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Caenorhabditis elegans DAZ-1 is expressed in proliferating germ cells and directs proper nuclear organization and cytoplasmic core formation during oogenesis

Rika Maruyama[a,b], Sachiko Endo[a], Asako Sugimoto[b], Masayuki Yamamoto[a,*]

[a]*Department of Biophysics and Biochemistry, Graduate School of Science, University of Tokyo, Bunkyo, 113-0032 Japan*
[b]*Laboratory for Developmental Genomics, RIKEN Center for Developmental Biology, Kobe, 650-0047 Japan*

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Abstract

The *deleted in azoospermia* (*DAZ*) family genes encode potential RNA-binding proteins that are expressed exclusively in germ cells in a wide range of metazoans. We have previously shown that mutations in *daz-1*, the only *DAZ* family gene in *Caenorhabditis elegans*, cause pachytene stage arrest of female germ cells but do not affect spermatogenesis. In this study, we report that DAZ-1 protein is most abundantly expressed in proliferating female germ cells, in a manner independent of the GLP-1 signaling pathway. DAZ-1 is dispensable in males but it is expressed also in male mitotic germ cells. Detailed phenotypic analyses with fluorescence microscopy and transmission electron microscopy have revealed that loss of *daz-1* function causes multiple abnormalities as early as the onset of meiotic prophase, which include aberrant chromatin structure, small nucleoli, absence of the cytoplasmic core, and precocious cellularization. Although the reduced size of nucleoli is indicative of a low translational activity in these cells, artificial repression of general translation in the germline does not phenocopy the *daz-1* mutant. Thus, we propose that DAZ-1 in *C. elegans* plays essential roles in female premeiotic and early meiotic germ cells, probably via regulating the translational activity of specific target genes required for the progression of oogenesis.

Keywords: *Caenorhabditis elegans*; Germ cells; *daz-1*; Meiosis; Oogenesis

Introduction

In many animals, primordial germ cells are separated from somatic cells early in development. These cells proliferate and differentiate at later stages to form sperm and oocytes, which transmit genetic information to the next generation. The *deleted in azoospermia* (*DAZ*) gene family has been suggested to play crucial roles in germline development and gametogenesis in both invertebrates and vertebrates (Eberhart et al., 1996; Houston and King, 2000; Karashima et al., 2000; Reijo et al., 1995; Ruggiu et al., 1997). The DAZ family proteins have the following common features: first, they share a high degree of similarity in the ribonucleoprotein (RNP)-type RNA recognition motif; second, they have a DAZ motif, another conserved domain of unknown function; and third, members of the DAZ family are expressed specifically in the germline. *Caenorhabditis elegans*, like *Drosophila*, has only one *DAZ* family gene in the genome (Eberhart et al., 1996; Karashima et al., 2000), whereas some mammals have multiple *DAZ* members (Saxena et al., 1996; Xu et al., 2001). In human, autosomal *DAZ* members (*DAZL* and *BOULE*) and a *DAZ* gene cluster on Y chromosome have been reported (Reijo et al., 1995; Saxena et al., 1996; Xu et al., 2001). Based on phylogenetic analysis of RNA recognition motifs in DAZ proteins from various species, the *DAZ* family members have been classified into two subfamilies: the ancestral *BOULE* subgroup (e.g., *Drosophila boule*, *C. elegans daz-1*, and human *BOULE*), and the

* Corresponding author. Department of Biophysics and Biochemistry, Graduate School of Science, University of Tokyo, Bunkyo, PO Hongo, 113-0032 Japan. Fax: +81 3 5802 2042.
E-mail address: myamamot@ims.u-tokyo.ac.jp (M. Yamamoto).

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DAZL subgroup thought to have arisen in the early vertebrate lineage (Xu et al., 2001).

Although the expression of *DAZ* family members is limited to the germline, their expression profiles as well as the loss-of-function phenotypes are diverse. For example, *Drosophila* BOULE localizes premeiotically to a perinucleolar region in spermatocytes and then translocates to the cytoplasm at the onset of meiosis (Cheng et al., 1998). Its mutation causes male sterility due to the arrest of primary spermatocytes at meiosis I (Eberhart et al., 1996). Mouse and human *BOULE*, which are putative orthologs of *Drosophila boule*, are also expressed in spermatocytes, but their subcellular localization is limited to the cytoplasm (Xu et al., 2001). In contrast, DAZL proteins in vertebrates are expressed in both male and female, from a much earlier stage of gametogenesis than BOULE. In *Xenopus*, *Xdazl* (*Xenopus DAZ*-like) is expressed in the germ plasm (Houston and King, 2000), and depletion of maternal *Xdazl* mRNA results in the lack of primordial germ cells (Houston et al., 1998). In mice and human, expression of DAZL begins early in development in germ stem cells and continues through meiotic divisions (Reijo et al., 2000; Ruggiu et al., 1997; Xu et al., 2001). Mice deficient in *Dazl* exhibit defects in germ cell development and survival and become sterile in both sexes (Ruggiu et al., 1997). Additionally, men with a deletion encompassing the Y-chromosome *DAZ* gene cluster show various spermatogenetic defects, which are detectable initially in the stem cell population (Reijo et al., 1995). DAZ is expressed in germ stem cells and spermatogonia, suggesting that the *DAZ* genes may also function early in the germline (Reijo et al., 2000; Xu et al., 2001).

We have previously reported that *C. elegans* has only one *DAZ* family gene, *daz-1* (Karashima et al., 2000). The amino acid sequence of the RNA recognition motif of *C. elegans* DAZ-1 is most similar to that of *Drosophila* BOULE (Karashima et al., 2000). However, whereas other BOULE group members are male specific, *C. elegans* DAZ-1 is specifically involved in oogenesis (Karashima et al., 2000). In hermaphrodites, *daz-1* mRNA is detectable in both mitotic germ cells and cells undergoing oogenesis, whereas it is nearly undetectable in male animals (Karashima et al., 2000). Consistent with abundant expression of *daz-1* in hermaphrodites, loss of *daz-1* function affects only oogenesis: *daz-1* hermaphrodites are sterile due to the arrest of oogenesis at the pachytene stage of meiotic prophase I, while spermatogenesis is affected by the *daz-1* mutation in neither hermaphrodites nor males (Karashima et al., 2000).

Despite the differences in expression profiles and loss-of-function phenotypes, at least some *DAZ* family genes appear to function interchangeably, as suggested by the observation that a human *DAZ* and *Xenopus Xdazl* transgenes can partially rescue a mouse *Dazl* and *Drosophila boule* mutant, respectively (Houston et al., 1998; Slee et al., 1999). Since DAZ family proteins have an RNA-recognition motif and can bind to RNA in vitro (Houston et al., 1998; Maegawa et al., 2002; Venables et al., 2001), it is presumable that their conserved biochemical function is to regulate mRNA translation. *Drosophila* BOULE has been indeed suggested to activate translation of *twine*, which encodes a meiotic type of Cdc25 phosphatase (Maines and Wasserman, 1999), although direct binding of BOULE to *twine* mRNA is yet to be shown. Furthermore, zebrafish *DAZL* (*zDAZL*) can activate translation of *Drosophila twine* or *zDazl* itself in transfection experiments, in a manner dependent on the 3′-UTR sequences (Maegawa et al., 2002). In addition, association of mouse and zebrafish DAZL with polyribosomes engaged in translation has been demonstrated (Maegawa et al., 2002; Tsui et al., 2000).

To gain insights into the function and evolution of the DAZ protein family, we extended our characterization of *C. elegans* DAZ-1. In this paper, we report that DAZ-1 protein is enriched in the cytoplasm of the mitotic and early meiotic germ cells in hermaphrodites, in a manner independent of the GLP-1 signaling pathway. In addition, we show that loss of *daz-1* function causes various abnormalities in the adult hermaphrodite germline, including aberrant nuclear and nucleolar morphology of the meiotic cells and the absence of a cytoplasmic core from the gonad. These results indicate that DAZ-1 in *C. elegans* plays essential roles at premeiotic and early meiotic stages in female germ cells in order to facilitate the proper progression of oogenesis.

