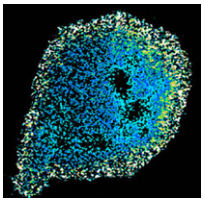
Patterning needs a little sweetener

N-linked glycosylation is a protein modification needed for protein folding in the endoplasmic reticulum (ER). If unfolded proteins accumulate in the ER, then the 'unfolded protein response' (UPR) is triggered, increasing folding rates and reducing translation rates. On p. 1745, Mattias Mannervik and colleagues describe the first embryonic patterning defects known to be caused by an inappropriate UPR. In their screen for maternal factors involved in embryonic patterning, they discovered a mutant – *wollknäuel* (*wol*) – that has reduced Dpp signalling, posterior segmentation defects due to a lack of the transcription factor Caudal, and defects in germband elongation and retraction. *wol* encodes ALG5, a UDP-glucose:dolichyl-phosphate glucosyltransferase involved in N-linked glycosylation, and its mutation causes the accumulation of unglycosylated proteins and triggers the UPR. One component of the UPR is the phosphorylation of the translation initiation factor eIF2α, which attenuates protein translation. These findings suggest that some mRNAs, such as *caudal*, are particularly sensitive to eIF2α phosphorylation, resulting in the *wol* patterning defects.

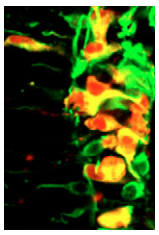
Reaction-diffusion mechanism for ancestral FGF signalling

The sea anenome *Nematostella vectensis* belongs to the Cnidaria phylum, which split from the Bilateria 600 million years ago. Similar to several basal bilaterian species, its larvae have an apical ciliary organ, which is believed to detect conditions suitable for metamorphosis. In their study of FGF signalling in *N. vectensis* development (see p. 1761), Fabian Rentzsch and colleagues used morpholino-mediated knockdown to analyse the function of two FGF ligands, NvFGFa1 and NvFGFa2, and of the NvFGFRa receptor. Their findings show that NvFGFa1 signalling via NvFGFRa is required for apical organ formation and that NvFGFa1 knockdown blocks metamorphosis. They also show that NvFGFa1 not only activates its own expression but also that of the antagonistic NvFGFa2, which possibly binds to NvFGFRa, without activating it, to restrict NvFGFa1's initially broad expression and to prevent ectopic organ formation. These findings provide the first known example of two FGF ligands that have activating and inhibiting effects consistent with a reaction-diffusion mechanism, and highlight an ancestral FGF signalling function.



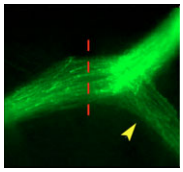
Syn4 and PCP give protrusive cell directions

Directed cell migration is crucially important for development, and is a feature of neural crest (NC) cells, which have remarkable migratory abilities. On p. 1771, Roberto Mayor and colleagues investigate how NC cells keep to the right path in zebrafish and *Xenopus* embryos, by studying the effects of a proteoglycan, Syndecan-4 (Syn4), on NC migration. Syn4, they report, is essential for directional NC migration, and directs NC cell movement by regulating the polarised formation of membrane protrusions, in a manner similar to that of non-canonical Wnt/planar cell polarity (PCP) signalling. To investigate how Syn4 orientates cell protrusions, the authors used in vivo FRET analysis to measure the localised activity of several small GTPases involved in cell migration. Syn4, they discovered, inhibits Rac activity, a small GTPase that controls cytoskeletal dynamics and cell adhesion, while PCP signalling activates RhoA, which also inhibits Rac in NC cells. Thus Syn4 and PCP signalling seemingly control directional NC migration by regulating membrane protrusions by inhibiting Rac at the back of the cell.



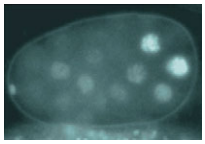
Notch and Sox: different routes to progenitor maintenance

During development of the chick nervous system, a combination of Notch signalling and SoxB1 transcription factors (Sox1, Sox2 and Sox3) maintains a pool of self-renewing stem and progenitor cells. On p. 1843, Jonas Muhr and colleagues investigate whether Notch and SoxB1 proteins suppress neuronal differentiation through the same, or different, pathways. By expressing dominant-negative components of these pathways in chick embryos, they show that, although Notch requires SoxB1 to maintain progenitor characteristics, SoxB1 activity blocks neurogenesis independently of Notch. Notch represses the activity of bHLH proneural proteins via the bHLH transcription factors Hes1 and Hes5, but, the researchers found, also represses E-proteins – the heterodimerizing partners of proneural proteins – through a Hes-independent mechanism. SoxB1 proteins, by contrast, seem to maintain progenitors by creating a molecular environment in which E-proteins and proneural proteins cannot promote neuronal differentiation. As Notch, Sox and bHLH proteins are also expressed in muscle and neural crest progenitor populations, the authors suggest their results could be of broader relevance.



Crossing a line in axon guidance

In bilaterally symmetric animals, the central nervous system is divided into two halves, and, during development, the proper formation of neuronal circuitry sometimes requires that axons choose whether they should project to the same side (ipsilateral) or to the opposite side (contralateral) of the embryonic midline. Many axon guidance molecules contribute to this decision, but little is known of their transcriptional regulation. Now in their study of the optic chiasm – the neuronal structure required for binocular vision – Eloísa Herrera and colleagues (p. 1833) report, for the first time, a link between a transcription factor (Zic2) and an axon guidance molecule (EphB1) in controlling axonal laterality. By manipulating *Zic2* expression in *EphB1*-expressing and *EphB1*-null mice, they show that Zic2 is sufficient to switch the contralateral trajectory of retinal axons to an ipsilateral one. Zic2 can do this via both EphB1-dependent and -independent mechanisms. From their findings, the authors propose that transcription factors can directly and sequentially activate different guidance receptors throughout an axon's journey.



How *nanos* is kept on hold

Many maternally provided transcripts play crucial roles in early development and often require tight translational regulation. During *C. elegans* embryogenesis, the maternal transcript *nanos-2* (*nos-2*) is translationally repressed until the germline founder cell, called P_4, is born. In their dissection of this process (see p. 1803), Kuppuswamy Subramaniam and co-workers have discovered that four additional proteins (OMA-1, OMA-2, MEX-3 and SPN-4) are involved in this repression of *nos-2*. These proteins bind to the 3′ UTR of *nos-2* and repress it at different developmental stages: OMA-1 and OMA-2 in oocytes, and MEX-3 and SPN-4 in the embryo. What eventually releases *nos-2* repression in P_4, the authors propose, is the competition between SPN-4 and POS-1 (a protein required for *nos-2* translation) to bind to *nos-2*. Thus, POS-1 works, not by activating translation, but by de-repressing it; as such, the authors believe that the relative concentrations of POS-1 and SPN-4 have a crucial role in initiating germ cell-specific developmental programmes.

Jenny Bangham

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Multiple maternal proteins coordinate to restrict the translation of *C. elegans nanos-2* to primordial germ cells

Shreyas Jadhav, Mainpal Rana and Kuppuswamy Subramaniam*

Although germ cell formation has been relatively well understood in worms and insects, how germ cell-specific developmental programs are initiated is not clear. In *Caenorhabditis elegans,* translational activation of maternal *nos-2* mRNA is the earliest known molecular event specific to the germline founder cell P_4. Cis-elements in *nos-2* 3′UTR have been shown to mediate translational control; however, the trans-acting proteins are not known. Here, we provide evidence that four maternal RNA-binding proteins, OMA-1, OMA-2, MEX-3 and SPN-4, bind *nos-2* 3′UTR to suppress its translation, and POS-1, another maternal RNA-binding protein, relieves this suppression in P_4. The POS-1: SPN-4 ratio in P_4 increases significantly over its precursor, P_3; and POS-1 competes with SPN-4 for binding to *nos-2* RNA in vitro. We propose temporal changes in the relative concentrations of POS-1 and SPN-4, through their effect on the translational status of maternal mRNAs such as *nos-2*, initiate germ cell-specific developmental programs in *C. elegans*.

KEY WORDS: *Caenorhabditis elegans*, Translational control, RNA-binding protein, 3′UTR, Germ cells, Nanos

INTRODUCTION

Many maternally expressed genes play crucial roles during early embryonic development. As these genes are transcribed prior to fertilization, regulation at post-transcriptional stages is key for their proper functioning. Studies on many maternal mRNAs have revealed a central role for translation regulation in the proper coordination of various early events of embryogenesis. For example, pattern formation in *Drosophila* embryo depends on the translational control of maternal mRNAs such as *oskar*, *nanos*, *caudal* and *hunchback* (Macdonald and Smibert, 1996; Dean et al., 2002). Similarly, the translational control of maternal mRNAs such as *glp-1*, *apx-1* and *pal-1* are essential for fate specification of certain blastomeres of *Caenorhabditis elegans* embryo (Evans and Hunter, 2005).

Genetic studies in flies point to the functioning of cascades of translational control during embryogenesis (Kuersten and Goodwin, 2003). For example, development of posterior structures depends on the restriction of *hunchback* translation to the anterior by Nanos (Sonoda and Wharton, 1999). Nanos translation, in turn, is restricted to the posterior by the action of Smaug and Oskar (Dahanukar et al., 1999; Smibert et al., 1999). Once again, translational control restricts Oskar to the posterior (Gunkel et al., 1998). However, barring a few examples of translational cascades characterized in *Drosophila*, their role during embryogenesis still remains largely unexplored. Protein factors involved in the translation of several maternal mRNAs are not known. Similarly, the target mRNAs for many maternal RNA-binding proteins have yet to be identified. Identification of these will be essential to obtain a complete picture of translational control in development.

