

The *Dr-nanos* gene is essential for germ cell specification in the planarian *Dugesia ryukyuensis*

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ABSTRACT Homologs of *nanos* are required for the formation and maintenance of germline stem cell (GSC) systems and for gametogenesis in many metazoans. Planarians can change their reproductive mode seasonally, alternating between asexual and sexual reproduction; they develop and maintain their somatic stem cells (SSCs) and GCSs from pluripotent stem cells known as neoblasts. We isolated a *nanos* homolog, *Dr-nanos*, from the expressed sequence tags (ESTs) of the sexualized form of *Dugesia ryukyuensis*. We examined the expression of *Dr-nanos* in asexual and sexualized planarians by *in situ* hybridization and analyzed its function using RNA interference (RNAi) together with a planarian sexualization assay. A *nanos* homolog, *Dr-nanos*, was identified in the planarian *D. ryukyuensis*. *Dr-nanos* expression was observed in the ovarian primordial cells of the asexual worms. This expression increased in proportion to sexualization and was localized in the early germline cells of the ovaries and testes. In X-ray-irradiated worms, the expression of *Dr-nanos* decreased to a large extent, indicating that *Dr-nanos* is expressed in some subpopulations of stem cells, especially in GSCs. During the sexualization process, worms in which *Dr-nanos* was knocked down by RNAi exhibited decreased numbers of oogonia in the ovaries and failed to develop testes, whereas the somatic sexual organs were not affected. We conclude that *Dr-nanos* is essential for the development of germ cells in the ovaries and testes and may have a function in the early stages of germ cell specification, but not in the development of somatic sexual organs.

KEY WORDS: *planarian, Nanos, germline stem cell, germ cell specification*

Introduction

Germ cells are specialized cells that transmit genetic information from generation to generation during sexual reproduction. They are totipotent stem cells that can differentiate into any cell type. Germ cells must form correctly in order to produce offspring and to ensure the survival of the species through the generations. There are at least 2 distinct modes of germline segregation in metazoans: germ cells can be specified by maternally inherited determinants (preformation) and by inductive signals (epigenesis) (Extavour and Akam, 2003; Johnson *et al.*, 2003).

The *nanos* gene encodes an RNA-binding protein containing a zinc finger motif, which is conserved in diverse metazoans. In *Drosophila*, *nanos* is required for abdominal segmentation (Lehmann and Nüsslein-Volhard, 1991; Wang and Lehmann, 1991) and

germ cell differentiation and maintenance (Forbes and Lehmann, 1991; Kobayashi *et al.*, 1996; Hayashi *et al.*, 2004). In nematoda, zebrafish, and mice, orthologs of *nanos* are reported to be involved in the migration of the primordial germ cells (PGCs) to the gonads, and in the transcriptional quiescence and mitotic arrest required for the maintenance of germline identity during migration (Subramaniam and Seydoux, 1999; Köprunner *et al.*, 2001; Tsuda *et al.*, 2003; Parisi and Lin, 2000; Suzuki *et al.*, 2007).

Freshwater planarians exhibit a robust regenerative ability

Abbreviations used in this paper: dsRNA, double strand RNA; EST, expressed sequence tag; GSC, germline stem cell; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; PGCs, primordial germ cells; RACE, 3' and 5' rapid amplification of cDNA ends; RNAi, RNA interference; RT-PCR, reverse transcription polymerase chain reaction; SSC, somatic stem cell; WISH, whole-mount *in situ* hybridization.

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Final, author-corrected PDF published online: 16 March 2012

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Dr-Nanos (D. ryukyensis)      IELCVFCRNNNEPFEMYVSHKVKDLNGKVTCPVLRNYTCPLCNSTGDFAHITIKYCPISSNSSKGLVE
DjNOS (D. japonica)         IELCVFCRNNNEPFEMYVSHKVKDLNGKVTCPVLRNYTCPLCNSTGDFAHITIKYCPISSNSSKSLVE
Smed-Nanos (S. mediterranea) IELCVFCRNNNEPFEMYVSHKVKDLNGKVTCPVLRNYTCPLCNSTGDFAHITIKYCPISSMNTTKQVSDNSLEEITRNQIAIKSRMSQSTLGIA
Nanos (Drosophila)         SRHCVFCENNEPEAVINSHSVRDNFNRVLCPKLRTYVCPICGASGDSAHITIKYCPKPKPITIMEDATIAESFRLAKSSYYKQQMKV
Nos1 (Zebrafish)           PKICVFCRNNNGAPEEVYGSVHLKTPDGRVVCPIILRAYTCPLCSANGDNAHTIKYCPLSKDQPAQRVLRKGGRAVGGKRVKIF
XCAT-2 (Xenopus)          HKGGCFRNSRREALSLYTSRLRALDGRVLCPLRGYTCPLCGANGDNAHTMRYCPLRRLRLDPQSNNSNPKLRH
Nanos1 (Mouse)            LQVCVFCRNNKEAVALYTTHTLKGPDGRVLCPLRRTYTCPLCGASGDNAHTIKYCPLSKVPPPTVRRPPRSNRDSLPSKLLR
NANOS1 (Human)           LQVCVFCRNNKEAMALYTTHTLKGPDGRVLCPLRRTYTCPLCGASGDNAHTIKYCPLSKVPPPPARPPRSARDGPPGKLLR

