Xenopus Nanos1 is required to prevent endoderm gene expression and apoptosis in primordial germ cells

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SUMMARY
Nanos is expressed in multipotent cells, stem cells and primordial germ cells (PGCs) of organisms as diverse as jellyfish and humans. It functions together with Pumilio to translationally repress targeted mRNAs. Here we show by loss-of-function experiments that Xenopus Nanos1 is required to preserve PGC fate. Morpholino knockdown of maternal Nanos1 resulted in a striking decrease in PGCs and a loss of germ cells from the gonads. Lineage tracing and TUNEL staining reveal that Nanos1-deficient PGCs fail to migrate out of the endoderm. They appear to undergo apoptosis rather than convert to normal endoderm. Whereas normal PGCs do not become transcriptionally active until neurula, Nanos1-depleted PGCs prematurely exhibit a hyperphosphorylated RNA polymerase II C-terminal domain at the midblastula transition. Furthermore, they inappropriately express somatic genes characteristic of endoderm regulated by maternal VegT, including Xsox17α, Bix4, Mixer, GATA4 and Edd. We further demonstrate that Nanos1 is required to repress VegT RNA in vitro and represses, along with Nanos1, VegT translation within PGCs. Repressed VegT RNA in wild-type PGCs is significantly less stable than VegT in Nanos1-depleted PGCs. Our data indicate that maternal VegT is an authentic target of Nanos1/Pumilio translational repression. We propose that Nanos1 functions to translationally repress mRNAs that normally specify endoderm and promote apoptosis, thus preserving the germline.

KEY WORDS: Xenopus, PGCs, Nanos/Xcat2, VegT, Germline determination, Endoderm, Translational repression

INTRODUCTION
A persistent question in developmental biology is how germ cell fate, with its characteristics of totipotency and immortality, is preserved in the context of somatic cell differentiation. In Xenopus, the germline is specified through the inheritance of germ plasm formed during oogenesis and asymmetrically segregated into the future germ cell lineage. At least three important activities appear to be required in primordial germ cells (PGCs) to protect them from a somatic fate: (1) repression of extant maternal messages encoding somatic determinants; (2) activation of sequestered maternal germline mRNAs; and (3) transient genome-wide suppression of mRNA transcription to ensure that somatic differentiation programs remain inactive when zygotic transcription is initiated in the rest of the embryo. Genetic studies have identified the conserved protein Nanos as being important in the first and last of these activities, although the molecular pathways in which it functions are largely uncharacterized.

Nanos encodes an RNA-binding protein with two CCHC zinc fingers that are evolutionarily conserved among all family members in organisms as diverse as sponge and humans (Curtis et al., 1997; Lai et al., 2011). Most recently, the structure of the zinc-finger region has been solved and shown to be required for RNA binding (Hashimoto et al., 2010). Together with Pumilio, Nanos is part of a translational repression complex in which Pumilio provides the RNA binding specificity and Nanos the repressive activity (Jaruzelska et al., 2003; Lai et al., 2011; Sonoda and Wharton, 1999; Sonoda and Wharton, 2001). Xenopus Pumilio can physically interact with Nanos1, as shown by in vitro pull-down assays, but evidence for direct interaction in vivo is lacking (Nakahata et al., 2001).

Loss of Nanos from Caenorhabditis elegans, Drosophila, zebrafish and mouse embryos results in multifaceted phenotypes including precocious cell divisions, ectopic expression of somatic genes, abnormal germ cell migration, and eventual loss of PGCs through apoptosis (Forbes and Lehmann, 1998; Kobayashi et al., 1996; Köprüuner et al., 2001; Sato et al., 2007; Tsuda et al., 2003). PGCs are normally transcriptionally repressed at times when somatic cells are initially expressing their gene program (Deshpande et al., 1999; Deshpande et al., 2005; Venkatarama et al., 2010). In Drosophila and C. elegans, loss of Nanos results in prematurely active transcription and the failure to establish germline-specific histone modifications typical of transcriptionally inactive chromatin (Schauer et al., 2003). Further, premature gene activation results in the misexpression of the somatic genes Sex-lethal, fushi tarazu and even skipped in the germline and, subsequently, cell death (Deshpande et al., 1999; Sato et al., 2007). However, the role of Nanos in transcriptional repression within the germline is unknown.