The development of primordial germ cells (PGCs) in *C. elegans* is a good example of an embryonic process involving complex translational control. In this organism, the maternal components required for germ line development are sequestered to a single cell at the first embryonic division itself (Seydoux and Strome, 1999). However, the formation of PGCs is postponed to a later stage. This is because, as shown in Fig. 1, the posterior lineage, which preserves germline-specific maternal components, gives rise to various somatic lineages during the first four divisions. Therefore, the maternal mRNAs essential for the activation of germ cell-specific developmental programs must remain translationally quiescent through various developmental events from oocyte until the formation of the germline founder cell P_4, which is born at the 28-cell stage. Although the CCCH-type zinc finger protein PIE-1 has been shown to be essential for RNA maintenance in germline blastomeres (Tenenhaus et al., 2001), it is not clear how the translational quiescence is maintained.

The maternal mRNA encoded by *nos-2*, a *C. elegans* member of the *nanos* family of germ cell regulators, is currently the only known mRNA whose translation is specifically activated in P_4 (Subramaniam and Seydoux, 1999; D'Agostino et al., 2006). Earlier results have shown that the translation of *nos-2* is repressed from oocytes until 28-cell embryo, and that this repression requires the functions of three distinct 3′UTR elements. It has also been shown that the CCCH-finger protein, POS-1, is essential for the activation of translation in P_4 (D'Agostino et al., 2006). Here, we report the identification of four additional maternal RNA-binding proteins, namely OMA-1, OMA-2, MEX-3 and SPN-4, which suppress *nos-2* translation in successive stages: OMA-1 and OMA-2 (suppress in oocytes), MEX-3 (in early embryo) and SPN-4 (in germline blastomeres). We find that these proteins suppress translation by directly binding to *nos-2* 3′UTR. Furthermore, our results presented here suggest that POS-1 activates *nos-2* translation in P_4 by competing out SPN-4 for binding to *nos-2* 3′UTR. Thus, temporal changes in the concentration of these maternal RNA-binding proteins appear to mediate the PGC-specific activation of *nos-2* translation.

MATERIALS AND METHODS

C. elegans strains

Worm strains were maintained as described (Brenner, 1974), except that all transgenic lines were kept at 25°C to avoid silencing of transgene expression in the germline (Strome et al., 2001). Transgenes were introduced into *unc-*

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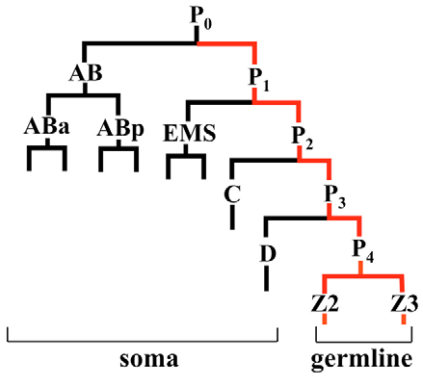

Fig. 1. A line diagram showing abbreviated embryonic lineage. The P lineage is shown in red. See Sulston et al. (Sulston et al., 1983).

119(–) strain by biolistic bombardment as described (Praitis et al., 2001), with the following modifications: 1 μm tungsten particles were used as the micro carrier with 1500-psi rupture discs. Mutant versions of the transgene were created by PCR and inserted into the plasmid, pKS111HisΔ5, which contains the GFP:H2B:*nos-2* 3′UTR (D'Agostino et al., 2006). The following strains were used:

EU769, spn-4(or25) unc-42(e270) V/nT1[let-?(m435)] (IV;V); JJ1014, mex-3(zu155) dpy-5(e61)/hT1 I; pos-1(zu148) unc-42(e270)/hT1 V; JJ462, +/nT1 IV; pos-1(zu148) unc-42(e270)/nT1 V.

RNAi screen

A list of genes that encode putative RNA-binding proteins was prepared based on annotations available at www.wormbase.org. Of these, 131 were part of a library of RNAi clones (see Table S1 in the supplementary material) (Kamath et al., 2003). Other target open reading frames (ORFs) were PCR amplified, inserted into the RNAi feeding vector, L4440 and introduced into *E. coli* HT115. These *E. coli* clones were used for inducing RNAi by the feeding method (Timmons et al., 2001) in transgenic worms carrying pKS111HisΔ5.

Protein expression and purification

Full-length ORFs of *mex-3* was PCR-amplified and inserted at the *Bam*HI site of pMAL-c4E, which expresses the inserted ORF as a fusion protein with the maltose-binding protein (MBP) (New England Biolabs). The ORF of *spn-4* was cloned between *Eco*RI and *Xho*I sites, and the ORFs of *oma-1* and *oma-2* between *Eco*RI and *Not*I sites of pGEX-4T1 vector. The *pos-1* ORF was inserted between *Eco*RI and *Bam*HI sites of pGEX-2T. The pGEX vectors express the cloned ORF as GST fusion protein (GE Lifesciences). Cloning techniques, including PCR, were carried out following standard protocols (Sambrook et al., 1989).

The transformants were grown in LB medium at 37°C until 0.5 OD at 600 nm before induction with 0.05 mM IPTG for 2 hours at 16°C. Cells were collected by centrifugation and lysed in lysis buffer [20 mM HEPES (pH 7.4), 0.5 M NaCl, 5 mM DTT, 0.02% Tween 20, 0.1 mM PMSF] by incubation on ice with 0.5 mg/ml of lysozyme, followed by 3 rounds of freeze-thaw cycles. The lysates were treated with 20 μg/ml of DNase I and cleared by centrifugation. Fusion proteins were purified from clear supernatants by affinity chromatography using either amylose resin (MBP:MEX-3) or glutathione-agarose (GST fusions) following manufacturers' protocols (MBP, New England Biolabs; GST, GE Lifesciences). Purified proteins were concentrated by ultrafiltration, added with glycerol to a final concentration of 50% and stored at –20°C.

Electrophoretic mobility shift assay

Radiolabeled RNA fragments used for EMSA were prepared by in vitro transcription of DNA template using T7 RNA polymerase (Fermentas) with α-^{32}P CTP (specific activity: 3000 Ci/mmole) following standard protocols (Sambrook et al., 1989). Full-length transcripts were purified from urea gel and quantitated using a liquid scintillation counter. Template DNAs were generated by PCR amplification using appropriate primers from pKS111HisΔ5. The T7 promoter sequence was incorporated to DNA templates through the forward PCR primer. Required mutations were also introduced through PCR primers. A 360 bp cDNA fragment encoding the splicing factor (GenBank accession # AW828516) of *Meloidogyne incognita*, a parasitic nematode, was used as template for generating the non-specific unlabeled RNA. This RNA is not GC rich and, using the M fold RNA folding program (Zuker, 2003), we found that it does not form long stretches of stable double-stranded structures (data not shown). Unlabeled RNA was prepared in the same manner as above except that the α-^{32}P CTP was replaced with CTP.

Binding reactions were carried out by incubating the appropriate RNA and protein in RNA-binding buffer [5 mM HEPES (pH 7.5), 25 mM KCl, 2 mM $MgCl_2$, 1 mM EDTA, 2 mM DTT, 3.5% glycerol, 0.25 mg/ml yeast tRNA] at room temperature (RT) for 20 minutes. RNA was denatured by first incubating at 75°C for 10 minutes and then at 37°C for a further 10 minutes, before adding to the binding reactions. All lanes contained identical amounts of RNA and protein, except where indicated. For competitions, protein was incubated simultaneously with radiolabelled RNA and indicated amounts of unlabeled RNA. The reaction mixtures were eletrophoresed at +4°C at 200 V on a 16 × 20 cm non-denaturing polyacrylamide gel in TBE buffer. The concentration of acrylamide-bisacrylamide mix in these gels was 3.5% in the case of MEX-3, 6% in the case of OMA-1 and OMA-2 fusion proteins, and 7.5% in the case of POS-1 and SPN-4 fusion proteins. Duration of electrophoresis varied, depending on the size of the RNA, from 4 to 20 hours. Following electrophoresis, the gel was dried and exposed to phosphor imager screen and imaged using a phosphor imager (Personal Molecular Imager FX, BioRad). Intensity of radioactive bands were quantitated using the Quantity One software (BioRad).

Pull down assay

This assay, similar to the affinity purification of fusion proteins described above, depends on the affinity of GST and MBP for their corresponding ligands, glutathione and amylose, respectively. For binding experiments with POS-1, glutathione-agarose beads were first washed three times in distilled water, then five times in RNA-binding buffer (RBB). Washed beads were incubated with GST::POS-1 at +4°C for 20 minutes with gentle agitation. Protein-bound beads were incubated with RNA in RBB for 20 minutes at room temperature. After the incubation period, the beads were collected by brief centrifugation and washed five times with RBB. The GST::POS-1 protein was eluted from beads with 20 mM glutathione and the bound RNA was separated by phenol:chloroform extraction. The RNA was then precipitated and separated on a 6% acrylamide gel containing 8 M urea. The gel was dried and exposed to phosphor imager screen as described earlier. Binding experiments with MEX-3 were performed in a similar manner, except that amylose resin and maltose were used as the solid matrix and eluant, respectively.