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Fig. 1. Alignment of the C-terminal region of the NANOS proteins from different species. NANOS zinc-finger domain is indicated by the black line; black boxes indicate identical residues in all aligned proteins; gray boxes indicate residues conserved in at least 5 of the aligned proteins; open boxes indicate the tail end region required for germ cell migration. The full-length cDNA and protein sequences of *Dr-Nanos* have been deposited in DDBJ/EMBL/GenBank databases (<http://www.ddbj.nig.ac.jp>) with the accession number AB650592.

that is mediated by the neoblasts—a type of pluripotent stem cells (Wolff and Dubois, 1948; Bagaña et al., 1989). Many planarians can be categorized according to their different reproductive modes: exclusively asexual, seasonally sexual, and/or exclusively sexual (Jenkins, 1967). Although sexual planarians do not reproduce asexually or by spontaneous fission, they can regenerate all their tissues, including the germline cells and somatic cells of the reproductive organs, after artificial amputation (Morgan, 1901; Newmark et al., 2008). Therefore, neoblasts apparently give rise to both somatic stem cells (SSC) and germline stem cells (GSCs). The GSCs are essential for the continuous production of gametes and are present in a variety of animals ranging from *Hydra* to insects and mammals (Lin, 1997; 1998). Reproducing in one of the modes described above by the influence of environmental conditions facilitates survival of the species. It is very important that a certain amount of GSCs are maintained during asexual reproduction in order to initiate the immediate formation of the germ cells following the switch to the sexual reproductive mode.

Recent studies of *nanos* in the planarians *Dugesia japonica* and *Schmidtea mediterranea* suggested that *nanos*-positive cells in asexual worms are GSCs, and in sexual worms, they are the oogonia and spermatogonia; the germ cells are differentiated from GSCs according to the development of the reproductive organs (Sato et al., 2006; Wang et al., 2007; Handberg-Thorsager and Saló, 2007). However, the function of the *nanos* gene during the change from asexual to sexual reproduction remains unknown.

To investigate the functions of the *nanos* gene during the conversion of the reproductive modes, we have established an experimental bioassay system to induce sexual switching (sexualization) (Kobayashi et al., 1999). In this system, the OH strain of *D. ryukyensis*, which has been maintained through asexual reproduction without gonads, can be induced to reproduce sexu-

ally by feeding worms of an exclusively oviparous species. During sexualization, the germline cells and the somatic cells of the reproductive organs, such as the ovaries, testes, genital pores, and yolk glands are differentiated in the same order and in similar positions during the developmental process of sexual individuals. This experimental system of sexualization enables us to follow the development of the germline cells in detail, and it is possible to evaluate the function of *nanos* during germ cell formation by a combination of RNA interference (RNAi) and this assay system. In this study, we isolated the *Dr-nanos* gene and characterized its function in germline cells and in reproductive organ formation.

Results

Identification of the *Dr-nanos* gene

The *nanos* homolog was isolated from the expressed sequence tags (ESTs) of the sexualized form of *D. ryukyensis* and was named *Dr-nanos*. *Dr-nanos* encodes a protein of 179 amino acids, with 2 conserved NANOS zinc finger motifs (Fig. 1). *Dr-Nanos* showed a high sequence identity with NANOS of *D. japonica* (DjNOS; 97.8%) and *S. mediterranea* (Smed-Nanos; 80.4%). However, the C-terminus domain, following the zinc finger motifs seen in the insects, did not exist in NANOS of the planarians.

Expression patterns of *Dr-nanos* in the asexual planarian

The *nanos*-positive cells observed in the asexual planarian worms were presumed to be the GSCs. To examine the expression pattern of *Dr-nanos*, we performed whole-mount *in situ* hybridization (WISH) in the asexual *D. ryukyensis* OH strain. *Dr-nanos* was expressed in the cells clustered along 2 ventral lines (Fig. 2A). In the anteroventral mesenchyme of the asexual worm, oogonia-like cells form small cell clusters known as the ovarian primordia