Key to understanding the molecular mechanisms underlying Nanos function in the germline is the identification and validation of the targeted mRNAs that are repressed. Pumilio binds the RNA target through a 3’UTR Pumilio binding element (PBE): UGUAAAUA (Wharton et al., 1998). Surprisingly, although general screens for the relevant mRNAs have been carried out with Nanos (Fox et al., 2005; Suzuki et al., 2010; Suzuki and Saga, 2008), only five authentic RNA targets of Nanos repression have been identified: (1) the cell cycle regulator cyclin B1, a target in frogs and flies (Asaoka-Taguchi et al., 1999; Dalby and Glover, 1993; Kadyrova et al., 2007; Lai et al., 2011); (2) the Drosophila promoter of apoptosis hid/ski (Hayashi et al., 2004; Sato et al., 2007); (3,4) the somatic determinants hunchback (Murata and
Nanos1 preserves germline fate

Wharton, 1995; Wreden et al., 1997) and bicoid (Wharton and Struhl, 1991); and (5) C. elegans fem-3, which regulates sex determination (Ahringer and Kimble, 1991; Zhang et al., 1997).

Xenopus nanos1 RNA is first detected in early stage oocytes, although it is not translated until after fertilization (Lai et al., 2011; Luo et al., 2011; Mosquera et al., 1993; Zhou and King, 1996). nanos1 RNA and protein persist until PGCs leave the endoderm at late tailbud stages (Lai et al., 2011; Luo et al., 2011; MacArthur et al., 1999). To determine the function of Nanos1 in Xenopus PGC development, we used antisense morpholino oligonucleotides (MOs) to block the translation of nanos1. Nanos1-depleted embryos showed a dramatic decrease in PGC number and, as expected, a subsequent loss of germ cells from the gonads. Importantly, the loss of PGCs could be rescued by injection of a mutated nanos1 RNA that was unaffected by the MO, demonstrating the specificity of the knockdown. PGCs without Nanos1 remained in the endoderm and eventually underwent apoptosis. Zygotic transcription, which is normally repressed in PGCs until neurula stages, was activated precociously at the start of gastrulation. Furthermore, PGCs deficient in Nanos1 inappropriately expressed somatic genes downstream of VegT that are required for endoderm specification. Pumilio was able to specifically bind VegT RNA in vitro and repress, along with Nanos1, VegT translation in PGCs. VegT RNA is significantly more stable in PGCs lacking Nanos1 than in wild-type PGCs. Our data point to maternal VegT RNA as an authentic target of Nanos1/Pumilio translational repression. From these results, we propose that Nanos1 is required to preserve Xenopus germline identity by translationally repressing RNAs that normally promote the endodermal developmental program and apoptosis.

MATERIALS AND METHODS

Xenopus embryos, MO injections and PGC isolation

Adult Xenopus laevis were purchased from Nasco. Embryos were generated as described (Sive et al., 2000). Twenty nanograms of Nanos1-MO or Nanos1-Ctrl-MO was injected into one-cell embryos. The nanos1-mut RNA (0.4 ng) used in the rescue experiments contained three mismatches within the MO complementary region and was not recognized by Nanos1-MO. MO sequences (Gene Tools) are: Nanos1-MO, GGCCATCCATGTGGGATTTCTGTG; Nanos1-Ctrl-MO, CGGCACTGCTTCTGCAAAATTCCTTCT. Images were collected from live embryos at about 15-20% epiboly.

Immunofluorescence microscopy

Immunofluorescence microscopy was performed as described (Lai et al., 2011). Primary antibodies were: affinity purified goat anti-Nanos1 antibody made against total recombinant expressed Nanos1 protein (Invitrogen), 2011). Primary antibodies were: affinity purified goat anti-Nanos1 antibody made against total recombinant expressed Nanos1 protein (Invitrogen), 1:50; rabbit anti-Xiswi [a gift from Dr Nelson Lau (Lau et al., 2009)], 1:1000; and H5 monoclonal antibody (Covance, #MMS-129R), 1:50. Secondary antibodies (Invitrogen) were: Alexa Fluor 488 donkey anti-rabbit IgG (H+L), 1:500; Alexa Fluor 555 donkey anti-goat IgG (H+L), 1:500; Alexa Fluor 488 donkey anti-mouse IgG (H+L), 1:1000; and Alexa Fluor 568 donkey anti-rabbit IgG (H+L), 1:1000.

