The *Caenorhabditis elegans* Homologue of Deleted in Azoospermia Is Involved in the Sperm/Oocyte Switch

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Submitted November 21, 2005; Revised April 3, 2006; Accepted April 19, 2006
Monitoring Editor: Marianne Bronner-Fraser

The Deleted in Azoospermia (DAZ) gene family encodes putative translational activators that are required for meiosis and other aspects of gametogenesis in animals. The single *Caenorhabditis elegans* homologue of DAZ, *daz-1*, is an essential factor for female meiosis. Here, we show that *daz-1* is important for the switch from spermatogenesis to oogenesis (the sperm/oocyte switch), which is an essential step for the hermaphrodite germline to produce oocytes. RNA interference of the *daz-1* orthologue in a related nematode, *Caenorhabditis briggsae*, resulted in a complete loss of the sperm/oocyte switch. The *C. elegans* hermaphrodite deficient in *daz-1* also revealed a failure in the sperm/oocyte switch if the genetic background was conditional masculinization of germline. DAZ-1 could bind specifically to mRNAs encoding the FBF proteins, which are translational regulators for the sperm/oocyte switch and germ stem cell proliferation. Expression of the FBF proteins seemed to be lowered in the *daz-1* mutant at the stage for the sperm/oocyte switch. Conversely, a mutation in *gld-3*, a gene that functionally counteracts FBF, could partially restore oogenesis in the *daz-1* mutant. Together, we propose that *daz-1* plays a role upstream of the pathway for germ cell sex determination.

INTRODUCTION

Germ cells in the nematode *Caenorhabditis elegans* go through two developmental decisions to become gametes: a decision between mitotic proliferation and meiotic development and a decision between sperm production and oocyte production. The mature gonad of *C. elegans* is a syncytial tube that contains >1000 germ nuclei at all stages of differentiation and shows a distal-to-proximal polarity in development (Hubbard and Greenstein, 2000). Although germ nuclei in mitosis and in early stages of meiosis do not constitute genuine cells, not being completely separated by plasma membrane, we conventionally call them "germ cells." Germ stem cells proliferate by mitosis at the distal end of the gonad. Meiosis and differentiation (spermatogenesis and oogenesis) occur in the more proximal regions. Male meiosis and spermatogenesis take place in the L4 larval stage in hermaphrodites. The germline switches from spermatogenesis to oogenesis (the sperm/oocyte switch) at the transition to the adult stage and produces oocytes thereafter. In contrast, males continue to produce sperm in the adulthood.

The primary regulator of the transition from mitosis to meiosis is the distal tip cell, which directs germ stem cells to keep proliferation via the GLP-1/Notch signaling pathway. RNA-binding proteins are known to function downstream of the GLP-1 pathway, in at least two redundant cascades to repress mitosis and/or promote entry to meiosis (reviewed in Kimble and Crittenden, 2005). GLD-1 and NOS-3 belong to one branch, and GLD-2 and GLD-3 belong to the other branch. GLD-1 is an RNA-binding protein carrying the

maxi-KH/STAR domain; GLD-2, an atypical cytoplasmic poly(A) polymerase; GLD-3, a KH-domain RNA-binding protein belonging to the Bicaudal-C family; and NOS-3, one of the three Nanos homologues in *C. elegans*. Accumulation of GLD-1 is likely to be a crucial step for the mitosis/meiosis decision and is regulated by FBF, NOS-3, and GLD-2. FBF is a generic name of two nearly identical RNA-binding proteins, FBF-1 and FBF-2, which are members of the conserved Pumilio and FBF translational repressors, and are proposed to repress translation of *gld-1* mRNA directly. GLD-1 in turn functions as a translational repressor of multiple targets during early meiotic phases of gametogenesis.

The sperm/oocyte switch of germ cells requires germline-specific control on two pivotal sex-determining factors, FEM-3 and TRA-2, and translational repression by RNA-binding proteins plays crucial roles here as well (reviewed in Ellis and Schedl, 2006). When spermatogenesis takes place, the female fate-inducer TRA-2 must be translationally repressed by GLD-1 and F-box protein FOG-2. The repression of TRA-2 then allows the male fate-inducer FEM-3 to be active. Conversely, FEM-3 has to be translationally down-regulated by FBF and NOS-3 at the sperm/oocyte switch. In regard to the sperm/oocyte switch, GLD-3 has been proposed to antagonize FBF through protein-protein interaction during spermatogenesis.