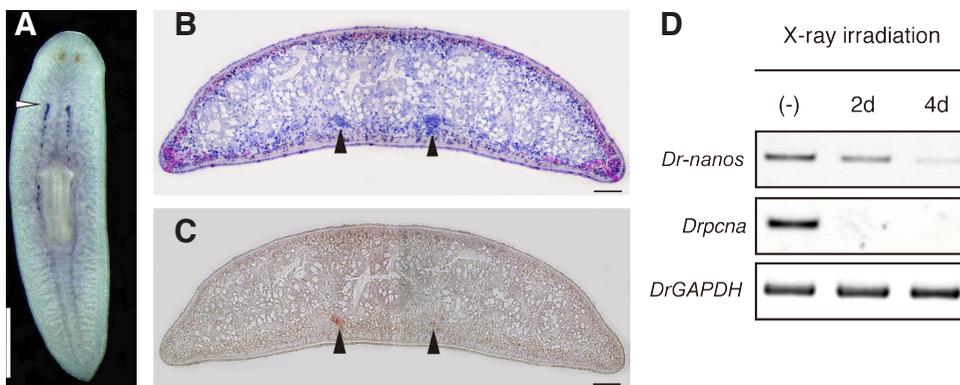


Fig. 2. Analysis of *Dr-nanos* expression in the asexual planarian *D. ryukyensis*. (A) Whole-mount *in situ* hybridization (WISH) in an intact planarian. *Dr-nanos* expression observed in the anteroventral region—the presumptive ovarian region—indicated by white arrowheads. Scale bar: 1 mm. (B,C) Transverse section of the region of the ovarian primordia: (B) HE stain and (C) *in situ* hybridization. *Dr-nanos* expression in the ovarian primordia is indicated by black arrowheads. Scale bars, 100 μm. (D) Expression levels of the *Dr-nanos*, *Drpcna*, and *DrGAPDH* genes after X-ray irradiation, examined by RT-PCR. (-), Not irradiated; 2d, 2 days after irradiation; 4d, 4 days after irradiation.

GAPDH genes after X-ray irradiation, examined by RT-PCR. (-), Not irradiated; 2d, 2 days after irradiation; 4d, 4 days after irradiation.

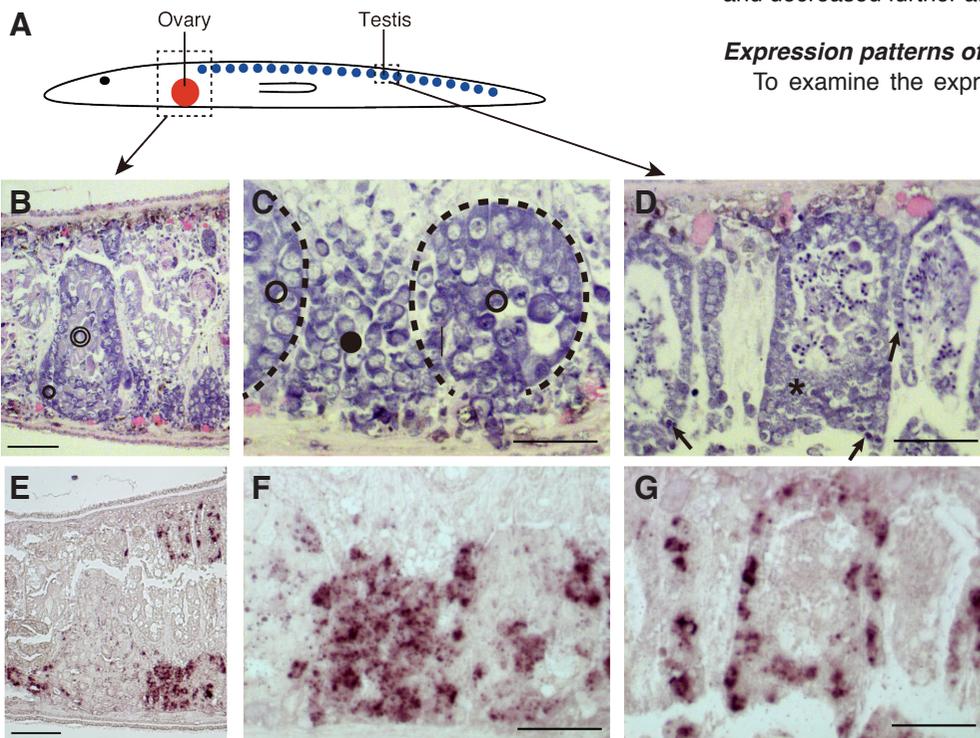
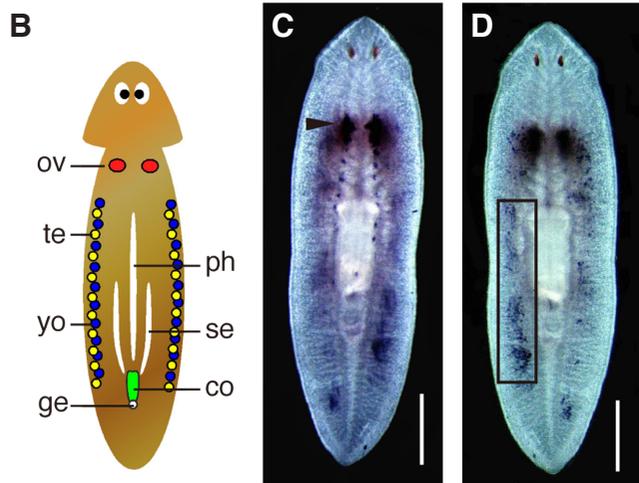
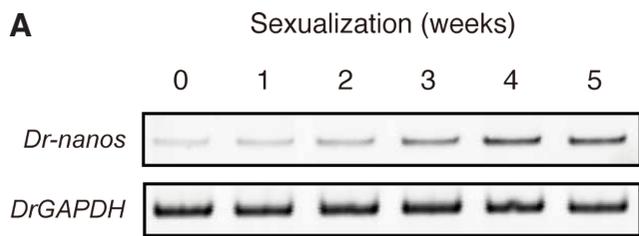