Whole-mount in situ hybridization (WISH) and histology

WISH was performed as described (Houston et al., 1998). The Xpat plasmid was linearized using NotI and the DrsRed plasmid was linearized by BanHI. The digoxigenin-labeled antisense probes were transcribed by T7 RNA polymerase (Promega). PGCs were stained using BM Purple (Roche). Histology was performed as described (Houston and King, 2000).

Reverse transcription PCR (RT-PCR)

PGC isolation, RNA extraction and RT-PCR were performed as described (Venkatarama et al., 2010) using 38 cycles with the following gene primers and annealing temperatures: ODC at 53°C (Xanthos et al., 2001); Xsox17α at 56°C (Hudson et al., 1997); Atp4 at 56°C (Hudson and Woodland, 1998); Bix4 at 56°C (Xanthos et al., 2001); Xbra at 56°C (Wilson and Melton, 1994); Mixer at 55°C (Henry and Melton, 1998); GATA4/5/6 at 56°C (Xanthos et al., 2001); Endodermin (Edd) at 56°C (Xanthos et al., 2001); Cerberus at 60°C (Darrats et al., 1997); XHex at 60°C (Chang and Hemmati-Brivanlou, 2000); Chordin at 55°C (Zhang et al., 1998); Oet91 at 56°C; and Xwnt11 at 56°C.

Xenopus Pumilio1 RNA-binding domain (Xpum1 RBD) expression and purification

The Xpum1 RBD (amino acids 825-1175) was cloned into pET28a vector (Novagen) and the plasmid transformed into E. coli BL21 (DE3). Protein expression was induced by 1 mM IPTG for 4-5 hours. The cells were collected by centrifugation (2300 g) and the cell pellet was resuspended in 1× binding buffer (included in the His-Protein Purification Kit, Novagen) and sonicated on ice. After centrifugation, the supernatant was loaded onto the prepared His-bind resin column according to the manufacturer’s instructions. After several washes, the protein was eluted in 500 μl elution buffer containing 200-300 mM imidazole.

Non-radioactive RNA electrophoresis mobility shift assay (EMSA)

VegT 3’UTR fragment (112 nt, 2468-2579) containing the PBE was cloned into pCR4-TOPO vector (Invitrogen) to produce TOPO-VegT PBE. The PBE mutant (UAAAAAA) was introduced by mutagenesis (QuickChange II site-directed mutagenesis kit, Stratagene), generating TOPO-VegT PBE mut. The RNA was transcribed using T7 RNA polymerase (Promega), biotinylated at the 3’ end (Thermo Scientific, 2016), and EMSA performed using the LightShift chemiluminescent RNA EMSA kit (Thermo Scientific) according to the manufacturer’s instructions.

In vivo reporter assay

Plasmid pCS2-Venus-DEADSouth 3’UTR was a gift from Dr Hidefumi Orii (Kataoka et al., 2006). The red reporter DsRED-DS3’UTR was generated by replacing Venus with the DsRED open reading frame. VegT 3’UTR (296 nt, 2336-2631) containing the PBE (UGUAAAUAU) was subcloned downstream of the DsRED open reading frame to produce DsRED-VegTPBE-DS3’UTR (In-Fusion HD EcoDry Cloning System, Clontech). DsRED-VegT PBE-DS3’UTR mutant was made by changing 3 nt within the PBE site (mutant PBE, UAAAAAA) using the QuickChange II site-directed mutagenesis kit.

Nanos1-MO was injected into one-cell stage embryos at the vegetal pole. At approximately the 32-cell stage, fluorescent reporters were injected at 100 pg/cell into the germ plasm residing in the ‘dark area’ of four vegetal blastomeres of control or Nanos1-depleted embryos (see Fig. 6B) (Ikenishi and Nakazato, 1986). Images were collected from live embryos at about stage 35 using a fluorescence stereomicroscope (Olympus SZX12).

Quantitative real-time PCR (qRT-PCR) and data analysis

PGCs from wild-type (WT) and Nanos1-depleted (Nanos1-MO) embryos were isolated as described (Venkatarama et al., 2010). RNA extraction and reverse transcription were performed as described (Lai et al., 2011). Real-time PCR was performed for 40 cycles using SsoAdvanced SYBR Green Supermix (#172-5260, Bio-Rad) in the CFX96 system (Bio-Rad). Primer sequences (5'-3') and annealing temperatures were: VegT forward AGAAACTGCTTGCAGGAA and reverse CCGGATCTTACACTGAGGA, 53°C; GAPDH forward CTCCCTCCGAAAGCCTCATC and reverse GGAAAGCCATTCCGGTTATT, 53°C. Primer specificity was monitored by analyzing melting curves. All samples were analyzed in duplicate and experiments were repeated three times.

