The 3′UTR of \textit{nanos2} directs enrichment in the germ cell lineage of the sea urchin

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**A B S T R A C T**

Nanos is a translational regulator required for the survival and maintenance of primordial germ cells during embryogenesis. Three nanos homologs are present in the genome of the sea urchin \textit{Strongylocentrotus purpuratus} (Sp), and each nanos mRNA accumulates specifically in the small micromere (sMic) lineage. We found that a highly conserved element in the 3′ UTR of \textit{nanos2} is sufficient for reporter expression selectively in the sMic lineage: microinjection into a Sp fertilized egg of an RNA that contains the GFP open reading frame followed by Sp \textit{nanos2} 3′ UTR leads to selective reporter enrichment in the small micromeres in blastulae. The same result was seen with \textit{nanos2} from the sea urchin \textit{Hemicentrotus pulcherrimus} (Hp). In both species, the 5′ UTR alone is not sufficient for the sMic localization but it always increased the sMic reporter enrichment when present with the 3′ UTR. We defined an element conserved between \textit{Hp} and \textit{Sp} in the \textit{nanos2} 3′ UTR which is necessary and sufficient for protein enrichment in the sMic, and refer to it as GNARLE (Global Nanos Associated RNA Lability Element). We also found that the \textit{nanos2} 3′ UTR is essential for the selective RNA retention in the small micromeres; GNARLE is required but not sufficient for this process. These results show that a combination of selective RNA retention and translational control mechanisms instills nanos accumulation uniquely in the sMic lineage.

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**Introduction**

Nanos is a RNA-binding protein containing two CCHC zinc-fingers, and was first described as a translational repressor in \textit{Drosophila} (Cho et al., 2006; Irish et al., 1989). Although the nanos sequence is not highly conserved, nanos orthologs have been found in the germ line of all animals tested (e.g. \textit{C. elegans} (Kraemer et al., 1999), \textit{Xenopus} (Lai et al., 2011) and planarians (Wang et al., 2007)). Translational repression by nanos is mediated through interaction with pumilio, which binds RNAs containing a conserved motif in their 3′ UTR, the Nanos Response Element (NRE); (Sonoda and Wharton, 1999; Wharton and Struhl, 1991). This function of nanos is involved in the regulation of various developmental processes; it was first characterized in \textit{Drosophila} to regulate the differentiation of the anterior–posterior body axis through translational repression of the gap gene hunchback (Wang and Lehmann, 1991), and later shown to be required also for the continued production of egg chambers during oogenesis (Wang et al., 1994) and for primordial germ cell migration (Forbes and Lehmann, 1998). Nanos is required in both the male and female germ line of \textit{Drosophila}; in the nanos mutant males, spermatogenesis is progressively affected and these males become sterile (Bhat, 1999). Similarly, nanos regulates primordial germ cell development and survival in \textit{C. elegans} (Subramaniam and Seydoux, 1999), sea urchins (Juliano et al., 2010), zebrafish (Koprunner et al., 2001) and mice (Tsuda et al., 2003). In addition to these conserved functions in the germ line, nanos also functions in other multipotent cells. For example, the nanos related gene \textit{Cnos1} in \textit{Hydra magmipapillata}, is expressed in both multipotent stem cells and germ-line cells, but not in somatic cells (Mochizuki et al., 2000). In the polychaete annelid, \textit{Platyneris dumerillii}, and the snail \textit{Ilyanassa obsoleta}, multipotent cells of the embryos expressed nanos (Rabinowitz et al., 2008; Rebscher et al., 2007). Moreover, nanos has other functions in development e.g. in the \textit{Drosophila} peripheral nervous system, in the dendritic arborization (da) neurons to maintain dendrite complexity (Brechbhel and Gavis, 2008), and at the larval neuromuscular junction (Menon et al., 2009).

