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The ontogeny of *nanos* homologue expression in the
oligochaete annelid *Tubifex tubifex*

by

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ABSTRACT

We have cloned and characterized the expression of a *nanos* homologue (designated *Ttu-nos*) from the oligochaete annelid *Tubifex tubifex*. *Ttu-nos* mRNA is distributed broadly throughout the early cleavage stages. *Ttu-nos* is expressed in most if not all of the early blastomeres, in which *Ttu-nos* RNA associates with pole plasm. *Ttu-nos* transcripts are concentrated to 2d and 4d cells. Shortly after 2d¹¹¹ (derived from 2d cell) divides into a bilateral pair of NOPQ proteloblasts, *Ttu-nos* RNA vanishes from the embryo, which is soon followed by the resumption of *Ttu-nos* expression in nascent primary blast cells produced by teloblasts. The resumption of *Ttu-nos* expression occurs only in a subset of teloblast lineages (viz., M, N and Q). After *Ttu-nos* expression is retained in the germ band for a while, it disappears in anterior-to-posterior progression. At the end of embryogenesis, there is no trace of *Ttu-nos* expression. Thereafter, growing juveniles do not show any sign of *Ttu-nos* expression, either. The first sign of *Ttu-nos* expression is detected in oocytes in the ovary of young adults (ca 40 days after hatching), and its expression continues in growing oocytes that undergo yolk deposition and maturation in the ovisac.

*Keywords:**nanos*

oligochaete annelid

Tubifex tubifex

Teloblast lineages

Germline

Oocyte

The *nanos* (*nos*) gene encodes an RNA binding protein with two highly conserved CCHC Zn-fingers in the C terminus. This gene was first identified as a maternal effect gene in the fruit fly *Drosophila melanogaster* (Nüsslein-Volhard et al., 1987). It is now well known that in *Drosophila*, a Nos protein interacts with maternal *hunchback* mRNA and represses its translation in posterior regions of the embryo (Tautz, 1988; Irish et al., 1989). Since then, *nos*-related genes have been identified in a number of animal species (Mosquera et al., 1993; Subramaniam and Seydoux, 1999; Torras et al., 2004; Extavour et al., 2005). In some animals, functional studies have been done to demonstrate that Nos-related proteins play a pivotal role in both somatic and germline development (Kobayashi et al., 1996; Tsuda et al., 2003; Agee et al., 2006; Rabinowitz et al., 2008; Juliano et al., 2009). On the other hand, studies on embryonic expression of *nos*-related genes using whole-mount *in situ* hybridization (WISH) have also been done in a variety of animals, and suggested that irrespective of their actual function in germline cells, *nos*-related genes serve as a good marker to identify germ cells as well as to study the process of germline formation in animals.

Among lophotrochozoans, *nos*-related genes have been identified in the polychaete annelids *Platynereis dumerilli* (Rebscher et al., 2007) and *Capitella* sp. I (Dill and Seaver, 2008), the oligochaete annelid *Pristina leidy* (Özpolat and Bely, 2015), the leech *Helobdella robust* (Kang et al., 2002), and the snails *Haliotis asinina* (Kranz et al., 2010) and *Ilyanassa obsoleta* (Rabinowitz et al., 2008). As in other animals, germline cells such as primordial germ cells (PGCs) express *nos*-related genes in these lophotrochozoans (except for the snails mentioned above). A recent study by Cho et al. (2014) has shown that female germline cells in *Helobdella* embryo express *piwi* and *vasa* in addition to *nos*, while male PGCs express *nos* only. This *Helobdella* (hermaphrodite) study prompted us to investigate *nos* expression pattern in another hermaphrodite annelid *Tubifex tubifex*, in which unlike those in *Helobdella*, both the male and female PGCs (located in segments X and XI, respectively) are known to express a *vasa* gene at the same time during embryogenesis (Oyama and Shimizu, 2007; Kato et al., 2012).

In this study, we have isolated a *nanos* homologue from *Tubifex tubifex* and examined its expression throughout the life cycle.

1. Results and discussion

1.1. Cloning of *Tubifex* homologues of *nanos*

Using degenerate PCR and subsequent 3'RACE, we isolated portions of two *nanos* (*nos*) homologues, *Ttu-nos1* and *Ttu-nos2*, from the oligochaete annelid *T. tubifex*. *Ttu-nos1* and *Ttu-nos2* cDNA fragments obtained were 1797 and 1794 base pairs long, respectively, and both contained a 3' untranslated region (UTR) and two putative CCHC-type Zn-finger domains in the coding region, displaying a high degree of homology with *nos*-related proteins of other metazoans, especially lophotrochozoans (Figs. 1A and B). Both cDNAs have identical amino acid sequences (Fig. 1A) though they share 97.8% nucleotide sequence identity with each other (not shown). By contrast, 3'UTR of the cDNAs have approximately 93% identical nucleotide sequences. Accession numbers for *Ttu-nos1* and *Ttu-nos2* are LC020234 and LC020235, respectively.

At present it is not known whether *Ttu-nos1* and *Ttu-nos2* are derived from the two different genes or result from alternative splicing of the same gene. Genomic Southern analysis would provide significant information to differentiate these possibilities.