In addition to the above-mentioned RNA-binding proteins, the single *C. elegans* member of the Deleted in Azoospermia (DAZ) protein family plays an important role in gametogenesis (Karashima *et al.*, 2000). Metazoan DAZ proteins, defined by their homology to human DAZ, are putative translational activators with a well-conserved RNP-type RNA recognition motif (RRM) (reviewed in Yen, 2004). Human DAZ is encoded by the DAZ gene cluster on the Y chromosome, which is deleted occasionally in azoospermic men (Reijo *et al.*, 1995). The DAZ gene family has been phylogenically divided into three subgroups (Xu *et al.*, 2001): The ancestral autosomal *BOULE* (*Drosophila boule*, *C. elegans*

This article was published online ahead of print in *MBC in Press* (<http://www.molbiolcell.org/cgi/doi/10.1091/mbc.E05-11-1067>) on April 26, 2006.

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daz-1, and mammalian *BOULE/Boule*); the vertebrate-specific autosomal *DAZL* (*DAZ*-like) (zebrafish *zDazl*, *Xenopus* *Xdazl*, and mammalian *DAZL/Dazl*); and the Y chromosome-linked *DAZ* found only in higher primates. Analyses of animals defective in some of these *DAZ* homologues have revealed the requirement of the *DAZ* family genes for gametogenesis. Knockout of mouse *Dazl* leads to both male and female sterility, with no mature gametes produced (Ruggiu *et al.*, 1997). Depletion of *Xdazl* mRNA causes a failure in the development of *Xenopus* primordial germ cells (PGCs) (Houston and King, 2000). The *Drosophila* *boule* mutant shows male-specific sterility due to a defect in G₂/M transition during spermatogenesis (Eberhart *et al.*, 1996). In *C. elegans*, germ cells in the *daz-1* hermaphrodite exhibit multiple abnormalities during female meiosis and eventually arrest at the pachytene stage in oogenesis (Karashima *et al.*, 2000; Maruyama *et al.*, 2005). Besides phenotypic analyses, there are a number of reports about biochemical characteristics and expression profiles of *DAZ* homologues, which altogether suggest that the *DAZ* family proteins bind to the 3' untranslated region (UTR) of target mRNAs and up-regulate their translation (Yen, 2004; Collier *et al.*, 2005).

In this article, we present evidence that *C. elegans* *DAZ* protein is involved in the sex determination of germ cells. RNA interference (RNAi) of a *daz-1* orthologue in *C. briggsae*, a nematode related to *C. elegans*, disrupted the sperm/oocyte switch completely. In addition, genetic combination of *daz-1* with a conditional or weak masculinization of germline (Mog) mutation indicated that *C. elegans* *daz-1* was also likely to play some role in the sperm/oocyte switch. Further analyses suggested that *DAZ-1* might function upstream of FBF and GLD-3. Thus, the *daz-1* gene may participate in a cascade of translational regulations responsible for the mitosis/meiosis decision and the sperm/oocyte switch.

MATERIALS AND METHODS

General Methods and Strains

Maintenance and genetic manipulation of *C. elegans* was carried out basically as described previously (Brenner, 1974). *C. briggsae* and *Caenorhabditis remanei* were handled similarly to *C. elegans* except that *C. remanei* was maintained on 3% agar plates. Wild-type refers to the Bristol strain N2 for *C. elegans*, the AF16 strain for *C. briggsae*, and the SB146 strain for *C. remanei*. Mutations in *C. elegans* used were as follows: (LG I) *gld-1(q485)*; (LG II) *daz-1(tj3)*, *fbf-1(ok91)*, *fbf-2(q704)*, *gld-3(ok308)*; (LG III) *fem-3(q20gf)*. The *ht2[bli-4(e937) let-? (q782) qIs48]* (I;III) translocation was used to balance *gld-1(q485)*, and the *mln1[dpy-10(e128) mIs13]* II chromosome was used to balance *daz-1(tj3)* and *gld-3(ok308)*. The *gld-3(ok308)* allele, initially isolated by the *C. elegans* Knockout Consortium, lacks bases 1966 through 2425 of the open reading frame (ORF) (Schedl, personal communication). The temperature-sensitive mutant *fem-3(q20gf)* was maintained at 15°C. Otherwise strains were maintained at 20°C.

