An Investigation of Nanos1 Function during Primordial Germ Cell Development in Xenopus Laevis.

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AN INVESTIGATION OF NANOS1 FUNCTION DURING PRIMORDIAL GERM CELL DEVELOPMENT IN *XENOPUS LAEVIS*

By

Fangfang Lai

A DISSERTATION

Submitted to the Faculty of the University of Miami in partial fulfillment of the requirements for the degree of Doctor of Philosophy

Coral Gables, Florida

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UNIVERSITY OF MIAMI

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy

AN INVESTIGATION OF NANOS1 FUNCTION DURING PRIMORDIAL GERM CELL DEVELOPMENT IN XENOPUS LAEVIS

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During Xenopus oogenesis, a variety of transcripts, which will be required later for
development, are produced, localized, and stored in oocytes. After fertilization, the
maternal transcripts are translated and used during early embryogenesis until zygotic
transcription at mid-blastula transition (MBT). nanos is one of the localized maternal
RNAs found in the germ plasm. Nanos is expressed in multipotent cells, stem cells, and
primordial germ cells (PGCs) of organisms as diverse as jellyfish and humans. One of the
well known molecular roles of Nanos is that it functions together with Pumilio to
translationally repress targeted mRNAs. We hypothesize that this function of Nanos is
required to preserve germline identity. In this thesis I present evidence from Loss-Of-
Function experiments that Xenopus Nanos1 is required to prevent endoderm gene
expression and apoptosis and thus to preserve PGC fate.

Xenopus nanos1 is transcribed in stage I oocytes, and this maternal message persists
only in the germ plasm through early embryogenesis until tailbud stage 33/34. nanos1
expressed in PGCs is strictly of maternal origin and it is not translated until fertilization.
Nanos1 protein could be detected until tailbud stage 33/34. As observed in other species,
morpholino knockdown of maternal Nanos1 resulted in a striking decrease in PGCs and
loss of germ cells from the gonads. Nanos1 deficient PGCs fail to migrate out of the endoderm and they appear to undergo apoptosis. What is novel here is that they inappropriately express somatic genes characteristic of endoderm regulated by maternal VegT including Xsox17-alpha, Bix4, Mixer, GATA4, and Edd, well before apoptosis. We further demonstrate that Pumilio specifically binds 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 maternal VegT RNA is an authentic target of Nanos1/Pumilio translational repression. This is the first time that a somatic determinant is identified as a target of Nanos/Pumilio repression, suggesting that the primary function of Xenopus Nanos1 in the germline is to prevent somatic differentiation.

Nanos depletion phenotypes have been well documented in various species. However, the mechanisms underlying these phenotypes are not all known. My thesis work addresses that PGCs arise from the endoderm mass without acquiring endoderm fates by restricting translational repression of VegT to the germline. These findings indicate how pivotal it is to identify the targets of Nanos/PUM translational repression in the germline to understand how the germline fate is preserved.

In addition to VegT, RNAs containing Pumilio binding element (PBE) may be targeted by Nanos1/PUM translational repression, such as cdk9 and Xwnt11. cdk9 PBE was bound by Pumilio and mediated translational repression of a reporter gene. However, Cdk9 protein was detected in wild type PGCs making it unlikely that cdk9 RNA repression in the germ plasm is relevant.
Maternal *Xwnt11* was found in the germ plasm and this RNA contains one canonical Pumilio binding element (PBE) and five non-canonical PBEs in the 3’UTR. The data from my work indicate that the five non-canonical PBEs are functional in Pumilio binding and translational repression. However, Nanos1 did not seem to be required in this repression event. It is possible that Pumilio binds *Xwnt11* and recruits other proteins to repress the translation and/or cause *Xwnt11* RNA degradation. Translational regulation of WNT pathway components are not well understood, so future work should be done to follow up on these findings.
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Chapter 1

Introduction

1.1. Germline formation in *Xenopus laevis*

Germ cells are the only cell population that can produce gametes so they are the foundation of sexual reproduction. Germ cells arise outside the gonad as primordial germ cells (PGCs) early during development in all animals. One of the essential questions in developmental biology is how PGCs are specified. So far, two mechanisms have been proposed (reviewed by Eddy, 2006). One is the inheritance of maternal cytoplasmic determinants contained within a subcellular domain called germ plasm. Germ plasm is found in *Drosophila, C. elegans,* zebrafish and *Xenopus.* The other mechanism involved in specifying PGCs is inductive signaling and involves the Bone Morphogenetic Protein (BMP) pathway in mouse and human.

In *Xenopus,* germ plasm is first identified in the mitochondrial cloud in pre-stage I oocytes (Heasman *et al.*, 1984). Beginning in stage II oocytes, germ plasm translocates to the vegetal pole and spreads along the vegetal cortex, through directional expansion of the mitochondria cloud (Wilk *et al.*, 2005). After fertilization and as the embryo divides, the vegetal pole localized germ plasm material is partitioned into separate blastomeres. Those cells that inherit sufficient germ plasm may become PGCs. Throughout early cleavage and the blastula stages (stage 9), germ plasm is found asymmetrically
Figure 1.1 Germline formation in *Xenopus laevis*. (A) Germ plasm was found in the mitochondria cloud in stage I oocyte, close to the nucleus. (B) A figure from Berekelya et al. (2005) shows the mitochondria cloud is located by the nucleus in stage I oocyte. (C) Germ plasm is localized in the vegetal pole in stage VI oocyte. (D) With embryo cleavage, germ plasm is inherited by vegetal blasomeres. (E) A figure from the literature shows the localization of germ plasm to the vegetal tip in 8-cell stage embryo. (F) Immunofluorescence using Xiwi antibody shows the localization of germ plasm to the vegetal tip in 8-cell stage embryo. (G) Around blastula stage, germ plasm is localized to the cell boundary. b.c., blastocole. g.p., germ plasm. (H) Around gastrula stage, germ plasm translocates to the cell boundary. (I) From early tailbud stage 25, PGCs begin active migration, first laterally, then dorsally, until they reach the dorsal mesentery. (J) PGCs migrate to the future gonads along the dorsal mesentery. (K) After PGCs arrive in the gonads, they begin to differentiate into gametes.

distributed within the cell and close to the plasma membrane. Cell divisions generate one daughter cell inheriting the germ plasm and preserving the ability to produce gametes while the other daughter cell follows somatic endoderm cell fates. As a result, the PGC number does not increase as do somatic cells. Only several PGCs (ranging from 3 to 7) are found before gastrula stage. As early gastrulation stages are reached (stage 10), the germ plasm translocates to a peri-nuclear position. PGCs divide symmetrically thereafter
with the result that the number of PGCs increases. However, cell division only occurs 2~3 times before PGCs migrate from the endoderm and into the genital ridges (stage 40) (Whitington and Dixon, 1975), suggesting that mitosis is slower in PGCs beginning at the gastrula stage (Dziadek and Dixon, 1975). It is likely that the slower pace of mitosis in PGCs ensures that each PGC receives a sufficient amount of germ plasm to maintain the germ cell fate (reviewed by Houston and King, 2000). During early tailbud stages (stage 24-26), PGCs begin active directed migration, first laterally to the body surface, and then dorsally into the dorsal crest of the endoderm, arriving there by early tadpole stages (stage 40) (Nishiumi et al., 2005). Germ cells subsequently migrate out of the endoderm and along the dorsal mesentery until they reach the gonads. Within the gonads, PGCs are induced to proliferate and undergo differentiation into functional gametes (Wylie and Heasman, 1976) (see Figure 1.1; reviewed in Wylie et al., 1985).

Germ plasm is a collection of mitochondria, endoplasmic reticulum, electron-dense germinal granules and specific localized RNAs and proteins. Our unpublished Mass-Spec results identified novel germ plasm components whose functions are unknown (Martinez and King, unpublished). The work about germ plasm component identification and corresponding function is ongoing. The localized RNAs include messenger RNAs, non-coding RNAs, and mitochondrial RNAs including large and small ribosomal RNAs (reviewed by King et al., 2005; Kashikawa et al., 2001; Kloc et al., 2001; Kobayashi et al., 1998). Some RNAs are associated within germinal granules and others are found in the germ plasm matrix (Kloc et al., 2001; Kloc et al., 2002). All available evidence points to germ plasm as the determinant of germ cell lineage. Removal of germ plasm or UV irradiation of the germ plasm containing region leads to embryo sterility (Buehr and
Blackler, 1970; Smith, 1966). Injection of vegetal cytoplasm containing germ plasm into UV irradiated embryos rescued the germ cell number (Smith, 1966; Wakahara, 1978).

To date, considerable efforts have gone towards identifying the functions of germ plasm components (see table 1.1). One example is the investigation of Nanos homolog function in different model organisms. *Drosophila* Nanos (Nos) is required for development of the abdomen as well as germline maintenance (Kobayashi et al., 1996; Sonoda and Wharton, 1999, 2001). $nos^{-/-}$ pole cells express somatic genes and undergo apoptosis resulting in the loss of germline. If apoptosis is inhibited, pole cells are incorporated into somatic tissues (Hayashi et al., 2004). Nanos homologs in *C.elegans*, zebrafish, and mouse also perform essential functions in maintaining germ cell survival (Köprüner et al., 2001; Saga, 2008, 2010; Subramaniam and Seydoux, 1999; Suzuki et al., 2008). Our latest work expanded the study of Nanos function to include the model system *Xenopus*. *Xenopus* Nanos1 only contains 128 amino acids. Two CCHC zinc fingers are located in the C-terminus. A domain containing 22 amino acids in the N-terminus is highly conserved in organisms as diverse as hydra and human, but, surprisingly, is not found in flies or worms (Lai et al., 2011; Mochizuki et al., 2000). One of our novel findings is that this N-terminal domain is required for Nanos1 to function as a translational repressor in the tethered function assay (Lai et al., 2011). Further, our unpublished observations suggest that this N-terminal region also carries a transcriptional repression function (Zhou, Luo and King, unpublished). Loss-of-Function studies reveal that *Xenopus* Nanos1 is required to prevent endoderm gene expression and cell death during germline development. Nanos1 depleted PGCs exhibit phosphorylated Ser2 in the CTD of RNA pol II (CTD-PSer2) prematurely during gastrulation and mis-express
Table 1.1 Germ plasm components and functions
(Proteins underlined are found in germinal granules.)

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<th>Function</th>
<th>Organism: Protein(s) (references)</th>
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<td>Translational Control</td>
<td></td>
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<tr>
<td>DEAD-box Helicases;</td>
<td><strong>Worm</strong>: GLH1-4 (Grüdel et al., 1996; Kuznicki et al., 2000; Spike et al., 2008), VBH-1 (Salinas et al., 2007), DRH-3 (Claycomb et al., 2009), CGH-1 (Audhya et al., 2005; Navarro et al., 2001), LAF-1 (Hubert et al., 2009); Fly: Vasa (Hay et al., 1988), Me31b (Nakamura et al., 2001); Frog: Hermes (King et al., 2005; Zearfoss et al., 2004), DEADSouth (MacArthur et al., 2000), XVGL1 (Komiya et al., 1994); Fish: Vasa (Braat et al., 2003)</td>
</tr>
<tr>
<td>RNA-binding Proteins and Complex Factors;</td>
<td></td>
</tr>
<tr>
<td>Repression of Translation;</td>
<td><strong>Worm</strong>: CAR-1 (Audhya et al., 2005; Squirrell et al., 2006), Nanos3 (Kraemer et al., 1999), Me31b (Nakamura et al., 2001), CGH-1 (Navarro et al. 2001); Fly: Trailer hitch (Wilhelm et al., 2005), Nanos (Kobayashi et al., 1996; Hayashi et al., 2004); Frog: Nanos (Lai et al., 2011; Lai et al., 2012);</td>
</tr>
<tr>
<td>De-capping Factors;</td>
<td><strong>Worm</strong>: DCAP-1 (Salinas et al., 2007; Lall et al., 2005), DCAP-2 (Lall et al., 2005), PATR-1 (Gallo et al., 2008); Fly: dDep1 (Lin et al., 2006)</td>
</tr>
<tr>
<td>Splicing Factors;</td>
<td><strong>Fly</strong>: Sm (Barbee and Evans, 2006), Fly: SmB, SmD3 (Amne, 2010); Frog: Sm (Bilinski et al., 2004)</td>
</tr>
<tr>
<td>Deadenylases;</td>
<td><strong>Worm</strong>: CCF-1 (Galoo et al., 2008)</td>
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<tr>
<td>Vasa-Stabilizers;</td>
<td><strong>Fly</strong>: Gus, Fsm (Styhler et al., 2002; Kugler et al., 2010)</td>
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<tr>
<td>Translation Factors;</td>
<td><strong>Worm</strong>: DCP-1 (Salinas et al., 2007); Lall et al., 2005, DCAP-2 (Lall et al., 2005), PATR-1 (Galoo et al., 2008); Fly: dDep1 (Lin et al., 2006)</td>
</tr>
<tr>
<td>Polymerases and Complex Factors.</td>
<td><strong>Worm</strong>: GLD-2 (Wang et al., 2002), GLD-4 (Schmid et al., 2009), GLS-1 (Rybarska et al., 2009), EGO-1 (Claycomb et al., 2009)</td>
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<td>Transcriptional Silencing</td>
<td><strong>Fly</strong>: Pge (Hanyu-Nakamura et al., 2008), GCL (Leatherman et al., 2002), <strong>Worm</strong>: PIE-1 (Tenenhaus et al., 2001)</td>
</tr>
<tr>
<td>RNAi Pathway</td>
<td></td>
</tr>
<tr>
<td>miRNA/piRNA biogenesis</td>
<td><strong>Worm</strong>: PRG-1 (Batista et al., 2008), CSR-1 (Claycomb et al., 2009), CDE-1 (van Wolfswinkel et al., 2009; Mello et al., 1996); Fly: Piwi (Megosh et al., 2006), Aubergine (Harris et al., 2001), Maeldrom (Findley et al., 2003), PAPI (Liu et al., 2011), Frog: Aubergine (Becalska et al., 2011), Xiw1 (Lau et al., 2009); Fish: Xiwi, Zili (Houwing et al., 2003), Dead end (Weidinger et al., 2003), Hen1 (Kamminga et al., 2010), DAZL (Takeda et al., 2009)</td>
</tr>
<tr>
<td>Germ granules assembly</td>
<td><strong>Fly</strong>: Tudor (Bardsley et al., 1993)</td>
</tr>
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<td>Cell Polarity</td>
<td><strong>Worm</strong>: PAR-1 (Guo and Kemphues, 1995); Fish: Buc (Bontems et al., 2009; Marlow and Mullins, 2008)</td>
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<td>Germ Plasm Localization</td>
<td><strong>Fly</strong>: Oskar (Breitwieser et al., 1996), Valois (Anne and Mechler, 2005), Frog: Xklp1 (Robb et al., 1996), Xpat (Machado et al., 2005), Pix-1 (Hames et al., 2008)</td>
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<td>Cell Signaling</td>
<td><strong>Frog</strong>: XALK4 (Fukui et al., 2003)</td>
</tr>
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<td>Anti-Apoptotic</td>
<td><strong>Frog</strong>: Survivin (Murphy et al., 2002)</td>
</tr>
<tr>
<td>Unknown Function</td>
<td><strong>Worm</strong>: DEPS-1 (Spike et al., 2008), MEG-1, MEG-2 (Leacock and Reinke, 2008; Kapelle and Reinke, 2011); Frog: GASZ (Yan et al., 2004), Dead end (Horvay et al., 2006)</td>
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endoderm specific genes. Nanos1 depleted PGCs enter apoptosis during their migration out of the endoderm, resulting in the loss of gonadal germ cells (Lai et al., 2012; see the details in Chapter 3). Furthermore, I found that the endoderm determinant VegT is a target of Nanos1/PUM translational repression in PGCs (Lai et al., 2012). This observation explains how Nanos1 prevents the germline from endoderm differentiation. How does Nanos1 function as a transcriptional repressor? Is that function related with its function as a translational repressor? What is the role of Nanos involved in the regulation of CTD-PSer2 in PGCs? What other germline specific RNA targets, i.e. apoptosis factors, are translationally repressed by Nanos1/PUM? Much remains to be determined.