Materials and methods

General methods and strains

Maintenance and genetic manipulation of *C. elegans* were carried out as described (Brenner, 1974). Strains used were as follows: wild-type *C. elegans* var. Bristol, strain N2; (LGI) *gld-2(q497)*, *gld-1(q485)*; (LGII) *daz-1(tj3)*; (LGIII) *glp-1(q46,q231)*, *unc-32(e189)*. Strains were maintained at 20°C, except for the temperature-sensitive mutant *glp-1(q231)*, which was maintained at 15°C. Their phenotypes were characterized at either 20°C or 25°C.

Production of anti-DAZ-1 antibodies

A fragment of *daz-1* cDNA corresponding to amino acid residues 159–499 was cloned into pET19b vector (Novagen) to create a 10xHis-DAZ-1 fusion construct. The fusion protein was expressed in *Escherichia coli*, purified by using Ni-NTA agarose (Qiagen), and used as the antigen to raise rabbit polyclonal antibodies (Sawady Technologies). DAZ-1-specific antibodies were purified by blot affinity purification.

Immunoblotting

Five hundred adult hermaphrodites or one thousand adult males were picked and boiled in 1× SDS sample

buffer for 30 min, which were then separated on a 10% SDS-polyacrylamide gel containing 9.9% acrylamide and 0.1% *N*,*N*-methylenebisacrylamide and transferred to Immobilon (MILLIPORE). The membrane was incubated with anti-DAZ-1 antibody (1:250) or with MH16 antibody (1:20,000) and detected using ECL Plus Western Blotting Detection System (Amersham Pharmacia Biotech). The quantitative densitometry of the bands was performed by NIH Image program (developed at the U.S. National Institutes of Health and available at http://rsb.info.nih.gov/nih-image/) by using the films whose signals were not saturated. For immunoblotting of the *glp-1(ts)* mutant, *glp-1* worms synchronized and maintained at 15°C were shifted to 25°C at young adult stages and subjected to sample preparation after 24 h.

Immunostaining

Fixation

For DAZ-1 staining, dissected gonads of L4 and adult worms were fixed with a mixture of 3% formaldehyde, 75% methanol, and 62 mM K_2HPO_4 [pH 7.2] for 10 min at −20°C and postfixed with 100% methanol for 10 min at −20°C. For HIM-3 (Zetka et al., 1999) and nucleoli staining, the dissected gonads were freeze-cracked and fixed with methanol for 10 min at −20°C, followed by acetone fixation for 4 min at the same temperature. For staining with BODIPY FL phallacidin (Molecular Probes), the dissected gonads were fixed as described previously (Strome, 1986). L1 to L3 larva were fixed with a mixture of 49% Bowin's fixative (71% saturated picric acid, 24% formaldehyde, and 5% glacial acetic acid), 49% methanol, and 1.2% 2-mercaptoethanol for 30 min at room temperature, frozen in liquid N_2, and quickly thawed under hot water (Nonet et al., 1997).

Staining

Fixed gonads or worms were incubated overnight at 4°C with following primary antibodies: affinity-purified anti-DAZ-1 antibodies (1:100 dilution), *S. cerevisiae* Nop1p antibody (1:100 dilution, a gift from J. Aris) (Aris and Blobel, 1988; MacQueen and Villeneuve, 2001), anti-lamin antibodies (1:400 dilution, a gift from Y. Gruenbaum) (Liu et al., 2000), and anti-HIM-3 antibodies (1:500 dilution, a gift from M. Zetka) (Zetka et al., 1999). As secondary antibodies, Alexa Fluor 488 goat anti-rabbit IgG (H + L) conjugate (Molecular Probes), Alexa Fluor 568 goat anti-mouse IgG (H + L) conjugate (Molecular Probes), and Cy3-conjugated goat anti-rabbit antibodies (Chemicon) were used at 1:100 dilution. For staining of DNA, DAPI was added to the final concentration of 2 μg/ml, and the sample was mounted with DABCO solution for microscopy. Staining with propidium iodide, and BODIPY FL phallacidin was performed as described (Jones et al., 1996; Strome, 1986).

Fluorescence and Nomarski microscopy

Epifluorescent images were captured with a Zeiss Axioplan2 microscope equipped with a cooled CCD camera (Hamamatsu Photonics) and stored digitally using the Fish Imaging software (Hamamatsu Photonics) program. Confocal images were captured by a Zeiss LSM 510 microscope as a series of stacked images. Nomarski images were captured with a Zeiss Axioplan2 microscope equipped with an Axiocam (Zeiss) and stored digitally using the Axiovision (Zeiss) software.

Electron microscopy

Worms were anesthetized with 8% ethanol in M9 buffer for 3–5 min, then cut into two or three pieces in the primary fixative (a mixture of 2% glutaraldehyde, 1% formaldehyde, 0.05 M sodium cacodylate, 0.2 M sucrose, 1 mM $MgCl_2$, pH 7.4). Primary fixation was continued for 2 h at room temperature and the pieces were postfixed with 1% osmium tetroxide in the same buffer for 2 h on ice. They were then stained en bloc with 2% uranylacetate for 2 h and dehydrated in a graded ethanol series. After ethanol was replaced with methyl methacrylate, samples were embedded in Rigolac mixture (Kushida, 1960). Thin sections were stained with uranyl acetate and lead citrate and subjected to electron microscopic observation using one-slot copper grids precoated with Formvar. The observation was done with Hitachi H-7000 at 75 kV. Fourteen gonads of N2 and 17 gonads of *daz-1* were examined.

RNAi

RNAi of *daz-1* was performed by a feeding method as described (Timmons and Fire, 1998): L4 hermaphrodites were plated onto NGM plates containing 0.8 mM IPTG seeded with *E. coli* (HT115/DE3) carrying a *daz-1* dsRNA-expressing plasmid, which was constructed by cloning a *daz-1* cDNA fragment (from yk62b4: a gift from Y. Kohara) into pPD 129.36 vector (a gift from A. Fire), and the worms were cultured at 25°C for 3 days. RNAi of *eft-3* was performed by a soaking method as described (Maeda et al., 2001). PCR products amplified from the cDNA clone yk190a8 (a gift from Y. Kohara) were used as a template for *eft-3* dsRNA synthesis. L4 worms were soaked in the dsRNA solution for 24 h, recovered on NGM plates, and incubated further at 25°C. Phenotypes of soaked worms were observed 30 h after the recovery from the dsRNA solution.

Injection of FITC-dextran

The 0.1% FITC-dextran (average molecular weight: 250,000) (SIGMA) suspended in M9 buffer was injected into gonads of adult worms, and the gonads were examined by confocal microscopy immediately after the injection.

Results

DAZ-1 is a germline-specific cytoplasmic protein expressed in the mitotic and early meiotic regions of the adult gonad

We previously reported that the *daz-1* gene was transcribed in the germline of L2 through adult worms (Karashima et al., 2000), but localization of DAZ-1 protein has been left undetermined. To analyze the spatial and temporal localization pattern of DAZ-1 during *C. elegans* development, we prepared polyclonal antibodies against a bacterially expressed DAZ-1 protein. In immunoblot analysis of the wild-type extract, the affinity-purified anti-DAZ-1 antibodies recognized triplet bands corresponding nearly to the expected molecular mass of DAZ-1 (56 kDa). This triplet was not detected in the *daz-1(RNAi)* extract (Fig. 1A), indicating that these bands were highly likely to represent DAZ-1 protein.

