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Germes is involved in translocation of germ plasm during development of *Xenopus* primordial germ cells

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ABSTRACT ***Germes*** **mRNA and protein are components of the germ plasm in** ***Xenopus laevis*****. Previously, based on phenotypic observations of tailbud embryos expressing intact and mutant** ***Germes,*** **it was suggested that** ***Germes*** **is involved in the organization of germ plasm (Berekelya** ***et al.,*** **2007). Recently, to observe the germ plasm in a living embryo, we generated transgenic** ***Xenopus*** **expressing EGFP fused with a mitochondrial targeting signal, because germ plasm is enriched with mitochondria (Taguchi** ***et al.,*** **2012). Using this transgenic** ***Xenopus,*** **we demonstrate that** ***Germes*** **plays an essential role in the translocation of germ plasm from the cortex to the perinuclear region in primordial germ cells during early gastrulation.**

KEY WORDS: *PGC, germline, DEADSouth, mitochondrial cloud, chromatoid body*

In early *Xenopus* development, the germline is generated by inheritance of specialized cytoplasm called 'germ plasm'. Germ plasm is present in the vegetal cortex of a fertilized egg, where it divides into about four blastomeres via the first two cleavages, and then distributes unequally to daughter cells until midblastula transition (MBT) (Taguchi *et al.*, 2012; Whitington and Dixon, 1975). Early in gastrulation (stage 10), germ plasm translocates from the cortex to the perinuclear region in primordial germ cells (PGCs) and divides equally into two daughter PGCs in later cell divisions. The germ plasm contains electron dense granules, many mitochondria, and specific mRNAs and proteins. Recently, we demonstrated that germ plasm contains determinants that are necessary and sufficient for germline specification (Tada *et al.*, 2012). To understand the mechanisms underlying germline specification in *Xenopus*, many RNA components of the germ plasm have been identified (Cuykendall and Houston, 2010; King *et al.*, 2005), including *Germes* mRNA (Berekelya *et al.*, 2003). The *Germes* gene is specifically expressed in the ovary. *Germes* mRNA has been detected in the entire cytoplasm of stage I oocytes and in the mitochondrial cloud of stage II oocytes. It is associated with the germ plasm until gastrulation and disappears after translocation to the perinuclear region. *Germes* encodes a protein with two leucine zipper motifs and an EF-hand motif (Berekelya *et al.*, 2003). It has been shown that Germes protein also localizes to the germ plasm by expression of EGFP-tagged Germes protein in oocytes (Berekelya *et al.*, 2007). A reduction in the number of PGCs has been observed in tailbud (stage 31–33) embryos overexpressing intact Germes (*GermesWT*) or Germes lacking both leucine zipper motifs (*GermesΔLZs*). Interestingly, in these tailbud embryos, some PGCs are larger and found inside the endodermal mass, indicating that active PGC migration is blocked from an early stage. Using *Xpat* mRNA and its protein as a germ plasm marker (Hudson and Woodland, 1998), it has been revealed that the germ plasm at stage 31–33 has a morphology similar to that at earlier stages. In addition, it has been shown that Germes protein interacts with dynein light chain 8 *in vitro* and *in vivo* (Berekelya *et al.*, 2007). These findings suggest that Germes is involved in PGC migration and germ plasm organization.

The *DEADSouth* gene encodes DEAD-box RNA helicase belonging to the DDX25 family, and its transcript has also been identified as an RNA component of the germ plasm in *Xenopus* (MacArthur *et al.*, 2000). Recently, we generated transgenic *Xenopus* expressing EGFP fused with a mitochondrial targeting signal (mito-EGFP) for observation of the germ plasm in living embryos (Taguchi *et al.*, 2012), because germ plasm is enriched with mitochondria (Venkatarama *et al.*, 2010; Elinson *et al.*, 2011). Using mito-EGFP embryos, we demonstrated that DEADSouth protein localizes to the germ plasm, where it is required for proper PGC development (Yamaguchi *et al.*, 2013). Interestingly, knockdown of *DEADSouth* mRNA causes expansion of the germ plasm, inhibition of germ plasm translocation and division of PGCs after MBT. As a result, a reduc-

Abbreviations used in this paper: MBT, midblastula transition.