1.2. Translational control during germline development

Translational regulation of gene expression in the germline plays a key role in preserving the germline identity. Germline specific translational repression assures that soma development does not occur in germ cells. In germ plasm bearing animals, the available evidence supports translational repression as crucial to preventing somatic specification, apoptosis, and somatic cell-cycle rates. In hermaphroditic C. elegans, translational repression controls the sperm to oocyte switch (Ahringer and Kimble, 1991; Zhang et al., 1997). Regional translational activation guarantees that germline specific genes are only expressed in germ cells; expression of germline genes in the soma could drive cancer formation (Janic et al., 2010).
1.2.1 Germline specific translational repression

In *Drosophila*, *C. elegans*, zebrafish, and *Xenopus*, germ cells form within the region of somatic endoderm. Therefore, it is very likely that some maternal RNAs required for soma specification may be inherited by germ cells and their expression has to be repressed in order to preserve germline identity. Although germ cells in mice and human are specified by inductive signaling between cells, the molecules specifically expressed in the germline and their functions are evolutionarily conserved (reviewed by Eddy, 2006). We know that either proteins or small non-coding RNAs can mediate translational repression in the germline.

**Pumilio/Nanos** functions as a translational repression complex (Sonoda and Wharton, 1999). This complex is evolutionarily conserved in the germline from worms to human (Kraemer et al., 1999; Jaruzelska et al., 2003). PUM/Nanos negatively regulate the translation of specific mRNAs that contain the Pumilio binding element (PBE) in their 3’UTRs (Sonoda and Wharton, 1999). A study of the human Pumilio1 homology domain (PUM-HD) crystal structure indicates that each of the eight PUM repeats recognizes and interacts with one RNA base of the canonical PBE (UGUANUA, where N could be any nucleotide) (Wang et al., 2002; White et al., 2001). Further, the specificity of the last three nucleotides (AUA) is not fixed and one mismatch can be tolerated (Cheong and Hall, 2005), which is then termed non-canonical PBE. The classical model of PUM/Nanos translational repression involves *hunchback* RNA (Figure 1.2A). The translational regulation of *hunchback* is crucial for polarity along the A/P body axis.
Table 1.2 Known targets of Nanos/Pumilio translational repression
(*, germline specific)

<table>
<thead>
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<th>Gene name</th>
<th>Function</th>
<th>Species</th>
<th>References</th>
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<tr>
<td>cyclin B*</td>
<td>Cell cycle regulation</td>
<td>Drosophila</td>
<td>Kadyrova et al., 2007</td>
</tr>
<tr>
<td>head involution defective (hid)*</td>
<td>Apoptosis factor</td>
<td>Drosophila</td>
<td>Sato et al., 2007</td>
</tr>
<tr>
<td>fem-3*</td>
<td>Control of the sperm to oocyte switch</td>
<td>C. elegans</td>
<td>Ahringer and Kimble, 1991; Zhang et al., 1997</td>
</tr>
<tr>
<td>hunchback</td>
<td>Body A/P axis establishment</td>
<td>Drosophila</td>
<td>Cho et al., 2006; Sonoda and Wharton, 2001</td>
</tr>
<tr>
<td>bicoid</td>
<td>Body pattern establishment</td>
<td>Drosophila</td>
<td>Wharton and Struhl, 1991</td>
</tr>
<tr>
<td>para</td>
<td>Sodium channel</td>
<td>Drosophila</td>
<td>Muraro et al., 2008</td>
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Pumilio recognizes and binds to the PBE in *hunchback* 3’UTR and Nanos is recruited by interacting with Pumilio through zinc fingers. Further, Brat joins this protein complex by interacting with Nanos through the zinc finger domain. Brat interaction with 4E-HP results in the translational repression of *hunchback* because 4E- HP does not bind eIF4G so that the translation initiation is inhibited (Figure 1.2A; Sonoda and Wharton, 2001; Cho et al., 2006). This translational regulation function of PUM/Nanos also plays a critical role in preserving germline identity. In the following section, we will review the known germline targets of PUM/Nanos translational repression (see table 1.2).

**Translational regulation of fem-3 (Figure 1.2C)**

*fem-3* is mainly expressed in the germline of *C.elegans* hermaphroditic adults (Rosenquist and Kimble, 1988). Ahringer and Kimble (1991) showed that the translational repression of *fem-3* controls the sperm to oocyte production switch. *fem-3*
Figure 1.2 The model of translational repression of hunchback and cyclinB. (A) In hunchback 3’UTR, Pumilio binds to the PBE and then recruits Nanos. Nanos brings Brat which interacts with 4E-HP. 4E-HP could not bind to eIF4G and thus the translation is impaired. (B) In the 3’UTR of cyclin B, PUM binds to PBE and recruits Nanos. Nanos interacts with NOT4 and thus brings the CCR4-pop2-NOT deadenylase complex. Deadenylation then happens and the translation is repressed. PUM, pumilio. PBE, Pumilio binding element. (C) C.elegans fem-3 is bound by FBF at the PME in the 3’UTR, recruiting Nanos3 and other unknown components, resulting in the translational repression. FBF, fem-3 binding factor. PME, point mutation element.

Gain-of-function produced excess amount of sperm without oocytes, while fem-3 loss-of-function resulted in only oocytes. By genetic analysis and titration experiments, they predicted a repressor would be found to bind fem-3 3’UTR and negatively regulate the
translation during the sperm-oocyte switch. Later, Zhang and their colleagues (1997) used a modified yeast-three-hybrid system and identified *fem-3* binding factor (FBF) to be the repressor that could bind the *fem-3* 3’UTR. *fhb* mRNA and protein are only expressed in the germline, and the expression peak happens during the sperm to oocyte switch. Further, they found that FBF depletion mimicked the phenotypes observed in *fem-3* gain-of-function mutants, that is, excess sperm without oocyte production. FBF contains eight tandem repeats that are conserved in *Drosophila* Pumilio (Barker et al., 1992), and together they form the PuF protein family. The highly conserved eight repeats with small flanking sequences at each end are required to bind *fem-3* RNA. The next question was what other components are interacting with FBF to regulate *fem-3* translation? In 1999, Kraemer and their colleagues used yeast two hybrid system and identified NANOS-3 physically interacting with FBF (Figure 1.2C). NANOS-3 interacts with FBF through an N-terminal domain other than the C-terminal zinc fingers domain, in contrast to *Drosophila* Pumilio which interacts with Nanos through the zinc finger domain (Sonoda and Wharton, 1999). Although the other two homologues, NANOS-1 and NANOS-2, do not directly interact with FBF, the three NANOS homologues function redundantly in controlling the sperm-oocyte switch. Depletion of NANOS1/NANOS3 or NANOS2/NANOS3 leads to the failure to switch to oogenesis, while elimination of all three homologues resulted in germline death. Therefore, *C.elegans* NANOS homologues are required not only for the switch from spermatogenesis to oogenesis but also for germline survival.

The function of FBF in translational repression was also revealed in germline stem cell studies in *C. elegans*. Stem cells choose between entering self-renewal or differentiation.
FBF negatively regulates the translation of *gld-1* mRNA, which controls meiotic entry by directly binding to the FBF binding element (FBE) in the 3’UTR and repressing GLD-1 translation. Repression of GLD-1 serves to block differentiation and thus maintain the germline stem cell population (Crittenden et al., 2002). The Cip/Kip cell cycle inhibitor CKI2 is also translationally repressed by FBF through the FBE in the 3’UTR of *cki2* RNA. Repression of CKI2 promotes cell division and maintains stem cell self-renewal in the germline (Kalchhauser et al., 2011).

**Translational regulation of apoptosis through hid**

*Head involution defective (hid)* belongs to the RHG gene family which functions upstream to activate Caspases during apoptosis in flies (Grether et al., 1995). In *Drosophila, nos*⁻/⁻ pole cells enter apoptosis during migration (Hayashi et al., 2004). Apoptosis was inhibited in *nos*-H99 pole cells, a *Drosophila* strain that contains a genomic deletion involving RHG genes, including *hid* (Hayashi et al., 2004). Sato and their colleagues (2007) did a search of the *hid* mRNA sequence and found a PBE-like sequence in the 3’UTR. *hid* mRNA was also expressed in the germline. Therefore, *hid* is very likely to be targeted by Nanos (Nos) repression in order to prevent apoptosis in pole cells. To test this hypothesis, they deleted maternal Nos and zygotic *hid* and found that apoptotic pole cells were significantly reduced, suggesting apoptosis in *nos*⁻/⁻ pole cells requires *hid* activity. Next, the expression of *hid* mRNA with or without PBE was expressed under *nos* promoter in pole cells. As a result, apoptosis occurred in pole cells expressing *hid* without PBE, but it was rarely observed when *hid* with PBE was
expressed. Therefore, *hid* translation was repressed by Nos in a PBE-dependent manner in the germline. Elimination of either Nos or the PBE sequence in *hid* 3’UTR resulted in *hid* translation and apoptosis in pole cells. It is known that Pumilio binds PBE and then recruits Nos, so Nanos/PUM translationally represses *hid* expression and thus promotes germline survival. Another RHG gene *sickle* (*skl*) was also repressed by Nos, but no evidence supports it as a substrate of Nanos/PUM translational repression. Thus, *skl* may be translationally repressed in pole cells by a protein complex including Nos.

**Translational regulation of cyclin B (Figure 1.2B)**

cyclin B is a maternal RNA inherited in the germline whose protein functions during mitosis. Cell division is blocked in *Drosophila* pole cells shortly after they are formed (Su et al., 1998). This pause in germline mitosis was terminated precociously in *pum* or *nos* embryos, and resulted in more pole cells (Asaoka-Taguchi et al., 1999; Deshpande et al., 1999). Over expression of Cyclin B drove premature mitosis in pole cells of wild type embryos (Asaoka-Taguchi et al., 1999). Furthermore, a search of *cyclin B* mRNA disclosed a PBE-like sequence in the 3’UTR. These findings strongly suggested that *cyclin B* was a target of PUM/Nanos translational repression. Later, Kadyrova and their colleagues (2007) identified the two PUM binding elements (PBEs) in *cyclin B* 3’UTR and both are required for sufficient translational repression. They also found that *CCR4* mutants presented premature accumulation of Cyclin B protein in the germline, suggesting *cyclin B* RNA might be translationally repressed through deadenylation. Additionally, Nanos interacted with NOT4 in yeast two hybrid experiments. However,
their data indicated that PUM was not necessary as long as Nanos was tethered to cyclin B 3’UTR. Furthermore, ectopic expression of Nanos in soma failed to impair somatic cell unless Osk was ectopically expressed. These results indicated that there must be another unknown germline restricted factor co-repressing cyclin B translation. The current model for cyclin B translational repression is that PUM binds to PBE and recruits Nanos through the interaction with Nanos zinc fingers; Nanos then brings in the CCR4-pop2-NOT deadenylase complex by interacting with NOT4 through the Nanos N-terminal region. Deadenylation results in the translational repression of cyclin B (Figure 1.2B).

Another observation worth our attention is that the relative position of the Nanos binding site to PBEs in cyclin B is different from that in hunchback. As a result, although it is the same protein-protein interaction (PUM/Nanos), different topographic structures are formed on specific RNAs, leading to subsequent recruitment of unique components as well as divergent consequences (Figure 1.2). The translational regulation of cyclin B1 by PUM was also studied in Xenopus oocytes (Nakahata et al., 2001; 2003). Nanos1 could physically bind to cyclin B1 (Lai et al., 2011). It’s likely that Nanos1/PUM translationally regulates cyclin B1 in Xenopus germline since mitosis is delayed in PGCs before they arrive in the gonads (Whittington and Dixon, 1975).

Mouse Nanos2 is found in the P-bodies of male gonocytes and it is required for P-body formation (Suzuki et al., 2010). P-bodies are a center for RNA degradation and turnover. Suzuki and his colleagues (2010) also found that Nanos2 physically interacted with the deadenylase complex CCR4 –NOT in vitro and regulated its localization to P-bodies in vivo. In a pull-down assay, Nanos2 specifically binds to some RNAs that are required
during meiosis in female germ cell differentiation. Therefore, in male gonocytes, Nanos2 binds to specific RNAs, such as Stra8 (Suzuki and Saga, 2008), and promotes their degradation in P-bodies by recruiting the CCR4-NOT deadenylase complex, thus suppressing meiosis and protecting male germline differentiation. Consistent with this hypothesis, Nanos2−/− male germ cells entered meiosis and exhibited features of female germline development.

**miRNAs** are small non-coding RNAs with a length of 21~23 base pair. Pri-miRNAs are transcribed by RNA polymerase II in the nucleus and processed to generate pre-miRNAs by the Drosha/DGCR8 protein complex. Pre-miRNAs are then exported through Exportin5 into the cytoplasm where Dicer operates on the pre-miRNAs producing double stranded miRNAs (reviewed by Saxe and Lin, 2011). The guide strand of the double stranded miRNAs then cooperates with the RNA-induced silencing complex (RISC) including Argonaute, to carry out the translational repression and/or degradation of specific RNAs. The targeted RNAs could be recognized by complementing the “seed” sequence in the 5’ end of miRNAs. miRNAs are required for primordial germ cell formation and germline stem cell maintenance (reviewed by Saxe and Lin, 2011). Deletion of miRNA biogenesis factor Dicer leads to sterility in *Drosophila* and *C.elegans* (Knight and Bass, 2001; Megosh et al., 2006; Park et al., 2007). miRNAs are ubiquitously distributed and they repress RNA translation through similar mechanisms both in the soma and germline. In human germ cells, NANOS1/PUMILIO2 physically interact with miRNA biogenesis factor GEMIN3 and they are co-localized in the chromatoid body where RNA degradation and turnover occurs (Ginter-Matuszewska
et al., 2011). These findings suggest that some RNAs may be specifically recognized and bound by NANOS1/PUMILIO2, translocated to the chromatoid body, and finally degraded by miRNA mediated RNA silencing. Since NANOS1 is expressed only in the germline, the RNA degradation mediated by miRNA in the chromatoid body is germ cell specific. A general theme that is emerging appears to be that cooperation with tissue specific components will restrict the function of miRNAs to particular RNA targets. Similar observations have also been found in cancer cells. In tumors, Pumilio binds to specific tumor-suppressing RNAs, such as p27, and causes a change of the RNA structure, which exposes the miRNAs recognition site resulting in the recruitment of miRNAs and subsequent degradation of the RNAs (Kedde et al., 2010).

As a guardian angel of germline identity, PUM/Nanos translationally represses a variety of messages that are required for somatic specification, mitosis and cell death. PUM usually functions as a bridge. It specifically binds to some RNAs by recognizing the PBEs and then recruits Nanos and other components, such as Brat, CCR4-NOT, or the miRNA-induced silencing complex. However, it is not understood how protein recruitment is carried out. Ginter-Matuszewska and their colleagues (2009) provide some clues to this process. They discovered that SNAPIN physically interacted with PUM2/Nanos1 in human spermatogonia. SNAPIN is expressed in all the tissues and it may have a role in vesicle fusion (Buxton et al., 2003; Vites et al., 2004). Therefore, it is speculated that SNAPIN may function as a multi-purpose adaptor that regulates and provides a scaffold for the interaction of PUM/Nanos with other proteins in the germline.
The examples of PUM/Nanos translational repression as described above show how important it is to identify other targeted messages specifically in the germline to understand how the germline fate is preserved during development. Thus, by searching the *Xenopus* laevis gene sequence database, we have obtained a list of potential targets of PUM/Nanos translational repression based on the PBE-like sequences in their 3’UTR (Murray R and King ML, unpublished). Focusing on RNAs localized to the vegetal pole and therefore, candidates for being mis-localized to the germline, we compiled a list of the most likely RNAs in table 1.3. Future work will verify by gel shift and *in vivo*

<table>
<thead>
<tr>
<th>Name</th>
<th>Function</th>
<th># PBEs</th>
<th># Non-canonical PBEs</th>
</tr>
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<tbody>
<tr>
<td>VegT</td>
<td>Endoderm determinant</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>cdk9</td>
<td>Transcription Activation</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td><em>Xwt11</em></td>
<td>Dorsal-Ventral Axis</td>
<td>1</td>
<td>5</td>
</tr>
<tr>
<td><em>Cyclin B1</em></td>
<td></td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td><em>Xotx1</em></td>
<td>Transcription Factor for Head Formation</td>
<td>1</td>
<td>4</td>
</tr>
<tr>
<td><em>Xlerk</em></td>
<td>Erythropoietin-Producing Hepatocellular Ligand</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td><em>Vg1</em></td>
<td>Mesoderm-inducing Factor</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td><em>Xvelo</em></td>
<td>Germ Plasm Organization</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td><em>xARHa</em></td>
<td>LDL-Receptor Adaptor Protein</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>β-TrCP-2</td>
<td>Ubiquitin Ligase Receptor</td>
<td>1</td>
<td>1</td>
</tr>
</tbody>
</table>

PBE: Pumilio binding element (UGUANAUA: “N” can be any nucleotide)
Non-canonical PBE: one mismatch in the last three nucleotides (AUA)
function assays, which candidates are true targets of Nanos1/PUM translational repression.