Using the same antibodies, expression of DAZ-1 protein was analyzed by immunostaining. In hermaphrodites, DAZ-1 was detected only in the germline of L2 through adulthood (Figs. 1B and C). This observation was consistent with the results of in situ hybridization against *daz-1* mRNA carried out previously (Karashima et al., 2000). With a closer look, however, the gradient of the abundance of DAZ-1 protein within adult gonads did not coincide with that of the mRNA. The mRNA was most abundant in the pachytene region (Karashima et al., 2000), but DAZ-1 protein was most abundant in the mitotic region and also in the transition zone, where germ cells enter meiosis (Fig. 1C, left panels). Progression of pachytene stage was accompanied by a decrease of DAZ-1 protein, and staining of DAZ-1 faded

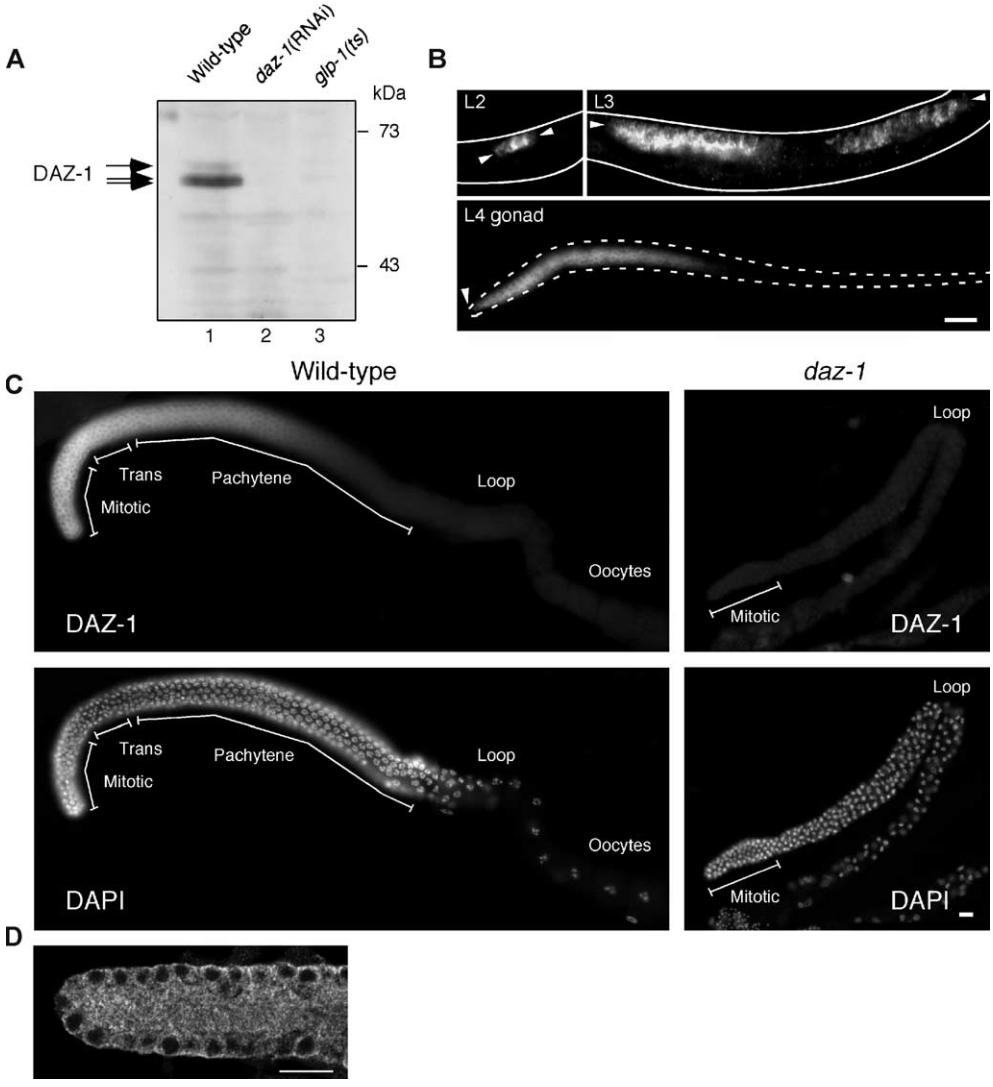

Fig. 1. Localization of DAZ-1 in wild-type hermaphrodites. (A) Immunoblot analysis of DAZ-1 protein in extracts of adult hermaphrodites. Extracts were prepared from wild-type (lane1), *daz-1(RNAi)* (lane2), and *glp-1(ts)* (lane3) worms. (B) Epifluorescent images of DAZ-1 in hermaphrodite larvae, stained with anti-DAZ-1 antibodies. Outlines of an L2 and an L3 larva (lines), an outline of a dissected L4 gonad (dotted line), and distal tips of the gonads (arrowheads) are indicated in the panels. (C) Anti-DAZ-1 and DAPI staining of gonads dissected from either a wild-type or a *daz-1* adult hermaphrodite. (D) A confocal image of the distal end of a gonad in a wild-type adult hermaphrodite, stained with anti-DAZ-1 (a middle focal plane). Scale bars: 10 μm throughout.

out around the middle of that stage. An observation by confocal microscopy under high magnification revealed that DAZ-1 localized to the cytoplasm of syncytial germ cells and to the cytoplasmic core (rachis) (Fig. 1D). No fluorescent signal was detected in the gonads of *daz-1*-deficient hermaphrodites (Fig. 1C, right panels), confirming that the observed staining was specific to DAZ-1.

In males, *daz-1* mRNA was barely detectable by in situ hybridization, and loss of *daz-1* function did not affect their fertility (Karashima et al., 2000). Rather unexpectedly, however, DAZ-1 protein was detected in the male germline (Fig. 2). As in hermaphrodites, DAZ-1 staining was detectable in the mitotic region and the transition zone of adult male gonads (Fig. 2A), and also in the germline of L3 and L4 larvae (data not shown). Quantification by Western blotting using the anti-DAZ-1 antibodies indicated that the amount of DAZ-1 protein per gonad of males was approximately 20% of that of hermaphrodites (Fig. 2B).

To examine whether DAZ-1 in the male germline has any function that we might have missed in our previous analysis, we examined the *daz-1* adult males carefully at 25°C, which is a more stringent condition for worms than the conventional culture temperature 20°C. The *daz-1* males appeared to be slightly abnormal at 25°C, in that their gonads were a little thinner than the wild type (data not shown). Nevertheless, these males mated readily with hermaphrodites and generated cross-progeny, suggesting that they could produce functional sperm. These results reinforce our conclusion that DAZ-1 plays no essential role in the male germline.

DAZ-1 expression in mitotic germ cells does not directly depend on GLP-1

In *C. elegans*, the GLP-1 signaling pathway, which consists of three components LAG-2, GLP-1, and LAG-1, regulates proliferation of germ cells and their entry into meiosis (Austin and Kimble, 1987; Christensen et al., 1996; Francis et al., 1995; Henderson et al., 1994; Kadyk and Kimble, 1998). A transmembrane protein, LAG-2, is a ligand expressed by the distal tip cell, which binds to the receptor GLP-1 located on the cell membrane of germ cells (Fitzgerald and Greenwald, 1995; Henderson et al., 1994). GLP-1 then activates a transcription factor LAG-1, which promotes mitotic proliferation of the germ nuclei (Christensen et al., 1996). When germ cells are sufficiently distant from the distal tip cell and the signaling from the GLP-1 pathway is no longer effective, they enter the meiotic pathway, in a manner dependent on the redundant function of GLD-1 and GLD-2 (Kadyk and Kimble, 1998).

To understand how expression of DAZ-1 is regulated, we examined DAZ-1 distribution in several germline mutants. In the *gld-2 gld-1* mutant, whose germ cells continue mitosis even in the proximal region (Kadyk and Kimble, 1998), DAZ-1 staining was observed throughout the gonad (Fig. 3A). In contrast, DAZ-1 expression was hardly detectable in young adults of the temperature-sensitive *glp-1* mutant shifted to the restrictive temperature, in which most germ cells have already undergone pachytene stage (Austin and Kimble, 1987) (Fig. 3C). The reduction of DAZ-1 protein in the *glp-1(ts)* mutant was confirmed by an immunoblot analysis (Fig. 1A). These results confirmed the observation in the wild type that DAZ-1 expression is maintained high in the proliferating germ cells but undergoes down-regulation in the pachytene germ cells.

