Caenorhabditis elegans atx-2 Promotes Germline Proliferation and the Oocyte Fate

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ABSTRACT

In the Caenorhabditis elegans germline, proliferation is induced by Notch-type signaling. Entry of germ cells into meiosis is triggered by activity of the GLD-1 and GLD-2 pathways, which function redundantly to promote meiosis and/or inhibit proliferation. Activation of the germline Notch-type receptor, GLP-1, ultimately inhibits the activities of the GLD-1 and GLD-2 pathways. We previously identified several ego (enhancer of glp-1) genes that promote germline proliferation and interact genetically with the GLP-1 signaling pathway. Here, we show that atx-2 is an ego gene. Our data suggest that ATX-2 is not a positive regulator of the GLP-1 signaling pathway and GLP-1 signaling is not the sole positive regulator of ATX-2 activity. Moreover, our data indicate that GLP-1 must have an additional function, which may be to repress activity of a third meiotic entry pathway that would work in parallel with the GLD-1 and GLD-2 pathways. In addition to its role in proliferation, ATX-2 acts downstream of FOG-2 to promote the female germline fate.

MANY animal tissues contain populations of proliferating and differentiating cells (Spradling et al. 2001). The presence of proliferating (stem) cells ensures that differentiation can continue without depleting the tissue. The Caenorhabditis elegans adult germline contains proliferating cells, which ensure the continued production of gametes. Germ cell precursors begin to proliferate in early larval development (L1 stage), and proliferation continues throughout adulthood (Schedl 1997; Hubbard and Greenstein 2000; Seydoux and Schedl 2001). Proliferation in the larva is maintained by signaling from cells in the somatic gonad, the distal tip cells (DTCs), and the anchor cell (AC; Kimble and White 1981; Pepper et al. 2003a). During midlarval development (L3 stage), proximal germ cells enter meiosis, and mitosis becomes confined to the distal germline (Hansen et al. 2004b). From this point onward, DTC signaling is solely responsible for maintaining proliferation (Pepper et al. 2003a).

Induction of germline proliferation is mediated by Notch-type signaling (see Schedl 1997; Seydoux and Schedl 2001; Pepper et al. 2003a). The germline expresses a Notch-type receptor, GLP-1, that is activated by the somatic DSL-type signal, LAG-2 (Crittenden et al. 1994; Henderson et al. 1994; Tax et al. 1994; Fitzgerald and Greenwald 1995). Interaction between LAG-2 and GLP-1 is thought to trigger proteolytic cleavage of the GLP-1 intracellular domain, GLP-1intra, for transport to the nucleus where it forms a transcriptional regulatory complex with the LAG-1 and LAG-3/SEL-8 proteins (see Mumm and Kopan 2000). LAG-1 is a CSL-type transcriptional regulator, and LAG-3/SEL-8 is a glutamine-rich protein hypothesized to tether LAG-1 and GLP-1 in their interaction (Schedl 2001). In the absence of GLP-1 signaling, germ cells undergo only 1 or 2 rounds of mitosis in L1 stage before prematurely entering meiosis (Austin and Kimble 1987; Lambie and Kimble 1991). In contrast, constitutive GLP-1 activity prevents proliferating germ cells from entering meiosis (Berry et al. 1997; Pepper et al. 2003b; Hansen et al. 2004b). Instead, germ cells overproliferate, forming a tumor.

The GLD-1 and GLD-2 pathways act redundantly to promote germ cell entry into meiosis and/or inhibit proliferation and function independently to regulate other aspects of germline development (Francis et al. 1995a,b; Kadyk and Kimble 1998). The gld-2(null) gld-1 (null) double mutant has an overproliferation/tumorous germline phenotype that is caused by a defect in meiotic entry (Kadyk and Kimble 1998; Hansen et al. 2004b). Genetic data indicate that the GLD-1 and GLD-2 pathways are repressed by GLP-1 signaling (Francis et al. 1995b; Kadyk and Kimble 1998; Hansen et al. 2004a). GLD-1 expression is inhibited in the distal end of the germline by the FBF translational inhibitor (Crittenden et al. 2002) and rises as cells move proximally away from the DTCs (Jones et al. 1996). The GLD-1 and...
GLD-2 pathways are both thought to regulate expression of target genes at a post-transcriptional level on the basis of their molecular identities. GLD-1 is a STAR/KH domain translational repressor (Jones and Schedl 1995; Jan et al. 1999; Clifford et al. 2000; Lee and Schedl 2001; Xu et al. 2001; Marin and Evans 2003). The GLD-1 pathway also includes NOS-3 (Hansen et al. 2004a), which shows similarity to the Droso phila translational regulator, Nanos (Kraemer et al. 1999; Subramaniam and Seydoux 1999). GLD-2 is the catalytic domain of poly(A) polymerase and presumably regulates gene expression at the post-transcriptional level (Wang et al. 2002). GLD-2 physically interacts with GLD-3, a KH domain RNA-binding protein (Eckmann et al. 2002) that may direct GLD-2 to the target mRNA. Both the GLD-1 and the GLD-2 pathways are hypothesized to increase expression of genes required for meiosis and/or decrease expression of genes required for proliferation.

In addition to the GLD-1 and GLD-2 pathways, evidence suggests a third pathway promotes meiotic entry in adults (Hansen et al. 2004b). The basic observation in support of a third pathway is that some germ cells enter meiosis even in the absence of GLD-1 and GLD-2 activity (Hansen et al. 2004b). Meiotic entry is relatively late and infrequent in these animals, suggesting that, at least under standard laboratory conditions, the third pathway has lower activity and becomes active later than the GLD-1 and GLD-2 pathways. Although poorly defined, this third pathway is downstream of GLP-1 signaling and positively regulated by NOS-3 (Hansen et al. 2004b). Consequently, NOS-3 activity apparently promotes meiotic entry via two different pathways.