Cloning of the *daz-1* Orthologues from *C. briggsae* and *C. remanei*

Total RNA from *C. briggsae* or *C. remanei* was prepared using TRIzol reagent (Invitrogen, Carlsbad, CA) and Poly(A)⁺ RNA was selected with Message Maker (Invitrogen). *Cb-daz-1* and *Cr-daz-1* cDNAs were cloned using Marathon cDNA Amplification kit (Clontech, Mountain View, CA) following manufacturer's instructions. For *C. briggsae* *daz-1*, two rounds of 3'-rapid amplification of cDNA ends (RACE) polymerase chain reaction (PCR) were performed with primers 5'-ATCCTATCTACCACTACAC-3' and 5'-ACCACTTCATCAACAGCAC-3', which were designed according to the expressed sequence tag sequences (GenBank accession nos. R04525 and R04900). Resultant DNA fragments were cloned into the pCR2.1-TOPO vector (Invitrogen). 5'-RACE PCR was performed using a primer 5'-CGTATGGTCCTGGTGAAGT-3'. For *C. remanei* *daz-1*, a fragment containing the RRM region was PCR amplified using primers 5'-ACGGTATCAAAAAGACCAA-3' and 5'-ACCATCAAGTGATTTAAACCATYTTNAC-3'. A single round of 5'- or 3'-RACE PCR was performed using primers 5'-GCTCAGCAGGTCAGATTCCGTTGT-3' and 5'-ACTCTCTTCAACACTTCCCAGCC-3', respectively. Full-length cDNA clones pCR2.1-TOPO-*Cb-daz-1* and pCR2.1-TOPO-*Cr-daz-1* were constructed based on the 5' and 3' clones.

The deduced sequences of full length *Cb-daz-1* and *Cr-daz-1* mRNAs were registered to DNA Data Bank of Japan/European Molecular Biology Labora-

tory/GenBank nucleotide sequence databases under the accession numbers AB236895 and AB236896, respectively.

RNA Interference

Templates for RNA synthesis were PCR-amplified using M13(-20) (5'-GTA-AAACGACGGCCAGT-3') and T7+M13-reverse (5'-GCGTAATACGACTCACTATAGGGCAGGAAACAGCTATGAC-3') primers, from yk64b4 (*daz-1*), pCR2.1-TOPO-*Cb-daz-1*, or pCR2.1-*Cr-daz-1*. Double-stranded (ds)RNA was synthesized *in vitro* with T7 RNA polymerase and purified as described (Maeda *et al.*, 2001). For phenotype analysis, delivery of dsRNA into animals was performed by microinjecting dsRNA solution into the gonad of young adult hermaphrodites (females in the case of *C. remanei*). F1 progeny laid 12–36 h after the injection were grown at 20°C for 5 d and examined. To obtain large amounts of RNAi animals for immunoblotting, RNAi by L1 soaking method was performed (Kuroyanagi *et al.*, 2000).

Construction of a Strain Expressing *DAZ-1-3xFLAG*

An XbaI-ScaI genomic DNA fragment of the *daz-1* locus was cloned from the cosmid F56D1 into the pBluescript vector (Stratagene, La Jolla, CA). Three tandem copies of the FLAG epitope were inserted into the EcoRV site in the *daz-1* ORF, creating pDAZ-1/3xFLAG. pDAZ-1/3xFLAG was mixed with pRF4[*rol-6(su1006)*], PvuII digested N2 genomic DNA, and injected into the gonads of *daz-1(tj3)/mln1[dpy-10(e128) mIs13]* adult hermaphrodites. Fertile animals homozygous for *daz-1(tj3)* and positive for the roller (Rol) phenotype were selected. The extrachromosomal array was integrated into the genome using UV irradiation as described previously (Mitani, 1995). One integrant allele obtained was named *btlS2*.

Preparation of Worm Extracts, Immunoprecipitation, and Reverse Transcription (RT)-PCR Analysis

Preparation of cytosol extracts from the *btlS2* strain, RT-PCR, subtraction, and cloning were done essentially as described previously (Lee and Schedl, 2001). T7 promoter-oligo(dT) primer (5'-AAGCAGTGGTATCAACGCAGAGT-AATACGACTCACTATAGGGC(T)₃₀VN-3') was used for the cDNA synthesis from mRNA samples. The PCR primers were 5'-ATCAACGCAGAGTAAT-ACGACTCACTATAG-3' (T7 promoter), 5'-TTGGAGAAGAATGGAATC-GAGGAAGGTAAAC-3' (*fbf-1*), 5'-TTGGAGAAGAATGGGATCGAGGAAGG-AAGC-3' (*fbf-2*), and 5'-GCTCATCAAGAAGCTCGCCAAGAGCTACGA-3' (*rpl-1*).

