NANOS-3 and FBF proteins physically interact to control the sperm–oocyte switch in Caenorhabditis elegans

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Background: The Caenorhabditis elegans FBF protein and its Drosophila relative, Pumilio, define a large family of eukaryotic RNA-binding proteins. By binding regulatory elements in the 3′ untranslated regions (UTRs) of their cognate RNAs, FBF and Pumilio have key post-transcriptional roles in early developmental decisions. In C. elegans, FBF is required for repression of fem-3 mRNA to achieve the hermaphrodite switch from spermatogenesis to oogenesis.

Results: We report here that FBF and NANOS-3 (NOS-3), one of three C. elegans Nanos homologs, interact with each other in both yeast two-hybrid and in vitro assays. We have delineated the portions of each protein required for this interaction. Worms lacking nanos function were derived either by RNA-mediated interference (nos-1 and nos-2) or by use of a deletion mutant (nos-3). The roles of the three nos genes overlap during germ-line development. In certain nos-deficient animals, the germ line died during larval development. This germ-line death did not require CED-3, a protease required for apoptosis.

Conclusions: The data suggest that NOS-3 participates in the sperm–oocyte switch through its physical interaction with FBF, forming a regulatory complex that controls fem-3 mRNA. NOS-1 and NOS-2 also function in the switch, but do not interact directly with FBF. The three C. elegans nanos genes, like Drosophila nanos, are also critical for germ-line survival. We propose that this may have been the primitive function of nanos genes.

Background

In the germ line and early embryo, post-transcriptional controls are responsible for key developmental decisions, including patterning and establishment of cell fates [1–4]. These controls are often mediated by specific regulatory elements in the 3′ untranslated region (UTR) that govern an mRNA’s translational activity, stability and cellular location.

The Caenorhabditis elegans FBF protein and Drosophila Pumilio define a family of related RNA-binding proteins called Puf proteins that are found throughout the eukaryotic kingdom [5]. Their functions are best understood in C. elegans and Drosophila. FBF binds specifically to a regulatory element of the fem-3 mRNA that controls the sperm–oocyte switch in C. elegans hermaphrodites [5–7]. Normally, hermaphrodites make sperm first and then switch to oogenesis. In gain-of-function mutants that disrupt a regulatory element in the fem-3 3′UTR, the switch does not occur, and sperm are made continuously. FBF is a repressor that binds specifically to that regulatory element in the fem-3 3′UTR. Pumilio was first identified as a gene that regulates patterning in the early Drosophila embryo [8], but more recently has been found to control germ-line stem-cell divisions [9,10]. Its role in patterning requires its specific interaction with regulatory elements in the 3′UTR of hunchback mRNA, leading to translational repression of that mRNA [11–14]. Although elegant genetic analyses establish that repression by Pumilio requires nanos gene activity, the molecular nature of this collaboration remains unknown.

