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

Nathalie Oulhen [a], Takaya Yoshida [b], Mamiko Yajima [a], Jia L. Song [a,1], Tetsushi Sakuma [b], Naoaki Sakamoto [b], Takashi Yamamoto [b], Gary M. Wessel [a,*]

[a] Department of Molecular and Cell Biology and Biochemistry, Brown University, 185 Meeting Street, Providence, RI 02912, USA
[b] Department of Mathematical and Life Sciences, Graduate School of Science, Hiroshima University, Higashi-Hiroshima, Japan

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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 *nanos*2 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 nanos*2 3′UTR leads to selective reporter enrichment in the small micromeres in blastulae. The same result was seen with *nanos*2 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 *nanos*2 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 *nanos*2 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.

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 (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 *Cnnos*1 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 (Brechbiel 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

* Correspondence to: 185 Meeting Street, Box G, Brown University, Providence, RI 02912, USA. Fax: 401.863.2421.
E-mail address: rhet@brown.edu (G.M. Wessel).
[1] Current address: Department of Biological Sciences, University of Delaware, Newark, DE 19716, USA.

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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., 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. *Nanos*1 and *nanos*2 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 *nanos*2 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 nanos*2 UTRs (Full length), *Hp nanos*2 5′ and 3′UTRs were amplified using the primers described in Supplementary Fig. S1A (Fujii et al., 2006). These *Hp nanos*2 UTRs were subcloned into pGreenLantern2-derived plasmid containing the GFP open reading frame and the T7 promoter. For 3′deletions, *Hp nanos*2 3′UTR was amplified from the full length using the primers presented in Supplementary Fig. S1B. For 5′deletions, *Hp nanos*2 3′UTR was amplified using the primers described in Supplementary Fig. S1C. For constructs A–C, *Hp nanos*2 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 nanos*2 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 promotor. *Sp nanos*2 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 nanos*2 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 nanos*2 3′UTR NRRE was amplified using the primers presented in Supplementary Fig. S2D.

For the *Renilla* luciferase constructs, *Sp nanos*2 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 promotor. The *Sp nanos*2 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 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 (Narishige; Tokyo, Japan) were