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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* (**e**nhancer 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; Hendersen *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-1^{intra}, 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^{intra} (Doyle *et al.* 2000; Petcherski and Kimble 2000; Seydoux and 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

Sequence data from this article have been deposited with the EMBL/GenBank Data Libraries under accession no. AY571963.

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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 Drosophila 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 proliferation/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* (**e**nhancer 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

Bristol (N2) and mutant phenotypes are as described by Hodgkin and Martinelli (1999) or as indicated. Nomenclature follows standard guidelines (Hodgkin and Martinelli 1999). Mutations used were as follows:

LGI: *gld-1(q485), gld-2(q497), hT2*;
LGII: *fog-2(q71* and *oz40), nos-3(oz231, 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: *gld-1(q485), gld-2 (q497), glp-1(q175),* and *nos-3(oz231)* (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, HIM-3 (Zetka *et al.* 1999), and REC-8 (Pasierbek *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°–22°) for ~48 hr. Seeded plates were stored at 15° 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 Maine 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)* germlines, 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. AY571963).

***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(+) unc-49*. 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°.

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 (Kodoyianni *et al.* 1992) that, at 20°, has nearly wild-type germline proliferation (Qiao *et al.* 1995). In these mutants, germ cell number is reduced (*e.g.*, ~50% of wild type at young adult stage), but >99% 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° 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°. We raised *glp-1(bn18ts)* animals on the appropriate dsRNA feeding strain at 20° 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

***atx-2* promotes germline proliferation**

Genotype	Temp (°)	% proliferating germlines	Range	*n*
Wild type	20, 25	100[a]	NA	—
atx-2(RNAi)	20	95	90–100	119
atx-2(RNAi)	25	93	86–98	186
glp-1(bn18ts) atx-2(RNAi)	20	16	11–25	199
glp-1(bn18ts)	20	>99[b]	NA	—

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 *glp-1(bn18ts)* were treated in parallel in each trial.

[a] Austin and Kimble (1987).

[b] 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)* phenocopy, 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 *glp-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 germline 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* (*atax*in-*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 cerevisae* protein, PBP1 [*p*rotein that *b*inds *p*oly(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 germline 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-