Immunofluorescence

Embryos permeabilized by the freeze-crack method and fixed in formaldehyde were immunostained as described (Subramaniam and Seydoux, 1999). The following primary antibodies were used: anti-POS-1 (Tabara et al., 1999) and anti-SPN-4. Anti-SPN-4 antibodies were obtained by affinity purification of polyclonal antiserum of rabbits immunized with GST:SPN-4. Immunofluorescence, as well as the GFP fluorescence from embryos was imaged using a fluorescence microscope (Zeiss Axioskop) and CCD camera (Axiocam HRm). Immunofluorescence signal intensities were quantitated by measuring pixel density of deconvoluted *z*-stack images using Axiovision software.

RESULTS

Identification of proteins that control *nos-2* translation

To identify proteins involved in the translational control of *nos-2* mRNA, we carried out an RNAi-based screen of genes predicted to encode proteins with RNA-binding motifs. To facilitate the monitoring of NOS-2 expression, we performed the RNAi on

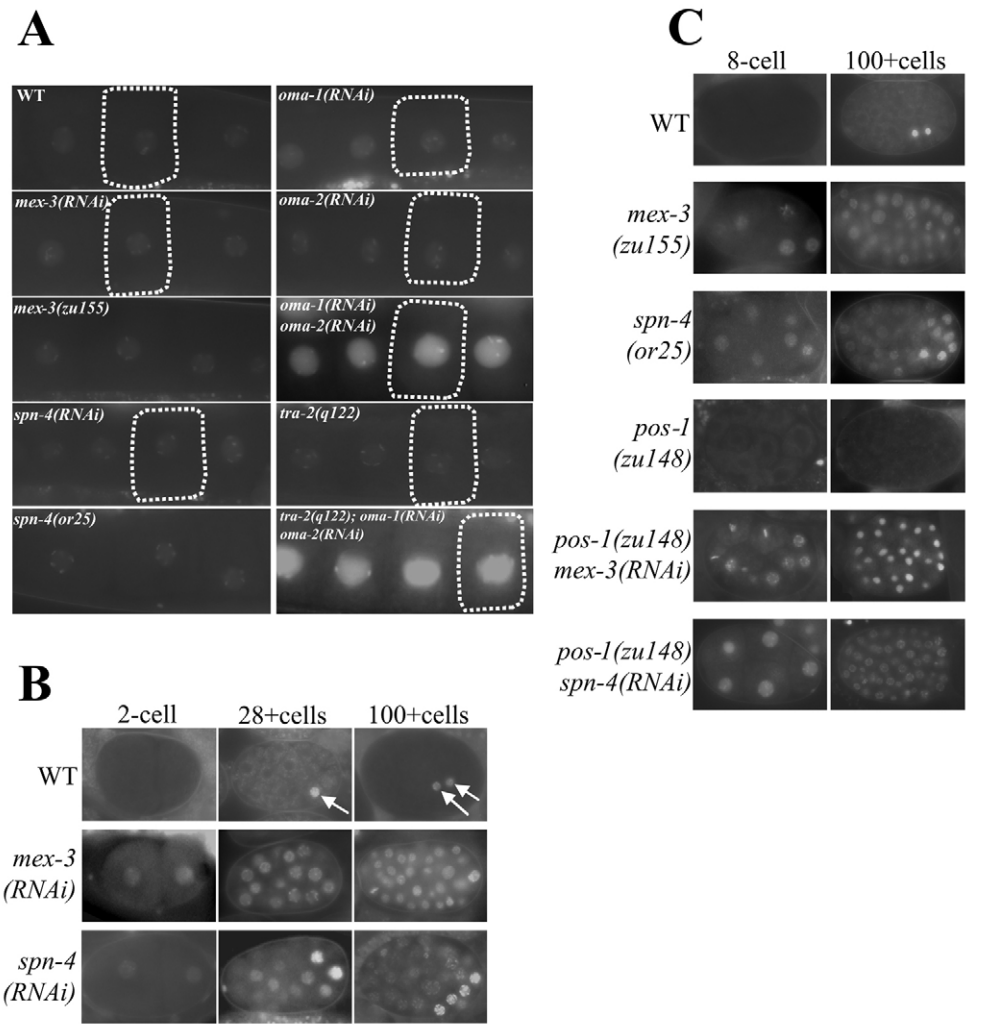

Fig. 2. Identification of proteins that control *nos-2* translation. (**A**,**B**) OMA-1, OMA-2, MEX-3 and SPN-4 are essential for the translation suppression of *nos-2* mRNA. Distribution pattern of GFP:H2B expressed under the control of *nos-2* 3′UTR in oocytes (A; a single oocyte in each panel is outlined) and embryos (B) is shown. Genes disrupted by RNAi treatment are indicated in each panel; WT, non-RNAi control. To facilitate visualization, we expressed GFP as a fusion protein with the histone H2B, which concentrates fluorescence signal in nuclei. (**C**) POS-1 acts as a de-repressor of *nos-2* translation. Epistasis analysis of GFP:H2B expression among *mex-3(–)*, *spn-4(–)* and *pos-1(–)* shown here reveals that POS-1 is not required for *nos-2* translation in the absence of repressors such as MEX-3 and SPN-4.

transgenic worms that express the GFP:H2B reporter under the control of *nos-2* 3′UTR (see Materials and methods for details). Expression pattern of GFP:H2B in embryos of these worms is reminiscent to that of the endogenous NOS-2 protein (D'Agostino et al., 2006). This screen identified four genes, namely *oma-1*, *oma-2*, *mex-3* and *spn-4*, the downregulation of which by RNAi resulted in misexpression of GFP:H2B (Fig. 2A,B). In the non-RNAi control embryos, GFP:H2B was not detected in any of the blastomeres until the 28-cell stage. Similar to endogenous NOS-2 expression, GFP:H2B first appeared at the 28-cell stage in the germline founder cell P_4. By contrast, in the case of *oma-1(RNAi) oma-2(RNAi)* 'double mutants', significantly higher levels of GFP:H2B were first detected in oocytes. As OMA-1 and OMA-2 are essential for oocyte maturation, their absence leads to oocyte arrest (Detwiler et al., 2001). To test whether the increased GFP:H2B expression was merely a result of accumulation of GFP in the arrested oocytes, we introduced the GFP:H2B:*nos-2* 3′UTR transgene into *tra-2(q122)* worms, another mutant in which the unmated hermaphrodites accumulate oocytes (Barton et al., 1987), and examined the expression of GFP:H2B in their oocytes. As shown in Fig. 2A, these oocytes did not show any increase in the level of GFP:H2B over wild-type control. By contrast, removal of OMA-1 and OMA-2 proteins in these worms by RNAi led to a dramatic increase in the level of GFP:H2B in their oocytes. Expression of GFP:H2B in *oma-1(RNAi)* and *oma-2(RNAi)* 'single mutants' were almost at the level of non-RNAi control oocytes, which is probably a result of functional redundancy between these two nearly identical genes (Fig. 2A). We conclude OMA-1 and OMA-2 function redundantly to suppress *nos-2* translation in the oocyte.

In *mex-3(RNAi)* embryos, GFP:H2B was present in all the blastomeres of embryos starting from the two-cell stage (Fig. 2B). We observed similar expression pattern in *spn-4(RNAi)* embryos as well, except that in these embryos GFP:H2B expression was significantly more pronounced in a few posterior blastomeres. Disruption of *mex-3* or *spn-4* by RNAi did not alter the background levels of GFP:H2B seen in the oocytes of control worms. We observed similar results in the genetic null alleles of these genes (Fig. 2A,C) (Draper et al., 1996; Gomes et al., 2001). From these results, we conclude *mex-3* and *spn-4* are essential for the suppression of *nos-2* translation in the early embryo and probably not essential in oocyte.

POS-1 de-represses, rather than activates, *nos-2* translation

Earlier we had shown that the CCCH-type zinc-finger protein POS-1 is required for the activation of *nos-2* translation in the primordial germ cells (PGCs) (D'Agostino et al., 2006). The POS-1 protein could function either by activating translation or by relieving the translational repression by repressors such as MEX-3 and SPN-4. To distinguish between these two possibilities, we determined the