A second cell fate choice in the C. elegans germline is the sperm/oocyte choice. In XX animals (hermaphrodites), germ cells produce sperm during larval development and switch to oocyte production in the L4 stage (see Jones et al. 1996; reviewed by Schedl 1997; Goodwin and Ellis 2002; Stothard and Pilgrim 2003). Sex determination in C. elegans depends on a well-characterized genetic regulatory cascade (Meyer 2000; Stothard and Pilgrim 2003). A set of “global” sex determination genes act in both the soma and the germline to promote either the male or the female fate, depending on the X chromosome:autosome (X:A) ratio. Additional regulators function in the XX germline to ensure the production of sperm despite the “female” X:A ratio. One set of regulators promotes the male fate during larval development (fog-2 and gld-1) and a second set of regulators promotes the switch to oogenesis in late L4 stage (the nos, fbf, and mog genes). Loss-of-function (lf) mutations in genes that promote the male fate can feminize the XX germline (a Fog phenotype) such that it produces only oocytes. Similarly, lf mutations in genes that promote the female fate can masculinize the XX germline (a Mog phenotype), such that it fails to switch to oogenesis and instead continues to produce sperm during adulthood. Several regulators of the prolifera-

tion/meiosis choice also function in the sperm/oocyte choice. For example, GLD-1 promotes the male fate, while NOS-3 and the FBFs promote the female fate (Francis et al. 1995b; Zhang et al. 1997; Kraemer et al. 1999).

Interestingly, two proteins may work in opposition with respect to one fate choice and together in another choice. For example, although GLD-1 and NOS-3 both promote meiotic entry, they promote different sexual fates (Francis et al. 1995a,b; Kraemer et al. 1999).

We previously identified a set of genes that promote germline proliferation by screening for genetic enhancers of a weak glp-1 loss-of-function mutation (Qiao et al. 1995; Smardon et al. 2000; J. Spoerke and E. Maine, unpublished data). These screens yielded alleles of lag-1 as well as several novel ego (enhancer of glp-1) genes. Enhancement of a weak glp-1 loss-of-function phenotype by the ego mutations suggests that these genes could be general positive regulators of Notch signaling (i.e., tissue nonspecific) or might function downstream of Notch signaling in a germline-specific manner. While lag-1 encodes a component of the GLP-1 signaling pathway (Christensen et al. 1996), the phenotypes of most ego genes suggest that they have other functions in addition to promoting GLP-1 signaling (Qiao et al. 1995). Indeed, ego-1 encodes an RNA-directed RNA polymerase (RdRP) that promotes not only proliferation but also specific aspects of meiosis and gametogenesis as well as RNA interference (RNAi; Smardon et al. 2000).

Here, we introduce a new regulator of germline development, atx-2. ATX-2 protein is the sole C. elegans relative of mammalian ataxin-2, a protein implicated in RNA metabolism (Shibata et al. 2000) and nervous system function (Imbert et al. 1996; Pulst et al. 1996; Sanpei et al. 1996). Functional genomic studies have previously shown that depletion of ATX-2 protein by RNAi causes embryonic lethality (Gonczy et al. 2000; Kamath et al. 2003) and sterility (Maeda et al. 2001); the former was also shown by Kiehl et al. (2000). Here, we show that ATX-2 functions in the germline to promote germ cell proliferation and the oocyte fate. We show that ATX-2 may function independently of GLP-1 signaling, as it is not a positive regulator of the GLP-1 signaling pathway and GLP-1 signaling cannot be the sole positive regulator of atx-2. Our data indicate that GLP-1 has a third function beyond suppression of the GLD-1 and GLD-2 pathways, providing support for a third meiotic entry pathway that acts in parallel with GLD-1 and GLD-2. Finally, we show that ATX-2 acts downstream of FOG-2 in the sex determination process to promote the sperm/oocyte switch. We discuss the mechanisms by which ATX-2 may regulate cell fate and the implications of our data for the role of GLP-1 signaling in the germline.

MATERIALS AND METHODS

Nematode strains: Standard culture conditions were used (Epstein and Shakes 1995). Wild-type strain C. elegans variant
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Bristol (N2) and mutant phenotypes are as described by Houd-kin and Martinelli (1999) or as indicated. Nomenclature follows standard guidelines (Houdskin and Martinelli 1999). Mutations used were as follows:

- LGI: gla-1(q485), gla-2(q497), hT2;
- LGII: fgo-2(q71 and a040), nos-3(a0231, q650);
- LGIII: dpy-19(e1259), unc-32(e189), glp-1(bn18ts, q175), ego-4 (om30), unc-49(e382), unc-69(e587), hT2.

The following alleles are known to be null: gla-1(q485), gla-2 (q497), glp-1(q175), and nos-3(a0231) (see Hansen et al. 2004b).

**Developmental analysis:** The C. elegans hermaphrodite contains two gonad arms with distinct, independently regulated populations of germ cells. Therefore, our data are reported in terms of numbers of gonad arms evaluated rather than numbers of animals. Proliferating vs. meiotic germ cells were distinguished on the basis of nuclear morphology and expression of marker proteins. Nuclei were visualized by staining with the DNA dye, DAPI, using standard methods (e.g., Qiao et al. 1995). The meiotic marker, HM-3 (Zetka et al. 1999) and hermaphrodite P(0)P(C (Paserbeka et al. 2001), which under our staining conditions is a marker for mitotic cells (Hansen et al. 2004b), were visualized by indirect immunofluorescence as described by Hansen et al. (2004a,b).

**RNA interference assays:** RNAi was performed by the feeding method of Timmons et al. (2001) or the injection method of Fire et al. (1998). In the former case, we used a feeding construct containing a (message-coding) portion of atx-2 genomic DNA cloned into the L4440 vector and transformed into Escherichia coli strain HT115 (Timmons et al. 2001). This construct was a kind gift of Julie Ahringer. Cells were seeded onto NGM-Lite plates (Sun and Lambie 1997) that contained appropriate concentrations of Ampicillin and IPTG and allowed to grow at room temperature (~20°C–22°C) for ~48 hr. Seeded plates were stored at 15°C and used within ~2 weeks of seeding. To ensure consistent expression of the dsRNA, the plasmid was routinely retransformed into HT115 rather than restreaked. L4 or adult animals were placed onto the plates and their progeny were raised continuously in the presence of the dsRNA. For injections, a portion of the atx-2 cDNA was cloned into pBluescript to generate pEL80. The cDNA insert was amplified by PCR, transcribed in vitro, annealed to produce dsRNA for injection, and injected at a final concentration of 500 ng/μl.

The efficacy of atx-2 RNAi varied from one experiment to another, as is typical for RNAi of most genes (see Maini 2001). To control for this variation, (1) wild-type animals were treated in parallel with mutants in each experiment (e.g., using the same batch of feeding plates or dsRNA and the same culture condition) and (2) the effect of atx-2(RNAi) on any given genotype was tested several times. We consistently observed a reduced number of proliferating cells in atx-2(RNAi) germ-lines, as evidenced by a smaller mitotic “zone” at the distal end and, in some cases, a complete loss of proliferation.