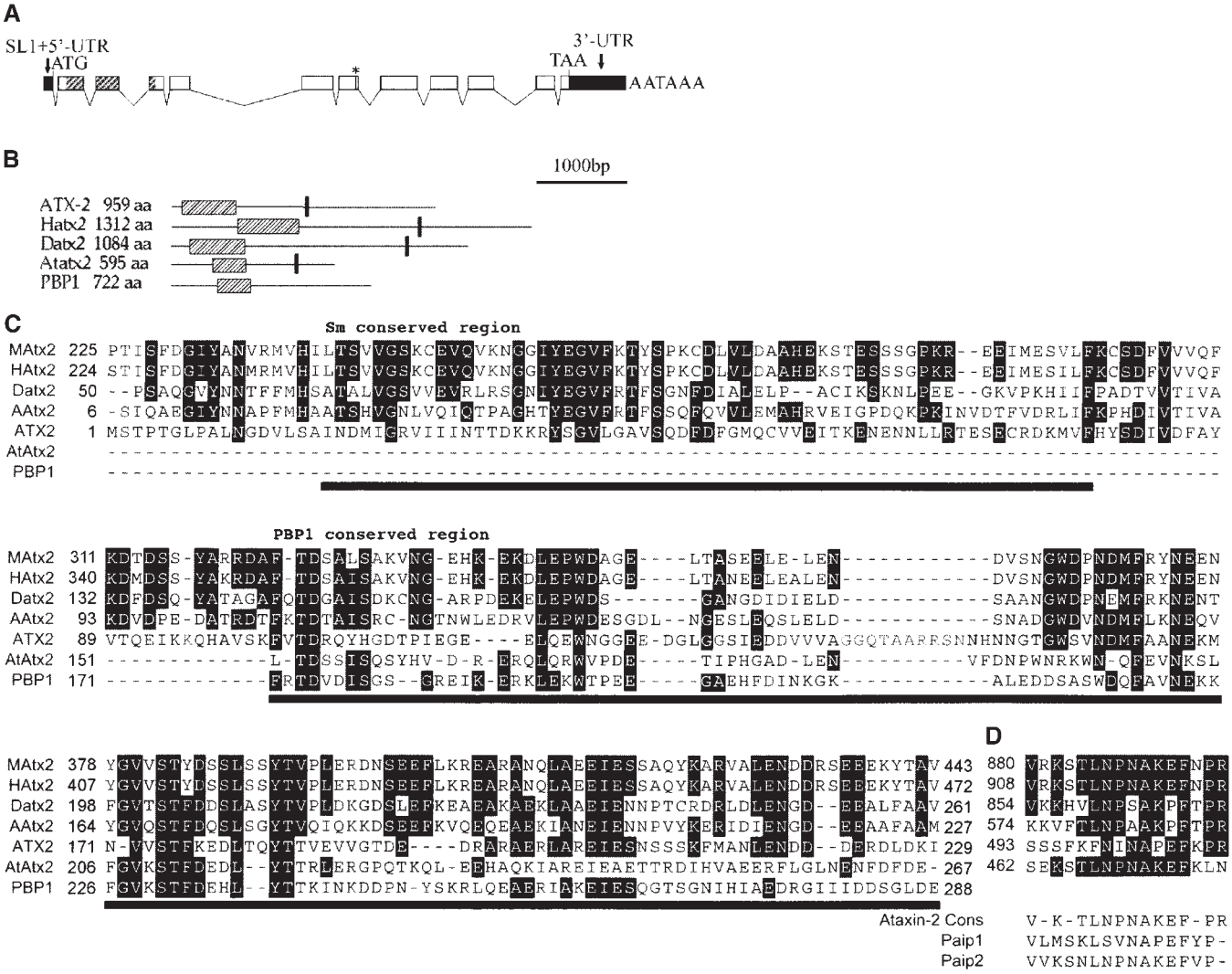

FIGURE 1.—Structure of the *atx-2* gene. (A) The *atx-2* message contains 12 exons (boxes) and a SL1 *trans*-spliced leader. 5′- and 3′-UTRs are indicated in black. ATX-2 is predicted to contain 959 amino acids. It differs from the D2045.1 prediction in containing 87 fewer amino acids at the amino terminus 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.

mals 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° (Table 1). This effect was slightly stronger at 25° (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. *rrf-1* encodes an RdRP (SMARDON *et al.* 2000) required for RNAi in the soma (SIJEN *et al.* 2001). *rrf-1* mutants are resistant to RNAi in the soma, but have normal sensitivity in the germline (SIJEN *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 (QIAO *et al.* 1995). We now report that the mitotic zone is subsequently lost by a substantial proportion of *ego-4(om30)* adults (~20% of germlines at ~48 hr into adulthood; $n = 61$), with all germ cells in these animals having prematurely entered