Here, we demonstrate that FBF physically interacts with C. elegans Nanos homolog, and characterize the biological roles of this and two other nanos orthologs present in the C. elegans genome. We originally identified NANOS-3 (NOS-3) using a yeast two-hybrid screen with FBF as bait. This physical interaction was confirmed using in vitro assays. Neither NOS-1 nor NOS-2 interacted detectably with FBF by either assay. Importantly, all three nanos (nos) genes play a role in controlling the sperm–oocyte switch. We suggest that NOS proteins act in a redundant fashion to control fem-3 post-transcriptionally, and that FBF and NOS-3 do so in a regulatory complex. Moreover, we found that the NOS proteins promote germ-line survival. Because Drosophila Nanos similarly
NOS-3 interacts with FBF. (a–c) The nos-3 mRNA and protein. (a) Schematic illustration of the nos-3 pre-mRNA and the nos-3(Δ3) mutation. Boxes, exons; lines, introns; blue, putative CCHC zinc fingers (each is indicated as a block; dark grey, minimal region required for interaction with FBF-1 (amino acids 192–428; see Figure 2); ATG and TAA, initiation and termination codons, respectively. NOS-3 contains polyglutamate stretches in its amino-terminal half, as does Drosophila Nanos [42]. Shown below (nos-3(Δ3)) is the chromosomal deletion nos-3(q650), which removes the minimal FBF-interaction domain, first zinc finger and half of the second zinc finger, and shifts the downstream sequence out of frame. The predicted deletion protein contains amino acids 1–286 plus 31 extra amino acids from the frameshift. (b) Schematic illustration of the proteins encoded by the three C. elegans nos genes, Drosophila nanos and Xenopus Xcat2. N, amino terminus; C, carboxyl terminus. The respective amino-acid identities and similarities in the CCHC region are indicated on the right. NOS-1 and NOS-2 contain 312 and 260 amino acids, respectively. (c) Alignment of amino-acid sequences in the CCHC regions of C. elegans NOS-3 and Drosophila Nanos. Dark and light shading, identical and similar amino acids, respectively; black dots, CCHC residues. (d) FBF-1–NOS-3 interaction in the yeast two-hybrid system. FBF-1 (amino acids 121–614), NOS-3 (amino acids 42–871), NOS-1 (amino acids 1–311), NOS-2 (amino acids 1–259), CPB-1, a C. elegans ortholog of cytoplasmic polyadenylation element binding protein (amino acids 1–560; C. Luitjens, J.K and M.W., unpublished data) and MS2 coat protein (amino acids 1–131; [43,44]) were tested in the yeast two-hybrid system, using fusions to the LexA DNA-binding domain and Ga4 transcriptional-activation domain. The third column shows β-galactosidase enzyme activity per µg of yeast lysate protein [34].

![Figure 1](image_url)

### Results

**NOS-3 and FBF proteins interact physically**

We used a fusion protein between FBF-1 and the DNA-binding domain of Lex A (Lex A–FBF-1) as bait in a yeast two-hybrid screen to identify nematode proteins that interact with FBF-1. Of the 20,000,000 independent cDNA transformants screened, 19 were found that activated both the HIS3 and lacZ reporter genes in the presence of LexA–FBF-1, but not in the presence of a fusion protein between LexA and the MS2 coat protein. Three independently isolated cDNAs were found to be derived from a single gene related in sequence to *Drosophila* Nanos. We refer to this nematode gene as nos-3.

The complete sequence of the longest nos-3 cDNA was determined, and its splicing pattern deduced by comparison with the genomic sequence [16] (Figure 1a). The 5′ and 3′ termini were determined by rapid amplification of cDNA ends (RACE) and oligo(dT)-primed RT–PCR, respectively. The nos-3 mRNA contains seven exons and encodes a protein of 871 amino acids. Sequence similarity between *C. elegans* NOS-3 protein and *Drosophila* Nanos was confined to the carboxy-terminal region, which contained two distinctive CCHC zinc fingers that are diagnostic of proteins in the NOS family (Figure 1a–c) [17]. These CCHC residues are required for *Drosophila* Nanos to regulate the nos-3 mRNA, and to bind non-specifically to RNA [18,19].

FBF-1 and NOS-3 interacted specifically in the yeast two-hybrid system: no interaction was detected when
regions are all required for binding to RNA [5].

(b) FBF-1 derivatives

(c) GST–NOS fusion proteins and FBF-1. Fusion proteins consisting of GST fused to NOS-1 (amino acids 1–311), NOS-2 (amino acids 1–259) or NOS-3 (amino acids 432–681) were linked to beads and incubated with 35S-labeled FBF-1 (amino acids 121–614) prepared by translation in vitro. Bound proteins were eluted and analyzed by electrophoresis. Protein immobilized on the beads is indicated above each lane. In (c,d) input (lane 1) indicates material before incubation with the GST protein (equivalent to 10% of the material analyzed in experimental lanes).