The threshold cycles (Ct) were determined by CFX Manager software (Bio-Rad). VegT expression was normalized to the expression of housekeeping gene GAPDH by ΔCt=ΔCt(VegT)−ΔCt(GAPDH). The relative fold change was calculated as 2−ΔΔCt, where ΔΔCt=ΔCt(Nanos1-MO)−ΔCt(WT). The statistical difference was determined by an unpaired Student’s t-test.

Whole-mount fluorescent in situ hybridization followed by TUNEL assay

Whole-mount fluorescent in situ hybridization (FISH) was performed exactly as described (Lai et al., 2011). The TUNEL assay protocol was taken from Henry and Gautier (Henry and Gautier, 1997) with a slight modification: after whole-mount FISH, embryos were refixed in 4% PFA.
in PBST. The TUNEL signal was detected using the TSA system (Cy3 tyramide, NEN Life Science Products) as described (Lai et al., 2011). Samples were analyzed using an inverted Zeiss LSM-510 laser-scanning confocal microscope equipped with argon ion, helium-neon and green-neon lasers. Fluorescein (FITC, Roche)-labeled Xpat antisense probe was transcribed by T7 RNA polymerase (Ambion, #AM 2082).

β-Gal assay
The β-Gal assay for lacZ expression was performed as described (Takeuchi et al., 2010).

**RESULTS**

**Embryos depleted of Nanos1 are deficient in PGCs**

To determine the function of *Xenopus nanos1* in the germline, we depleted Nanos1 protein using a morpholino antisense oligonucleotide (MO) approach. Nanos1-MO blocked the translation of *nanos1* in a dose-dependent fashion as determined in an in vitro translation assay. Neither a mutant of *nanos1* with 5′ sequences non-complementary to the MO (*nanos1-mut*) nor a control MO with five base changes (Nanos1-Ctrl-MO) had any effect on *nanos1* translation (data not shown). We have previously shown that during early developmental stages, Nanos1 is found within germ plasm, colocalizing with the germ plasm marker Xiwi (Lai et al., 2011; Lau et al., 2009; Wilczynska et al., 2009). Importantly, whereas Xiwi was readily detected, Nanos1 protein was not observed in embryos previously injected with Nanos1-MO (20 ng) after staining with anti-Nanos1 and anti-Xiwi antibodies (Fig. 1A). By contrast, Nanos1-Ctrl-MO (20 ng)-injected embryos could not be distinguished from uninjected siblings. No differences were detected in the external development of embryos injected with either Nanos1-MO or Nanos1-Ctrl-MO, indicating the lack of toxicity of the MOs used.

The fate of PGCs in embryos depleted of Nanos1 activity was followed by whole-mount in situ hybridization (WISH) with Xpat probe, a specific marker for PGCs (Hudson and Woodland, 1998; Machado et al., 2005). Significant differences in numbers were detected in the experimental population of PGCs starting at tailbud stage 26, with a dramatic decline by stage 35/36 (Fig. 1B, C). Xpat expression as detected by RT-PCR was prematurely lost by stage 30 instead of stage 39 (data not shown). From stage 26 to 39, PGCs actively migrate towards the dorsal aspect of the gut, from which they will eventually leave the endoderm (Nishiumi et al., 2005). The loss-of-PGC phenotype was rescued by co-injection with the *nanos1-mut* transcript (0.4 ng) in which three mismatches had been introduced to prevent recognition and repression by Nanos1-MO (P=0.012, one-way ANOVA) (Fig. 1B, C). Thus, our data show that Nanos1-MO acts specifically to knock down nanos1 gene function. Loss of Nanos1 activity results in the loss of PGCs at the time when PGCs are normally at the end of their interlude in the endoderm and not before stage 26.
An alternative explanation to a loss of PGCs is that the premature loss of Xpat instead reflects its instability in the absence of Nanos1. To address this concern, we carried out a histological examination of developing gonads at a time when they are richly populated by germ cells (Fig. 1D). We observed a significant loss of germ cells in the Nanos1-deficient tadpoles (n=5). The example shown in Fig. 1D represents the greatest number of germ cells detected after Nanos1 knockdown (~4-5 oocytes), as compared with the dense cluster of germ cells found in control tadpoles. Importantly, the loss of gonadal germ cells was rescued by introducing a nanos1 mutant message that is not recognized by the Nanos1-MO. Thus, Xpat is a reliable indicator for loss of PGCs. Taken together, we conclude that Nanos1 is required to maintain the PGC lineage.