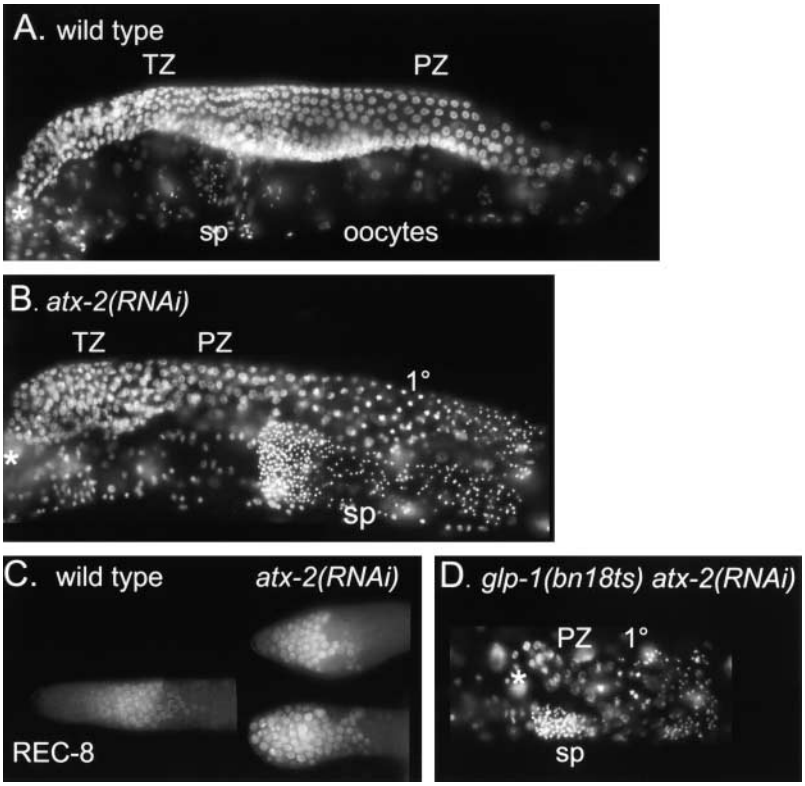

FIGURE 2.—*atx-2(RNAi)* produced proliferation and sex determination defects. One arm of the hermaphrodite gonad in (A) wild-type, (B) *atx-2 (RNAi),* and (D) 20° *glp-1(bn18ts) atx-2(RNAi)* animals is shown. Asterisk indicates the distal end of each germline. (A) Mitotic cells are present at the distal end. Meiotic cells are present; the transition (leptotene-zygotene) and pachytene "zones" are indicated (TZ and PZ). Spermatogenesis is complete, and mature sperm (sp) are present at the proximal end of the gonad. Oocytes are present. (B) Spermatogenesis is ongoing; germ cells have failed to switch to oogenesis. 1°, primary spermatocytes. (C) The distal region of wild-type and *atx-2(RNAi)* germlines is shown. The region of strong nucleoplasmic REC-8 staining corresponds to the mitotic germ cell nuclei (HANSEN *et al.* 2004b). The region of REC-8 staining is consistently smaller in *atx-2(RNAi)* germlines than in the wild-type germlines. (D) No proliferating germ cells are present. Sperm (sp), primary spermatocytes (1°), and a few pachytene nuclei (PZ) are visible. Asterisk indicates the distal end of the germline. Photos in A, B, and D were taken at the same magnification.