(e) Interaction between GST–NOS derivatives and FBF-1. GST–NOS-3 was incubated with 35S-labeled FBF-1 (amino acids 121–614) prepared by translation in vitro. Bound proteins were eluted and analyzed by electrophoresis. Protein immobilized on the beads is indicated above each lane. In (c,d) input (lane 1) indicates material before incubation with the GST protein (equivalent to 10% of the material analyzed in experimental lanes). Bound protein eluted and analyzed by electrophoresis. Protein immobilized on the beads is indicated above each lane. In (c,d) input (lane 1) indicates material before incubation with the GST protein (equivalent to 10% of the material analyzed in experimental lanes).

Regions required for the FBF-1–NOS-3 interaction. (a) NOS-3 domains required for the interaction with FBF-1 in the yeast two-hybrid system. The Gal4 activation-domain plasmid carrying wild-type (WT) NOS-3 or the indicated truncated derivatives (the amino-acid residues expressed are indicated in parentheses) was introduced into yeast expressing LexA–FBF-1 (121–614), a fusion protein between the DNA-binding domain of LexA and amino-acid residues 121–614 of FBF-1. None indicates a strain containing the activation-domain vector without an insert. Dark grey, minimal region required for interaction with FBF-1; light grey, region that enhances interaction with FBF-1; blue blocks, putative C CHC zinc fingers. (b) FBF-1 region required for interaction with NOS-3 in the yeast two-hybrid system. Full-length FBF-1 or the indicated truncated derivatives carried in the activation-domain plasmid were introduced into yeast containing LexA–NOS-3 (42–681). Green, the eight consecutive Puf repeats of FBF; pink anvil, Csp1; pink oval; Csp2. The eight Puf repeats, Csp1 and Csp2 regions are all required for binding to RNA [5]. (c) Interaction between GST–NOS fusion proteins and FBF-1. Fusion proteins consisting of GST fused to NOS-1 (amino acids 1–311), NOS-2 (amino acids 1–259) or NOS-3 (amino acids 432–681) were linked to beads and incubated with 35S-labeled FBF-1 (amino acids 121–614) prepared by translation in vitro. Bound proteins were eluted and analyzed by electrophoresis. (d) GST–FBF-1 fusion proteins and NOS-3 derivatives required for their interaction. In (e) treatment, an aliquot of the lysate was removed and deproteinized to detect endogenous RNAs using electrophoresis and staining with ethidium bromide. The lysate was incubated with GST–NOS-3 beads, and bound protein eluted and analyzed by electrophoresis. Lane 1, protein retained, no RNase A treatment; lane 2, protein retained, with RNase A treatment; lane 3, RNA in lystate, no RNase A treatment; lane 4, RNA in lystate, with RNase A treatment.

The regions of NOS-3 and FBF-1 required for their interaction were identified by analyzing deletion derivatives of each protein in the yeast two-hybrid assay (Figure 2a). For NOS-3, a 249 amino-acid segment (residues 432–681, dark grey shading) that lies amino-terminal to the zinc-finger region was sufficient for binding FBF-1 (Figure 2a; Δ5). Inclusion of an additional 239 amino acids of NOS-3 further upstream (amino acids 192–431, light grey shading) enhanced the interaction to the level observed with the complete protein (Figure 2a; Δ3, light grey shading). The enhancing region did not interact significantly with FBF-1 (Figure 2a, Δ4 and Δ6). The interaction between the minimal FBF-binding domain of NOS-3 (Δ5, amino acids 432–681) and FBF-1 was specific: it was detected with either NOS-3 or FBF as the LexA fusion, and was not detected with either control or mutant NOS-3
proteins as partners (data not shown). For FBF-1, the region required for binding to NOS-3 was determined through comparable yeast two-hybrid studies, using LexA–NOS-3 and a collection of FBF-1 derivatives (Figure 2b). FBF-1 consists of eight repeats of approximately 40 amino acids; short conserved sequences, Csp1 and Csp2, are found amino-terminal to the first repeat and carboxy-terminal to the last repeat, respectively. These features are diagnostic of Puf proteins generally [5]. The eight conserved Puf repeats and flanking Csp regions of FBF-1 were sufficient for interaction with NOS-3 (Figure 2b, Δ2), as they are for RNA binding [5]. Removal of Csp2, on the carboxy-terminal side of the Puf repeats, abolished binding (Figure 2b, Δ3 and Δ4). Partial removal of Csp1, which greatly reduces FBF-1 binding to the fem-3 RNA [5], had little or no effect on the interaction with NOS-3 (Figure 2b, Δ1).

