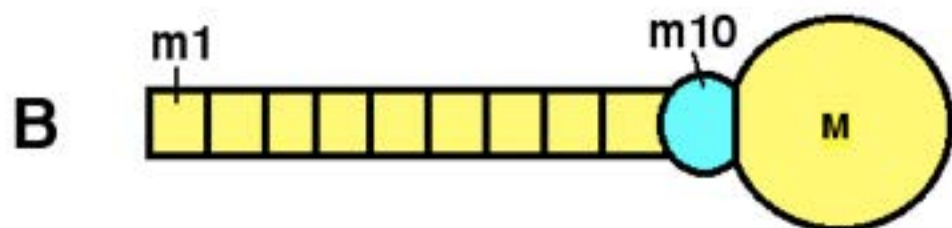
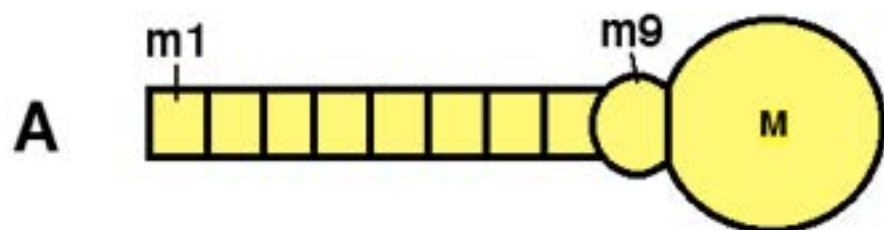
100 50 0 0 50 100

**E****Unoperated** ($n = 25$)

100 50 0 0 50 100



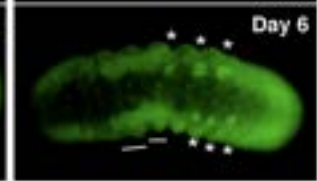
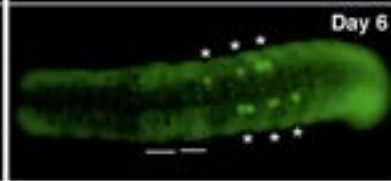
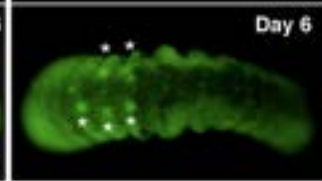
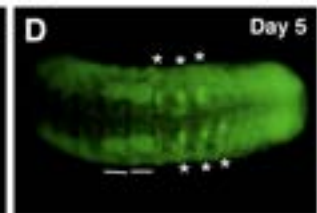
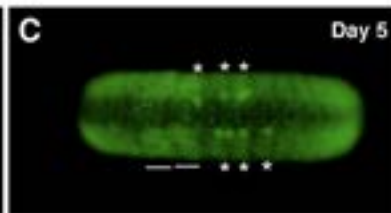
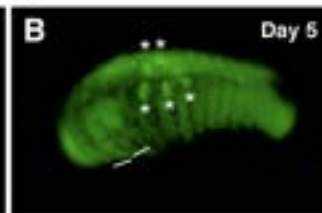


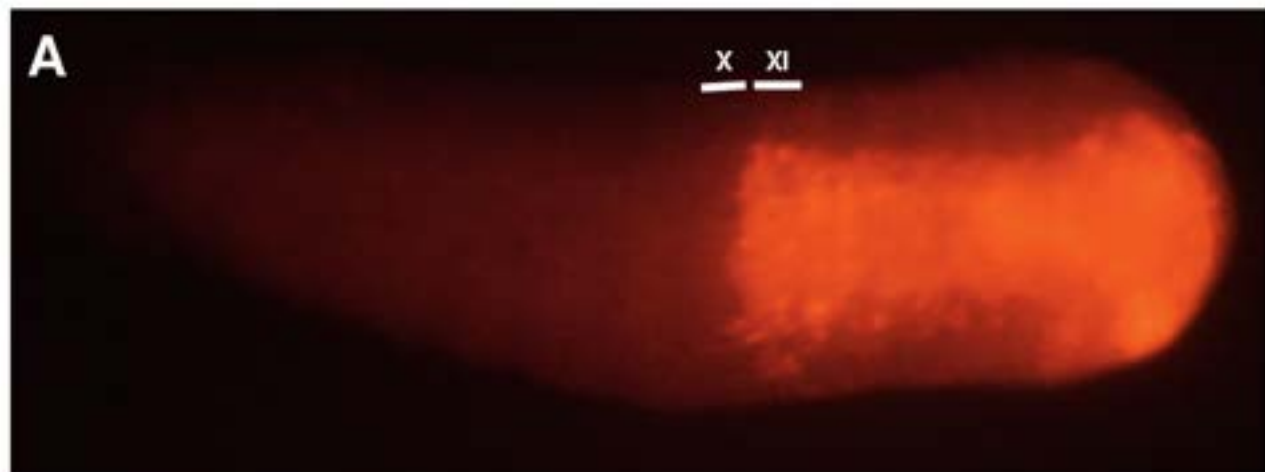
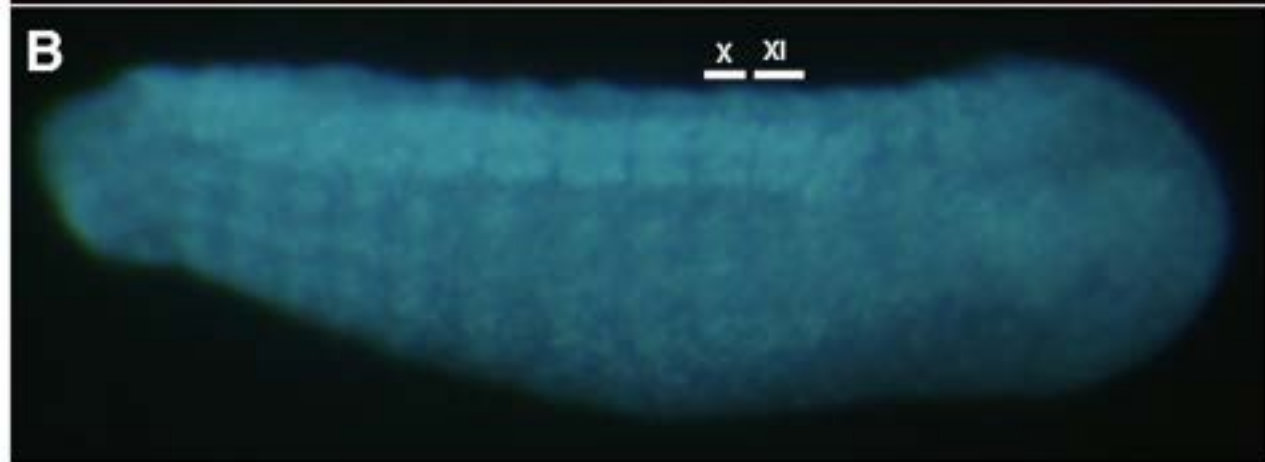


A

130—
100—
75—
50—
35—

**B**



AX XI**B**X XI**C**X XI