Differential Nanos 2 protein stability results in selective germ cell accumulation in the sea urchin

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Abstract

Nanos is a translational regulator required for the survival and maintenance of primordial germ cells. In the sea urchin, Strongylocentrotus purpuratus (Sp), Nanos 2 mRNA is broadly transcribed but accumulates specifically in the small micromere (sMics) lineage, in part because of the 3′ UTR element GNARLE leads to turnover in somatic cells but retention in the sMics. Here we found that the Nanos 2 protein is also selectively stabilized; it is initially translated throughout the embryo but turned over in the future somatic cells and retained only in the sMics, the future germ line in this animal. This differential stability of Nanos protein is dependent on the open reading frame (ORF), and is independent of the sumoylation and ubiquitylation pathways. Manipulation of the ORF indicates that 68 amino acids in the N terminus of the Nanos protein are essential for its stability in the sMics whereas a 45 amino acid element adjacent to the zinc fingers targets its degradation. Further, this regulation of Nanos protein is cell autonomous, following formation of the germ line. These results are paradigmatic for the unique presence of Nanos in the germ line by a combination of selective RNA retention, distinctive translational control mechanisms (Oulhen et al., 2013), and now also by defined Nanos protein stability.

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1. Introduction

Nanos is a RNA binding protein which was first identified in Drosophila as a translational repressor required in anterior-posterior patterning (Cho et al., 2006; Irish et al., 1989). Subsequently it was found to be essential for germ line determination and Nanos orthologs have been found in the germ line of all animals tested (e.g. Caenorhabditis elegans (Kraemer et al., 1999), Xenopus (Lai et al., 2011) and planarians (Wang et al., 2007)). Nanos functions through its interaction with Pumilio, which binds RNAs containing a conserved motif in their 3′UTR, the Pumilio Response Element (PRE) (Sonoda and Wharton, 1999; Wharton and Struhl, 1991). Although only a limited number of mRNAs have been identified as Nanos/Pumilio targets, these mRNAs encode proteins with key cellular and developmental functions. These Nanos-targeted mRNAs include cyclin B (Asaoka-Taguchi et al., 1999; Dalby and Glover, 1993; Kadyrova et al., 2007; Lai et al., 2011), hif (Hayashi et al., 2004; Sato et al., 2007), hunchback (Murata and Wharton, 1995; Wreden et al., 1997), fem 3 (Ahringer and Kimble, 1991; Zhang et al., 1997), VegT (Lai et al., 2012), and CNOT6 (Swartz et al., 2014). This list may expand significantly; using a database from Xenopus laevis, a series of additional candidates of Nanos/Pumilio targeted mRNAs in the germline have been generated based on scanning for PRE consensus sequences in the 3′UTR sequences (Lai and King, 2013).

The Nanos protein has two conserved Cys-Cys-His-Cys zinc-finger motifs that are indispensable for its function and it was found that each amino acid of the CCHC motif is important for Nanos function in Drosophila (Arrizabalaga and Lehmann, 1999; Curtis et al., 1997). This zinc-finger domain was crystallized for the first time using the zebrafish Nanos (Hashimoto et al., 2010), which revealed that its basic surface is directly involved in RNA binding. In contrast to Nanos, its binding partner protein, Pumilio, is not limited to the germ line and instead is uniformly distributed e.g. in the Drosophila embryo (Macdonald, 1992). Pumilio is a member of the Puf family (Pum and FBF), in which members typically contain eight Puf repeats, each 36 amino acids in length (Goodwin, 2001). The eight repeats together with the C terminal conserved sequence form the Puf domain, which is critical for binding both the PRE in RNAs, and its cofactors, such as Nanos (Edwards et al., 2001; Wang et al., 2001).

The expression of germ-line factors is essential for the reproductive future of the organism, and as such, these factors are highly regulated. Indeed, ectopic expression of these genes often induces cell cycle and developmental defects (Luo et al., 2011; Wu and Ruvkun, 2010) and Nanos is thought to be "toxic" outside of its
normal domain (Lai and King, 2013). In *Drosophila, C. elegans, Danio, Xenopus*, sea urchin, and mouse, appropriate expression of Nanos in the germ line requires its unique 3′ untranslated region (UTR) (D’Agostino et al., 2006; Gavis et al., 1996a, 1996b; Kloc et al., 2000; Koprunner et al., 2001; Oulhen et al., 2013; Saito et al., 2006; Suzuki et al., 2010). Furthermore, in *Xenopus* oocytes, the *Nanos* mRNA contains a secondary structural element - just downstream of the AUG start site which is both necessary and sufficient to prevent ribosome scanning of this mRNA until after fertilization (Luo et al., 2011). Thus, many different levels of regulation are imposed upon Nanos expression, yet no post-translational steps of regulation of the Nanos protein have yet to be identified.