**cDNA analysis:** Partial atx-2 cDNA sequence is available from the C. elegans EST (Expressed Sequence Tag) Project. We obtained two cDNAs, yk1040b12 and yk63e6, from the C. elegans EST Project and sequenced them to determine the rest of the atx-2 cDNA sequence. The cDNA sequence differs from the predicted D2045.1 open reading frame (ORF) as described in the text and Figure 1 and has been deposited in GenBank (accession no. AF571965).

**ego-4 mapping:** Previous mapping placed ego-4 within ~1 map unit to the right of glp-1 on LGIII (Qiao et al. 1995). Using three-factor mapping, we placed ego-4 (om30) just to the left of unc-49. Of 29 non-Dpy-19 Unc-49 recombinants picked from a dpy-19 + + unc-49+/+ glp-1(bn18ts) ego-4 (om30) + strain, two were glp-1(bn18ts) ego-4 (om30) unc-49 and 27 were glp-1 (bn18ts) ego-4(+). Using single nucleotide polymorphism (SNP) mapping, we placed ego-4 (om30) to the right of an SNP at position 29778 in clone R01H10. We assayed this polymorphism in 32 Unc-32 non-Ego-4 recombinants recovered from an unc-32 glp-1(ts) ego-4 (om30)/+ + + strain where the ego-4 chromosome is from the N2 wild-type background and the wild-type chromosome is from the polymorphic wild-type strain, CB4856. In 2/32 cases, recombination had occurred to the right of the SNP, indicating the ego-4 lies to the right of the SNP. We used RNAi to assay the 40 predicted open reading frames in the region between the R01H10 SNP and unc-49 for ego activity. Depletion of one predicted gene product, D2045.1/ATX-2, enhanced glp-1(bn18ts) at 20°C.

**RT-PCR:** Total RNA was isolated from a group of 30 dpy-19 (e1259) ego-4 (om30) hermaphrodites and a group of 30 dpy-19 (e1259) control animals, each grown to 1 day past L4 stage, using a TRIZOL-based method. The RNA was resuspended in 30 μl of DEPC-treated water for each genotype, and RT-PCR was performed using 1, 2, and 5 μl of the RNA as template. The different amounts of template were used to ensure that amplification was in the linear range. RT-PCR was performed using the manufacturer’s instructions (Superscript One Step RT-PCR, Invitrogen, Carlsbad, CA). The region amplified by RT-PCR contained five exon splice sites so that genomic DNA contamination, if present, could be detected.

**RESULTS**

**Identification of atx-2 as an ego gene:** We previously identified several genes that promote germline proliferation and/or inhibit meiotic entry by screening for genetic enhancers of a weak glp-1 loss of function (ego mutations; Qiao et al. 1995). Specifically, we used the temperature-sensitive glp-1(bn18ts) mutation (Kodovini et al. 1992) that, at 20°C, has nearly wild-type germ-line proliferation (Qiao et al. 1995). In these mutants, germ cell number is reduced (e.g., >50% of wild type at young adult stage), but >95% of germlines maintain proliferation (Qiao et al. 1995). We screened for recessive enhancer mutations that would produce a severe Glp-1 loss-of-function phenotype at 20°C in the glp-1(bn18ts) background.

To initiate molecular studies of the ego-4 gene, we mapped it relative to genetic markers and SNPs and then used RNAi to test each gene in the interval for enhancement of glp-1 (see MATERIALS AND METHODS.) By analogy with our genetic screen, we asked whether RNAi-mediated knockdown of each gene product enhanced glp-1(bn18ts) at 20°C. We raised glp-1(bn18ts) animals on the appropriate dsRNA feeding strain at 20°C and monitored their germline development. One ORF, D2045.1 (also called atx-2), behaved like an ego gene in this assay. Mitotic proliferation was not maintained into adulthood in most glp-1(bn18ts) atx-2(RNAi) germlines, but instead all germ cells prematurely entered meiosis (Table 1; Figure 2D; data not shown). For example, proliferation was absent from 84% of glp-1(bn18ts) atx-2(RNAi) germlines (on average) at ~24 hr into adulthood (Table 1). In these germlines, the proliferating germ cells had all entered meiosis at an earlier point in development.
TABLE 1

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Temp (°)</th>
<th>Range</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type</td>
<td>20, 25</td>
<td>100*</td>
<td>NA</td>
</tr>
<tr>
<td>atx-2(RNAi)</td>
<td>20</td>
<td>95</td>
<td>90–100</td>
</tr>
<tr>
<td>atx-2(RNAi)</td>
<td>25</td>
<td>93</td>
<td>86–98</td>
</tr>
<tr>
<td>gfp-1(bn18ts)</td>
<td>20</td>
<td>16</td>
<td>11–25</td>
</tr>
<tr>
<td>atx-2(RNAi)</td>
<td>20</td>
<td>&gt;99</td>
<td>NA</td>
</tr>
</tbody>
</table>

RNAi was done by feeding. Animals were raised on feeding plates and scored at ~48 hr postadult molt. n, number of gonad arms examined; NA, not applicable; % proliferating germlines, the percentage of germlines containing a population of proliferating germ cells. The percentage listed is the average of three or more independent experiments. The range of percentage of proliferating germlines in the independent experiments is listed. Wild type and gfp-1(bn18ts) were treated in parallel in each trial.

† Qiao et al. (1995).

