Functional reconstruction of NANOS3 expression in the germ cell lineage by a novel transgenic reporter reveals distinct subcellular localizations of NANOS3

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Abstract

Mutations of RNA-binding proteins such as NANOS3, TIAL1, and DND1 in mice have been known to result in the failure of survival and/or proliferation of primordial germ cells (PGCs) soon after their fate is specified (around embryonic day (E) 8.0), leading to the infertility of these animals. However, the mechanisms of actions of these RNA-binding proteins remain largely unresolved. As a foundation to explore the role of these RNA-binding proteins in germ cells, we established a novel transgenic reporter strain that expresses NANOS3 fused with EGFP under the control of *Nanos3* regulatory elements. NANOS3–EGFP exhibited exclusive expression in PGCs as early as E7.25, and continued to be expressed in female germ cells until around E14.5 and in male germ cells throughout the fetal period with declining expression levels after E16.5. NANOS3–EGFP resumed strong expression in postnatal spermatogonia and continued to be expressed in undifferentiated spermatogonial cells in adults. Importantly, the *Nanos3*–EGFP transgene rescued the sterile phenotype of *Nanos3* homozygous mutants, demonstrating the functional equivalency of NANOS3–EGFP with endogenous NANOS3. We found that throughout germ cell development, a predominant amount of NANOS3–EGFP co-localized with TIAL1 (also known as TIAR) and phosphorylated eukaryotic initiation factor 2 α , markers for the stress granules, whereas a fraction of it showed co-localization with DCP1A, a marker for the processing bodies. On the other hand, NANOS3–EGFP did not co-localize with Tudor domain-containing protein 1, a marker for the intermitochondrial cements, in spermatogenic cells. These findings unveil the presence of distinct posttranscriptional regulations in PGCs soon after their specification, for which RNA-binding proteins such as NANOS3 and TIAL1 would play critical functions.

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Introduction

The germ cell lineage is the source of totipotency, ensuring the creation of new organisms in most multicellular species. Understanding the precise molecular mechanisms underpinning the germ cell development, therefore, represents one of the most critical challenges in life science.

In mice, and presumably in all mammals, the germ cell fate is not an inherited trait by the structure generally called germ plasm from the egg (preformation), as seen in some model organisms, but is induced in pluripotent cells by signaling activities from adjacent tissues (epigenesis; Hayashi *et al.* 2007). In mice, it has been demonstrated that in response to BMP4 from the

extraembryonic ectoderm, a subset of proximal epiblast cells express *Prdm1* (also known as *Blimp1*) and *Prdm14*, encoding two of the PR (PRDI-BF1 and RIZ homology) domain-containing transcriptional regulators, at around embryonic day (E) 6.25–6.5 (Ohinata *et al.* 2005, 2009, Yamaji *et al.* 2008), and these *Prdm1*- and *Prdm14*-positive cells increase their numbers, move posteriorly during gastrulation, and go on to form a cluster of primordial germ cells (PGCs), the exclusive source of both oocytes and spermatozoa, with characteristic alkaline phosphatase (AP) activity (Ginsburg *et al.* 1990) and *Dppa3* (also known as *stella* or *Pgc7*) expression (Saitou *et al.* 2002, Sato *et al.* 2002), at the base of the incipient allantois at around E7.25. Reflecting the function of the germ line as the transducer of

genetic and epigenetic information, PGC specification has been found to involve repression of the somatic program and re-acquisition of potential pluripotency, followed by genome-wide epigenetic reprogramming (Saitou 2009a). PRDM1 orchestrates transcriptional events critical for all three of these activities (Kurimoto *et al.* 2008), whereas PRDM14 seems essential for the latter two (Yamaji *et al.* 2008).

The established PGCs then initiate migration, moving through the developing hindgut endoderm and mesentery, eventually colonizing the embryonic gonads at around E10.5, where they proliferate and initiate a complex differentiation program either into oocytes or into spermatozoa (Sasaki & Matsui 2008). Notably, to date, a number of genes, whose mutations apparently do not affect PGC specification but impair the subsequent survival of PGCs during their migration, have been identified (see review Saitou (2009b)). These genes include *Nanos3* (Tsuda *et al.* 2003), *Tial1* (also known as *Tiar*; Beck *et al.* 1998), and *Dnd1* (Youngren *et al.* 2005). Interestingly, all these genes encode evolutionally conserved RNA-binding proteins with important implications in germ cell development, suggesting that although modes of germ cell specification differ among organisms, PGCs rely on conserved molecular pathways for their survival soon after their specification. It is particularly noteworthy that all these gene products localize at the germ plasm in a diverse range of model organisms with preformed germ cell specification (Wang & Lehmann 1991, Weidinger *et al.* 2003, Gallo *et al.* 2008). However, the mechanisms by which these proteins function in PGCs in mice remain poorly understood, in part due to the difficulty of analyzing small numbers of PGCs *in vivo*.

As a foundation to explore the role of these RNA-binding proteins in germ cell development, we generated transgenic lines expressing NANOS3 fused with EGFP under the control of *Nanos3* regulatory elements. The transgenic lines not only recapitulated the expression of *Nanos3* faithfully throughout germ cell development but also rescued the sterile phenotype of *Nanos3* knockout mice. NANOS3–EGFP distributed in a granular pattern in the cytoplasm of PGCs as early as E7.5 and co-localized either with phosphorylated eukaryotic initiation factor 2 α (p-EIF2A) and TIAL1, or with DCP1A, markers for the stress granules (SGs) or the processing bodies (PBs) respectively (see review Anderson & Kedersha (2006, 2008)). Notably, NANOS3–EGFP did not show co-localization with Tudor domain-containing protein 1 (TDRD1), a component of the intermitochondrial cements (IMCs), which are other cytoplasmic RNA–protein granules, in spermatogonial cells (Chuma *et al.* 2006). These findings reveal the presence of specific posttranscriptional regulation of RNAs in PGCs soon after their specification, and thus the NANOS3–EGFP transgenic line constitutes a valuable tool for the analysis of RNA metabolism in PGCs.