PGCs deficient in Nanos1 activity undergo apoptosis

Is the PGC loss in nanos1 morphant embryos due to apoptosis or does it reflect PGC transdifferentiation into normal endoderm? Embryos injected with Nanos1-Ctrl-MO or Nanos1-MO and uninjected siblings were subjected to the TUNEL assay. PGCs were identified by FISH with Xpat and all double-labeled cells were scored positively for apoptosis. As expected, apoptosis was not detected at time points before the decline in PGC numbers observed in the Nanos1-MO embryos (stage 18, n=52 PGCs; Fig. 2A). In early tailbud embryos (stages 26-30), when Nanos1-deficient embryos showed a 27.2-56.3% decline in PGC numbers, 9.1% of PGCs were apoptotic (n=154 PGCs; Fig. 2B). Control-MO embryos were indistinguishable from uninjected embryos, with 1.8% apoptotic PGCs (n=340 PGCs). As apoptosis occurs relatively quickly (reviewed by Majno and Joris, 1995), at any one time we would expect only a few, and not all, PGCs to be TUNEL positive.

To follow the fate of Nanos1-depleted PGCs later in development, we used lacZ fused with the 3′ UTR of DEADSouth (NLD) as a germline lineage tracer (Takeuchi et al., 2010). The DEADSouth 3′ UTR contains germ plasm localization information that restricts the reporter protein expression to PGCs. Any RNA remaining in the somatic cells is degraded, most probably by microRNA activity (Kataoka et al., 2006; MacArthur et al., 2000). Therefore, the expression of lacZ is PGC specific. After MOs were injected at the one-cell stage, the NLD message was injected into the vegetal blastomeres at the 32-cell stage. Embryos were allowed to develop until stages 46-48, fixed and processed for β-Gal staining (Takeuchi et al., 2010) (Fig. 3A). As expected, control embryos showed lacZ-positive cells migrating within the dorsal mesenchyme (Nishiumi et al., 2005). By contrast, Nanos1-depleted embryos revealed very few lacZ-positive cells, a phenotype that was rescued by injection of nanos1-mut (0.4 ng) (P<0.001, one-way ANOVA) (Fig. 3B,C). A few PGCs were found in the gut tissue in both control and experimental embryos, but never at other ectopic locations. Histological examination of rare lacZ-positive cells in gut tissue revealed round cells that displayed no evidence of cellular differentiation and that subsequently disappeared from this tissue (data not shown) (Ikenishi et al., 2007). These data indicate that Nanos1-deficient PGCs undergo apoptosis and do not contribute to normal gut tissue. Further, we saw no evidence of abnormal migration, as occurs in Xdazl or XDead end morphants (Horvay et al., 2006; Houston and King, 2000), prior to PGCs becoming apoptotic, arguing that apoptosis is the primary reason that mutant PGCs do not leave the endoderm.

PGCs deficient in Nanos1 exhibit CTD-PSer2 prematurely

During the period when PGCs are transcriptionally silent, they do not exhibit the phosphorylated form of serine 2 (PSer2) in the C-terminal domain (CTD) of RNA polymerase II (Pol II), an event that is highly correlated with transcriptional elongation (Hirose and Ohkuma, 2007; Venkatarama et al., 2010). However Pol II CTD-PSer5 is present in all blastomeres even prior to the midblastula transition (MBT), signifying that initiation complexes are formed and machinery poised for transcription (Venkatarama et al., 2010). We hypothesized that in the absence of Nanos1, Pol II CTD-Ser2 is prematurely phosphorylated at the MBT, permitting possible misexpression of somatic genes in PGCs, similar to Drosophila nanos mutants (Deshpande et al., 2005; Venkatarama et al., 2010).
To assess the phosphorylated state of CTD-Ser2 in PGCs we performed double immunofluorescence using the H5 monoclonal antibody, which is specific for the PSer2 epitope, together with the anti-Xiwi antibody to identify germ plasm. CTD-PSer2 was detected during early gastrulation in 71% of the PGCs counted in Nanos1-MO-injected embryos from three independent experiments (n=44/62). By contrast, PGCs in Nanos1-Ctrl-MO- and uninjected WT embryos expressed the PSer2 epitope at significantly lower frequencies (n=11/45, 24% Nanos1-Ctrl-MO; n=9/48, 19% WT). One such experiment is shown in Fig. 4. We conclude that Nanos1 is required to prevent zygotic transcription in PGCs.