Three Nanos homologs are present in the genome of the sea urchin, *Strongylocentrotus purpuratus* (Sp). *Nanos 1* mRNA is only present in the ovaries (Swartz, unpublished data). *Nanos 2* and *Nanos 3* accumulate in the small micromeres (SMics) (Juliano et al., 2010), cells that contribute to the germ line (Yajima and Wessel, 2010), but whose gene is transcriptionally active in many cells of the embryo. Nanos 2 is the only Nanos paralog that accumulates in the SMics until gastrulation when the Nanos 3 transcript then accumulates specifically also in the small micromere lineage. Previous data indicated that in sea urchins, the stability and the translation of *Nanos 2* RNA depended on its UTRs, both 5′ and 3′ sequences (Oulhen et al., 2013). In this study, we found that Nanos 2 protein accumulation is regulated by an additional mechanism that is post-translational; Nanos is translated in all cells of the embryo but the protein accumulates only in the germ line. This is the first documented case of such a germ cell specific, post-translational event for Nanos and may reflect the key and selective roles that Nanos plays in early development and in germ line determination.

2. Material and methods

2.1. Animals

The sea urchin *Strongylocentrotus purpuratus* and the starfish *Patiria miniata* adults were housed in aquaria with artificial seawater (ASW) at 16 °C (Coral Life Scientific Grade Marine Salt; Carson, CA). Sea urchin gametes were acquired by either 0.5 M KCl injection or by shaking. Eggs were collected in ASW or filtered seawater and sperm was collected dry. Embryos were cultured in filtered seawater at 16 °C. Sea star gametes were acquired by opening up the animals. Oocytes were collected in filtered seawater and sperm was collected dry. To obtain mature oocytes, the full-grown immature oocytes were incubated for an hour in filtered seawater at 16 °C (Foltz et al., 2004; Wessel et al., 2010).

2.2. Immunofluorescence

Untreated embryos were cultured as described above and samples were collected at indicated stages of development for immunofluorescence. To test the ubiquitylation and sumoylation pathways, embryos were treated respectively with 25 μM MG132 (C2211, Sigma Aldrich, St. Louis, MO) added 10 h after fertilization or 10 μM Gingkoic acid (345887, EMD Millipore, Billerica, MA) added 12 h after fertilization. These treated embryos were fixed at mesenchyme blastula stage. Immunofluorescence was performed as described earlier; the *Sp Nanos 2* affinity-purified antibody was diluted to 1:500 and used as described (Juliano et al., 2010). The Sp Vasa affinity-purified antibody was diluted to 1:500 (Voronina et al., 2008). Sumo antibody was purchased from Cell Signaling Technology (4930), (Danvers, MA) and from Abcam (ab3742) (Cambridge, MA), and both were used at 1:100 dilution. Ubc9 antibody (AV43021) was purchased from Sigma Aldrich (St. Louis, MO) and used at dilution 1:100. For each immunofluorescence, the primary antibody was incubated at 4 °C overnight, and an anti-rabbit Alexa Fluor 488 was used as the secondary antibody (Life Technologies, Carlsbad, CA), diluted by 1:500 in blocking buffer, for two hours at room temperature. Images were captured using a LSM 510 laser scanning confocal microscope (Carl Zeiss, Inc.; Thornwood, NY).

2.3. Plasmid constructions

The Renilla luciferase constructs, *Xenopus β-globin* 3′ UTR and *Sp cyclin B* 3′ UTRs were amplified (Supplemental Fig. 5A1) and cloned into a plasmid containing the *Sp Nanos 5′ UTR, Renilla luciferase* open reading frame, and a SP6 promoter. The GFP constructs, *Sp Nanos 2* 5′ and ΔGNARLE 3′UTRs, were amplified and cloned next to the GFP open reading frame, and the T7 promotor as described previously (Wessel et al., 2013). *Nanos 2* ORF was amplified and inserted in frame in C-terminal of the GFP ORF (Supplemental Fig. 5B). A mutant control was made by adding a T between the GFP ORF and the *Nanos 2* ORF (Supplemental Fig. 5C), to create a stop codon after the GFP ORF and to change the open reading frame of *Nanos 2*. Mutations of the seven lysines and the zinc finger domains found in *Nanos 2* ORF were accomplished using the QuickChange II Site-directed mutagenesis kit (Agilent Technologies, Santa Clara, CA), with the primers listed in Supplemental Fig. 52. The deletion constructs of *Nanos ORF* were made using the primers listed in Supplemental Fig. 53.