Results

Generation of NANOS3–EGFP mice

To efficiently monitor the expression and subcellular localization of NANOS3, an essential RNA-binding protein in the germ cell lineage, we decided to generate a transgenic reporter strain that expresses NANOS3 fused with EGFP under the control of *Nanos3* regulatory elements. Prior to the generation of the reporter strain, we examined the position of NANOS3 with which the EGFP tag should be fused. For this purpose, we made cDNA constructs expressing NANOS3 fused with EGFP either at its N- or C-terminus, and transfected these cDNAs into ES cells. We found that NANOS3 fused with EGFP at its N-terminus (EGFP–NANOS3) did not show consistent expression in ES cells and localized in a diffuse manner both at the nucleus and at the cytoplasm with apparently weak expression levels, whereas NANOS3 fused with EGFP at its C-terminus (NANOS3–EGFP) exhibited robust expression and predominantly cytoplasmic localization (data not shown). The cytoplasmic localization of NANOS3–EGFP is in good agreement with the fact that *Nanos* is a component of germ plasm in diverse organisms (Wang & Lehmann 1991, Subramaniam & Seydoux 1999, Seydoux & Braun 2006) and with the localization of endogenous NANOS3 in PGCs in mice as detected by a specific antibody we generated (see below). These findings strongly suggest that NANOS3–EGFP but not EGFP–NANOS3 recapitulates the proper subcellular localization of NANOS3 protein. We therefore decided to construct a bacterial artificial chromosome (BAC) bearing the *Nanos3* gene with EGFP fused with its 3-prime end (Fig. 1A) by ‘recombineering’ technology (see Materials and Methods). A segment of the BAC with ~32-kb upstream and ~20-kb downstream elements of *Nanos3–EGFP*, which apparently does not harbor any other genes, was used for transgenic mouse production by the pronuclear injection. We generated 15 independent transgenic lines, among which three lines showed apparently good and consistent fluorescence in the germ cell lineage, and we chose one line that exhibited the brightest expression for further analysis.

Specific expression of NANOS3–EGFP in the germ cell lineage

We went on to determine whether NANOS3–EGFP shows specific expression in the germ cell lineage by immunofluorescence double-staining methodology. We first looked at the expression of NANOS3–EGFP in the genital ridges at E13.5, the stage at which the sex is easily discriminated by the morphology of the genital ridges (Capel 2000). We found that NANOS3–EGFP is expressed specifically in the cells that are positive for mouse vasa homolog (MVH), a highly conserved marker of the germ cell lineage, both in the male

Figure 1 Generation and characterization of the *Nanos3-EGFP* transgenic mouse strain. (A) Schematic representation of the *Nanos3-EGFP* transgene. The genomic locus and exon-intron structures of the *Nanos3* gene are shown. The position at which the sequences of the fluorescent reporter are recombined is also shown. (B) Co-staining of NANOS3-EGFP (upper panel, green) with MVH (middle panel, red) in the male (left column) and female (right column) genital ridges at E13.5. The merged images with Hoechst (white) are shown on the lower column. Magnified images are included in the upper-right corner. Scale bar, 50 μ m. (C and D) Co-staining of NANOS3-EGFP (left column, green) with PGC markers (middle column, red) such as AP2 γ at E7.5 (C) and POU5F1 (Oct3/4) at E8.75 (D). The merged images with Hoechst (white) are shown on the right column. Scale bars, 50 μ m.

and female (Extavour & Akam 2003, Seydoux & Braun 2006; Fig. 1B). Consistent with the findings by the ES cell transfection experiments, NANOS3-EGFP was localized predominantly at the cytoplasm and weakly in the nucleus. We noted that NANOS3-EGFP at the cytoplasm in PGCs exhibited a somewhat granular distribution (Fig. 1B insets, see also below). We then went on to examine the earlier expression of NANOS3-EGFP and found that it was detectably expressed at E7.5 in the posterior extraembryonic

mesoderm in the cells that were exclusively positive for AP2 γ , a transcription factor specifically expressed in PGCs (Kurimoto *et al.* 2008; Fig. 1C). We found that a cluster of NANOS3-EGFP-expressing cells starts to be detectable as early as E7.25 at the base of the incipient allantois, and these cells then disperse and show a migrating phenotype toward the developing endoderm from around E7.5 (data not shown and Fig. 1C). At E8.75, when PGCs migrate in the developing hindgut, we detected NANOS3-EGFP

expression in the cells that were positive for POU5F1 (OCT4), a key protein for pluripotency and a marker for PGCs, in the hindgut endoderm (Fig. 1D). These findings are consistent with the previous reports showing that *Nanos3* starts to be expressed in a subset of *Prdm1*- and *Dppa3*-positive PGCs at around E7.25 (Yabuta *et al.* 2006), and that NANOS3 is expressed in gonadal PGCs at E13.5 in both sexes (Tsuda *et al.* 2003, Suzuki *et al.* 2007). Thus, the NANOS3-EGFP transgenic line successfully recapitulates germ cell-specific expression of the endogenous *Nanos3* gene.