PGCs deficient in Nanos1 activity express somatic genes

Previous results showed that the maternal determinant for endoderm, VegT RNA, is present in PGCs and would, of necessity, have to be repressed (Venkatarama et al., 2010). Do PGCs depleted of Nanos1 activity now mistakenly initiate endoderm specification and thus lose Xpat expression (Machado et al., 2005)? To explore this possibility, we isolated PGCs from uninjected, Nanos1-Ctrl-MO- or Nanos1-MO-injected embryos. We asked whether PGCs now expressed endoderm or mesoderm markers as assessed by semi-quantitative RT-PCR on isolated PGCs. We choose Xsox17 α (Hudson et al., 1997) and Bix4 (Casey et al., 1999) to detect endoderm as both are direct downstream targets of maternal VegT and both are required for endoderm fate. Both were assessed to determine whether other fates were initiated besides endoderm (Smith et al., 1991).

Knockdown of Nanos1 resulted in expression of the endoderm markers Xsox17α and Bix4, but not of Xbra, in stage 10/11 PGCs (Fig. 5A). This suggested that PGCs deficient in Nanos1 enter an endoderm, but not a mesoderm, differentiation program. Further, Xsox17α and Bix4 were expressed at times comparable to endoderm expression during gastrulation. As expected, Bix4 was not detected in the excised endoderm core sample (circled in Fig. 5) as it is expressed during gastrulation within the endomesoderm just outside the core (Casey et al., 1999). ornithine decarboxylase
VegT translationally repressed by Nanos1/Pumilio

To better understand which specific differentiation programs are active in Nanos1-depleted PGCs, we examined gene expression at a later time point when distinctive germ layer expression patterns are established. At stage 15 neurula, additional downstream gene targets of maternal VegT were detected in PGCs, including the endoderm-specific markers Mixe and Edd (Fig. 5C). GATA4 was detected, but not GATA5/6, Xhex, Chordin or Cerberus, which are all expressed in more anterior mesendoderm and involved in anterior patterning (Bouwmeester et al., 1996; Gove et al., 1997; Henig et al., 1998; Henry and Melton, 1998; Kelley et al., 1993; Smithers and Jones, 2002; Tao et al., 2005; Weber et al., 2000; Xanthos et al., 2001). Xwnt11, an anterior dorsalizing factor that lies upstream of Xhex activity, was not expressed in Nanos1-depleted PGCs. The RT-PCR results in Fig. 5C indicate that Nanos1-depleted PGCs were differentiating toward posterior gut endoderm and not other regions. The most likely explanation for our results is that Nanos1 is required to repress VegT expression in PGCs.

VegT is translationally repressed by Nanos1/Pumilio in PGCs

A search of the VegT 3'UTR revealed a canonical PBE: UGUAUAUA. As a first step in determining whether Nanos1/Pumilio could repress VegT translation, we examined whether the VegT PBE could be recognized and bound by Xenopus Pumilio. Xenopus Pumilio1 containing the RNA-binding domain (Xpum1 RBD, amino acids 825-1175) was tested with biotinylated VegT PBE RNA (112 nt, 2468-2579) in an EMSA. Xpum1 RBD (20 ng) was able to bind VegT PBE (1 ng) but not the VegT PBE mutant (1 ng) (Fig. 6A). This binding interaction was competed by unlabeled VegT PBE RNA (250 ng). We conclude that Pumilio can specifically bind the VegT PBE in vitro.