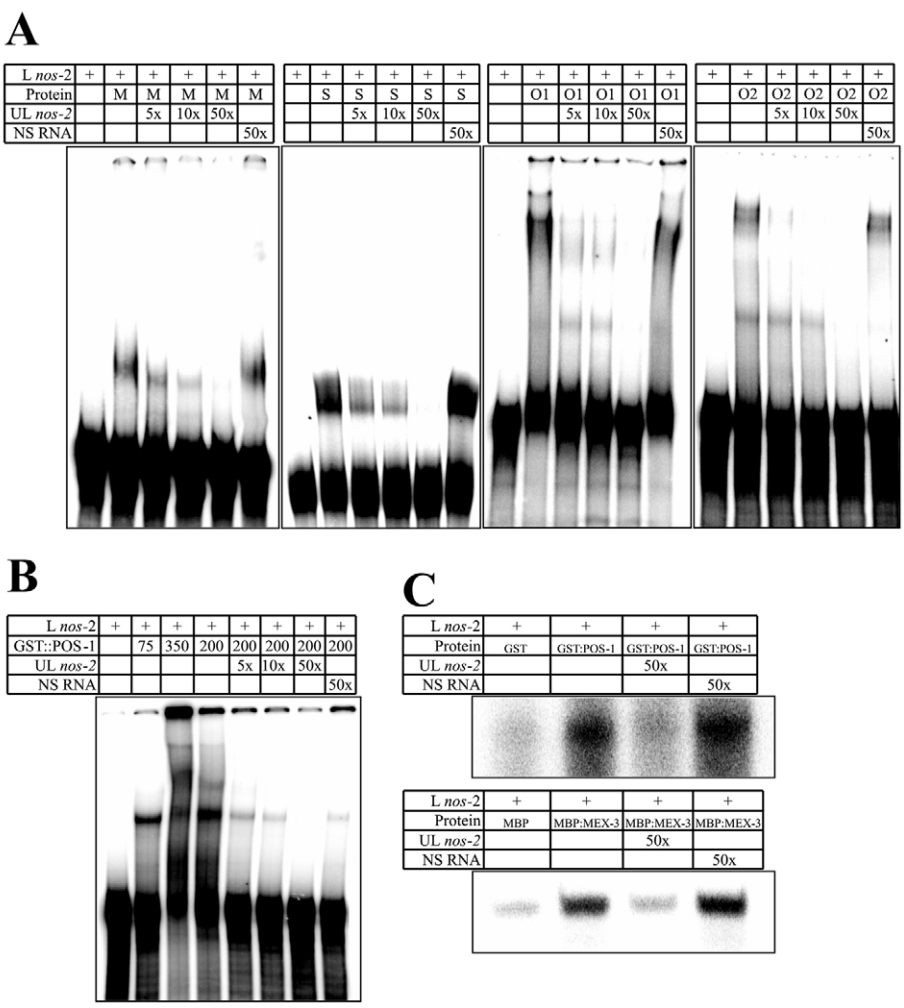

Fig. 3. MEX-3, SPN-4, OMA-1, OMA-2 and POS-1 physically interact with *nos-2* 3′UTR. (**A**) Electrophoretic mobility patterns of radiolabeled 200 bp *nos-2* 3′UTR RNA in the presence of MBP:MEX-3 (M), GST:SPN-4 (S), GST:OMA-1 (O1) and GST:OMA-2 (O2). L *nos-2*, radiolabeled 200 bp *nos-2* 3′UTR; UL *nos-2*, unlabeled *nos-2* 3′UTR; NS RNA, unlabeled non-specific RNA; 5×, 10× and 50×, number of times molar excess over L *nos-2*. (**B**) Electrophoretic mobility shift with GST:POS-1. Three different concentrations of GST-POS-1 were used: 75, 350 and 200 ng/μl. Comparison of lanes 2-4 indicates multimerization of this protein-RNA complex at higher protein concentrations. (**C**) Binding of radiolabeled *nos-2* 3′UTR RNA to solid matrix in presence of the indicated components (see Materials and methods for details).

epistatic relationship among these genes. If POS-1 were to be required for the activation of *nos-2* translation, then the ectopic GFP:H2B expression observed in embryos lacking MEX-3 or SPN-4 should be dependent on POS-1. However, if it functions as a derepressor, then GFP:H2B expression in *mex-3(RNAi)* or *spn-4(RNAi*) embryos would not be dependent on POS-1. To ensure complete absence of POS-1 protein, we used the null allele, *pos-1(zu148)* (Tabara et al., 1999), rather than *pos-1(RNAi)*, in these epistasis analyses. The pattern of GFP:H2B observed in *mex-3(RNAi) pos-1(zu148)* embryos was similar to that of *mex-3(RNAi)* and that in *spn-4(RNAi) pos-1(zu148)* embryos was similar to that of *spn-4(RNAi)* (Fig. 2C), indicating that the POS-1 protein is not required for *nos-2* translation in the absence of MEX-3 or SPN-4. We conclude that POS-1 derepresses, rather than activates, *nos-2* translation.

All five proteins, OMA-1, OMA-2, MEX-3, SPN-4 and POS-1, directly bind to *nos-2* 3′UTR

To begin to investigate the mechanism(s) of the translational control, we tested whether any of the proteins identified by the RNAi screen, including POS-1, physically interact with *nos-2* 3′UTR. For this, we expressed these proteins as GST (OMA-1, OMA-2, SPN-4 and POS-1) or MBP (MEX-3) fusion in bacteria and purified using affinity chromatography. The purified recombinant proteins were then tested for their ability to bind radiolabeled 200 bp minimal *nos-2* 3′UTR RNA in electrophoretic mobility shift assay (EMSA). This 200 bp RNA has been shown earlier to be sufficient for the endogenous expression pattern of NOS-2 (D'Agostino et al., 2006). As shown in Fig. 3, all five proteins retarded the electrophoretic mobility of *nos-2* RNA. In case of MEX-3, SPN-4, OMA-1 and OMA-2, incubation with 50-fold molar excess of unlabeled *nos-2* 3′UTR RNA completely abolished the shift of the radiolabeled RNA, whereas a similar molar excess of non-specific unlabeled RNA did not affect this mobility shift (Fig. 3A). In the case of POS-1, although the non-specific RNA did reduce the radioactive signal corresponding to mobility shift, its effect was far less than the unlabeled *nos-2* 3′UTR RNA (Fig. 3B). To validate these results further, we performed an alternative RNA-binding assay for POS-1 and MEX-3. This assay depends on the affinity of the fusion tags GST and MBP for their corresponding ligands covalently linked to a solid matrix (see Material and methods for details). Even in this assay, 50-fold molar excess of unlabeled *nos-2* 3′UTR RNA significantly reduced the amount of radiolabeled RNA bound to POS-1 or MEX-3. By contrast, a similar molar excess of a non-specific unlabeled RNA did not alter binding of the radiolabeled RNA (Fig. 3C). We used PUF-8, another RNA-binding protein, as a negative control; this protein did not bind *nos-2* 3′UTR RNA in either of the in vitro binding assays (data not shown). Based on the above results, we conclude that the five proteins identified in our RNAi screen can specifically and directly bind to the *nos-2* 3′UTR in vitro. As OMA-1 and OMA-2 share a high degree of sequence homology and their electrophoretic mobility shift patterns were identical, we tested only OMA-2 in the subsequent EMSAs.

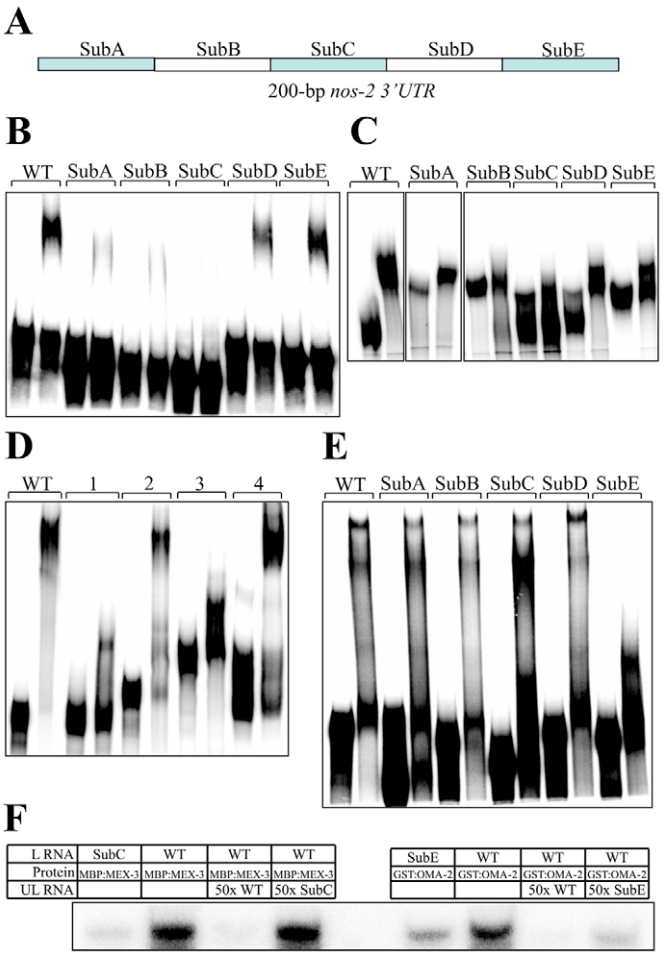

Fig. 4. Determination of *nos-2* 3′UTR regions that are critical for interaction with the various proteins. (**A**) Schematic illustration of the five regions of *nos-2* 3′UTR that were mutated by substitution. SubA begins immediately downstream of the stop codon. (**B-E**) Electrophoretic mobility shifts of various mutant versions of radiolabeled *nos-2* 3′UTR by MBP:MEX-3 (B), GST:SPN-4 (C) and GST:OMA-2 (D,E). The first lane in each set is the mobility of RNA in the absence of protein. Radiolabeled RNA used in D contained the wild-type version of the following regions only: 1, SubA-C; 2, SubB-E; 3, SubA-D; 4, SubD-E, whereas those in other panels contained the 200 bp *nos-2* 3′UTR with the indicated regions substituted with $(TG)_{15}$. WT in all panels indicate the wild-type version of the 200 bp *nos-2* 3′UTR. (**F**) Binding of radiolabeled WT and mutant *nos-2* 3′UTR RNA to solid matrix in presence of the indicated components (see Materials and methods for details). L RNA, radiolabeled RNA; UL RNA, unlabeled RNA.