GLP-1 expression is limited to the distal region of the gonad in wild-type worms, but it expands to the whole gonad in the *gld-2 gld-1* double mutant (Kadyk and Kimble, 1998). Because the area expressing DAZ-1 apparently coincided with that for GLP-1 (Crittenden et al., 1994), we examined whether expression of DAZ-1 is under the control of GLP-1. In the *gld-2 gld-1;glp-1* triple mutant, germ cells continue mitosis in the absence of *glp-1* function (Kadyk and Kimble, 1998). DAZ-1 was present throughout the gonad in this triple mutant (Fig. 3B). These results indicate that GLP-1 is not directly relevant to the expression of DAZ-1 in the mitotic germ cells.

Germ cells deficient in daz-1 show abnormal appearance of chromosomes in early prophase of meiosis I

In *daz-1* hermaphrodites, oogenesis is defective and germ nuclei arrest at the pachytene stage (Karashima et al.,

Fig. 2. Localization of DAZ-1 in wild-type males. (A) Anti-DAZ-1 and DAPI staining of a gonad dissected from a wild-type male. Arrowheads indicate the distal end of the gonad. Scale bar: 10 μm. (B) Immunoblot analysis of possible DAZ-1 protein and paramyosin (MH16) as a control somatic protein. Each lane was loaded with a protein extract prepared from either 500 hermaphrodites (lane1) or 1000 males (lane2) (i.e., 1000 gonads each). The ratio of DAZ-1 protein in hermaphrodite and male was quantified to be 1:0.18, whereas that of MH16 was quantified to be 1:1.8.

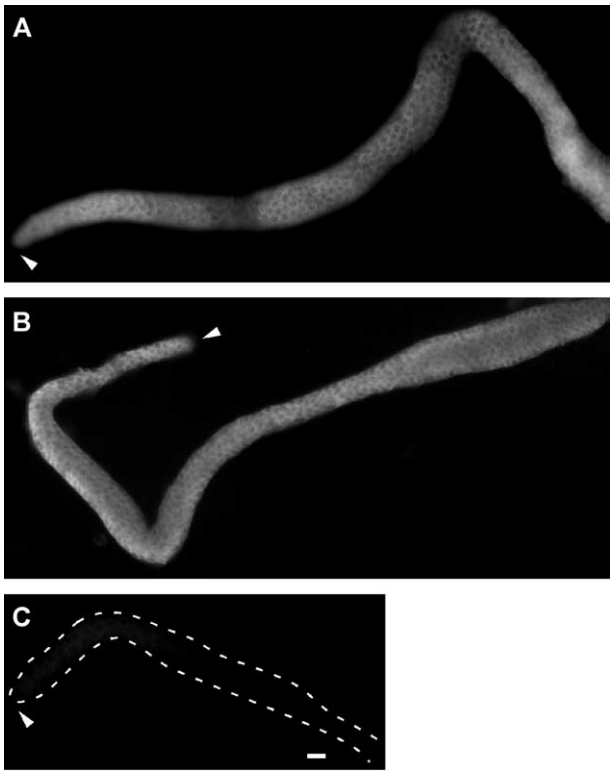

Fig. 3. Localization of DAZ-1 in germline mutants. Anti-DAZ-1 staining of gonads dissected from the *gld-2 gld-1* (A), *gld-2 gld-1;glp-1* (B), and *glp-1(ts)* (C) mutants. The *glp-1(ts)* worms synchronized and maintained at 15°C were shifted to 25°C at young adult stages and stained after 24 h, when all germ nuclei entered meiosis. An outline of the gonad (dotted line in panel C) and distal tips of the gonads (arrowheads) are indicated. Scale bar: 10 μm.

2000). Since the above analyses revealed that DAZ-1 protein was more abundant in the mitotic region and the transition zone than the pachytene region, we speculated that the germ cells deficient in *daz-1* might also be affected at earlier stages. To detect possible earlier defects in germline development, we performed close cytological analysis of germ cells in *daz-1* hermaphrodites. In this analysis, we cultured wild-type and *daz-1* worms at 25°C since it is known that some phenotypes are enhanced and become more penetrant at this high temperature.

In wild-type worms, germ cells entering meiosis at the transition zone exhibit a crescent-shaped chromatin region, where pairing of homologous chromosomes is taking place (Dernburg et al., 1998). As meiotic prophase progresses, paired chromosomes gradually condense and form thread-like chromatin structures at the pachytene stage (Albertson et al., 1997). In the proximal gonad, germ cells enter the diplotene/diakinesis stage, where chromosomes can be seen as six highly condensed bivalents (Albertson et al., 1997).

Our precise observation of *daz-1*-deficient germ cells revealed no apparent defect in the mitotic nuclei, although DAZ-1 was most abundantly expressed in the mitotic region. However, abnormally condensed nuclei were observed in the region immediately proximal to the mitotic region (Figs. 4B and D), which were distinct from the wild-type nuclei carrying a crescent-shaped chromatin region (Fig. 4C). Nuclei with this abnormal appearance were spread up to the loop region of the gonad. In the proximal half of a gonadal arm, nuclei showed thread-like chromosomes indicative of the pachytene stage (Fig. 4B), although overcondensed and undercondensed chromosomes were often observed among them (Fig. 4F). No diplotene/diakinesis nuclei were observed in *daz-1* hermaphrodites. Analysis of the gonad of *daz-1* males grown at 25°C did not reveal any detectable defect, as examined by DAPI staining (data not shown). These observations suggest that, by the loss of DAZ-1 function, female germ cells become aberrant at the onset of meiotic prophase, but they manage to advance up to the pachytene stage.

The daz-1 germ nuclei apparently can assume the meiosis-specific chromatin structure

Since the earliest cytological abnormality of *daz-1* germ cells was detected at the transition zone, where pairing of homologous chromosomes occurs, we further characterized the chromatin structure in this zone and thereafter by immunostaining of a meiosis-specific protein HIM-3 (Zetka et al., 1999) and by transmission electron microscopy (TEM). HIM-3 is a component of the chromosome axis that arises at the interface of aligned homologous chromosomes (Zetka et al., 1999). In *daz-1* hermaphrodites, HIM-3 was detected in the region immediately proximal to the mitotic region and was distributed towards the proximal end of the gonad (Figs. 5C and D), as was the case in the wild type (Zetka et al., 1999) (Figs. 5A and B). Under higher magnification, HIM-3 was detectable on the condensed chromosomes in both wild-type and *daz-1* worms (Figs. 5E and F), although HIM-3 staining appeared to be more compact in the *daz-1* pachytene nuclei than in the wild type (Figs. 5G and H). Furthermore, formation of a synaptonemal complex was detected by TEM in the *daz-1* pachytene nuclei (Fig. 5I). Thus, despite obvious abnormalities of the nuclear morphology, some meiosis-specific chromosomal events such as synapsis of homologous chromosomes and loading of HIM-3 onto the chromosome axis were not significantly obstructed by the *daz-1* deficiency.

Meiotic daz-1 germ cells undergoing oogenesis carry an abnormally small nucleolus

In *C. elegans*, germ cells undergoing oogenesis exhibit a prominent nucleolus, which gives them a “fried-egg” appearance when observed by Nomarski microscopy. We noticed that the size of the nucleolus in female *daz-1* germ cells, as observed under the Nomarski microscope, appeared significantly smaller than that of the wild type (Figs. 6A and B). No significant size difference was observed between the premeiotic nucleoli of *daz-1* germ cells and those of the wild type (data not shown; see below).