**Piwi/piRNA**

Piwi belongs to the Piwi/Argonaute protein family that is known to be a member of the RNA induced silencing complex. Unlike Argonaute, vertebrate Piwi is mainly expressed in the germline. Piwi is required for the pole cell formation in flies, as Piwi functions to localize OSK and VASA to the pole plasm. Increased Piwi expression induced more pole cell numbers (Megosh et al., 2006). Zebrafish Piwi homolog, Ziwi, functions to protect germ cells from death; Zili is required in germline differentiation and meiosis (Houwing et al., 2007; Houwing et al., 2008). Piwi and Piwi-interacting RNAs, piRNAs, function in the epigenetic regulation and silencing of transposons protecting the integrity of the germline genome. Piwi also functions in the translational regulation of germline specific RNAs. Sea urchin Piwi homolog, Seawi, is a component of the translationally silencing ribonucleoprotein (RNP) complex that binds bep4 mRNA. The speculation is that Seawi binding temporally and spatially regulates bep4 translation within the micromeres resulting in the correct localization of β-catenin, required for embryonic axis establishment (Rodriguez et al., 2005). Mouse Piwi homologs, Miwi and Mili, were found to interact with the cap-binding complex (Grivna et al., 2006; Unhavaithaya et al., 2009). Miwi co-fractionates with polysomes, suggesting that Miwi may be involved in translational regulation (Grivna et al., 2006). Mili deletion resulted in the degradation of specific RNAs as well as loss of germline stem cell self-renewal. Mili may have a
function in protecting translation of some RNAs that will be required for the germline stem cell maintenance (Unhavaithaya et al., 2009). The role of Piwi in translational regulation is not completely understood. How are those specific RNAs recognized by Piwi? Are piRNAs involved in this translational regulation? What other proteins are involved that may cooperate with Piwi? These questions remain unanswered in the field.

1.2.2 Germline specific translational activation

Maternal transcripts are destined to be degraded during the mid-blastula transition (MBT, maternal to zygotic transition), and this process is mediated by miRNAs. miRNAs are zygotically transcribed during MBT, ubiquitously expressed in the embryo including the germline. Germ cells remain transcriptionally quiescent when the maternal to zygotic transition occurs in somatic cells. A variety of maternal transcripts are required to play crucial roles in germline development, and these must be protected from the wholesale elimination of maternal transcripts and specifically activated in the germline. Two proteins, Dead-end and DAZ, have been discovered to have such a function that prevents germline specific maternal RNAs from miRNA mediated degradation.

**Dead-end**, a unique germ plasm component found only in the vertebrate germline, is an RNA binding protein with ATPase activity (Horvay et al., 2006; Weidinger et al., 2003; Youngren et al., 2005). Dead-end loss-of-function results in the loss of primordial germ cells (PGCs) in zebrafish and *Xenopus* (Horvay et al., 2006; Weidinger et al., 2003). Mutations in *Ter*, the mouse *Dead-end* homolog, caused PGC loss as well as germ cell
Figure 1.3 Dead-end activates zebrafish *nanos* translation by antagonizing miRNA activity. In the 3’UTR of *nanos*, Dead-end binds to the URRs and hides the miRNA targeting site, resulting in the stabilization of the RNA as well as translation.

tumors (Youngren et al., 2005). Therefore, Dead-end activity is required for germline maintenance and survival. Recent studies in zebrafish revealed a function of Dead-end in counteracting miRNA mediated RNA degradation (Chen et al., 2010; Kedde et al., 2007; Mickoleit et al., 2011).

Dead-end recognizes and binds to the uridine-rich regions (URRs) in the 3’UTR of specific RNAs (Kedde et al., 2007). Dead end binding blocks the access of miRNA to sites in the 3’UTR, thus prohibiting the degradation of the RNA (Kedde et al., 2007). Deletion of URRs in the 3’UTR resulted in RNA degradation. In zebrafish, maternal *nanos* message is ubiquitously distributed in the embryo and its somatic localization is eliminated by the fifth day of development by miR-430 mediated RNA degradation (Figure 1.3; Köprunner et al., 2001; Mishima et al., 2006). *nanos* degradation in the
germline is prevented by Dead-end binding to its 3’UTR, resulting in the germline specific expression (Kedde et al., 2007). Similar mechanisms were also utilized for the germline specific expression of TDRD7, Geminin, and HuB (Chen et al., 2010; Kedde et al., 2007; Mickoleit et al., 2011). Elimination of germline specific gene expression in the soma is essential for normal development. In summary, Dead-end protects specific maternal transcript from degradation and assures their germline specific expression by antagonizing miRNA function.

DAZ is abbreviated for Deleted in AZoospermia, a gene required for human male fertility (Reijo et al, 1995). Together with DAZ-Like (DAZL) and BOULE, they form the DAZ gene family in humans (Chai et al., 1997; Dorfman et al., 1999; Saxena et al., 1996; Seboun et al., 1997; Xu et al., 2001; Yen et al., 1996). DAZL homologues have been identified in many species, including Drosophila, C.elegans, Xenopus, zebrafish, and mice (Hashimoto et al., 2004; Houston and King, 2000; Reijo et al., 1996; Xu et al., 2001). DAZL is an RNA binding protein and required for germline development (Hashimoto et al., 2004; Houston and King, 2000; Reijo et al., 1995; Ruggiu et al., 1997). In the germline, there are specific RNAs translationally repressed during early development that need to be activated at specific developmental stages. DAZL plays a role in the translational activation of germline RNAs, such as Tdrd7. These RNAs contain DAZL-binding motifs in the 3’UTRs. DAZL binds to the 3’UTR and recruits poly-(A) binding proteins (PABPs) that will initiate translation (Collier et al., 2005). Takeda and his colleagues (2009) have found that zebrafish DAZL could protect specific RNAs, such as Tdrd7, from RNA degradation mediated by miR430 in the germline.
DAZL binds to specific regions in the 3’UTR, which blocks the miRNA targeting sites and thus stabilizes the RNA. RNAs are subsequently polyadenylated, resulting in translational activation.

Therefore, through blocking miRNA mediated RNA degradation, Dead end and DAZL help to restrict the expression of specific genes in the germline, crucial for the preservation of germline fate. This mechanism explains why the same gene such as *nanos* may have different expression patterns in different organisms. *Xenopus nanos1* is specifically localized in the germ plasm, while zebrafish *nanos* is ubiquitously distributed in the embryo (Kloc et al., 2000; Köprunner et al., 2001). Germline specific translational activation assures the gene function is preserved in germ cells during its clearance in the soma. Identification of those specific RNAs protected by Dead end or DAZL in the germline will help us understand what kind of molecular networks function to preserve the germ cell fate.

### 1.2.3. Concluding Remarks

Maintenance of germline fate is one of the most critical events during embryo development because germ cells are the only cell population that can pass genetic information to the offspring. To preserve germline identity, various molecules, such as proteins and small non-coding RNAs, cooperate in a network to regulate germ cell development.
Pum/Nanos mediated translational repression is germline specific during early embryogenesis. The function of this complex is evolutionarily conserved across species from worms to humans (Kraemer et al., 1999; Jaruzelska et al., 2003). In adults, Pum/Nanos also functions in the nervous system to carry out translational repression of specific RNAs, such as *Drosophila* voltage-gated sodium channel *paralytic* (*para*). In *Drosophila* motorneurons, Pum recognizes and binds to the PBE in the *para* mRNA open reading frame (ORF), recruits Nanos and then Brat, resulting in the repression of *para* translation (Muraro et al., 2008). Pum also functions to regulate the global protein synthesis in neurons by repressing the translation of *eIF4E* (Chen et al., 2008). Human Pum 1 and Pum 2 can bind to the PBE in the 3’UTR of oncogene E2F3 mRNA and thus repress the E2F3 protein expression as well as ‘off scale’ cell proliferation; Pum also facilitates miRNA-mediated regulation of E2F3 expression; Pumilio/miRNA cooperation on translational regulation of E2F3 prevents cancer formation (Miles et al., 2011).

Mouse Pumilio2 (PUM2) was found in the neuromuscular junction. PUM2 can specifically bind to the PBE in the 3’UTR of acetylcholinesterase (AChE) and repress AChE translation in neuromuscular synapse (Marrero et al., 2011). Further, our unpublished observations indicate that *Xenopus* Pumilio1 is involved in the translational regulation of *Xwnt11* in the germline. This regulation is through non-canonical PBEs in the *Xwnt11* 3’UTR, instead of the canonical PBE (Lai and King, unpublished, see Chapter 4). Therefore, the same molecules, PUM/Nanos, may function in different tissues at particular developmental stages through distinctive mechanisms. Identification of the RNA targets of PUM/Nanos and the protein complexes that are recruited to this complex
will undoubtedly contribute to uncovering essential networks operating during development.

The investigation of the function of small non-coding RNAs (sRNAs) is a new field and the research in this field has been developing very quickly in recent years. sRNAs are known to function in transposon silencing to protect the genome integrity. Their function in translational repression and/or degradation of specific messages is also well known. The cooperation of sRNAs and germline components in regulating the translation of specific RNAs is becoming a new research area. Tissue specific operators bind to targeting RNAs, resulting in a structural change in the RNA and thus concealing/exposing miRNA recognition signals resulting in RNA stabilization/degradation. This may be a general mechanism in regional translational activation/repression. Finding out those tissue specific operators and their corresponding RNA targets will help us understand more about the regulatory network during development.
Chapter 2

*nanos1* RNA and Protein are only Expressed in Primordial Germ Cells during Early Embryogenesis

**Summary**

*nanos1* RNA is transcribed in pre-stage I oocytes and associated with germinal granules. This maternal RNA persists only in the germline until embryonic stage 34. There is no zygotic transcription of *nanos1* in the germline during early embryogenesis but the zygotic transcript could be detected in the head at tadpole stage 56. Although *nanos1* RNA is translationally repressed during oogenesis, it is translated after fertilization. Nanos1 protein was detected exclusively in the germ plasm by immunostaining. Nanos1 persisted in the germline until embryogenesis stage 34 when the primordial germ cells (PGCs) are migrating out of the endoderm.

**Background**

*nanos* RNA is identified to be a germ plasm component in *Drosophila, C.elegans, zebrafish,* and *Xenopus* (Forristall et al., 1995; Kobayashi et al., 1996; Köprunner et al., 2001; Subramaniam and Seydoux, 1999). *Drosophila nanos (nos)* RNA is distributed in a gradient pattern in the oocyte through diffusion and anchorage in the pole plasm by
F-actin cytoskeleton (reviewed by Kugler and Lasko, 2009). Unlocalized nos is translationally repressed through two mechanisms mediated by Smaug: cap-dependent translational repression and deadenylation (reviewed by Richter and Lasko, 2011). Osk allows nos translation in the pole plasm by preventing Smaug/CCR4 mediated deadenylation (Zaessinger et al., 2006). Maternal nanos RNA is ubiquitously distributed in the zebrafish one-cell stage embryo but becomes restricted to the germline through miR430 mediated degradation in the soma (Köprunner et al., 2001; Mishima et al., 2006). Mouse nanos1 is transcribed maternally, but nanos2 and nanos3 are zygotically expressed (Haraguchi et al., 2003; Tsuda et al., 2003). Human Nanos1 is ubiquitously expressed, but Nanos2 and Nanos3 expression are restricted to the germline and brain; the protein expression of NANOS 1, 2, 3 is only found in the germline except that NANOS 3 is also found in the brain (Julaton and Reijo Pera, 2011).

Xenopus nanos1 was previously found in oocytes and primordial germ cells (PGCs) during early embryogenesis and Nanos1 protein could be detected until gastrula stage 12 (Forristall et al., 1995; MacArthur et al., 1999; Mosquera et al., 1993). By using more sensitive techniques, nanos1 RNA and protein expression was detected until tailbud stage 34 when PGCs are migrating out of the endoderm.

Results

2.1. Germline nanos1 RNA is maternally derived

As multiple nanos family members have been described in mouse (Haraguchi et al., 2003; Tsuda et al., 2003), we searched the Xenopus tropicalis genome for possible nanos
members in addition to Nanos1. Three candidates were identified and tested for expression in germ cells, embryos or in adult X. tropicalis tissues using primers specific to each candidate. There was no evidence for expression for any of these putative nanos family members. However, Nanos1 was detected in adult testis and brain in addition to the previously described expression sites: oocytes and PGCs (Lai et al., 2011; Mosquera et al., 1993). Using the more sensitive method of RT-PCR, maternal nanos1 RNA was found to persist until tailbud stage 33/34 (Figure 2.1A; Figure 2.3).

The extended expression of nanos1 raised the question of whether it was strictly of maternal origin or whether it was also zygotically transcribed at stage 14 when PGCs first become transcriptionally active (Venkatarama et al., 2010). In mouse, all three Nanos members are zygotically expressed although Nanos1 is maternally expressed as well (Haraguchi et al., 2003; Tsuda et al., 2003). To answer this question, laevis/ borealis hybrids were created to allow the detection of any zygotically transcribed nanos. In the X. laevis (female) and X. borealis (male) hybrid embryos, borealis nanos1 was not detected by RT-PCR, indicating that zygotic transcription of nanos1 did not occur (Figure 2.1B). To rule out the possibility that no detection of borealis nanos1 is because the borealis genome is transcriptionally inactivated in hybrids, those results were confirmed by the reverse hybrid experiment, using laevis (male) and borealis (female) hybrids (Brun and Kobel, 1977). Analysis of genomic DNA confirmed authentic hybrids had been generated. The mid-blastula transition occurred normally in these hybrids as demonstrated by testing known zygotic genes including, ornithine decarboxylase (ODC) and Bix4 (Figure 2.1C). From the data presented above, the conclusion is that nanos1 RNA is strictly of maternal origin in the early embryo and it persists not only for months
Figure 2.1. Expression of nanos1 during development. (A) nanos1 RNA was detected by RT-PCR in embryogenesis until stage 33/34 (tailbud). ODC served as a loading control. (B and C) nanos1 expression is strictly maternal. (B) Zygotic transcription of nanos1 RNA was examined using Xenopus laevis (female) and borealis (male) hybrid embryos. RT-PCR analysis using species specific primers for laevis nanos1 (X.l.) or borealis nanos1 (X.b.) at the indicated stages. Note that while laevis Nanos1 was present as expected, borealis nanos1 RNA was not detected in the hybrid embryos at any stage. (C) laevis nanos1 RNA was not detected in Xenopus laevis (male) and borealis (female) hybrid embryos at any stage tested. In each case, hybrid embryos were confirmed to be authentic hybrids by PCR amplification of species specific genomic DNA.
during oogenesis but three days into embryogenesis. *nanos1* is zygotically expressed later in the brain and testis.

