



The 3'UTR of *nanos2* directs enrichment in the germ cell lineage of the sea urchin

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ABSTRACT

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 *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 *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 nanos2* 3'UTR leads to selective reporter enrichment in the small micromeres in blastulae. The same result was seen with *nanos2* from the sea urchin *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 *Hp* and *Sp* in the *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 *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 *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. *C. elegans* (Kraemer et al., 1999), *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 *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 *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 *C. elegans* (Subramaniam and Seydoux, 1999), sea urchins (Juliano et al., 2010), zebrafish (Koprunker 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 *Cnno1* in *Hydra magnipapillata*, is expressed in both multipotent stem cells and germ-line cells, but not in somatic cells (Mochizuki et al., 2000). In the polychaete annelid, *Platynereis dumerilii*, and the snail *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 *Drosophila* peripheral nervous system, in the dendritic arborization (da) neurons to maintain dendrite complexity (Brechtel 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

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cycle and developmental defects (Luo et al., 2011; Wu and Ruvkun, 2010). In *Drosophila*, *Celegans*, 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., 1996). 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, 2011). 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 (Fujii 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.

Material and methods

Animals

Strongylocentrotus purpuratus adults were housed in aquaria with artificial seawater (ASW) at 16 °C (Coral Life Scientific Grade Marine Salt; Carson, CA). 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. *Hemicentrotus pulcherrimus* were harvested from Seto inland sea or from Tateyama Bay and their gametes were obtained by coelomic injection of 0.55 M KCl. Fertilized eggs were cultured in filtered sea water (FSW) containing 50 µg/ml of streptomycin sulfate and 100 µg/ml of penicillin G potassium at 16 °C.

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 (Fujii 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 Sall, 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 GNARLE 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 Etensohn, 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 (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.

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'-GCAAGAAACAACGGAGAGAGC-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