In this study, we prepared WISH probes for both *Ttu-nos1* and *Ttu-nos2* and used them to examine the spatiotemporal distribution pattern of transcripts of these homologues. We found that both probes gave identical staining patterns in WISH preparations (data not shown). It should be mentioned, however, that embryos hybridized with a *Ttu-nos1* probe were stained more intensely than those hybridized with a *Ttu-nos2* probe even if both embryos were processed under identical conditions including RNA probe concentration. In the following sections, we will describe the expression patterns of *Ttu-nos1* only, and refer to *Ttu-nos1* as *Ttu-nos*.

1.2. Maternal *Ttu-nos* transcripts are distributed broadly in early embryos

Whether they are stored in the ovisac of the adult worm or packed in the cocoon concurrently with oviposition, eggs (oocytes) undergoing maturation division exhibit intense signals of *Ttu-nos* expression throughout the egg (Figs. 3A and C), with concentration in meiotic spindles in metaphase II eggs (Fig. 3C). When pole plasm domains form at both poles, the mRNA accumulated to the pole plasm domains (Fig. 3D). During early cleavage stages (up to 22-cell stage; see Fig. 2E), the mRNA was present in all blastomeres (Figs. 3E-H) though 2d cell and its progeny (such as 2d², 2d¹² and

2d¹¹²) often appeared to exhibit stronger signals of *Ttu-nos* staining than other micromeres (Figs. 3I and J). At stage 11 when 2d¹¹¹ divides into a bilateral pair of proteloblasts NOPQ (Fig. 2F), however, no signals of *Ttu-nos* expression were detected in blastomeres located on the surface of the embryo (except for internally located primary m blast cells; Fig. 3K). This apparently suggests that maternal transcripts of *Ttu-nos* decay abruptly around this stage.

In eggs (including ovisac eggs) and embryos processed for WISH with a sense probe, no staining was detected from ovisac eggs (Fig. 3B) to stage 18 embryos (data not shown).

1.3. Zygotic expression of Ttu-nos occurs in the ectodermal and mesodermal germ bands but not in other cells

After disappearance of maternal *Ttu-nos* transcripts from surface cells derived from micromeres, *Ttu-nos* expression resumed first in primary m blast cells (see Fig. 4C) and then in primary n blast cells (Figs. 4A-C), concurrently with the onset of germ band (GB) formation. As development proceeds up to stage 16, both the ectodermal and mesodermal GBs elongate and exhibit lineage-specific distribution patterns of *Ttu-nos*-expressing cells, as described below. During these embryonic stages, there is no indication of *Ttu-nos*-positive cells in other regions of the embryo.

Mesodermal GBs. By stage 12b, M teloblasts undergo cell division seven times to form initial mesodermal GBs (Goto et al., 1999). As Fig. 4C shows, such GBs were found to be *Ttu-nos*-positive. As development proceeds, mesodermal GBs on both sides of the embryo elongate by addition of primary m blast cells from M teloblasts. The GB elongation continues during stages 13-16 (Goto et al., 1999).

Ttu-nos-expression was detected only in nascent m blast cells, viz. those lining in the portion of the GB proximal to the M teloblast; most anteriorly located *Ttu-nos*-expressing m blast cells coincided with the anterior margin of ectodermal teloblasts O and P. By contrast, **more anteriorly located** m blast cells, which are overlain by the ectodermal GB, did not appear to exhibit staining signals (see Figs. 6E and F). As described below, stained cells belonging to the ectodermal GB domain seen in embryos at stages 14-16 are all located at the surface of the embryo, suggesting that it is unlikely that they are mesodermal.

Ectodermal GBs. It is not until the production of proteloblast OP and teloblast Q that *Ttu-nos*-expressing cells are recognizable at the surface of the embryo (Figs. 4A-C). Such surface cells are located in a "gulf" formed on the anterior side of cells N, OP and Q (Figs. 4D and E; also see Fig. 2H). On both sides, a cell adjacent to N teloblast exhibited stronger signal than the remaining cells. When proteloblast OP divides into teloblasts O and P (Figs. 2H and I), ectodermal GBs begin to form on both sides of the embryo (Fig. 2I). As Figs. 4F-I show, as far as nascent cells are concerned, intensity of *Ttu-nos* expression is different among the four ectoteloblast lineages (n, o, p and q): n-blast cells exhibited strongest signal of *Ttu-nos* staining; q blast cells are recognized as *Ttu-nos*-positive cells though expression signal from each cell lineage is weak. In contrast to these lineages, either o or p blast cells do not appear to be stained significantly in WISH preparations. It should be noted that *Ttu-nos*-expressing cells are distributed discontinuously along the n bandlet. For example, in the embryo (stage 13) shown in Fig. 5, two primary n blast cells proximal to the N teloblast exhibit strong signals of *Ttu-nos* staining, and they are succeeded by four consecutive blast cells exhibiting very weak signals, which are followed by four consecutive cells with strong signals. We examined closely 15 embryos (stage 13) that were stained for *Ttu-nos* similarly, and found that these embryos all exhibited similar blast cell distribution patterns in the n bandlets. This observation may suggest that *Ttu-nos* expression is down-regulated at a distance of a few cells from the N teloblast. Since the first division of a primary n blast cell occurs at a distance of seven cells from the N teloblast (Arai, 2000), it is likely that this putative down-regulation of *Ttu-nos* expression occurs significantly earlier than the first division in a primary n blast cell.