### 2.2. Nanos1 protein is restricted in the germline during early embryogenesis.

To determine the developmental time period when Nanos1 protein is present in the germ plasm, our lab generated a polyclonal antibody against Nanos1 recombinant protein. *nanos1* RNA is translationally silenced during oogenesis (MacArthur *et al.*, 1999), therefore stage VI oocytes served as a negative control (Figure 2.2A). During early cleavage and blastula stages, Nanos1 was found close to the cell periphery in a germ plasm-like pattern (Figure 2.2B,C) where it co-localized with Xiwi (Figure 2.2B,C; Lau *et al.*, 2009; Wilczynska *et al.*, 2009). Xiwi has only been detected in PGC germ plasm where it persists until late neurula stage (Wilczynska *et al.*, 2009). From gastrula stage on, Nanos1 was detected predominantly in a perinuclear location (Figure 2.2 D,E,F,G,H,I,J,K ). *Xpat* RNA served as a germ plasm indicator for tailbud stages (from stage 28 to stage 37/38) (Hudson and Woodland, 1998). Nanos1 protein co-localized with *Xpat* RNA until stage 34 after which Nanos1 was no longer detected (Figure 2.2L, L’, M, M’; Figure 2.3). Therefore, Nanos1 is potentially available to repress translation of germ plasm RNAs while PGCs are in the endoderm (see Figure 2.3 for summary of expression).
Figure 2.2 *Nanos1* protein expression during *X. laevis* development. Endogenous Nanos1 was detected by confocal immunofluorescence with anti-Nanos1 antibody (red in all images). (A) Nanos1 was not expressed in stage VI oocytes. n, nucleus. (C) Nanos1 protein was concentrated in a sub-cellular cytoplasmic domain close to the cell periphery in stage 8 embryos. PGCs are outlined with dashed white line. (B, E, G, I) Nanos1 protein co-localizes with the germ plasm at stages 4 (8 cell), 10.5, 12, and 13. Xiwi, a germ plasm specific protein, was detected by anti-Xiwi antibody (green). (D, F, H, J, K) Hyperacetylated histone H4 (Penta), a nuclear marker, was detected by immunostaining (Green). Nanos1 protein (red) was found only in perinuclear locations in stages 10.5, 12, 13, 14, and 28. (L and M) Stages 33/34 and 37/38. *Xpat* RNA was detected by whole mount in situ hybridization using fluorescein tyramide (green). L’ and M’, same sample as in L and M. Nanos1 protein could no longer be detected in PGCs after stage 34. C-K, images were from the endoderm mass. L and M, lateral view, with anterior to the left. Scale bars are as indicated.
Figure 2.3 Summary of expression pattern of *Xenopus nanos1* RNA and protein during oogenesis and early embryogenesis. *nanos1* RNA was transcribed in stage I oocyte and this maternal transcripts persist through early embryogenesis until stage 34. Nanos1 protein was translated after fertilization. This protein is available to function in the germline through early embryogenesis before PGCs migrate out of the endoderm.

**Discussion**

To study a gene’s function, it is necessary to know where and when this gene is expressed. Using laevis/borealis hybrid experiments, the data presented above support that *Xenopus nanos1* is strictly of maternal origin during early embryonic development. It is unlikely that chromatin imprinting caused the failure of detection of *nanos1* paternal transcripts, since no evidence for imprinting has ever been found in frogs (de la Casa-Esperon and Sapienza, 2003). Therefore, the detection of increased *nanos1* RNA level after stage 14 is not due to zygotic transcription. Since *nanos1* RNA is associated within the germinal granules in the germ plasm, it may be protected from RNA extraction at stage 14, for some unknown reason, resulting in reduced detection level at this stage.

*nanos1* is translated after fertilization (Luo et al., 2011). Nanos1 protein expression is only detected in the germ plasm until tailbud stage 33/34 when PGCs are migrating out of the endoderm. As a translational repressor (Lai et al., 2011), Nanos1 may translationally
repress RNAs required for somatic differentiation, cell death, and/or mitosis, and thus help to preserve germline fate when PGCs are still in the endoderm. The investigation of Nanos1 function during PGC development will be discussed in chapter 3.
Chapter 3

*Xenopus* Nanos1 is Required to Repress Endoderm Gene Expression and Apoptosis during PGC Development

**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 loss of germ cells from the gonads. Lineage tracing and TUNEL staining reveals 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 express a hyperphosphorylated RNA Pol II-CTD at the mid-blastula transition. Furthermore, they now inappropriately express somatic genes characteristic of endoderm regulated by maternal VegT including *Xsox17-alpha*, *Bix4*, *Mixer*, *GATA4*, and *Edd*. We propose that Nanos1 functions to translationally repress RNAs that normally specify endoderm and promote apoptosis, thus preserving the germline.
Background

A persistent question in development 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 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 in the C terminus 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 although without specificity (Hashimoto et al., 2010). Together with Pumilio, Nanos is part of a translational repression complex where 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 (Pum) 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* (*C. 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; Köprunner 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 premature active transcription and the failure to establish germ-line-specific histone modifications, typical of transcriptionally inactive chromatin (Schaner et al., 2003). Further, premature gene activation results in the mis-expression of somatic genes *Sex-lethal (Sxl), fushi tarazu*, and *even-skipped* in the germ line and subsequently, cell death (Deshpande et al., 1999; Sato et al., 2007).

However, what role Nanos plays in transcriptional repression within the germline is unknown.

*Xenopus nanos1* RNA is first detected in early staged oocytes, although it is not translated until after fertilization (see Chapter 2 this thesis; Mosquera et al., 1993; Lai et al., 2011; Luo et al., 2011; 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 anti-sense morpholino oligos (MO) to block the translation of *nanos1*. Nanos1 depleted embryo 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 *nanos1-mut* RNA, not affected by the MO,
demonstrating the specificity of the knockdown. PGCs deficient in Nanos1 inappropriately expressed somatic genes required for endoderm specification including Xsox17a. Further, zygotic transcription, normally repressed in PGCs until neurula stages, was activated precociously at the start of gastrulation. PGCs without Nanos1 remained in the endoderm and eventually underwent apoptosis. Lineage tracing revealed that even at later stages (st.47), ectopic PGCs were primarily found in gut tissue. From these results, we propose that Nanos1 is required to preserve Xenopus germline identity by translationally repressing RNAs that normally promote the endoderm developmental program and apoptosis.

Results

3.1. Nanos1 specific morpholino (Nanos1 MO) blocks nanos1 translation both in vitro and in vivo.

To determine the function of Xenopus nanos1 in the germline, we depleted Nanos1 protein by using a morpholino oligonucleotides (MO) approach. Morpholino is an anti-sense technology. It can recognize and hybridize with the sense RNA at the translation starting site and thus block the translation and, in this way, it knocks down the gene function. Nanos1-MO blocked the translation of nanos1 in a dose dependent fashion as determined in an in vitro translation assay (Figure3.1A). Neither a mutant of nanos1 with non-complimentary 5’ sequences to the MO (nanos1-mut) nor a control MO with five
Figure 3.1. Nanos1 Morpholino blocks nanos1 RNA translation. (A) In vitro translation of wild-type nanos1 or a morpholino-resistant mutant (nanos1-mut) in the absence or presence of Nanos1-MO. Nanos1-MO blocks translation of nanos1 but not nanos1-mut RNA in a dose-dependent manner. The non-specific control morpholino had no effect. This experiment was repeated at least three times. (B) Nanos1-MO blocks the translation of endogenous nanos1. Confocal analysis showing the expression of Nanos1 (red) and Xiwi (germ plasm marker, green) at the 8-cell stage of embryos previously injected with Nanos1-Ctrl-MO or Nanos1-MO at the 1-cell stage. Uninjected embryos served as controls. Diagram indicates area picture. Images staining Nanos1 were adjusted of brightness and contrast at the same level. Scale bar, 130μm.

base changes (Nanos1-Ctrl-MO) had any effect on nanos1 translation. It has been previously shown that during early stages, Nanos1 is found within germ plasm co-localizing with the germ plasm marker Xiwi (Lai et al., 2011; Lau et al., 2009; Wilczynska et al., 2009). Importantly, while 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 (Figure 3.1B). In contrast, Nanos1-Ctrl-MO (20 ng) injected embryos could not be distinguished from their 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.

3.2. Nanos1 knockdown results in loss of PGCs as well as gonadal germ cells.

The fate of PGCs in embryos depleted of Nanos1 activity was explored by Xpat whole mount in situ hybridization (WISH) \[Xpat, a specific marker for PGCs (Hudson and Woodland, 1998)\]. 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 (Figure 3.2A,C). Xpat expression was prematurely lost at stage 30 instead of stage 39 from PGCs deficient in Nanos1 as shown by RT-PCR (Figure 3.2B). It is during this time that PGCs more actively migrate toward the dorsal aspect of the gut from which they will eventually leave the endoderm (Nishiumi et al., 2005). The loss-of- PGC phenotype could be rescued by co-injection with the nanos1-mut transcript (0.4 ng) where 3 nt changes had been introduced preventing recognition and repression by the Nanos1-MO (One-way ANOVA analysis, p=0.012) (Figure 3.2C). 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 normal PGCs are at the end of their interlude in the endoderm.
Figure 3.2 Nanos1 depleted embryos are deficient in PGCs. (A) Knockdown of Nanos1 results in loss of PGCs. WISH-Xpat analysis of stage 37/38 wild type (WT), Nanos1-Ctrl-MO injected, and Nanos1-MO injected embryos. Note: Nanos1-MO embryos were rescued by nanos1-mut RNA showing specificity of morpholino. Scale bar, 100µm. (B) Xpat signal, normalized to ODC, declines prematurely in Nanos1 depleted embryos compared to the control groups. This experiment was repeated twice. (C) Histogram summarizing results of PGC loss at different stages after Nanos1 knockdown. Differences were significant by one-Way ANOVA (**, p<0.01; ***, p<0.001). Error bars indicate standard errors. (D) H & E staining of tadpole sections showing gonads from wild type (stage 54), Nanos1-Ctrl-MO (stage 55), Nanos1-MO (stage 54), and Rescue (stage 56) embryos. Note loss of germ cells (red arrows) from Nanos1-MO compared to Nanos1-Ctrl-MO injected embryos and it could be rescued. Scale bar, 20µm.
One possible explanation for the premature loss of *Xpat* is that Nanos1 is required for *Xpat* stability and its loss reflects that and not the loss of PGCs. To address this concern, we carried out a histological examination of developing gonads at a time when they are richly populated by germ cells (Figure 3.2D). We observed a significant loss of germ cells in the Nanos1 deficient tadpoles (n=5). The example shown in Figure 3.2D represents the most germ cells observed in a cross section after Nanos1 knockdown (~4-5 oocytes), compared to the dense cluster of germ cells found in control tadpoles and rescued tapole. Thus, *Xpat* WISH is a reliable indicator for loss of PGCs. Taken together, these results indicate that Nanos1 is required to maintain the PGC lineage.

### 3.3. PGCs deficient in Nanos1 activity undergo apoptosis.

The next question is whether PGC loss in *nanos1* morphant embryos was due to apoptosis or whether PGCs transdifferentiated into endoderm. Embryos injected with Nanos1-Ctrl-MO or Nanos1-MO and uninjected siblings were subjected to the TUNEL assay. The specificity of TUNEL assay was monitored by positive and negative controls for TUNEL, respectively (Figure 3.3A). PGCs were identified by fluorescent in situ hybridization with *Xpat* and all double labeled cells scored positively for apoptosis. Apoptosis was not detected at time points before the decline in PGC numbers observed in the Nanos1-MO embryos (st. 18, n = 52 PGCs, Figure 3.3B). In early tailbud embryos (st. 26-30) when Nanos1 deficient embryos showed a 27.2% to 56.3% decline in PGC numbers respectively, 9.1 % PGCs were apoptotic (n =154 PGCs, Figure 3.3C). Control-MO embryos were indistinguishable from uninjected embryos with 1.8% apoptotic PGCs.
Figure 3.3 Nanos1 depleted PGCs enter apoptosis. (A) TUNEL negative control, no terminal deoxynucleotidyl transferase (TDT) in the TUNEL assay. TUNEL positive control, TURBO DNase treatment of samples for 30min at 37°C before the TUNEL assay. Scale bar, 200μm. (B) PGCs deficient in Nanos1 are not apoptotic throughout early development (st.18). WISH-Xpat (germ plasm marker, green) and TUNEL staining (red) of wild type, Nanos1-Ctrl-MO and Nanos1-MO injected embryos. White arrowheads indicate control TUNEL positive cells in ectodermal region. Scale bar, 100μm. (C) TUNEL positive PGCs are detected during tailbud stage (st.28) in Nanos1 depleted embryos, but not in control embryos. Yellow arrowheads, TUNEL positive PGCs. Scale bar, 150μm.
(n = 340 PGCs). As apoptosis occurs relatively quickly (reviewed in Majno and Joris, 1995), at any one time we could expect only a few, but not all PGCs to be TUNEL positive as the total number of PGCs decline.

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). Expression of LacZ was targeted to PGCs by the 3’UTR of DEADSouth (Kataoka et al., 2006; MacArthur et al., 2000). After MOs were injected at the one-cell stage, the NLD message was injected into the vegetal blastomeres at the 32-cell stage at a dose of 1 ng/blastomere. Embryos were allowed to develop until stage 46-47, fixed and processed for β-Gal assay (Takeuchi et al., 2010; Figure 3.4A). As expected, control embryos showed LacZ positive cells present in the dorsal mesenchyme (Nishiumi et al., 2005). Nanos1 depleted embryos revealed the loss of LacZ positive cells and this phenotype was rescued by nanos1-mut (0.4 ng) (p<0.001, one-way ANOVA) (Figure 3.4B,C). A few ectopic PGCs were only found in the gut tissue in both control and experimental embryos. Histological examination of the few surviving “PGCs” in the gut tissue revealed round cells that displayed no evidence of cellular differentiation into gut endoderm and they would eventually die (data not shown; Ikenishi et al., 2007). These data strongly suggest that PGCs deficient in Nanos1 do not contribute to normal gut tissue.

3.4. Available apoptosis inhibitors are not effective in Xenopus system.

An interesting question is whether Nanos1 depleted PGCs could survive as endoderm cells if apoptosis is inhibited. To address this question, I tried two general caspase
Figure 3.4 Nanos1 depleted PGCs fail to migrate from the endoderm. (A) Schematic of experimental design for lineage tracing of PGCs. Morpholino oligos were injected at stage 1. Germline lineage tracer *NLD* mRNA was injected into germ plasm containing blastomeres at the 32-cell stage. Embryos were allowed to develop until st.46/47 and stained for LacZ. (B) Whole mount lineage analysis at st.46/47 showing LacZ positive PGCs (blue) in wild type, Nanos1-Ctrl-MO, and Nanos1-MO injected embryos. PGCs migrating in the dorsal mesenchyme are circled (red). PGCs were not detected in Nanos1 depleted embryos in this region. Black arrows indicate wild type ectopic PGCs in gut. Sagittal paraffin section of wild type embryo, stained with Eosin; PGCs are indicated by blue arrows. Scale bar, 100µm. (C) Histogram summarizing results of lineage traced PGCs in st.46/47 tadpoles. Ectopic PGCs within the gut (red bar) occurred in both control and experimental embryos. Statistical analysis was done only for PGCs in the genital ridges (green bar). ***, p<0.001, Student’s t-test for two group analysis; one-way ANOVA for three group analysis. Error bars indicate standard error for the green bars.
inhibitors, Z-VAD-FMK and Q-VD-OPh (MP Biomedicals, LLC). These two apoptosis inhibitors were injected, respectively, together with the germline lineage tracer NLD. However, the loss of LacZ positive cells was not rescued in Nanos1 depleted embryos (Figure 3.5). Further, I found that these two drugs failed to inhibit apoptosis in *Xenopus* embryos induced by doxorubicin, paclitaxel, or staurosporine. In other words, the available apoptosis inhibitors are not effective in the *Xenopus* system. Thus, an effective apoptosis inhibitor may help to answer the question whether PGCs will be incorporated into endoderm tissue and become somatic cells if they are not allowed to enter apoptosis.

![Figure 3.5 Apoptosis inhibitor Q-VD-OPh is not effective to block death in PGCs deficient in Nanos1.](image)

**Figure 3.5 Apoptosis inhibitor Q-VD-OPh is not effective to block death in PGCs deficient in Nanos1.** Histogram summarizing results of lineage traced PGCs in st.46/47 tadpoles from β-gal assay. *, p<0.05, one-way ANOVA analysis of PGC number in the genital ridges. Error bars indicate standard errors of PGC number in genital ridges. Plum, ectopic PGC number in the gut. Blue, PGC number in the genital ridges.