RNA-Binding Assays

The 3' UTRs of *fbf* cDNAs and a 365-nt cDNA for the *rpl-1* mRNA 3' region were cloned into vector pCR2.1-TOPO (Invitrogen), PCR-amplified with M13 primers, and transcribed *in vitro* by T7 RNA polymerase. [α -³²P]ATP/UTP or biotin-UTP labeling mix (Roche Diagnostics, Indianapolis, IN) was added for labeling reaction. Biotin-RNA pull-down assay was performed as described, using extracts prepared from the *daz-1(tj3)*; *btlS2* strain (Lee and Schedl, 2001). For gel retardation assay, the GST-*DAZ-1* protein was expressed and purified from *Escherichia coli* BL21(DE3) harboring part of the *daz-1* ORF (amino acids 1–241) cloned in the pGEX-KG vector (GE Healthcare, Little Chalfont, Buckinghamshire, United Kingdom). Binding reaction mixtures contained 4 ng/ μ l [³²P]RNA, 0–25 ng/ μ l protein, 1 mM dithiothreitol, 1 mM EDTA, 5% glycerol, 15 mM HEPES, pH 7.4, 75 mM KCl, 1.5 mM MgCl₂, 100 mM NaCl, 0.5% Triton X-100, and 500 ng/ μ l yeast tRNA. Unlabeled competitor RNA was added where needed. Reaction mixtures were incubated at room temperature for 20 min, supplemented with 7.5 μ g/ μ l heparin, and run on a 3.6% native polyacrylamide gel containing 0.5× Tris borate-EDTA.

Immunoblotting

Immunoblotting was performed following standard procedures. Primary antibodies used were anti-FLAG M2 mouse monoclonal antibody (mAb) (Sigma-Aldrich, St. Louis, MO), anti-actin MAB1501 mouse mAb (Chemicon International, Temecula, CA), and anti-FBF polyclonal antibodies (see below). Incubation of antibodies was performed in Can Get Signal reagent (Toyobo Engineering, Osaka, Japan), and signals were detected using an ECL or ECL Plus kit (GE Healthcare). Rabbit anti-FBF polyclonal antibodies were raised against the N-terminal quarter of FBF-1 (amino acids 1–153) and affinity purified using the FBF-1 protein blotted on the Immobilon membrane (Millipore, Billerica, MA). Our antibody is likely to recognize both FBF-1 and FBF-2.

Fixation and Staining of Gonads

Extruded gonads were fixed with 3.2% formaldehyde/80% methanol/6.2 mM KH₂PO₄, pH 7.2, at -20°C and rehydrated with phosphate-buffered saline (PBS) at room temperature. For plain 4,6-diamidino-2-phenylindole (DAPI) staining, DAPI was added to the final concentration of 2 μ g/ml. For antibody staining, samples were treated with blocking solution (3% bovine serum albumin in PBS/0.5% Tween 20) for 1 h at room temperature, incubated with primary antibody at 4°C overnight, and incubated with secondary antibody. DAPI was then added to the final concentration of 2 μ g/ml. Primary antibodies used were anti-FLAG M2 mAb (Sigma-Aldrich) and anti-OMA-2 antibody (Detwiler *et al.*, 2001). Secondary antibodies used were Cy3-conju-

Figure 1. DAZ homologues from three *Caenorhabditis* species. (A) Alignment of the deduced amino acid sequences of Cb-DAZ-1, Cr-DAZ-1, and *C. elegans* DAZ-1. Black boxes indicate identical residues and shaded boxes indicate similar residues. The RRM is underlined and amino acid residues conserved in the BOULE subfamily proteins are marked with asterisks. The putative DAZ motif is double underlined. (B) Exon-intron structures of Cb-daz-1, Cr-daz-1, and daz-1. Exons are shown by boxes, introns, by broken lines, and 5' and 3' UTR sequences, by lines. The RRM motif is indicated by split black boxes, and the putative DAZ motif, by a shaded box, in each gene.

gated goat anti-rabbit IgG and Cy3-conjugated goat anti-mouse IgG (Chemicon International). For whole mount staining, worms were fixed with -20°C methanol and stained with 10 ng/ml DAPI in PBS. Images were captured by a Zeiss Axioplan 2 microscope equipped with a Hamamatsu cooled charge-coupled device camera and fluorescence in situ hybridization software.