We next examined whether ego-4 is in fact the same gene as atx-2/D2045.1. ego-4 is identified by a single allele, om30 (Qiao et al. 1995). The ego-4(om30) phenotype is similar to the atx-2(RNAi) phenotype, but not identical (see below). Therefore, we considered two possibilities: (1) om30 may be a partial loss-of-function mutation in atx-2 or (2) atx-2 and ego-4 may be different genes with related functions that happen to be located close together. To investigate these possibilities, we sequenced the atx-2 gene from gfp-1(bn18ts) ego-4(om30) animals. We did not detect a sequence change within the atx-2 transcribed region or within ~1 kb up- or downstream of the gene (see MATERIALS AND METHODS; data not shown). We also asked whether atx-2 mRNA levels are reduced in ego-4(om30) mutants. To test this possibility, we did RT-PCR with RNA isolated from ego-4(om30) and control animals (see MATERIALS AND METHODS). atx-2 mRNA levels were equivalent in the two RNA populations (data not shown). Therefore, the ego-4 (om30) mutation does not lower expression of atx-2 mRNA. Overall, atx-2 and ego-4 appear to be different genes that interact at the genetic level to promote germ-line proliferation; however, genetic analysis of atx-2 will resolve this point.

atx-2 cDNA sequence: The D2045.1 ORF was originally annotated by the C. elegans genome sequencing project and predicted to encode a protein distantly related to mammalian ataxin-2 (Kiehl et al. 2000; Satterfield et al. 2002). D2045.1 was subsequently named atx-2 (ataxin-2 related) by Kiehl et al. (2000). To facilitate molecular studies and better understand the relationship between C. elegans ATX-2 and mammalian ataxin-2, we determined the atx-2 cDNA sequence (Figure 1; see MATERIALS AND METHODS). The atx-2 cDNA contains 3619 nucleotides, including an SL1 trans-spliced leader, and differs from the predicted D2045.1 ORF at the 5’ end and in exon 10 (see MATERIALS AND METHODS). On the basis of comparison of the cDNA and genomic sequences, the atx-2 gene contains 12 exons and spans 6430 nucleotides from the trans-splice acceptor sequence through the polyadenylation site (Figure 1A). The SL1 leader sequence serves as a reliable indicator of the 5’ end of the mRNA.

The predicted ATX-2 protein contains 959 amino acids and, as previously described by Satterfield et al. (2002), shares two regions of sequence conservation with mammalian ataxin-2 (Figure 1, B–D). In addition, ATX-2 and ataxin-2 are both glutamine rich. Proteins that contain the two conserved regions are also present in insects and plants (Figure 1B). We will refer to these proteins, collectively, as “ataxin-2 related proteins.” The conserved regions have been named the ATX2-N (amino-terminal) and ATX2-C (carboxy-terminal) domains (Satterfield et al. 2002). We point out that these names reflect the relative positions of the domains within the protein and that the ATX2-C domain is typically located in the middle of the protein. The ATX2-N domain is related in sequence to a portion of a Saccharomyces cerevisiae protein, PBP1 [protein that binds poly(A)-binding protein; Mangus et al. 1998; Figure 1C]. The ATX2-C domain essentially contains a PAM2 motif (Figure 1D), which has been shown to mediate binding of certain mammalian (Paip) proteins to poly(A)-binding protein (PABP; Khaleghpour et al. 2001; Roy et al. 2002). Interestingly, yeast PBP1 does not contain a PAM2 motif, despite its interaction with PABP (Mangus et al. 1998).

atx-2 activity promotes germ line proliferation: We characterized the effect of atx-2(RNAi) in a wild-type genetic background using the feeding and injection methods (Fire et al. 1998; Timmons et al. 2001; see MATERIALS AND METHODS). In the first case, wild-type animals were raised on the bacterial feeding strain and monitored for germline developmental defects. In the second case, animals were injected with dsRNA and their progeny were monitored. The progression of germ cells from mitosis through meiotic stages and into gametogenesis was monitored on the basis of chromosome morphology and expression of the REC-8 and HIM-3 proteins (see MATERIALS AND METHODS). REC-8, a cohesin component, is strongly expressed in mitotic germ cells (Pasierbek et al. 2001; Hansen et al. 2004b). HIM-3, an outer component of the synaptonemal complex, is strongly expressed in meiotic germ cells, beginning in the transition zone (Zetka et al. 1999; Hansen et al. 2004b). atx-2 (RNAi) consistently produced a smaller “mitotic zone” than did controls: at 24 hr after L4 stage, the mitotic zone extended 11 cell diameters from the distal tip cell (n = 7 gonad arms; SE = 0.5 cell diameters) vs. 19.5 cell diameters for control animals treated with gfp dsRNA and wild-type germlines (Figure 2, A–C; Hansen et al. 2004b). Consistent with this reduction, some ani-
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Figure 1.—Structure of the atx-2 gene. (A) The atx-2 message contains 12 exons (boxes) and a SL1 trans-spliced leader. 5′ and 3′ UTRs are indicated in black. ATX-2 is predicted to contain 959 amino acids. It differs from the D2045.1 prediction in containing 87 fewer amino acids at the amino terminus and an additional 20 amino acids in exon 10. The PBP1 homology domain is shaded; exon 7 contains the PAM2 motif (*). See text for more details. (B) Schematic of ataxin-2 related proteins from several organisms. Homology is limited to the PBP1 (hatched box) and PAM2 (heavy line) regions. (C and D) Alignment of conserved domains from ataxin-2-like proteins. (C) ATX2-N domain and (D) PAM2 motifs from mouse ataxin-2 (MAtx2; accession no. NP_033151), human ataxin-2 (HAtx2; accession no. NP_002964), Drosophila melanogaster Datx2 (accession no. NP_732033), Anopheles gambiae ataxin-2 related protein (AAtx2; accession no. EAA05947), C. elegans ATX-2, and Arabidopsis thaliana ataxin-2 related protein (AtAtx2; accession no. NP_566471). The Sm and PBP-1 homology domains within ATX2-N are underlined.

In mammals eventually lost the mitotic zone altogether. For example, by ~48 hr after the adult molt (~60 hr after L4), the mitotic zone was absent from ~5% of germlines (on average) at 20°C (Table 1). This effect was slightly stronger at 25°C (Table 1), as is typical for the RNAi response of many genes (Maine 2001). The distal end of atx-2(RNAi) gonad arms is often spade-like in shape compared with wild-type gonad arms; the reason for this morphological difference is unknown.

We observed the same set of atx-2(RNAi) germline defects in both wild-type (N2) and rrf-1 mutant backgrounds. rff-1 encodes an RdRP (Smardon et al. 2000) required for RNAi in the soma (Stjøen et al. 2001). rff-1 mutants are resistant to RNAi in the soma, but have normal sensitivity in the germline (Stjøen et al. 2001). Therefore, this result suggests that ATX-2 promotes germline development by functioning in the germline itself rather than in somatic cells.