In relation to the above-mentioned observation, it is of interest to note that *hunchback* (*hb*) protein is detected in O and P teloblasts but not in N and Q teloblasts in *Tubifex* embryos (Shimizu and Savage, 2002). Given that acting with Pumilio, Nos protein suppresses translation of *hb* mRNA (Barker et al., 1992; Murata and Wharton, 1995), the absence of *hb* protein in N and Q lineage could be explained by the restricted expression of *Ttu-nos* in these two lineages, though *Ttu-nos* mRNA and *hb* protein are present in blast cells and teloblasts, respectively.

During stages 14 and 15, ectodermal GBs elongate and undergo segmentation; morphogenetic events leading to segmentation of the GB occur in anterior-to-posterior progression (Nakamoto et al., 2000). In stage 14 embryos, cells that express *Ttu-nos*

intensively are localized at the ventral margin of the GB; in the remaining region, *Ttu-nos*-positive cells are intermingled with *Ttu-nos*-negative cells (Figs. 4H and I). It should be noted that *Ttu-nos*-expressing cells located at the ventral margin of the GB are organized in a chain of clusters in the anteriormost portion of the GB (Fig. 4I). This cell cluster formation is one of the earliest events accompanying segmentation. As development proceeds, similar cell clusters form along the ventral margin of the GB in anterior-to-posterior progression; by the beginning of stage 15, cell cluster formation completed in the posteriormost portion of the GB (Fig. 6C). With time, each cell cluster grows in size; when the GB on both sides coalesces with each other along the ventral midline, each cell cluster appears to be incorporated in a hemiganglion (Figs. 6A, D and E). If one sees transverse sections of GBs, it is found that cells exhibiting strong signals are **restricted** to the epithelial layer and ganglion (Fig. 6F).

During stages 16-18 when embryos undergo body elongation accompanied by dorsalward expansion of the ectodermal GB (Figs. 2M-R), *Ttu-nos*-positive cells gradually disappeared from the embryo. Cessation of *Ttu-nos* expression began at the anteriormost segment (Fig. 7A) and progressed in an anterior-to-posterior fashion (Figs. 7B-D). At the end of embryogenesis (at stage 18), there are found no positive cells in the embryo (data not shown).

1.4. *Ttu-nos* expression in germline cells

It has been reported that *nos*-related genes are expressed in primordial germ cells (PGCs) during embryonic development in a variety of animals (Kang et al., 2002; Extavour et al., 2005; Rebscher et al., 2007; Dill and Seaver, 2008; Özpolat and Bely, 2015). In *Tubifex*, presumptive PGCs (pre-PGCs), which arise from segmental mesoderm, are recognized as *vasa*-expressing large cells located on the ventral side of the embryo. They are distributed in a variable set of midbody segments including genital segments X and XI (Oyama and Shimizu, 2007; Kato et al., 2013). During the course of the aforementioned observations on embryos (at stages 15-18) subjected to *Ttu-nos* staining, we failed to detect any stained mesodermal cells in at least segments X and XI. In this regard, it is interesting to note that in *Capitella* and *Helobdella*, *nos* expression persists in PGCs when *nos* expression in the surrounding somatic tissues diminishes (Kang et al., 2002; Dill and Seaver, 2008). Conceivably, in these animals, decline of *nanos* expression in somatic tissues might lead to "visualization" (or

emergence) of stained PGCs in WISH preparations. There is a possibility that in *Tubifex* as well, pre-PGCs located in *Ttu-nos*-expression-diminishing region appear as *Ttu-nos* positive cells for a short time. To test this possibility, we prepared embryos which had been doubly stained for *Ttu-nos* transcripts and fluorescein dextran (Fl-D used as a tracer of the mesodermal lineage in which pre-PGCs are included; see Experimental procedures). Fig. 8 shows a ventral view of a double-stained embryo in which *Ttu-nos* expression in the ectoderm persisted in segment XIII but had diminished in more anterior segments. In this embryo, pre-PGCs (stained in red) seen in segments X-XII do not appear to exhibit *Ttu-nos* staining, suggesting that it is unlikely that pre-PGCs in embryos at stages 15-18 are *Ttu-nos*-positive.

On the other hand, as described above, every primary m blast cell (derived from M teloblast) is evidently *Ttu-nos*-positive (see Fig. 4C). Given that primary m blast cells are rather distantly located precursor of pre-PGCs (Oyama and Shimizu, 2007), it is safe to say that pre-PGCs could be regarded as cells that have already undergone zygotic *Ttu-nos* expression.