We introduced a series of deletions in the 200 bp minimal *nos-2* 3′UTR and tested them in EMSA to identify the specific sequences that are responsible for the interaction. Deletion of any part of the minimal UTR abolished or significantly reduced the binding of MEX-3, SPN-4 and POS-1 (data not shown), indicating that the entire sequence of the minimal UTR may be essential for efficient interaction of these three proteins. Alternatively, it is also possible that the distance between different sequence elements within the UTR, rather than the whole of the minimal UTR sequence, is crucial for proper interaction. To address this, we substituted 30 bp stretches with a non-specific sequence [$(TG)_{15}$] of the same length and tested them in EMSA (Fig. 4). Of the five substitutions tested, SubB and SubC significantly reduced the mobility shift by MEX-3 and SPN-4 proteins (Fig. 4B,C), suggesting that the wild-type sequences of SubB and SubC are crucial for the binding of these two proteins. These results were further confirmed by the pull-down assay described above. In this assay, unlabeled SubC substitution did not compete with labelled wild-type RNA as efficiently as the unlabeled wild-type RNA for binding to MEX-3. Consistently, the binding of radiolabeled SubC substitution was poorer when compared with the wild type (Fig. 4F). Surprisingly, none of the substitutions had an appreciable effect on POS-1 binding (data not shown). By contrast, OMA-2 bound the region defined by SubD and SubE substitutions as efficiently as the 200 bp 3′UTR (Fig. 4D). Consistent with this, in the substitution analysis, only SubE significantly reduced OMA-2 binding (Fig. 4E). The binding of SubE substitution was significantly weaker in the pull-down assay as well (Fig. 4F). These results indicate that the SubE region is sufficient for OMA-2 interaction with *nos-2* 3′UTR.

Remarkably, SubB and SubC regions contain two 8 bp direct repeats (DR1 and DR2), which have been well conserved in the *nos-2* 3′UTR among the three *Caenorhabditis* species for which sequence information is available [Fig. 5A and D'Agostino et al. (D'Agostino et al., 2006)]. To test whether these two repeats are essential for the RNA-protein interactions, we replaced these repeats with $(TG)_4$ and tested in EMSA. Mutations in only one of either direct repeats reduced the mobility shift by MEX-3, which was further weakened when both repeats were simultaneously mutated (Fig. 5B). These results indicate that the two direct repeats are essential for the binding of MEX-3. In the case of SPN-4, although the double mutant and the DR1 mutant significantly reduced the shift, DR2 mutations did not affect the mobility shift, indicating a crucial role for DR1 in SPN-4 binding (Fig. 5B). Together these results suggest that both MEX-3 and SPN-4 may bind the same region of *nos-2* 3′UTR.

Binding to *nos-2* 3′UTR is essential for the translational suppression by MEX-3, SPN-4 and OMA-2

If the direct repeats DR1 and DR2 are required for the interactions with MEX-3 and SPN-4 proteins, and if these proteins controlled *nos-2* translation by direct interaction with *nos-2* 3′UTR, then DR1 and DR2 mutations should have the same effect on *nos-2* translation as that of the removal of these proteins. To test this, we prepared GFP:H2B:*nos-2* 3′UTR transgene constructs with the same DR1 and DR2 mutations used in the EMSA experiments and generated transgenic lines expressing the mutant constructs. Mutations in either one of the repeats led to weak GFP:H2B expression in all cells and stronger expression in a few cells at the posterior of the embryo – a pattern identical to the *spn-4(RNAi)* embryos. Although DR2 mutations did not affect the in vitro binding of SPN-4 to *nos-2* 3′UTR, these results indicate that both the direct repeats are probably crucial for the interaction in vivo, where potential competitors are probably present (see below). By contrast, GFP:H2B expression was uniformly stronger in all cells of the embryo when both direct repeats were simultaneously mutated – a pattern strikingly similar to the removal of MEX-3 (Fig. 5C). This observation is remarkably consistent with the EMSA results described in the previous section, in which the double mutant RNA showed considerably weaker interaction with MEX-3 than did either of the single mutants. In summary, the removal of MEX-3 and SPN-4 or mutations in the RNA sequence that is essential for their binding both have very similar effects on

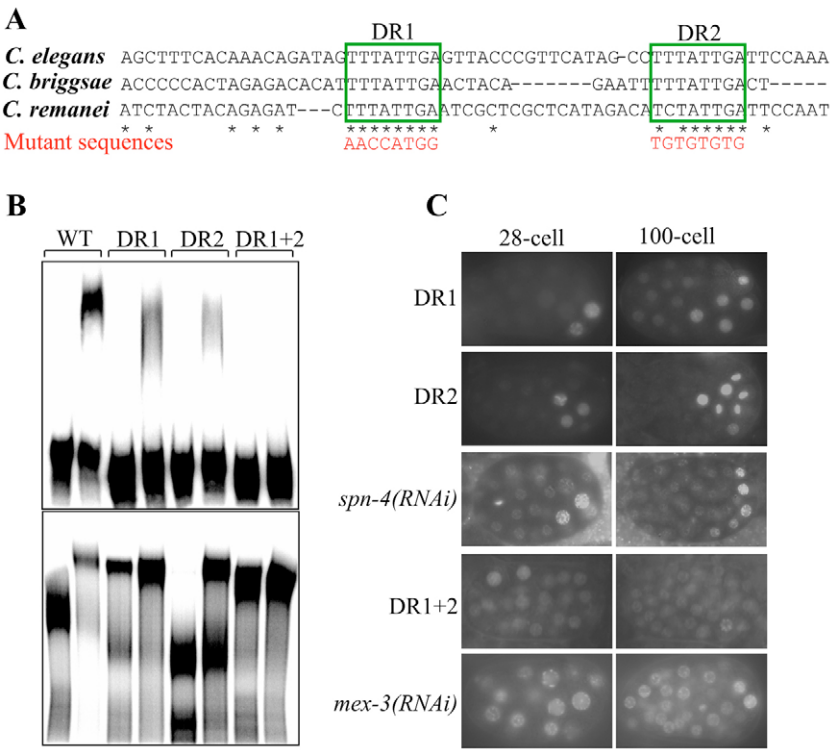

Fig. 5. Binding to *nos-2* 3′UTR is essential for the translation suppression activity of MEX-3 and SPN-4. Two 8 bp direct repeats present in *nos-2* 3′UTR are critical for the binding of MEX-3 and SPN-4. (**A**) Alignment of the *nos-2* 3′UTR of the indicated species (D'Agostino et al., 2006). Only the region with two 8 bp direct repeats (DR1 and DR2; boxed) is shown. Stars indicate bases conserved in all three species. Sequences of mutations used in B,C are shown in red. (**B**) Electrophoretic mobility shifts by MBP:MEX-3 (top) and GST:SPN-4 (bottom) of the various mutant versions of radiolabeled *nos-2* 3′UTR. (**C**) Expression pattern of GFP:H2B in embryos of transgenic worms carrying the GFP:H2B:*nos-2* 3′UTR transgene bearing the indicated mutations, or following *spn-4(RNAi)* or *mex-3(RNAi)*. The GFP:H2B distribution pattern in DR1 and DR2 is similar to that of *spn-4(RNAi)* and the pattern in DR1+DR2 is similar to that of *mex-3(RNAi)*.

nos-2 translation. From these results, we conclude MEX-3 and SPN-4 suppress the translation of *nos-2* mRNA by directly binding to *nos-2* 3′UTR.

As described earlier, SubE region is sufficient for OMA-2 interaction. Mutation of this region in the GFP:H2B:*nos-2* 3′UTR transgene did not express GFP:H2B in any stage during germ cell development (D'Agostino et al., 2006), which is contradictory to the effect of removal of OMA-1 and OMA-2 by RNAi. As the SubE region contains a potential polyadenylation signal and the cleavage site, we reasoned that mutations that affect this entire region would probably interfere with the core translational machinery, leading to complete absence of translation. To reveal potential subdomains within SubE that might be crucial for OMA-2 interaction more specifically, we generated RNA probes containing smaller substitutions (10-bp) of SubE and tested them in EMSA with GST:OMA-2. As shown in Fig. 6B, SubE-Δ27 more severely reduced the mobility shift than the other substitutions. Based on this, we generated a GFP:H2B:*nos-2* 3′UTR transgene construct carrying SubE-Δ27 substitution and introduced into worms. Quite remarkably, these transgenic worms strongly expressed GFP:H2B in oocytes that showed striking similarity to the expression pattern in *oma-1(RNAi) oma-2(RNAi)* (Fig. 6C). These results indicate that OMA-1 and OMA-2 suppress *nos-2* translation in oocytes by directly binding to the SubE region of *nos-2* 3′UTR.