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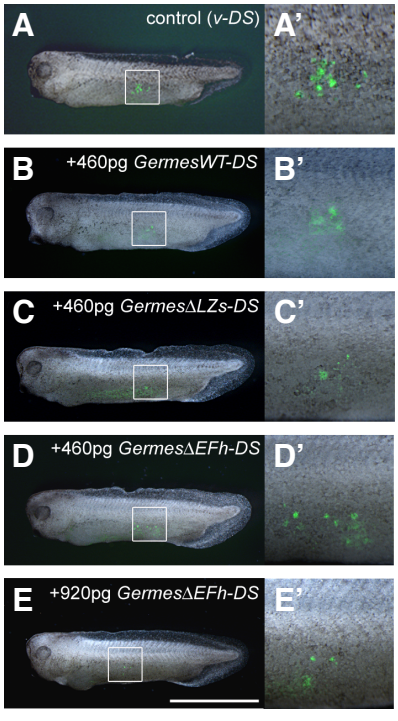
tion in the number of PGCs is observed in tailbud-stage embryos. The phenotype of *DEADSouth*-knockdown embryos is similar to that of embryos overexpressing *GermesWT* or *GermesΔLZs*. To understand the mechanisms underlying germ plasm organization, it is important to investigate the phenotypes of embryos overexpressing *GermesWT* or *GermesΔLZs* for comparison with those of *DEADSouth*-knockdown embryos, particularly at stages 7–12 that are much earlier than the tailbud stage. Although it has been very difficult to directly observe the germ plasm in isolated PGCs, the use of mito-EGFP *Xenopus* enables such observations (Taguchi *et al.*, 2012; Yamaguchi *et al.*, 2013). Here, we demonstrate that translocation of the germ plasm is inhibited by overexpression of *GermesWT* or *GermesΔLZs*, and by knockdown of *DEADSouth*, suggesting that both Germes and DEADSouth proteins are involved in the translocation of germ plasm during MBT.

Results and Discussion

To investigate the function of *Germes* in PGC development, we generated three constructs, including intact Germes (*GermesWT-DS*), Germes lacking the EF-hand motif (*GermesΔEFh-DS*), and Germes lacking both leucine zipper motifs (*GermesΔLZs-DS*) according to Berekelya *et al.* (2007). To ensure PGC-specific expression, the open reading frames (ORFs) were followed by the 3' untranslated region (UTR) of the *DEADSouth* gene that is expressed in a PGC-specific manner (Kataoka *et al.*, 2006). Each mRNA (460 pg) was co-injected into the vegetal pole of a fertilized egg with *Venus-DEADSouth 3' UTR* mRNA (*v-DS*, 460 pg) as a PGC tracer (Kataoka *et al.*, 2006). At stage 32, significantly reduced numbers of PGCs were observed in embryos injected with *GermesWT-DS* or *GermesΔLZs-DS*, compared with those injected with only *v-DS* (*GermesWT-DS*, 9.2±3.2; *GermesΔLZs-DS*, 10.5±3.5; and *v-DS*, 17.9±2.6) (Fig. 1 A-C,F). Embryos injected with 460 pg *GermesΔEFh-DS* mRNA showed no reduction in PGC numbers (16.1±2.9) (Fig. 1 D,F). These effects were identical to those described in a previous report in which PGCs were visualized by whole-mount *in situ* hybridization using the PGC-specific gene *Xpat* (Berekelya *et al.*, 2007). Furthermore, we examined the effect of *GermesΔEFh-DS* when twice the amount of mRNA (920 pg/embryo) was injected. Interestingly, *GermesΔEFh-DS* also resulted in a significant reduction of PGC numbers (8.9±1.9) (Fig. 1 E,F), although the injected embryos developed normally. This result indicated that overexpression of *GermesWT-DS*, *GermesΔLZs-DS,* and *GermesΔEFh-DS* resulted in similar reductions of PGC numbers, but are different in terms of the magnitude of their effect. These differences might have occurred because Germes functions are regulated by multiple domains (see below).