NANOS3-EGFP in male and female gonads

Having established a transgenic line precisely reporting *Nanos3* expression, we next went on to determine the NANOS3-EGFP expression both in the male and in the female after E12.5. Indeed, *Nanos3* expression after E12.5 has not yet been fully explored (Tsuda *et al.* 2003, Suzuki *et al.* 2007, Lolicato *et al.* 2008). We found that in the female germ cells, NANOS3-EGFP expression continued to be strong until E13.5, but declined in an anterior-to-posterior order at around E14.5, presumably coincident with the onset of meiosis

Figure 2 NANOS3-EGFP expression in the male and female gonads. (A–C) Immunofluorescence analysis of NANOS3-EGFP (upper panel, green) using female (A) and male (B) fetal gonads at the indicated stages. The merged images with Hoechst (white) are shown on the lower panel. NANOS3-EGFP declines in an anterior-to-posterior order as shown in (C). A, anterior; P, posterior. Scale bars, 50 μ m. (D) Immunofluorescence analysis of NANOS3-EGFP (upper panel, green) in postnatal testes at the indicated stages. The merged images with Hoechst (white) are also shown on the lower panel. Scale bar, 50 μ m. (E) Magnified images of seminiferous tubules (at 1 day (left), 1 w (middle), and 2 w (right)) shown in the dotted boxes in the lower panel of (C). Arrow, Sertoli cell; double arrow, myoid cell; yellow arrowheads, spermatogonia; and arrowheads, NANOS3-EGFP-negative spermatogonia.

in PGCs (Fig. 2A and C; Menke *et al.* 2003). Thereafter, we did not detect NANOS3–EGFP expression either in the fetal germ cells or in the adult oocytes (Fig. 2A and data not shown). On the other hand, in the male germ cells, NANOS3–EGFP showed strong expression until E14.5, but subsequently waned, also in an anterior-to-posterior order, and exhibited extremely weak expression at E16.5 (Fig. 2B and C), a time point at which almost all male germ cells enter mitotic arrest (Western *et al.* 2008). However, we consistently observed a low level of NANOS3–EGFP expression throughout the fetal period (Fig. 2B).

In the testes of newborn mice, the seminiferous tubules, which are surrounded by a monolayer of myoid cells, are known to contain only gonocytes and Sertoli cells. Gonocytes uniformly show a round shape with large, pale-staining nuclei and are separated from the basement membrane, whereas Sertoli cells are distinguished by their smaller, ovoid nuclei arranged perpendicularly to the basement membrane. Within a few days *post partum* (ppt), gonocytes resume proliferation, and migrate to the basement membrane with pseudopods, and are generally defined as spermatogonia at around 1-week ppt. At around 2-week ppt, the spermatogonia begin meiotic divisions, and spermatocytes at the pachytene stage begin to be observed in the seminiferous tubules (Russell *et al.* 1990 and see Fig. 2E and its legend).

We found that in the newborn gonocytes, the NANOS3–EGFP re-initiates robust expression, and at 1-week ppt, it shows expression in almost all

spermatogonia defined by the morphological criteria (Fig. 2D and E, yellow arrowheads). At 2-week ppt, the NANOS3–EGFP was expressed in a subset of spermatogonia (Fig. 2D and E), but was not expressed in other germ cells attaching to the basement membrane (Fig. 2D and E, right panel, white arrowheads), and was not expressed in spermatocytes undergoing meiotic divisions (Fig. 2D and E). The number of the NANOS3–EGFP-positive cells per section of a seminiferous tubule decreased gradually with age. From the morphological criteria, these NANOS3–EGFP-positive cells looked like undifferentiated type-A spermatogonia (Fig. 2D and Russell *et al.* 1990, and see below).

NANOS3–EGFP in the spermatogonia of adult testis

Morphological studies have classified undifferentiated type-A spermatogonia into at least three subtypes based on their topographical arrangements: single (designated as A_s), paired (A_{pr}), and aligned spermatogonia (A_{al} ; Russell *et al.* 1990).

To gain insight into whether NANOS3–EGFP is indeed expressed in undifferentiated type-A spermatogonia – and if so, within which subtypes – we went on to investigate the topographical arrangements of the NANOS3–EGFP-positive cells in the seminiferous tubules by whole-mount immunofluorescence analysis. Since the NANOS3–EGFP mainly localized in the cytoplasm, it should be possible to visualize the chains of the NANOS3–EGFP-positive cells if NANOS3–EGFP was indeed present. As shown in Fig. 3A, we found that

Figure 3 NANOS3–EGFP is expressed in the undifferentiated type-A spermatogonia. (A) Distribution of NANOS3–EGFP-positive cells on the surface of a seminiferous tubule of a 1-year-old mouse observed by confocal microscopy (three-dimensional projection). Scale bar, 100 μ m. (B) Quantification of the NANOS3–EGFP-positive clusters. The horizontal axis represents the numbers of NANOS3–EGFP-positive cells comprising each cluster. The vertical axis indicates the frequency of each cluster per scanned area (~ 215 mm²). (C) Co-staining of NANOS3–EGFP (b, green) with GFRA1 (c, red) in adult testes. The merged images of NANOS3–EGFP with Hoechst (white), or with Hoechst and GFRA1 are shown in (a) and (d) respectively. Scale bar, 10 μ m.

at the basal surface of the seminiferous tubules, the NANOS3–EGFP-positive cells are distributed as a cluster of cells, with each cluster bearing a distinct number of cells. We then quantified the number of cells in each NANOS3–EGFP-positive cluster, revealing that the distribution showed clear peaks at the numbers of $2^{N(N=0-4)}$ (Fig. 3B). This finding strongly suggests that the NANOS3–EGFP is expressed in undifferentiated type-A spermatogonia of all three types: A_s , A_{pr} and A_{al} .

