

Xenopus germline *nanos1* is translationally repressed by a novel structure-based mechanism

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SUMMARY

The translational repressor Nanos is expressed in the germline and stem cell populations of jellyfish as well as humans. Surprisingly, we observed that unlike other mRNAs, synthetic *nanos1* RNA translates very poorly if at all after injection into *Xenopus* oocytes. The current model of simple sequestration of *nanos1* within germinal granules is insufficient to explain this observation and suggests that a second level of repression must be operating. We find that an RNA secondary structural element immediately downstream of the AUG start site is both necessary and sufficient to prevent ribosome scanning in the absence of a repressor. Accordingly, repression is relieved by small in-frame insertions before this secondary structure, or translational control element (TCE), that provide the 15 nucleotides required for ribosome entry. *nanos1* is translated shortly after fertilization, pointing to the existence of a developmentally regulated activator. Oocyte extracts were rendered fully competent for *nanos1* translation after the addition of a small amount of embryo extract, confirming the presence of an activator. Misexpression of Nanos1 in oocytes from unlocalized RNA results in abnormal development, highlighting the importance of TCE-mediated translational repression. Although found in prokaryotes, steric hindrance as a mechanism for negatively regulating translation is novel for a eukaryotic RNA. These observations unravel a new mode of *nanos1* regulation at the post-transcriptional level that is essential for normal development.

KEY WORDS: *Xenopus*, *nanos* family, Translational repression, RNA secondary structure, Germline

INTRODUCTION

The germ cell lineage and somatic fates of the early embryo are initially specified by maternal RNAs localized to the vegetal pole of *Xenopus* oocytes (King et al., 2005; Kloc et al., 2001; Zhang et al., 1998). These maternal determinants are active well before zygotic transcription begins, highlighting the importance of post-transcriptional mechanisms in regulating early development. Translation of the endoderm determinant *VegT* is initiated during maturation and appears to be regulated by cytoplasmic polyadenylation together with a factor that both stabilizes and promotes its translation (Souopgui et al., 2008; Stennard et al., 1999). *Vg1*, which is required for mesoderm formation and left-right asymmetry, is translated soon after its localization is complete during mid-oogenesis (reviewed by King et al., 2005). Prior to localization, *Vg1* is bound by a trans-acting repressor at a 3'UTR site (Colegrove-Otero et al., 2005a). Thus, the translational regulation of essential somatic cell determinants is accomplished through trans-acting factors binding to the UTR via mechanisms that are common to eukaryotic RNAs (Johnstone and Lasko, 2001; Schier, 2007; Richter and Sonenberg, 2005). By contrast, little is known about how expression of the germ cell determinants is regulated, although such regulation is crucial for establishing the next generation.

One evolutionarily conserved component of germ cells that is required for maintenance and self-renewal is *nanos* (reviewed by Shen and Xie, 2010; Subramaniam and Seydoux, 1999; Wang and Lin, 2004). In vertebrate and invertebrate species, the Nanos family

of proteins function as translational repressors that are essential to maintain the germline precursors – the primordial germ cells (PGCs) (Curtis et al., 1997; Kadyrova et al., 2007; Lai et al., 2011; Tsuda et al., 2003). *Xenopus nanos* RNA (*nanos1*; formerly called *Xcat2*) is transcribed during early oogenesis and becomes localized to the germ plasm, a subcellular compartment bearing the germ cell determinants. There, *nanos1* is packaged into germinal granules, which are diagnostic structures of germ plasm that are considered to provide a mechanism for long-term storage of RNAs and proteins (Forristall et al., 1995; Kloc et al., 2002; Tsuda et al., 2003; Zhou and King, 1996). *nanos1* is not translated during the 4 to 6 months required for oogenesis, but Nanos1 protein is detected during early development (Lai et al., 2011). Thus, *nanos1* and other germline RNAs are activated sometime during development but the mechanisms remain unknown (reviewed by King et al., 2005; Kloc et al., 2001).

Although the repressive activity of Nanos is required for PGCs to maintain their identity in the presence of somatic determinants, the somatic cells must also require Nanos function to be restricted to the germline to allow somatic cell determination (Jadhav et al., 2008; Kobayashi et al., 1996; Köprunner et al., 2001; Tsuda et al., 2003). Therefore, one might expect a robust and overlapping translational repression of *nanos* transcripts, as best documented in *Drosophila* and *C. elegans*. *Drosophila* Nanos is restricted to the posterior pole of the oocyte and early embryo through both translational repression of unlocalized *nanos* mRNA and translational activation of localized *nanos* message at the posterior pole (Forrest and Gavis, 2003; Gavis and Lehmann, 1992; Gavis and Lehmann, 1994). The regulation of *nanos* germline expression in *Drosophila*, *C. elegans* and mouse requires the 3'UTR (Gavis et al., 1996; Jadhav et al., 2008; Suzuki et al., 2010). Interestingly, the 3'UTR translational control element (TCE) of *Drosophila nanos* forms a structure containing two hairpins, each of which binds a distinct repressor. These stem-loops act independently of each other to repress translation at different

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times in development: in embryonic somatic cells and in the oocyte, respectively (Forrest et al., 2004; Kalifa et al., 2006). Similarly, *C. elegans nos-2* is translationally regulated to permit expression exclusively in the germline lineages by two independent stem-loops in the 3'UTR (Jadhav et al., 2008; Subramaniam and Seydoux, 1999; D'Agostino et al., 2006). Thus, in many respects, both the function and regulation of germline *nanos* have been conserved.

Surprisingly, we observed that unlike any other known mRNA, synthetic *nanos1* RNA translates very poorly after injection into *Xenopus* oocytes. Clearly, the current model of simple sequestration of *nanos1* within germinal granules is insufficient to explain this observation and suggests that a second level of repression must be operating. Here, we describe a novel mechanism for regulating *nanos1* RNA expression. We present evidence that an RNA secondary structural element in the ORF is sufficient to repress the translational initiation of *nanos1* RNA and the expression of a reporter. Consistent with this model, we find that insertions between the start codon and the TCE relieve repression. Misexpression of *nanos1* in oocytes results in abnormal development, highlighting the importance of translational control mediated by the TCE during oogenesis. Such a structural mechanism that operates independently of a repressor for negative regulation of translation, although common in prokaryotes, is completely new for eukaryotic RNA (reviewed by Kozak, 2005). These observations unravel a novel mode of *nanos1* regulation at the post-transcriptional level that is essential for normal development.

MATERIALS AND METHODS

Plasmids

Deletion, insertion and substitution mutants (see Fig. 2A, mutants S1-S4 and CS) of *nanos1* (Mosquera et al., 1993) were generated using the QuickChange Mutagenesis Kit (Stratagene). Inserted nucleic acid sequences were from equivalent positions of the β -globin ORF. The β -globin transcripts were generated from pSPX β M (Krieg and Melton, 1984). Constructs of *nanos1* and β -globin with exchanged UTRs were generated by two-step PCR. For Myc-*nanos1*, the *nanos1* coding region was subcloned into the pCS2-Myc vector between *EcoRI* and *XhoI* sites. *nanos1*-Myc was generated by subcloning the 5'UTR and ORF of *nanos1* into the pCS2-Myc vector between *BamHI* and *ClaI* sites. For primer sequences, see Table S1 in the supplementary material.