To analyze the interaction between FBF-1 and NOS-3 in vitro, we performed affinity chromatography experiments using fusions of glutathione-S-transferase (GST) to NOS-3 or FBF-1 (Figure 2c,d). To this end, 35S-labeled FBF-1 was produced by translation in vitro, and incubated with beads carrying GST fusions to NOS-1, NOS-2 or NOS-3. Radiolabeled FBF-1 bound to GST–NOS-3, but not to the other GST fusion proteins or to GST alone (Figure 2c, compare lane 5 with lanes 2–4). We also reversed the NOS and FBF-1 protein components (Figure 2d). Immobilized GST–FBF-1 was incubated with either a mixture of nearly full-length, 35S-labeled NOS-3 (amino acids 80–871) or an amino-terminally deleted form of the protein (amino acids 681–871) lacking the portion of NOS-3 required to interact with FBF-1 in the two-hybrid system. The nearly full-length NOS-3 bound to GST–FBF, whereas the deleted version did not (Figure 2d).

The in vitro translation system used to produce 35S-labeled protein contains RNAs, including tRNA and rrNA, as well as the mRNA added exogenously. In principle, although no RNAs containing fem-3 sequences are present in the lysate, FBF-1 and NOS-3 might co-occupy an RNA through independent, non-specific RNA–protein interactions. To test this possibility, we treated a lysate containing 35S-labeled FBF-1 with high levels of ribonuclease A (RNase A) before incubation with GST–NOS-3 (Figure 2e). RNase A treatment had little effect on the binding of GST–NOS-3 to FBF-1 (Figure 2e, lanes 1,2), yet resulted in the virtually complete degradation of rRNA in the lysate (Figure 2e, lanes 3,4). Furthermore, NOS-3 bound RNA non-specifically in the three-hybrid system, binding similarly to an irrelevant stem–loop structure (an iron response element) and the fem-3 3' UTR (data not shown). Further more, if the CCHC zinc-finger region is responsible for its non-specific RNA binding of C. elegans NOS, as is the case for Drosophila Nanos [18], the regions of NOS-3 that bind RNA are separable from those binding FBF-1. We conclude that NOS-3 and FBF-1 proteins interact with one another, and that the interaction requires the central portion of NOS-3 and the Puf repeat region of FBF-1. In contrast, neither NOS-1 nor NOS-2 interacts detectably with FBF-1, consistent with the fact that they lack the interaction domain present in NOS-3.

The nos-3 mRNA and protein are present in the germ line and present throughout development

FBF is required in C. elegans hermaphrodites for the sperm–oocyte switch: in its absence, sperm are made continuously and the switch to oogenesis does not occur [5]. We found that nos-3 is expressed at the right time and place to participate with FBF in the sperm–oocyte switch (Figure 3). A 3.3 kb nos-3 mRNA, which corresponds well to its predicted length (3,207 nucleotides), was present in wild-type animals, but barely detectable in animals lacking a germ line (Figure 3a, lanes 1,2). Therefore, nos-3 mRNA is likely to reside primarily in the germ line, and to be expressed only at a low level in somatic tissue. Furthermore, nos-3 mRNA was present throughout development, increasing in abundance at the L4 larval stage, just before oocytes first appear (Figure 3a, lanes 3–9).