The expression of germ-line determination genes is highly regulated, and ectopic expression of these genes often induces cell
cycle and developmental defects (Luo et al., 2011; Wu and Ruvkun, 2010). In Drosophila, C. elegans, zebrafish, and mouse, translation of nanos in the germ line requires its 3′ Untranslated Region (UTR) (D’Agostino et al., 2006; Gavis et al., 1996b; Koprunner et al., 2001; Saito et al., 2006; Suzuki et al., 2010). In Drosophila, the nanos 3′UTR mediates its RNA localization to the posterior pole of the syncytial embryo (Gavis et al., 1996a) and the protein rumpelstiltskin (rump), a heterogeneous nuclear ribonucleoprotein (hnRNP), binds nanos RNA directly to regulate its localization (Jain and Gavis, 2008). More recently, the argonaute family member,aubergine (aub) was also found to be a nanos RNA localization factor, independent of its function in RNA silencing. Aub interacts with nanos mRNA in vivo and co-purifies with rump in an RNA-dependent manner (Becalska et al., 2011). This nanos RNA localization element includes a 90 nucleotide translational control element (TCE) which mediates its translational repression (Gavis et al., 1996b) by forming two stem-loops which act independently of each other to repress translation at different times in development. Smaug and Glorund bind to each of the stem loop to control its translation, respectively, during oogenesis (Kalifa et al., 2006) and embryogenesis (Smibert et al., 2006). Similarly, in C. elegans, nanos2 is translationally regulated by two independent stem loops in its 3′UTR (D’Agostino et al., 2006). Thus, nanos protein translation is highly safeguarded and may reflect its toxicity outside of the germ line, or multipotent cell environment.

Three nanos homologs are present in the genome of the sea urchin, Strongylocentrotus purpuratus (Sp), and each of them are expressed with differential timing in the small micromeres (Juliano et al., 2010), cells that contribute to the germ line (Yajima and Wessel, 2006). These cells are formed during embryogenesis at the 32-cell stage after two unequal cleavage divisions. In the blastula, the small micromeres reside at the vegetal plate where they divide once before being transported to the tip of the archenteron during gastrulation. The eight small micromere descendants are then partitioned into the left and right coelomic pouches, with the adult rudiment forming on the left side in the larva. Nanos1 and nanos2 are the first de novo mRNAs and proteins to accumulate in the sMics, and in both S. purpuratus and Hemicentrotus pulcherrimus (Hp), are required for adult rudiment formation (Fuji et al., 2009). In this study, we found that at least part of the mechanism for nanos2 RNA selective accumulation in the sMics stems from a post-transcriptional step of rapid mRNA turnover in all cells of the embryo, except the sMics, and that this essential information results from an RNA element in the 3′UTR of the mRNA that is highly conserved over the ~20 million years separating the last common ancestor of S. purpuratus and H. pulcherrimus. This element leads to mRNA turnover in all cells except the sMics, and is independent of miRNA-mediated decay.

Plasmid constructions

For the GFP construct with Hp nanos2 UTRs (Full length), Hp nanos2 5′ and 3′UTRs were amplified using the primers described in Supplementary Fig. S1A (Fuji et al., 2006). These Hp nanos2 UTRs were subcloned into pGreenLantern2-derived plasmid containing the GFP open reading frame and the T7 promoter. For 3′ deletions, Hp nanos2 3′UTR was amplified from the full length using the primers presented in Supplementary Fig. S1B. For 5′ deletions, Hp nanos2 3′UTR was amplified using the primers described in Supplementary Fig. S1C. For constructs A–C, Hp nanos2 3′UTR A–C regions were amplified using the primer sets (primers Δ7 F and Δ4 R to amplify region A; primers Δ8 F and Δ4 R to amplify region B; primers Δ7 F and Δ5 R to amplify region C). These PCR products were digested with XbaI and SalI, and inserted into the corresponding sites. For internal deletions of Hp nanos2 3′UTR, inverse PCR was carried out with 5′3′UTR-GFP using the primer sets presented in Supplementary Fig. S1D.

For the GFP constructs, Sp nanos2 5′ and 3′UTRs were amplified using the primers described in Supplementary Fig. S2A (Juliano et al., 2010). These Sp nanos2 UTRs were subcloned in a plasmid containing the GFP open reading frame, and the T7 promoter. Sp nanos2 3′UTR GNARLE region was amplified using the primers presented in Supplementary Fig. S2B, and inserted in the GFP containing plasmid. To make the Sp nanos2 3′UTR ΔGNARLE construct, two EcoRI restriction site were introduced in the UTR, at the beginning and at the end of GNARLE using the primers described in Supplementary Fig. S2C. Mutations were made using the QuickChange II Site-directed mutagenesis kit (Agilent Technologies, Santa Clara, CA). The plasmid was then digested by EcoRI to remove GNARLE, and then ligated. Sp nanos2 3′UTR NRRE was amplified using the primers presented in Supplementary Fig. S2D.