Oocytes, embryos and micro-injection

Oocytes and embryos were obtained as described (Sive et al., 2000). RNAs for injection were prepared from the plasmid templates described above using the mMACHINE mMACHINE Kit (Ambion).

In vitro translation

In vitro translation with either reticulocyte lysates or wheat germ extracts was carried out according to the manufacturer's instructions (Promega). Translation-competent oocyte and embryo extracts were prepared essentially as described (Murray, 1991). For the translation assay, a mixture of amino acids (Promega), creatine kinase (1 mg/ml final concentration; Sigma) and capped mRNA was added to the extract.

Immunoprecipitation

Synthetic *nanos1* transcripts were injected into *Xenopus* oocytes or 1-cell embryos. Samples were subjected to immunoprecipitation with goat anti-Nanos1 antibody and Protein G beads (Roche).

Western blotting

Western blots were performed as previously described with goat anti-Nanos1 (1:100) or rabbit anti-Myc-HRP (1:2000; Invitrogen) primary antibody and rabbit anti-goat-HRP secondary antibody (1:1000) (Venkatarama et al., 2010). All blots consistently showed a high molecular weight non-specific band, which was used as a loading control.

Sucrose gradient analysis

Wheat germ translation extract (50 μ l; Promega) was mixed with 0.2 μ g of radiolabeled transcripts and incubated at 20°C for 20 minutes. The reactions were stopped by addition of cold sucrose gradient buffer [(20 mM Hepes pH 7.5, 90 mM KCl, 1.5 mM MgCl₂ and 500 μ g/ml cycloheximide (Sigma) or 2 mM GMP-PNP (Sigma)] and layered onto a 10–30% linear sucrose gradient. The gradient was then centrifuged at 39,000 rpm (188,000 g) for 4 hours at 4°C in an SW41 rotor. The gradient was collected from the bottom in 0.5 ml fractions and the ribosomal RNA profile was determined by UV₂₆₀. Radioactivity of each fraction was measured by scintillation.

RNase probing

RNase probing was performed as previously described (Darfeuille et al., 2007).

Immunofluorescence and confocal imaging

Immunofluorescence and confocal imaging were performed as described previously (Venkatarama et al., 2010).

Host transfer

Host transfer was performed as described by Mir and Heasman (Mir and Heasman, 2008).

RESULTS

nanos1 RNA is poorly translated after injection into oocytes

Xenopus oocytes have long been used to express injected mRNAs from a wide variety of sources, including viruses (Brown, 2004). Therefore, in experiments addressing the function of *nanos1*, we were surprised to find that although *nanos1* RNA was translated in reticulocyte lysates, it was poorly translated, if at all, after injection into oocytes (Fig. 1A). One explanation is that *nanos1* RNA and protein are degraded if present outside their normal cellular location within germ plasm. To explore this possibility, we injected radiolabeled *nanos1* transcripts and isolated oocytes at different time points to assess the percentage that remained (see Fig. S1 in the supplementary material). We found that *nanos1* RNA was comparable in stability to that of control β -globin mRNA (see Fig. S1A in the supplementary material). Next, we synthesized radiolabeled *nanos1* protein by in vitro translation and injected it into oocytes. Nanos1 was still detected at least 6 hours after injection (see Fig. S1B in the supplementary material). These results showed that both *nanos1* RNA and protein were stable, suggesting that injected *nanos1* RNA is poorly translated in oocytes.

Translational repression of *nanos1* is not dependant on UTRs

Repression of maternal transcripts is commonly regulated through TCEs in the UTRs that serve as binding sites for proteins or small RNAs. Previous work from our laboratory suggested that the TCE did not lie within the 3'UTR, as substitution with the 3'UTR of β -globin did not result in translation (MacArthur et al., 1999). To confirm these published results, and to identify which sequences constitute the *nanos1* TCE, a set of constructs was generated representing the various combinations of UTRs and ORFs from *Xenopus* β -globin and *nanos1* (Fig. 1B). These transcripts were tested for translation in stage VI oocyte extracts supplemented with radioactive methionine. β -globin is a particularly good control for these experiments as β -globin and Nanos1 have the same number of methionines, their UTRs are similar in length, and the proteins are of similar mass. Thus, we can judge translational efficiency directly from radiolabel-incorporation data. We first asked whether

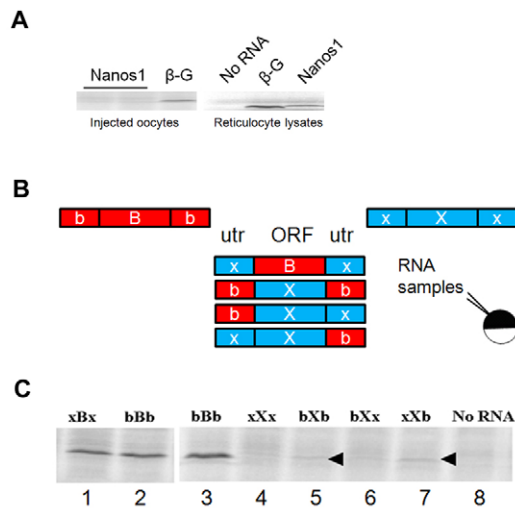


Fig. 1. Repression of *nanos1* translation in *Xenopus* oocytes is mediated by the ORF. (A) (Left) *nanos1* transcripts were co-injected with [³⁵S]methionine into oocytes and incubated overnight at 18°C. β -globin (β -G) served as control. (Right) *nanos1* and β -globin were translated in reticulocyte lysates. (B) Schematic of the transcripts tested and the experimental design. ORFs (uppercase) and UTRs (lowercase) were from *nanos1* [X] or β -globin [B]. (C) RNAs with UTR substitutions (labeled in accordance with B) were assayed in oocyte extracts. Arrowheads mark the position of *nanos1*. See also Fig. S1 in the supplementary material.

the *nanos1* UTRs were sufficient to repress translation of the β -globin ORF in oocyte extracts. These hybrid constructs were equally well translated as the β -globin RNA itself (Fig. 1C, compare lane 1 with lanes 2 and 3). Similarly, substitution of the *nanos1* UTRs with those of β -globin failed to relieve repression of the *nanos1* ORF (Fig. 1C, compare lanes 5–7 with 4). Our results strongly suggested that the TCE lies within the *nanos1* ORF itself.