The glial cell line-derived neutropic factor is known to be essential for the maintenance of spermatogonial stem cells, and its receptor, GFRA1, is reasonably thought to be a spermatogonial stem cell marker (Meng *et al.* 2000, He *et al.* 2007). To gain molecular evidence that NANOS3–EGFP is expressed in spermatogonia, we performed double immunofluorescent staining for NANOS3–EGFP and GFRA1, and found that 89 out of 118 (75.4%) GFRA1-positive clusters were also positive for NANOS3–EGFP (Fig. 3C), providing further support for the idea that NANOS3–EGFP is expressed in the undifferentiated population of the spermatogonia in adult testis. Additionally, we found weak fluorescence of NANOS3–EGFP in elongating spermatids, which would be ectopic expression, since we did not detect *Nanos3* in these cells by *in situ* hybridization (data not shown).

Rescue of the sterile phenotype of the *Nanos3* mutants by the *Nanos3*–EGFP transgene

We next went on to examine whether NANOS3–EGFP expressed from the transgene is functionally equivalent to the endogenous NANOS3 protein. First, we found that the subcellular distribution of the endogenous NANOS3 protein as detected by an anti-NANOS3 rabbit polyclonal antibody that we raised was indistinguishable from that of NANOS3–EGFP detected by an anti-EGFP antibody: both exhibited finely granular distribution in the cytoplasm (Fig. 4A and B). This finding suggests that the *Nanos3*–EGFP transgene recapitulates not only the precise expression pattern of the *Nanos3* gene but also the subcellular distribution of the NANOS3 protein.

Secondly, we examined whether the NANOS3–EGFP can rescue the sterile phenotype of *Nanos3* homozygous^{−/−} mutant mice. For this purpose, we performed several generations of intercrosses between the *Nanos3*–EGFP transgenic line and the *Nanos3* heterozygous^{+/-} mutants, and obtained *Nanos3* homozygous^{−/−} mutants heterozygous or homozygous for the *Nanos3*–EGFP transgene (*Nanos3*–EGFP^{+/-}; *Nanos3*^{−/−} or *Nanos3*–EGFP^{+/+}; *Nanos3*^{−/−} respectively). As reported previously, in the *Nanos3*^{−/−} mutants with no transgene, the testes and ovaries were reduced in size and showed a completely germ

Figure 4 The *Nanos3*–EGFP transgene rescues the germ cell-less phenotype of *Nanos3* knockout mice. (A) A representative image of the subcellular localization of endogenous NANOS3 (middle, red) co-stained with DPPA3–EGFP (left, green) in PGCs at E9.5. The merged image with Hoechst (white) is shown on the right. Scale bar, 4 μ m. (B) A representative image of the subcellular localization of NANOS3–EGFP (left, green). The merged image with Hoechst (white) is shown on the left. Scale bar, 4 μ m. (C) Histological sections of 9-week-old testes (a) and ovaries (b) of *Nanos3* heterozygous^{+/-}, homozygous^{−/−}, and homozygous^{−/−} mice bearing the *Nanos3*–EGFP transgene heterozygously (*Nanos3*^{−/−}; *Nanos3*–EGFP^{+/-}; left, middle, and right column respectively), stained with hematoxylin and eosin. Scale bars, 100 μ m. (D) Gross appearance of pups obtained by intercrossings of the *Nanos3*^{−/−}; *Nanos3*–EGFP^{+/-} mice.

cell-less phenotype (Fig. 4C, middle column; Tsuda *et al.* 2003). In contrast, we found that the testes and ovaries of the *Nanos3-EGFP^{+/-}; Nanos3^{-/-}* mice were normal in size (data not shown), and, strikingly, histological analysis revealed that the germ cells were

produced in an apparently normal fashion both in the male and in the female, as in the case of a littermate control (Fig. 4C, right and left column respectively). We then evaluated the fertility of *Nanos3-EGFP^{+/-}; Nanos3^{-/-}* males and females, leading to the finding

Figure 5 Subcellular localization of NANOS3-EGFP, TIAL1, PUM2, p-EIF2A, and DCP1A in PGCs analyzed by confocal microscopy. (A) Co-staining of NANOS3-EGFP (green, left) and TIAL1 (red, second left) in PGCs at E8.75 (a), E9.5 (b), and E11.5 (c). Merged images of NANOS3-EGFP and TIAL1 (second right), and those further merged with Hoechst (white, right) are also shown. Arrowheads indicate prominent co-localization of NANOS3-EGFP with TIAL1. Scale bar, 4 μ m. (B) Co-staining of NANOS3-EGFP (green, left) and PUM2 (red, middle) in PGCs at E11.5. Merged images of NANOS3-EGFP and PUM2 (second right), and those further merged with Hoechst (white, right) are also shown. Scale bar, 4 μ m. (C) Co-staining of NANOS3-EGFP (green, left) and p-EIF2A (red, middle) (a) or DCP1A (b) in PGCs at E8.75. Merged images of NANOS3-EGFP and p-EIF2A or DCP1A (second right), and those further merged with Hoechst (white, right) are also shown. Arrowheads indicate prominent co-localization of NANOS3-EGFP with p-EIF2A (a) or DCP1A (b). Scale bar, 4 μ m. (D) Western blot analysis of the expression levels of TIAL1 in PGCs and gonadal somatic cells (soma) at E12.5. MVH and GAPDH were used as the PGC marker and the loading control respectively. M, male; F, female.

that both these mice are fully fertile and delivered pups with a similar litter size as the littermate controls (Fig. 4D). Moreover, the pups grew normally, and we were able to maintain the *Nanos3-EGFP*^{+/+}; *Nanos3*^{-/-} line by internal crossing. Collectively, these findings clearly demonstrate that NANOS3-EGFP is functionally equivalent to the endogenous NANOS3 protein.