RESULTS

Identification of the daz-1 Orthologues in *C. briggsae* and *C. remanei*

To obtain insight into the function of *C. elegans* daz-1 by comparison, we cloned cDNAs of the putative daz-1 orthologues in *C. briggsae* and *C. remanei*, which are two nematodes evolutionarily close to *C. elegans*, using the 5' and 3'

RACE methods. They were named *Cb-daz-1* and *Cr-daz-1*, respectively, and their gene structures were deduced from the cDNA sequences and the draft genome sequences of *C. briggsae* and *C. remanei* (Stein *et al.*, 2003) (GSC BLAST Search: <http://genome.wustl.edu/tools/blast/>). The predicted open reading frames of *Cb-* and *Cr-daz-1* encoded proteins with 224 and 452 amino acid residues, respectively (Figure 1A). Both of them possessed the consensus feature of the DAZ family proteins, namely, one RNA-recognition motif followed by the DAZ motif (Figure 1B).

Although the three orthologues showed considerable amino acid identity throughout the protein, some regions revealed a higher degree of identity (Table 1). The N-termi-

Table 1. Percentage of identity between regions of *Caenorhabditis* DAZ-1 proteins

Region	N-terminal ^a	RRM ^b	Middle ^c	DAZ motif ^d	C-terminal ^e	Total
Cb/Cr	66.7	88.4	60.0	29.3	3.9	37.6
Cb/Ce	53.7	84.9	39.1	20.5	3.0	29.9
Cr/Ce	64.5	89.5	52.5	51.2	47.9	58.4

Regions were defined as follows. Numbers indicate corresponding amino acid residues.

^a Cb-DAZ-1: 1–67, Cr-DAZ-1: 1–60, DAZ-1: 1–62.

^b Cb-DAZ-1: 68–153, Cr-DAZ-1: 61–146, DAZ-1: 63–148.

^c Cb-DAZ-1: 154–192, Cr-DAZ-1: 147–178, DAZ-1: 149–187.

^d Cb-DAZ-1: 193–207, Cr-DAZ-1: 179–217, DAZ-1: 188–224.

^e Cb-DAZ-1: 208–225, Cr-DAZ-1: 218–452, DAZ-1: 225–499.

Figure 2. RNAi for *Cb-daz-1* and *Cr-daz-1*. (A, B, E, and F) Gonads were dissected from the following adult animals and stained with DAPI. (C and D) Whole animals were stained with DAPI. (A) *C. briggsae* wild-type hermaphrodite with no treatment. (B) *C. briggsae Cb-daz-1(RNAi)* F1 hermaphrodite. (B) *C. briggsae* P0 hermaphrodite with mock RNAi treatment. (D) *C. briggsae Cb-daz-1(RNAi)* P0 hermaphrodite. (E) *C. remanei* wild-type female with no treatment. (F) *C. remanei Cr-daz-1(RNAi)* F1 female. Arrows indicate germ nuclei at the diakinesis stage during oogenesis. Arrowheads indicate the distal end of each gonad. Bar, 50 μm.

nal region preceding the RRM was fairly identical. The RRM region was highly conserved. *C. elegans* DAZ-1 was previously classified into the BOULE subclass of the DAZ protein family (Xu *et al.*, 2001). The residues specifically conserved in the BOULE subfamily were also found in the Cb- and Cr-DAZ-1 proteins (Figure 1A). Compared with the RRM, the DAZ motif is less conserved among DAZ homologues (Xu *et al.*, 2001). Consistently, the DAZ motifs in the three orthologues showed more divergence from each other. Although moderate matches of amino acid residues were observed among them, most of the identical residues were different from the conventional DAZ motif constituents, which may indicate less functional importance of this region in worm DAZ proteins. The C-terminal region was structurally more diverged than the N-terminal region. DAZ-1 is unique among the DAZ family proteins in that it carries a long C-terminal stretch after the DAZ motif. Cr-DAZ-1 turned out to have also a relatively long C-terminal region, which was significantly homologous to *C. elegans* DAZ-1. In contrast, Cb-DAZ-1 was much smaller, composed of only 224 amino acids with a very short C-terminal stretch, hence being closer to *Drosophila* Boule (228 amino acids) in size (Eberhart *et al.*, 1996). No pieces of DNA corresponding to the missing region in Cb-DAZ-1 were found in the currently