The TCE lies within the first 75 nucleotides of the ORF

We reasoned that the TCE might lie in close proximity to the start codon and form a structure involved in repression. As a first step, we analyzed full-length *nanos1* RNA using MFOLD RNA structure prediction software (Zuker, 2003) to compute the most probable structures based on the free energies of the folds. Of the 35 separate predictions returned, 16 (45.7%) revealed one large and two small stem-loop structures within the 5'UTR and first 93 nucleotides (nt) of the *nanos1* ORF (Fig. 2A; see Fig. S2B in the supplementary material). Interestingly, the region 15 nt upstream of the AUG start site in *X. laevis* is identical to that in *X. borealis* and virtually identical to that in *X. tropicalis*, species that are separated from *X. laevis* by 50 and 120 million years, respectively (Graf, 1996), and that have much longer 5'UTRs (see Fig. S2A in the supplementary material). Structure predictions for *nanos1* in *X. borealis* and *X. tropicalis* also revealed stem-loop structures around, and just downstream of, the start site (see Fig. S2B–D in the supplementary material).

We tested the functional significance of the large putative stem-loop by deleting these sequences (nt 8–73 in TCE Δ) and monitoring translation after injection into oocytes. Removal of these sequences completely relieved repression, as indicated by western blot analysis with anti-Nanos1 antibody (Fig. 2B). We then asked

whether the *nanos1* 5'UTR plus the first 75 nt (TCE) placed upstream of Myc sequences would be sufficient to repress translation after injection into oocytes. Indeed, these sequences were sufficient to completely block translation (Fig. 2C). Taken together, these results reveal a TCE within the first 75 nt of the *nanos1* ORF that is required to repress *nanos1* translation and is sufficient to repress a reporter.

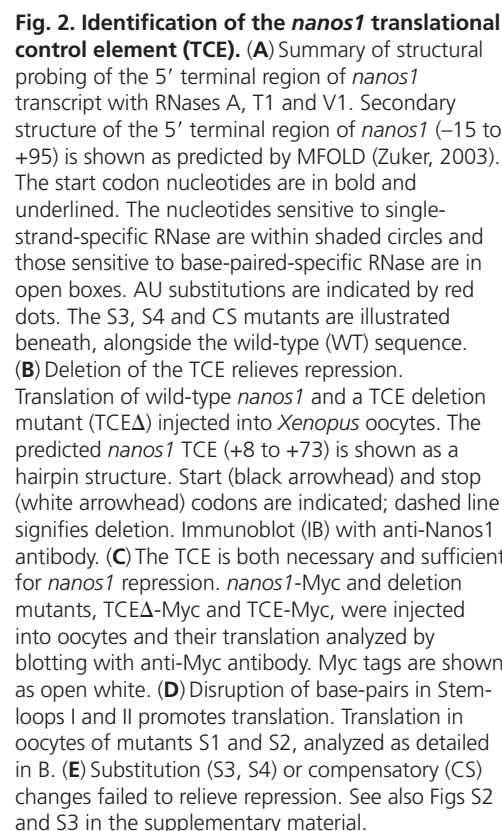
The TCE forms a secondary RNA structure

To determine whether the TCE we identified did indeed form a secondary structure as predicted by MFOLD, we performed an enzymatic structural analysis of *nanos1* transcripts. RNases A and T1 cut at single-stranded residues 3' of C and U and 3' of G, respectively, whereas RNase V1 digests base-paired nucleotides. ³²P-labeled *nanos1* RNA was digested with one of the three RNases and the resulting products analyzed on sequencing gels (see Fig. S3 in the supplementary material). In general, the results of the enzymatic structural analyses were consistent with the MFOLD program prediction of unpaired RNA regions or loop structures (Fig. 2A, black circles). RNases A and T1 hit most of the C and G single-stranded nucleotides, including those in the loops, and a few of the base-paired nucleotides at the end of the stems of the predicted structure. It is likely that 'breathing' at the end of stems exposed those nucleotides to attack by the single-strand-specific RNases. The predicted Stem I around the AUG site was validated in this assay as RNase V1 attacked four nucleotides within this region. Importantly, the large predicted stem in Stem-loop II was also recognized by RNase VI. Therefore, our analysis detected at least two stretches of nucleotides that appeared to form stable stems around the start codon.

To further probe the predicted secondary structures around the start codon, two mutants were made to disrupt the GC-rich stem regions by substituting AU sequences along one side of the predicted stems, as indicated in Fig. 2A (red dots). As shown by Fig. 2D, the AU mutants S1 and S2 were translated after injection into oocytes. The S1 mutant, with AU substitutions only downstream of the AUG site, was not as effective (translation was 27% of Δ TCE levels) in relieving repression as the S2 mutant with its additional substitutions in the 5'UTR (83.5% of Δ TCE levels). We conclude from these studies that the TCE forms a secondary RNA structure and that the conserved 5'UTR sequence plays a role in stabilizing it. We next made a compensatory change (Fig. 2A, CS) to this area, exchanging the left side (8–19 nt) of the predicted stem for the right (61–73 nt), thus preserving the predicted stem, but not its sequence. This mutant did not support translation (Fig. 2E). However, duplication of the sequence (i.e. two right or two left side sequences; Fig. 2A, S3, S4) in the GC-rich stem region did not support translation either (Fig. 2E). Taken together, we interpret these findings to suggest that the left half of the predicted Stem II is base-paired but not necessarily with the sequences predicted by MFOLD.

Translational repression is sensitive to the distance between the AUG and TCE

What role does the TCE play in *nanos1* repression? *nanos1* repression could act through a repressor that specifically binds the TCE. Alternatively, the structure alone could sterically prevent translational initiation events without the need for a repressor. In other studies defining the function of *nanos1* in the germline, we noticed that epitope tags cloned in-frame 5' to the *nanos1* ORF were translated (Fig. 3A). We hypothesized that the 5' tags might be disrupting the TCE structure and/or preventing a repressor from binding. Alternatively, the addition of extra in-frame sequences in



The positioning of the 40S ribosomal subunit at the start codon requires ~15 nt downstream of the first nucleotide at the P site (Pestova et al., 2001; Kozak, 1977). We propose that the structure-based TCE, lying just 4 nt downstream of the start codon, interferes