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

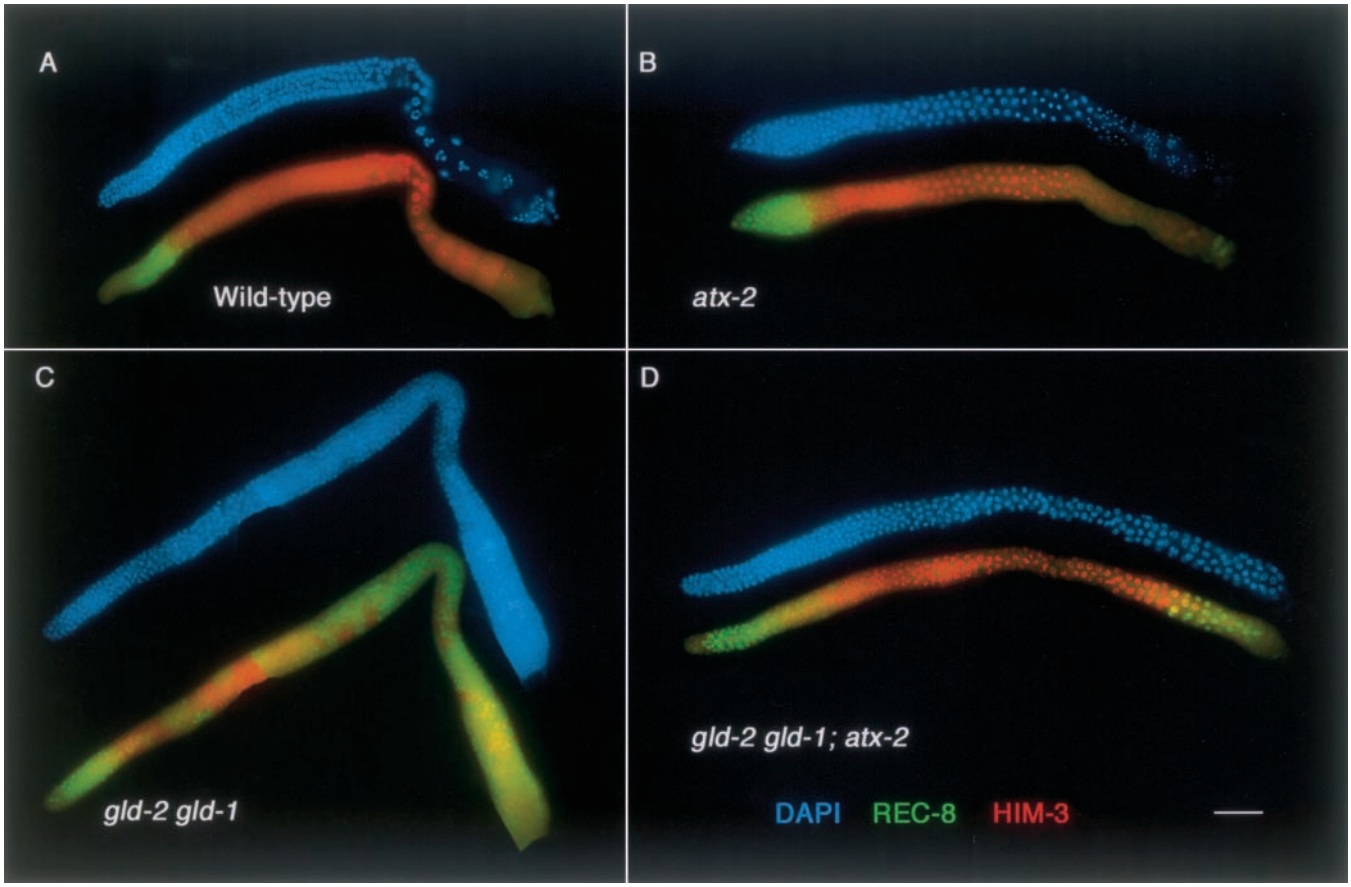

Figure 3.—Depletion of ATX-2 promotes meiotic entry. One arm of the adult hermaphrodite germline is shown in each panel. Chromosomes were colabeled with the DNA dye, DAPI, and antibodies against HIM-3 and REC-8. In (A) wild-type and (B) *atx-2(RNAi)* germlines, extensive meiotic cells are present and 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

TABLE 2

Tests for suppression of meiotic entry defects by *atx-2(RNAi)*

Genotype	% suppressed germlines	*n*
gld-2(q497) gld-1(q485)	0[a]	—
gld-2(q497) gld-1(q485); atx-2(RNAi)[b]	95	55
gld-2(q497) gld-1(q485); glp-1(q175)	0[a]	—
gld-2(q497) gld-1(q485); glp-1(q175) atx-2(RNAi)[b]	68	53
gld-2(q497); nos-3(oz231); glp-1(q175)	0[a]	—
gld-2(q497); nos-3(oz231); glp-1(q175) atx-2(RNAi)[c]	17	70

Consistent results were obtained in independent RNAi experiments; 100% of *gld-2 gld-1* 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-1* > *gld-2 gld-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 gld-1; glp-1* or *gld-2; nos-3; glp-1* synthetic tumorous mutants.

[a] Defined here as zero, on the basis of criteria in Hansen *et al.* (2004b), Table 1.

[b] Experiments were done by feeding at 25°.

[c] Experiments were done by injection; animals were raised at 20°.

partially offset the loss of GLD-1 and GLD-2 activity, and more meiotic entry would occur. Alternatively, ATX-2 might repress a third meiotic pathway. If so, then depletion of ATX-2 would increase the activity of this third pathway, in turn increasing the level of meiotic entry (Figure 5C).