As described above, oocytes in the ovisac (Fig. 3A) contain *Ttu-nos* mRNA, which is to be used as maternal transcripts during early cleavage stages. This implies that *Ttu-nos* mRNA must be transcribed at a certain stage of oogenesis. To find out when transcription of maternal *Ttu-nos* mRNA begins, we examined juveniles and adults for *Ttu-nos* expression. It is known that juveniles of *T. tubifex* grow up to adulthood within 40 days after hatching under laboratory conditions (at 22°C; Shiomi, 2014). We reared juveniles for 5, 14, 28 or 40 days before fixation and processed them for *Ttu-nos* staining (see Experimental procedures). We found that positive signals of *Ttu-nos* staining were detected exclusively in 40-day-old specimens (i.e., adults). *Ttu-nos* mRNA was localized in ovaries in segment XI (Figs. 9A-D); no positive cells were detected in segment X where testis is located (see Figs. 9A and E). As Figs. 9B-D show, positive cells, which had relatively large nuclei, were located far from the basal (ventral) portion of the ovary. In *Tubifex*, oogonia (in the multiplication period) occupy the most ventral portion of the ovary and extend toward the dorsal side generating oocytes (Hirao, 1964). (Usually, 7-8 oocytes are released from the dorsal portion of the ovary to the ovisac, where the oocytes undergo yolk deposition and maturation; see Hirao, 1964) Judging from their location in the ovary and their large nuclear size, it is safe to say that *Ttu-nos* is expressed in oocytes but not in oogonia.

1.5. Comparison with other lophotrochozoans

The embryonic expression pattern of *Ttu-nos*, revealed in this study, is summarized as follows. *Ttu-nos* mRNA is distributed broadly during early cleavage stages up to 22-cell stage. *Ttu-nos* is expressed in most if not all of the early blastomeres (up to 22-cell stage) though *Ttu-nos* transcripts are concentrated to pole plasm-bearing cells (i.e., 2d and 4d). *Ttu-nos* expression declines to background levels around the time of NOPQ proteloblast formation (at 28-cell stage; see Fig. 2G). The resumption of *Ttu-nos* expression occurs in nascent blast cells produced from a subset of teloblast lineages (viz., m, n and q). After *Ttu-nos* expression is retained in the GB for a while, it disappears in anterior-to-posterior progression. At the end of embryogenesis, there is no trace of *Ttu-nos* expression.

Initial broad distribution of *nos* transcripts and subsequent concentration to specific blastomeres have also been reported in other lophotrochozoans such as *Haliotis*, *Ilyanassa*, *Capitella* and *Helobdella* (Kang et al., 2002; Dill and Seaver, 2008; Rabinowitz et al., 2008; Kranz et al., 2010), suggesting that this early distribution pattern of *nos* mRNA has been conserved widely among lophotrochozoans. The behavior of *nos* transcripts during subsequent development is diverse among lophotrochozoans. As in *Tubifex*, maternal transcripts in *Ilyanassa* and *Helobdella* appear to be cleared from the embryo during cleavage stages (Kang et al., 2002; Rabinowitz et al., 2008) while *nos* mRNA in *Haliotis* and *Capitella* retains even at late embryonic stages (Dill and Seaver, 2008; Kranz et al., 2010). Interestingly, as in *Tubifex*, zygotic *nos* expression resumes in elongating GBs in *Helobdella*; later the expression gradually disappears in an anterior-to-posterior fashion (Kang et al., 2002). Unlike *Ttu-nos* expression in the GBs, *nos* expression in *Helobdella* is reportedly uniform along the length of the GB (Kang et al., 2002). In *Capitella*, the mesodermal bands (derived from the mesodermal precursors) undergo *nos* expression and subsequent decline (Dill and Seaver, 2008).

2. Experimental procedures

2.1. Embryos, juveniles and adults

Embryos of the freshwater oligochaete *T. tubifex* were obtained as described previously (Shimizu, 1982) and cultured at 22°C. For experiments, embryos were

freed from cocoons in the culture medium (Shimizu, 1982). Newly hatched juveniles were reared to adulthood under laboratory conditions (at 22°C) as described previously (Shiomi, 2014). Briefly, they were put in a plastic petri dish containing a sand (tiny particles) layer and tap water, and fed with a small amount of yeast suspension once a week. Unless otherwise stated, all experiments were carried out at room temperature (20-22°C).

2.2. RNA Isolation and cDNA synthesis

Total RNA was isolated from *Tubifex* embryos by using ISOGEN (Nippon Gene) according to the manufacturer's recommendations. Poly (A)⁺ RNA was isolated from total RNA using Oligotex-MAG (TaKaRa) according to the manufacturer's instructions. Isolated total RNA and poly (A)⁺RNA were dissolved in RNase-free sterilized H₂O and stored at -80°C until use. First-strand cDNA was synthesized with a *NotI*-dT18 primer using Time Saver cDNA Synthesis Kit (Amersham Pharmacia Biotech) according to the manufacturer's protocols.

2.3. Degenerate PCR and 3' Rapid amplification of cDNA ends (RACE)

To clone the *nanos* (*nos*) gene of *T. tubifex*, we designed degenerate PCR primers based on the amino acid sequences conserved among the *nos* class genes of *Drosophila melanogaster*, *Capitella* sp. I, and *Helobdella robusta*. A forward primer, F1, corresponded to amino acid sequence CVFCRNN; a reverse primer, R1, corresponded to HTIKYCP. Nucleotide sequences of these primers were 5'-TG YGTNTTYTG YMGNAAYAA-3' (F1) and 5'-GGRCARTAYTTDATNGTRTG-3' (R1). Detailed protocols and amplification parameters for the degenerate PCR are available upon request.