POS-1 competes with SPN-4 for binding to *nos-2* 3′UTR

Two lines of evidence suggest a potential competition between SPN-4 and POS-1 for binding to *nos-2* 3′UTR. First, RNAi epistasis described earlier indicates that POS-1 acts to relieve the translational repression by SPN-4. Second, both these proteins are present in the germline blastomeres until P_4 is born (Tabara et al., 1999; Ogura et al., 2003). Therefore, we decided to test this potential competition more directly. For this, we added both proteins simultaneously to the binding reactions and observed the changes in electrophoretic mobility shift patterns when the relative concentrations of these two proteins were varied. As shown in Fig. 7A, an increase in the POS-1 to SPN-4 ratio decreased the intensity of the band corresponding to the RNA-SPN-4 complex with a concomitant increase in the intensity of RNA-POS-1 complex. If binding of one protein was independent of the other, then a 'super shift' resulting from the simultaneous binding of both proteins should have been observed. By contrast, we observed partitioning of RNA between the two proteins in a concentration-dependent manner, indicating that POS-1 and SPN-4 may indeed compete with each other for binding *nos-2* 3′UTR. Next, we quantified the fluorescence intensities of P_3 and P_4 cells in embryos immunostained with antibodies against POS-1 and SPN-4, and calculated the POS-1 to SPN-4 ratio in these cells. Significantly, this ratio in P_4 was about ninefold higher than P_3 (Fig. 7B,C). Taken together, these results suggest that the higher POS-1 to SPN-4 ratio in P_4 enables POS-1 to overcome the *nos-2* translation repression by SPN-4.

DISCUSSION

We have identified four proteins, OMA-1, OMA-2, MEX-3 and SPN-4, that suppress *nos-2* translation. Although OMA-1 and OMA-2 suppress in the oocyte, MEX-3 and SPN-4 suppress in the embryo. Through a combination of genetic and biochemical experiments, we provide evidence that these proteins suppress *nos-2* translation by directly interacting with its 3′UTR. Firstly, disruption of their expression by RNAi activates *nos-2* translation prematurely. Second, these proteins interact specifically with *nos-2* 3′UTR in vitro. Finally, 3′UTR mutations that abolish in vitro interaction activate translation prematurely in vivo in a pattern that is remarkably similar to the removal of these proteins by RNAi. In addition, our epistatic analysis shows that the CCCH-finger protein POS-1, which is required for *nos-2* translation in the germline founder cell P_4 (D'Agostino et al., 2006), acts as a derepressor, rather than as an activator, of *nos-2* translation.

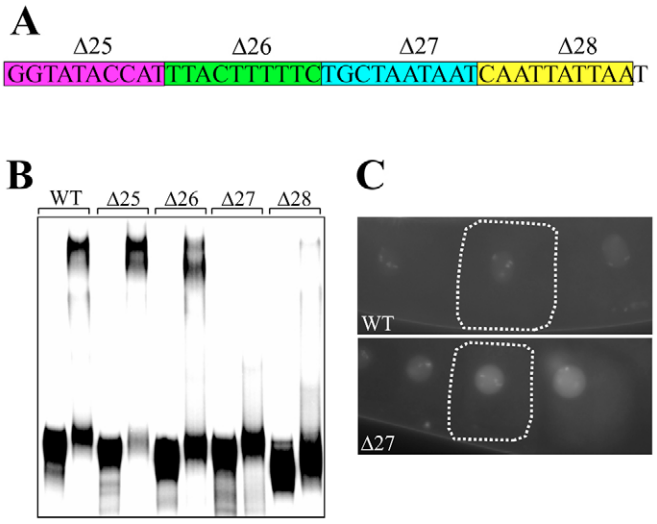

Fig. 6. Interaction with *nos-2 3′*UTR is essential for the translation suppression activity of OMA-2. (**A**) Sequence of the SubE region. Sequences targeted by substitution analysis in EMSA are boxed and named. (**B**) Electrophoretic mobility shift by OMA-2 of radiolabeled *nos-2* 3′UTR bearing the indicated mutations. The first lane in each set is the mobility of RNA in the absence of protein. (**C**) Expression pattern of GFP:H2B in embryos of transgenic worms carrying the GFP:H2B:*nos-2* 3′UTR transgene with wild-type sequence (WT) or bearing Δ27 mutation.

Results of our in vitro binding studies show that POS-1 and SPN-4 compete with each other for binding to the same region of *nos-2* 3′UTR. Significantly, the POS-1: SPN-4 ratio increases about ninefold in P_4 over its mother P_3. Based on these results, we propose that the translational status of *nos-2* in the embryonic germline depends on the relative concentrations of POS-1 and SPN-4. According to our model, the POS-1: SPN-4 ratio is below the threshold required for translational activation in earlier stages, which subsequently increases above this threshold in P_4, resulting in the activation *nos-2* translation.

Translational repression in oocytes

Two closely related CCCH-finger proteins, OMA-1 and OMA-2, are expressed only in the female germline and are enriched in oocytes. Consistent with their expression pattern, these two proteins function in oocyte maturation. Although no direct downstream target has been reported, based on the presence of CCCH-type RNA-binding motifs, they have been proposed to regulate the translation of downstream target mRNAs (Detwiler et al., 2001). Our results show that the *nos-2* mRNA is one of their direct targets. First, simultaneous removal of OMA-1 and OMA-2 leads to *nos-2* translation in oocytes. Second, both proteins interact in vitro with a short region of *nos-2* 3′UTR in a sequence-specific manner. Finally, a mutation in this region (Δ27) that severely reduces OMA-2 binding activates translation in vivo. In addition to these two proteins, at least one other protein must be involved in translation repression in oocytes, for mutations in a stem-loop at the 5′ region of *nos-2* 3′UTR abolishes this repression (D'Agostino et al., 2006) and neither of these proteins interacts with the stem loop. Surprisingly, MEX-3 and SPN-4, two other RNA-binding proteins that interact with *nos-2* 3′UTR and suppress translation in the embryo (see below), are unable to suppress *nos-2* translation in the oocyte, although both are present in oocytes (Draper et al., 1996; Ogura et al., 2003).

Translational repression in the early embryo

Absence of GFP:H2B in the somatic blastomeres of transgenic embryos indicates that the translational repression mechanisms must operate in these cells until the 16-cell stage, by which *nos-2* mRNA is degraded in these cells. At least four proteins seem to be involved in this repression: depletion of the two nearly identical CCCH-finger proteins MEX-5 and MEX-6 (Schubert et al., 2000; D'Agostino et al., 2006), the KH-domain protein MEX-3 or the RRM protein SPN-4 results in the activation of translation in all cells of the early embryo. A couple of observations indicate that the role of MEX-5 and MEX-6 on *nos-2* translation is probably mediated via their effects on the expression of POS-1 and MEX-3: (1) embryos lacking MEX-5 and MEX-6 express POS-1 ectopically in the anterior cells (Schubert et al., 2000) and this ectopic POS-1 is essential for the misexpression of GFP:H2B observed in these embryos (Schubert et al., 2000; D'Agostino et al., 2006); and (2) the level of MEX-3 is significantly lower in embryos lacking MEX-6 (Huang et al., 2002). While MEX-3 directly interacts with *nos-2* 3′UTR, it is not clear whether MEX-5 or MEX-6 bind *nos-2* 3′UTR. We have earlier reported that the *nos-2* mRNA degradation in the somatic cells is delayed in *mex-5(RNAi) mex-6(RNAi)* embryos (D'Agostino et al., 2006). A similar delay has also been observed in *mex-3(RNAi)* embryos (M.R. and K.S., unpublished). By contrast, MEX-3 and SPN-4 appear to play a more direct role in the control of *nos-2* translation. These proteins physically interact with *nos-2* 3′UTR in vitro, and mutations in the 3′UTR that disrupt this interaction show strikingly similar effects on translation in vivo as the RNAi depletion of these proteins, indicating that they probably interact with the 3′UTR in vivo and that this interaction is essential for the translation control of *nos-2* mRNA.

At the two-cell stage, both MEX-3 and SPN-4 appear to be essential to suppress *nos-2* translation, for absence of either one leads to activation of GFP:H2B expression. Both these proteins are present in both cells of the two-cell embryo and they have been shown earlier to interact physically (Huang et al., 2002). Therefore, it is possible they act together to suppress *nos-2* translation at the two-cell stage. However, at later stages, they appear to function independently. For example, at the four-cell stage, although MEX-3 is primarily present only in the anterior two cells (Draper et al., 1996), SPN-4 is restricted to the posterior two cells (Ogura et al., 2003). This spatial restriction of MEX-3 appears to restrict its translational repressor activity to the anterior cells, at least in the case of *pal-1* mRNA (Huang et al., 2002). In a similar fashion, MEX-3 may repress *nos-2* mRNA only in the anterior cells. However, as the accumulation of SPN-4 on P granules of *mex-3(RNAi)* embryos is significantly reduced (S.J. and K.S., unpublished), we think MEX-3 may also have an indirect role on the repression of *nos-2* translation in the posterior cells. This might explain the expression of GFP:H2B in all cells of *mex-3(RNAi)* embryos. Consistent with its spatial distribution pattern, SPN-4 appears to repress *nos-2* translation primarily only in the posterior cells. In *spn-4(RNAi)* embryos, the levels of GFP:H2B was significantly higher in the posterior cells when compared with the anterior cells. Low levels of GFP:H2B seen in the anterior cells probably results from perdurance of the protein produced at the two-cell stage of these embryos. Thus, these proteins appear to act together at the two-cell stage and independently at later stages to repress *nos-2* translation.