Distinct subcellular distribution of *Nanos3-EGFP* in the germ cell lineage

Having demonstrated the function of NANOS3-EGFP, we set out to determine the nature of the NANOS3/NANOS3-EGFP-containing cytoplasmic granules throughout germ cell development. First, we decided to compare the localization of NANOS3-EGFP with that of TIAL1, an evolutionarily conserved RNA-binding protein and a core component of SGs, which are cytoplasmic aggregates of stalled translational pre-initiation

complexes that accumulate during stress, such as heat shock, oxidative stress, ischemia, or viral infection (Anderson & Kedersha 2006, 2008). Interestingly, it has been shown that in *Tial1* knockout mice, PGCs fail to survive in the migration period, a phenotype similar to that of *Nanos3* knockout mice (Beck *et al.* 1998).

We found that TIAL1 is expressed both in PGCs and in neighboring somatic cells at a similar level, and is localized in the nucleus as well as in the cytoplasm (Fig. 5A). Since it has been reported that TIAL1 is highly and preferentially expressed in PGCs at E12.5–14.5 (Beck *et al.* 1998), we further examined the expression level of TIAL1 by western blot analysis by using PGCs and neighboring gonadal somatic cells at E12.5 separated by magnetic cell sorting (see Materials and Methods). We could not detect any significant difference of the expression level of TIAL1 between PGCs and the somatic cells (Fig. 5D). Interestingly, however, we found that the localization of TIAL1 in the cytoplasm was generally much more consistent and prominent in PGCs

Figure 6 Subcellular localization of NANOS3-EGFP, TIAL1, p-EIF2A, DCP1A, PUM2, and TDRD1 in spermatogonia. Co-staining of NANOS3-EGFP (green, left) and TIAL1 (a), p-EIF2A (b), DCP1A (c), PUM2 (d), and TDRD1 (e) (red, middle) in adult spermatogonial cells. Merged images of NANOS3-EGFP and each protein (second right), and those further merged with Hoechst (white, right) are also shown. Arrowheads indicate prominent co-localization of NANOS3-EGFP with each protein. Scale bar, 4 μ m.

than in their somatic neighbors (Fig. 5A). Importantly, NANOS3-EGFP showed good co-localization with TIAL1 in the cytoplasm in migrating PGCs at E8.75 and E9.5, and in rapidly proliferating PGCs in the gonads at E11.5 (Fig. 5A). We also compared the localization of NANOS3-EGFP with that of p-EIF2A, an essential trigger for SG formation and an exclusive marker for SGs (Anderson & Kedersha 2006, 2008), and found that some of the NANOS3-EGFP-positive granules are indeed positive for p-EIF2A in PGCs at E8.75 (Fig. 5Ca). These findings indicate that the NANOS3/NANOS3-EGFP-containing cytoplasmic granules are similar structures to SGs in cultured mammalian cells.

The PB is another distinct cytoplasmic RNA-protein granule containing components of the 5'-3' mRNA decay machinery, the nonsense-mediated decay pathway, and the RNA-induced silencing complex (Parker & Sheth 2007). It is known that SGs and PBs are distinct structures, but they share many components and often interact with one another (Anderson & Kedersha 2006, 2008). We therefore examined whether NANOS3-EGFP is also a component of PBs by immunostaining the migrating PGCs with DCP1A, an exclusive marker for PBs, and found that a fraction of NANOS3-EGFP-positive granules are DCP1A positive (Fig. 5Cb). In PGCs, NANOS3-EGFP did not show good co-localization with PUM2, an evolutionarily conserved protein known to associate with Nanos (Fig. 5B; Sonoda & Wharton 1999, Lolicato *et al.* 2008). Collectively, these results suggest that NANOS3-EGFP may regulate posttranscriptional gene expression in both SG- and PB-like structures in PGCs.

We next investigated the nature of the NANOS3/NANOS3-EGFP-positive granules in adult spermatogonial cells. Similarly to the NANOS3/NANOS3-EGFP-positive granules in PGCs, these granules were positive for TIAL1 and p-EIF2A, and a fraction of them were also DCP1A positive (Fig. 6a-c). In these cells, in contrast to the case in PGCs, NANOS3 showed prominent co-localization with PUM2 (Fig. 6d).

In spermatogenic cells, there exists another class of RNA-protein granules known as IMCs. Recently, the IMCs have been revealed as the site of Piwi-interacting small RNA biogenesis, which appears to be essential for transposon silencing and proper spermatogenesis (see reviews Kotaja & Sassone-Corsi (2007) and Klattenhoff & Theurkauf (2008)). To examine the relationship between the NANOS3 and EGFP granules and the IMCs, we immunostained the NANOS3-EGFP-positive spermatogonia with an antibody against TDRD1, a specific component of the IMCs (Chuma *et al.* 2005). We found that, essentially, the TDRD1-positive granules do not co-localize with NANOS3-EGFP-positive foci (Fig. 6e). Finally, we performed immunoelectron microscopic analysis of the localization of TDRD1 and NANOS3-EGFP. As reported previously, TDRD1-positive immunogold labels were detected in the IMCs, whereas

Figure 7 Immunoelectron microscopic localization of NANOS3-EGFP and TDRD1 in spermatogonia. Upper left, staining of spermatogonia with anti-TDRD1 antibody. A black arrowhead indicates TDRD1-positive intermitochondrial cements. Upper right, a magnified view of the boxed area in the left. Lower left, staining of NANOS3-EGFP-positive spermatogonia with anti-EGFP antibody. White arrowheads indicate NANOS3-EGFP-positive cytoplasmic areas. A black arrow indicates an intermitochondrial cement, which is negative for NANOS3-EGFP. Lower right, a magnified view of the boxed area in the left. Scale bar, 500 nm.