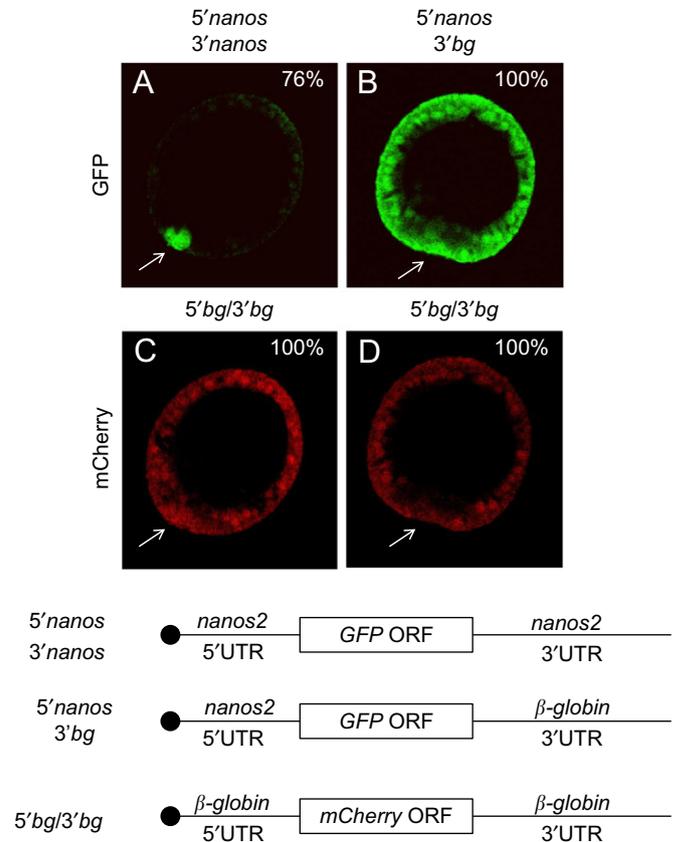


Fig. 1. *Sp nanos2* 3'UTR is sufficient for the selective protein accumulation in the small micromeres. Synthetic mRNA containing the *GFP* open reading frame flanked by (A) *Sp nanos2* 5' and 3'UTRs or (B) *Sp nanos2* 5'UTR and *Xenopus β-globin* 3'UTR were co-injected with *mCherry* mRNA (C and D) containing *Xenopus β-globin* 5' and 3'UTRs in *Sp* fertilized eggs. GFP (green) and mCherry (red) fluorescence were assayed in the same embryos 24 h post-fertilization at mesenchyme blastula: A and C represent the same embryo, B and D represent another one. For GFP images, A and B were obtained using the same settings (laser intensity, pin-hole opening). For mCherry images, C and D were also taken using the same settings. *bg* indicates *β-globin* UTRs. The arrows are pointing toward the small micromeres. The blastula are presented in the same orientation in the subsequent figures. Approximately one hundred blastulas were visualized after injection of each construct, the corresponding percentages of representative embryos are indicated in the right corner. Cartoons of the injected RNAs are presented at the bottom (the black circle representing the m⁷GTP cap).

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 pulcherrimus* 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 ($\Delta 1/2/3$), and with one 5'deletion ($\Delta 7$), 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 $\Delta 4$, $\Delta 5$, $\Delta 6$ and reached 0% in the $\Delta 9$ 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 containing only the nucleotides located between 859 and 1257, was sufficient to give a selective GFP expression in most of the injected embryos (Fig. 3A).

These results are complemented by experiments using additional *Hp nanos2* 3'UTR deletion constructs (Fig. 3B). Deletion of the nucleotides 880 to 1236 (ΔA) completely abolished the selective expression of GFP in the small micromeres. Similarly, partial deletions in this element at the 3'end (ΔB) or at the 5'end (ΔC) did not give a selective GFP expression in any of the injected embryos. Altogether,

<i>Hp nanos2</i> 3'UTR deletions		N	N sMic (%)
Full length	739–1790	99	74 (74.7)
$\Delta 1$	739–1692	184	131 (71.1)
$\Delta 2$	739–1573	152	119 (78.2)
$\Delta 3$	739–1394	148	107 (72.2)
$\Delta 4$	739–1257	156	104 (66.2)
$\Delta 5$	739–1033	176	100 (56.8)
$\Delta 6$	739–879	133	9 (6.7)
$\Delta 7$	859–1790	99	79 (79.7)
$\Delta 8$	1011–1790	125	1 (0.8)
$\Delta 9$	1236–1790	58	0 (0.0)
$\Delta 10$	1370–1790	112	0 (0.0)

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. N sMic indicates the number of injected embryos having a protein enrichment in the small micromeres, the corresponding percentages are represented in the parentheses (%).

<i>Hp nanos2</i> 3'UTR deletions		N	N sMic (%)
Full length	739–1790	88	79 (88.6)
A	859–1257	70	42 (60.0)
B	1011–1257	63	1 (0.8)
C	859–1033	60	6 (10.0)

<i>Hp nanos2</i> 3'UTR deletions		N	N sMic (%)
Full length	739–1790	77	44 (57.1)
ΔA	880–1236	60	0 (0.0)
ΔB	1033–1236	62	0 (0.0)
ΔC	880–1011	58	0 (0.0)

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 numbers of injected embryos having a GFP 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 (%).

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 Liability 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 (Δ GNARLE) (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* Δ GNARLE 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