For the Renilla luciferase constructs, Sp nanos2 5′ and 3′UTRs were amplified (Supplementary Fig. S2E). These UTRs were then subcloned in a plasmid containing the Renilla luciferase open reading frame, and a SP6 promoter. The Sp nanos2 3′UTR NRRE and ΔGNARLE were amplified from the corresponding GFP construct described above (Supplementary Fig. S2E).

In vitro RNA synthesis

Capped sense RNAs were 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 contain: 20% glycerol, 1 × 10^12 copies of a GFP RNA, 1.10^12 copies of the mCherry RNA. Approximately 2 pl of each RNA mixture was injected into each fertilized egg.

Morpholino approach

The morpholino against dicer 5′ GGACTCGATGGTGGCTCATC- CATTC 3′ was previously described (Song et al., 2011). Each embryo received approximately 24 nM of the dicer morpholino.

Microinjections

Microinjections of zygotes were performed as previously described (Cheers and Ettensohn, 2004). In brief, eggs were de-jellied 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 (Mashhi; Tokyo, Japan) were...
pulled on a vertical needle puller for injections (Narishige; Tokyo, Japan). Injected embryos were cultured in sea water at 16 °C.

Whole mount RNA in situ hybridization (WMISH)

Antisense DIG-labeled RNA probes against GFP and mCherry 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 1 h. All steps were carried out in 96-well round-bottom PVC plates (ThermoFisher Scientific; Rockford, IL). Samples were imaged on a Zeiss Axiovert 200 M microscope equipped with a Zeiss color AxioCam MRc5 camera (Carl Zeiss, Inc.; Thornwood, NY).

Real-time quantitative PCR (QPCR)

RNA was extracted from 100 mock-injected and 100 Sp-nanos morpholino-injected embryos, collected at 24 hpf, using the RNeasy Micro Kit (Qiagen; Valencia, CA). cDNA was prepared using the TaqMan Reverse Transcription Reagents kit (Applied Biosystems; Foster City, CA). QPCR was performed on the 7300 Real-Time PCR system (Applied Biosystems; Foster City, CA) with the SYBR Green PCR Master Mix Kit (Applied Biosystems; Foster City, CA). Sp-nanos 2 primer set is described in (Juliano et al., 2006): F (5'-GCAAGAACAACGGAGAGGC-3') and R (5'-CCGCA-TAATGGACAGGTGTA-3'). 4 embryo equivalents were used as template. Experiments were run in triplicate and the data were normalized to ubiquitin RNA levels.

Reporter fluorescence

Injected embryos were cultured as described above and samples were collected at indicated stages of development. Embryos were imaged on an LSM 510 laser scanning confocal microscope (Carl Zeiss, Inc.; Thornwood, NY).

Dual luciferase assay

Strongylocentrotus purpuratus fertilized eggs were injected, as described above, with a solution containing 1 × 10^12 copies of a Renilla luciferase RNA, 1 × 10^12 copies of a Firefly luciferase 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).

Results

The nanos2 3'UTR directs selective protein accumulation in the small micromeres.

Transcript and protein of nanos2 accumulate selectively in the small micromere lineage in two sea urchin species, H. pulcherrimus (Hp) and S. purpuratus (Sp) (Fujii et al., 2009; Juliano et al., 2010). In analyzing these mRNAs we found a region of the 3'UTR that was highly conserved between these two species. To test if the nanos2 3'UTRs were involved in this selective accumulation, two constructs were developed, both contained the Sp nanos2 5'UTR and GFP open reading frame (ORF). The Sp nanos2 3'UTR was fused to the first one, and the Xenopus β-globin 3'UTR was fused to the second one (Fig. 1). The corresponding RNAs were in vitro transcribed, and injected into Sp fertilized eggs. An RNA comprising mCherry ORF flanked by Xenopus β-globin UTRs was co-injected as a control reporter. At the mesenchyme blastula (MB) stage (approximately 24 h post fertilization), the mCherry protein was found in all cells. In contrast, the co-injected GFP-encoding fluorescence was enriched in the small micromeres when it contained the Sp nanos2 5' and 3'UTRs. The GFP signal resulting from the Xenopus β-globin 3'UTR RNA, however, accumulated uniformly in all cells. The same observations were made by injecting Hp constructs in Hemicentrotus pulcherimus fertilized eggs (data not shown). These results indicate that the nanos2 UTRs are required for the selective translation of the encoded protein in the small micromeres.