To determine whether Nanos1 represses VegT translation in PGCs, we developed an in vivo fluorescent reporter assay (Fig. 6B). The DEADSouth 3'UTR contains germ plasm localization information as described above. Therefore, Venus-DEADSouth 3'UTR was introduced into the PBE site (Kataoka et al., 2006) and DsRED-DEADSouth 3'UTR both serve as germ line lineage tracers. A 296 nt fragment of the VegT 3'UTR containing the PBE was cloned into DsRED-DEADSouth 3'UTR, generating DsRED-VegT PBE-DS3'UTR. Three point mutations were introduced into the PBE site (UGUAUAUA), generating DsRED-VegT PBE mut-DS3'UTR (PBE mut, UAAAAAAA). One point mutation (G2A) in the PBE site has been shown to result in a 33-fold lower efficiency of Pumilio binding (Cheong and Hall, 2006). The images shown in Fig. 6C were taken at stage 34/35 at a time when PGCs have migrated laterally towards the body surface. The surface location of PGCs (outlined by dashed line) were identified by Xiwi immunostaining (green). Merged images are shown at the top, with separate channels beneath. Images were taken from the endoderm core. Scale bars: 50 μm.
The absence of the DsRED signal in reporters bearing the PBE could be explained by repression or by targeted DsRED RNA degradation, or both. WISH with the DsRED antisense probe revealed that the DsRED-VegT PBE-DS3’/H11032 UTR RNA was indeed degraded in wild-type PGCs, whereas the reporter lacking the PBE was unaffected (Fig. 7A). These data suggest that the VegT PBE and Pumilio binding mediate the translational repression of DsRED in part by destabilizing the reporter RNA. To address whether Nanos1 is required for this activity, we compared the endogenous VegT RNA level in PGCs with and without Nanos1 knockdown. The qRT-PCR results (Fig. 7B) indicate that VegT RNA is significantly more stable in Nanos1-depleted PGCs than in wild-type PGCs at stage 10 (1.63-fold increase, P=0.03). Importantly, the VegT level was similar in both wild-type and Nanos1-depleted PGC samples at stage 8, well after nanos1 is translated (Fig. 7B). We conclude that the Nanos1-Pumilio complex is required and sufficient to repress VegT translation in PGCs. Further, the data support repression as the primary cause and RNA degradation as secondary to eliminating germline VegT activity. Our findings identify a new downstream target for Nanos1/Pumilio repression and explain how PGCs avoid differentiation as endoderm.

**DISCUSSION**

Nanos1 impacts gene expression at two levels, promoting both transcriptional and translational repression. Our studies show that *Xenopus* Nanos1 is required to prevent PGCs from expressing an endoderm gene program and undergoing cell death (Fig. 8). Importantly, our work defines a new target for translational repression by Nanos1 and Pumilio, the endoderm determinant VegT. Given that Nanos strictly partitions with the *Xenopus* germline (Lai et al., 2011), our results explain how VegT repression is restricted to PGCs while neighboring blastomeres express VegT and are specified as endoderm.

**PGCs are lost from nanos1 morphants**

Knockdown of Nanos1 resulted in a dramatic decrease in PGC number and the subsequent loss of germ cells from the gonads, similar to what has been observed in other organisms (Forbes and Lehmann, 1998; Kobayashi et al., 1996; Köprunner et al., 2001; Sato et al., 2007; Tsuda et al., 2003). In Nanos1-depleted embryos of *Drosophila*, *C. elegans*, zebrafish, mouse and now *Xenopus* (this work), germ cells enter programmed cell death indicating that Nanos function as an anti-apoptotic factor has been conserved across species. In *Drosophila* germ cells, Nanos translationally
Nanos1 preserves germline fate

Ectopic migration has been observed in Nanos-depleted germ cells of Drosophila, zebrafish and mice. In zebrafish, migration is initiated early during gastrulation and nanos is required for PGC directed active movement (Köprunner et al., 2001). After Nanos1 knockdown, germ cells migrate abnormally to somites and the head region. However, our data have shown that Xenopus PGCs deficient in Nanos1 do not migrate to ectopic locations, but remain in the endoderm. Thus, Nanos1 involvement in Drosophila and zebrafish PGC migration appears to differ from that in Xenopus.

It is worth noting that not only Nanos1-depleted germ cells remaining in the endoderm undergo apoptosis, but wild-type PGCs that failed to migrate do so as well (Ikemishi et al., 2007; Köprunner et al., 2001). It is likely that a mechanism is in place that triggers apoptosis in any PGC that ‘veers’ off course from the normal migration pathway. Such observations contrast with those of Wylie et al. (Wylie et al., 1985), who found that Xenopus labeled PGCs placed in the blastocoel were able to incorporate normally into somatic tissue belonging to any of the three primary germ layers. Why wild-type PGCs remaining in the endoderm did not adopt an endoderm fate is not clear.