**atx-2(RNAi) enhances the ego-4 proliferation defect:** In a glp-1(+) background, ego-4(om30) mutants have a reduced number of germ cells, although young adults typically retain a mitotic zone (Qi et al. 1995). We now report that the mitotic zone is subsequently lost by a substantial proportion of ego-4(om30) adults (~20% of germ lines at ~48 hr into adulthood; n = 61), with all germ cells in these animals having prematurely entered...
meiosis. Moreover, when a mitotic zone is present, it typically is smaller than wild type (data not shown).

Given that atx-2(RNAi) and ego-4(om30) both produce a proliferation defect and interact with glp-1, we asked whether atx-2 RNAi enhances the ego-4(om30) phenotype. We raised atx-2(RNAi) and ego-4(om30) atx-2(RNAi) animals in parallel and examined them for proliferating germ cells. ego-4(om30) atx-2(RNAi) animals consistently had a more severe proliferation defect than did either ego-4(om30) or atx-2(RNAi) animals. For example, the mitotic zone was present in only ~40% of ego-4(om30) atx-2(RNAi) germlines (on average) at ~48 hr into adulthood; in the remaining ~60% of germlines, all cells had prematurely entered meiosis (n = 40). In contrast, a mitotic zone was present in 98% of atx-2(RNAi) controls that were done in parallel (n = 56) and 80% of ego-4(om30) animals (above). Therefore, depletion of ATX-2 enhanced the ego-4(om30) proliferation defect.

**atx-2(RNAi) suppresses the gld-2 gld-1 meiotic entry defect:** We investigated the relationship between ATX-2 and GLD-1/GLD-2 by testing whether atx-2(RNAi) can suppress the gld-2 gld-1 meiotic entry defect. gld-2 gld-1 germlines are tumorous because few germ cells enter meiosis. They contain predominantly mitotic cells, which are REC-8 positive and HIM-3 negative. However, a few meiotic nuclei are often present, which are REC-8 negative and HIM-3 positive (Hansen et al. 2004a; compare Figure 3, A and C). We asked whether atx-2(RNAi) suppresses the gld-2 gld-1 meiotic entry defect/germline tumor. Indeed, gld-2 gld-1,atx-2(RNAi) germlines show extensive meiotic entry (Table 2; Figure 3D). All arms contained a distal mitotic region and most had some proliferating cells in the proximal end of the gonad, while the remaining arms completely lacked proximal proliferating cells. Therefore, atx-2(RNAi) at least partially suppresses the gld-2 gld-1 meiotic entry defect. We also note that these germlines are somewhat underproliferative compared with wild type. The requirements for gld-1 and gld-2 activity later in meiotic prophase progression were apparently not suppressed by atx-2(RNAi), because gld-2 gld-1,atx-2(RNAi) germ cells did not complete meiosis (Figure 3D).

We know that ATX-2 cannot simply be a positive regulator of GLP-1 signaling, because gld-2 gld-1; atx-2(RNAi) and gld-2 gld-1; glp-1 animals do not have equivalent germlines. Instead, we observed significantly more meiotic entry in gld-2 gld-1; atx-2(RNAi) germlines than Hansen et al. (2004b) observed in gld-2 gld-1; glp-1 germlines. By the same logic, GLP-1 signaling cannot be the sole positive regulator of atx-2 expression (although it could be a positive regulator). One possibility is that ATX-2 may promote proliferation and/or inhibit meiosis independent of GLP-1 signaling. For example, ATX-2 may act in parallel with GLP-1 signaling to negatively regulate targets downstream of the GLD-1 and/or GLD-2 pathways, or, alternatively, a third meiotic entry pathway.

Given the genetic interaction between ego-4 and atx-2, we asked whether ego-4(om30) suppresses the gld-2 gld-1 meiotic entry defect. We constructed a gld-2 gld-1/hT2; dpy-19 ego-4(om30)/hT2 balanced strain and examined
Figure 3.—Depletion of ATX-2 promotes meiotic entry. One arm of the adult hermaphrodite germline is shown in each panel. Chromosomes were colabeled with the DNA dye, DAPI, and antibodies against HIM-3 and REC-8. In (A) wild-type and (B) atx-2(RNAi) germlines, extensive meiotic cells are present and HIM-3 expression is strong. Note that oocytes are not present in B, and ectopic sperm are located distal to the loop. (C) The tumorous gld-2 gld-1 germline has very few meiotic germ cells as evidenced by extensive REC-8 staining and little HIM-3 staining. (D) gld-2 gld-1; atx-2(RNAi) animals contain extensive meiotic germ cells as evidenced by extensive HIM-3 staining. REC-8 staining indicates the presence of a mitotic zone in the distal germline.

gld-2 gld-1; dpy-19 ego-4 progeny for the presence of pachytene cells and suppression of the tumor. We found no suppression of the meiotic entry defect (n = 40).

A third meiotic entry pathway? To further investigate the relationship between atx-2 and meiotic entry, we tested whether atx-2(RNAi) increases the level of meiotic entry in gld-2 gld-1; glp-1 triple mutants. In the absence of GLD-1/GLD-2 pathway activities, GLP-1 is not required to maintain germ cell proliferation (Kadyk and Kimble 1998). We find a substantial increase in meiotic entry in gld-2 gld-1; glp-1 atx-2(RNAi) germlines compared with gld-2 gld-1; atx-2(RNAi) germlines (Figure 4, A–C; Table 2). In most cases, the distal mitotic region is completely absent as evidenced by the lack of REC-8 staining and the presence of HIM-3 staining extending all the way to the distal end.

This result further demonstrates that ATX-2 is active in the absence of GLP-1 activity, confirming that ATX-2 is not a positive regulator of GLP-1 signaling and GLP-1 signaling cannot be the sole positive regulator of atx-2 activity. Importantly, this result reveals another role for GLP-1 signaling in addition to suppression of the GLD-1 and GLD-2 pathways. For example, GLP-1 signaling may repress a third meiotic entry pathway. In the absence of GLP-1 signaling, this pathway would be more active. Hyperactivation of this third pathway in combination with atx-2(RNAi) would result in a greater degree of meiotic entry than atx-2(RNAi) alone.