To isolate the 3' portion of *nos* transcripts, 3' RACE was performed using gene specific primers (GSPs) and an adapter primer. Nucleotide sequences of these primers were 5'-TGTGTGTTTTGTCGGAATAACGGCG-3' (GSP1), 5'-ATAACGGCGAAGCTGAAGCCGTCTA-3' (GSP2), and 5'-AACTGGAAGAATTCGCGGCC-3' (adaptor primer). Amplification was carried out with Elongase Enzyme Mix (Invitrogen) and cDNA synthesized with *NotI*-dT18 primer. Detailed protocols and amplification parameters for the 3' RACE are available upon request.

2.4. Whole-mount *in situ* hybridization (WISH)

DIG-labeled RNA probes were prepared according to the protocols described by Matsuo et al. (2005). An antisense and sense riboprobes were synthesized with T7 and T3 RNA polymerases, respectively.

Embryos, juveniles and young adults which were to be processed for WISH were fixed with 4% formaldehyde according to the methods described by Matsuo et al. (2005). After a brief rinse in PBST (PBS plus 0.1% Tween-20), embryos were freed from vitelline membrane with fine forceps. Fixed juveniles and adults were transferred to PBST, and cut into short fragments comprising five to six segments with a razor blade; fragments containing genital segments (X and XI) were collected, and fine forceps were used to remove layers of epithelium and muscle from the dorsal half of each fragment. Ovisacs containing growing oocytes were also dissected out from segments XIII-XV (together with the intestine attached to the ventral side of the ovisac). This treatment was carried out to facilitate penetration of proteinase K and riboprobes into the mesodermal tissues on the ventral side of the embryo. Embryos and fragments (of juveniles and adults) thus obtained were dehydrated with a graded series of methanol, and stored at -20°C in methanol until use.

For WISH, embryos, juveniles and adults that had been stored in methanol were rehydrated and processed according to the protocols described by Matsuo et al. (2005), except that juveniles and adults were treated with proteinase K ($2\ \mu\text{g}/\text{ml}$) for 20 min and that hybridization (24 h) and subsequent wash were both performed at 60°C . Hybridization signals were detected by chromogenic reaction using BCIP and NBT.

Whole-mount processed (stained) embryos were fixed with 3.5% formaldehyde in phosphate buffer (pH 7.4) for 12 h, mounted in PBST, and observed under incident light using an Olympus BX50 microscope. Some stained embryos were treated with $1\ \mu\text{g}/\text{ml}$ Hoechst 33258 for 20 min, and observed on a Leica MZFLIII fluorescence stereomicroscope. Some stained embryos were cleared according to the method described by Matsuo and Shimizu (2006) and observed with transmitted light.

2.5. Cell lineage tracing in WISH preparations

To label mesodermal cells specifically in WISH preparations, we injected a lineage tracer Fluorescein Dextran (Fl-D; Molecular Probes) into 4d cells of stage 8

embryos, which were cultured for 4 days before fixation. Tracer injection and subsequent embryo culture were performed according to the methods described previously (Kato et al., 2013). It has been demonstrated that fluorescent lineage tracers such as Fl-D injected into 4d cells (at stage 8) are inherited by all mesodermal cells including PGCs during development (see Kato et al., 2013). To verify that injected Fl-D distributed to mesodermal cells, we observed fixed embryos briefly under a fluorescence microscope. They were stored at -20°C in methanol until use.

To detect Fl-D and *Ttu-nos* transcripts in the same embryo, we utilized a double staining method modified from the triple staining method developed for *Xenopus* embryos by Koga et al. (2007). Briefly, rehydrated embryos were first subjected to WISH as described above, and stained for *Ttu-nos* transcripts. After immersion in 0.1 M glycine-HCl (pH 2.0) for 40 min to inactivate alkaline phosphatase (ALP) which was carried over from WISH, stained specimens were processed for immunostaining with anti-fluorescein Fab fragments conjugated to ALP (Roche) and a Fast Red tablet containing naphthol substrate and Fast Red chromogen (Roche). ALP activity products were precipitated in red in anti-fluorescein staining and in blue in WISH. Detailed protocols for anti-fluorescein/Fast Red immunostaining are available upon request.