Using affinity-purified antibodies, we detected NOS-3 protein throughout the germ-line tissue during larval development (Figures 3b,c; data not shown). Staining with antibodies to NOS-3 was specific, in that it was dramatically reduced in nos-3 deletion mutants (compare Figure 3b with Figure 3e). At all stages of germ-line development, NOS-3 was predominantly cytoplasmic: this was evidenced by the dark, non-staining ‘holes’ corresponding to nuclei in the germ line at the larval L3 stage (Figure 3b). The distribution of NOS-3 overlapped with regions of the cytoplasm containing P granules, detected using anti-PGL-1 antibodies (Figure 3c,d; [20]); however, within the cytoplasm, NOS-3 staining was uniform and diffuse throughout. At the late L4 stage, NOS-3 was detected in germ cells in mitosis and meiotic pachynete, but decreased in the spermatogenic region (Figure 3h). In these experiments, mitotic cells were detected using antibodies against a phosphorylated form of histone H3 (Figure 3i; green) and sperm were marked with an anti-sperm antibody (Figure 3i; blue).

In addition to staining in the larval gonad, NOS-3 expression was also observed in embryos. Specifically, NOS-3 was found in P1–P4, early embryonic blastomeres that ultimately generate the germ line (Figure 3j–l, data not shown), and later in germ-line progenitor cells, Z2 and Z3 (data not shown). Although the distribution of NOS-3 in P cells was punctate, it did not precisely coincide with P granules (Figure 3j–k).

NOS functions in the hermaphrodite sperm–oocyte switch

If NOS-3 acts with FBF to repress fem-3 to cause the hermaphrodite switch from spermatogenesis to oogenesis,
then animals lacking nos-3 should produce sperm continuously and not switch to oogenesis. To test this prediction, we isolated a deletion of the nos-3 gene (see Materials and methods). The nos-3(Δ) mutation (Figure 1a) removes 1381 nucleotides from the nos-3 coding region; the predicted NOS-3(Δ) protein lacks the FBF-interacting region, all of the first zinc finger and half of the second, and truncates the protein 526 amino acids before the normal carboxyl terminus. The mRNA produced by nos-3(Δ) mutants was approximately 1.8 kb, in keeping with its predicted length of 1,826 nucleotides (data not shown). As with progeny of nos-3 mutants generated by RNA-mediated interference (RNAi), nos-3(Δ)/+ heterozygotes (data not shown). We conclude that nos-3 can indeed influence the sperm–oocyte switch, but that it is not normally essential.

One possible explanation for the rare occurrence of nos-3 mutants with defects in the sperm–oocyte switch is redundancy. The C. elegans genome contains three predicted nos homologs, nos-1, nos-2 and nos-3 (Figure 1b). Each C. elegans NOS protein contains two putative CCHC zinc fingers at the carboxyl terminus, as well as additional conserved amino acids. The spacing of CCHC residues in nos-3, but not in nos-1 or nos-2, is identical to that in Drosophila Nanos (Figure 1b). To test whether the three nos genes might encode redundant functions, we used RNAi [21] to reduce nos-1 and nos-2 expression, and the nos-3(Δ) mutant to remove nos-3. To obtain all possible ‘mutant’ combinations, nos-1 and nos-2 RNAs were injected either singly or in combination into either wild-type or nos-3(Δ) animals. The results are summarized in Table 1, and key defects are shown in Figure 4.
Reduction of either nos-1 or nos-2 strongly enhanced the defect in the sperm–oocyte switch in the nos-3(Δ) mutant (Table 1, column 2). The sperm–oocyte switch failure in nos-1 nos-3 and nos-2 nos-3 double mutants was similar to that observed in FBF-deficient [5] or fem-3(gf) [6] mutants: excess sperm accumulated over a larger portion of the gonad than normal and no oocytes were produced (Table 1, Figure 4a). In contrast, the nos-1, nos-2 and nos-3 single mutants were essentially wild-type for the sperm–oocyte switch (Table 1, column 2). The simplest interpretation is that these three nos paralogs are largely redundant in their control of the sperm–oocyte switch.