Next, we used embryos from transgenic *Xenopus* expressing EGFP with the mitochondrial targeting signal (mito-EGFP) to examine the effects caused by overexpression of *Germes-DS* and *Germes* mutants. At stage 12, a significant reduction of PGC numbers was observed in mito-EGFP embryos injected with *GermesWT-DS* (7.8±0.7, $P<0.05$) or *GermesΔLZs-DS* (6.3±1.9, $P<0.01$) mRNAs, compared with that in the control (11.0±2.6). However, we did not observe significantly reduced PGC numbers in embryos injected with *GermesΔEFh-DS*, even at a dose of 920 pg/embryo (Fig. 1G). We also investigated the size distribution of PGCs at stage 12 (Fig. 2). The average diameters of PGCs from mito-EGFP embryos injected with *GermesWT-DS* (460 pg/embryo), *GermesΔLZs-DS* (460 pg/embryo) or *GermesΔEFh-DS* (920 pg/embryo) were 59.6±24.4 μm, 56.9±21.2 μm and 57.6±18.1 μm, respectively. The diameters of these PGCs were significantly larger than those of control PGCs (48.4±8.0 μm). Moreover, these phenotypes were very similar to those of *DEADSouth* knockdown (Yamaguchi *et al.*, 2013).

We also examined the morphology and intracellular localization of the germ plasm in PGCs from mito-EGFP embryos injected with *GermesWT-DS* or *Germes* mutant mRNAs. In uninjected PGCs,



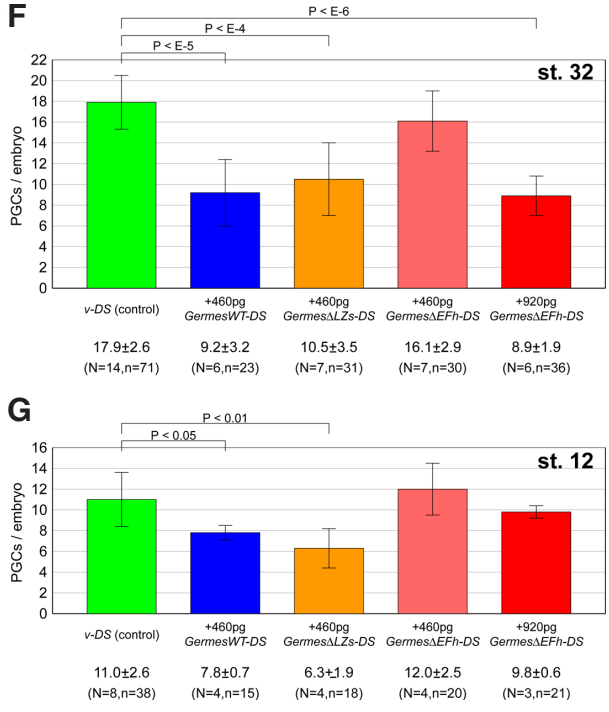


Fig. 1. Effect of overexpression of *GermesWT-DS*, *GermesΔLZs-DS* or *GermesΔEFh-DS* in PGCs. (A–E) *Representative examples of tailbud embryos injected with mRNA of* v-DS *only,* v-DS *plus* GermesWT-DS, v-DS *plus* GermesΔLZs-DS, *or* GermesΔEFh-DS, *respectively. The amount of injected mRNA is indicated. Anterior is toward the left. Scale bar, 1 mm.* **(A'–E')** *High magnifications of the areas indicated in* **(A–E)**, *respectively.* **(F)** *The number of PGCs per embryo at stage 32 injected with the indicated mRNAs. PGC numbers were determined by externally counting from both sides.* **(G)** *The number of PGCs per embryo at stage 12. Mito-EGFP transgenic embryos were injected with the indicated mRNAs, and then dissociated at stage 12. EGFP-positive and large cells were counted as PGCs. N and n indicate the number of experiments and total embryos examined, respectively. Error bars indicate standard deviation. One-tailed* P*-values were calculated by the Student* t*-test for significance.*