### 3.5. PGCs deficient in Nanos1 express PSer2-CTD prematurely.

During the period when PGCs are transcriptionally silent, they do not express the phosphorylated form of Ser2 in the carboxyl terminal domain (CTD) of RNA polymerase
Figure 3.6 Nanos1 depleted PGCs exhibit PSer2-CTD prematurely. (A) Confocal analysis of wild type, Nanos1-Ctrl-MO, and Nanos1-MO injected embryos at st.11. Double immunostaining with H5 monoclonal antibody (PSer2-CTD, green) and rabbit anti-Xiwi antibody (germ plasm, red). Merged images are shown at the top, with separate images at the bottom. Scale bar, 50µm. Images were taken from the endoderm core (see the diagram in Figure 3). (B) Summary of data showing PGCs deficient in Nanos1 expressing PSer2-CTD (74%) compared to wild type (21%) and Nanos1-Ctrl-MO (16%) injected embryos, respectively. Differences of H5 positive PGC number in each group were highly significant by one-way ANOVA (**, p<0.01). This experiment was repeated three times.

II, an event highly correlated with transcriptional elongation (Hirose and Ohkuma, 2007; Venkatarama et al., 2010). However Pol II PSer5-CTD is present in all blastomeres even prior to the MBT signifying that initiation complexes are formed and machinery poised for transcription (Venkatarama et al., 2010). The hypothesis was that in the absence of
Nanos1, Pol II CTD-Ser2 was prematurely phosphorylated at the MBT permitting the subsequent mis-expression of Xsox17α in PGCs (Deshpande et al., 2005). Premature transcription at stage 9/10 rather than 14 would allow maternal VegT, still present at this stage, to initiate endoderm fates in PGCs (Venkatarama et al., 2010).

To determine whether Nanos1 deficient PGCs now express PSer2-CTD prematurely, double IF was performed using the H5 monoclonal antibody specific for the PSer2 epitope together with the anti-Xiwi antibody to identify germ plasm. PSer2-CTD was detected during early gastrulation in 71% of the PGCs counted in Nanos1-MO injected embryos from three independent experiments (n = 44/62). In contrast, PGCs in Nanos1-Ctrl-MO injected and uninjected WT embryos expressed the PSer2 epitope at significantly lower frequencies (n = 11/45, 24% ctrl MO; n =9/48, 19% WT). One such experiment is shown in Figure 3.6. From these results, the conclusion is that zygotic transcription was prematurely activated in PGCs after Nanos1 knockdown.

3.6. PGCs deficient in Nanos1 activity express somatic genes.

Nanos1 is a potent translational repressor and it may be required to prevent somatic determinants from being expressed in the germ line (Lai et al., 2011). Previous results showed that the maternal determinant for endoderm, VegT RNA, is present in PGCs and would necessarily have to be repressed (Venkatarama et al., 2010). Do PGCs depleted of Nanos1 activity now mistakenly initiate endoderm specification and thus lose Xpat expression? To explore this possibility, PGCs were isolated from uninjected, Nanos1-Ctrl-MO, or Nanos1-MO injected embryos. The question is if PGCs now expressed
endoderm or mesoderm markers as assessed by semi-quantitative RT-PCR on the isolated PGCs. $X_{sox17\alpha}$ (Hudson et al., 1997) and $Bix4$ (Casey et al., 1999) were chosen to detect endoderm as both are direct downstream targets of maternal VegT and both are required for endoderm fate. The mesoderm marker $X_{bra}$ was also assessed to determine if other fates were initiated besides endoderm (Smith et al., 1991).

Knockdown of Nanos1 resulted in the expression of the endoderm markers $X_{sox17\alpha}$ and $Bix4$ but not $X_{bra}$ in stage 10/11 PGCs (Figure 3.7A). This suggested that PGCs deficient in Nanos1 enter an endoderm, but not a mesoderm, differentiation program. Further, $X_{sox17\alpha}$ and $Bix4$ were expressed at times comparable with endoderm expression during gastrulation. As expected, $Bix4$ was not detected in the excised endoderm core sample (circled in Figure 3.7) as it is expressed during gastrulation within the endomesoderm just outside the core (Casey et al., 1999). $ODC$ (ornithine decarboxylase) served as an internal control. To confirm these RT-PCR results in vivo, we examined whole embryos by fluorescent in situ hybridization (Tyramide) to detect $X_{sox17\alpha}$ RNA and identified germ plasm by immunostaining with anti-Xiwi antibody (Figure 3.7B). These experiments indicated that in the absence of Nanos1, PGCs expressed $X_{sox17\alpha}$, coincident with endoderm specification at gastrulation.

To better understand what specific differentiation programs were active in Nanos1 depleted PGCs, we examined gene expression patterns 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 endoderm specific markers $Mixer$, and $endodermin$ ($Edd$) (Figure 3.7C). $GATA\ 4$ was detected, but not the $GATA\ 5/6$ family members, $X_{hex}$, $Chordin$, or $Cerberus$, genes all expressed in
Figure 3.7. PGCs deficient in Nanos1 activity express endoderm specific genes. RT-PCR analysis of PGCs or endoderm cells isolated from the endoderm core from st.11 (circled in red surrounded by endomesoderm, blue in diagram) (A) and st.15 embryos (C). P: primordial germ cells; E: endoderm; WE: whole embryo. Xpat: germ plasm marker; Xsox17α: endoderm marker; Bix4: endomesoderm marker; Xbra: mesoderm marker; ODC: ornithine decarboxylase, internal control. Mixer, GATA4-6, Edd, endoderm specific genes; chordin, dorsalizing factor; cerberus, head inducing factor; Xhex, anterior patterning factor; Oct91, pluripotent factor; Xwnt11, dorsal-ventral axis establishment factor. *, resulting from endoderm cell contamination. (B) Confocal analysis of wild type (WT), Nanos1-Ctrl-MO, and Nanos1-MO injected embryos at stage 11. Xsox17α RNA (red) was detected by whole-mount fluorescent in situ hybridization. PGCs were identified by Xiwi immunostaining (green). PGCs are outlined by dashed line. Merged images are shown at the top, with separate images at the bottom. Scale bar, 50µm. Images were taken from the endoderm core.
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, upstream of Xhex activity and an anterior dorsalizing factor, was not expressed
in Nanos1 depleted PGCs. Interestingly, the pluripotent factor Oct-91 was expressed by
both endoderm and PGCs regardless of Nanos activity. The RT-PCR results in Figure
3.7C indicate that Nanos1 depleted PGCs were differentiating towards posterior gut
denderm and not other regions. The most likely explanation for our results is that
Nanos1 is required to repress VegT expression in PGCs.

Discussion

Nanos is required for germline maintenance and survival and this observation has been
well documented in many model organisms (Forbes and Lehmann, 1998; Kobayashi et al.,
1996; Köprunner et al., 2001; Sato et al., 2007; Tsuda et al., 2003). However, the
underlying mechanisms about how Nanos functions to maintain the germline identity are
not well understood. In Xenopus, my thesis work demonstrates that knockdown of
Nanos1 resulted in PGC loss as well as gonadal germ cell deficiency. These phenotypes
could be rescued by injection of Nanos1 message indicating the specificity of the
knockdown technique. Nanos1 depleted PGCs exhibited precocious expression of PSer2-
CTD as well as zygotic transcription of somatic genes. PGCs deficient in Nanos1 entered
apoptosis during the migration out of the endoderm. Further, my thesis work determined
that VegT is a target of Nanos1/Pumilio translational repression. Therefore, Nanos1
functions to preserve the germline identity by preventing somatic differentiation programs.

**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 represses two activators of caspase, *hid* (*head involution defective*) and *skl* (*sickle*) protecting germ cells from cell death (Sato et al., 2007). In *Drosophila*, if apoptosis is prevented by genetic means, PGCs are incorporated into endoderm and express somatic specific genes (Hayashi et al., 2004). The few *Drosophila nanos* mutant pole cells that migrate into the gonads, fail to develop (Hayashi et al., 2004). In the mouse, Nanos3⁺/⁻ PGCs die even though the Bax-dependent apoptotic pathway is blocked. These results suggest multiple “death” pathways may be operating in PGCs. Interestingly, somatic gene expression does not occur in Nanos3⁺/⁻ PGCs, indicating different functions among the three mouse *nanos* genes (Suzuki et al., 2008). Nanos1 may carry out all these different functions, as it is the only *nanos* member in *Xenopus*. An important question is whether blocking apoptosis in *Xenopus* Nanos1 deficient PGCs will cause PGCs to trans-differentiate into endoderm. Alternatively, will
they continue to migrate into the gonads, but fail to generate functional gametes, as occurs in *Drosophila nanos* mutants? Our attempts to block apoptosis in *Xenopus* were repeatedly unsuccessful leaving this question open for future work.

**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). The first molecular aberration we observed in Nanos1 deficient PGCs was the premature expression of CTD-PSer2 at the MBT. Thus, Nanos1 is in some way linked to the suppression of CTD-Ser2 phosphorylation. Cdk9, the kinase in P-TEFb, contains a PBE in its 3’UTR suggesting it as a candidate for Nanos1/PUM translational repression. However, our unpublished data indicate that Cdk9 protein is expressed in the nucleus of wild type PGCs during gastrulation, and thus it is unlikely to be the limiting factor. Therefore, it will require further work to establish how Nanos is linked to the repression of CTD-Ser2 phosphorylation and whether it depends on Nanos1 function as a translational inhibitor.

**Expression of endoderm genes in Nanos1 morphants**

The second molecular aberration we observed in Nanos1 deficient PGCs was the expression of the endoderm transcription factor *Xsox17α*. Its expression occurred 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 premature CTD-PSer2 staining in PGCs that we observed. Endoderm specific markers *Mixer*, and *Edd*, and *GATA4*, a potent inducer of endoderm specific gene expression, were detected in Nanos1 depleted PGCs (Henry and Melton, 1998; Kelley et al., 1993; Xanthos et al., 2001). Interestingly, we found that zygotic expression of *Oct91* in PGCs does not depend on Nanos1 activity (Figure 3.7C). *Oct91*, a homolog of *Oct3/4* in mammals, functions as a pluripotent factor to preserve uncommitted states of the early blastomeres (Cao et al., 2007; Henig et al., 1998) and the germline (Lai and King, unpublished; Venkatarama et al., 2010). It is possible that the pluripotent program initiated by Oct91 is incompatible with the mis-expressed endoderm program and, as a result, contributes to triggering apoptosis in Nanos1 morphants.

Other genes expressed in more anterior endoderm (*GATA5, Xhex, Cerberus*), presumptive mesoderm (*GATA6*), or prechordal plate and notochord (*chordin*) were not detected in Nanos1 deficient PGCs at later stages (Bouwmeester et al., 1996; Gove et al., 1997; Smithers and Jones, 2002; Weber et al., 2000; Xanthos et al., 2001). Although we do not know the exact transcription sites now active in Nanos1 deficient germ cells, our data is most consistent with transcription being activated in a targeted, regional manner for posterior endoderm specification, the location of normal PGC development. Such inappropriate gene expression would explain why PGCs did not leave the endoderm and migrate into ectopic locations as has been noted for *nanos* mutants in zebrafish and *Drosophila*. Importantly, injection of nanos1 RNA completely rescued this phenotype. We conclude that, in *Xenopus*, Nanos1 is required and sufficient to block endoderm gene expression in the germline.
Chapter 4

RNA Targets of Nanos1/Pumilio Translational Repression

Summary

Three potential targets of Nanos1/Pumilio translational repression, VegT, cdk9, and Xwnt11, were investigated using both an in vitro binding assay (RNA electrophoresis mobility shift assay, EMSA) as well as an in vivo fluorescent reporter assay. The data indicate that maternal VegT RNA is an authentic target of Nanos1/Pumilio translational repression and subsequent degradation. Nanos1 plays a role in destabilizing VegT RNA in PGCs. Although cdk9 contains a functional PBE, Cdk9 protein was detected in wild type PGCs during gastrulation, which makes it unlikely to be translationally repressed in the germ plasm. Further, my work shows that five non-canonical PBEs in the 3’UTR of Xwnt11, but not the canonical PBE, function in Pumilio binding and translational repression. However, in contrast to our findings with VegT, Nanos1 was not required to block Xwnt11 translation in vivo. My data suggest that Xwnt11 is translationally repressed in the germline by Pumilio but requires unknown factors to cause repression.

Background

Nanos and Pumilio function together to translationally repress specific RNAs. Pumilio binds the RNA target through a 3’UTR Pumilio Binding Element (PBE): UGUANAUA
(N can be any nucleotide) (Cheong and Hall, 2005; Wang et al., 2002; Wharton et al., 1998; White et al., 2001). Key to understanding the molecular mechanisms underlying Nanos function in the germline is the identification and validation of the targeted mRNAs repressed. 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 six authentic RNA targets of Nanos/Pumilio repression have been identified: [1] the cell cycle regulator cyclin B1, a target in frogs and flies (Lai et al., 2011; Asaoka-Taguchi et al., 1999; Dalby and Glover, 1993; Kadyrova et al., 2007), [2] the Drosophila promoter of apoptosis hid/skl (Hayashi et al., 2004; Sato et al., 2007), [3&4] the somatic determinants hunchback (Wreden et al., 1997; Murata and Wharton, 1995) and bicoid (Wharton and Struhl, 1991), [5] C.elegans fem-3, which regulates sex-determination (Ahringer and Kimble, 1991; Zhang et al., 1997); and [6] paralytic (para), Drosophila voltage-gated sodium channel which regulates sodium current in Motoneurons (Muraro et al., 2008). Failure of repressing these targets would result in germline loss and soma defects.

In Xenopus, germ plasm was identified in the vegetal pole, colocalized with many somatic RNAs, such as VegT. During the germ cell formation, somatic RNAs which determine the soma differentiation may be incorporated into the germline. Venkatarama et al. (2010) verified the presence of endoderm determinant VegT RNA in PGCs before mid-blastula transition (MBT). In order to prevent germ cells from somatic differentiation, VegT translation has to be repressed. Sequence searching for Pumilio binding element (PBE: UGUANAU. “N” could be any nucleotide.) in Xenopus gene database generated a list of potential targets of Nanos1/PUM translational repression in
the germ plasm (Murray R and King ML, unpublished). These potential targets contain at least one PBE in the 3’UTR, such as VegT (see table 1.3). In this chapter, using RNA EMSA and an in vivo fluorescent reporter assay for translation, we show that VegT is an authentic target of Nanos1/PUM translational repression in PGCs, and Xwnt11 is a target of Pumilio mediated translational repression in the germline.

Results

4.1. Nanos1/Pumilio represses VegT translation in PGCS.

4.1.1 VegT PBE is functional for Pumilio binding

The endoderm determinant VegT RNA was found in PGCs (Venkatarama et al. (2010). This master transcription factor initiates endoderm differentiation in somatic cells during mid-blastula transition (MBT). To preserve the germline totipotency, VegT translation in the germ cells has to be repressed. A search of the VegT 3’UTR revealed a canonical PBE, UGUAAUA. As a first step in determining if Nanos1/Pumilio could repress VegT translation, we asked whether the VegT PBE could be recognized and bound by Xenopus Pumilio. The Xenopus Pumilio1 RNA binding domain (Xpum1 RBD, 825aa–1175aa) was expressed in BL21 (DE3) strain (Novagen) and purified by His-binding to an affinity resin column (Novagen) (Figure 4.1A). The VegT 3’UTR containing the PBE (2468–2579, 112nt) was biotinylated at the 3’ end. The EMSA results
(Figure 4.1B) show that Xpum1 RBD (20 ng) could bind VegT PBE (1 ng) but did not bind the VegT PBE mutant (1 ng, PBE mut, UAAAAAAA). This binding interaction could be competed by unlabeled VegT PBE RNA (250 ng). Therefore, Pumilio can bind to the VegT PBE in vitro, suggesting that this interaction may mediate translational repression of VegT RNA in PGCs.