Fig. 4. Analysis of *Dr-nanos* expression in germline cells. (A) Scheme of a sagittal section of a sexualized planarian. The dashed boxes indicate the ovarian region (B) and testicular region (D). (B–D) HE stain. (E–G) In situ hybridization of a sagittal section for *Dr-nanos*. (B, E) Region around the ovaries; (C, F) higher magnification in the peripheral region of ovaries. The dashed lines separate each ovary. (D, G) Testes. Closed circle, early oogonia; open circles, oogonia; double circle, oocytes; arrow, spermatogonia; asterisks, spermatocyte. Scale bars, 100 μm .

Fig. 3 (Left). Analysis of *Dr-nanos* gene expression in sexualized worms. (A) Comparison of *Dr-nanos* expression levels during sexualization, examined by RT-PCR. (B) Scheme of a sexualized planarian: ph, pharynx; ov, ovary; te, testis; yo, yolk gland; se, seminal vesicle; co, copulatory apparatus; and ge, genital pore. (C,D) *Dr-nanos* expression in sexualized worms detected by WISH: (C) ventral views; (D) dorsal views. Expression in ovaries is indicated by arrowheads. The black box indicates expression in the testes. Scale bars, 1 mm.

(Fig. 2B). Then, using *in situ* hybridization on sections from these cells, *Dr-nanos* signals were specifically detected in the cells of the ovarian primordia (Fig. 2C). In many cases, sexualized worms developed several supernumerary ovary pairs along the ventral nerve cords, which reached the pharyngeal level (Sakurai, 1981; Kobayashi and Hoshi, 2011). The cells indicated by WISH may be in the presumed ovarian region. Meanwhile no such cell clusters were observed in the dorsal region, which is the presumed testicular region, and the *Dr-nanos* signal was not detected in this region.

It is well known that X-ray irradiation causes the loss of regenerative ability by the rapid and specific destruction of the neoblasts (Wolff and Dubois, 1948; Bagnuà *et al.*, 1989; Orii *et al.*, 2005). To compare the characteristics of the neoblasts and *Dr-nanos*-positive cells, we examined changes in *Dr-nanos* mRNA levels 2 and 4 days after irradiation. Two days after X-ray irradiation, elimination of the neoblasts was confirmed, because *Drpcna*, which is necessary for DNA replication, was almost eliminated. The mRNA levels of *Dr-nanos* were largely decreased after 2 days of X-ray irradiation, and decreased further after 4 days (Fig. 2D).

Expression patterns of *Dr-nanos* in germline cells

To examine the expression pattern of *Dr-nanos* in germline cells, the change in *Dr-nanos* mRNA levels was examined by comparative RT-PCR during the process of sexualization. The expression of *Dr-nanos* increased during sexualization (Fig. 3A).

WISH of the sexualized worms revealed that *Dr-nanos* was expressed in the ovaries in the anteroventral area and in the testes in the bilateral dorsal area (Fig. 3 C,D). *In situ* hybridization observed on sections indicated that *Dr-nanos* mRNAs were expressed in the ovaries and testes. In the early stages of planarian gonad development, the oocytes are located in the center of the ovary and the oogonia are located in the ventral periphery. The most peripheral oogonium-like cells outside an ovarian mass may be early oogonia or proliferated germline stem cells (Fig. 4B, C). Similarly, in the testes, the spermatogonia and spermatocytes are observed in the periphery, and the spermatid and sperm are located in the center of the testes (Fig. 4D). *Dr-nanos* was mainly expressed in the more peripheral cells