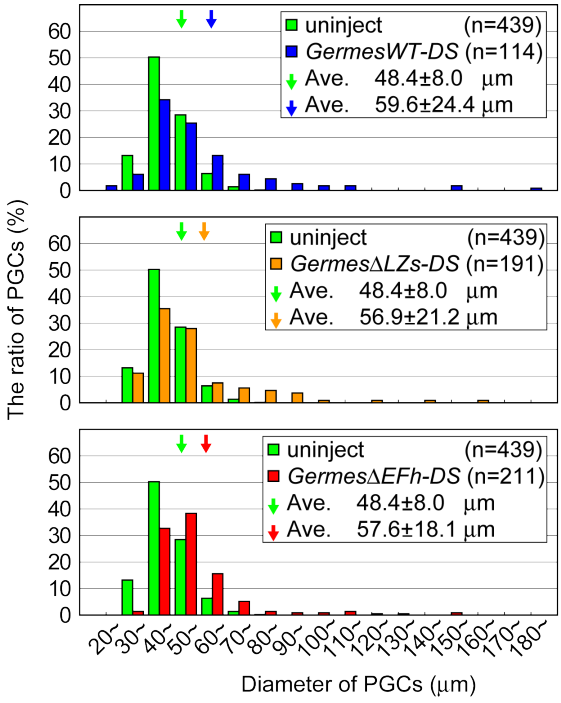


Fig. 2. Size distribution of PGCs from stage 12 embryos injected with *GermesWT-DS*, *GermesΔLZs-DS* or *GermesΔEFh-DS* mRNAs. *PGCs were isolated from the injected mito-EGFP embryos to measure their diameter. Total numbers of isolated PGCs (n) are shown as 100%. The size distribution of PGCs in embryos injected with 460 pg* GermesWT-DS *(blue), 460 pg* GermesΔLZs-DS *(orange), or 920 pg* GermesΔEFh-DS *(red) is compared with that in uninjected embryos (green). n indicates total PGC numbers. Arrows indicate average diameters in the indicated experiments.*

the germ plasm was present in the cortex at stage 7, and then moved to the perinuclear region at stage 9. In embryos injected with *GermesWT-DS* or *Germes* mutants, many large PGCs, which retained the germ plasm in the cortex, were observed at stage 12 (Fig. 3A) in contrast to perinuclear localization of the germ plasm in most PGCs of uninjected embryos. Quantitative analysis demonstrated that 32.6%, 30.9% and 18.4% of PGCs in embryos injected with *GermesWT-DS*, *GermesΔLZs-DS* or *GermesΔEFh-DS* (920 pg), respectively, contained germ plasm in the cortex at stage 12, compared with only 6.8% of PGCs in uninjected embryos (Fig. 3B). These differences were significant ($P<0.01$, *U*-test). Interestingly, we could not detect any alteration in the PGCs of injected embryos until stage 7. The mislocalization of germ plasm in these PGCs was very similar to that in *DEADSouth*-knockdown PGCs (Yamaguchi *et al.*, 2013). To elucidate the relationship among Germes, germ plasm translocation, and mitosis, we individually cultured PGCs isolated from stage 8 embryos injected with *GermesWT-DS*, and followed PGC mitosis for 11 hours (Fig. 4A). All five experiments (about 12 PGCs each/female/experiment) showed that mitosis of PGCs overexpressing *GermesWT-DS* was significantly inhibited compared with that in the control (35.7±21.7% vs. 46.3±20.4%; $P<0.05$, paired samples *t*-test). Interestingly, in non-dividing PGCs, mislocalization of germ plasm was observed more frequently in