The three nos genes are critical for germ-line survival

In addition to a defect in the sperm–oocyte switch, nos-deficient animals had an additional and dramatic defect. In nos-1 nos-2 double mutants and, at a higher frequency, in nos-1 nos-2 nos-3 triple mutants, adults were found with no detectable germ line (Table 1, column 3; Figure 4b); nos-1 nos-2 males were similarly lacking a germ line (data not shown). To elucidate the basis of this phenotype, we examined the germ lines of nos-1 nos-2 and nos-1 nos-2 nos-3 mutants during development. Most newly hatched larvae contained two germ-line precursor cells, as normal, but some had three or four germ-line precursor cells (25%, n = 63). The simplest interpretation is that these extra germ-line precursor cells arose by abnormally early cell division. Therefore, the nos genes are not required for germ-line specification during embryogenesis, but may prevent germ-line mitoses before hatching. Subsequently, in the larval L1 stage, germ-line precursor cells divided normally, but by L2 or L3, germ-line nuclei had acquired an unusual ‘crinkly’ or granular appearance (Figure 4c). By L4, these affected germ lines were much smaller than normal and, by adulthood, germ cells were no longer detectable (Figure 4b). We conclude that the three nos genes function together in promoting germ-line survival. Furthermore, because animals lacking a germ line were found after removal of nos-1 and nos-2, but not with other double mutants, we speculate that nos-1 and nos-2 might be more critical for this control.

To determine whether the nos genes promote germ-line survival by preventing the programmed cell death pathway, we reduced nos-1 and nos-2 activities in a ced-3 mutant background. For this experiment, ced-3(n717), a strong loss-of-function allele [22], was used. The ced-3 gene encodes a homolog of the interleukin-1β converting enzyme (ICE) protease that is required for programmed cell death [23]. We found that ced-3(n717); nos-1(RNAi) nos-2(RNAi) mutant adults lacked germ lines (data not shown). Therefore, the ced-3-dependent pathway of programmed cell death is not essential for the defect in germ-line survival of nos-deficient animals. We conclude that a novel, nos-dependent pathway can control the decision between cell death and survival in germ-line tissue.

Overlapping but non-identical functions of the nos genes

The percentage of sterile animals among nos-1(RNAi) and nos-2(RNAi) single mutants was remarkably similar to that observed among nos-1(RNAi) nos-3(Δ) and nos-2(RNAi) nos-3(Δ) double mutants. Nevertheless, the spectrum of sterile phenotypes was different (Table 1). For example, the most prominent sterile phenotype of nos-2 single mutants was a small germ line with some sperm and undifferentiated cells, whereas that of nos-2 nos-3 double mutants was a larger germ line with an excess of sperm and no oocytes (see Table 1 legend). A simple interpretation is that the functions of nos-1 and nos-2 overlap with...
that of nos-3 in controlling the sperm–oocyte switch, but that the individual nos genes also have distinct functions in the germ line. Because the effects of nos-1 and nos-2 that we report here were obtained using RNAi, and this method can vary from gene to gene with respect to how completely it reduces function, the quantitative impact of each nos gene on germ-line functions will require the isolation of null mutants in nos-1 and nos-2.

Other nos defects
In addition to defects in the sperm–oocyte switch and germ-line survival, nos-deficient animals displayed germ-line defects that were not clear cell-fate transformations and also displayed ambiguous somatic gonadal malformations (Table 1, column 4 and legend). Surprisingly, however, no defects were observed in either the embryonic specification of the germ line or embryonic viability, despite the localization of NOS-3 protein to germ-line precursor cells in the embryo.