2.4. In vitro RNA synthesis

Capped sense RNA was synthesized using the mMessage mMachine® T7 or Sp6 Kit (Ambion, Austin, TX) yielding RNA concentrations between 0.5 and 2 μg/μl. Each RNA was co-injected with *mCherry* flanked with *β-globin* UTRs. Injection solutions contained: 20% glycerol, 1 × 10^12^ copies of either a GFP RNA, or of the *mCherry* RNA. Approximately 2 pl of each RNA mixture was injected into each fertilized egg.

2.5. Microinjections

Microinjections of zygotes were performed as previously described (Cheers and Ettensonh, 2004). In brief, eggs were de-jelled with acidic sea water (pH 5.0) for 10 min, washed with filtered sea water three times, rowed with a mouth pipette onto protamine sulfate-coated 60 × 15 mm petri dishes, fertilized in the presence of 1 mM 3-AT, and injected using the Femto Jet® injection system (Eppendorf; Hamburg, Germany). 1 × 90 mm glass capillaries with filaments (Narishige: Tokyo, Japan) were pulled on a vertical needle puller for injections (Narishige; Tokyo, Japan). Injected embryos were cultured in sea water at 16 °C.

2.6. Morpholino approach

Morpholinos against *Sp Sumo* (5′-ACTTCCTTTGTGCAGTGGT-CATGA-3′) were purchased from Genetools (Philomath, OR). Morpholino injection solutions include 20% glycerol and 1 mM 10,000 MW Dextran conjugated to Texas Red® (Life technologies, Carlsbad, CA). A morpholino against the sea star *Patiria miniata dysferlin* (5′-TCCAGACAGTACCATACATGCGACAT-3′) was used as a non-relevant control (Oulhen et al., 2014). For injection in sea star oocytes, the sea urchin, Sp SCP2 morpholino was used as a control.
(5′-GGACATACTGTCAGGTCGTGCCAA-3′).

2.7. Dual luciferase assay

Strongylocentrotus purpuratus fertilized eggs were injected, as described above, with a solution containing 1 × 10^12 copies of each RNA, 20% glycerol, and 1 mM Alexafluor 488-dextran to allow visualization of injected eggs. For each measurement, 100 injected embryos were collected at the blastula stage. Renilla and Firefly luminescence were measured using the Dual luciferase assay kit (Promega) in a Lumat LB 9501 luminometer (Berthold Technologies, Germany).

2.8. GFP Reporter Fluorescence

Injected embryos were cultured as described above and samples were collected at indicated stages of development. Live embryos were imaged on an LSM 510 laser scanning confocal microscope (Carl Zeiss, Inc.; Thornwood, NY). Approximately one hundred blastulae were visualized for each construct and time point.

2.9. Whole mount RNA in situ hybridization (WMISH)

Sequences used to make antisense WMISH probes for Sp Sumo and Sp Ubc9 were amplified from a cDNA library and cloned into pGEMT-EZ. The Sp Sumo probe template (789 bases) includes the entire open reading frame plus 522 bases of the 3′ UTR, the primer set is as follows: F (5′-ATGCGTTTAGAAGTGAAGCCAAGTAATG-3′) and R (5′-ACCTACATGACATACATGCAATGAGTTAG-3′). The Sp Ubc9 probe template (510 bases) includes the entire ORF and was amplified with the following primers: F (5′-ATGCCTGATGCAGATCAGTATCG-3′) and R (5′-CTACGCCTTGCTGTTTACGGTTG-3′). A probe against GFP (Oulhen and Wessel, 2013) was used to visualize GFP RNA and GFP Sp Nanos 2 RNA (Fig. 1).

Antisense DIG-labeled RNA probes were constructed using a DIG RNA labeling kit (Roche; Indianapolis, IN). WMISH experiments were performed as previously described (Minokawa et al., 2004) and the alkaline phosphatase reaction was carried out for 5 h. Samples were imaged on a Zeiss Axiovert 200 M microscope equipped with a Zeiss color AxioCam MRc5 camera (Carl Zeiss, Inc.; Thornwood, NY).