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Figure legends

Fig. 1. Characterization of *Ttu-nos1*, a *nanos (nos)* homologue from *Tubifex tubifex*. (A) Alignment of the putative CCHC-type zinc finger domain of TtuNos1 with known *nos*-related proteins. Conserved C and H residues are highlighted in yellow and red, respectively. Numbers in parentheses indicate the percentage amino acid identity with TtuNos1. (B) Molecular phylogenetic analysis of the *nos* zinc finger domain. The following sequences were used: CgiNos1 (accession number EKC30400), CteNos (DAA06318), GgaNos1 (XP_003641548), DjaNos (BAD88623), DmeNos (AAA28715), DryNos (BAK57419), EflNos (BAB19253), HpuNos (BAE53723), HroNos (AAB63111), HvuNos2 (BAB01492), IobNos (ABV54788), MmuNos1 (NP848508), NveNos1 (AAW29070), NveNos2 (AAW29071), OlaNos1 (ABU63571), PduNos (CAJ28985), PleNos (ADE44350), SsaNos (NP001135057), SpuNos2 (NP001073023), *Ttu-nos1* (LC020234), *Ttu-nos2* (LC020235) and XlaXcat (NP001081503). The phylogenetic tree was generated by the neighbor joining method using PAUP*4.0b10. *Cel*Nos1 (NP496358) was used as an outgroup. Numbers are bootstrap values (as percentages of 1000 replications). Lengths of branches are drawn to the scale indicated. Species abbreviations: *Cel* *Caenorhabditis elegans* (nematode); *Cgi* *Crassostrea gigas* (mollusc); *Cte* *Capitella teleta* (annelid); *Dja* *Dugesia japonica* (planaria); *Dme* *Drosophila melanogaster* (fruit fly); *Dry* *Dugesia ryukyuensis* (planaria); *Efl* *Ephydatia fluviatilis* (sponge); *Gga* *Gallus gallus* (chick); *Hpu* *Hemicentrotus pulcherrimus* (sea urchin); *Hro* *Helobdella robusta* (annelid); *Hvu* *Hydra vulgaris* (cnidaria); *Iob* *Ilyanassa obsoleta* (mollusc); *Mmu* *Mus musculus* (mouse); *Nve* *Nematostella vectensis* (cnidaria); *Ola* *Oryzias latipes* (medaka); *Pdu* *Platynereis dumerilii* (annelid); *Ple* *Pristina leidy* (annelid); *Ssa* *Salmo salar* (salmon); *Spu* *Strongylocentrotus purpuratus* (sea urchin); *Ttu* *Tubifex tubifex* (annelid); *Xla* *Xenopus laevis* (frog).

Fig. 2. Summary of *Tubifex* development. Diagrammatic illustration of selected stages of embryonic development. (A-D) Animal pole views of embryos at stages 1-cell (A), 4-cell (B), 8-cell (C) and 10-cell (D). (E) Stage 8 embryo. Posterior view with dorsal to the top. Three cells (2d¹¹, 4d and 4D) of the D cell line come to lie in the future midline. (F) Stage 11 embryo with ectoteloblast precursors (NOPQl, NOPQr), mesoteloblasts (Ml, Mr) and endodermal precursors (E^D). (G-I) Stage 12

embryos undergoing ectodermal teloblastogenesis. Dorsal views with anterior to the top. N teloblast is generated first and located ventralmost (stage 12a), and the Q teloblast, which is generated next, is located dorsalmost (stage 12b); finally the O and P teloblasts are generated by almost equal division of their precursor cell, at which point teloblastogenesis is complete (stage 12c). During stage 12, germ bands begin to form and with time, elongate via addition of tiny blast cells generated from teloblasts (M, N and Q) and proteloblasts (OPQ and OP). Note that mesodermal germ bands are overlain by the ectodermal germ bands except for the portion proximal to the M teloblasts (I). For brevity of the figure, germ bands are omitted in (G) and (H). (J-L) Left side views (upper) plus ventral views (lower) of embryos (with anterior to the left) at stages 13-15 undergoing gastrulation that consists of ventralward movement of elongating germ bands (shaded) and spreading of micromere-derived epithelial cells (not depicted here) over the endoderm. The germ band is associated, at its anterior end, with an anteriorly located cluster of micromeres (called a micromere cap), and it is initially located at the dorsal side of the embryo (J). Along with their elongation, the germ bands on both sides of the embryo gradually curve round toward the ventral midline (K) and finally coalesce with each other along the ventral midline (L). (M-R) Left side views of embryos (with anterior to the left) undergoing body elongation, which begins in the anteriormost region of the embryo (M), continues in an anterior-to-posterior fashion (N-Q), and completes in the caudal end at stage 18 (R). Body elongation is accompanied by formation of segmental ectoderm, which is accomplished by dorsalward expansion of germ bands (shaded). Anterior is to the left; dorsal is to the top. Asterisks indicate the position of buccal cavity. pr, prostomium. (For details of morphogenetic movements, see Shimizu and Nakamoto, 2001.)

Fig. 3. Expression of *Ttu-nos* detected by *in situ* hybridization with an antisense riboprobe. (A)-(E), (G) and (I)-(K), uncleared specimens viewed with incident light; (F) and (H), cleared specimens viewed with transmitted light. (B) illustrates a specimen that was hybridized with a sense probe, and serves as a control of (A). (A) and (B) Oocytes in ovisacs isolated together with intestine (asterisks). Anterior is to the left and dorsal is to the top. Horizontal lines in (A) indicate three consecutive segments (XII, XIII and XIV); (B) shows segment XIV only. The ovisac shown in (A) contained four oocytes though the boundaries between oocytes were not illustrated well.