A 357 nucleotide element located in the nanos2 3'UTR is sufficient for selective reporter accumulation in the small micromeres.

To test which part of the nanos2 3'UTR is important for the selective protein expression, ten deletion mutants were made using the Hp nanos2. All constructs contained the Hp nanos2 5'UTR, followed by GFP, and then by different regions of Hp nanos2 3'UTR. RNAs were injected in Hp fertilized eggs, and the GFP signal was monitored at the blastula stage (Fig. 2). In Hp, the full length nanos2 3'UTR is 1051 nucleotide long, from nucleotide position 739 of the nanos2 transcript to 1790. Of the embryos injected with the RNA
comprising the full length Hp nanos2 3’UTR, 74.7% had a selective GFP signal in the small micromeres at the blastula stage (Fig. 2 Full length). Similar percentages were obtained with three 3 deletions (Δ1/2/3), and with one 5 deletion (Δ7), demonstrating that the last 396 nucleotides and the first 120 (859–739) nucleotides of Hp nanos2 3’UTRs were expendable for the selective protein expression. This percentage progressively decreased after injection of Δ4, Δ5, Δ6 and reached 0% in the Δ9 mutant. These results show that the sequence located between nucleotides 859 and 1236 in Hp nanos2 3’UTR is essential for the selective protein expression in the small micromeres. Moreover, the RNA with a 3’UTR located between 859 and 1257, was sufficient to give a selective GFP expression in any of the injected embryos. Altogether, these results indicate that nucleotides 880 to 1236 represent the element required for selective protein expression in the small micromeres. This element will be noted GNARLE in the following experiments: Global Nanos Associated RNA Lability Element.

GNARLE is conserved, necessary, and sufficient for selective protein expression in the small micromeres.

A sequence of 388 nucleotides in the 3’UTR of the nanos2 transcript from Sp aligned closely with the Hp GNARLE (80% identity: Fig. 4). GNARLE contains two highly conserved regions, highlighted in gray, which shares 92 and 82% nucleotide identity between Hp and Sp, greater even than the coding region of nanos between these species, whereas regions of the 3’UTR flanking GNARLE are not conserved between these species. To test if Sp nanos GNARLE is involved in the protein accumulation in the small micromeres, we injected RNAs containing Sp nanos2 5’UTR, the GFP ORF and Sp nanos2 3’UTR either full length, GNARLE only, or deleted in GNARLE (ΔGNARLE) (Fig. 5). The GFP protein produced after injection of the full length Sp nanos2 3’UTR RNA was strongly enriched in the small micromeres (Fig. 5A). Interestingly, the Sp nanos2 GNARLE RNA gave a similar GFP enrichment in the small micromeres (Fig. 5B), in contrast to the Sp nanos2 ΔGNARLE RNA in which GFP fluorescence was detected at a strong level in all cells (Fig. 5C). Similar results were obtained after injection of the Hp-derived RNA in Sp (Fig. S3) although the selectivity of with or without the GNARLE was not as great as seen in Sp. Perhaps this minor enrichment difference reflects an importance in the regions of sequence for which differences between the species occur. Overall however, these results suggest that the GNARLE is necessary and sufficient for selective protein enrichment in the small micromeres in the sea urchin Sp.