Discussion
The work reported here demonstrates that FBF and NOS-3 proteins interact physically, and that both participate in a single cell-fate decision, the sperm–oocyte switch in the hermaphrodite germ line. We suggest that FBF and NOS-3 function together in a macromolecular complex to regulate the switch in cell fate from spermatogenesis to oogenesis in C. elegans. FBF binds specifically to a regulatory site in the 3′ UTR of fem-3 mRNA [5], whereas NOS-3 binds RNA non-specifically (this work; also previously shown for Drosophila Nanos in vitro [18]). The FBF–NOS-3 interaction does not require RNA. These findings taken together lead to a simple model. We suggest that recruitment of NOS-3 by FBF stabilizes a regulatory complex on the fem-3 3′UTR (Figure 5a, left). This model cannot, however, explain the involvement of NOS-1 and NOS-2 in control of the sperm–oocyte switch, because neither NOS-1 nor NOS-2 detectably bound FBF. We therefore speculate that NOS-1 and NOS-2 form complexes with FBF indirectly, requiring either another protein or the fem-3 3′UTR to form a stable complex (Figure 5a, right). The relative contributions of protein–protein and protein–RNA interactions may differ between NOS-3 and the other two NOS proteins, such that NOS-1–FBF and NOS-2–FBF interactions, in the absence of RNA or the bridging protein, escape detection. Of course, more complex models are possible and include a possible association of NOS-1 and NOS-2 with other Puf proteins.

In Drosophila, translational repression of hunchback expression by the Puf protein, Pumilio, requires nos function [14,18]. As with NOS-1 and NOS-2, no physical interaction between the Pumilio and Drosophila Nanos proteins has been reported. The FBF–NOS-3 interaction leads us to suggest that proteins of the Puf and NOS families may
Models of NOS function in C. elegans. (a) A Puf–NOS–RNA regulatory complex. We propose that NOS and Puf proteins interact in a repressive complex that binds to a regulatory element (open box) in the 3′UTR. In this model, Puf proteins make sequence-specific contacts with RNA by their eight Puf repeats [5,11,13]; NOS contacts RNA non-specifically through its CCHC region ([18], this work), thereby stabilizing the complex. Left, FBF and NOS-3 interact directly. This Puf–NOS binding may further stabilize the complex. Right, PUF and NOS proteins interact indirectly through a bridging molecule, which could be either RNA or protein. In this model, we suggest the existence of a protein X. This may be the case with Drosophila Nanos and Pumilio, as no direct interaction has been reported to date. Similarly, we suggest that NOS-1 and NOS-2 may interact with Puf proteins in this fashion. (b) Multiple functions of nos genes in C. elegans. The nos genes in C. elegans are required for germ-line survival and for the sperm–oocyte switch. Regulation of the switch hinges on control of fem-3 mRNA; the mRNA targets (X) that mediate survival of the germ line have not been identified. The question marks indicate as yet unknown partners of NOS proteins. Germ-line survival is proposed to be the ancestral function of the nos genes (see text).

Generally mediate their effects by participating in protein–protein complexes bound to RNA. This view predicts that Drosophila Nanos functions in a complex with RNA-bound Pumilio, as proposed [13,18].

Regulation by NOS proteins in C. elegans is likely to be combinatorial, involving a network of protein–protein interactions and multiple mRNA targets (Figure 5b). This prediction follows from our finding that distinct developmental processes are regulated by NOS-related proteins in C. elegans. Here, we have focused on the sperm–oocyte switch and germ-line survival but, in addition, we have observed a defect in the mitotic arrest of germ-line precursor cells as well as post-embryonic defects in development of the somatic gonad. Furthermore, a problem with incorporation of germ-line precursor cells into the somatic gonad in animals has been observed in animals deficient for nos-1 and nos-2 [24]. Although FBF is required for the sperm–oocyte switch, it plays no apparent role in other nos-mediated effects, implying that one of the seven other puf orthologs in the C. elegans genome may provide these other functions or be redundant with FBF. Similarly, the Drosophila pumilio and nos genes have separate, but overlapping roles in germ-line development [10]. The various roles of C. elegans nos are likely to be mediated by regulating discrete target mRNAs. Although most of those targets have not yet been identified, they are likely to be distinct from fem-3 mRNA, because other nos defects, such as germ-line death, are not observed in fem-3 mutants [6,25]. The combinatorial nature of regulation by NOS prompts an analogy to well-documented principles of transcriptional regulation, in which distinct protein–protein interactions between transcriptional regulators discriminate among various target DNAs and yield specific biological outcomes.