Figure 3. Synthetic phenotype of the *C. elegans daz-1* mutation or RNAi and the *fem-3(q20gf)* mutation. (A–E) DAPI-stained images of extruded gonads from adult hermaphrodites are shown. (A) Wild-type *C. elegans* at 20°C. (B) The *daz-1* mutant at 20°C. (C) The *fem-3(q20gf)* mutant at 15°C. (D) The *fem-3(q20gf)* mutant at 25°C. (E) The *daz-1; fem-3(q20gf)* double mutant at 15°C. (F and G) Whole mount DAPI-stained images of adult hermaphrodites cultured for 5 d at 15°C after RNAi treatment. (F) Wild-type *C. elegans* treated with *daz-1* RNAi. (G) The *fem-3(q20)* mutant treated with *daz-1* RNAi. Arrows indicate germ nuclei at the diakinesis stage during oogenesis. Arrowheads indicate the distal end of each gonad. Bar, 50 μm.

available *C. briggsae* genome sequence. However, the assumed positions for splicing were all identical in the ORFs of the three species, indicating that the worm DAZ genes are likely to have evolved from a single parental gene (Figure 1B).

RNAi against the *daz-1* Orthologues Causes Defects in Female Gametogenesis

To assess the functions of *Cb-daz-1* and *Cr-daz-1*, we disrupted the gene activity by RNAi (Fire *et al.*, 1998). F1 progeny laid by *C. briggsae* P0 injected with *Cb-daz-1* dsRNA were sterile, producing neither oocytes nor embryos in the adult stage. We then examined germ nuclei in *Cb-daz-1(RNAi)* F1 animals by DAPI staining. In wild-type *C. briggsae*, the array of germ cells seems similar to that of *C. elegans*, showing mitotic proliferation at the distal end and gametogenesis in more proximal regions (Haag *et al.*, 2002) (Figure 2A). Sperm are produced in the larval stage and the sperm/oocyte switch occurs at the transition to the adult stage. The chromatin in sperm is highly condensed after the pachytene stage and can be easily distinguished from that of diakinesis oocytes, which has a rod-like morphology. DAPI staining

Figure 4. Binding of the DAZ-1 protein to *fbf* mRNAs. (A) Expression pattern of DAZ-1-3xFLAG in the *daz-1; btl-2* strain. Images of an extruded gonad from the *daz-1; btl-2* strain, stained with either anti-FLAG antibody (top) or DAPI (bottom), are shown. Arrows indicate germ nuclei at the diakinesis stage during oogenesis. Arrowhead indicates the distal end of the gonad. Bar, 50 μ m. (B) Coprecipitation of *fbf* mRNAs with DAZ-1. (a) Detection of DAZ-1-3xFLAG in the anti-FLAG immunoprecipitate from a *btl-2* worm extract by immunoblotting. Tubulin is shown as a negative control. (b) Concentration of *fbf-1* and *fbf-2* mRNAs in the anti-FLAG immunoprecipitate, assayed by RT-PCR. A nonspecific binder *rpl-1* is shown as a control. (C) Features of the 3' UTR sequences of *fbf-1* and *fbf-2* mRNAs. (A/C/U)GUUC sequences are doubly underlined and U-rich tracts, singly. Sequences used as the RNA probes are shown in uppercase. Dotted nucleotides were replaced by the complementary ones in mutant competitors used in E. (D) Binding of DAZ-1 to the 3' UTR of *fbf* mRNAs. The 3' UTR fragment of *fbf-1*, *fbf-2*, or *rpl-1* mRNA was biotin labeled, added to a *btl-2* worm extract, and pulled down. DAZ-1-FLAG in the biotin-RNA precipitate was detected by immunoblotting using anti-FLAG antibody. S, sense-strand mRNA; AS, anti-sense-strand RNA. Tubulin is shown as a negative control. (E) In vitro reconstruction of DAZ-1-*fbf* mRNA complex. Gel retardation assay was performed to monitor the binding of GST-DAZ-1 protein (0–25 ng/ μ l) to 32 P-labeled sense strand 3' UTR RNAs (2 ng/ μ l). Competitors are unlabeled sense RNA (13–100 ng/ μ l): F1, *fbf-1*; F2, *fbf-2*; F1mut, *fbf-1* with GUnC mutated into CAnG; F2mut, *fbf-2* with GUnC mutated into CAnG; and R, *rpl-1*.