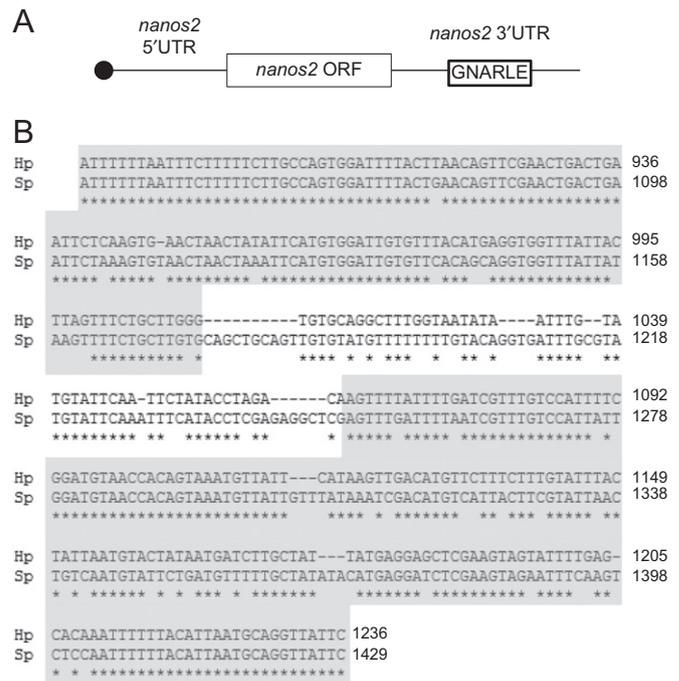


Fig. 4. (A) Schematic representation of *nanos2* RNA, indicating the location of GNARLE in the 3'UTR. (B) Alignment of *Hp* and *Sp nanos2* GNARLE using clustalW2. The stars below the sequences represent the identical nucleotides between *Hp* and *Sp*. The highly conserved regions are highlighted in gray.