Note distinct and uniform staining in oocytes in (A) but not in (B). (C) and (D) 1-cell stage. Animal pole view. *Ttu-nos* RNA is concentrated to the **metaphase II** spindle at the animal pole (C) and to the pole plasm (D). (E) and (F) 8-cell stage. Animal pole view. The largest cell 1D inherits a large portion of *Ttu-nos* transcripts. (G) and (H) 10-cell stage. Left-side view with anterior to the top. The largest cell 2D and its sister cell 2d inherits a large portion of *Ttu-nos* transcripts. (I) Embryo undergoing 3d cell formation. Note intense staining in $2d^1$ (derived from 2d), 3d (arrowhead) and other micromeres. (J) 22-cell stage. Posterior view with dorsal to the top. Note intense staining in $2d^{11}$ (derived from $2d^1$), $2d^2$ (arrowhead), $2d^{12}$ (double arrowhead), and descendants of other micromeres (arrows). (K) Stage 11 embryo shortly after division of $2d^{11}$ (derived from $2d^{11}$) into a pair of NOPQ proteloblasts. Posterior view with dorsal to the top. Arrowheads indicate the shadow of staining signals emanating from internally located mesodermal blast cells that had most recently formed from M teloblasts. Note the absence of *Ttu-nos* staining in NOPQ. Scale bar in (A): (A-K) 200 μm .

Fig. 4. Expression of *Ttu-nos* during teloblastogenesis and germ band formation. All embryos except (C) were uncleared and viewed with incident light. Square brackets indicate domains in which ectoteloblasts (N, O, P and Q) and/or proteloblast OP are present. Note that contours of either ectoteloblasts or OP are not depicted here. Arrowheads indicate primary n blast cells adjacent to N teloblasts. Arrows indicate primary q blast cells adjacent to Q teloblasts. (A-C) Stage 12b embryo shortly after division of OPQ into OP and Q. (A) Anterior view with dorsal to the top; (B, C) dorsal view with anterior to the top. *Ttu-nos*-positive cells are seen in front of ectoteloblast domains (A and B). Note bundlets of positive cells extending anteriorly from M teloblasts whose contour is indicated by broken lines. (D-I) Embryos at stages 12b (D, E), 13 (F, G) and 14 (H, I) viewed (with dorsal to the top) from right side (D, F, H) and left side (E, G, I). Note weak staining signals seen in the domains of ectoteloblasts (square brackets) and more posterior portions. These signals are emanating from internally located mesodermal cells, but not from surface cells. A, anterior; P, posterior. Scale bar in (A): (A-I) 200 μm .

Fig. 5. *Ttu-nos*-expression in the n blast cell bundlet in stage 14 embryo. (A) Bright

field and (B) fluorescence (Hoechst 33258) images of the same embryo are shown. (C) is a digital superimposition of (A) and (B). Uncleared specimen. Right side view with anterior to the right. Horizontal lines with numerals indicate three consecutive portions of the n bundlet; the portion 1 is located proximal to the N teloblast (not depicted here). Note that n blast cells in the portion 2 exhibit much weaker staining signal than those in other portions. Arrows indicate a primary q blast cell adjacent to Q teloblast (not depicted). Scale bar in (A): (A-C) 200 μm .

Fig. 6. Expression of *Ttu-nos* in embryos undergoing gastrulation. (A-D) Stage 15 embryo viewed from antero-ventral (A), right side/ventral (B), right side/posterior (C) and ventral (D). (A) Dorsal is to the top; (B-D) anterior is to the top. Uncleared specimen. (E and F) Stage 16a embryo stained doubly for *Ttu-nos* transcripts (blue) and fluorescein dextran (red) which had been injected into 4d cell (at stage 8) as a tracer of the mesodermal lineage (see Experimental procedure). D, dorsal; V, ventral. (E) Left side view with anterior to the top. Note *Ttu-nos*-positive cells in ventral ganglia. (F) A transverse section of the middle region (at the level of segment X) of the embryo shown in (E). Note the absence of *Ttu-nos*-positive cells from the mesodermal region comprised of the progeny of m blast cells (red). Scale bar in (A): (A-F) 200 μm .

Fig. 7. Expression of *Ttu-nos* in embryos undergoing body elongation. Embryos at stages 16a (A), 16b (B), 17a (C) and 17b (D) are viewed from left side with anterior to the left. Asterisks indicate the position of buccal cavity. Note that expression in the ectodermal germ bands declines in anterior-to-posterior progression. Scale bar in (A): (A-D) 200 μm .