Moreover, the RNA containing Sp nanos2 5’UTR and Xenopus β-globin 3’UTR demonstrates that the 5’UTR by itself is not sufficient to give a protein enrichment in the small micromeres (Fig. 5D). Of note here is that an RNA containing Xenopus β-globin 5’UTR and Sp nanos2 3’UTR did not give any detectable fluorescence using the same

Fig. 2. A proximal element in the Hp nanos2 3’UTR is required for selective protein accumulation in the small micromeres. Synthetic mRNAs were made using Hp nanos2 5’UTR and GFP followed by different deletions of Hp nanos2 3’UTR. These RNAs were injected into Hp fertilized eggs, and the number of injected embryos having a GFP signal enriched in the small micromeres at the blastula stage was monitored under the fluorescence scope. N indicates the number of injected embryos used for each RNA construct. N sMic indicates the number of injected embryos having a protein enrichment in the small micromeres, the corresponding percentages are represented in the parentheses (%).

Fig. 3. The nucleotides localized between the position 880 and 1236 of Hp nanos2 3’UTR are essential for protein enrichment in the small micromeres. Synthetic mRNAs were made using Hp nanos2 5’UTR and the GFP ORF followed by different deletions of Hp nanos2 3’UTR (A and B). These RNAs were injected in Hp fertilized eggs, and the number of injected embryos having a protein signal enriched in the small micromeres at the blastula stage was monitored under the fluorescence microscope. N indicates the number of injected embryos used for each RNA construct. N sMic indicates the number of injected embryos having protein enrichment in the small micromeres, the corresponding percentages are represented in parentheses (%).

Fig. 4. (A) Schematic representation of nanos2 RNA, indicating the location of GNARLE in the 3’UTR. (B) Alignment of Hp and Sp nanos2 GNARLE using clustalW2. The stars below the sequences represent the identical nucleotides between Hp and Sp. The highly conserved regions are highlighted in gray.
detection parameters on the microscope as used for adjacent experiments (Fig. 5I). Nevertheless, by increasing the detection sensitivity, a GFP signal was still found enriched in the small micromeres (Fig. 5L), in contrast to the GFP signal obtained everywhere after injection of the GFP RNA containing the *Xenopus* β-globin 5′ and 3′ UTRs (Fig. 5J and M). These results indicate that Sp nanos2 5′UTR plays a substantial role in stimulation of protein synthesis.

Protein synthesis obtained with Sp nanos2 UTRs was tested at the same time using a luciferase assay (Fig. 6). Sp fertilized eggs were injected with RNAs containing Sp nanos2 5′UTR followed by the Renilla luciferase (Rluc) ORF. Three 3′UTRs were tested: Sp nanos2 3′UTR full length, the GNARLE alone, or the 3′UTR ΔGNARLE. At the mesenchyme blastula stage, a high level of Rluc was measured after injection of the ΔGNARLE RNA, and this ratio was set to 100%. In contrast, RNAs containing Sp nanos2 3′UTR full length or the GNARLE only, gave respectively an activity of 56% and 52%. Moreover, the control Rluc RNA, containing the *Xenopus* β-globin 5′ and 3′ UTRs, only gave an activity of 6%. First, these results indicate that there is more Rluc produced when the injected RNA does not contain the GNARLE. Secondly, the protein

Fig. 5. Sp GNARLE is necessary and sufficient for selective protein accumulation in the small micromeres. Synthetic mRNAs containing GFP open reading frame were injected in Sp fertilized eggs. The GFP ORF was preceded by either (A–D) Sp nanos2 5′UTR or (I and J) *Xenopus* β-globin 5′UTR, and followed by either (A, I and L) Sp nanos2 full length 3′UTR, (B) Sp nanos2 GNARLE 3′UTR, (C) Sp nanos2 ΔGNARLE 3′UTR, or (D, J and M) *Xenopus* β-globin 3′UTR. Uninjected embryos (K, N and Q) were used as a control. Each GFP mRNA was co-injected with a mRNA containing mCherry ORF (E, F, G, H, O and P) flanked by *Xenopus* β-globin 5′ and 3′ UTRs. GFP (green) and mCherry (red) fluorescence were assayed in the same embryos 24 h post-fertilization at mesenchyme blastula following microinjection of synthetic mRNAs. For GFP images, A, B, C, D, I, J, and K were obtained using the same settings (laser intensity, pin-hole opening) at the microscope. L and M were obtained by increasing the laser intensity on the embryos shown in I and J respectively. For mCherry images, all the pictures were also taken using the same settings (E, F, G, H, O, P and Q). bg indicates β-globin UTRs. Approximately one hundred blastulas were visualized after injection of each construct, the corresponding percentages of representative embryos are indicated in the right corner.
The 3' UTR of nanos2 also directs selective RNA retention in the small micromeres