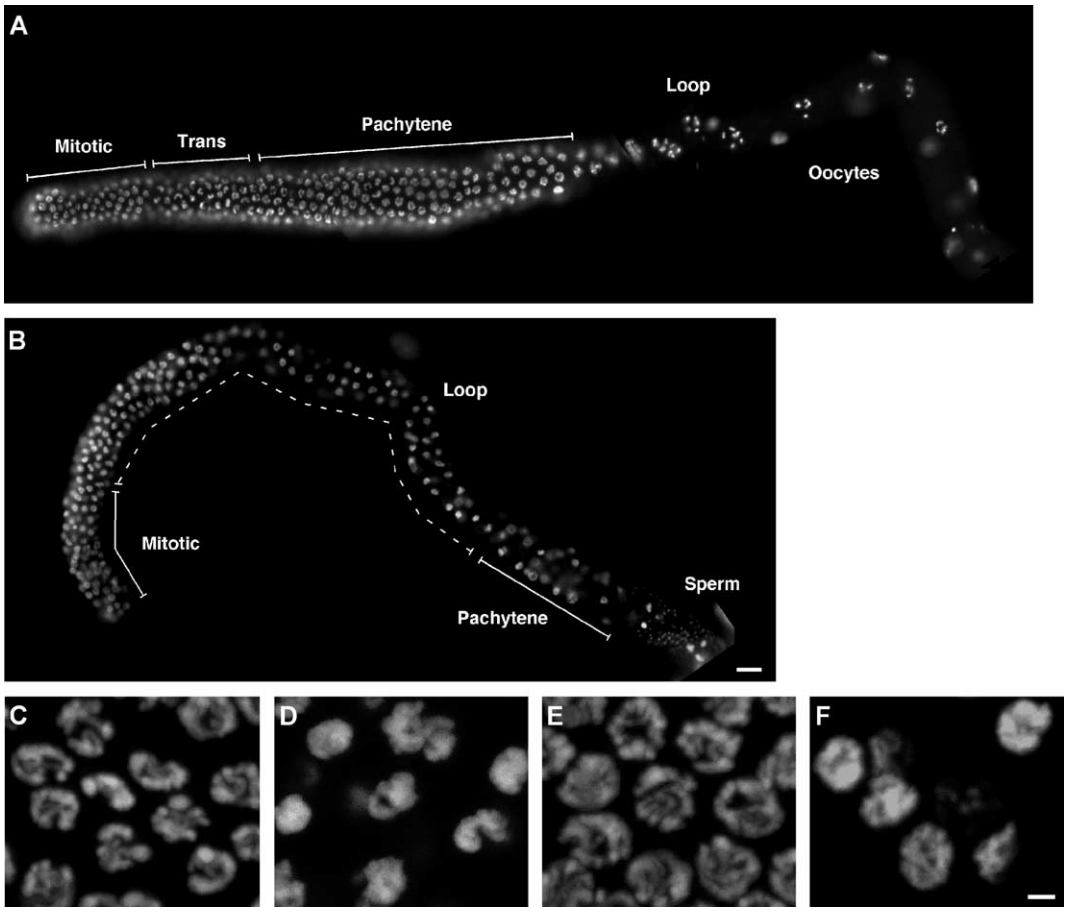

Fig. 4. Abnormal chromosome morphology in *daz-1* female germ cells. (A and B) Epifluorescent images of DAPI-stained gonads dissected from either a wild-type (A) or a *daz-1* (B) adult hermaphrodite. Scale bar: 10 μm. (C–F) PI-stained germ nuclei in adult hermaphrodites. Projected images captured by confocal microscopy are shown. (C) The transition zone in the wild type, showing crescent-shaped chromatin regions. (D) The corresponding area in the *daz-1* mutant, where chromosomes appear to be abnormally condensed. (E) The pachytene stage in the wild type, showing chromosomes with thread-like morphology. (F) The corresponding stage in the *daz-1* mutant, which shows chromosomes condensed to varied extent from nucleus to nucleus. Scale bar: 2 μm.

We examined the nuclear and nucleolar morphology also by double immunostaining with a nucleoli-specific antibody mAbD77 (anti-Nop1p) (Aris and Blobel, 1988; MacQueen and Villeneuve, 2001) and an anti-Ce-lamin antibody (Liu et al., 2000), the latter of which stains nuclear membrane. The results confirmed the smaller size of meiotic *daz-1* nucleoli (Figs. 6C and D). In addition, this analysis revealed that meiotic *daz-1* nuclei in the further downstream were morphologically abnormal: their size was variable and the shape was often distorted (Figs. 6E and F). In these abnormal nuclei, the size of the nucleolus appeared even smaller compared with the mutant nuclei seen around the middle of the distal arm.

We analyzed the mutant nuclear morphology more quantitatively by observing serial sections under TEM. In the mitotic region of female gonads, the nuclear and nucleolar structures were indistinguishable between the wild-type and the *daz-1* mutant (Table 1). In the wild type, both the nucleus and the nucleolus enlarged in diameter during meiotic prophase, in a way that the nucleolus/nucleus ratio increased (Table 1). However, while the diameter of *daz-1* nuclei increased 1.5 times at the loop region relative to the mitotic region, as in the wild type, the increase of the diameter of *daz-1* nucleoli was only marginal, if any, compared to the 1.8 times increase in the wild type (Table 1).

The nucleolus/nucleus ratio of meiotic germ cells in wild-type males appeared smaller than that of oogenetic germ cells in hermaphrodites (Fig. 6I). The size of the nucleolus in *daz-1* males, grown at 25°C, was comparable to that of wild-type males grown at the same temperature. However, outlines of the mutant nucleoli were less clear compared to the wild type, as examined under the Nomarski microscope (Figs. 6I and J). Taken together, although it is possible that DAZ-1 may play roles in determining nucleolar morphology of meiotic germ cells in both sexes, its function is obviously far more crucial in hermaphrodites than in males.

Reduction of the general translational activity in germ cells does not phenocopy the daz-1 deficiency

The nucleolus is a subcompartment of the nucleus where rRNA transcription and ribosome assembly occur (Olson et