Fig. 1. Nanos 2 protein is enriched in the small micromeres. Synthetic mRNAs containing the GFP ORF alone (A) or the GFP ORF fused in frame with the Nanos 2 ORF (B) were injected in Sp fertilized eggs. In each case, the GFP alone, or the GFP Nanos 2, the ORF was surrounded by Nanos 2 5′ and 3′UTRs. GFP fluorescence was assayed 18 hours post-fertilization at mesenchyme blastula. A and B were obtained using the same settings (e.g. laser intensity, pin-hole opening) at 400 × magnification. Approximately one hundred blastulae were visualized after injection of each construct. After injection of each construct, whole mount in situ hybridization was also performed using probes against GFP (C and D; not necessarily the same embryos for both tests) at the mesenchyme blastula stage. Synthetic mRNAs containing Renilla luciferase (Rluc) ORF alone or Rluc ORF fused in frame with the Nanos 2 ORF (E) were injected in Sp fertilized eggs. An mRNA containing the Firefly luciferase (Fluc) ORF flanked by Xenopus β-globin 5′ and 3′UTRs was co-injected. Luminescence was measured in mesenchyme blastulae. The ratio Rluc/Fluc was determined and the results are shown in percentages considering the ratio obtained for Rluc ORF as 100%. Error bars indicate the standard deviation from three technical replicates. Significance was assessed with the use of Student t-test (P < 0.05). Synthetic mRNAs containing the GFP ORF alone (F), the GFP ORF fused in frame with the Nanos 2 ORF (G) or the GFP ORF followed by a stop codon and an unframed Nanos 2 ORF (H) were injected in Sp fertilized eggs. In each case, the ORF was surrounded by Nanos 2 5′ and 3′UTRs. GFP fluorescence was assayed 20 hours post-fertilization at mesenchyme blastula. F, G and H were obtained using the same settings (laser intensity, pin-hole opening).

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2.10. Cell dissociation and measurement of autonomy

14 hours post fertilization embryos were dissociated as described in Juliano et al. (2014), and cultured for 6 additional hours on a fluorodish. The single cells were then transferred to a protamine sulfate coated dish, and fixed with PFA 4% in filtered sea water. Immunofluorescence was used to co-label the cells using the affinity purified antibody against the Sp Vasa (as explained above), and an antibody against GFP (for Nanos-GFP) used at dilution 1:100 (A10262 from Invitrogen (Carlsbad, CA)).

3. Results

3.1. Nanos 2 protein is post-translationally enriched in the small micromeres

The sea urchin S. purpuratus Nanos 2 protein accumulates exclusively in the small micromeres as seen by a Sp Nanos-specific antibody (Juliano et al., 2010). This exclusivity may be explained by unique Nanos transcription in the sMics although preliminary results show that it is transcribed more broadly (Yajima, Swartz and Oulhen, personal communication). Alternatively, a broadly transcribed Nanos 2 mRNA may only be retained in the sMics since many mRNAs appear to be degraded selectively in the soma by a CNOT6-dependent mechanism, one that is lacking in the sMics (Gustafson and Wessel, 2010; Oulhen and Wessel, 2013; Swartz et al., 2014). Indeed, Nanos 2 contains an element in its 3’UTR, GNARLE (Global Nanos Associated RNA Lability Element), essential for the selective enrichment of its mRNA in the small micromeres (Oulhen and Wessel, 2014; Oulhen et al., 2013). Removal of GNARLE however, results in uniform RNA retention, and translation of the GFP reporter protein throughout the embryo. For example, injecting into fertilized eggs the mRNA synthesized in vitro containing the GFP ORF fused to Nanos 2 5’UTR and ΔGNARLE 3’UTR, resulted in robust and uniform GFP accumulation throughout the embryo (Fig. 1A). However, when the Nanos 2 ORF is also fused in frame to the C terminus of the GFP in this construct surrounded by Nanos 2 5’UTR and ΔGNARLE 3’UTR, low levels of protein were detected throughout the somatic cells during early development, followed by selective retention only within the small micromeres (Fig. 1B). In situ hybridization indicates that both of these injected RNAs were present uniformly in embryos (Fig. 1C and D), suggesting that an ectopic expression of a Nanos 2 reporter mRNA does not lead to an ectopic expression of its protein. These data indicate that even though the expression of the endogenous Nanos 2 is regulated by differential mRNA stability as previously shown, the embryos use an additional, post-translational mechanism that maintains a high protein retention in the PGCs and a low protein level in the somatic cells.

Nanos protein expression was then tested using a luciferase assay for quantitation independent of confocal imaging (Fig. 1E). The Renilla luciferase ORF was used alone, or in frame with Nanos 2, and injected in Sp fertilized eggs. Both in vitro transcribed mRNAs were co-injected with a control RNA containing the Firefly luciferase ORF, flanked by the Xenopus β-globin 5’ and 3’UTRs. Both Rluc and Fluc activities were then measured at the mesenchyme blastula stage. Rluc was highly expressed and the corresponding ratio of Rluc/Fluc was set to 100%. In contrast, Rluc Nanos 2 gave a significantly lower luciferase activity, indicating that the Nanos 2 open reading frame itself does not alter accumulation or translation dynamics of the selective expression in the small micromeres. The regulation for selective reporter accumulation instead functions once the Nanos protein is translated. In contrast to the GFP protein that continued to accumulate during development (Supplemental Fig. S4), the GFP Nanos 2 protein is maintained at overall low levels in the soma but is highly enriched in the small micromeres.