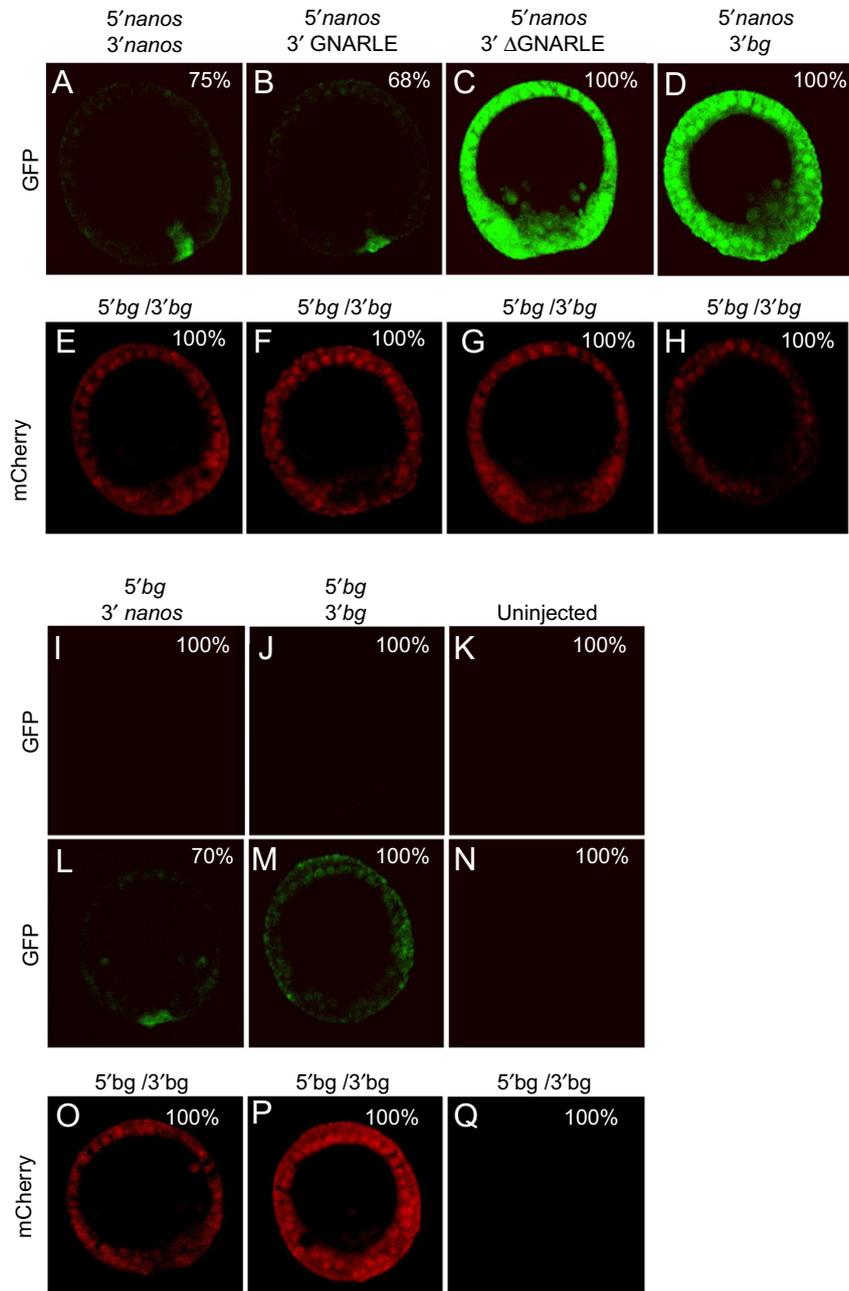


Fig. 5. *Sp* GNARLE is necessary and sufficient for selective protein accumulation in the small micromeres. Synthetic mRNAs containing *GFP* open reading frame were injected in *Sp* fertilized eggs. The *GFP* ORF was preceded by either (A–D) *Sp nanos2* 5'UTR or (I and J) *Xenopus β-globin* 5'UTR, and 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.

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

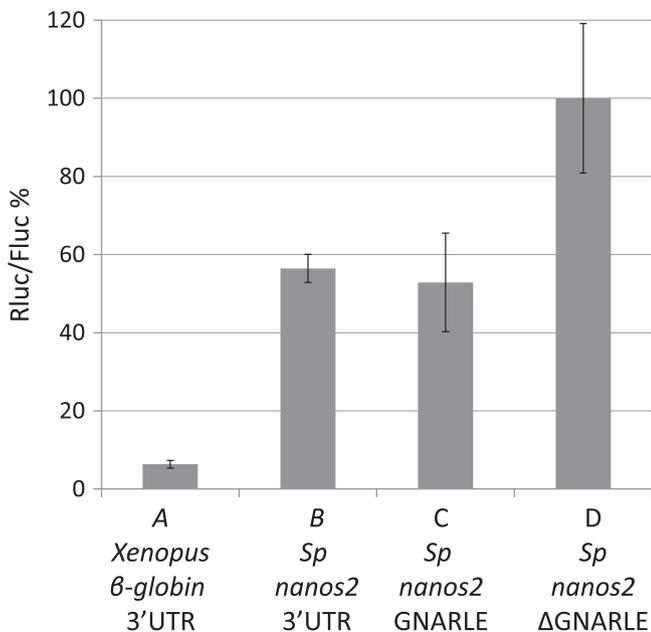


Fig. 6. Deletion of the GNARLE increases protein synthesis. Synthetic mRNAs, containing *Renilla* luciferase (Rluc) ORF preceded by (A) *Xenopus* β -globin 5'UTR or (B–D) *Sp nanos2* 5'UTR and followed by (A) *Xenopus* β -globin 3'UTR, (B) *Sp nanos2* full length 3'UTR, (C) *Sp nanos2* GNARLE 3'UTR, or (D) *Sp nanos2* Δ GNARLE 3'UTR, 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 at mesenchyme blastula. The ratio Rluc/Fluc was determined, and the results are shown in percentages considering the ratio obtained for *Sp* Δ GNARLE (D) as 100%. Error bars indicate the standard deviation from three technical replicates.

synthesis obtained with *Xenopus* β -globin UTRs is lower than the one obtained with *nanos2* UTRs. These results quantitatively supported the conclusions made by observations of fluorescence *in vivo* (Fig. 5).

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 Δ GNARLE. 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 Δ GNARLE 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 Stability 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 pulcherrimus*, which diverged less than 20 million years ago (Lee, 2003).