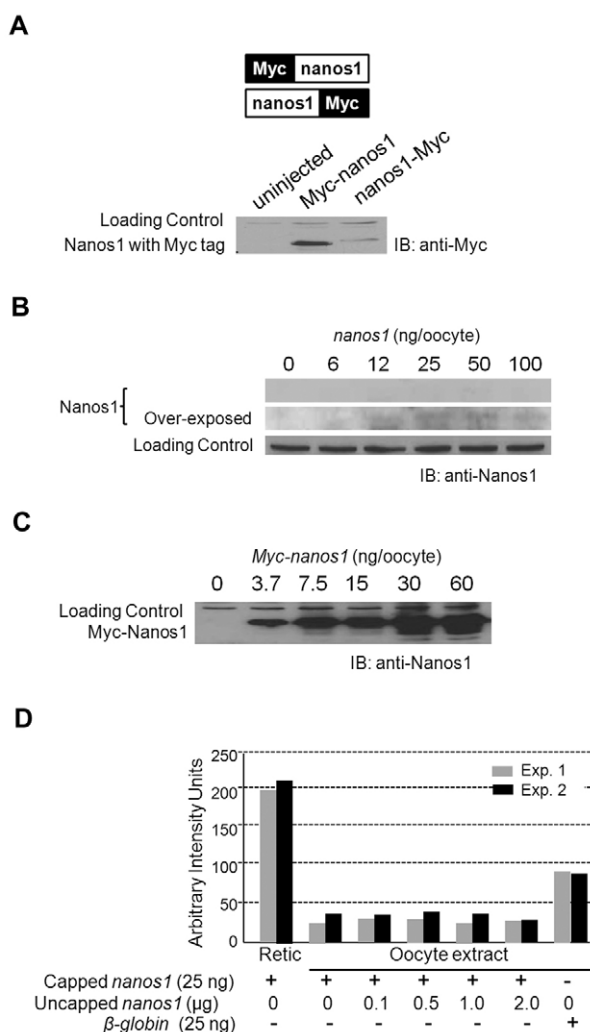


Fig. 3. Repression of *nanos1* in oocytes does not depend on trans-acting factors. (A) *Xenopus* oocytes were injected with either *nanos1*-Myc or Myc-*nanos1* transcripts and incubated at 18°C overnight. Translation products were detected by blotting (IB) with anti-Myc antibody. (B,C) *nanos1* repression is not relieved in vivo by excess levels of *nanos1* mRNA. Oocytes were injected with increasing amounts of synthetic capped *nanos1* (B) or capped Myc-*nanos1* (C) as indicated, incubated at 18°C overnight, and products analyzed by blotting with anti-Nanos1 antibodies. Myc-*nanos1* served as a positive control. Note that *nanos1* repression was not relieved at any concentration of *nanos1* mRNA. (D) Histogram showing the results from two independent in vitro competition experiments. Twenty-five nanograms of capped *nanos1* transcripts were translated for 90 minutes at room temperature in oocyte extract containing [³⁵S]methionine. Uncapped *nanos1* transcripts were added to the translation mix in increasing amounts as indicated. As positive controls, 25 ng of capped *nanos1* and β-globin RNAs were translated in reticulocyte lysates and oocyte extracts, respectively. Even in the presence of an 80-fold excess of uncapped *nanos1* RNA, repression was not relieved.

with initiation events because there is insufficient space for the 40S ribosomal subunit to bind. Two predictions follow from this model. First, only insertions of 12 nt or longer before the TCE would be sufficient for ribosome loading and thus relief of repression. Subsequent elongation steps are predicted to overcome the negative ΔG of the TCE (Kozak, 2005; Takyar et al., 2005). Second, we

would expect the *nanos1* transcript to be severely impaired in binding to the 43S pre-initiation complex. To test the first prediction, 3, 6 and 15 nt were inserted in-frame between the start codon and the TCE of *nanos1* (Fig. 4A). Transcripts containing the insertions were injected into oocytes and tested for their translational efficiency as before. As predicted by the model, insertions of 3 or 6 nt had no effect, whereas 15 nt relieved repression to levels comparable to that of the TCE deletion mutant (Fig. 4A).

nanos1 translation is blocked at initiation

To test the second prediction of our model, we used a ribosomal loading assay to determine whether the 43S complex would associate with *nanos1* mRNA. Initial experiments using oocyte extracts were not informative as the positive control did not accumulate in 80S complexes in the presence of cycloheximide, suggesting that translation was inefficient. However, wheat germ extract was found to mimic the results obtained in oocytes: *nanos1* was only translated if the TCE was deleted or nucleotides were inserted before the TCE (Fig. 4B). Therefore, wild-type and mutant ³⁵S-labeled *nanos1* transcripts were incubated for 15 minutes in wheat germ extract with cycloheximide, followed by fractionation on a 10–30% linear sucrose gradient. As expected, in the presence of cycloheximide ~10% (*n*=3) of the Myc-tagged *nanos1* transcripts accumulated in the 80S complex (Fig. 4C, white arrow). By contrast, only 1% (*n*=3) of the *nanos1* transcripts fractionated with the 80S peak (Fig. 4C, arrowhead), confirming that a step in initiation was blocked. Importantly, both the insertion and TCE deletion mutants of *nanos1* showed increased association with the 80S complex (8%, *n*=2) that was comparable to the control Myc-*nanos1* transcript (Fig. 4D).

To more precisely define the initiation step at which *nanos1* translation was stalled, we tested the ability of the 43S complex to bind radiolabeled *nanos1* transcripts in the presence of GMP-PNP, a non-hydrolysable GTP analog. The recruitment of the 60S subunit is dependent on GTP hydrolysis. Including the GMP-PNP in the translation mix prevents 60S subunit recruitment and increases the amount of the 43S complex that is associated with mRNA. Therefore, in the presence of GMP-PNP, mRNAs that are competent to initiate will accumulate within the 48S initiation complex. As expected, a significant proportion (14%, *n*=3) of the Myc-labeled *nanos1* transcripts was associated with the 40S ribosomal subunit (Fig. 4E, white arrow). A small, but significant, amount (1%, *n*=3) of *nanos1* RNA co-sedimented with the 43S peak, comparable to the amount observed in the 80S peak in the presence of cycloheximide (compare Fig. 4C with 4E, arrowhead). These results support the conclusion that *nanos1* RNA expression is attenuated at a very early translational initiation step: loading of the 40S ribosomal subunit. Furthermore, as wheat germ extract is unlikely to contain a repressor that specifically recognizes the TCE, these findings also lend support to the conclusion that the TCE secondary structure alone is required for the observed repression.

Translation of injected *nanos1* transcripts occurs prior to first cleavage

Oocyte maturation and fertilization are pivotal times for the activation of stored maternal mRNAs (Colegrove-Otero et al., 2005b; Richter, 2007; Standart and Minshall, 2008). To determine when *nanos1* translation is initiated during development, *nanos1* transcripts were injected into oocytes that were subsequently induced to mature with progesterone, or were injected into fertilized eggs. Embryos were collected 75 minutes post-fertilization and at each division