NANOS3-EGFP-positive labels were observed in the vicinity of the cytoplasm (Fig. 7). We therefore conclude that the NANOS3/NANOS3-EGFP-positive cytoplasmic granules are distinct from the IMCs.

Discussion

We have here reported the generation of a transgenic strain that recapitulates the endogenous expression and subcellular localization of NANOS3, and rescues the germ cell-less phenotype of *Nanos3* homozygous^{-/-} mutant mice. Nanos is an evolutionarily conserved RNA-binding protein essential for germ cell development and is a component of germ plasm in organisms, in which germ cells are specified by germ plasm-based 'preformation' (Wang & Lehmann 1991, Subramaniam & Seydoux 1999, Seydoux & Braun 2006). NANOS3 is a mammalian homolog of Nanos, whose function is critical for the survival of PGCs immediately after their specification (Tsuda *et al.* 2003). However, the mechanism by which NANOS3 functions in PGCs remains totally unresolved. The *Nanos3-EGFP* transgenic strain, which, for the first time, revealed the precise subcellular localization of NANOS3 in PGCs and spermatogonia, should thus serve as a critical model for exploring the mechanisms of action of conserved RNA-binding proteins in PGCs in mice.

The *Nanos3-EGFP* transgene bears ~32-kb upstream and ~20-kb downstream sequences of the *Nanos3* gene, and these sequences were sufficient for recapitulating *Nanos3* expression. These sequences are considerably shorter compared to those utilized for other germ cell reporters, such as *Blimp1*-mEGFP/Venus, *Prdm14*-mVenus, and *stella*-EGFP (Payer *et al.* 2006, Ohinata *et al.* 2008, Yamaji *et al.* 2008). It may therefore be possible to isolate 'the core elements' for the germ cell-specific expression of *Nanos3* by generating and evaluating transgenic embryos bearing successively shorter transgene elements. Such works would be important not only for the identification of the gene regulatory network for germ cell specification but also for the development of germ cell-specific gene regulation tools.

We found a weak expression of NANOS3-EGFP in male germ cells at E16.5–18.5, while a previous work reported that NANOS3 was undetectable during this period by western blot analysis using whole gonad samples (Suzuki *et al.* 2007). This difference may be attributable either to a difference in protein stability between NANOS3 and NANOS3-EGFP or to the difference in sensitivity between the two detection methods. Since germ cells are surrounded by ~tenfold more somatic cells in the gonads around these stages, it might be difficult to detect low levels of protein expression in germ cells by western blot analysis using whole gonad samples.

One of the critical findings of the present work is that a predominant amount of NANOS3/NANOS3-EGFP shows specific localization in the TIAL1- and p-EIF2A-positive SG-like structures, and a fraction is localized in DCP1A-positive PB-like structures both in PGCs and in spermatogonia (Figs 5 and 6). SGs are cytoplasmic RNA-protein complexes that are formed in response to environmental stress (e.g. heat, oxidative conditions, u.v. irradiation, and hypoxia), whereas PBs are distinct cytoplasmic sites of mRNA degradation that arise as a consequence of such stresses (Anderson & Kedersha 2006, 2008, Parker & Sheth 2007). It has been proposed that SGs and PBs are inter-related dynamic structures in that mRNA released from disassembled polysomes by signals arising from a stressor is sorted and remodeled at SGs, from which selected transcripts are delivered to PBs for degradation. Thus, both these structures would seem to play critical roles in the cellular response to and survival after environmental stresses. However, most of the studies on SGs and PBs have been conducted in cultured mammalian cells, and the roles of these cytoplasmic granules *in vivo* have not been fully elucidated. Our identification of well-developed SG- and PB-like structures, especially in PGCs as early as E7.5, suggests that PGCs rely on an intricate posttranscriptional regulation of gene expression soon after their fate is determined. Considering that both *Nanos3* and *Tial1* mutants show defective PGC survival and/or proliferation, NANOS3- and TIAL1-positive SG-like

structures may control translational events essential for PGC survival and/or proliferation.

In many species, including *Drosophila melanogaster* and *Caenorhabditis elegans*, Nanos has been shown to repress the translation of target mRNAs through binding to the 3'UTR of these targets. This translational repression seems to be accomplished, at least in part, by the interaction with Pumilio and/or by recruitment of the CCR4-NOT de-adenylation complex to target mRNAs (Kadyrova *et al.* 2007). The localization of NANOS3/NANOS3-EGFP in the SG- and PB-like granules may indicate that NANOS3 regulates translational activity and/or degradation of target mRNAs in PGCs. However, we did not detect co-localization of NANOS3/NANOS3-EGFP with PUM2 in PGCs (Fig. 5B), suggesting that NANOS3 may act with other Pumilio homologs or that Pumilio proteins may be dispensable for the function of NANOS3 in PGCs. Consistently, *Pum2* mutant mice show only a partial testicular developmental defect (Bowles & Koopman 2007), and three homologs of *Drosophila Pumilio*, *Pum1*, *Pum2*, and *Puf-A* have been identified and have been shown to be expressed in PGCs (Kurimoto *et al.* 2008, Kuo *et al.* 2009).