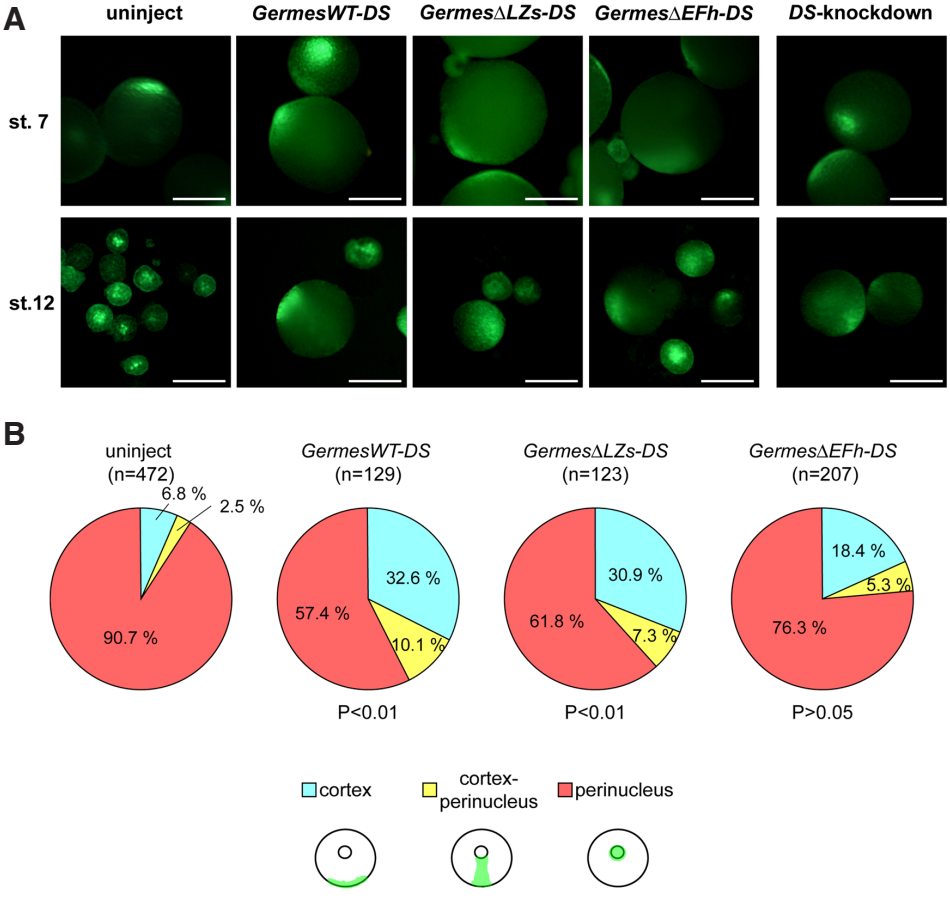


Fig. 3. Overexpression of *GermesWT-DS*, *GermesΔLZs-DS*, or *GermesΔEFh-DS* affects translocation of the germ plasm. (A) *Localization of the germ plasm in PGCs isolated from mito-EGFP embryos at stages 7 (upper) and 12 (lower), which were injected with* GermesWT-DS *(460 pg),* GermesΔLZs-DS *(460 pg) or* GermesΔEFh-DS *(920 pg) mRNAs. For comparison, PGCs from uninjected and DEADSouth-knockdown (*DS-*knockdown; Yamaguchi* et al., *2013) embryos are also shown. The images represent the severe phenotypes of PGCs in the experiment. Scale bars, 100 μm. Note that, at stage 12, PGCs overexpressing* GermesWT-DS, GermesΔLZs-DS, *or* GermesΔEFh-DS, *and* DS-*knockdown PGCs are relatively large and contain the germ plasm beneath the cell membrane.* **(B)** *Ratio of PGCs with three localization patterns of germ plasm from stage 12 embryos uninjected or injected with* GermesWT-DS *(460 pg),* GermesΔLZs-DS *(460 pg) or* GermesΔEFh-DS *(920 pg) mRNAs. According to the localization patterns, PGCs were classified into three groups: cortex, perinucleus, and intermediate (cortex-perinucleus) as shown at the bottom of the panel. P-values were calculated by the Mann-Whitney* U*-test for significance.*