3.2. Selective Nanos 2 protein stability is independent of the sumoylation and ubiquitination pathways

Protein modifications such as sumoylation (Geiss-Friedlander and Melchior, 2007; Johnson, 2004) or ubiquitination (Komander and Rape, 2012) could be respectively stabilizing Nanos 2 in the small micromeres, or degrading it in the non-small micromere cells. One gene coding for Sumo was found in the sea urchin (SPU_018833). By immunofluorescence, we found that the Sumo protein is enriched in the small micromeres in blastulæ (Fig. 2A, C and Supplemental Fig. S5), coincident with the selective accumulation of Nanos 2 protein. The sequence of the sumoylation E2-conjugating enzyme Ubc9 (SPU_005107) was also found in the Sp genome. In contrast to Sumo, the Ubc9 protein was found uniformly throughout development (Supplemental Fig. S6). In situ hybridizations using probes against Sumo and Ubc9 indicate that both transcripts are expressed throughout the embryo (Supplemental Fig. S7). To test if the enrichment of Nanos 2 in the small micromeres depends on Sumo expression, embryos were co-injected with the transcript coding for the GFP Nanos 2 protein, and a morpholino against Sumo. This morpholino significantly decreased the level of Sumo protein in the small micromeres at blastula stage (Supplemental Fig. S8), although it had no effect on the enrichment of GFP Nanos 2 in the small micromeres (Fig. 2F).

To test a potential role of Sumo and Ubiquitin on Nanos 2 expression, embryos were incubated with a sumoylation inhibitor, Ginkgolic acid (Fukuda et al., 2009), and the level of the endogenous Nanos 2 protein was analyzed by immunofluorescence. Inhibition of the sumoylation pathway also did not affect the enrichment of the endogenous Nanos protein in the small micromeres (data not shown) nor did inhibition of the ubiquitilation pathway with MG132 (Supplemental Fig. S9).

Both sumoylation and ubiquitilation occur on the lysines of the target proteins and seven lysines are present in the Nanos 2 protein. As another test for sumoylation or ubiquitilation on Nanos 2 enrichment in the small micromeres, five mutant constructs were crafted and injected (Fig. 3). Mutations of the first (Fig. 3A), the second (Fig. 3B), the third and fourth (Fig. 3C), the fifth (Fig. 3D), or mutations of the sixth and seventh lysines (Fig. 3E) did not affect the expression of GFP Nanos 2 in the small micromeres. Mutations of the seven lysines together led to an abnormally low expression of Nanos 2 protein throughout the embryo, perhaps as a result of misfolded protein structure that is quickly targeted for degradation in all cells (data not shown). Altogether, these results suggest that Nanos 2 enrichment in the small micromeres is not driven by protein modifications such as sumoylation or ubiquitilation.

3.3. Selective Nanos 2 accumulation is regulated by elements in its N-terminal region

To test which domain of the Nanos 2 ORF may be involved in enrichment of the protein in the small micromeres, each of the two zinc finger motifs were first mutated. It was previously shown that mutation of either the fourth cysteine of the first zinc-finger (C4), or the fourth cysteine of the second zinc-finger (C8) into a serine leads to a strong abdominal phenotype (Nanos-null)
Following a similar mutagenesis strategy for the GFP Nanos 2 protein, and then injecting the cognate synthetic transcripts of these sequences, we found that the Nanos 2 accumulation remained unchanged. The signal still remained enriched in the small micromeres (Fig. 4) relative to the wild type Nanos 2 protein and appeared independent of Nanos function.

To determine what parts of the Nanos protein may be responsible for the selective accumulation in the germ line, we employed the GFP fusion protein strategy. We constructed deletion mutants whose cognate mRNAs were injected after fertilization, and the absence or presence of the green fluorescence in the somatic cells was analyzed in blastulae. Interestingly, GFP fused to the first 68 amino acids of Sp Nanos 2 (N term 2) is specifically retained in the PGCs, similarly to Sp Nanos 2 full length (Figs. 5 and 7). However, the first 54 amino acids (N term 3) are not sufficient to degrade Sp Nanos 2 in the somatic cells. These results indicate that the first 68 amino acids of Sp Nanos 2 represent the minimal

in Drosophila embryos (Arrizabalaga and Lehmann, 1999). Following a similar mutagenesis strategy for the GFP Nanos 2 protein, and then injecting the cognate synthetic transcripts of these sequences, we found that the Nanos 2 accumulation remained unchanged. The signal still remained enriched in the small micromeres (Fig. 4) relative to the wild type Nanos 2 protein and appeared independent of Nanos function.