The existence of a third meiotic entry pathway that is active in late L4 larvae/adults has previously been hypothesized on the basis of two lines of evidence: (1) meiosis occurs in gld-2 gld-1 double mutants (albeit delayed and at a low level) whereas meiosis is completely absent in germlines with ligand-independent, constitutive GLP-1 signaling, and (2) the gld-2 gld-1 tumorous phenotype is enhanced by a weak glp-1 gain-of-function allele (Hansen et al. 2004b; see below). Our data provide independent evidence that a third pathway is likely to exist. Our data are consistent with two general alternatives for how ATX-2 might interact with meiotic entry pathways. ATX-2 might act directly downstream of GLD-1 and/or GLD-2 or might act in parallel with GLD-1 and/or GLD-2 pathways to regulate common targets (Figure 5, A and B). In either case, depletion of ATX-2 would...
Consistent results were obtained in independent RNAi experiments; 100% of gld-2 and gld-2; nos-3 double mutants have a meiotic entry defect (tumorous phenotype) that, surprisingly, is partially enhanced by glp-1 null mutations, e.g., glp-1(q175) null (Hansen et al. 2004b). Although all three genotypes cause a severe meiotic entry defect, they can be ranked with respect to the proportion of meiotic nuclei, as follows: gld-2; nos-3; glp-1 > gld-2 > gld-2; glp-1; glp-1 (Hansen et al. 2004b). n, number of gonad arms examined; % suppressed germlines, the percentage of germlines with a decrease in the proportion of proliferating nuclei (REC-8 positive, HIM-3 negative), a corresponding increase in the proportion of meiotic nuclei (REC-8 negative, HIM-3 positive; see Hansen et al. 2004b), and the presence of pachytene nuclei, which are never observed in gld-2, gld-1, gld-2; glp-1 or gld-2; nos-3; glp-1 synthetic tumorous mutants.

atx-2(RNAi) does not suppress glp-1(oz112gf): We wanted to investigate whether GLP-1 signaling might be a positive regulator of ATX-2 activity or, alternatively, ATX-2 activity is completely independent of GLP-1. To investigate this question, we asked whether atx-2(RNAi) can suppress a ligand-independent, constitutive glp-1 gain-of-function mutation. The glp-1(oz112gf) allele encodes a ligand-independent receptor that produces a germline tumor (Berry et al. 1997). In the most extreme case, animals carrying two glp-1(oz112) alleles plus a third wild-type allele [glp-1(oz112/oz112/+)], germ cells never enter meiosis (Hansen et al. 2004b). If GLP-1 signaling positively regulates ATX-2 activity, then we reasoned that atx-2(RNAi) should at least partially suppress glp-1(oz112gf). Alternatively, if ATX-2 is regulated independently of GLP-1, then atx-2(RNAi) may not suppress glp-1(oz112gf).

We tested whether atx-2(RNAi) can restore meiosis to glp-1(oz112/oz112); glp-1(+) germlines. We found that glp-1(oz112/oz112/+ ) atx-2(RNAi) animals have a similar phenotype, indicating that atx-2(RNAi) does not suppress glp-1(gf) (data not shown). This result suggests that ATX-2 activity is not regulated by GLP-1 signaling. We verified that oz112 animals are sensitive to RNAi by treating them with nce-1 dsRNA. Loss of nce-1 function prevents cell division in all tissues (Boxem et al. 1999). We found that glp-1(oz112) animals responded properly to nce-1(RNAi), indicating that they are sensitive to RNAi (data not shown). Our results suggest that ATX-2 may function independent of GLP-1 signaling, a hypothesis consistent with other data presented above. However, the caveat remains that atx-2(RNAi) may not fully deplete ATX-2. Thus, if glp-1(oz112) is suppressed only in the complete absence of ATX-2 activity, then we may not see suppression under our conditions (see discussion).

atx-2 promotes the oocyte fate: In addition to its role in proliferation, atx-2 functions in sex determination by promoting the oocyte fate. The atx-2(RNAi) germline often fails to switch from spermatogenesis to oogenesis, but instead continues to produce sperm during adult-
C. elegans atx-2 Regulates Germ Cell Fate

Figure 4.—Extensive meiosis occurs in gld-2 gld-1; glp-1 atx-2(RNAi) and gld-2; glp-1 atx-2(RNAi); nos-3 germlines. One arm of the adult hermaphrodite germline is shown in each panel. (A) The characteristic gld-2 gld-1; glp-1 tumorous phenotype (Kadyk and Kimble 1998; Hansen et al. 2004b). (B) Suppression of the gld-2 gld-1; glp-1 tumorous phenotype by atx-2(RNAi). The mitotic region is absent, consistent with the absence of GLP-1 signaling. Note that the extent of meiotic entry is higher here than in gld-2 gld-1; atx-2(RNAi) animals (Figure 3D). (C) Another example of suppression of the gld-2 gld-1; glp-1 tumor by atx-2(RNAi). (D) Suppression of the gld-2; glp-1; nos-3 meiotic entry defect by atx-2(RNAi). Extensive meiosis is visible. The distal mitotic region is absent.

hood. Consequently, sperm and/or primary spermatocytes eventually extend up around the loop region, rather than being confined to the spermatheca (Figures 2B and 3B). These defects are similar to those produced by mutations in previously identified genes that promote the oocyte fate, including the mog (masculinization of the germline) genes (Graham and Kimble 1993; Graham et al. 1993). The continued production of sperm during adulthood indicates a defect in the spermoocyte switch rather than a defect in oogenesis (Graham and Kimble 1993). Moreover, sperm counts indicated that these germlines contained more sperm [average 390 ± 66 (SE) at 24–48 hr postadult molt; n = 7 gonad arms] than the ~150 sperm produced by wild-type germlines. In atx-2 RNAi feeding experiments, an average of ~72% of germlines at 25° and ~44% of germlines at 20° were Mog (Table 3). In atx-2 RNAi injection experiments, ~77% of germlines were Mog at 20° (Table 3).

The atx-2(RNAi) Mog defect suggests that ATX-2 promotes the oocyte fate. We did not observe any sign of masculinization of the XX soma; therefore we conclude that ATX-2 functions specifically in germline sex determination (data not shown). Sex determination in the XX germline involves tissue-specific regulatory mechanisms that repress the global feminizing mechanism during larval development and then allow the germline to become female at approximately the LA/adult molt (Schedl 1997). The first known step in germline-specific regulation is repression of tra-2 expression by the FOG-2/GLD-1 complex (Clifford et al. 2000). In the absence of TRA-2 activity, the germline is male. To switch to oogenesis, the FOG-2/GLD-1 complex must be inactivated (by a mechanism that is unclear at present). In mutants that lack fog-2 activity, tra-2 activity is thought to be abnormally high in the XX larval germline, which precludes the male fate and causes the germline to instead be female (Schedl and Kimble 1988; Jan et al. 1999; Clifford et al. 2000).