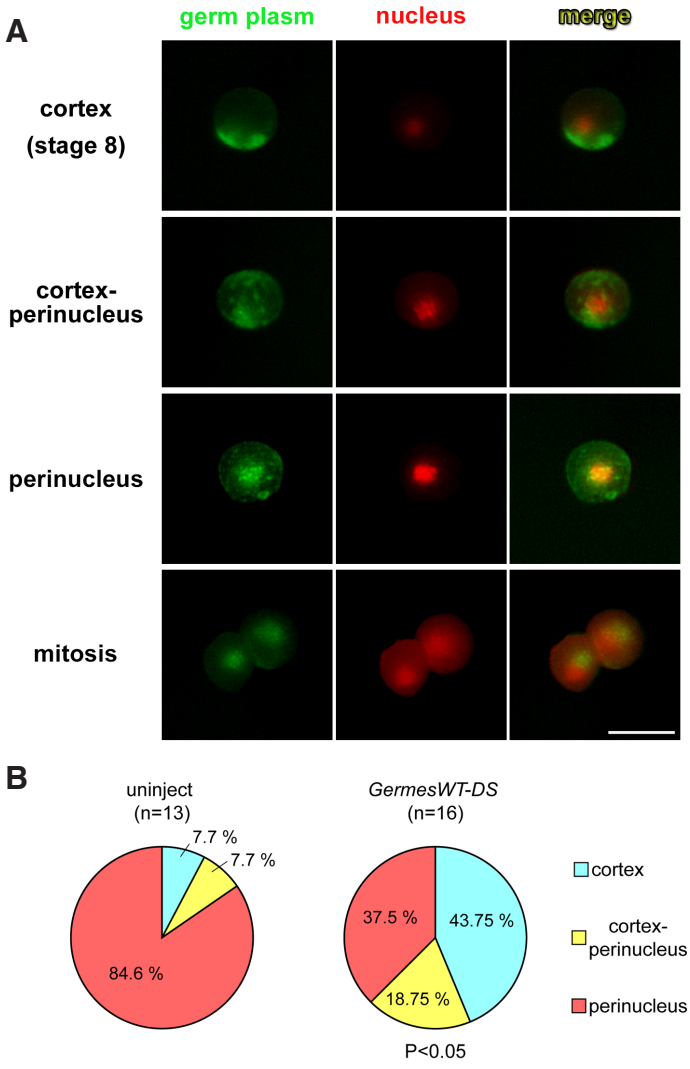


Fig. 4. Overexpression of *GermesWT-DS* results in mitotic inhibition of PGCs with mislocalized germ plasm. (A) *Behavior of germ plasm (green) during mitosis of a single cultured PGC from normal mito-EGFP transgenic embryos at stage 8. Note that mitosis occurs after translocation of germ plasm from the cortex to the perinuclear region. Mitosis was observable at about 9 hours after isolation. Nuclei were labeled by injection of mCherry-NLS-*DEADSouth *3′ UTR mRNA (red).* **(B)** *Ratio of non-divided PGCs with three localization patterns of germ plasm after culture. The PGCs from stage-8 embryos uninjected or injected with* GermesWT-DS *(460 pg) were cultured individually for 11 hours. Classification of non-divided PGCs and statistical analysis were performed as described in Fig. 3. Scale bar, 100 μm.*

PGCs overexpressing *GermesWT-DS* than that in control PGCs (Fig. 4B). This difference was also significant ($P<0.05$, *U*-test). Conversely, perinuclear localization of the germ plasm was observed in all divided PGCs in control and overexpression of *GermesWT-DS.* Translocation of the germ plasm occurred prior to the initial equal division of PGCs after MBT. Taken together, these observations strongly suggested that abnormal translocation of germ plasm by overexpression of *GermesWT-DS* or *Germes* mutants resulted in inhibition of equal division of PGCs. It was likely that these large PGCs could not move normally in the endodermal mass as described previously (Berekelya *et al.*, 2007). Based on observations of the PGCs from tailbud embryos (at stage 31–33) injected with *GermesWT* or *GermesΔLZs* mRNAs, Berekelya *et al.,* (2007) suggested that *Germes* is involved in the organization and functioning of germ plasm. In their study, it was unclear why overexpression of *GermesΔEFh* caused no phenotypic effects, because the EF-hand domain is thought to bind calcium ions to regulate physiological roles. Here, we demonstrated that overexpression of *GermesΔEFh* also caused phenotypic effects similar to those of *GermesWT* or *GermesΔLZs* overexpression. The effects of their overexpression might be caused by competition for multiple Germes-interacting proteins including dynein light chain 8 (Berekelya *et al.*, 2007).