It has recently been shown that in *C. elegans*, germ granules and PBs are distinct structures, and germ granules may be parallel to SGs, in part because a *C. elegans* homolog of TIAL1, TIA-1, is localized exclusively in germ granules (Gallo *et al.* 2008). The predominant localization of NANOS3/NANOS3-EGFP at TIAL1-positive SG-like structures in PGCs may therefore suggest that the NANOS3-positive granules correspond to germ plasm-like structures in mice, although they may not be structures easily distinguishable by conventional electron microscopic analyses (Fig. 5; Eddy 1975). On the other hand, we showed that the NANOS3- and TIAL1-positive granules are essentially different from TDRD1-positive IMCs in spermatogonia (Fig. 6e). However, Tudor, the founder protein of the Tudor domain-containing protein family, localizes exclusively at germ plasm (pole plasm) in *D. melanogaster* (Boswell & Mahowald 1985). It may therefore be the case that in mice, reflecting the different mode of germ cell specification and more complicated epigenetic regulations (Sasaki & Matsui 2008, Saitou 2009a), strategies for posttranscriptional regulation of gene expression have diverged. If so, precise clarification of these strategies will be an important future challenge.

Materials and Methods

Embryo isolation and staging

All the animals were treated with appropriate care according to the RIKEN ethics guidelines. Embryos were isolated in PBS(–). Noon of the day when the vaginal plugs of mated females were identified was scored as E0.5. For a more accurate staging, embryos younger than E8.0 were classified according to the

morphological landmarks (Downs & Davies 1993), and we matched the embryonic days to embryonic stages as follows: E7.25, early bud; E7.25–E7.5, mid bud; E7.5, late bud; E7.75, early head fold; and E8.0, late head fold. Embryos older than E8.25 were staged according to the somite numbers (St) as follows: E8.25, 2–4 St; E8.5, 6–8 St; E8.75, 10–12 St; E9.0, 14–16 St; E9.25, 18–20 St; and E9.5, 22–24 St.

Generation of *Nanos3*–EGFP transgenic line

A BAC bearing the *Nanos3* genomic locus with a 107-kb upstream and 88-kb downstream region (RP23-298K21, C57BL/6 background) was purchased from the BACPAC Resources Center (Children's Hospital Oakland Research Institute, Oakland, CA, USA). The EGFP sequence was recombined in-frame just before the stop codon of the *Nanos3* gene by Red/ET Recombineering (Gene Bridges, Dresden, Germany) according to the protocol provided by the manufacturer. To remove the genes in proximity to *Nanos3* from the transgene, the recombined BAC was digested with BstBI and NruI, and a ~53-kb DNA fragment containing a ~32-kb upstream and ~20-kb downstream region of *Nanos3* was separated by pulsed field gel electrophoresis, and extracted and purified by agarase I digestion and gel filtration using CL-4B sepharose (Yang *et al.* 1997). The purified construct was then injected into pronuclei of B6DBA F2 zygotes to generate transgenic mice, which were genotyped by PCR using the primer set Arm1-5'e/F, GGAAGCCCCCTG-GACCTTCA; Nos3-3'UTR/R, ATCGCTGACAAGACTGTGGC; EGFP-N1-810/R, TGGTGCAGATGAACTTCAGG. The selected transgenic line has been maintained by crossing with BDF1 mice four to five times.

Nanos3 knockout mice, and *Dppa3*–EGFP and *Prdm14*–mVenus transgenic mice

The generation of the *Nanos3* knockout mice was described previously (Tsuda *et al.* 2003). The *Nanos3* heterozygous $^{+/-}$ mice were maintained by crossing with BDF1 mice six to seven times. The *Nanos3* homozygous $^{-/-}$ mice were obtained by intercrossing of the *Nanos3* $^{+/-}$ mice. *Dppa3*–EGFP transgenic mice (C57BL/6 background) and *Prdm14*–mVenus transgenic mice (BDF1 background) were established as reported previously (Seki *et al.* 2007, Yamaji *et al.* 2008).

Histological analysis

Dissected ovaries and testes were fixed in Bouin's fixative overnight at room temperature, dehydrated, embedded in paraffin, sectioned at 8 μ m, and stained with hematoxylin and eosin. Images were taken with a Leica DM4500B upright microscope.

Indirect immunofluorescence analysis

For whole-mount indirect immunofluorescence analysis of mouse embryos, isolated embryos were fixed in 4% paraformaldehyde (PFA) in PBS for 2 h at 4 °C, washed thrice with

PBS–0.1% Triton X-100 (PBTx), and blocked with PBTx with 0.1% bovine serum albumin (BSA) (Sigma–Aldrich) overnight at 4 °C. Embryos were then incubated with primary antibodies in the blocking solution for 3 days at 4 °C, washed eight times with PBTx at 4 °C, incubated with secondary antibodies and Hoescht in the blocking solution overnight at 4 °C, washed eight times with PBTx at 4 °C, and mounted in VECTASHIELD (Vector Laboratories, Burlingame, CA, USA) for observation by confocal microscopy (FV1000; Olympus, Tokyo, Japan).

For indirect immunofluorescence analysis of cryosections of fetal and postnatal gonads, isolated gonads were fixed in 4% PFA in PBS for 2 h at 4 °C and washed thrice with PBS–0.2% Tween 20 (PBT). After treatment with sucrose, samples were embedded in OCT compound (Sakura Finetek USA Inc., Torrance, CA, USA) and stored at 80 °C. Sections at 10 μ m were cut on a cryostat (HM505E; Microm, Walldorf, Germany), thaw-mounted onto MAS–GP-coated glass slides (Matsunami, Kishiwada, Japan), and dried completely. After several washes in PBS, the sections were blocked with PBT with 0.1% BSA for 1 h at RT, incubated with primary antibodies in the blocking solution for 2 h at RT, washed thrice with PBT at 4 °C, incubated with secondary antibodies and Hoescht in the blocking solution for 1 h at RT, washed thrice with Hoescht at 4 °C, and mounted in VECTASHIELD for observation by confocal microscopy.