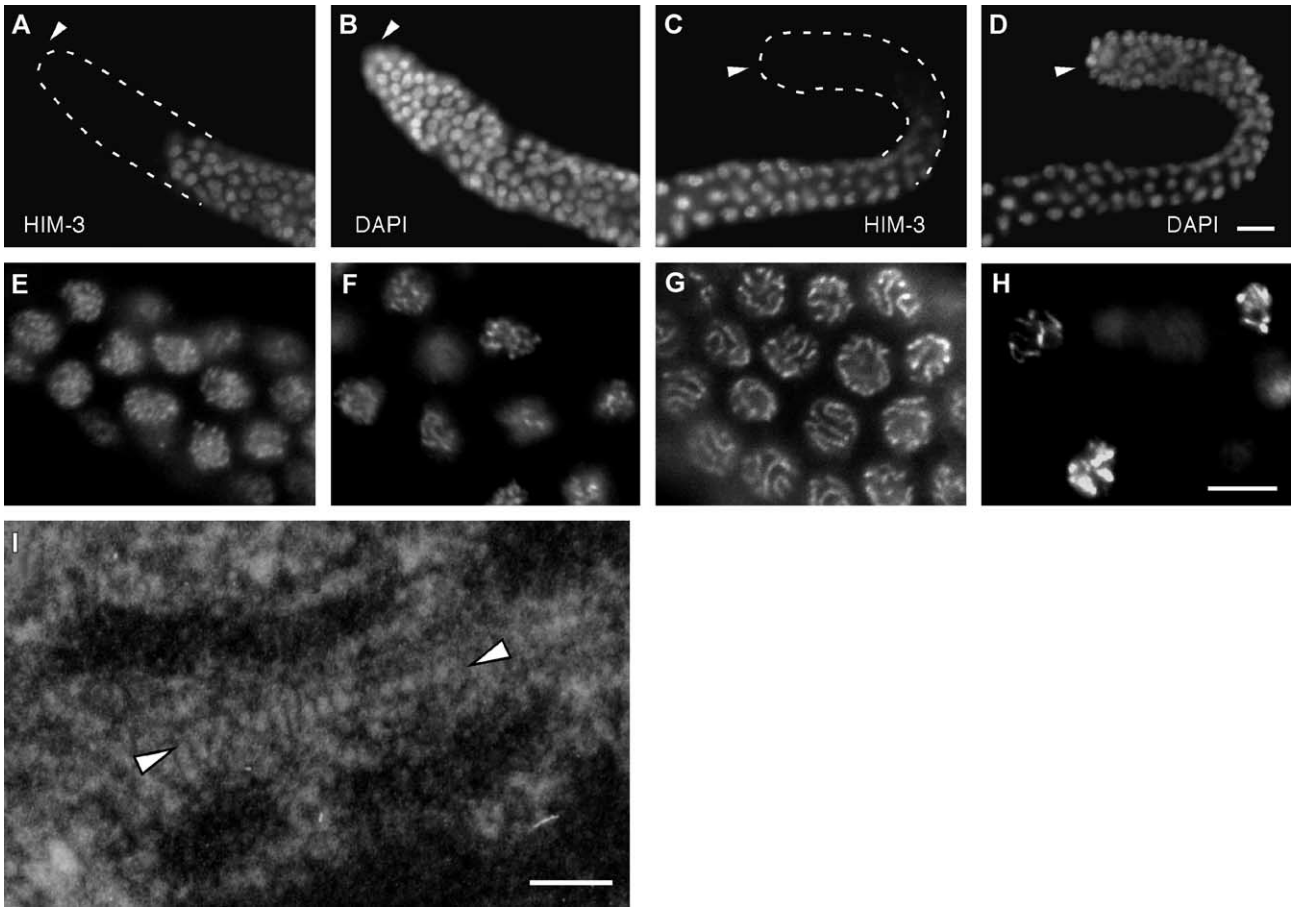

Fig. 5. Meiotic chromatin in the *daz-1* hermaphrodite germline. (A and B) The distal region of a gonad dissected from a wild-type adult hermaphrodite. Anti-HIM-3 staining (A) and DAPI staining (B) are shown. (C and D) The distal region of a gonad dissected from a *daz-1* adult hermaphrodite. Anti-HIM-3 staining (C) and DAPI staining (D) are shown. Outlines of the gonads (dotted lines in panels A and C) and their distal tips (arrowheads) are indicated. Scale bar: 10 μm. (E–H) Anti-HIM-3-stained germ nuclei in adult hermaphrodites. (E) The transition zone in the wild type. (F) The corresponding area in the *daz-1* mutant. (G) The pachytene stage in the wild type. (H) The corresponding stage in the *daz-1* mutant. Scale bar: 5 μm. (I) A TEM image of a meiotic *daz-1* nucleus, revealing part of a synaptonemal complex, as indicated between the two arrowheads. Scale bar: 0.1 μm.

al., 2000), and its size reflects the activity of ribosome synthesis and the level of translation (Frank and Roth, 1998; Kurata et al., 1978). For example, cells of the *C. elegans ncl-1* mutant exhibit enlarged nucleoli (Hedgecock and Herman, 1995) and have more abundant rRNA and proteins than the wild type (Frank and Roth, 1998). Thus, we speculated that the translational activity might be decreased in the *daz-1* gonad. To address the possibility that the observed *daz-1* phenotypes resulted from a decrease in translational activity, we examined the effects of functional suppression of the translational machinery in the adult gonad. The *eft-3* gene encodes elongation factor 1α, an essential component of the translational machinery. EFT-3 activity in adult gonads was inhibited by soaking RNAi, as described in Materials and methods. The nucleoli of germ cells in the *eft-3(RNAi)* adults were smaller than those of the wild type throughout the gonad (Figs. 6K and L), consistent with the hypothesis that the size of nucleoli reflects the level of the translational activity. Remarkably, unlike mitotic *daz-1* germ cells, which were indistinguishable from the wild type, mitotic *eft-3(RNAi)* germ cells possessed abnormally small nucleoli. In addition, the overall phenotype of the *eft-3(RNAi)* was different from that of the *daz-1* mutant: the *eft-3(RNAi)* animals had fewer germ cells than the mutant, and these cells did not arrest at a particular stage, while *daz-1* germ cells arrested uniformly at the pachytene stage (data not shown). As shown below, the cytoplasmic core was absent from the gonad of the adult *daz-1* mutant, but the core was present in *eft-3 (RNAi)* animals, although it was thinner than that of the wild type (data not shown). These observations suggest that a reduction in the general translational activity cannot account for the germline phenotypes observed in the *daz-1* mutant.

DAZ-1 is required for the formation of an anucleate cytoplasmic core in the gonad

In wild-type worms, germ nuclei in the syncytium are located around an anucleate cytoplasmic core called the rachis (Figs. 7A, E, and G). The syncytial germ nuclei cellularize at the loop region of the gonad during oogenesis. The cytoplasmic core is supposed to supply cytoplasmic

constituents to developing proximal gametes (Gibert et al., 1984). We noticed by Nomarski microscopy that the cytoplasmic core was missing in the gonad of *daz-1* hermaphrodites (Fig. 7B). Visualization of cell membranes by actin staining confirmed the absence of the cytoplasmic core from the *daz-1* gonad (Figs. 7C and D). In wild-type worms, small cytoplasmic compartments that might be precursors of the cytoplasmic core were observed at the distal end of the gonad (Fig. 7C), but such compartments were not detectable in *daz-1* worms (Fig. 7D). Analysis by TEM indicated that germ cells occupied the core region in the gonad of *daz-1* hermaphrodites (Figs. 7E and F). Furthermore, to examine whether *daz-1* germ cells were cellularized or not, FITC-dextran was injected into the distal arm of an adult hermaphrodite gonad. While FITC-dextran spread throughout the cytoplasmic core and the cytoplasm of germ cells in the wild-type germline (Fig. 7G), the signal in *daz-1* was detected only in the space between germ cells (Fig. 7H), except for the cells in which the dye was directly injected (data not shown). These results indicate that the *daz-1* germline is cellularized already in the distal region of the gonad.

The cytoplasmic core is also present in the gonad of males, though less prominent (Gibert et al., 1984). An apparently normal cytoplasmic core was observed in the gonad of *daz-1* males, indicating that DAZ-1 is necessary to form a cytoplasmic core only in the gonad of hermaphrodites (data not shown).