Endogenous nanos2 transcripts are localized in the small micromeres in these 2 sea urchin species, H. pulcherrimus and S. purpuratus (Fujii et al., 2009; Juliano et al., 2010). Preliminary results of transcriptional regulation of the nanos2 gene suggest that nanos2 is more broadly transcribed in the embryo (unpublished observations, M. Yajima), suggesting that a post-transcriptional regulation is required to prevent nanos2 expression outside of the small micromeres. To test if nanos2 UTRs are required for the localization of nanos2 transcript in the small micromeres, we injected two RNAs containing the GFP ORF flanked by either Sp or Hp nanos2 5' and 3' UTRs (Fig. 7A). In contrast to the β-globin control RNA which is detected everywhere, the RNAs containing nanos2 UTRs from Sp or Hp were both found selectively in the small micromeres at the MB stage. Analysis of earlier stages of development using the Sp nanos2 UTR sequence indicated that the injected RNA is present in every cell during the first few hours after fertilization (Fig. S4), and then is degraded in the non-small micromere cells; i.e. the RNA is protected from degradation in the small micromeres. These results suggest that the nanos2 UTRs are regulated in the small micromeres differently than in other cells of the embryo.

We next tested if the 5' and 3' UTR of nanos2 are required for its RNA retention in the small micromeres, or if only one of them was sufficient. To test separately nanos2 5'UTR and 3'UTRs, we made two constructs for each sea urchin species: the first one containing nanos2 5'UTR followed by GFP ORF and Xenopus β-globin 3'UTR, and the second one containing the Xenopus β-globin 5'UTR followed by the GFP ORF and nanos2 3'UTR (Fig. 7B). Only the RNAs containing nanos2 3' UTR are retained selectively in the small micromeres at the MB stage. RNAs containing only nanos2 5'UTR were found everywhere, similar to the control and the Sp-derived and Hp-derived constructs gave similar results. These results show that in sea urchin, nanos2 3'UTR is sufficient for the early and selective retention of the RNA in the small micromeres.

The GNARLE is required but not sufficient for the selective RNA retention in small micromeres.

To test if the GNARLE involved in the selective protein expression is also essential for RNA retention in the small micromeres, we injected Sp fertilized eggs with RNAs containing nanos2 5'UTR, GFP ORF followed by the nanos2 3'UTR full length, the GNARLE only or the 3'UTR ΔGNARLE. RNA retention was monitored by WMISH (Fig. 8A) and similar results were obtained using Sp nanos2 or Hp nanos2. The RNA which contains only the GNARLE did not give a strong RNA retention in the small micromeres but is still selectively retained in the small micromere cells. In contrast, the RNA ΔGNARLE is more stable than the RNA containing the full length 3'UTR or the GNARLE, and is present at a high level in the entire blastula. These results suggest that the GNARLE is required for the retention of nanos2 RNA in the small micromeres, but is not as effective as the full length 3'UTR in this phenotype.

To better define the element sufficient for the selective RNA retention in the small micromeres, we arbitrarily made several constructs containing GNARLE and its surrounding nucleotides. After RNA injection followed by WMISH, we identified a minimal sequence: the NRRE for Nanos2 RNA Retention Element. This sequence of Sp nanos2 3'UTR includes 55 nucleotides before and 69 nucleotides after the GNARLE (Figure S5). The RNA retention obtained after injection of RNAs containing the Sp nanos2 5'UTR, followed by the GFP ORF and different parts of Sp nanos2 3'UTR: the full length, the NRRE or the GNARLE is presented in Fig. 8B. As previously described in Fig. 8A, the RNA containing the full length 3'UTR gave a strong selective RNA retention in the small micromeres, in contrast to the GNARLE which only gave a partial RNA retention. Interestingly, the NRRE RNA gave a pattern very similar to the one obtained with the full length 3'UTR. These results indicate that the NRRE is the element important for RNA retention in small micromeres.