To address the role of atx-2 in the sperm-oocyte switch, we investigated whether atx-2(RNAi) can suppress
mutations in \textit{fog-2}. We raised \textit{fog-2(oz40)} and \textit{fog-2(q71)} null mutants in the presence of \textit{atx-2} dsRNA and found that most of them produced sperm. Some \textit{atx-2(RNAi)}; \textit{fog-2(−)} animals produced both sperm and oocytes whereas others produced only sperm (Table 3). Therefore, depletion of \textit{ATX-2} suppressed the \textit{Fog-2} phenotype. On the basis of this result, \textit{atx-2} may act downstream of \textit{fog-2} to promote the female fate. In animals that produce sperm and oocytes (rather than only sperm), we suspect that the RNAi was less effective at reducing \textit{ATX-2} protein levels. In fact, the efficacy of the RNAi was relatively weak in this particular set of experiments (see Table 3 controls).

We next tested whether \textit{atx-2(RNAi)} could suppress feminizing mutations in genes that act downstream of \textit{fog-2}. We found that \textit{atx-2(RNAi)} could not suppress the \textit{tra-2(q122)} gain-of-function allele (which is deleted for one \textit{GLD-1} binding site in the 3′-UTR; \textit{Jan et al.} 1999) at 25° (\(n = 164\)) or loss-of-function mutations in \textit{fem-1} (\(n = 24\)). The simplest interpretation of these data is that \textit{atx-2} acts upstream of \textit{tra-2} or as a positive regulator of \textit{tra-2}. This hypothesis needs to be tested using an \textit{atx-2 (null)} mutation (see \textit{discussion}).

**DISCUSSION**

The \textit{C. elegans} \textit{atx-2} gene promotes germline proliferation and the oocyte fate. We have examined \textit{atx-2} function using RNAi to deplete the \textit{ATX-2} protein. We find that \textit{atx-2} activity prevents premature meiotic entry. \textit{ATX-2} does not upregulate \textit{GLP-1} signaling activity, and \textit{GLP-1} signaling cannot be the sole positive regulator of \textit{ATX-2} activity. Therefore, \textit{atx-2} may work independently of and in parallel with the \textit{GLP-1} signaling pathway to promote proliferation and/or repress meiotic entry. On the basis of our data, \textit{GLP-1} signaling has another function beyond repression of \textit{GLD-1} and \textit{GLD-2} activity, suggesting that these two pathways are not the only targets of \textit{GLP-1} signaling and substantiating the idea of a third meiotic entry pathway. In addition to its role in proliferation, \textit{atx-2} functions in sex determination to promote the sperm-oocyte switch in \textit{XX} animals. \textit{ATX-2} appears to act downstream of the \textit{XX} germ-line masculinizing gene, \textit{fog-2}, to either promote activity of a feminizing gene (e.g., \textit{tra-2}) or limit activity of a masculinizing gene (e.g., \textit{fem-3}). The dual role of \textit{ATX-2} in the proliferation/meiosis and male/female choices is consistent with the pattern observed for other regulators in the \textit{C. elegans} germline. For example, \textit{NOS-3}, \textit{FBE}, \textit{GLD-1}, and \textit{GLD-2} function in later aspects of development subsequent to meiotic entry (\textit{Francis et al.} 1995a,b; \textit{Kadyk and Kimble} 1998; \textit{Kraemer et al.} 1999).

Mammalian \textit{ataxin-2} was first studied because it is associated with the human neurodegenerative disease, spinocerebellar ataxia (\textit{Imbert et al.} 1996; \textit{Pulst et al.} 1996; \textit{Sanpei et al.} 1996). Neurodegeneration is triggered by polyglutamine expansions that lead to formation of protein plaques. On the basis of molecular features of the \textit{ataxin-2} protein, as well as its ability to bind to RNA-binding proteins (\textit{Shibata et al.} 2000; \textit{Khaledpour et al.} 2001; \textit{Roy et al.} 2002), \textit{ataxin-2} has been hypothesized to function in RNA metabolism (\textit{Satterfield et al.} 2002). We propose that \textit{C. elegans} \textit{ATX-2} acts at the post-transcriptional level to regulate gene expression for germline proliferation and female sex determination. A role for \textit{ATX-2} in the \textit{C. elegans} nervous system is not known given that RNAi (by feeding and injection) is very inefficient in nervous tissue.
ATX-2 identifies another regulatory mechanism that promotes germline proliferation: GLP-1 activity restricts expression of the GLD-1 meiotic entry pathway and is absolutely required for maintenance of germline proliferation in an otherwise wild-type background (Austin and Kimble 1987; Francis et al. 1995a; Hansen et al. 2004a,b). GLP-1 activity is also suspected to restrict activity of the GLD-2 pathway (Kadyk and Kimble 1998). We suggest that ATX-2 may promote germline proliferation and/or prevent meiotic entry via a mechanism that is partially or completely independent of GLP-1 signaling. For example, ATX-2 may function in parallel with GLP-1 signaling in the germline. ATX-2 clearly does not promote GLP-1 activity and, if GLP-1 signaling is a positive regulator of ATX-2 activity, then it cannot be the sole positive regulator. Moreover, the inability of atx-2(RNAi) to suppress glp-1(oz112) suggests that GLP-1 may have no role in regulating ATX-2. We note, however, that atx-2(RNAi) may not fully eliminate ATX-2 activity. An atx-2(null) mutation would allow us to know with certainty that the loss of ATX-2 function does or does not suppress glp-1(gf).

Suppression of the tumorous gld-2 gld-1 meiotic entry defect by atx-2(RNAi) is consistent with several models. First, ATX-2 activity may be directly repressed by the GLD-1 and/or GLD-2 pathways (Figure 5A). Second, ATX-2 may act in parallel with GLD-1 and/or GLD-2 to regulate common target genes (Figure 5B). GLD-1 and GLD-2 presumably upregulate expression of genes required for meiotic entry and/or repress expression of genes required for proliferation. The identities of these target genes are unknown, although evidence suggests that the GLD-2 pathway also promotes gld-1 translation (Hansen et al. 2004a). ATX-2 would presumably work in opposition to the GLD-1 and GLD-2 pathways, upregulating expression of genes that are required for proliferation and/or repressing expression of genes required for meiosis. If they regulate common targets, then presumably GLD-1 and GLD-2 activity would override ATX-2 absolutely required for maintenance of germline proliferation.