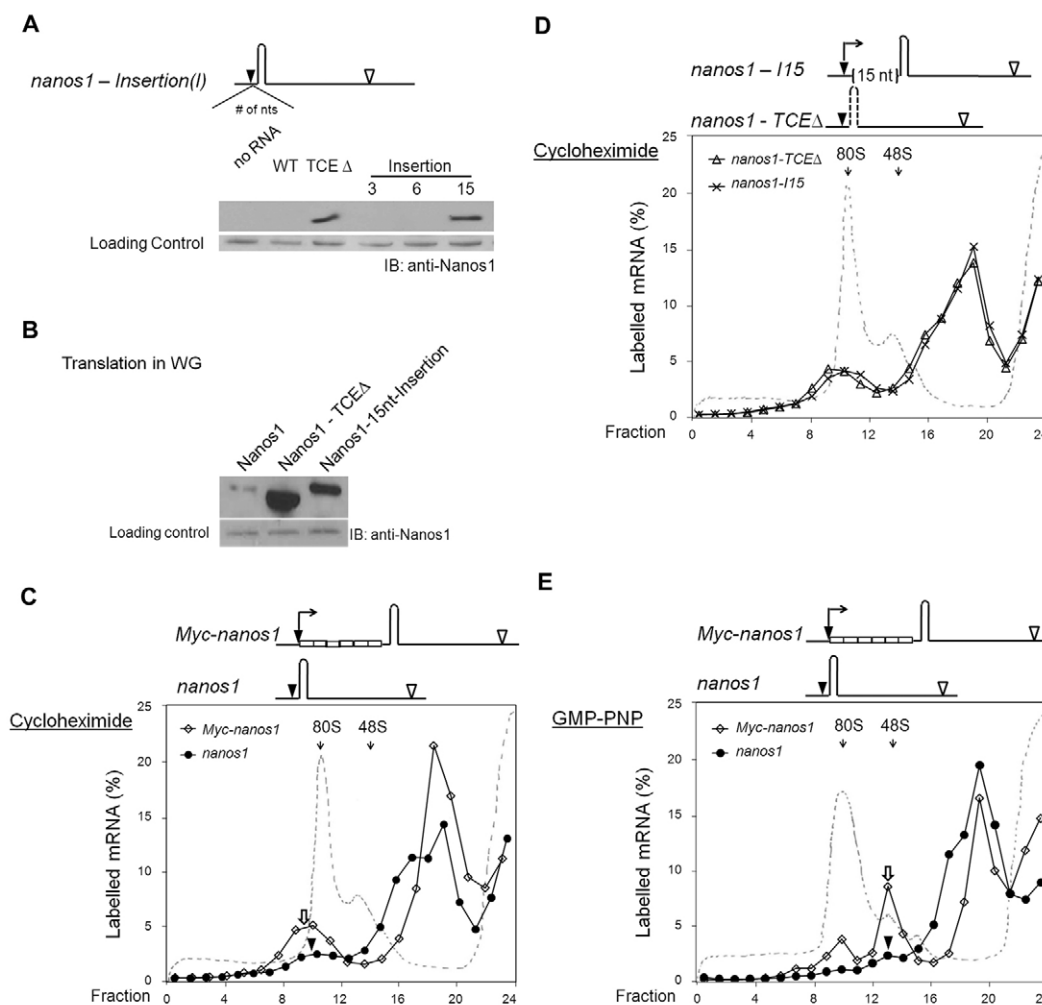


Fig. 4. Translation of *nanos1* RNA is blocked at initiation. (A) Translation of wild-type (WT) *nanos1* and insertion mutants in *Xenopus* oocytes. WT *nanos1* and TCE Δ are described in Fig. 2B and the insertion mutants are as illustrated. Nucleotides from equivalent positions of β -globin were inserted in between the start codon and the TCE. Translation products from injected oocytes were analyzed by blotting with anti-Nanos1 antibody. (B) Translation of *nanos1* and *nanos1* mutants in wheat germ extracts. One microgram of synthetic mRNA was translated in wheat germ extracts and the resulting products analyzed as in A. (C–E) Ribosomal loading assay performed with radiolabeled *Myc-nanos1*, *nanos1*, *nanos1*-TCE Δ or *nanos1*-15nt-insertion (*nanos1*-I15) transcripts. Transcripts were incubated in wheat germ extract in the presence of cycloheximide (500 μ g/ml) (C,D) or GMP-PNP (2 mM) (E) at 18°C for 15 minutes and the reaction mixture fractionated on 10–30% linear sucrose gradients. The labeled mRNA in the fractions is expressed as a percentage of total counts recovered and is plotted against the fraction number. The dashed line denotes the A_{254} absorption profile. Compare the regions indicated by the white arrows and black arrowheads (see text).

cycle up to the 32-cell stage. Nanos1 protein levels were determined by immunoprecipitation and western blotting with anti-Nanos1 antibody. *nanos1* RNA remained repressed during maturation events (Fig. 5A), but Nanos1 protein was detected before first cleavage and reached maximum levels by the 2-cell stage (Fig. 5B). These levels persisted through to the 32-cell stage, which was the last stage examined (data not shown).

To gain further insight into the molecular mechanism that might govern *nanos1* translational activity, we employed *in vitro* translation assays using oocyte and embryo extracts (Murray, 1991). Each extract translated β -globin RNA equally well (data not shown). We asked whether the addition of embryo to oocyte extract would render oocyte extract competent for *nanos1* translation or whether oocyte extract would repress translation in embryo extracts. As shown in Fig. 5C (lanes 1 and 2), the efficiency of *nanos1* translation was much greater in embryo extract than in oocyte extract, as expected from the *in vivo* assays (Fig. 5B).

Strikingly, addition of 5 or 15 μ l of embryo extract to oocyte extract supported *nanos1* translation to levels that were indistinguishable from using embryo extract alone (Fig. 5C, compare lanes 3 and 4 with lane 2). These results are consistent with an activator being present in stage 2 embryos. Endogenous Nanos1 protein was found to accumulate to detectable levels as early as the 8-cell stage (Fig. 5D). These results are consistent with *nanos1* translation being activated shortly after fertilization, but not at maturation, and suggest the presence of a developmentally regulated activator.

Can *nanos1* RNA be translated in somatic cells or is its activation restricted to the germline? Immunofluorescence microscopy of *nanos1*-injected embryos revealed that *nanos1* protein accumulated in somatic cells, especially at the animal pole (Fig. 5E), suggesting that either a common activator is present in somatic cells or that more than one activator is competent to activate *nanos1* translation.