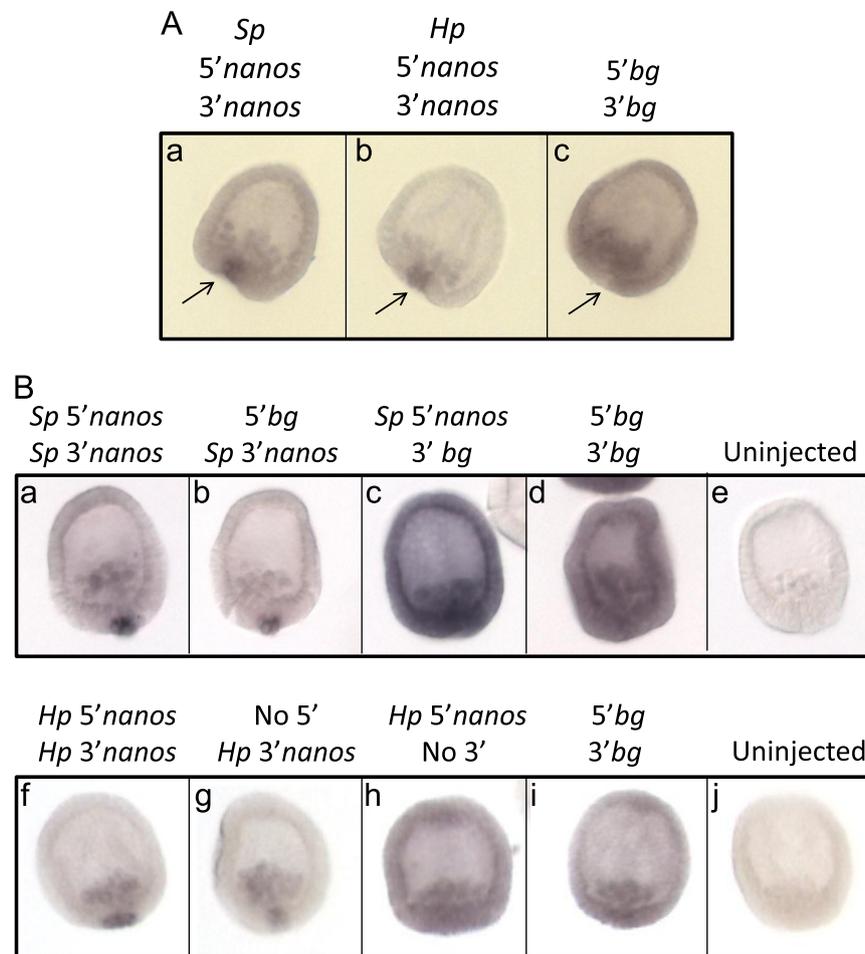


Fig. 7. *Sp* and *Hp* *nanos2* 3'UTRs are sufficient for the selective retention of RNA in the small micromeres. *In situ* hybridization on *Sp* mesenchyme blastula embryos using a probe against GFP, after injection of synthetic mRNAs in *Sp* fertilized eggs. (A) Synthetic mRNAs were made using the GFP ORF flanked by (a) *Sp nanos2* 5' and 3'UTRs, (b) *Hp nanos2* 5' and 3'UTRs, or (c) *Xenopus β-globin* UTRs. The arrows are pointing toward the small micromeres. (B) One set of synthetic mRNAs was made using the GFP ORF flanked by (a) *Sp nanos2* 5' and 3'UTRs, (b) *Xenopus β-globin* 5'UTR and *Sp nanos2* 3'UTR, (c) *Sp nanos2* 5'UTR and *Xenopus β-globin* 3'UTR, or (d) *Xenopus β-globin* 5' and 3'UTRs. A second set of synthetic mRNAs was made using the GFP ORF surrounded by (f) *Hp nanos2* 5' and 3'UTRs, (g) *Hp nanos2* 3'UTR, (h) *Hp nanos2* 5'UTR, or by (i) *Xenopus β-globin* UTRs. Uninjected embryos are shown in (e) and (j). Approximately one hundred blastulas were visualized after injection of each construct, the representative embryos are presented.