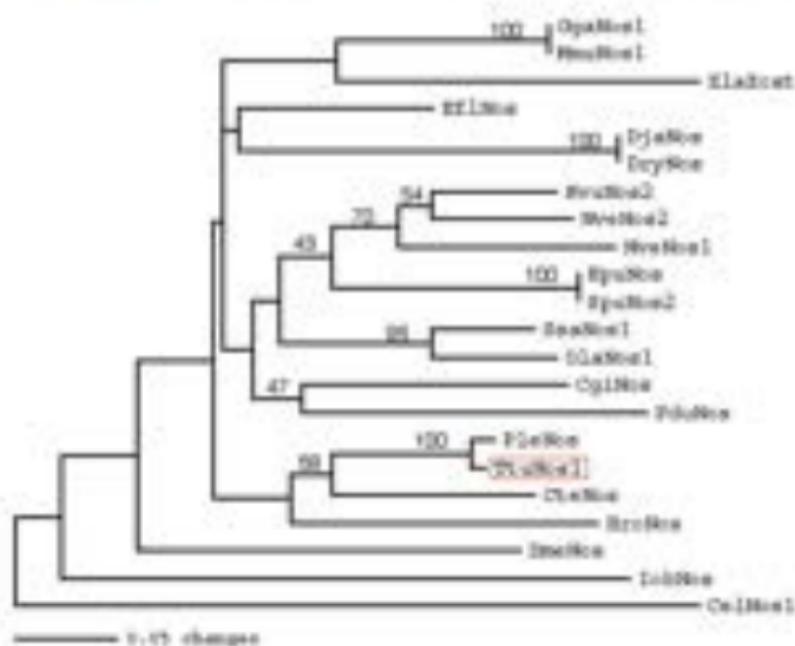
Fig. 8. Stage 16c embryo that was double-stained for *Ttu-nos*-expressing cells (blue) and fluorescein dextran (Fl-D)-labeled mesoderm (red). Ventral view with anterior to the top. (B) is an enlargement of (A). The embryo whose 4d cell had been injected with Fl-D at stage 8 was allowed to develop for five days (up to stage 16c) before fixation, and processed for double-staining as described in Experimental procedures. Vertical lines with roman numerals in (B) indicate the position of segments IX-XIV. Arrowheads indicate pre-PGCs in segments X-XII. In this embryo, *Ttu-nos*-positive cells in the ectoderm were confined to segment XIII and more posterior segments (B).

Scale bar in (A): (A) 200 μm ; (B) 100 μm .

Fig. 9. Expression of *Ttu-nos* in genital segments of a young adult worm. Fragments containing genital segments that were dissected out from adults were processed for WISH as described in Experimental procedures. (A) Dorsal view of genital segments (X and XI), showing a pair of spermatheca (asterisks) in segment X and a pair of ovaries in segment XI. The image shown here was captured using an Olympus microscope. Square brackets indicate the dorsal portion of the ovary, which exhibited intensive *Ttu-nos* expression. (B-D) Enlargement of the ovaries shown in (A). (B) Bright field and (C) fluorescence (Hoechst 33258) images are digitally superimposed in (D). Asterisks indicate the basal (ventral) portion of the ovary. (E) Diagrammatic illustration of the reproductive system in *Tubifex tubifex*. Sperm funnel and vas deferens are omitted. Dorsal view of segments IX-XIII is shown. Asterisks indicate sperm sac. Note spermathecae (st) in segment X and ovaries in segment XI. os, ovisac; ts, testis. After Hirao (1964) and Jaana (1982). Scale bar in (A): (A) 200 μm ; (B-D) 100 μm .

Ttu00a1 C V F C R H S G E A E A Y V T S H G L K D F S G A Y T C P I L T I Y T C P I C G A S S G S A N T I R Y C P (100)
 Ttu00a2 C V F C R H S G E A E A Y V T S H G L K D F S G A Y T C P I L T I Y T C P I C G A S S G S A N T I R Y C P (100)
 Fla00a C V F C R H S G E A E A Y V T S H G L K D F S G A Y T C P I L T I Y T C P I C G A S S G S A N T I R Y C P (98)
 Cta00a C V F C R H S G E A E A Y V T S H G L K D A G K I V C P V L T I Y T C P I C G A S S G S A N T I R Y C P (83)
 Hsu00a C V F C R H S K E F P C V A M G L V E D E K Q G V T C P I L T I Y T C P I C G A S S G S A N T I R Y C P (77)
 Cgi00a C V F C R H S G E T V A I T S S H V L K E D S G R V C P I L R K Y T C P L G V S S G S A N T I R Y C P (68)
 Fdu00a C V F C T S G E S E V I T T S H V L K E D S G R V C P I L R A Y T C P L G A S S G S A N T I R Y C P (64)
 Iob00a C F P L A C K E Q A D I V A N S L P L A G S G V C P I L Q Q Y R C V C H A T S G K A N T I R Y C P (51)
 Daa00a C V F C R H S E F K A V I N S H V S D S P S V L C K L S T Y V C P I C G A S S G S A N T I R Y C P (64)
 Dja00a C V F C R H S E F P D G S V S K V E L L S G K Y T C P V L R S Y T C P L G A S S G S A N T I R Y C P (62)
 Bru00a1 C V F C R H S G E S K V Y S S H V L K E D A G S T T C P I L R A Y T C P L G A S S G S A N T I R Y C P (70)
 Efi00a C V F C R H S G E S E F Y T S H V L K E D A S K Y T C P V L R A Y T C P L G A S S G S A N T I R Y C P (77)
 Spu00a C V F C R H S G E S S F A S K L E S G I T T C P I L R A Y T C P L O T T S G S A N T I R Y C P (71)
 Oia00a1 C V F C R H S G A F E E Y S T H I L K T O S S V L C P I L R A Y T C P L C A S S G S A N T I R Y C P (70)
 Xia0cat C G P C R H S E A L S L T T S H R L R A L D S S V L C P V L R S Y T C P L G A S S G S A N T I R Y C P (60)
 Hsu00a1 C V F C R H S K E A V A L Y T T H I L S P D S G S V L C P V L R S Y T C P L G A S S G S A N T I R Y C P (70)

A



B