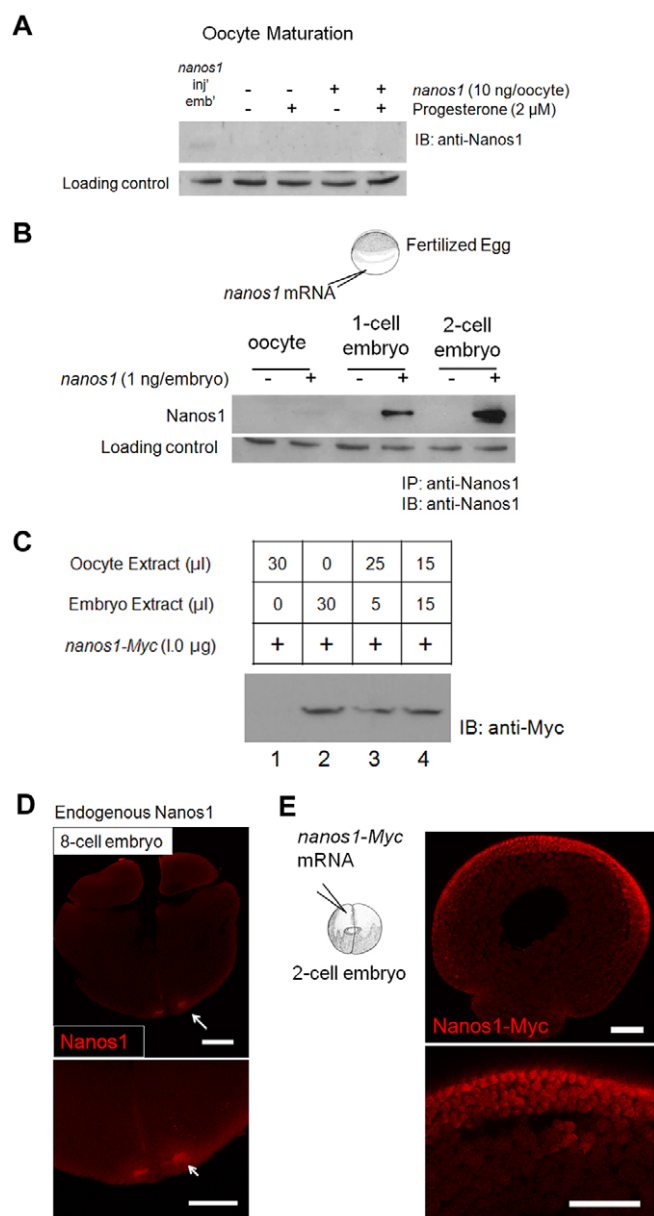


Fig. 5. *nanos1* repression is relieved only after fertilization.

(A) *nanos1* remains repressed during oocyte maturation events. *Xenopus* oocytes were injected with 10 ng of *nanos1* transcript per oocyte and incubated at 18°C. To trigger maturation events, oocytes were incubated with progesterone (2 μ M) and collected until at least 50% displayed germinal vesicle breakdown. Samples were analyzed by blotting with anti-Nanos1 antibody. *nanos1*-injected embryos served as a positive control. (B) Injected *nanos1* transcripts are efficiently translated in embryos before first cleavage. One nanogram of capped transcript was injected into 1-cell stage embryos soon after fertilization. Embryos were collected at the indicated stages and protein extracts analyzed by blotting after immunoprecipitation (IP) with anti-Nanos1 antibody. (C) *nanos1-Myc* was translationally active in the presence of embryo extract in a dose-independent fashion. One microgram of capped transcript was translated in vitro with oocyte and/or embryo extract. Samples were analyzed by blotting with anti-Myc antibody. (D) Endogenous Nanos1 was easily detected in the germ plasm by the 8-cell stage. Confocal immunofluorescence with anti-Nanos1 antibody. (E) Confocal immunofluorescence of embryos previously injected with *nanos1-Myc* RNA shows Nanos1 accumulation in somatic cells. Scale bars: 200 μ m.

Misexpression of unlocalized *nanos1* in oocytes results in abnormal development

Maternal *nanos1* is transcribed and localized to the germ plasm during early oogenesis. The germ plasm containing *nanos1* RNA is subsequently localized to the vegetal pole and asymmetrically distributed into the future germ cells (Mosquera et al., 1993). However, Nanos1 protein was not detected in germ plasm until early cleavage (the 8-cell stage), some 4 months after its RNA is localized (Fig. 5D) (MacArthur et al., 1999), consistent with *nanos1* being under tight negative control. Our data suggest that unlocalized *nanos1* RNA is not translated outside the context of germ plasm, pointing to a repressive mechanism in addition to physical sequestration in the germinal granules. Is this repressive mechanism essential for normal development or is premature Nanos1 expression from unlocalized mRNA tolerated in the oocyte? To address this, we forced Nanos1 expression in stage VI oocytes by placing Myc tags upstream of the *nanos1* ORF (*Myc-nanos1*) and then assessed any developmental consequences by host transfer (Fig. 6A,B). Oocytes injected with Myc tags alone developed normally (Fig. 6C,D). The *Myc-nanos1*-injected embryos were indistinguishable from the uninjected controls until stage 11, at which time they displayed large blastopores that failed to close (data not shown). These embryos remained round, with their endodermal mass exposed. Those that survived gastrulation went on to display severely incomplete neural tube closure at tailbud stages (Fig. 6C,D). From these results, we conclude that Nanos1 expressed outside the germ plasm is tolerated by the oocyte (maturation is normal), but not by the somatic cells that inherit the ectopically expressed Nanos1 during development. These results underscore the importance of TCE structure-based repression to ensure that unlocalized maternal *nanos1* RNA is not translated.

DISCUSSION

We have identified a novel structure-based mechanism for the translational repression of germline *nanos1* RNA that ensures its inactivity during oogenesis, a feature essential for normal development. We propose that ribosome scanning is sterically prevented by a structural element of the RNA without the involvement of a repressor. Several lines of evidence support this model. First, we have shown that a 73 nt TCE immediately downstream of the AUG site is both required to repress translational initiation of *nanos1* RNA and sufficient to repress a reporter. Second, enzymatic probing and nucleotide substitutions revealed regions around the start site that were resistant to single-strand-specific RNase attack. Third, repression was relieved and initiation complexes were formed only after the insertion of sufficient nucleotides (15, but not 3 or 6 nt) between the start site and the TCE to permit ribosome scanning, a critical test of our model. Fourth, both in oocytes and in oocyte extracts, *nanos1* repression persisted even in the presence of a vast excess of *nanos1* RNA, but was relieved by the addition of a small amount of embryo extract, ruling out the involvement of a soluble repressor. After fertilization, endogenous *nanos1* is translated, strongly suggesting the presence of a developmentally regulated activator.