For whole-mount indirect immunofluorescence analysis of seminiferous tubules of adult testes, first, the tunica albuginea was removed from the testes in PBS with 0.02% sodium azide and 0.5 mM CaCl₂. Almost all interstitial cells were removed manually using fine forceps. Then, untangled seminiferous tubules were recovered and fixed in 4% PFA in PBS with 0.5 mM CaCl₂ for 3 h at 4 °C. For staining with anti-TDRD1 and anti-NANOS3 antibodies, the samples were fixed in 2% PFA in PBS with 0.5 mM CaCl₂ for 1 h at 4 °C. The fixed samples were washed thrice with PBT and blocked with PBT with 1% ECL advance blocking reagent (GE Healthcare, Amersham Biosciences) for 2 h at 4 °C. The seminiferous tubules were incubated with primary antibodies in the blocking solution overnight, washed four times with PBT at 4 °C, incubated with secondary antibodies and PBT in the blocking solution overnight at 4 °C, washed four times with PBT at 4 °C, and mounted in VECTASHIELD for observation by confocal microscopy.

The primary antibodies used were as follows: anti-NANOS3 rabbit polyclonal antibody (raised in rabbits using recombinant NANOS3 as an antigen, affinity purified, and used at a dilution of 1:500); anti-TDRD1 rabbit polyclonal, 1:1000 (a kind gift from Nakatsuji; Chuma *et al.* 2006); anti-GFP rat monoclonal, 1:500 (clone GF090R, cat# 04404-84, Nacalai, Kyoto, Japan); anti-GFP rabbit polyclonal, 1:500 (cat# 598, MBL, Aichi, Japan); anti-AP2 γ rabbit polyclonal, 1:500 (H-77, cat# sc-8977, Santa Cruz Biotechnology, Santa Cruz, CA, USA); anti-Oct3/4 mouse monoclonal, 1:100 (clone C-10, cat# sc-5279, Santa Cruz Biotechnology); anti-DDX4/MVH rabbit polyclonal, 1:500 (cat# ab13840, Abcam, Cambridge, UK); anti-GFR α 1 goat polyclonal (cat# AF560, R&D Systems, Minneapolis, MN, USA); anti-phospho-EIF2A-Ser51 rabbit polyclonal, 1:100 (cat# 119A11, Cell Signaling Technology, Beverly, MA, USA); and anti-TIAR/TIAL1 mouse

monoclonal, 1:100 (clone 6, cat# 610352, BD Biosciences, San Jose, CA, USA). The following secondary antibodies from Molecular Probes (Eugene, OR, USA) were used at a dilution of 1:500: Alexa Fluor 488 goat anti-rat IgG; Alexa Fluor 488 goat anti-rabbit IgG; Alexa Fluor 568 goat anti-rabbit IgG; Alexa Fluor 568 goat anti-mouse IgG; Alexa Fluor 488 donkey anti-rabbit IgG; and Alexa Fluor 568 donkey anti-goat IgG.

Immunoelectron microscopy

Immunoelectron microscopy was performed as reported previously (Chuma *et al.* 2006). In brief, testes were fixed in 2% PFA/0.02% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) and embedded in epoxy resin. Sections of 90 nm were incubated with anti-GFP rabbit polyclonal (MBL) or with anti-TDRD1 antibodies, followed by 15-nm gold-labeled secondary antibody (AuroProbe EM Anti-Rabbit IgG (H+L), 15 nm Gold; GE Healthcare, Waukesha, WI, USA). After postfixation with 2% glutaraldehyde in PBS, sections were stained with uranyl acetate and lead citrate, and were examined by using an electron microscope (Hitachi H-7650; Hitachi).

Isolation of PGCs and western blot analysis

Genital ridges were dissected out from *Prdm14-mVenus* transgenic embryos (Yamaji *et al.* 2008) at E12.5 and dissociated in 500 µl of pre-warmed 0.05% Trypsin solution with 0.53 mM EDTA (cat# 15400; Gibco BRL) for 5 min at 37 °C in a CO₂ incubator. The reaction was stopped by the addition of an equal volume of the stop solution (250 µl of 10 mg/ml DNase I (cat# 11284932001; Roche Applied Science) in PBS plus 250 µl of DMEM with 10% FBS). The resulting cell suspension was filtrated through a 35-µm pore-size nylon mesh (Cat# 2235; FALCON/BD Biosciences) and washed twice in 1 ml of 5 mg/ml BSA, 2 mM EDTA, PBS. PGCs were enriched by magnetic-activated cell sorting (MiniMACS (Miltenyi Biotec, Bergisch-Gladbach, Germany)) according to the manufacturer's protocol with anti-GFP rat MAB, 1:500. The separated cells were washed twice in 1 ml of 0.1% polyvinyl alcohol in PBS and lysed in 2× Laemmli's sample buffer. The resulting populations of germ cells were around 95% pure, as judged by the fluorescence of mVenus protein. Proteins were separated by 10% SDS-PAGE and blotted onto an Immobilon-P Transfer Membrane (Millipore, Bedford, MA, USA). Immunodetection was performed by an ECL Plus Western Blotting Detection System (cat# RPN2132; GE Healthcare).

The primary antibodies used were as follows: anti-MVH rabbit polyclonal, 1:000; anti-TIAR/TIAL1 mouse monoclonal, 1:1000; and anti-Gapdh mouse monoclonal, 1:5000 (clone 6C5, cat# ab8245; Abcam). The following HRP-conjugated secondary antibodies from GE Healthcare were used: anti-mouse IgG sheep polyclonal, 1:10 000 (cat# NA9310) and anti-rabbit IgG donkey polyclonal, 1:20 000 (cat# NA9340).

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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