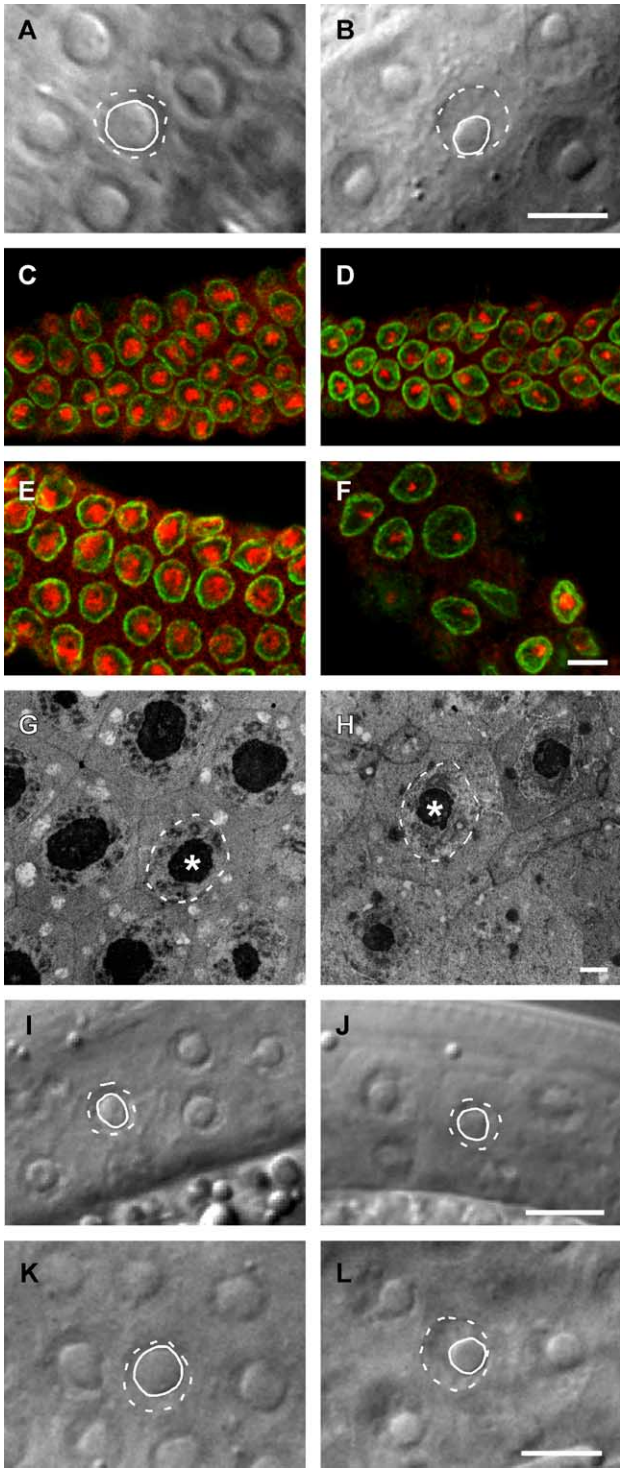

Fig. 6. Small nucleoli in meiotic *daz-1* germ cells. (A and B) Nomarski images of meiotic germ nuclei in a wild-type (A) and a *daz-1* (B) adult hermaphrodites. An outline of a nucleolus (line) and an outline of the nucleus holding it (dotted line) are indicated in each panel. Scale bar: 5 μm. (C–F) Projected confocal images of germ nuclei stained with mAbD77 (red: nucleoli) and anti-Ce-lamin (green: nuclear membrane). (C) The transition zone in the wild type. (D) The corresponding area in the *daz-1* mutant. (E) The late pachytene stage in the wild type. (F) The loop region of the *daz-1* mutant. Scale bar: 5 μm. (G and H) TEM images of meiotic germ nuclei in a wild-type (G) and a *daz-1* (H) adult hermaphrodites. An outline of a nucleus (dotted line) and the nucleoli within it (asterisk) are indicated in each panel. Scale bar: 1 μm. (I–L) Nomarski images of meiotic germ nuclei in a wild-type male (I), a *daz-1* male (J), a mock RNAi hermaphrodite (K), and an *eft-3 (RNAi)* hermaphrodite (L). An outline of a nucleolus (line) and an outline of the nucleus holding it (dotted line) are indicated in each panel. Scale bar: 5 μm.

Discussion

Regulation of daz-1 expression

We have previously reported that transcription of *daz-1* is limited to the germline and regulated developmentally (Karashima et al., 2000). In this paper, we have extended expression analysis to the spatial and temporal distribution of DAZ-1 protein. Comparison of the distribution patterns of *daz-1* mRNA and its protein product indicates that *daz-1* expression is regulated at both transcriptional and posttranscriptional levels. The transcriptional control appears to be central in determining the tissue- and developmental stage-specific expression of *daz-1*: *daz-1* transcripts are present only in the germline of L2 through adulthood in hermaphrodites (Karashima et al., 2000). The posttranscriptional control appears to be critical for the spatial distribution of DAZ-1 protein within the adult gonad: DAZ-1 protein is most abundant in the distal mitotic region and the transition zone and decreases gradually towards the pachytene region,

Table 1
The diameter of nucleoli and nuclei in wild-type and *daz-1* gonads

Diameter (μm, mean ± S.D.)				
		Mitotic (*n*)	Loop (*n*)	Percentage of increase
Wild type	nucleoli	1.2 ± 0.1 (6)	2.2 ± 0.2 (6)	180
	nuclei	2.4 ± 0.1 (7)	3.6 ± 0.1 (4)	150
	ratio	0.50	0.61	120
daz-1	nucleoli	1.2 ± 0.1 (5)	1.3 ± 0.1* (4)	110
	nuclei	2.3 ± 0.2 (6)	3.5 ± 0.2 (6)	150
	ratio	0.52	0.37	71

Nucleolar and nuclear diameters for both stages were measured from the single TEM image of a longitudinal section of a gonad.
* The size of nucleoli in the loop region of *daz-1* was significantly smaller than that of the wild type (Student's *t* test: *P* value = 0.00006).

although *daz-1* mRNA is present throughout the adult germline.

In *C. elegans,* proteins that exhibit spatially limited distribution in the gonad are often regulated at the translational level. For example, *glp-1* mRNA is present throughout the germline but GLP-1 protein is detected only in the most distal region of the gonad (Crittenden et al., 1994). This localized translation is accomplished by translational repression of *glp-1* mRNA in the proximal gonad, through binding of GLD-1 and other unknown translational repressors to the 3′-untranslated region (UTR) of this mRNA (Marin and Evans, 2003). We have shown in the present study that although both GLP-1 and DAZ-1 expression is restricted to the distal region of the gonad, DAZ-1 expression is not directly dependent on the GLP-1 pathway. Thus, it is an interesting question whether or not *daz-1* expression is translationally controlled by a similar mechanism to *glp-1*, and if so, whether these two genes share the same machinery for translational control.

DAZ-1 function in the female germline development

Consistent with ample expression of DAZ-1 in mitotic and early meiotic germ cells, we have found that loss of its function causes three types of abnormalities around the onset of meiosis, as summarized below.

The first is abnormal chromosome morphology in germ cells at meiotic prophase. In the *daz-1* mutant, most nuclei at the transition zone are not crescent shaped but excessively condensed, and they eventually arrest at the pachytene stage. It has been also noted, however, that at least some meiosis-specific chromosome structures are established in the mutant, as indicated by the localization of HIM-3 on meiotic chromosomes and the formation of synaptonemal complexes.

The second is smaller size of nucleoli in meiotic germ cells. The nucleoli of *daz-1* germ cells do not enlarge during meiotic prophase, unlike those of the wild type, implying that they may have only a low translational activity. Possible involvement of DAZ family proteins in translation has been inferred from the association of mouse and zebrafish DAZL proteins with polysomes (Maegawa et al., 2002; Tsui et al., 2000). However, a reduction in the general translation activity in the germline cannot account for the *daz-1* phenotypes, as demonstrated by RNAi of the *eft-3* gene in this study. These observations lead us to speculate that the *daz-1* mutation is likely to affect translation of some specific gene(s) required for the progression of oogenesis. Indeed, DAZ proteins in other organisms such as *Drosophila* BOULE and zebrafish zDAZL have been implicated in the translational activation of particular genes (Maines and Wasserman, 1999). It remains to be clarified whether the reduction of the size of nucleoli in *daz-1* germ cells is a direct result of the translational repression of the target gene(s) by DAZ-1, or rather an indirect downstream event.

The third is a lack of the cytoplasmic core and the aberrant cellularization of the germline of *daz-1* hermaphrodites. The anucleate cytoplasmic core is prominent in the pachytene region in wild-type worms, where RNA molecules are actively transported from germ nuclei to the core (Gibert et al., 1984). Spatially controlled distribution and/or activation of molecules, within the cytoplasmic core and in germ cells neighboring it, are regarded important to achieve the distal-proximal polarity in the gonad, which facilitates proper germline development. If the cytoplasmic core is missing and the germline is cellularized, developing germ cells and oocytes will not receive molecules required for the progression of oogenesis and maturation of oocytes. This may explain why *daz-1* female germ cells fail to proceed beyond pachytene.

It is plausible that the above three abnormalities are interdependent. For example, a low translational activity may result in both reduced nucleoli size and a shortage of protein accumulation necessary for the development of the cytoplasmic core. Alternatively, a lack of the cytoplasmic core and the precocious cellularization of the germline may result in an inefficient transport of molecules directing meiotic events, including proper morphological changes of the chromatin and the nucleus. Interestingly, it has been shown that RNAi of the *ima-3* gene, which encodes an importin α involved in the nuclear transport, causes phenotypes partially similar to *daz-1*, i.e., the cytoplasmic core is lost, the chromatin structure becomes aberrant, and germ cells do not progress beyond pachytene in female meiosis, with spermatogenesis unaffected (Geles and Adam, 2001). Thus, active flow of molecules between germ cells and the cytoplasmic core and within themselves appears to be crucial for oogenesis.