**Figure 4.1 Xenopus Pumilio could recognize and bind VegT PBE in vitro.** A. Column purified Xenopus Pumilio1 RNA binding domain (Xpum1 RBD) was analyzed on SDS-PAGE gel and stained by Coomassie Brilliant Blue. B, Non-radioactive RNA EMSA analysis shows that Xenopus Pumilio1 RNA binding domain (Xpum1 RBD) could bind biotin labeled VegT PBE but not the mutant. This binding reaction could be competed by unlabeled VegT PBE.
4.1.2 *VegT* PBE is able to mediate translational repression in the germline

To determine whether Nanos1 represses *VegT* translation in PGCs, an *in vivo* fluorescent reporter assay was developed in our lab (Figure 4.2A). The DEADSouth 3’UTR contains germ plasm localization information. Unlocalized reporter RNA containing DEADSouth 3’UTR in the somatic cells is degraded probably by microRNA so that the reporter protein expression is restricted to germ cells (Kataoka et al., 2006; MacArthur et al., 2000). Therefore, Venus-DEADSouth 3’UTR (Kataoka et al., 2006) and DsRED-DEADSouth 3’UTR both serve as germline lineage tracers by late tailbud Stage. A 296nt fragment of the *VegT* 3’UTR containing the PBE was cloned into the DsRED-DEADSouth 3’UTR, generating DsRED-VegT PBE-DS3’UTR. Three nucleotide mutations were introduced into the PBE site, generating DsRED-VegT PBE mut-DS3’UTR (PBE mut, UAAAAAAA) that is incompetent to bind RNA. One point mutation (G2A) in the PBE site results in a 33-fold lower efficiency of Pumilio binding (Cheong and Hall, 2006). The images shown in Figure 4.2B were taken at st.34/35 when PGCs have migrated laterally toward the body surface allowing the fluorescent signal to be observed directly from live embryos with the aid of a fluorescent stereomicroscope. Our results indicate that *VegT* PBE is required to mediate the repression of DsRED translation in the germline (93.10%, n= 19 embryos). This repression could be released either by mutation in the PBE site (83.15%, n= 18 embryos) or by Nanos1 knockdown (86.58%, n= 26 embryos). These results strongly suggest that Nanos1 is required to repress *VegT* translation in PGCs in a PBE dependent manner. Further, to clarify that the
Figure 4.2 Nanos1 represses VegT translation in PGCs. A, Schematic of fluorescent germline lineage tracer construction design. Venus-DEADSouth 3'UTR and DsRED-DEADSouth 3'UTR are germline lineage tracer. VegT PBE or mutant is subcloned to the downstream of DsRED ORF. B, Schematic of experimental design. Morpholino was injected at one-cell stage and reporter RNAs were injected into the germ plasm residing area at 32-cell stage. C, in vivo fluorescent reporter assay indicates that VegT PBE can mediate the translational repression of DsRED. This repression could be released either by PBE mutation or Nanos1 depletion. Pictures were taken under fluorescent stereomicroscope (Olympus SZX12) when embryos were alive (st.34/35). Scale bar, 100 μm.
absence of DsRED signal results from no expression of DsRED protein in DsRED-VegT PBE-DS3’UTR RNA injected embryos; immunofluorescence experiment was carried out to detect DsRED protein in PGCs as shown in Figure 4.3. The results in Figure 4.3 indicate that there was no DsRED protein expression in the germline with VegT PBE in the 3’UTR of the reporter RNA, except two PGCs (white arrows). This leakage of DsRED expression was observed in 6.9% examined PGCs (Figure 4.2C). This translational repression of DsRED was relieved by Nanos1 knockdown.
4.1.3 Nanos1 depletion stabilizes endogenous VegT RNA

The absence of the DsRED signal in reporters bearing the PBE could be explained by translational repression or by targeted DsRED RNA degradation, or both. WISH with

![Figure 4.4 VegT RNA is stabilized in PGCs after Nanos1 knockdown. (A) VegT PBE mediates the degradation of the reporter message DsRED. PGCs are indicated by whole mount in situ hybridization, using Xpat anti-sense probe (top panel) or DsRED anti-sense probe (bottom panel). Arrow heads, PGCs. Scale bar, 50µm. (B) Fold VegT expression from real-time PCR. Expression of VegT in Nanos1 knockdown samples is normalized to that of the wild type samples, respectively. The real-time PCR was done in duplicate, and experiments were repeated three times showing the similar pattern. Error bars indicate standard errors. *, p<0.05, unpaired student’s t-test.](image)
the DsRED anti-sense probe revealed that indeed the DsRED-VegT PBE-DS3’UTR RNA was degraded in wild type PGCs while the reporter lacking the PBE was unaffected (Figure 4.4A). These data suggest that VegT PBE and Pumilio binding mediate the translational repression of DsRED in part by promoting the degradation of the reporter RNA. Was Nanos1 required for this activity? To address this question, we compared the endogenous VegT RNA level in PGCs with and without Nanos1 knockdown. The qRT-PCR results in Figure 4.4B 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 (Figure 4.4B). We conclude that Nanos1/PUM 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 germ plasm target for Nanos1/PUM repression and explain how PGCs avoid differentiation as endoderm.

4.2. Cdk9 is translated in PGCs.

4.2.1 Pumilio binds the cdk9 PBE

Loss of Nanos1 resulted in the premature phosphorylation of Ser2-CTD with the consequence of endoderm gene expression in germ cells. Thus Nanos1 is in some way
Figure 4.5 *cdk9* PBE is functional in Pumilio binding and translational repression in the germline. A, Non-radioactive RNA EMSA analysis shows that *Xenopus* Pumilio1 RNA binding domain (Xpum1 RBD) could bind biotin labeled *cdk9* PBE, but not the PBE mutant, and this binding reaction could be competed by unlabeled *cdk9* PBE. B, *in vivo* fluorescent reporter assay indicates that *cdk9* PBE can mediate the translational repression of DsRED. This repression could not be released either by PBE mutation or Nanos1 depletion. Images were taken under fluorescent stereomicroscope (Olympus SZX12) when embryos were live (st.34/35). White arrowheads, DsRED signal expressed in the PGCs. Scale bar, 50 μm.

linked to the suppression of Ser2-CTD phosphorylation in both vertebrate and invertebrate germlines. By sequence analysis, Cdk9, a component of *Xenopus* P-TEFb, is
a candidate for Nanos1/PUM translational repression. Thus, one possibility for transcriptional silencing in the *Xenopus* germline is that Nanos1 indirectly represses Pol II transcription by suppressing *cdk9* translation.

RNA EMSA was performed to detect whether the PBE (UGUAAAUA) in *cdk9* 3’UTR could be bound by Pumilio. A fragment (152nt, 1658–1809) of the *cdk9* 3’UTR was cloned into the pCR4-TOPO vector. The plasmid was linearized by SpeI and the RNA was transcribed by T7 RNA polymerase (Ambion, #AM1344) and biotinylated at the 3’end according to the manufacturer’s instructions (Thermo Scientific, #20160). The EMSA results shown in Figure 4.5A reveal that Pumilio could bind *cdk9* PBE containing fragment but not the PBE mutant, and further, that this binding reaction could be competed by unlabeled *cdk9* PBE. We conclude that Pumilio can bind *cdk9* RNA.

### 4.2.2 *cdk9* PBE is functional in translational repression

To determine whether the *cdk9*-PBE is able to mediate translational repression, a fragment (429nt, 1381–1809) of *cdk9* 3’UTR containing PBE was cloned downstream of DsRED, generating DsRED-*cdk9* PBE-DS3’UTR. Mutations in the PBE site were introduced to generate DsRED-*cdk9* PBE mut-DS3’UTR (PBE mut, CCGGAAAA). The reporter RNAs were injected into embryos at the 32-cell stage and the images were taken during st.34/35. The results show that *cdk9* PBE could mediate the translational repression of DsRED in the germline (Figure 4.5B). However, this repression was not released by either mutation in the PBE site or by Nanos1 knockdown.
Figure 4.6. Cdk9 protein is expressed in wild type PGCs. Confocal immunofluorescence analysis shows that Cdk9 protein (shown in red) is detected in wild type PGCs’ nucleus. PGCs were identified by immunostaining using rabbit-anti-Xiwi antibody (shown in green). Pictures were taken from the endoderm core of st.11 embryos (region within the red circle). PGCs were outlined with white dashed line. WT, wild type. Scale bar, 50 μm.

4.2.3 Cdk9 protein is expressed in wild type PGCs during gastrulation

Although cdk9 PBE is functional in translational repression in the germline, cdk9 RNA may be not localized in the germ plasm in PGCs. Thus, we are not sure whether endogenous Cdk9 protein is expressed in PGCs, and/or where it is localized if expressed. To find out the answer to these questions, immunofluorescence was performed using goat anti-Cdk9 antibody (Santa Cruz, sc-484-G, 1:20 dilution) and rabbit anti Xiwi antibody (Xiwi, germ plasm marker) on wild-type and Nanos1 depleted embryos at st.11.5. The confocal results indicate that Cdk9 protein was present in the nucleus of wild type PGCs
and Nanos1 knockdown did not affect Cdk9 expression or localization pattern in PGCs. Therefore, cdk9 mRNA translation is not repressed in the germline (Figure 4.6).

4.3. **Xwnt11 is translationally repressed in the germline.**

4.3.1 **Xwnt11 3’UTR is able to mediate translational repression in PGCs**

Maternal Xwnt11 RNA was identified in the germ plasm and a canonical PBE is located in the 3’UTR (Ku and Melton, 1993). Xwnt11 protein functions in the dorsal-ventral axis establishment. So our question is whether Xwnt11 is translationally repressed by Nanos1/Pum in order to preserve the germline totipotency. Since no effective Xwnt11 antibody is commercially available, the *in vivo* fluorescent reporter assay was applied to address this question.

Xwnt11 3’UTR full length (FL, 1348~2133) was cloned into a region downstream of the DsRED open reading frame (ORF), producing DsRED-Xwnt11 FL-DS3’UTR. The reporter message made from this construct was injected into the embryos at the 32-cell stage and analyzed at stage 34/35. The results in Figure 4.7 indicate that the Xwnt11 3’UTR (FL) could mediate the translational repression of DsRED and that repression persisted even in the absence of Nanos1 (Figure 4.7). From these we conclude Xwnt11 3’UTR can mediate translational repression in the germline and Nanos1 is not required.
4.3.2 The translational control element in Xwnt11 3’UTR contains five non-canonical PBEs

To determine what sequences are responsible for the translational repression of the reporter gene DsRED, the Xwnt11 3’UTR was divided into three fragments, F1 (273nt, 1348~1620), F2 (260nt, 1621~1880), and F3 (253nt, 1881~2133). F1 contains no PBE. One canonical PBE (UGUAAUA) is found in F2. F3 contains five non-canonical PBEs (Figure 4.8A). [Note: If there is only one nucleotide change in one of the last three nucleotides (AUA) of the PBE, we labeled it as a non-canonical PBE.] The EMSA results
A, Schematic of Xwnt11 mRNA. 3'UTR fragments F1, F2 (containing canonical PBE), and F3 (containing five non-canonical PBEs). B, Non-radioactive RNA EMSA analysis shows that Xenopus Pumilio1 RNA binding domain (Xpum1 RBD) could bind biotin labeled Xwnt11 3'UTR fragment (F3) but not through the canonical PBE (F2). This binding reaction could be competed by unlabeled Xwnt11 3'UTR F3.

Figure 4.8 Xenopus Pumilio could recognize and bind the non-canonical PBEs in Xwnt11 3'UTR in vitro. A, Schematic of Xwnt11 mRNA. 3'UTR fragments F1, F2 (containing canonical PBE), and F3 (containing five non-canonical PBEs). B, Non-radioactive RNA EMSA analysis shows that Xenopus Pumilio1 RNA binding domain (Xpum1 RBD) could bind biotin labeled Xwnt11 3'UTR fragment (F3) but not through the canonical PBE (F2). This binding reaction could be competed by unlabeled Xwnt11 3'UTR F3.

shown in Figure 4.8B reveal that Pumilio could bind Xwnt11 3’UTR F3 (1952–2051, 100nt) but not F2 (1653–1747, 95nt). Next, these three fragments were introduced downstream of the DsRED ORF, respectively, generating DsRED-Xwnt11 F1/F2/F3-DS3’UTR. Results of the in vivo reporter assay indicated that Xwnt11 3’UTR F1 or F2 did not mediate the translational repression of DsRED in the germline, but F3 did (Figure 4.9A,B). However, Nanos1 knockdown did not relieve the repression mediated by F3.

Therefore, from the evidence presented above, we conclude that Xwnt11 is very likely to be translationally repressed in the germline since this RNA is present in the germ plasm. The speculation is that Pumilio binds to the non-canonical PBEs, probably recruiting other components but not Nanos1, and represses Xwnt11 translation in the germline.
Figure 4.9 Xwnt11 translation is repressed in the germline through 3’UTR fragment F3, and this repression is not released by Nanos1 knockdown. **A**, Xwnt11 3’UTR F1 and F2 failed to mediate the repression of DsRED translation in PGCs. White arrowheads, PGCs also expressing DsRED signal. Scale bar, 50 μm. **B**, Xwnt11 3’UTR F3 can mediate the translational repression of DsRED in the germline, and Nanos1 knockdown is not sufficient to release this repression. Pictures were taken under fluorescent stereomicroscope (Olympus SZX12) when embryos were alive (st.34/35). Scale bar, 50 μm.
Discussion

Nanos1/PUM represses VegT translation and promotes its degradation.

My work has identified a novel target for Nanos1/Pumilio repression in the maternally localized VegT RNA. Our reporter assay may be more reliable in assessing Nanos1/Pumilio mediated repression than simply asking if VegT protein is absent by Immunofluorescence (IF). The reason is because VegT RNA is translated during the oocyte maturation (Stennard et al., 1999) and the protein could be present within nuclei as they are formed. Since PGCs are transcriptionally repressed during gastrulation (Venkatarama et al., 2010), PGCs can tolerate some level of VegT protein being present. The data presented above further support a role, either direct or indirect, for Nanos1 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/PUM could play a direct role recruiting the CCR4-pop2-NOT deadenylation complex, a situation found in the translational regulation of cyclin B in flies (Kadyrova et al., 2007). Another possible interpretation is that Nanos1/Pumilio binds to maternal VegT and thus blocks its translation in PGCs; maternal VegT is eliminated by microRNA mediated degradation during MBT. Ultimately, Nanos1/PUM 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 where Nanos1/PUM repression is not primarily based on degradation of VegT RNA. Exactly how Nanos represses VegT RNA remains unknown.

**Cdk9 is expressed in PGCs.**

The data in Figure 4.5 suggest that the cdk9 3’UTR contains a functional Pumilio binding element (PBE), and that the PBE could mediate translational repression in the germ plasm. However, endogenous cdk9 translation is not repressed as protein was detected in the nucleus of PGCs (Figure 4.6), even though we cannot rule out the possibility that the detected Cdk9 protein is maternal. Further, Nanos1 knockdown had no effect on Cdk9 expression pattern. Therefore, it is unlikely that cdk9 is translationally repressed in PGCs, or if it is, its repression cannot account for the observed transcriptional repression in PGCs. Thus, it is still unclear how Nanos1 knockdown is related with premature exhibition of PSer2-CTD in PGCs. Further work is required to establish how Nanos1 is linked to the repression of the phosphorylation of Ser2-CTD in the germline.

**Xwnt11 is translationally repressed in the germline.**

*Xwnt11* is a germ plasm component and maternal Xwnt11 is required for dorsal-ventral axis establishment (Ku and Melton, 1993; Tao et al., 2005). My thesis work suggests that Xwnt11 RNA is translationally repressed in the germline. The data in Figure 4.8 indicate that Xwnt11 RNA is an authentic target of Pumilio binding, and this binding is through, as many as five non-canonical PBEs. These non-canonical PBEs could mediate the translational repression of the reporter message DsRED. Unexpectedly, the canonical
PBE failed to be recognized by Pumilio and, consistently, failed to mediate the translational repression of the reporter gene in vivo. A sequence search of the five non-canonical PBEs shows that four of the five canonical PBEs have a G, instead of an A, at position 6 or 8 [UGUANA(6)UA(8)]. The amino acids that will recognize these two base positions are glutamine and cysteine-serine. According to Cheong and Hall (2006), glutamine and cysteine-serine will bind relatively well to either A or G. In other words, G is tolerated at the positions 6 and 8. Therefore, theoretically, the non-canonical PBEs could be recognized as well as bound by Pumilio. The non-canonical PBEs in the Xwnt11 3’UTR may be bound by multiple Pumilio proteins, resulting in a unique scaffold structure that recruits some unknown components but not Nanos1. This protein complex would then repress Xwnt11 translation. As previously shown, in addition to Nanos/PUM, Brat is required for hunchback translational regulation; CCR4-pop2-NOT deadenylase complex is recruited for Cyclin B degradation in flies (Sonoda and Wharton, 2001; Kadyrova et al., 2007). To conclude, Xwnt11 RNA is translationally repressed in the germline and Pumilio binding is involved in this event. Unknown proteins other than Nanos1 may be also involved and it needs further work to find out what they are.