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region required for the selective accumulation of the protein in the PGCs. This element will be then referred as NPRE (Nanos Protein Retention Element).

Importantly, GFP fused to as little as 45 amino acids (amino acid 55-99) of Sp Nanos 2 (N term 4) is degraded in every cell of the blastula (Figs. 6, 7, and Supplemental Fig. S10). The protein is translated, and accumulates early in development throughout the embryo but became undetectable before reaching the blastula stage (Supplemental Fig. S11). In contrast, deletion of this element (N term 5) led to Nanos 2 stabilization, with the protein accumulating abundantly throughout the entire blastula. These results indicate that the N terminal domain of Sp Nanos 2 (amino acid 55-99) is necessary and sufficient for the degradation of the protein. This 45 amino acid element (MNKNMVSLGPVSTSQSTTASTGFMPQLPLNIAEITELSKMRG) will be then referred as NPDE (Nanos Protein Degradation Element). Interestingly, the NPDE leads to the degradation of Nanos 2 in both the somatic cells and the PGCs, showing the importance of the NPRE to prevent the degradation of the protein specifically in the PGCs. Several additional deletion mutants were tested; fusion proteins containing only the last 133

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amino acids (C term 1), were highly expressed in the entire blastula, just as seen with GFP alone (Supplemental Fig. S12 and S13). However, the construct N term 9 (amino acid 81–140; Supplemental Figs. S12 and S14), like the N term 2 (amino acid 1–68; Fig. 7) that do not contain the complete NPDE, were effectively degraded in the somatic cells, suggesting that the NPDE contains at least two independent functional motifs (amino acid 55–68, and 81–99). Altogether, these data suggest that multiple elements are present in the N terminal region of Nanos 2 to target its degradation in the soma, but to protect the protein from degradation in the PGCs.

3.4. Autonomous accumulation of Nanos 2 protein in the PGCs

It was previously shown that Nanos mRNA autonomously accumulates in the small micromere lineage after dissociation of 16 cell stage embryos and in vitro culture. Nanos transcription was similarly regulated in whole embryos and in isolated cells (Yajima and Wessel, 2012). To test if Nanos 2 protein also accumulates autonomously in the PGCs, the GFP Nanos 2 full length mRNA surrounded by Sp Nanos 2 5′ UTR and ΔGNARLE 3′UTRs was injected into fertilized eggs. The embryos were dissociated into single cells at 14 h, before Nanos 2 protein selectively accumulates in the small micromeres (Supplemental Fig. S4). At 20 h, the Nanos 2 protein was identified by immunofluorescence and found selectively and highly expressed in the PGCs (Fig. 8). Similar observations were obtained by directly observing the Nanos-GFP fluorescence in live dissociated cells (data not shown). Altogether these results indicate that Nanos 2 expression is autonomously regulated, not only at the transcription level as previously shown, but also at the protein stability level.

Fig. 6. Nanos 2 N terminal domain contains an element that causes its degradation at the blastula stage. Synthetic mRNAs containing the GFP ORF alone (D, H) or fused with the Nanos 2 full length ORF (A, E) were injected into Sp fertilized eggs. Deletion mutants were also injected: GFP fused to the NPDE (N term 4: B, F) and GFP fused to Nanos 2 ORF deleted in the NPDE (N term 5: C, G). In each case, the ORF was surrounded by Nanos 2 5′ and ΔGNARLE 3′UTRs. A control RNA containing mCherry flanked with β-globin UTRs was co-injected with each deletion construct. Images were taken at mesenchyme blastula stage. The GFP and mCherry fluorescence were imaged using respectively the same settings: GFP (A to H), and mCherry (A’ to H’) at 400 × magnification.
4. Discussion

The specific expression and function of Nanos in germ line development is seen widespread: in the planarian flatworms (Wang et al., 2007), the nematode Caenorhabditis elegans (Krämer et al., 1999; Subramaniam and Seydoux, 1999), Drosophila (Bhat, 1999; Forbes and Lehmann, 1998; Kobayashi et al., 1996; Sato et al., 2007), sea urchin (Juliano et al., 2010; Yajima and Wessel, 2011), zebrafish (Beer and Draper, 2013; Draper et al., 2007; Koprunner et al., 2001), Xenopus (Houston and King, 2000; Lai et al., 2012; Luo et al., 2011; Mosquera et al., 1993), mouse (Haraguchi et al., 2003; Saga, 2010; Tsuda et al., 2003), and human (Julaton and Reijo Pera, 2011; Wu et al., 2013). Loss of Nanos expression induces cell cycle and developmental defects resulting in the loss of PGCs through apoptosis (Forbes and Lehmann, 1998; Kobayashi et al., 1996; Koprunner et al., 2001; Sato et al., 2007; Tsuda et al., 2003). In contrast, ectopic expression of Nanos often leads to embryonic lethality (Luo et al., 2011). Furthermore, Nanos expression is also observed in cancer cells. A study in Drosophila showed that ectopic expression of germ line genes such as the orthologs of Piwi, Vasa,