Activation of *nos-2* translation in the germline founder cell

Activation of *nos-2* translation in the germline founder cell P_4 depends on the presence of POS-1: although *nos-2* mRNA is present in *pos-1(RNAi)* embryos until the birth of PGCs, NOS-2 protein is

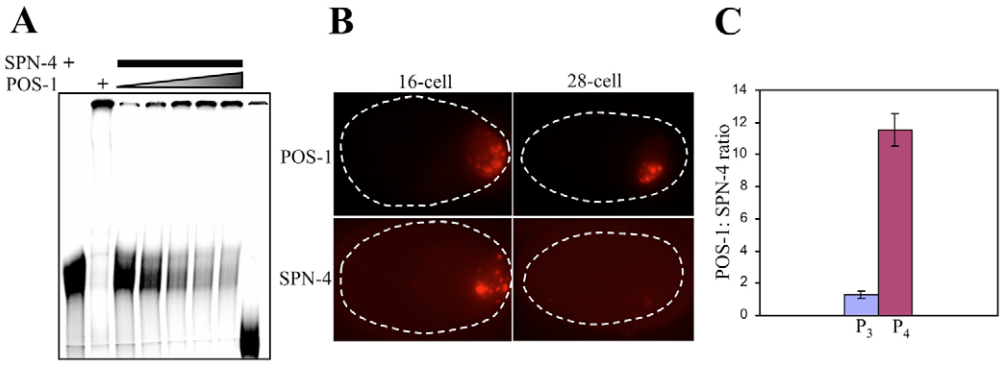

Fig. 7. POS-1 competes with SPN-4 for binding to *nos-2* 3′UTR. (**A**) Electrophoretic mobility shift of the radiolabeled 200 bp *nos-2* 3′UTR incubated with GST:SPN-4 alone (lane 1), GST:POS-1 alone (lane 2) or with increasing concentration of GST:POS-1 at a constant concentration of GST:SPN-4 (lanes 3-7). No protein was added to RNA in lane 8. Lanes 1 and 3-7 contain 2 μl of GST:SPN-4 per lane. Amounts of GST:POS-1 in lane 2 are 4 μl and in lanes 3-7 are 2, 4, 6, 8 and 10 μl, respectively. (**B**) Representative examples of 16- and 28-cell embryos immunostained with anti-SPN-4 and anti-POS-1 antibodies. (**C**) Bar graph showing average POS-1: SPN-4 ratios obtained by quantitation of immunofluorescence signals from 12 embryos for the indicated stages.

not detected at any stage during embryogenesis (D'Agostino et al., 2006). However, POS-1 is not required for *nos-2* translation in the absence of the repressors MEX-3 and SPN-4. Similarly, premature activation of translation caused by a 3′UTR mutation does not require POS-1 (D'Agostino et al., 2006). These observations clearly indicate that POS-1 functions as a derepressor, rather than as an activator, of *nos-2* translation. Surprisingly, even though POS-1 protein is continuously present in the P lineage starting from the two-cell stage (Tabara et al., 1999), it does not activate *nos-2* translation until the 28-cell stage. One possible explanation for this is that POS-1 requires an unknown P_4-specific factor for its derepressor activity. Alternatively, the ratio of POS-1 concentration to that of a repressor such as SPN-4 may determine the translational status and this ratio in P_4 probably tilts in favour of derepression. Our results support the second model: (1) in vitro, both SPN-4 and POS-1 bind to *nos-2* 3′UTR, and POS-1 competes with SPN-4 for binding to *nos-2* 3′UTR in a concentration-dependent manner; and (2) quantitation of immunofluorescence signals indicate that POS-1: SPN-4 ratio increases in the P lineage. We propose that the POS-1: SPN-4 ratio increases in P_4 above the threshold required for the activation of *nos-2* translation. Genetic mutants or other means that alter this ratio will be essential to validate this model.

Translation regulation by MEX-3, SPN-4 and POS-1

All three proteins, MEX-3, SPN-4 and POS-1, regulate the translation of a few other maternal mRNAs. The target mRNAs identified so far are *pal-1*, which is negatively regulated by MEX-3 and SPN4 (Hunter and Kenyon, 1996; Huang et al., 2002); *glp-1*, which is negatively regulated by POS-1 and positively regulated by SPN-4 (Ogura et al., 2003); *skn-1*, which is negatively regulated by SPN-4 (Gomes et al., 2001); and *apx-1*, which is positively regulated by POS-1 (Tabara et al., 1999). In addition, MEX-3 suppresses the translation of *rme-2* in the germline stem cells of adult gonad (Ciosk et al., 2004). Of these, the translational regulation by direct binding of the 3′UTR has been demonstrated only in the case of *glp-1* mRNA. SPN-4 and POS-1 proteins bind at different sites within *glp-1* 3′UTR and mediate opposite effects on translation. Although SPN-4 binds the temporal control region (TCR) and promotes translation, POS-1 binds the spatial control region (SCR) and suppresses translation. Asymmetric distribution of the two proteins in the two-cell embryo – SPN-4 is present in both cells, but POS-1 is restricted to the posterior cell – ensures restriction of *glp-1* translation to the anterior (Ogura et al., 2003).

Comparison of the translation control of *glp-1* and *nos-2* mRNAs reveal striking diversity in the translation regulation mediated by these two proteins. Although the relative concentration of SPN-4 and POS-1 is crucial for the translation of both these mRNAs, the final outcome is opposite: a higher POS-1: SPN-4 ratio suppresses *glp-1* (Ogura et al., 2003), but activates *nos-2*. It is not clear at the moment how they promote translation of one mRNA while inhibiting the translation of another. Some clues emerge from the comparison of the 3′UTR sequences of *glp-1* and *nos-2*. There are some important differences between these two 3′UTRs. First, both SPN-4 and POS-1 bind distinct and relatively short regions of the *glp-1* 3′UTR. By contrast, they require the entire 200 bp of *nos-2* 3′UTR for maximal binding. Second, the two 8 bp direct repeats, which are crucial for SPN-4 binding of *nos-2* 3′UTR, are not present in the SPN-4 binding element (TCR) of *glp-1* 3′UTR. Finally, 3′UTRs of the two mRNAs do not share any significant similarity at the sequence or secondary structure level. Based on these observations, we propose the final outcome of translation regulation depends on the type of 3′UTR sequence these proteins bind. Binding of one specific 3′UTR sequence could lead to association with an additional protein factor that might positively influence the translation machinery, while the binding of a different RNA sequence could lead to association with a different protein factor that might negatively influence the translational machinery. Identification of protein partners of SPN-4 and POS-1, and additional target mRNAs with which these two proteins directly interact, will be helpful to test this hypothesis.

Significantly, MEX-3, SPN-4 and POS-1 have been shown to interact among them (Huang et al., 2002; Ogura et al., 2003). In addition, these three proteins and the *nos-2* mRNA associate with P granules (Draper et al., 1996; Subramaniam and Seydoux, 1999; Tabara et al., 1999; Ogura et al., 2003). Presently, it is not clear whether these interactions play any role on the translation control of *nos-2* or any other mRNA. Experiments focused on determining the importance of these interactions will be an interesting challenge and will help us understand the mechanism(s) by which these proteins differently influence the translation of different target mRNAs. Such an understanding will help us explain the role of P granule-like structures present in the germ cells of many organisms.

Translation regulation of nanos gene family members

Members of the *nanos* gene family are the evolutionarily conserved regulators of germ cell development (Kobayashi et al., 1996; Forbes and Lehmann, 1998; Subramaniam and Seydoux, 1999; Koprunner et al., 2001; Tsuda et al., 2003). In addition to their function, even the basic aspects of the regulation of their expression have been conserved: (1) the *Drosophila, C. elegans* and zebrafish members are controlled at the translation level by mechanisms that require 3′UTR; (2) in both *Drosophila* and *C. elegans*, translation repression in oocytes and embryos are mediated by two distinct regions of the 3′UTR (Forrest et al., 2004; D'Agostino et al., 2006). However, there is at least one major difference between the translation regulation of *Drosophila nanos* and *C. elegans nos-2*. The protein factors that control these two mRNAs do not share either sequence or functional (other than the regulation of *nanos*) similarity. The worm proteins OMA-1 and OMA-2, which bind *nos-2* 3′UTR and suppress translation in oocytes, are CCCH-type zinc-finger proteins and are essential for oocyte maturation (Detwiler et al., 2001). By contrast, the fly protein Glorund, which binds *nanos* 3′UTR and suppress translation in oocytes, is a hnRNP family protein and does not appear to be essential for oocyte maturation (Kalifa et al., 2006). Similarly, the fly protein Smaug, which represses *nanos* translation in embryos, is essential for nuclear divisions (Dahanukar et al., 1999). By contrast, MEX-3 and SPN-4, which repress the worm *nos-2* in the embryo, do not resemble Smaug at the sequence level and are not involved in cell division. Consistently, the cis-elements of the two 3′UTRs also do not share sequence similarity. These differences possibly reflect the fundamental difference in the process of embryogenesis in these two species. The fly zygote undergoes a series of nuclear divisions and forms a multinucleate syncytium. During the ensuing cellularization, the first cells to form are the PGCs, known in the fly as pole cells. By contrast, the worm zygote does not form a syncytium. Instead, it undergoes an asymmetric cell division generating a larger anterior cell called AB and a smaller posterior cell called P_1. Although P_1 inherits the maternally synthesized germ cell components, unlike the fly pole cells, P_1 is not a PGC. As mentioned earlier, the P lineage produces one somatic daughter at each of first four rounds of cell division before becoming committed to PGC fate (Fig. 1). Therefore, the developmental contexts in which PGCs arise in these two species are different. Consequently, the RNA-binding proteins available at these different contexts for the translation control of *nanos* mRNA may not be similar. In addition, at least some of the mechanistic details may also have diverged. For example, although Smaug mediates translation repression by blocking translation initiation (Nelson et al., 2004), it also promotes mRNA degradation by recruiting deadenylation complex (Semotok et al., 2005). Whereas such a mechanism may operate in the somatic blastomeres of worm embryo, an additional mechanism that does not involve RNA degradation is essential in the P lineage to suppress translation, as *nos-2* mRNA is preserved in this lineage until the birth of PGCs.