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. Recently, 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

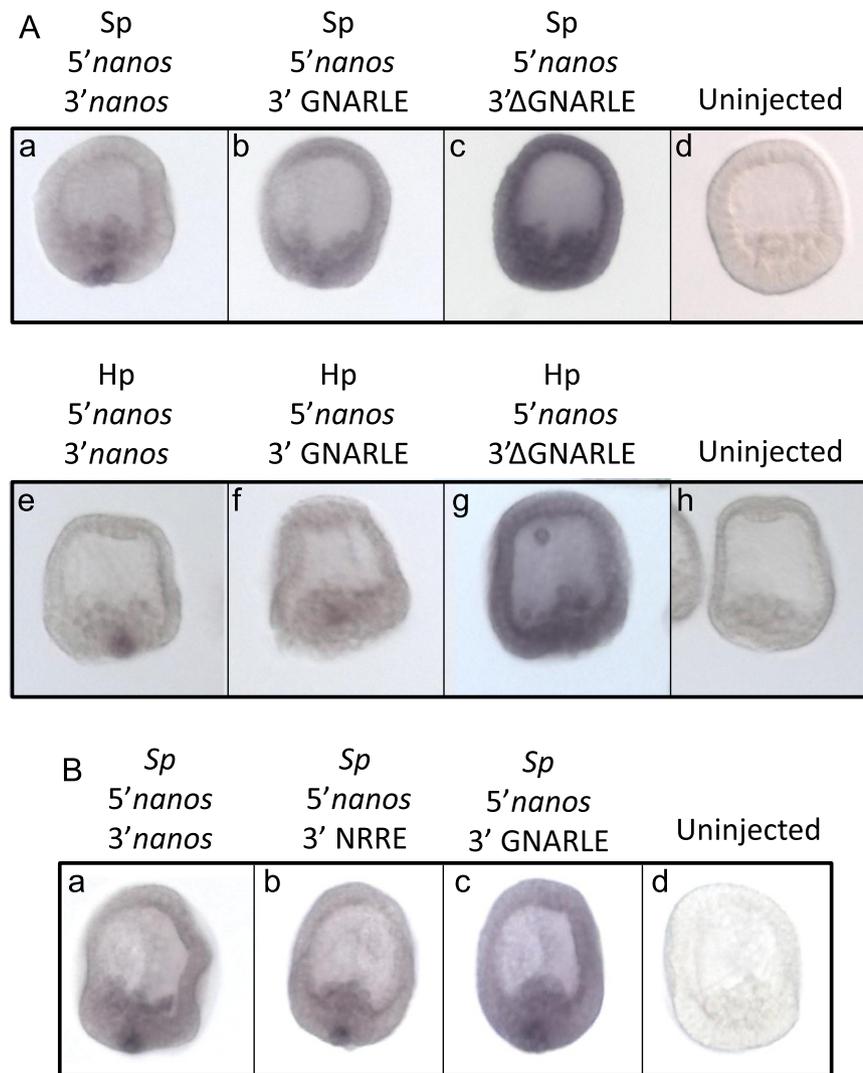


Fig. 8. The GNARLE is not sufficient for RNA retention in small micromeres. *In situ* hybridization on *Sp* mesenchyme blastula, using a RNA *in situ* probe against GFP, after injection of synthetic mRNAs in *Sp* fertilized eggs. (A) Synthetic mRNAs were made using the GFP ORF surrounded by (a and e) *nanos2* 5' and 3'UTRs, (b and f) *nanos2* 5'UTR and GNARLE 3'UTR, (c and g) *nanos2* 5'UTR and ΔGNARLE 3'UTR. UTRs from *Sp nanos2* (a–c) were used in one set of synthetic mRNAs, and UTRs from *Hp nanos2* (e–g) were used in a second set. Uninjected embryos are shown in (d) and (h). (B) Synthetic mRNAs were made using the GFP ORF surrounded by (a–c) *Sp nanos2* 5'UTR, and either (a) *Sp nanos2* full length 3'UTR, (b) *Sp NRRE* 3'UTR, (c) *Sp GNARLE* 3'UTR. Uninjected embryos are shown in (d). Approximately one hundred embryos were visualized after injection of each construct, and representative embryos are presented.

of *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

Supplementary data associated with this article can be found in the online version at <http://dx.doi.org/10.1016/j.ydbio.2013.01.019>.

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