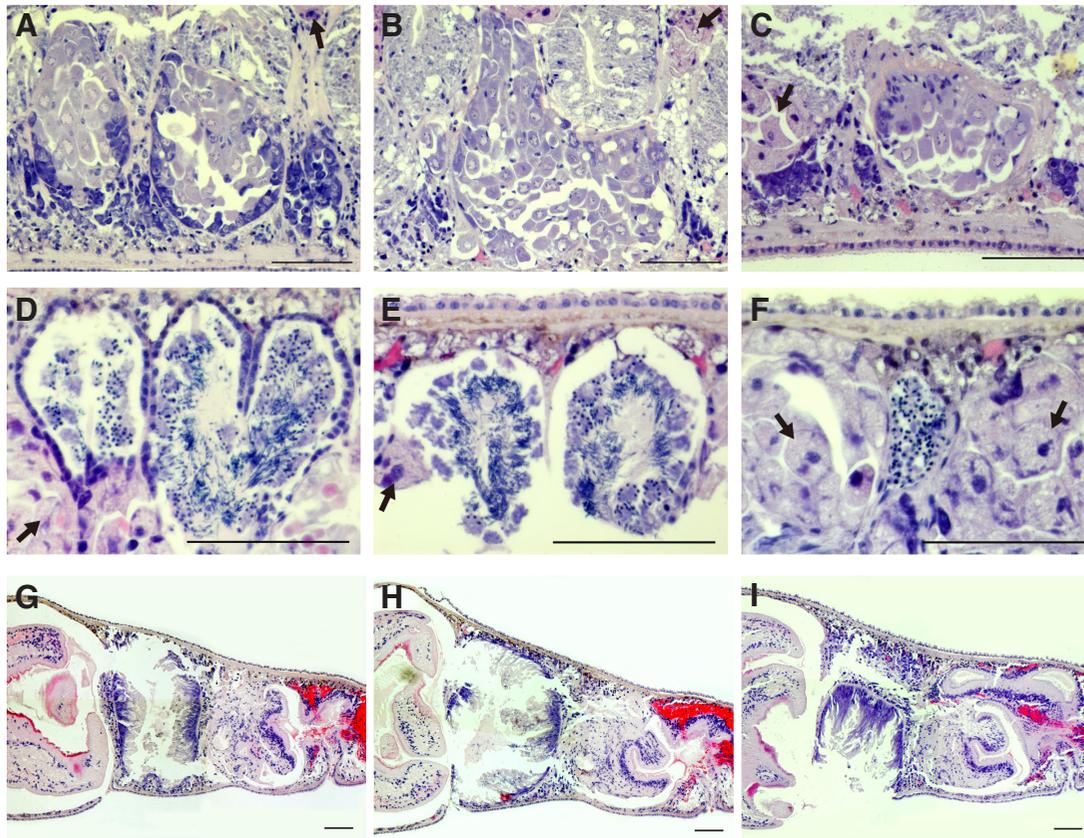


Fig. 5. Analysis of *Dr-nanos* function during maintenance of sexual worms. (A-F) HE staining of sagittal sections of the acquired sexual worms with *Dr-nanos* RNAi. (A-C) Ovaries, (D-F) testes, and (G-I) copulatory apparatus. (A, D, and G) Day 0 with dsRNA feeding; (B) ovary at 20 days and (C) 40 days; (E) testes at 20 days and (F) 30 days; (H) copulatory apparatus at 20 days and (I) 50 days. Arrows indicate yolk glands. Scale bars, 100 μm .

of the ovaries, i.e., the early oogonia or GSCs. Its expression was weaker in the oogonia than in the GSCs, and it was not detected at all in the oocytes (Fig. 4 E,F). In the testes, *Dr-nanos* was expressed as expected in the spermatogonia; however, expression signals were not detected in the spermatids or in the sperm inside the testes (Fig. 4G).

Functional analysis of *Dr-nanos* during the maintenance of sexual worms

To identify which process of germ cell formation is regulated by *Dr-nanos*, we performed an RNAi knockdown on *Dr-nanos* in mature sexualized worms with fully developed reproductive organs and germ cells. Twenty days after *Dr-nanos* silencing, spermatids and sperm were observed inside the testes, but no spermatogonia or spermatocytes were detected in the periphery (Fig. 5E). Similarly, the ovaries contained oocytes but no oogonia (Fig. 5B). Despite this, the copulatory apparatus (Fig. 5H) and the yolk glands (Fig. 5 B,E) had developed normally with the correct morphology. The testes became smaller at 30 days (Fig. 5F) and had completely disappeared at 40 days. The ovaries were not changed at 30 days, became extremely small at 40 days (Fig. 5C), and finally, were hardly visible at 50 days. The copulatory apparatus (Fig. 5I) and the yolk glands (Fig. 5 C,F) did not regress at 50 days.

Functional analysis of *Dr-nanos* during germ cell formation

To investigate the role of *Dr-nanos* in the process of germ cell

formation from germline precursor, we performed RNAi on *Dr-nanos* with the sexualization assay system. In the *Dr-nanos*(RNAi) worms, the testes did not develop, and no primordial or immature testes were observed (Fig. 6B). The gene encoding the Y-box protein DrC_00456, a homologue of *DeY1* in *Dugesia etrusca* (Salveti et al., 2002) and *MSY4* in *S. mediterranea* (Wang et al., 2010), all of which are specific markers for the testes, was not detected by WISH anywhere in the body (Fig. 6G). Moreover, there seemed to be few oogonia in the ventral periphery of the ovary (Fig. 6A). Meanwhile, the somatic reproductive organs (copulatory apparatus and yolk glands) were morphologically normal (Fig. 6 B,C).

Furthermore, after being maintained with RNAi food for a month following the RNAi sexualization, the *Dr-nanos*(RNAi)-sexualized worms that lacked testes were maintained for 5 months and fed their usual food (chicken liver without double strand RNA (dsRNA)). Most individuals returned to morphologically asexual reproduction involving repeated fission and regeneration. Histological observations revealed that the copulatory apparatus had disappeared, the morphological ovary no longer existed, and instead, only the oogonia or stem cells were observed.