We investigated the relationship between ATX-2 and the hypothetical third pathway by asking whether *atx-2 (RNAi)* suppresses the meiotic entry defect/tumorous phenotype of *gld-2; nos-3* and *gld-2; nos-3; glp-1* animals (Hansen *et al.* 2004b). Genetic data suggest that NOS-3 promotes meiotic entry by regulating not only GLD-1 but also a second target, which presumably is a component of the third pathway (Hansen *et al.* 2004b). For example, the meiotic entry defect is greater in *gld-2 gld-1; nos-3* than in *gld-2 gld-1* animals. We sought to determine whether ATX-2 might repress this hypothetical third pathway targeted by NOS-3.

Hansen *et al.* (2004b) have shown that the different synthetic tumorous genotypes are associated with different amounts of meiotic entry, as follows: *gld-2; nos-3* > *gld-2; nos-3; glp-1* > *gld-2 gld-1* > *gld-2 gld-1; glp-1*. We first asked whether *atx-2(RNAi)* suppresses the meiotic entry defect in *gld-2; nos-3* animals. However, it was difficult to determine the extent of suppression by *atx-2(RNAi)* because *gld-2; nos-3* germlines have substantial meiotic entry on their own (data not shown). Therefore, we tested whether *atx-2(RNAi)* could suppress the *gld-2; nos-3; glp-1* meiotic entry defect. Indeed, we found that some gonad arms showed strong suppression by *atx-2(RNAi)* as evidenced by the lack of proliferating cells (Table 2; Figure 4D). We take this result as an indication that ATX-2 may not be a negative regulator of the third pathway, and instead model 1 or 2 (Figure 5, A and B) would be more likely to be correct. However, two caveats to this conclusion are that *nos-3(null)* mutants do not completely eliminate the activity of the third pathway (Hansen *et al.* 2004b) and *atx-2(RNAi)* may not fully eliminate ATX-2 activity. Therefore, we cannot eliminate the possibility that ATX-2 represses the third meiotic entry pathway.

***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)* and *glp-1(oz112/ oz112/+)* 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 *ncc-1* dsRNA. Loss of *ncc-1* function prevents cell division in all tissues (Boxem *et al.* 1999). We found that *glp-1(oz112)* animals responded properly to *ncc-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-