In eukaryotes, the known mechanisms for mRNA-specific translational repression require trans-acting factors that interact with cis-regulatory regions (TCEs), which are most commonly found in the 3'UTR (reviewed by Livingstone et al., 2010). TCEs for which structure is a critical feature are not common, but have been described for *nanos* repression in *Drosophila* and *C. elegans* (D'Agostino et al., 2006; Gavis et al., 1996; Kalifa et al., 2006). Perhaps the best-known structural element controlling translation in eukaryotes is the iron-response element (IRE), a stem-loop in the

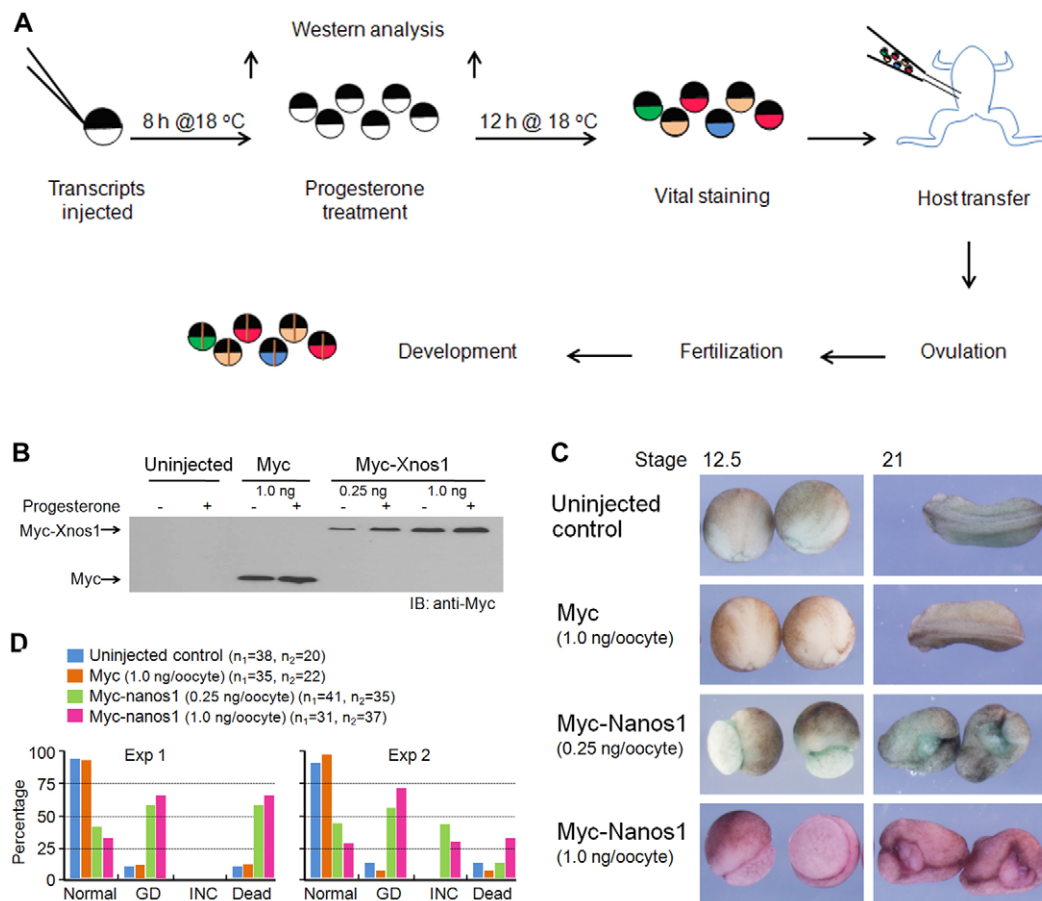


Fig. 6. Misexpression of *nanos1* in oocytes results in abnormal development. (A) Schematic of host transfer experiment. (B) Following the procedure in A, oocytes were injected with either Myc-*nanos1* RNA or control Myc RNA and analyzed by blotting with anti-Myc antibody. RNAs were translated in a dose-dependent fashion. (C) *Xenopus* embryos from experiment 2 (see D) showing representative phenotypes that result from *nanos1* expression in oocytes. Uninjected oocytes or Myc-injected oocytes served as controls and were normal. Embryos observed at stage 12.5 fail to close their blastopores. Surviving embryos display incomplete neural tube closure at stage 21. (D) The distribution of phenotypes from two independent experiments. Embryos with a gastrulation defect (GD) were counted at stage 12.5 and those with incomplete neural tube closure (INC) or that were dead were counted at stage 21. The key includes the total number of embryos in each category for each experiment.

5'UTR of the ferritin gene and other genes that regulate iron homeostasis (Theil and Eisenstein, 2000). The IRE appears to be unique within eukaryotes as it does not inhibit initiation by interfering with the formation of the initiation complex, but rather is recognized by the iron-response protein, and together they sterically inhibit scanning of the 43S initiation complex.

Our model (Fig. 7) depends on the predicted structures in the TCE being sufficiently stable to prevent the initiation complex from forming. Stem-loops with a free energy of at least -50 kcal/mol inhibit ribosomal scanning in COS cells (Kozak, 1986). Stem-loops I and II in *Xenopus laevis* are predicted to have a free energy of -17 and -56 kcal/mol, respectively. Thus, based on thermodynamic considerations alone, the RNA secondary structures surrounding the AUG, including the 15 nt 5'UTR, are sufficient to prevent ribosome scanning and initiation events. However, disrupting the predicted Stem II by substituting a repeat of one side did not relieve repression, suggesting that this GC-rich region can form alternative structures of substantial stability (Fig. 2E). Consistent with this interpretation, disruption of the base-pairing by AU substitutions did 'loosen' the TCE as revealed by increased translational efficiency. The predicted structure might simply not be accurate. Our data also suggest a role of the

evolutionarily conserved *nanos1* 5'UTR in contributing to the repressive ability of the TCE, perhaps by further stabilizing the TCE. Low levels of *nanos1* translation could be detected after RNA injection into oocytes. We cannot rule out the possibility that additional repressor-based mechanism(s) are operating, such as the physical sequestration of *nanos* within germinal granules. Although novel in eukaryotes, structural inhibition of translation has been demonstrated repeatedly in the regulation of prokaryotic mRNAs (Chowdhury et al., 2006; Serganov, 2009; Kozak, 2005).

Toe-printing analysis has established that the binding cleft of the 40S subunit requires 15 nt downstream of the first nucleotide at the P site (Pestova et al., 2001). The proposed TCE within the *nanos1* ORF is just 4 nt from the start codon. An insertion of 3 or 6 nt, which would result in only 7 or 10 nt between the start codon and the TCE, would not allow for 40S subunit positioning and efficient translation, as predicted from our model. However, an insertion of 15 nt would allow ribosome binding and efficient translation. An alternative, but less likely, explanation is that the addition of more nucleotides changed the structure so profoundly as to allow translation. We expect that such binding and subsequent translocation of the mRNA through the P site generates sufficient force to disrupt the TCE (Takyar et al., 2005).

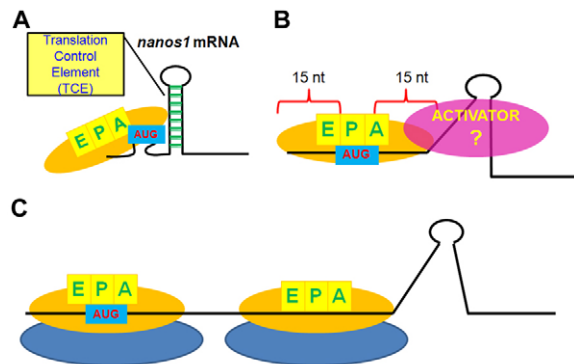


Fig. 7. Model for *nanos1* translational repression. (A) The secondary structure formed by the first 73 nt in the *nanos1* ORF sterically prevents efficient loading of mRNA on the 43S pre-initiation complex, blocking translation in oocytes and in wheat germ extracts. (B,C) A developmentally regulated activator becomes available soon after fertilization, disrupts the secondary structure (B) and allows ribosome loading and efficient translation (C). Note that disruption of the secondary structure by deletion, insertion or substitution or by putative endogenous helicases allows loading of the P site and formation of the 48S initiation complex. 40S (yellow) and 60S (blue) ribosomal subunits. EPA, sites in 40S subunit required for initiation.