Fig. 7. Summary of the expression of GFP Nanos 2 fusion proteins imaged at mesenchyme blastula stage. The names of the injected constructs are indicated on the left, the corresponding amino acids fused in frame to the C terminal of the GFP are presented by rectangles on the right. The number surrounded the rectangles indicate the amino acid present at the beginning of each construct. The location of the zinc finger motifs (CCHC) is shown in blue. Nanos 2 contains 7 lysines, their location is indicated by the small red circles, above the full length sequence. A schematic blastula at the end of the construct indicates where the corresponding GFP fluorescence was detected.

Fig. 8. The selective retention of Nanos 2 protein is autonomous. Synthetic mRNAs containing the GFP Nanos 2 full length ORF were injected in Sp fertilized eggs. The resulting early blastulae (14 h post fertilization), were dissociated into single cells and cultured for an additional 6 h. At 20 h, cells were fixed to test the presence or absence of Nanos protein in the PGCs (D) and the somatic cells (G). Vasa was used as a control to identify the PGCs (E,H). Undissociated mesenchyme blastulae were used as a control (A,B). Images were taken using respectively the same microscope settings (laser intensity, pin-hole opening) for (A,D,G), and for (B,E,H) at 400 × magnification. With Metamorph, the green fluorescence (Nanos expression) was quantified in 11 PGCs and 34 somatic cells (J). Significance was assessed with the use of Student t-test (P < 0.05).
Nanos and Aubergine contributed significantly to growth and survival of malignant brain tumors (Janic et al., 2010); inactivation of these germ cell genes even resulted in tumor regression. In humans, some genes that are predominantly expressed in germ-line cells become aberrantly activated in various malignancies, and these genes could be potential targets to treat tumors in humans (Wu and Ruvkun, 2010). Understanding the mechanisms regulating Nanos expression will address basic processes in germ-cell development and likely contribute more broadly than originally anticipated.

In the sea urchin embryo, we find that Nanos 2 protein accumulation depends on the UTRs of the transcript for mRNA stability (Oulhen et al., 2013), on translational regulation (Oulhen et al., 2013), and now on the selective stability of the protein in the sMics, post translationally. Mutations of either the zinc finger motifs present in the C terminal region of Nanos 2, or the 7 lysines, that could be targeted by Sumo or Ubiquitin, do not affect the stability of the protein in the germ line. However, the Nanos Protein Degradation Element (NPDE) present in the N terminal region of Nanos 2 destabilizes the protein in both the somatic cells and the PGCs. This element is well conserved between urchin species, the amino acid sequence of the NPDE in Sp is 93% identical to the corresponding sequence found in the urchin *Hemicentrotus pulcherimus* that diverged from Sp approximately 20 million years ago (Lee, 2003). Thus we conclude that the post-translational mechanism of selective Nanos protein accumulation and degradation is a broad phenomenon. The NPDE in post-translational regulation is probably recognized by one or multiple proteases starting between 12 and 13 h (blastula) until at least 18 h (mesenchyme blastula) after fertilization. Potential proteases for this region were predicted using PROSPER (Supplemental Fig. S15) and predicted protease sites were predicted within the two motifs identified functionally in the NPDE (either between the amino acids 55-68, or 81-99). Identification of the protease(s) that regulates Nanos 2 stability in the somatic cells is currently under investigation. The Nanos Protein Retention Element present in the N terminal region also appears required to protect the protein from degradation in the PGCs; this element could interact with one or more proteins specifically localized in the small micromeres to sterically block the binding or the activity of the protease responsible for Nanos 2 degradation. The NPDE and NRPE may also be useful technicall in that at least this embryo and its close relatives, one could target rapid turnover of proteins by attaching the NPDE, or enhance selective protein accumulation in the PGCs by attaching the NPRE.