Discussion

Germline stem cells express *nanos* in *D. ryukyensis*

Nanos proteins contain 2 conserved NANOS zinc finger motifs, which are known to be necessary for the formation of germ cells

and for abdominal segmentation in *Drosophila*. It is reported that the C-terminal region following these motifs, which is conserved in insects, is required for abdominal segmentation and the migration of the PGCs to the gonads during embryogenesis but is not required for germ cell formation (Arrizabalaga and Lehmann, 1999). In this study, a *nanos* ortholog was identified in *D. ryukyuensis* as *Dr-nanos*, which encoded a protein with 2 conserved NANOS zinc finger motifs. However, the C-terminal domain in NANOS did not exist in *Dr-nanos*, as observed in 2 *nanos* genes identified in different planarians (Sato *et al.*, 2006; Wang *et al.*, 2007; Handberg-Thorsager and Saló, 2007). It is suggested that the function of *nanos* in planarians is limited to the formation of germ cells.

In asexual worms, a functional ovary does not exist. Instead, GSCs are formed and maintained in the prospective ovarian region and may contribute to switching between the reproductive modes in these worms. *Dr-nanos* was expressed in the early oogonia and spermatogonia of the sexualized worms and in the oogonia-like cells of the primordial ovaries of the asexual worms. Furthermore, *Dr-nanos* expression signals were detected in the cells of the presumed ovaries, whereas they were not detected in the prospective testicular region. In 2 other planarian species, *D. japonica* and *S. mediterranea*, *nanos* ortholog genes were found to be *Djnos* and *Smed-nanos*, respectively. The expression signals of *Djnos* and *Smed-nanos* in the asexual worms were distributed in the prospective testes and ovarian regions (Sato *et al.*, 2006; Wang *et al.*, 2007; Handberg-Thorsager and Saló, 2007). The male GSCs in the asexual form of *D. ryukyuensis* may be non-existent or too small to be observed, and it is expected that the GSCs or spermatogonia (without GSC formation) develop from the neoblasts at the beginning of the sexualization process.

Dr-nanos function during germline development

The results of the *Dr-nanos* knockdown in the mature sexualized worms, along with the expression pattern of *Dr-nanos*, indicated that *Dr-nanos* is necessary for the formation and the maintenance of the oogonia, spermatogonia, and GSCs. Additionally, it appears

that *Dr-nanos* is not involved in the later process of gametogenesis because the oocytes, spermatids, and sperm were present, even when the cells were eliminated before meiosis. The results of the *Dr-nanos* knockdown during the sexualization process showed that the spermatogonia and the cells at subsequent developmental stages did not appear, and that the testes had not formed at all. This supports the possibility that *Dr-nanos* is essential during the early stages of germline specification in the testes. Meanwhile, the ovaries were not eliminated completely, and the oogonia had decreased. This may be because GSCs are present only in the presumed ovarian region, which is developed by sexualization, in asexual worms. However, after a certain time, the oogonia may not be formed, as the replenishment of the GSC supply (formed from the neoblasts) or the GSC turnover by self-renewal does not occur. Moreover, the suppression of *Dr-nanos* expression did not influence the formation of the somatic reproductive organs, which showed that *Dr-nanos* has a specialized key role in germline development.

Incidentally, the sexualized *Dr-nanos*(RNAi) worms, which lack testes, induced the regression of the ovaries and the copulatory apparatus and became asexually reproductive after discontinuation of the gene silencing (data not shown). Ovary formation occurs earlier than testes formation during the sexualization process. Most likely, the formation of the ovary is induced by signals at the beginning of the sexualization process, but the maintenance of the ovaries, copulatory apparatus, and yolk glands (i.e., maintenance of the sexual state) might depend on the testes.

Comparison of germline stem cell (GSC) regulation between planarian species

In the asexual worm, the expression of *Dr-nanos* was only observed in the ovarian primordia; however, in *D. japonica* and *S. mediterranea*, *nanos* mRNA was expressed not only in the ovarian primordia, but also in the prospective testes region (Sato *et al.*, 2006; Wang *et al.*, 2007; Handberg-Thorsager and Saló, 2007). Their phenotypes in the sexual worm after RNAi treatment differed from the phenotype of *D. ryukyuensis*, which