The steric hindrance model predicts that an activator unwinds the TCE and permits an initiation complex to form (Fig. 7). Interestingly, *nanos1* RNA injected into embryos was translated and its protein accumulated in somatic cells, indicating that the putative activator might not be restricted to the germline. We have ruled out three known germline helicases – DeadSouth, Centroid and Vasa – as well as eIF4F (Jaramillo et al., 1990) and the unwinding RNA-editing enzyme originally discovered in *Xenopus* embryos (Bass and Weintraub, 1988). Although we know of no examples, it is possible that the activator is an RNA that disrupts the TCE secondary structure. Fertilization initiates complex signaling events including calcium influx, activation of a kinase cascade and the translation of specific mRNAs (Richter, 2007). A possible scenario is that fertilization triggers the activity of a reserved cytoplasmic factor or stored mRNA within the germ plasm, which in turn activates *nanos1* translation. The identity of such a putative activator is currently under intensive investigation.

Nanos1 synthesis occurs prior to the complete segregation of the germline during gastrulation, yet its presence in the somatic lineages is not tolerated and results in abnormal development. How then is Nanos1 activity confined to the germline? The germ plasm matrix could function as a scaffolding platform that anchors the germ cell determinants, including *nanos1* RNA and Nanos1 protein. We have not observed *nanos1* RNA or protein outside of PGCs or, for that matter, germ plasm (Lai et al., 2011) (Fig. 5D). Alternatively, any *nanos1* RNA outside the germ plasm would have to be efficiently degraded, a mechanism described for *nanos* in *Drosophila* and zebrafish (Bashirullah et al., 1999; Mishima et al., 2006). Both *Drosophila* and zebrafish have inefficient modes of *nanos* RNA localization, necessitating a post-transcriptional control mechanism (Gavis et al., 2008; Giraldez et al., 2006). In zebrafish, maternal mRNAs are degraded by zygotic miR-430 during early embryogenesis. *Xenopus* miR-427 has been shown to resemble the orthologous zebrafish miR-430 in loss-of-function analyses and in its expression pattern and target specificity (Giraldez et al., 2006;

Lund et al., 2009). However, we found ectopically expressed *nanos1* RNA and protein to be stable within somatic cells (Fig. 5E; data not shown).

Interestingly, ectopic expression of Nanos1 protein in oocytes did not affect oocyte maturation or early cleavage stages. However, it did cause severe gastrulation defects and, at lower levels of lethality, incomplete neural tube closure. These are likely to be gain-of-function phenotypes. Nanos1 functions as a translational repressor of *nanos*-response element (NRE)-containing mRNAs such as *VegT* (Wharton et al., 1998; Nakahata et al., 2001) (our unpublished observations). We speculate that Nanos1 expressed in somatic cells represses regulatory pathways that are important for development, resulting in the observed embryonic abnormalities. Our results are consistent with a requirement for maternal *nanos1* to be translationally repressed everywhere outside of the germ plasm. Identification of mRNAs targeted by Nanos1 for repression will be important in understanding the gain-of-function phenotype.

The model presented here is appealing because it offers a simple explanation for how somatic cells prevent translation of any unlocalized *nanos1* mRNA. Nanos family members are potent repressors of somatic cell fates, functioning as repressors of translation and perhaps transcription as well (Curtis et al., 1997; Deshpande et al., 2005; Kadyrova et al., 2007; Lai et al., 2011). The need to restrict Nanos translation to the germline is crucial, with misexpression resulting in embryonic lethality. Structural inhibition of translation would provide a robust means of repression as it would be intrinsic to the transcript and independent of the proper localization of the message or the proper expression/stability of a repressor. Reliance on the stability of a repressor over long periods of time, as occurs during oogenesis, might present too high a biological risk. We predict that other mRNAs that encode regulatory proteins and are stored over extended periods of time would be excellent candidates for using a structure-based mechanism of translational repression. It will be of great interest to identify such mRNAs that contain complex RNA structures in close proximity to the start codon.

Acknowledgements

We thank Drs Lasko, Lipshitz, Gavis, Steitz, Rotundo and Malhotra for encouragement and advice and especially Drs Deutscher and Houston for their generosity in sharing reagents and methods. This work was supported by NIH grant GM33932 to M.L.K. Deposited in PMC for release after 12 months.

Competing interests statement

The authors declare no competing financial interests.

Supplementary material

Supplementary material for this article is available at <http://dev.biologists.org/lookup/suppl/doi:10.1242/dev.056705/-DC1>

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Table S1. Primer sequences

Construct	Primer sequences (5' to 3')
<i>Myc-nanos1/pCS2+</i>	GCTACGAATTCAATGGATGGCGGTCTCTGCTT TCGATCTCGAGTCAGTGTCTCAGCTTTGGGT
<i>nanos1-Myc/pCS2+</i>	GACTGAGGATCCCAGAACAATTCCAACATGGA GACTGTATCGATCGTGTCTCAGCTTTGGGTTA
<i>nanos1-TCEΔ/pSPORT1</i>	CAATTCCAACATGGATCAGCCTCAGAGAGAAG CTTCTCTCTGAGGCTGATCCATGTTGGAATTG
<i>nanos1-3 nt insertion/pSPORT1</i>	CAATTCCAACATGGATT TGGG CGGTCTCTGCTTTGAC GTCAAAGCAGAGACCGCCCAAATCCATGTTGGAATTG
<i>nanos1-6 nt insertion/pSPORT1</i>	CAATTCCAACATGGATT TGACAGG CGGTCTCTGCTTTGAC GTCAAAGCAGAGACCGCCTGTCAAATCCATGTTGGAATTG
<i>nanos1-15 nt insertion/pSPORT1</i>	CAATTCCAACATGGATT TGACAGCACATGAT GGCGGTCTCTGCTTTG CAAAGCAGAGACCGCCATCATGTGCTGTCAAATCCATGTTGGAATTG
<i>nanos1-TCEΔ-Myc/pCS2+</i>	CAATTCCAACATGGATCAGCCTCAGAGAGAAG CTTCTCTCTGAGGCTGATCCATGTTGGAATTG
<i>nanos1-TCE-Myc/pCS2+</i>	GGGGGCTGCAGCATCGATGAGAAGGCGAAAG CTTTCGCCTTCTCATCGATGCTGCAGCCCC

β-globin sequences inserted to create the mutants used in Fig. 4 are in bold.