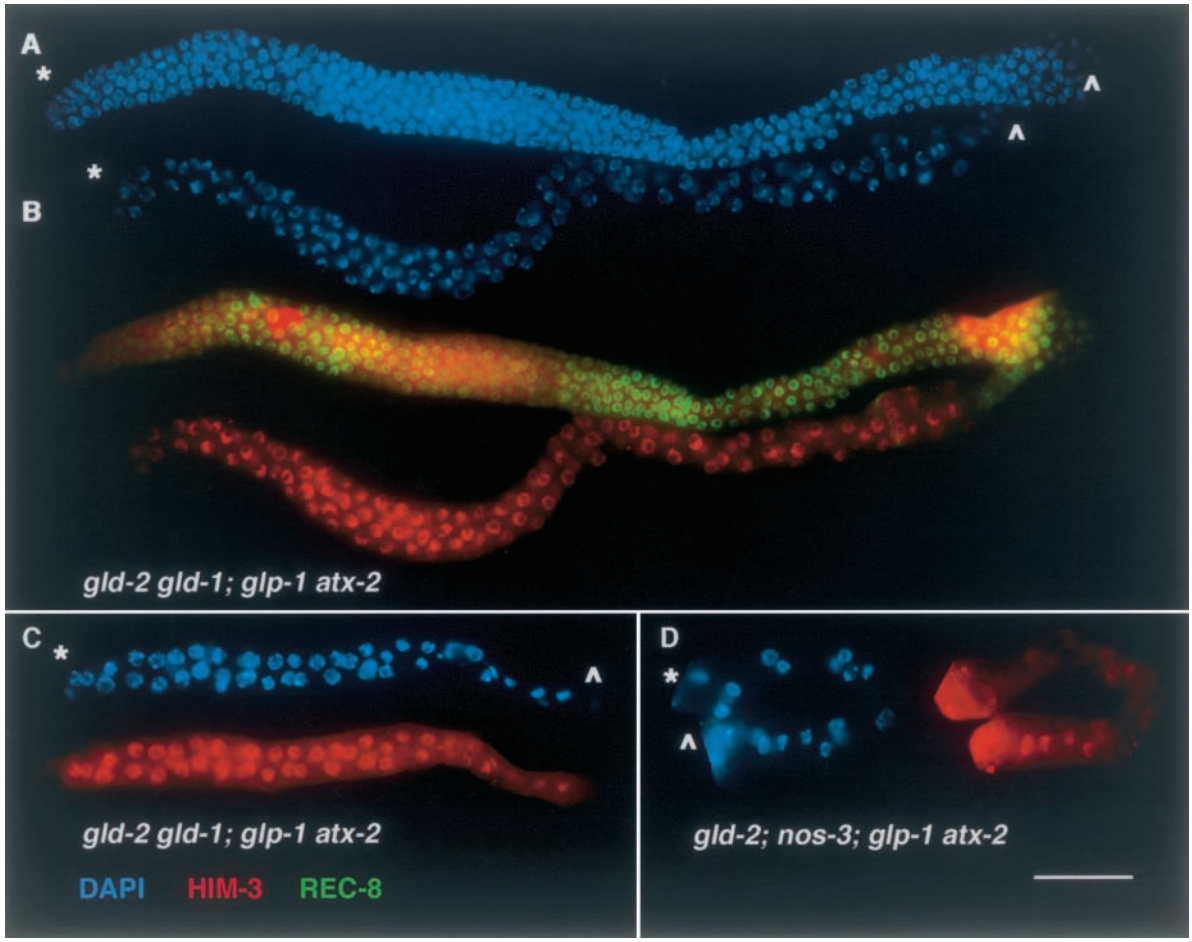

Figure 4.—Extensive meiosis occurs in *gld-2 gld-1; glp-1 atx-2(RNAi)* and *gld-2; glp-1 atx-2(RNAi); nos-3* germlines. One arm of the adult hermaphrodite germline is shown in each panel. (A) The characteristic *gld-2 gld-1; glp-1* tumorous phenotype (Kadyk and Kimble 1998; Hansen *et al.* 2004b). (B) 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* (*m*asculinization *o*f the *g*ermline) genes (Graham and Kimble 1993; Graham *et al.* 1993). The continued production of sperm during adulthood indicates a defect in the sperm-oocyte 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 L4/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