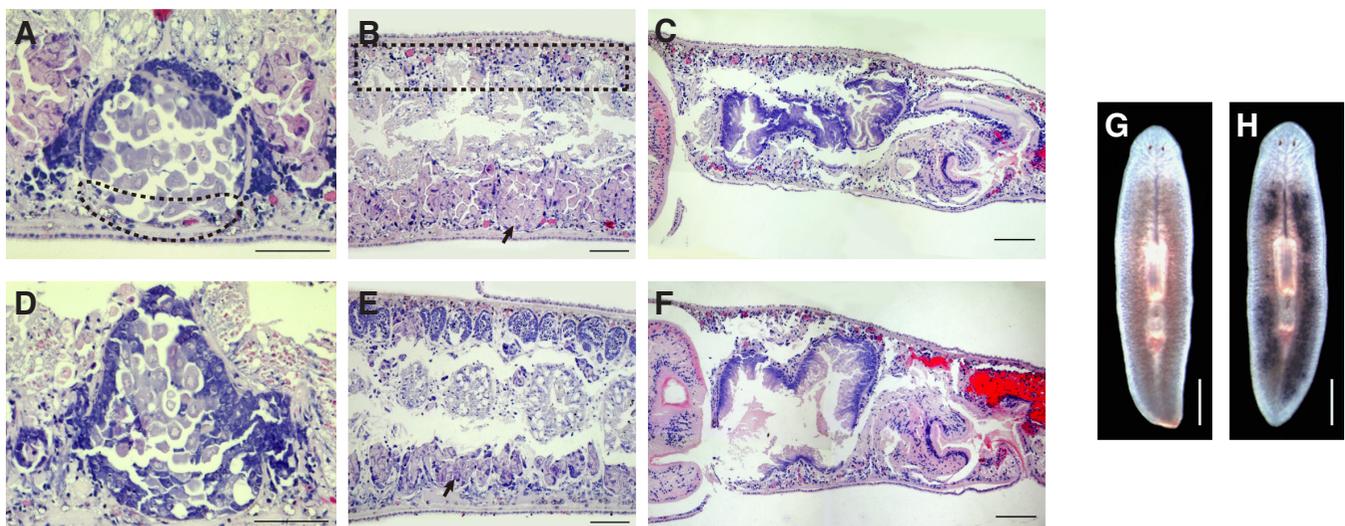


Fig. 6. Analysis of *Dr-nanos* function during sexualization. (A-F) HE staining of sagittal sections of the sexualizing worms at 40 days with *Dr-nanos* RNAi. **(A-C)** *Dr-nanos*(RNAi) worm and **(D-F)** EGFP(RNAi) control worm. **(A, D)** Ovaries; **(B, E)** region around the testes; and **(C, F)** copulatory apparatus. Arrows indicate yolk glands. The affected regions are enclosed with a dotted line. Scale bars, 100 μ m. **(G,H)** WISH for *DrC_00456*, a specific marker for the testes. **(G)** *Dr-nanos*(RNAi) worm and **(H)** EGFP(RNAi) control worm. Scale bars, 1 mm.

lacked testes and had abnormal ovaries. All the reproductive organs—the ovaries, testes, and copulatory apparatus—were not developed in the *Smed-nanos(RNAi)* sexual worms, whereas no special phenotype was observed during the sexualization process in the *Djnos(RNAi)* worms. In a comparison of reproductive abilities, both *D. ryukyensis* and *D. japonica* can switch their reproductive modes, but *S. mediterranea* cannot exercise experimental sexualization. Furthermore, *D. ryukyensis* OH strains change from the asexual to sexual reproductive modes more easily and promptly than *D. japonica*. Therefore, the planarian system for germ cell specification such as the spatial and temporal regulation of the GSCs differs among planarian species. From these results, it could be demonstrated that the asexual worm form of *D. ryukyensis* contains only ovarian GSCs and retains the asexual reproductive state more than *D. japonica* and *S. mediterranea*, which instead have both ovarian and testicular GSCs. Therefore, differences in their *nanos* gene expression patterns and phenotypes of *nanos* RNAi would be caused by the GSC condition in each planarian species. Among them, we considered the *nanos* functions in the early stages of germ cell specification, which are common to the planarian species. The molecular mechanism controlling NANOS has not yet been fully clarified, except for in *Drosophila*; therefore, our sexualization experiment carried out on *D. ryukyensis* is a powerful tool to study the development of neoblasts into testicular GSCs. This research will provide further understanding of NANOS in the metazoans.

Dr-nanos, the *nanos* homolog in *D. ryukyensis* is expressed in the presumed GSCs and immature germline cells. However, the expression of *nanos*-positive GSCs and the phenotypes resulting from the knockdown of *nanos* are different according to the species and may depend on the difference in time points when the GSCs are regulated, the germ cell formation process, and reproductive mode of each species. In the asexual worm form of *D. ryukyensis*, only the ovarian GSCs and not the testicular GSCs are specified; therefore, the cell development can be observed during the specification process as the neoblasts convert to testicular GSCs during *D. ryukyensis* sexualization experiments. In order to study the mechanisms underlying the germ cell developmental process in *D. ryukyensis*, further studies must be conducted to clarify the regulation of the *nanos* gene and the function of the NANOS protein.

Materials and Methods

Animals

An exclusively fissiparous strain, the OH strain of the planarian *D. ryukyensis*, was provided by Dr. S. Ishida of Hirosaki University, Hirosaki, Japan. The planarians were maintained at 20°C in dechlorinated tap water and were fed chicken liver. The OH strain was completely sexualized using the feeding procedure described by Kobayashi et al., (1999).

Identification and cloning of *Dr-nanos* genes

Clones of the *nanos* homolog, *Dr_sW_003_K04* and *Dr_sW_028_K12* in the *D. ryukyensis* ESTs (Ishizuka et al., 2007), included the complete *Dr-nanos* open reading frame. The full-length sequences of *Dr-nanos* were obtained using 3' and 5' rapid amplification of cDNA ends (RACE) using a SMART™ RACE cDNA Amplification Kit (Clontech, Mountain View, CA, USA).