Does DAZ-1 function in males?

In males, DAZ-1 protein is expressed in the mitotic and early meiotic germline, as in hermaphrodites but at a much lower level. This is rather surprising because we have previously shown that DAZ-1 is dispensable for the

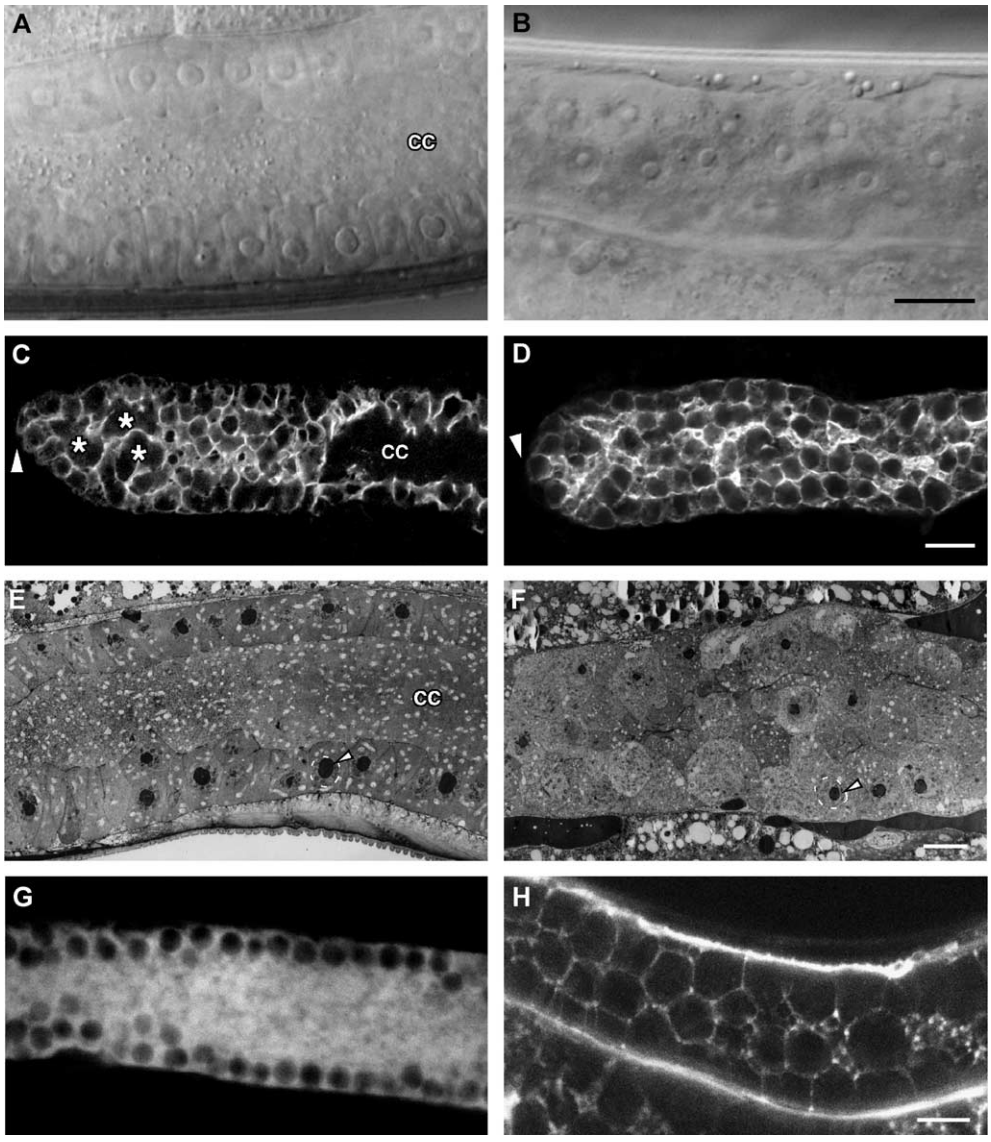

Fig. 7. A lack of the cytoplasmic core in the gonad of *daz-1* hermaphrodites. (A and B) Nomarski images of the distal arm of a gonad in a wild-type (A) and a *daz-1* (B) adult hermaphrodites. The cytoplasmic core (CC in panel A) is indicated. Scale bar: 10 μm. (C and D) Confocal images at a middle focal plane of the distal region of a gonad in a wild-type (C) and a *daz-1* (D) adult hermaphrodites, stained with BODIPY FL phallacidin visualizing actin. Distal tips of the gonads (arrowhead), the cytoplasmic core (CC), and cytoplasmic compartments (asterisks) are indicated. Scale bar: 10 μm. (E and F) Longitudinal TEM images of the distal arm of a gonad in a wild-type (E) and a *daz-1* (F) adult hermaphrodites. An outline of a nucleus (dotted line) and the nucleoli within it (arrowhead) is indicated in each panel. The cytoplasmic core (CC in panel E) is also indicated. No cytoplasmic core has been observed in the *daz-1* gonad in any TEM serial sections or at any focal planes of confocal microscopy. Scale bar: 5 μm. (G and H) Confocal microscopy of the distal region of a gonad in the wild-type (G) or the *daz-1* (H) adult hermaphrodite, which was injected with FITC-dextran. Images of a middle focal plane are shown. Scale bar: 10 μm.

production of sperm (Karashima et al., 2000). In this study, we have observed that the gonad of adult *daz-1* males is slightly thinner than the wild type and that the morphology of nucleoli is weakly affected at 25°C, although it produces fertile sperm. Thus, DAZ-1 appears to play a function, although minor, in male germline development. Why is the requirement of DAZ-1 so different between male and female germlines? One possibility may be that the male germline has a factor(s) that functions redundantly with DAZ-1. Another possibility may be that DAZ-1 executes the same function in spermatogenesis and oogenesis but the dependency of oogenesis on this function is quantitatively more crucial. For instance, if DAZ-1 is involved in the production of cytoplasmic constituents, possibly via translational activation and/or formation of the cytoplasmic core, *daz-1* mutation will be more damaging to oogenesis, which calls for a much larger amount of cytoplasm.

Comparison with other DAZ family proteins

Based on the sequence similarity of the RNA-recognition motifs, Xu et al. (2001) have proposed to divide the DAZ

family into two subgroups, namely, BOULE and DAZL. They infer that the BOULE group is the more ancient, that the DAZL group derived from BOULE by duplication in the ancestor of vertebrates, and that DAZ, which belongs to the DAZL group, was generated in primates by duplication of DAZL. The BOULE members other than *C. elegans* DAZ-1, i.e., *Drosophila boule* and murine and human BOULE, are expressed in testis but neither ovary nor primordial germ cells (Cheng et al., 1998; Xu et al., 2001). In contrast, all the DAZL members appear to be expressed in both male and female germlines since the primordial germ cells stage (Houston and King, 2000; Johnson et al., 2001; Maegawa et al., 1999; Ruggiu et al., 1997; Xu et al., 2001). *C. elegans* DAZ-1 is undetectable in germ cell precursors in the embryo but emerges in both male and female germ cells as they start proliferation at the early larval stage. This expression profile is rather similar to that of the DAZL subgroup. In addition, the function of *C. elegans* DAZ-1 in vivo is apparently female specific, contrasting with the male specificity of *Drosophila boule*. Thus, the expression profiles and the loss-of-function phenotypes of the DAZ family members do not necessarily correlate with the subgrouping that relies on sequence similarity. This divergence of the in vivo function even within the BOULE subgroup implies that the role of the DAZ family members might have been subjected to rapid modification in the course of evolution. However, because some DAZ members can substitute for others in different species, at least partially (Houston et al., 1998; Slee et al., 1999; Xu et al., 2003), they appear to maintain a common biochemical function, presumably as translational regulators utilizing their conserved RNA-binding activity. Further characterization of DAZ family members, including identification of the downstream target genes, is awaited to understand how this family controls germline development and how it has evolved.

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