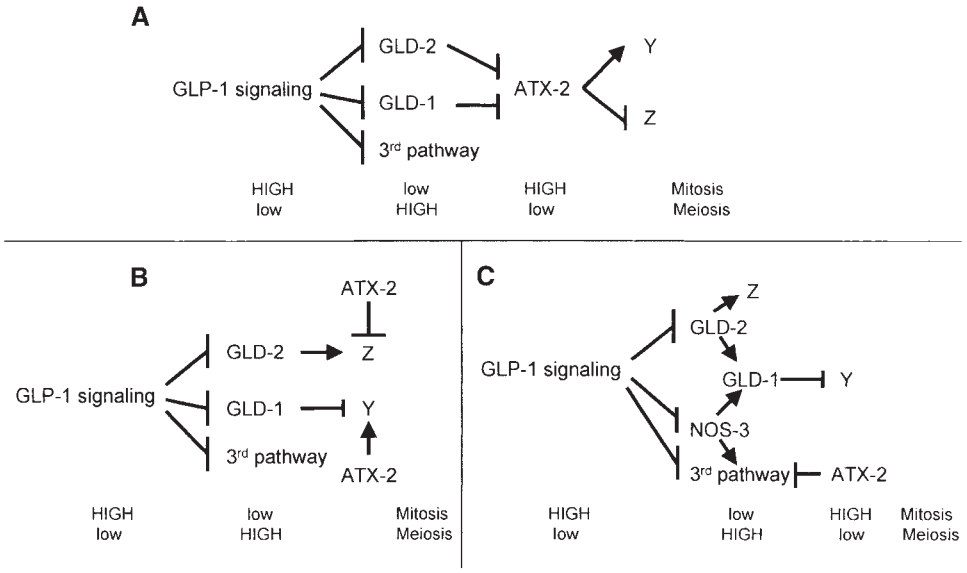

FIGURE 5.—Models for the regulation of germline proliferation and meiotic entry. In each case, GLP-1 signaling is shown to repress three meiotic entry pathways. For simplicity, the targets of the third pathway are not shown. (A) Model 1. ATX-2 activity is repressed by GLD-1 and GLD-2. ATX-2 may positively regulate genes required for proliferation (Y) and/or negatively regulate genes required for meiosis (Z). (B) Model 2. ATX-2 acts in parallel with the GLD-1 and GLD-2 pathways to regulate common targets. For example, ATX-2 upregulates Y, a negative target of GLD-1, and represses Z, a positive target of GLD-2. Other possibilities exist. (C) Model 3. ATX-2 represses the third meiotic entry pathway. Components of the third pathway are not known, therefore we simply indicate it as lying downstream of NOS-3. Since ATX-2 can suppress *gld-2 gld-1* double mutants, we have shown ATX-2 as interacting with the third pathway at a point downstream of NOS-3. See text.

mutations in *fog-2*. We raised *fog-2(oz40)* and *fog-2(q71)* null mutants in the presence of *atx-2* dsRNA and found that most of them produced sperm. Some *atx-2(RNAi); fog-2(−)* animals produced both sperm and oocytes whereas others produced only sperm (Table 3). Therefore, depletion of ATX-2 suppressed the Fog-2 phenotype. On the basis of this result, *atx-2* may act downstream of *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 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 *atx-2(RNAi)* could suppress feminizing mutations in genes that act downstream of *fog-2*. We found that *atx-2(RNAi)* could not suppress the *tra-2(q122)* gain-of-function allele (which is deleted for one GLD-1 binding site in the 3′-UTR; JAN *et al.* 1999) at 25° ($n = 164$) or loss-of-function mutations in *fem-1* ($n = 24$). The simplest interpretation of these data is that *atx-2* acts upstream of *tra-2* or as a positive regulator of *tra-2*. This hypothesis needs to be tested using an *atx-2 (null)* mutation (see DISCUSSION).

DISCUSSION

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

Mammalian ataxin-2 was first studied because it is associated with the human neurodegenerative disease, spinocerebrellar ataxia (IMBERT *et al.* 1996; PULST *et al.* 1996; 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 ataxin-2 protein, as well as its ability to bind to RNA-binding proteins (SHIBATA *et al.* 2000; KHALEGHPOUR *et al.* 2001; ROY *et al.* 2002), ataxin-2 has been hypothesized to function in RNA metabolism (SATTERFIELD *et al.* 2002). We propose that *C. elegans* ATX-2 acts at the post-transcriptional level to regulate gene expression for germline proliferation and female sex determination. A role for ATX-2 in the *C. elegans* nervous system is not known given that RNAi (by feeding and injection) is very inefficient in nervous tissue.

TABLE 3

***atx-2* promotes the oocyte fate**

Genotype	Temp (°)	% sperm + oocytes	% Mog	% Fog	% other[a]	*n*
atx-2(RNAi) feeding[b]	20	56	44	0	0	424
atx-2(RNAi) injection	20	23	77	0	0	75
atx-2(RNAi) feeding[b]	25	28	72	0	0	186
fog-2(q71 or *oz40)*	NA	0	0	100[c]	0	—
atx-2(RNAi)[d]	20	82	18	0	0	50
fog-2(oz40); atx-2(RNAi)[d]	20	56	6	25	12.5	16
fog-2(q71); atx-2(RNAi)[d]	20	62	38	0	0	16
fog-2 mock RNAi[d]	20	0	0	100	0	40

n, number of gonad arms examined; NA, not applicable.

[a] No gametes were produced.

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

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

[d] 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.

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 activity to allow meiotic entry. Third, ATX-2 may negatively regulate a third meiotic entry pathway, as discussed below (Figure 5C).

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 germlines have some meiotic germ nuclei whereas *glp-1(oz112gf)* tumorous germlines 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-

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 *fbf* 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 *gld-2 gld-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 germline 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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