Although Drosophila nos is best known for its role in patterning the early embryo (reviewed in [1,4,26,27]), it is also required for various aspects of germ-line development [10,15,28]. As C. elegans and Drosophila nos both control germ-line survival, we propose that the ancestral function of the nos genes may have been protection against germ-line death. It seems likely that the specialized roles of nos, such as the sperm–oocyte switch in nematodes and axis formation in Drosophila, were later evolutionary additions, in which a primordial regulator of germ-cell function was co-opted for other purposes, as proposed earlier by Forbes and Lehmann [10]. NOS homologs have been identified in a range of species, including vertebrates, and some are expressed in the germ line [29,30]. We speculate that these NOS proteins may be required generally for germ-line survival, as well as for idiosyncratic functions peculiar to individual species.

Conclusions

The three C. elegans nos genes function redundantly to control the sperm–oocyte switch and germ-line survival. FBF and NOS-3 bind to each other and both regulate the hermaphrodite sperm–oocyte switch. We suggest that these two RNA-binding proteins are physically associated in a regulatory complex that governs expression of fem-3 mRNA. All three C. elegans nos genes are also critical for germ-line survival. We propose that the primordial function of nos genes in evolution is protection against germ-line cell death, and that their role in the sperm–oocyte switch was an idiosyncratic, evolutionary accretion.

Note added in proof

Sonada and Wharton (in press; Genes and Development) have demonstrated that Drosophila Nanos and Pumilio form a tertiary complex with target RNA.

Materials and methods

Nomenclature and sequence analysis

The nos-3 gene corresponds to Y53C12B.3 in the C. elegans database; nos-1 and nos-2 correspond to RO3D7.7 and ZK1127.1, respectively. CPB-1 is C04H1.1. Sequence comparisons and analyses were performed using the Wisconsin GCG package version 8.1 for VMS.
The yeast two hybrid-screen was carried out using yeast strain LE392. A portion of the phage library was converted to plasmid by passage through E. coli strain R4AE, a derivative of the strain BNN132 [32], which expresses the Cre recombinease.

Isolation of deletion mutants

To induce deletion mutations, L4 haphemorphid proteins were treated with trimethylpsoralen and UV light as described [41]. Dispersing from mutagenized animals were cultured in 1152 groups of 50. After one generation, DNA was prepared from each population, and PCR used to identify populations with animals carrying deletions in nos-3. Primers were: VK109 (nucleotides 26965–26986), VK110 (nucleotides 26917–27128), VK111 (nucleotides 29982–30004), and VK112 (nucleotides 30152–30173), where numbers are derived from the cosmid and span nearly the entire coding region of nos-3. Populations carrying a deletion were repeatedly subdivided until homozygotes carrying the deletion were obtained. Each deletion mutant was outcrossed against wild-type N2 at least six times before further characterization. Deletion endpoints were determined by sequencing PCR products that spanned the region deleted.

Acknowledgements

We thank Kuppuswamy Subramaniam and Geraldine Seydoux for communicative results before publication; Ruth Lehmann for thought-provoking discussions; and the two reviewers for excellent suggestions. We are grateful to members of the Kimble and Wickens labs for scientific and technical suggestions as well as comments on the manuscript; K. Geles and S. Adams for valuable advice on purification of bacterially expressed proteins; Susan Strome for PGL-1 antibodies; and Sam Ward for anti-sperm antibodies. We appreciate Laura Vanderplow in the Biochemistry Media Lab for invaluable assistance in preparing figures. This work was supported by a Biotechnology Training Pre-doctoral Fellowship (B.K.), NIH
research grants (M.W., R.B., J.K.) and the American Heart Association (R.B.). J.K. is an investigator with the Howard Hughes Medical Institute.

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