Taken together, three potential targets of Nanos1/PUM translational repression in the germline were investigated: VegT, cdk9, Xwnt11. Although all of them contain a canonical PBE, the results are different. The data support VegT as an authentic target of Nanos1/PUM translational repression. VegT RNA may be translationally repressed and eventually degraded. Even though a functional PBE is present in the cdk9 3’UTR, Cdk9 protein is expressed in PGCs. For Xwnt11, instead of the canonical PBE, perhaps as
many as five non-canonical PBEs are responsible for the PUM binding and translational repression. Nanos1 was not required for the repression of Xwnt11 translation in our reporter assay. These results suggest that different mechanisms are utilized in the translational regulation of different RNAs, even though the same scaffold protein is involved as the RNA binding protein, Pumilio. The diversity of the regulatory mechanisms is a reflection of the highly organized regulatory network in the germline. As the only cell line that can pass the genetic information to the next generation, germ cells are under extensive regulation. Any messages that may affect germline totipotency preservation will be repressed. Different messages bear unique regulatory codes in their sequences. Therefore, to guarantee the repression of various selfish messages, a variety of regulatory mechanisms function as a network in the germline.
Chapter 5

Discussion and Significance

Nanos1 impacts gene expression at two levels, promoting both transcriptional and translational repression. My thesis work demonstrates that *Xenopus nanos1* RNA is of maternal origin during early embryogenesis; and that the protein is available to function in the germ plasm from one cell stage to embryonic stage 34; and that *Xenopus* Nanos1 is required to prevent PGCs from expressing an endoderm gene program and undergoing cell death. Importantly, the work presented here defines a new target for translational repression by Nanos1/Pumilio, the endoderm determinant *VegT*. Given that Nanos1 strictly partitions within the *Xenopus* germline, our results explain how *VegT* repression is restricted to PGCs, while neighboring blastomeres express *VegT* and are specified as endoderm. My thesis work also proposes that the dorsalizing factor *Xwnt11* is translationally repressed in the germ plasm by a protein complex containing Pumilio but not Nanos1.

5.1. Migration in Nanos1 depleted PGCs

The fact that Nanos is required for germline survival has been shown in many species (Forbes and Lehmann, 1998; Kobayashi et al., 1996; Köprunner et al., 2001; Sato et al., 2007; Tsuda et al., 2003). As presented in my work, this phenotype is also observed in
Xenopus after Nanos1 knockdown. The underlying mechanism that triggers apoptosis in Nanos1 depleted PGCs remains open to investigation. Mis-migration has been found in Nanos depleted PGCs of Drosophila, zebrafish, and mice. In zebrafish, migration is initiated quite early during gastrulation and nanos is required for PGC directed active movement (Köprunner et al., 2001). After Nanos knockdown, germ cells migrate abnormally to somites and the head region. However, our data have shown that Xenopus PGCs deficient in Nanos1 did not migrate to ectopic locations, but remained in the endoderm. At stage 26-28 when PGCs normally begin their directed migration out of the endoderm, they are observed as clearly separated from each other. PGCs from Nanos1-MO injected embryos showed a diffused Xpat signal but were well separated in the endoderm, like wild-type PGCs. In contrast, after knockdown of Xdazl or XDead end where PGC migration is affected, PGCs are clustered with intense Xpat staining at these stages (Horvay et al., 2006; Houston et al., 1998; Houston and King, 2000). Based on these observations, we would argue that apoptosis is the primary reason for Nanos1-depleted PGCs to fail leaving the endoderm and not abnormal migration per se. Thus, Nanos1 involvement in Drosophila and zebrafish PGC migration appears to be quite different from that of Xenopus.

It is worth noting that not only Nanos1 depleted germ cells remaining in the endoderm underwent apoptosis, but wild-type PGCs that failed to migrate do so as well (Ikenishi 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 are in contrast to those of Wylie et al. (1985) who found that Xenopus labeled PGCs placed in the blastocoel were able to normally incorporate into somatic
tissue belonging to any of the three primary germ layers. Why wild-type PGCs remaining in the endoderm did not acquire an endoderm fate is not clear, but may be related to the amount of germ plasm inherited by individual PGCs.

5.2. Transcriptional activation in PGCs deficient in Nanos1

Germ cells remain transcriptionally quiescent when somatic transcription has been activated. DNA and histone modifications at the level of chromatin structure have been reported to cause global transcriptional silencing in the germline of flies, worms, and mice, but not frogs (Duncan et al., 2008; Katz et al., 2009; Schaner et al., 2003; Shilatifard, 2008; Stancheva et al., 2002; Venkatarama et al., 2010; Yin and Lin, 2007). Another common target for global transcriptional repression is the kinase Positive Transcription Elongation Factor b (P-TEFb) that 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 start 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). Loss of nanos in Drosophila results in the premature phosphorylation of CTD-Ser2, and ectopic expression of Nanos impairs transcription and CTD phosphorylation in somatic cells, but how this is mechanistically related to Pgc sequestration of P-TEFb function is not known (Deshpande et al., 2005; Hanyu-Nakamura et al., 2008). Similarly, depletion of Xenopus Nanos1 leads to premature expression of P-Ser2 and zygotic transcription at MBT. Although our data showed Cdk9 protein is present in the nucleus of wild type PGCs
during gastrulation, we cannot rule out the possibility that Nanos1 has some function in the assembly of active P-TEFb. Unpublished work in our lab shows that Nanos1 is capable of repressing a CMV driven Luciferase reporter injected into embryos, suggesting a direct role of Nanos1 in global transcriptional repression (Luo and King, unpublished observations). To conclude, what roles Nanos1 plays in germline transcriptional silencing requires further work.

5.3. Repression of somatic gene expression is the primary function of Xenopus
Nanos1

In *Drosophila*, *nos*−/− pole cells are eliminated by apoptosis triggered by the expression of maternal RNAs *hid* (*head involution defective*) and *skl* (*sickle*) (Sato et al., 2007). Deletion of these proapoptotic activators results in somatic gene expression in *nos*−/− pole cells and their incorporation into somatic tissues (Hayashi et al., 2004). Therefore, apoptosis has been proposed to be the primary function of Nanos during germline development. However, in *Xenopus*, apoptosis was not observed in Nanos1 depleted PGCs until tailbud stages (stage 28), far later than when somatic genes were mis-expressed during gastrulation (stage 10–11). Therefore, we propose that the primary function of *Xenopus* Nanos1 in the germline is in preventing somatic gene expression.

5.4. Translational repression of maternal somatic RNAs in the germline

In *Drosophila*, *C.elegans*, zebrafish, and *Xenopus*, germ cells are specified by inheriting germ plasm that contains maternal RNAs and proteins (reviewed by Eddy, 2006). Among
those maternal RNAs, there are somatic determinants, such as *VegT*, apoptosis factors, such as *hid*, cell cycle factors, such as *cyclin B*, and dorsal-ventral axis establishment factors, such as *Xwnt11*. In order to protect the germ cell identity, those RNAs that play a role in soma development and apoptosis activation need to be repressed during PGC development by germline specific regulators, such as Nanos. Although mouse and human use a different germ cell specification mechanism, a variety of germline specific molecules and their functions are highly evolutionarily conserved (reviewed by Eddy, 2006; reviewed by Voronina et al., 2011).

5.4.1 Restricted translational repression of maternal VegT in PGCs

My thesis work identified *VegT* as an authentic target of Nanos1/PUM translational repression. Nanos/PUM repression has been implicated in blocking or modulating the cell-cycle, apoptosis, and meiosis in the germline (see table 1.2; Lai et al., 2011; Asaoka-Taguchi et al., 1999; Dalby and Glover, 1993; Kadyrova et al., 2007; Hayashi et al., 2004; Sato et al., 2007; Wreden et al., 1997; Wharton and Struhl, 1991; Ahringer and Kimble, 1991; Zhang et al., 1997), and now we add the first example of a somatic determinant as a target. In *Xenopus*, embryonic patterning is regulated by maternal RNAs localized to the vegetal pole, the same region where germ plasm is found. How PGCs arise from the endodermal region, but avoid the endoderm differentiation programs, is a biological dilemma not previously explained in other systems. *VegT* is the endoderm determinant and functions in endoderm differentiation; *VegT* loss of function disrupts the endoderm germ layer specification (Zhang et al., 1998). Ectopic expression of Nanos1 in
somatic cells severely impaired normal embryonic development (Lai and King, unpublished observations). The strict localization of nanos1 to the germ plasm, while Pumilio is found everywhere, explains how the repression of VegT translation can occur exclusively in the germline, without impairing endoderm development. In contrast, zebrafish nanos1 is expressed in the whole embryo and is gradually restricted to PGCs by microRNA-mediated degradation of nanos1 RNA in the soma (Mishima et al., 2006).

5.4.2 Germline specific regulation of Xwnt11 translation

Maternal Xwnt11 RNA was identified as a germ plasm component by Ku and Melton (1993). Maternal Xwnt11 protein is required and sufficient to activate the canonical Wnt pathway which is necessary for the dorsal-ventral axis establishment in Xenopus embryonic development (Tao et al., 2005). To date, there is no evidence to suggest any function for Xwnt11 in germline development. We hypothesize that Xwnt11 is translationally repressed in the germ plasm. The rationale for this hypothesis is that primordial germ cells have to stay totipotent before they arrive in the gonads and undergo gametogenesis. Therefore, any somatic specification factors, such as Xwnt11, that may potentially interfere with preserving totipotency need to be repressed in the germline. Ectopic expression of Nanos1 in somatic cells impaired dorsal development, resulting in incomplete neural tube closure, and this phenotype suggests ectopic Nanos1 may impair Wnt signaling (Lai and King, unpublished; Luo et al., 2011). The data from my work strongly suggests that maternal Xwnt11 translation is negatively regulated in the germline and that the translational control element resides in the 3’UTR. My data also show that
Pumilio is able to recognize and bind to, maybe, as many as five non-canonical PBEs in the 3’UTR of Xwnt11. These findings suggest that Pumilio likely mediates the repression of endogenous Xwnt11 translation. In theory, all five non-canonical PBEs in Xwnt11 3’UTR are functional for Pumilio binding (Chapter 4), but which ones are authentic for Pumilio binding and translational repression function in the germline needs to be determined by further work. Nanos1 is not required in the translational repression of Xwnt11 in contrast to VegT repression that does require Nanos1. Even the canonical PBEs can mediate the translational regulation of different RNAs through different mechanisms. For example, Drosophila hunchback and cyclin B both are translationally repressed but by different protein complexes (Cho et al., 2006; Kadyrova et al., 2007; Sonoda and Wharton, 2001). Non-canonical PBE bearing RNAs offer yet another level of specific regulation. Since Pumilio is ubiquitously distributed in the embryo, there must be some unknown germline specific proteins recruited so that the translational repression of Xwnt11 could be restricted only in the germline without impairing dorsal-ventral axis establishment. After fertilization, maternal Xwnt11 in somatic cells is polyadenylated; polyadenylation allows Xwnt11 translation in somatic cells (Schroeder et al., 1999). Therefore, two speculated mechanisms of translational repression of Xwnt11 in PGCs are: 1) germline Xwnt11 3’UTR is bound by Pumilio and other germ plasm specific proteins and thus the polyadenylation machinery is inaccessible to the RNA; 2) Pumilio binds to Xwnt11 3’UTR and then recruits deadenylase, such as the CCR4 deadenylase complex, resulting in deadenylation of the RNA. Exactly how Xwnt11 is translationally repressed in the germline needs further work.
Figure 5.1 Model of germline totipotency preservation in *Xenopus* PGCs. *Xenopus* Nanos1 preserves the germline fate through multiple functions: preventing endoderm gene expression by repressing VegT translation, blocking RNA Pol II transcription at MBT, and inhibiting apoptosis. Unknown proteins together with Pumilio prevent PGCs from dorsalization by repressing *Xwnt11* translation.

Taken together, the data presented in my thesis support the primary function of *Xenopus* Nanos1 as preventing endoderm differentiation programs, and thus preserving germline totipotent fate. What role Nanos1 plays in blocking apoptotic pathways and supporting transcriptional repression in vertebrate systems remains an active area of research. Unknown germline specific proteins, together with Pumilio, translationally repress *Xwnt11*, thus preventing PGCs from dorsalization (Figure 5.1). Clearly, we still have a long way to go in understanding the regulatory network that preserves the germline identity. The diverse phenotypes described for *nanos1* mutants underscores the
importance of finding the RNA targets of Nanos1 repression and of determining the pathways these repressed targets operate in during embryogenesis.

**Significance**

The best understood molecular role of Nanos is its interaction with Pumilio (PUM) to negatively regulate targeted mRNAs at a posttranscriptional level. Therefore, to identify the targets of Nanos/PUM in germ cells is certainly essential to understand the function of Nanos during germline development and how the germline is preserved. Here my thesis work supports *VegT* as an authentic target of Nanos1/PUM translational repression and *Xwnt11* as a potential target. For the very first time in a vertebrate, we understand how germline identity is protected from somatic specification.

While different mechanisms for preserving germline fate may be applied in different species, so far two genes have been proposed to play a crucial role in the germline development from flies, worms, frogs and mice to humans: Nanos and *Deleted in Azoospermia (DAZ)*. Nanos is expressed in multipotent cells, stem cells, and primordial germ cells (PGCs) of organisms as diverse as jellyfish and humans. Ectopically expressed germline specific genes, such as Nanos, drove brain cancer growth in flies and the cancer growth was suppressed by inactivating any of the germline genes, suggesting a strategy of combating cancer by germline gene suppression (Janic et al., 2010). Therefore, I believe my thesis work will be of general interest to researchers in other disciplines as well, such as stem cell biology and cancer biology. In the long term, basic studies in
model systems will contribute to a better understanding of the genes and their functions required for human germ cell development.
Chapter 6

Materials and Methods

Obtaining embryos and embryo injection

Adult *Xenopus laevis* frogs were purchased from *Nasco*. *Xenopus laevis* female frogs were induced to ovulate by injecting 500U hCG. After artificial fertilization using *laevis* sperm, embryos were dejellied and collected at specific stages following the procedures detailed in Sive et al. (2000). Embryos were staged according to Nieuwkoop and Faber (1956). Collected embryos were immediately frozen on dry ice, stored for RNA extraction for future use in PCR reactions.

To knockdown endogenous *nanos1* translation, 20 ng of Nanos1-MO was injected at the vegetal pole before the 2-cell stage. Injection of Nanos1-Ctrl-MO (20ng) served as a control. The *nanos1-mut* RNA (0.4 ng) used in the rescue experiments had 3 base pair mismatches within the MO binding site and was not recognized by the Nanos1-MO.

Morpholino sequence (Gene tools):

Nanos1-MO: CGCCATCCATGTGGGAATTGTTCTG;
Nanos1-Ctrl-MO: CGCCATGATcTTGcAAaTcTTCTG, with the 5 base pair mismatches within the MO binding site in lower case.
Histology

Staged tadpoles were fixed overnight at 4°C in Bouin’s solution (Sigma) and stored in ethanol at -20°C. The tadpoles were dehydrated through a series of ethanol-butanol solutions, embedded in paraffin, sectioned at 10-µm and mounted on slide glasses. The specimens were de-paraffinized through a series of xylene, hydrated through a series of ethanol–water solutions, and subjected to H&E staining.

Immunofluorescence

Staged embryos within their vitelline membranes were fixed in Dent’s (80% Methanol, 20% DMSO) at -20°C overnight and then bleached in 0.5X Standard Saline Citrate (SSC) solution with 5% formamide and 1% H₂O₂ for 1~2 hours under a fluorescent light. Bisected embryos were washed in 0.3% Triton-X100/PBS, 2X 30min at room temperature. Embryos were then incubated in Blocking solution [1X PBS, 2% BSA (2mg/mL), 0.1% Triton X-100] for 15 min at room temperature, followed by incubation in Blocking solution with 10% donkey serum for 30 min at room temperature with rocking. Primary antibody incubation was done in Blocking solution overnight at 4°C, and after four times1hr washes in 1X PBS/0.1% Tween20 (PBST), AlexaFluor-labeled secondary antibody was added and samples incubated overnight at 4°C in the dark. Embryos were washed as above and then dehydrated with ethanol. The above steps were done in 24-well plastic plates. Then, samples were transferred to a small cassette (see Figure 6.1) which is suitable for analysis using inverted lens on confocal. Samples were
analyzed in Murray’s Clear (2:1 benzylbenzoate:benzylalcohol) using an inverted Zeiss LSM-510 Confocal Laser Scanning Microscope equipped with Argon ion, Helium-Neon and Green-Neon lasers.