Recently, we showed that translocation of the germ plasm is sensitive to nocodazole, but insensitive to cytochalasin D, and that α-tubulin is co-localized with germ plasm (Taguchi *et al.*, 2012). These findings indicate that germ plasm translocates from the cortex to the perinuclear region through the microtubule system, but not the microfilament system. This observation is in good agreement with that of Germes protein interacting with dynein light chain 8 *in vitro* and *in vivo* (Berekelya *et al.*, 2007). Germ plasm may be transported along microtubules to the perinuclear region by a dynein motor. However, the directionality of microtubules for germ plasm translocation remains to be determined.

It is very interesting that the phenotypes caused by overexpression of *GermesWT*, *GermesΔLZs,* or *GermesΔEFh* were very similar to those of *DEADSouth*-depleted embryos. Because DEADSouth is an RNA helicase with some similarity to eIF4A, it may be involved in translation of germ plasm-specific mRNAs (MacArthur *et al.*, 2000). The subcellular region-specific translation of such mRNAs may be coupled to germ plasm translocation with Germes. Although we have not determined whether Germes associates with DEADSouth directly or indirectly, both proteins are obviously indispensable for proper maintenance and translocation of the germ plasm. After translocation, interactions between the germ plasm and nucleus would be required for germline specification. To understand the mechanisms underlying germline specification, it would be necessary to delineate more components of the germ plasm and their interactions with each other.

Materials and Methods

Xenopus *embryos*

Eggs and embryos from wild-type *Xenopus laevis* were prepared as described previously (Kataoka *et al.*, 2006). Female transgenic *Xenopus laevis* expressing EGFP fused with a mitochondrial targeting signal (mito-EGFP) were also used in this study (Taguchi *et al.*, 2012; Yamaguchi *et al.*, 2013).

Preparation of constructs

The *Germes* ORF was amplified by a nested PCR method using ovarian cDNA from *Xenopus laevis* as a template, and was exchanged for the *Venus* ORF of pCS2-Venus-DEADSouth 3'UTR (pCS2-Germes-DEADSouth 3'UTR). For the initial amplification, the sequences of the forward and reverse primers were CCTGTGACTCCTATATATGTGCTGGAACAT and CAAGTTTGGCATGTACAAGGTATACTCTGG, respectively. The PCR product was subjected to a secondary amplification using the nested forward and reverse primers, CTTTTTGCAGGATCCATGTGGCAAATGCTGAAATATGTGGT and CTCATCACTGAATTCTTAAGTATGGCTTAATTTTTGCGCCCT, respectively (sequences overlapping with the pCS2-Venus-DEADSouth 3'UTR are underlined). pCS2-GermesΔLZs-DEADSouth 3'UTR and pCS2-GermesΔEFh-DEADSouth 3'UTR constructs were generated by inverse PCR and In-Fusion technology (Clontech) using pCS2-Germes-

DEADSouth 3'UTR as a template. The amino acid sequences of GermesWT, GermesΔLZs and GermesΔEFh used in this study were identical to those in a previous report by Berekelya *et al.,* (2007).

Preparation and microinjection of mRNA

Template plasmids were linearized by digestion with *Not*I and used as templates for *in vitro* mRNA synthesis with an mMESSAGE mMACHINE SP6 kit (Ambion). The mRNA was microinjected into the cortical region at the vegetal pole of a fertilized egg with a Nonoject II microinjector (Drummond Scientific Company). Each mRNA (460 pg, 4.6 nL of 100 ng/μL mRNA) was injected into a fertilized egg. The same amount of *v-DS* mRNA was co-injected as a PGC tracer.

Observation of PGCs and germ plasm

PGCs were observed and counted externally in *v-DS*-co-injected embryos at stage 32 (Kataoka *et al*., 2006). The PGCs were also isolated manually from dissociated transgenic embryos, counted, and examined for intracellular localization of the germ plasm (Yamaguchi *et al*., 2013). PGCs from stage 8-transgenic embryos were uninjected or injected with 460 pg *GermesWT-DS* mRNA, and then individually seeded on 1% agarose-coated dishes containing 55% Leibovitz's L-15 medium (Gibco) supplemented with 5% fetal bovine serum, and then cultured at 25 °C for 11 hours. Germ plasm behavior was observed in PGCs from embryos injected with 460 pg *mCherry-NLS* (SV40 nuclear localization signal)-*DEADSouth* 3' UTR mRNA for nuclear staining (Taguchi *et al*., in preparation).

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