Analysis of ATX-2 suggests an additional function for GLP-1 signaling: One striking observation is the significantly higher proportion of meiotic germ cells in gld-2 gld-1; glp-1 atx-2(RNAi) germlines than in gld-2 gld-1; atx-2(RNAi) germlines. On the basis of these results, GLP-1 signaling must have (at least) one other function in addition to repressing the GLD-1 and GLD-2 pathways. One obvious possibility is that GLP-1 activity represses the proposed third meiotic entry pathway (Hansen et al. 2004b). Two previous lines of evidence suggested the existence of a third pathway. First, gld-2 gld-1 tumorous germ lines have some meiotic germ nuclei whereas gld-2 gld-1; glp-1(oz112gf) tumorous germ lines do not; second, a weak glp-1(gf) allele enhances the tumorous phenotype of gld-2 gld-1 animals [gld-2 gld-1; glp-1(ar202gf)] animals show less meiotic entry than gld-2 gld-1 animals; Hansen et al. 2004b). Our data provide independent evidence for the existence of a third pathway insofar as they reveal a third function for GLP-1. By this logic, GLP-1 signaling represses the third meiotic entry pathway in gld-2 gld-1; atx-2(RNAi) animals, thereby allowing proliferation to occur at the distal end. The third pathway is no longer repressed in gld-2 gld-1; glp-1 atx-2(RNAi) animals and is free to promote meiotic entry throughout the germline.

The relationship between atx-2 and ego-4: atx-2 RNAi strongly enhanced the proliferation defect associated with ego-4(om30). This result is consistent with enhancement of glp-1(ts) by both ego-4(−) and atx-2(RNAi), indi-

### Table 3

<table>
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<th>Genotype</th>
<th>Temp (°C)</th>
<th>% sperm + oocytes</th>
<th>% Mog</th>
<th>% Fog</th>
<th>% other</th>
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* n, number of gonad arms examined; NA, not applicable.

* Data are the average of four or more independent feeding experiments; % Mog germlines ranged from 18 to 83% at 20°C and 50 to 97% at 25°C.

* Schedl and Kimble (1988); Clifford et al. (2000).

* Wild type and fog-2 were treated in parallel, with wild type serving as a control for efficacy of the RNAi in this specific set of experiments. Mock RNAi was done by feeding HT115 cells containing the L4440 vector to fog-2 mutants to make sure that this treatment per se did not suppress the Fog phenotype.
cating that all three genes promote germline proliferation. *atx-2* and *ego-4* are also both required for embryogenesis (Qiao et al. 1995; Gonczy et al. 2000; Kiehl et al. 2000; Kamath et al. 2003). Although phenotypic and mapping data suggest that *atx-2* and *ego-4* may be the same gene, we could not locate the om30 lesion within *atx-2*. We considered that om30 might lie in a distant regulatory site that influences *atx-2* transcription. However, we did not find any reduction in *atx-2* transcript levels in *ego-4(om30)* animals. Our data are most consistent with the hypothesis that *ego-4* and *atx-2* are distinct genes that promote germline proliferation.

**atx-2 and sex determination:** To promote the switch from spermatogenesis to oogenesis, ATX-2 might positively regulate a feminizing gene, such as *tra-2*, or repress a masculinizing gene, such as *fem-3* (see reviews by Schedl 1997; Goodwin and Ellis 2002; Stothard and Pilgrim 2003). Regulation of both *tra-2* and *fem-3* has been described in some detail (see Pouti et al. 2001; Goodwin and Ellis 2002). Alternatives are that ATX-2 may promote the activity of another feminizer, e.g., *tra-3* or one of the *mog*, *nos*, or *fsh* genes, or repress the activity of a masculinizer, e.g., one of the *fem* or downstream *fog* genes (see Stothard and Pilgrim 2003). Indeed, *fem-3* is under particularly tight regulation, being controlled at the level of RNA metabolism (by the MOG proteins), translation (by the NOS and FBF proteins), and at the level of protein function (by TRA-2; see Goodwin and Ellis 2002). An *atx-2(null) mutation would allow us to determine true epistasis relationships between *atx-2* and these genes. In any event, ATX-2 appears to act in the same “direction” as NOS-3 to promote the oocyte fate while it acts in opposition to NOS-3 to promote proliferation.

**Regulation of *atx-2* activity in the germline:** Our observations raise the question of how *atx-2* activity is regulated. If *atx-2* lies directly downstream of GLD-1 and/or GLD-2 in the regulation hierarchy, then *atx-2* activity may be directly repressed by GLD-1 and/or GLD-2 in the transition zone. Alternatively, ATX-2, GLD-1, and GLD-2 may converge to regulate common targets. If so, then GLD-1 and GLD-2 activity may simply override ATX-2 to allow meiotic entry (see above). In this case, ATX-2 activity *per se* may not decrease as cells enter meiosis. Instead, regulation of target mRNAs by GLD-1/ GLD-2 may supersede their regulation by ATX-2. Finally, if ATX-2 represses a third meiotic pathway, then its target(s) would presumably be distinct from GLD-1 and GLD-2 targets (since this pathway would be active in *glb-2 glf-1* double mutants).

Kohara and colleagues have described the *atx-2* mRNA expression pattern as part of their expressed sequence tag project. (Data are available at the Nematode Expression Pattern Database, http://nematode.lab.nig.ac.jp.) They detect *atx-2*mRNA throughout the larval and adult germline. This broad distribution of *atx-2* transcripts may reflect constitutive expression of *atx-2* in the germ-line and is consistent with ATX-2 protein being present throughout the germline (as has been observed for NOS-3; Kraemer et al. 1999), although other explanations are possible.

**Other functions of *atx-2***: Previous studies have demonstrated an essential role for *atx-2* in embryogenesis (Gonczy et al. 2000; Kiehl et al. 2000; Kamath et al. 2003). We likewise note substantial embryonic lethality associated with *atx-2(RNAi)* (data not shown). The requirement for ATX-2 activity in the early embryo is likely to be satisfied by maternal expression. The presence of *atx-2*mRNA in the proximal germline is consistent with incorporation of *atx-2* mRNA and/or protein into oocytes. Analysis of the role of *atx-2* in embryogenesis is likely to be a fruitful avenue of research.

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