Discussion

Nanos has a dominant function in cells to repress translation of mRNAs containing a Nanos Response Element (NRE). The best example of NRE in cells is in the cyclin B mRNA, and in this way, primordial germ cells are slow to divide during embryogenesis (Asaoka-Taguchi et al., 1999). Only after reaching the gonad and depleting the nanos protein do the germ cells begin to divide more rapidly. Ectopic nanos expression has a detrimental effect in embryos (e.g. Luo et al., 2011) and it is not surprising then that the nanos gene is carefully regulated at every level. Here we show a dramatic regulatory step at retention of the mRNA. We found that nanos2 3' UTR contains a Global Nanos Associated RNA Lability Element (GNARLE), which is required for selective RNA retention and protein enrichment in the small micromeres. We find this functionality conserved in two sea urchin species, Strongylocentrotus purpuratus and Hemicentrotus pulcherinus, which diverged less than 20 million years ago (Lee, 2003).
The GNARLE is required for RNA retention in the small micromeres and its deletion leads to a high stabilization of the injected RNA in the entire embryo, meaning that this element is required for the degradation of the RNA in the non-small micromere cells. In terms of nanos regulation, mRNA retention and translation globally would be detrimental since the cyclin mRNAs needed for rapid embryo cell division would be repressed by nanos and its ubiquitous pumilio partner protein (in preparation). On the other hand, the GNARLE by itself is not sufficient for optimal RNA retention in the small micromeres, suggesting that the surrounding nucleotides defined in the NRRE are important to protect the RNA from degradation in the small micromeres. A combination of degradation in the non-small micromeres, and protection in the small micromeres, seems to be required for this strong selective RNA retention. Moreover, even if the GNARLE does not give a strong selective RNA retention, this element is sufficient for high protein enrichment in the small micromeres, suggesting a role of the GNARLE in inhibiting the translation in the non-small micromeres, and/or stimulating the translation in the small micromeres. Therefore, the GNARLE plays a role in both promoting selective RNA retention and translational activity of nanos in the small micromeres.

Several overlapping mechanisms may contribute to selective nanos protein accumulation. The GNARLE may interact specifically with one or several proteins involved in RNA stability and/or protein translation. This element could be binding, directly or indirectly, proteins such as nucleases, or deadenylases to destabilize the transcripts in the non-small micromere cells, and proteins which could inhibit translation of nanos in the non-small micromere cells. Moreover, in various organisms, the nanos 3'UTR is also known to be regulated by the binding of miRNAs and piRNAs in its 3'UTR. During zebrafish embryogenesis, nanos expression is required for the germ-line development, and is restricted to the primordial germ cells. MicroRNA-430 targets the 3'UTR of nanos1 RNA to reduce its polyA tail length, its stability and its translation in the somatic cells (Mishima et al., 2006; Takeda et al., 2009). In Drosophila, nanos is expressed as a gradient that emanates from the posterior pole of the embryos. The piRNAs pathway was shown to be required for nanos mRNA deadenylation and decay as well as translational repression in Drosophila embryos (Rouget et al., 2010). Aubergine, one of the argonaute proteins in the piRNA pathway, is present in a complex with the RNA binding protein smaug, the deadenylase CCR4, nanos mRNA and piRNAs that target nanos 3’UTR. Small RNAs from different developmental stages of the sea urchin S. purpuratus embryos were recently identified (Song et al., 2011). However, perturbation of the miRNA formation by injection of a Dicer morpholino in Sp embryos did not affect the level

The GNARLE is required for RNA retention in the small micromeres and its deletion leads to a high stabilization of the injected RNA in the entire embryo, meaning that this element is required for the degradation of the RNA in the non-small micromere cells. In terms of nanos regulation, mRNA retention and translation globally would be detrimental since the cyclin mRNAs needed for rapid embryo cell division would be repressed by nanos and its ubiquitous pumilio partner protein (in preparation). On the other hand, the GNARLE by itself is not sufficient for optimal RNA retention in the small micromeres, suggesting that the surrounding nucleotides defined in the NRRE are important to protect the RNA from degradation in the small micromeres. A combination of degradation in the non-small micromeres, and protection in the small micromeres, seems to be required for this strong selective RNA retention. Moreover, even if the GNARLE does not give a strong selective RNA retention, this element is sufficient for high protein enrichment in the small micromeres, suggesting a role of the GNARLE in inhibiting the translation in the non-small micromeres, and/or stimulating the translation in the small micromeres. Therefore, the GNARLE plays a role in both promoting selective RNA retention and translational activity of nanos in the small micromeres.