Several results converge to indicate that the selective accumulation of Nanos 2 in the germ cells is controlled by post-translational mechanisms. First, the GFP is fused to the N terminal of Nanos 2 ORF, exempting a translational regulation. As shown in Xenopus, *Nanos 1* mRNA contains a Translation Control Element (TCE) downstream of its ATG (Luo et al., 2011). This repression happens when the Nanos 1 ORF is on the N terminal of the Myc ORF reporter, and does not occur when the Nanos 1 ORF is on the C terminus of the Myc ORF; the ribosomes presumably are already recruited to the mRNA by the Myc ORF and the full Myc Nanos ORF was translated despite the TCE. Secondly, Nanos 2 ORF does not seem to contain a consensus sequence or a structural element that could inhibit its translation. As demonstrated in Fig. 1H, the transcript containing the GFP ORF followed by a stop and an unframed Nanos 2 ORF can be translated in the whole blastula, suggesting that the sequence of the ORF does not inhibit translation, and that it’s the protein itself after being synthesized who is the target of this regulation. Thirdly, we see overall Nanos protein expression throughout the early embryo and then rapid degradation everywhere except in the germ line. Finally, we see the same results using luciferase as a reporter as well as multiple Nanos-deletion constructions to provide confidence in this conclusion of post-translational regulation.

These multiple control steps in Nanos accumulation emphasize its functional importance at many different levels. Recently it was shown in this embryo that Nanos regulates selective mRNA turnover in the egg – to – embryo transition. Nanos 2 accomplishes this feat by interacting with its partner Pumilio to degrade transcripts in the PGCs such as CNOT6, encoding a deadenylase that is responsible for clearing of the maternally stored mRNAs in the future somatic cells, while retaining these transcripts in the germ line (Swartz et al., 2014). Mis-regulation of CNOT6 by interfering with Nanos function is lethal. In *Drosophila*, the Nanos/Pumilio complex is known to control the translation of specific mRNAs like *cyclin B* (Asaoka-Taguchi et al., 1999). A common sequence motif is found in the 3’UTR of Pumilio mRNA targets, the consensus includes a highly conserved 8 nucleotide core motif: UGU(A/U)UUA (Gerber et al., 2006). We found that in at least two sea urchin species, *Strongylocentrotus purpuratus*, and *Lytechinus variegatus*, the *cyclin B* 3’UTR contains 7 out of these 8 highly conserved nucleotides (Supplemental Fig. S16A). Using a luciferase assay, we also found that Nanos 2 specifically inhibits the translation of the RNA containing the *cyclin B* 3’UTR (Supplemental Fig. S16B). Together, these results demonstrate that the restriction of Nanos 2 protein to the PGCs is indeed essential to prevent degradation of transcripts such as CNOT6 or *cyclin B* in somatic cells.

Vasa protein is also enriched in the sMics post-translationally (Gustafson et al., 2011; Voronina et al., 2008). This protein appears to be rapidly degraded in the somatic cells, but is retained in the sMics by differential ubiquitylation mechanisms. Thus, we surmised that the Nanos 2 protein may be regulated by a similar strategy that might form a unifying concept of germ cell selection. However, having deleted each of the lysines in Nanos individually or in combination, and use of inhibitors of ubiquitylation suggests that ubiquitylation is unlikely a mechanism in the regulation of Nanos 2 selective accumulation. We did however discover that Sumo is enriched in the germ cell lineage in sea urchin. It was previously reported in *Drosophila* that transcripts of five genes involved in the sumoylation pathway were found throughout the early embryo, and then predominantly expressed in the nuclei of pole cells in late stage embryos and then in gametogenic cells (Hashiyama et al., 2009). In the green mud crab, results suggest that Sumo-1 may have special import during gametogenesis. Sumo-1 was shown to be more abundantly expressed in the crab ovary than in other tissues, and it was also expressed in testis development (Dai et al., 2012). Although the role of Sumo is not well understood, studies with mutant animals have demonstrated that the sumoylation pathway is also critical for embryonic development (Lomeli and Vazquez, 2011). For example in C. elegans, *Sumo-1* is required for germline development and the authors described the transcription factor Lin-11 as a substrate for sumoylation (Broday et al., 2004). Sumoylation of transcription factors has been correlated with inhibition of transcription (Gill, 2005) and evidence also suggests that Sumo has multiple roles in chromatin regulation (Sriakumar et al., 2013). Sumoylation of histones appears to mediate gene silencing through recruitment of histone deacetylase and heterochromatin protein 1 (Shiio and Eisenman, 2003). In most organisms, the specification of the germ line depends on mechanisms that also inhibit the expression of somatic genes (Seydoux and Braun, 2006). Thus, the enrichment of Sumo in the small micromere lineage could be crucial to inhibit transcription in the PGCs, especially when considering that these cells appear to be transcriptionally silent (Swartz et al., 2014).
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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.ydbio.2016.07.007.

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