Loss of transcriptional repression in nanos1 morphants

In many organisms, including Xenopus, PGCs are protected from acquiring somatic fates in part by their state of transcriptional repression while somatic expression programs are initiated (Batchelder et al., 1999; Hanyu-Nakamura et al., 2008; Venkatarama et al., 2010; Zhang et al., 2003). A common target for global transcriptional repression is the kinase Positive transcription elongation factor b (P-TEFb), which phosphorylates CTD-Ser2 thus initiating transcriptional elongation events. In Drosophila, the germline protein Pgc physically interacts with P-TEFb and inhibits its recruitment to transcription sites, resulting in transcriptional repression. PIE-1 performs a similar function in C. elegans (Batchelder et al., 1999; Hanyu-Nakamura et al., 2008; Zhang et al., 2003). The first molecular aberration we observed in Nanos1-deficient PGCs was the premature appearance of CTD-PSer2 at the MBT. Loss of nanos in Drosophila also results in the premature phosphorylation of CTD-Ser2, but how this is mechanistically related to Pgc sequestration of P-TEFb function is not known. Thus, Nanos1 is in some way linked to the suppression of CTD-Ser2 phosphorylation in both the vertebrate and invertebrate germlines. Cdk9, which encodes the kinase in P-TEFb, contains a PBE in its 3’UTR, suggesting that it might be a target for Nanos1/Pumilio translational repression. However, our unpublished data indicate that CDK9 protein is expressed in the nucleus of wild-type PGCs during gastrulation and thus is unlikely to be the limiting factor. Further work will be required to establish how Nanos1 is linked to the repression of CTD-Ser2 phosphorylation and whether this depends on Nanos1 function as a translational inhibitor.

Expression of endoderm genes in nanos1 morphants

The second molecular aberration that we observed in Nanos1-deficient PGCs was the expression of the endoderm transcription factor Xsox17α, during gastrulation and well before PGC numbers declined. The expression of Xsox17α and Bix4 in Nanos1-depleted PGCs indicated VegT activity on their respective promoters and that zygotic transcription had indeed occurred prematurely. These results are entirely consistent with the observed premature CTD-PSer2 staining in PGCs. The endoderm-specific markers Mixer and
endoderm differentiation programs, is a dilemma not previously explained in other systems. The strict localization of nanos1 to the germ plasm, while Pumilio is not regulated, explains how the repression of VegT translation can occur exclusively in the germline. By contrast, zebrafish nanos1 is initially expressed throughout the whole embryo and is gradually restricted to PGCs by microRNA-mediated degradation of nanos1 RNA in the soma (Mishima et al., 2006).

Our data further support a role, either direct or indirect, for Nanos and Pumilio in destabilizing VegT RNA within PGCs. VegT RNA stability was related to both the presence of Nanos1 and the VegT PBE in our studies. The absence of Nanos could result indirectly in stabilization of VegT RNA, as translated RNAs are protected from degradation by the translation machinery. Alternatively, Nanos1/Pumilio could play a direct role in recruiting the CCR4-NOT deendylation complex, as demonstrated in the mouse germline (Suzuki et al., 2010). Ultimately, Nanos1/Pumilio binding leads to message degradation. However, for VegT RNA the degradation process occurred over several stages of embryogenesis and begins well after Nanos1 has been synthesized (Luo et al., 2011). Therefore, we favor the model whereby Nanos1/Pumilio repression is not primarily based on degradation of VegT RNA.

Taken together, our data indicate that the primary function of Xenopus Nanos1 is to prevent endoderm differentiation programs and thus preserve germ line totipotent fate (Fig. 8). What role Nanos1 plays in blocking apoptotic pathways and in supporting transcriptional repression in vertebrate systems remains an active area of research. Clearly, further functions for Nanos1 in the germline remain to be discovered. The diverse phenotypes described for nanos1 mutants underscore the importance of identifying the RNA targets of Nanos1 repression and of determining the pathways within which these repressed targets operate during embryogenesis.

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Competing interests statement

The authors declare no competing financial interests.

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