Several overlapping mechanisms may contribute to selective nanos protein accumulation. The GNARLE may interact specifically with one or several proteins involved in RNA stability and/or protein translation. This element could be binding, directly or indirectly, proteins such as nucleases, or deadenylases to destabilize the transcripts in the non-small micromere cells, and proteins which could inhibit translation of nanos in the non-small micromere cells. Moreover, in various organisms, the nanos 3'UTR is also known to be regulated by the binding of miRNAs and piRNAs in its 3'UTR. During zebrafish embryogenesis, nanos expression is required for the germ-line development, and is restricted to the primordial germ cells. MicroRNA-430 targets the 3'UTR of nanos1 RNA to reduce its polyA tail length, its stability and its translation in the somatic cells (Mishima et al., 2006; Takeda et al., 2009). In Drosophila, nanos is expressed as a gradient that emanates from the posterior pole of the embryos. The piRNAs pathway was shown to be required for nanos mRNA deadenylation and decay as well as translational repression in Drosophila embryos (Rouget et al., 2010). Aubergine, one of the argonaute proteins in the piRNA pathway, is present in a complex with the RNA binding protein smaug, the deadenylase CCR4, nanos mRNA and piRNAs that target nanos 3’UTR. Small RNAs from different developmental stages of the sea urchin S. purpuratus embryos were recently identified (Song et al., 2011). However, perturbation of the miRNA formation by injection of a Dicer morpholino in Sp embryos did not affect the level
nanos2 transcript significantly, indicating that miRNAs are not responsible for the decay of nanos2 RNA outside of the small micromeres (Fig. S6).

Our results show that Sp nanos2 5'UTR is not sufficient for selective RNA retention or protein enrichment but it strongly increases the level of protein synthesis compared to the Xenopus β-globin 5'UTR. This strong expression is independent of the 3'UTR used. The sea urchin translational machinery might be recognizing and scanning more efficiently the Sp nanos2 5'UTR than Xenopus β-globin 5'UTR which is used as a control in RNA injection experiments. Sp nanos2 5'UTR seems to be important to increase protein translation and could also be a useful tool to over-express protein after RNA injection in sea urchin embryos.

In many cases, translation is controlled by cis-regulatory sequences within the 5' and 3'UTRs of the transcripts (Chatterjee and Pal, 2009), but the open reading frames could also be important to regulate gene expression. For example, in Xenopus, nanos (Xcat2) is transcribed during early oogenesis and becomes localized to the germ plasm, a subcellular compartment bearing the germ cell determinants. nanos RNA localization depends on a cis-acting element within its 3'UTR (Kloc et al., 2000). Recently, a new mechanism was discovered to be regulating nanos translation in Xenopus oocytes. Nanos contain an RNA secondary structural element immediately downstream of the AUG start site which is both necessary and sufficient to prevent ribosome scanning during oogenesis (Luo et al., 2011). This inhibition could be relieved after fertilization by a developmentally regulated activator to allow the ribosome loading. The nanos2 open reading frame was not included in the injected RNAs used in this study, but its function in nanos2 expression is currently under investigations.

We show here that in the sea urchin, nanos2 3'UTR is sufficient for its selective protein enrichment. This study provides new insights on how gene expression is regulated in the sea urchin small micromeres. Another well-known protein selectively expressed in the sea urchin small micromeres is vasa, a DEAD box RNA helicase. In early development, vasa mRNA is present uniformly throughout all cells of the embryos, but vasa protein accumulates selectively in the small micromeres (Voronina et al., 2008). It was shown recently that vasa coding sequence is sufficient for its selective enrichment. The E3 ubiquitin ligase, gustavus, binds vasa to induce its proteolysis in all cells except the small micromeres (Gustafson et al., 2011). Thus, regulation of the various germ line components necessary to direct...
this cell type to contribute to the germ line already has multiple different pre- and post-transcriptional regulatory mechanisms essential for the process. Indeed, use of these many regulatory steps may in itself be a selection mechanism for culling cells deficient in any one of a diverse set of determination pathways.

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Appendix A. Supporting information

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References