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Supplementary material

Supplementary material for this article is available at http://dev.biologists.org/cgi/content/full/135/10/1803/DC1

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Table S1. List of open reading frames screened by RNAi for effects on GFP:H2B-*nos-2* 3′UTR transgene expression

S. number	ORF	RNAi phenotype	GFP:H2B
1	F53G12.5a; *mex-3*	100% embryonic lethal (emb)	In all cells from two-cell stage
2	R119.4	Wild-type (WT)	Wild-type pattern
3	W01B11.3	Wild type	Wild-type pattern
4	C44E4.4	10% emb; remaining 100% sterile	Wild-type pattern
5	M01A10.3	2% emb; L2 arrest	Wild-type pattern
6	M01E11.5	Wild type	Wild-type pattern
7	B0414.5	50% emb; remaining wild type	Wild-type pattern
8	T08B2.5a	Wild type	Wild-type pattern
9	F26B1.2	50% emb; remaining wild type	Wild-type pattern
10	F37E3.1	L2 arrest	Wild-type pattern
11	C37A2.1	L2 arrest	Wild-type pattern
12	C48B6.2	80% sterile	Wild-type pattern
13	F26A3.2	Slow growth	Wild-type pattern
14	K07A12.4	Wild type	Wild-type pattern
15	C12C8.3a	Low brood; dumpy (dpy) and sterile	Wild-type pattern
16	C17E4.5	10% emb; the rest slow development	Wild-type pattern
17	F46A9.6	10% dpy	Wild-type pattern
18	Y106G6H.2	100% sterile	Wild-type pattern
19	F45H11.2	Wild type	Wild-type pattern
20	F28D9.1	Wild type	Wild-type pattern
21	R09B3.3	Wild type	Wild-type pattern
22	R09B3.2	Wild type	Wild-type pattern
23	R06C1.4	Wild type	Wild-type pattern
24	W08E3.1	95% emb	Wild-type pattern
25	T01D1.2a	50% emb	Wild-type pattern
26	W07E6.4	100% emb	Wild-type pattern
27	F52C6.3	Generally very sick, but not sterile	Wild-type pattern
28	F52C6.4	Wild type	Wild-type pattern
29	F09D1.1	100% emb	Wild-type pattern
30	ZK430.7	Very weak growth	Wild-type pattern
31	F59A6.6	Wild type	Wild-type pattern
32	EEED8.7a	Wild type	Wild-type pattern
33	F56D1.7	100% sterile	Wild-type pattern
34	C18A3.5a	Wild type	Wild-type pattern
35	F21H12.5	Wild type	Wild-type pattern
36	H12I13.4	Wild type	Wild-type pattern
37	C30B5.3	5% emb	Wild-type pattern
38	C30B5.4	50% emb	Wild-type pattern
39	T28D9.10	20% emb	Wild-type pattern
40	T28D9.2a	Wild type	Wild-type pattern
41	F32A5.1	50% emb; some uncs; 70% sterile	Wild-type pattern
42	C08B11.5	100% emb	Wild-type pattern
43	F28C6.6	Wild type	Wild-type pattern
44	F44G4.4	Wild type	Wild-type pattern
45	ZK1067.6	Wild type	Wild-type pattern
46	F35H8.5	Wild type	Wild-type pattern
47	C14A4.4	Slow development; 100% sterile	Wild-type pattern
48	M28.5	100% emb	Wild-type pattern
49	D2089.1	80% emb	Wild-type pattern
50	D2089.4	Wild type	Wild-type pattern
51	R06F6.1	100% emb	Wild-type pattern
52	W02B12.2	Wild type	Wild-type pattern
53	F07A11.6a	10% emb; wild type	Wild-type pattern
54	Y54E2A.11	10% emb; L1 arrest	Wild-type pattern
55	R05H10.2	Wild type	Wild-type pattern
56	K02F3.11	Wild type	Wild-type pattern
57	R10E9.1	Wild type	Wild-type pattern
58	R74.5	Wild type	Wild-type pattern
59	R07E5.3	100% emb	Wild-type pattern
60	R07E5.14	100% emb	Wild-type pattern
61	M88.5	Wild type	Wild-type pattern
62	F35G12.2	Wild type	Wild-type pattern
63	T04A8.6	Slow growth; 5%	Wild-type pattern
64	B0336.9	50% emb; remaining wild type	Wild-type pattern
65	F25B5.4	Wild type	Wild-type pattern
66	F25B5.7a	Wild type	Wild-type pattern
67	F31E3.5	Wild type	Wild-type pattern
68	ZK418.8	10% emb	Wild-type pattern
69	B0280.1	Wild type	Wild-type pattern

70	K04G7.10	Wild type	Wild-type pattern
71	C07H6.4	2% emb	Wild-type pattern
72	ZK652.1	100% emb	Wild-type pattern
73	K12H4.8	Wild type	Wild-type pattern
74	C30A5.2	Wild type	Wild-type pattern
75	C50C3.6	100% emb	Wild-type pattern
76	B0303.15	Very slow growth	Wild-type pattern
77	R08D7.3	Wild type	Wild-type pattern
78	T20G5.11	Wild type	Wild-type pattern
79	M03C11.7	50% emb; L2 arrest	Wild-type pattern
80	Y48A6B.3	Wild type	Wild-type pattern
81	C18D11.4	Mostly sterile	Wild-type pattern
82	F56A8.6	70% emb; L3 arrest	Wild-type pattern
83	T12D8.2	Wild type	Wild-type pattern
84	F42A6.7a	30% sterile	Wild-type pattern
85	K08D10.3	50% emb; unc and wild type	Wild-type pattern
86	K08D10.4	Wild type	Wild-type pattern
87	C11D2.4	Wild type	Wild-type pattern
88	C33H5.12	Wild type	Wild-type pattern
89	K07H8.9	Wild type	Wild-type pattern
90	K07H8.10	Slow growth	Wild-type pattern
91	F08B4.7	L3 arrest	Wild-type pattern
92	D1046.1	95% sterile	Wild-type pattern
93	C01F6.5	Wild type	Wild-type pattern
94	F23B2.6	Wild type	Wild-type pattern
95	W08D2.7	L2 arrest	Wild-type pattern
96	F32B6.3	60% sterile	Wild-type pattern
97	C47E12.7	Arrest at L1 or L2	Wild-type pattern
98	K08F4.2	About 90% sterile	Wild-type pattern
99	T11G6.8	Wild type	Wild-type pattern
100	ZK593.7	100% sterile	Wild-type pattern
101	F54D1.1	Wild type	Wild-type pattern
102	B0035.12	Wild type	Wild-type pattern
103	F58B3.7	Wild type	Wild-type pattern
104	M18.7	Wild type	Wild-type pattern
105	ZK795.3	Arrest at L3	Wild-type pattern
106	T23F6.4	Slow growth 100% sterile	Wild-type pattern
107	Y57G11A.5	Wild type	Wild-type pattern
108	Y41E3.11	L2-L3 arrest	Wild-type pattern
109	T22F3.4	L2-L3 arrest	Wild-type pattern
110	M03F8.3	100% emb	Wild-type pattern
111	ZC404.8*; *spn-4*	100% emb	In all cells starting at 2-cell stage; stronger in posterior cells beyond 28-cell stage
112	C26F1.4	L3 arrest	Wild-type pattern
113	C12D8.11	Wild type	Wild-type pattern
114	K07C5.4	L2 arrest	Wild-type pattern
115	K07C5.6	100% emb	Wild-type pattern
116	Y32F6A.3	Slow and sterile	Wild-type pattern
117	C52E4.3	L2 arrest	Wild-type pattern
118	ZK863.7	Wild type	Wild-type pattern
119	T07F10.3	Wild type	Wild-type pattern
120	T10G3.6	100% sterile	Wild-type pattern
121	C50B8.1	Wild type	Wild-type pattern
122	C15H11.3	Wild type	Wild-type pattern
123	T01C3.7	L2 arrest	Wild-type pattern
124	F28F8.3	Wild type	Wild-type pattern
125	F55A4.4	Wild type	Wild-type pattern
126	F55A4.5	Wild type	Wild-type pattern
127	T07D1.4	Wild type	Wild-type pattern
128	T22B2.4	Wild type	Wild-type pattern
129	H28G03.1	Wild type	Wild-type pattern
130	R03G5.1	100% sterile	Wild-type pattern
131	F18H3.3a	Wild type	Wild-type pattern

*This RNAi clone from the RNAi library did not contain ZC404.8 ORF. We PCR-amplified the correct insert from wild-type cDNA and inserted in L4440 vector and used in the RNAi screen.