**Primary antibodies:** Affinity purified anti-Nanos1 antibody was made in goats against total recombinant expressed Nanos1 protein (Invitrogen), 1:50; rabbit anti-Xiwi (a gift from Dr. Nelson Lau), 1:1000; Hyperacetylated Histone H4 (Penta) rabbit antibody (Upstate, cat#06-946) 1:1000; rabbit anti DsRED antibody (Clontech, #632496), 1:200; H5 monoclonal antibody, 1:50 (Covance, #MMS-129R); Goat anti CDK9 IgG (Santa Cruz, sc-484-G), 1:20. **Secondary antibodies** (Invitrogen): AlexaFluor 488 donkey anti rabbit IgG (H+L), 1:500; AlexaFluor 555 donkey anti goat IgG (H+L), 1:500; AlexaFluor 568 donkey anti rabbit IgG (H+L), 1:1000; AlexaFluor 568 donkey anti rabbit IgG (H+L), 1:1000; AlexaFluor 488 donkey anti mouse IgG (H+L), 1:1000.

**In vitro** translation using rabbit reticulocyte lysate system

*nanos1* and *nanos1-mut* RNA were transcribed *in vitro* according to the manufacturer’s instructions (Ambion, mMESSAGE mMACHINE kit). **In vitro** translation was performed
in a rabbit reticulocyte lysate system according to the manufacturer’s instructions (Promega), using $^{35}$S-Methionine. The results were analyzed using a PhosphoImager (Storm 840, molecular dynamics) and quantified using Image J (NIH).

**In vivo fluorescent reporter assay**

Plasmid pCS2-Venus-DEADSouth 3’UTR is a gift from Dr. Oriias (Kataoka et al., 2006). The green reporter Venus-DS3’UTR (VD) serves as a germline lineage tracer. The red reporter DsRED-DS3’UTR (RD) was generated by replacing Venus with the DsRED open reading frame. *VegT* 3’UTR (2336–2631, 296nt) containing PBE (UGUAAAUA) was subcloned downstream of DsRED ORF to generate DsRED-VegT PBE-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, UAAAAAAA). [*cdk9* PBE containing fragment and *Xwnt11* 3’UTR fragments were subcloned downstream of DsRED ORF in the same way as described for VD.] The reporter construct DsRED-VegT PBE-DS3’UTR was linearized by XhoI and reporter message was made by *in vitro* transcription using mMESSAGE mMACHINE kit (Ambion, #AM1340). Nanos1 MO was injected into one-cell stage embryo at the vegetal pole. Around 32-cell stage, fluorescent reporters were injected at a dose of 200pg/cell into 4 vegetal blastomeres of either the uninjected control or Nanos1 knockdown embryos. Images were taken of live st.34 embryos using a fluorescent stereomicroscope (Olympus SZX12).
Non-radioactive RNA electrophoresis mobility shift assay

VegT 3’UTR fragment (2468–2579, 112nt) containing the PBE was cloned into pCR4-TOPO vector (Invitrogen), producing TOPO-VegT PBE. The RNA was transcribed using T7 RNA polymerase (Promega) and biotinylated at the 3’ end according to the manufacturer’s instructions (Thermo Scientific).

20 ng Xpum1 RBD was incubated with 1 ng Biotin-VegT PBE in 1 X binding buffer (10 mM Tris pH 7.5, 50 mM NaCl, 1 mM EDTA, 0.5 mM DTT, 10% glycerol, 200 ng of yeast tRNA, and 200 ng of BSA). After incubation for 20 min at room temperature, samples were loaded onto a 4% polyacrylamide gel and run for 40 minutes at 100 V in 0.5X TBE buffer (45mM Tris-HCl, 45mM Boric acid, 1mM EDTA, pH 8.3). The gel and nylon membrane as a sandwich were placed in a clean electrophoretic transfer unit, and the RNA transferred at 35 V for 30 min. The nylon membrane was subsequently UV-crosslinked and the biotin-labeled RNA was detected by chemiluminescence according to manufacturer’s instructions (Thermo Scientific). The same methods were used in the EMSA analysis of the cdk9 3’UTR PBE-containing fragment as well as the Xwnt11 3’UTR fragments.

PGC isolation

PGCs were isolated as described by Venkatarama et al. (2010) with slight modification. A saturated solution of DiOC₆(3) was made in DMSO. A stock solution was made from the saturated solution at 2:1000 dilution in 0.1X MMR. The working solution was made
from the stock solution at 24:1000 in 0.1X MMR. For staining, 4-cell stage embryos were incubated in the working solution for 30 min, and then washed 10 times in 0.1X MMR. Stained embryos were incubated in the dark until the appropriate stage was reached.

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

PGCs from wild type embryos (WT) and Nanos1 depleted embryos (Nanos1 MO) were isolated as described by Venkatarama et al. (2010), respectively. The RNA extraction and reverse transcription were performed as described in Lai et al. (2011). Real-time PCR was done for 40 cycles using SsoAdvanced™ SYBR® Green Supermix (#172-5260, BioRad Laboratories Inc.) in Bio-Rad CFX96™ system (BioRad Laboratories Inc.). Primer sequences are listed as follows: 

\[ \text{VegT (forward) 5'}-\text{AGAAACTGCTGTCGGGAA-3'}, \text{ (reverse) 5'}-\text{CGGATCTTACACTGAGGA-3'}, 53^\circ \text{C}; \]
\[ \text{GAPDH (forward) 5'}-\text{CTCCTCTCGCAAAGGTCATC-3'}, \text{ (reverse) 5'}-\text{GGAAAGCCATTCCGGTTATT-3'}, 53^\circ \text{C}. \]

Primer specificity was monitored and ascertained by analyzing the melting curve. All samples were done in duplicate and experiments were repeated three times.

The threshold cycles (C_\text{T}) were determined by CFX manager software (BioRad). VegT expression was normalized to the expression of housekeeping gene GAPDH, by calculating \( \Delta C_\text{T}=C_\text{T}(\text{VegT})- C_\text{T}(\text{GAPDH}) \). The relative fold change was calculated as Power (2, -\( \Delta\Delta C_\text{T} \)), where \( \Delta\Delta C_\text{T}=\Delta C_\text{T} \) (Nanos1 MO) - \( \Delta C_\text{T} \) (WT). The statistical difference was determined by an unpaired Student’s t-test.
Reverse transcription polymerase chain reaction (RT-PCR)

Total RNA and genomic DNA were separately extracted using TRIzol® Reagent (Invitrogen #15596-026) and RNA was reverse transcribed to cDNA (SuperScript® III First-Strand Synthesis System for RT-PCR, Invitrogen #18080-051). PCR was done for 32 cycles and the products were analyzed by electrophoresis on a 1% agarose gel. RT-PCR primers: ODC 53°C (Xanthos et al., 2001); *Xenopus laevis* Xnos1: forward 5’-CTGCAGCCTCAGAGAGAAGG-3’, reverse 5’-CCACACAAAGGGAAGCTGTA-3’, 58°C; *Xenopus borealis* Xnos1: forward 5’-AGCCCAGTTAGGGAAGTGTG-3’, reverse 5’-GCAGGTGTAGCCCCTAGTA-3’, 55°C; *Xsox17α* at 56°C (Hudson et al., 1997); Xpat 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); GATA 4/5/6 at 56°C (Xanthos et al., 2001); Endodermin (Edd) at 56°C (Xanthos et al., 2001); *Cerberus* at 60°C (Darras et al., 1997); Xhex at 60°C (Chang and Hemmati-Brivanlou, 2000); Chordin at 55°C (Zhang et al., 1998); Oct91 at 56°C; Xwnt11 at 56°C.

Quantitation was done using Image J software (NIH)

Whole-mount in situ hybridization

Staged embryos were fixed in MEMFA and whole-mount in situ hybridization was performed as described by Houston et al. (1998). The Xpat plasmid was linearized using NotI and the digoxigenin labeled anti-sense probe was transcribed by T7 RNA polymerase (Ambion, #AM 2082).
Whole-mount fluorescent in situ hybridization and immunofluorescence

Staged embryos were fixed in MEMFA (2 mM MOPS pH 7.0, 0.2 mM Sodium Acetate, 0.1 mM EDTA (pH 8.0), 2.0 mM EGTA, 1.0 mM MgSO₄, 3.7% formaldehyde, freshly made in DEPC-treated H₂O) overnight at 4°C, bleached and then stored in methanol at -20°C. After rehydration by stepwise transfer into 100%, 75%, 50%, 25% ethanol in PBST, and into PBST for 2X 10min, embryos were then permeabilized in 10µg/mL protease K made in PBST and digestion was terminated by washing in PBST for 2X 5min. Embryos were re-fixed in 4% paraformaldehyde (PFA) in PBST for 20 min at room temperature, followed by washing in PBST for 2X 5min. After incubation in prehybridization buffer for 4h at 60°C, embryos were transferred into hybridization buffer containing 1µg/mL FITC-labeled probe and incubated overnight at 60°C. Then embryos were washed by replacing with fresh hybridization buffer for 10min, and 2X SSC/0.1%Tween20 (SSCT) for 30min three times, at 60°C, and 10min in SSCT at room temperature, followed by 2X 10min washing in 1X Maleic Acid buffer/0.1%Tween20 (MABT). Embryos were blocked in Blocking solution (2% blocking reagent (Roche) in MABT) for 1h at room temperature, and then incubated in Blocking solution containing anti-FITC-POD (Roche) (1:1000) overnight at 4°C. Embryos were washed in MABT 4X 1h and PBS 2X 10min at room temperature, then incubated in 1/50 volume of fluorescein tyramide in 1×Plus Amplification Diluent (Perkin Elmer) for 30 min, and the reaction was terminated by washing in PBST for 2X 10min. Immunofluorescence was done afterwards as described above.
**Whole-mount Fluorescent in situ hybridization, followed by TUNEL assay**

Staged embryos were fixed in MEMFA overnight at 4°C, bleached and then stored in methanol at -20°C. Whole-mount fluorescent in situ hybridization was performed as described by Lai et al. (2011). After rehydration into PBST, embryos were permeabilized by protease K and then re-fixed in 4% paraformaldehyde (PFA). After incubation in prehybridization buffer for 4h at 60°C, embryos were transferred into hybridization buffer containing 1µg/mL FITC-labeled probe and incubated overnight at 60°C. Then embryos were washed and blocked in Blocking solution [2% blocking reagent (Roche)] for 1h at room temperature, and then incubated in Blocking solution containing anti-FITC-POD (Roche) (1:1000) overnight at 4°C. After 4 times washes in MABT and 2 times washed in PBS, the specimens were then incubated in 1/50 volume of fluorescein tyramide in 1×Plus Amplification Diluent (Perkin Elmer) for 30 min, and the reaction was terminated by 2 times washing in PBST. Embryos were re-fixed in 4% PFA/PBST for 20 min and washed in PBST for 2X 30 min, followed by washing in PBS for 2X 15 min at room temperature. Samples were then transferred into Terminal Deoxynucleotidyl Transferase (TdT, Invitrogen) buffer diluted in 1X PBS and incubated for 30 min at room temperature, followed by incubation with 1X TdT buffer containing 150U/mL TdT and 0.5µL DIG-labeled dUTP (Roche) overnight at room temperature. Embryos were then washed 2 X 1 h in PBS/EDTA 1 mM, at 65°C, followed by 4X 30min in PBS at room temperature. Detection was carried out using TSA system (Perkin Elmer). Samples were washed twice in MABT, blocked in Blocking solution for 1h at room temperature, and then incubated in Blocking solution containing anti-DIG-POD (1:1000, Roche) antibody overnight at
4°C. Embryos were washed as above and then incubated in 1/50 volume of Cy3 tyramide in 1×Plus Amplification Diluent for 30 min, and the reaction was terminated by washing in PBST 2X 10min. Samples were cleared in Murray’s Clear and analyzed using an inverted Zeiss LSM-510 Confocal Laser Scanning Microscope equipped with Argon ion, Helium-Neon and Green-Neon lasers. Fluorescein (FITC, Roche) labeled Xpat anti-sense probe was transcribed by T7 RNA polymerase (Ambion, #AM 2082).

Negative control for TUNEL: no terminal deoxynucleotidyl transferase was included in the TUNEL assay.

Positive control for TUNEL: embryo samples were pre-treated with TURBO DNase (Ambion) for 30 minutes at 37°C before the TUNEL assay.

**β-gal assay**

NLD plasmid was obtained as a generous gift from Dr. Tohru Komiya. NLD plasmid was linearized by Sal I and transcribed by T7 RNA polymerase (Ambion, #AM 1344).

Nanos1 MO or Nanos1-Ctrl-MO was injected at the vegetal pole of one-cell stage embryos as described above. At 16-cell stage, NLD mRNA was injected to the vegetal blastomeres at a dose of 1ng/cell. At stage 46, embryos were collected for β-Gal assay as described by Takeuchi et al. (2010). Embryos were fixed in MEMFA for 25 min at room temperature, followed by washing 5 times in 1X PBS for 10 min each. Samples were then incubated in Bluo-gal solution for 6 hours at room temperature. The enzymatic reaction was stopped by washing 3 times in PBS. The samples were then fixed in Bouin’s solution (Sigma) overnight at room temperature and washed in 70% ethanol. After
bleaching, the samples were dehydrated to 100% methanol and PGCs were counted in Murray’s clearing medium using a Leica dissecting microscope.

**Xenopus pumilio1 RNA binding domain (Xpum1 RBD) protein expression and purification**

Xpum1 RBD (825aa~1175aa) was cloned into the pET28a expression vector. Plasmids were transformed into the E.coli BL21 (DE3) strain. One colony was inoculated into 100ml LB medium with shaking overnight at 37°C. Next morning, the bacteria medium was diluted (1:10) into fresh LB broth until OD600 reaches 0.6. Subsequently, protein expression was induced by 1mM IPTG for 4~5hrs.

The collected cell pellet was thawed on ice around 10 min. The pellet was completely resuspended in 1X binding buffer (included in the His-protein purification kit, Novagen) and sonicated on ice. After centrifuge at 14,000g for 20 min at 4°C, the supernatant was transferred into a fresh tube and loaded onto the prepared His-bind resin column. (Preparation of the column is according to the manufacturer’s instructions). The column was washed with binding buffer and wash buffer. Protein was eluted in 500uL of buffer containing 200mM~300mM imidazole. The protein sample was concentrated using Amicon centricon (5KD, 4000g) and dialysised against 1X PBS (pH 7.4) at 4°C for overnight. Protein sample was aliquoted and stored in PBS/50% glycerol at -80°C.
**Xenopus laevis and Xenopus borealis inter-species hybrids**

*Xenopus laevis* female was induced to ovulate and the eggs were artificially fertilized by *Xenopus borealis* sperm in 0.05 DoBoers solution (0.11M NaCl, 0.0013M KCl, 0.00044M CaCl₂, pH7.2). The hybrids were grown in 0.05 DoBoers solution and collected at different stages until tadpole stage.

Adult *Xenopus borealis* frogs were purchased from *Xenopus Express*. Borealis female eggs have been shown to strongly block cross fertilization due to their thick jelly coat. An egg-transfer technique designed by Burn and Kobel (1977) was used: *Xenopus borealis* female (donor) was induced to ovulate with 600U hCG; the donor was sacrificed at the appearance of eggs in the cloaca; and the eggs from the body cavity were collected into DB solution. *Xenopus laevis* ovulating female (previously induced by 900U hCG) was immobilized with 900mg/L MS222 and *borealis* eggs were transferred into her coelom by a small incision in the ventral body wall. After recovery, ovulation continued and the donor eggs appeared about two hours later (distinguished by the smaller size of *borealis* eggs). *Laevis* female was gently stripped and *borealis* eggs were collected and fertilized by *laevis* sperm in 1X MMR for 10min. Embryos were cultured in 0.1X MMR and collected at different stages for analysis.
References


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