In situ hybridization and histological analysis

WISH was carried out as described previously (Umesono et al., 1997; Higuchi et al., 2008). Digoxigenin-labeled riboprobes were synthesized using an *in vitro* Transcription Kit (Roche Applied Science, Indianapolis, IN, USA).

In situ hybridization of tissue sections was performed as previously described by Kobayashi et al., (1998); the fixative and the hybridization buffer were modified as below. Recipients were treated with 2% (v/v) hydrochloric acid in 20 mM HEPES(4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid)-buffered 5/8 Holtfreter's solution (pH 7.4) for 5 min at 4°C, which was then replaced with fixative (4% paraformaldehyde in 20 mM HEPES-buffered 5/8 Holtfreter's solution, pH 7.4) for 12–16 h, at 4°C. Fixed samples were dehydrated gradually through an ethanol series, cleared with xylene, and embedded in Paraplast™ Plus (Sherwood Medical, St. Louis, MO, USA). Transverse and sagittal sections were cut at a thickness of 4 µm. Sections were used for *in situ* hybridization or stained with hematoxylin and eosin (HE). Sexualized worms were fully matured by feeding with chicken liver several times, after complete sexualization, using the feeding procedure described by Kobayashi et al., (1999).

X-ray irradiation

The planarians were placed on wet filter paper on ice and exposed to 10 Gy of X-ray irradiation using an ultrasoft X-ray generator SOFTEX E-3 (Softex Co., Ebina, Japan).

Reverse transcription polymerase chain reaction (RT-PCR)

Total RNA was isolated using Sepasol (Nacalai Tesque, Kyoto, Japan), treated with DNase (Promega Corp., Madison, WI, USA), and transcribed using a ReverTra Ace kit (Toyobo, Tokyo, Japan). RT-PCR for the GAPDH gene was also performed as an internal amplification control. *Drpcna* is a homolog of *Djpcna*, which is present in proliferative- and X-ray-sensitive cells that correspond to the neoblasts (Orii et al., 2005). PCR amplification of cDNA was performed using a set of specific oligonucleotide primers:

Dr-nanos

Forward: 5'-GACAAATATGAATCCTTCACTT-3'

Reverse: 5'-GAATTTGAAGAAATTGGACAATA-3'

DrGAPDH

Forward: 5'-AGTCGATGGTCATGCAATGTCTGT-3'

Reverse: 5'-TAATATTCTGAGCTGCACCTCTGCC-3'

Drpcna

Forward, 5'-CCTCGGAAGTAACGACTCTC-3'

Reverse, 5'-ATAGCCTCACCCATCTGACT-3'.

RNAi gene silencing

Fragments of *Dr-nanos* were amplified by PCR and were cloned into pGEM® T-vectors (Promega Corp., Madison, WI, USA). The following primers were used:

Dr-nanos dsRNA

forward, 5'-GCTACGAGCTCACTTTGGCAATCGGTAAAC-3'

reverse, 5'-AGTTCAGGTACCTTGATTGTATGAGCAAAATCACC-3'.

Two templates for dsRNA synthesis that added a T7 promoter to one end of each were amplified by PCR using the plasmid described above using 2 sets of primers. dsRNAs were synthesized from each template using T7 RiboMAX™ (Promega Corp., Madison, WI, USA).

dsRNA template-1

forward, 5'-GTAAAACGACGGCCAGT-3';

reverse, 5'-CTCAAGCTATGCATCCAACG-3';

dsRNA template-2

forward, 5'-ATTGGGCCCCGACGTCGCATG-3';

reverse, 5'-GCGTAATACTACTACTATAGGGCTCAAGCTATGCATCCAACGCG-3'.

For 10 animals, 4–10 µg/10 µl dsRNA was mixed with 20 µl 70% liver, 7.5 µl 2% ultra-low gelling temperature agarose, and red food coloring, and allowed to solidify on ice.

To silence the *nanos* function and to maintain homeostasis in the sexualized worms, RNAi food was fed to the mature sexualized worms every 3 days, and the worms were subsequently maintained for 50 days. Two animals were fixed every 10 days, and were observed histologically. Control animals were fed RNAi food with enhanced green

fluorescent protein (EGFP) dsRNA, which is not found in planarians.

To silence the *nanos* function during sexualization, RNAi food was fed to the asexual worms twice a day every 3 days. After 5 days, minced *Bdellocephala brunnea* was fed to the worms every day for 40 days, and 20 µg dsRNA was mixed with *B. brunnea* twice a week. Four animals were fixed every 10 days, and were observed histologically. After RNAi treatment during the sexualization process, planarians were maintained on their usual food (chicken liver).

RT-PCR was performed to confirm gene suppression using the primers described above. These primers were designed to contain the region used in the RNAi.

Acknowledgments

We would like to thank Dr. Sachiko Ishida, Hirosaki University, for his gift of the OH strain. We also thank Dr. Phil Newmark, for advice and discussions concerning the expression of *nanos* and its function between planarian species. This work was supported in part by grants from the Ministry of Education, Culture, Sports, Science, and Technology awarded to M.M (16086209), and from the Keio Leading-edge Laboratory of Science & Technology Research Grant for the Ph.D. Program awarded to H.N.

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