revealed that *Cb-daz-1(RNAi)* adult hermaphrodites produced only sperm continuously, which resulted in excessive sperm packed up to the loop of the gonad (Figure 2B). This Mog phenotype was reminiscent of a defect in the sperm/oocyte switch in *C. elegans* (Barton *et al.*, 1987). Furthermore, *Cb-daz-1* RNAi could cause the P0 animals to exhibit a similar Mog phenotype. When adult hermaphrodites actively producing eggs were injected with *Cb-daz-1* dsRNA, the P0 animals ceased oogenesis and reinitiated spermatogenesis $\approx$ 3 d after the injection (Figure 2D). This observation suggests that *Cb-daz-1* is continuously required for the determination of germ cell sex during the larval and adult stages.

In *C. remanei*, female F1 progeny laid by P0 females injected with *Cr-daz-1* dsRNA were also sterile. The gonad of the *Cr-daz-1(RNAi)* female progeny seemed similar to that of *C. elegans daz-1(RNAi)* hermaphrodites except that they did not produce sperm before oogenesis. Unlike wild-type females (Figure 2E), germ cells in the *Cr-daz-1(RNAi)* females were arrested at the pachytene stage (Figure 2F). This phenotype was the same as that of *C. elegans* hermaphrodites deleted in *daz-1*. *Cr-daz-1(RNAi)* male progeny seemed to be fully fertile. They could mate with wild-type females and produced viable progeny.

The above-mentioned observations indicate that DAZ homologues in *C. briggsae* and *C. remanei* are required for female gametogenesis, as is the case for *C. elegans*, but that there may be some subtle differences in the phenotypes among the three worm species.

The *daz-1* Mutation Enhances Masculinization of Weak Mog Mutants in *C. elegans*

The terminal phenotype of the *C. elegans daz-1* mutant was pachytene-arrest during oogenesis, and this phenotype was not rescued by a feminizing mutation that fixes the germline to an oogenic mode (Karashima *et al.*, 2000), implying that *daz-1* was unlikely to be involved in the germ cell sex determination pathway. However, subsequent analysis has revealed that loss of *daz-1* function causes anomalies in nuclei at much earlier stages of oogenesis (Maruyama *et al.*, 2005). Inspired by the RNAi phenotype found in *C. briggsae*, we investigated whether *C. elegans daz-1* could have any role in sex determination.

We tested whether the *daz-1* mutation could enhance masculinization in the *fem-3(q20gf)* mutant (Figure 3). This *fem-3* allele carries point mutations in the 3' UTR, which result in temperature-sensitive gain-of-function (gf) (Barton *et al.*, 1987; Ahringer and Kimble, 1991). At the restrictive temperature 25°C, *fem-3(q20gf)* hermaphrodites lose translational repression of *fem-3* mRNA, and hence cannot switch from spermatogenesis to oogenesis, giving a Mog phenotype (100% Mog; $n = 95$) (Figure 3D). They can fulfill the sperm/oocyte switch and produce oocytes at the permissive temperature 15°C (0% Mog; $n = 115$) (Figure 3C).

Although the major phenotype of the *daz-1(tj3)* single mutant was an oogenic pachytene arrest at both 20°C (Figure 3B) and 15°C (0% Mog; $n = 120$), *daz-1(tj3); fem-3(q20gf)*

hermaphrodites did not show this phenotype even at 15°C, the permissive temperature for the *fem-3(gf)* single mutant. Instead, production of sperm continued even in adulthood and most of the gonad was eventually filled up with sperm (99% Mog; $n = 125$) (Figure 3E). Furthermore, *daz-1* RNAi of the adult *fem-3(gf)* mutant terminated ongoing oogenesis and caused reinitiation of spermatogenesis at 15°C (Figure 3G), as was seen for *Cb-daz-1* RNAi in *C. briggsae*. In addition to the *fem-3(gf)* mutant, *daz-1* mutation enhanced masculinization in the *fbf-1(ok91)* mutant. The *fbf-1(ok91)* mutant is supposed to be weakly derepressed in translation of *fem-3*: 99% of *fbf-1(ok91)* mutant animals produce oocytes normally but with an increase in sperm production compared with the wild type (Crittenden *et al.*, 2002). However, *daz-1(tj3) fbf-1(ok91)* hermaphrodites produced only sperm throughout the life (100% Mog; $n = 124$). These observations supported that *C. elegans* DAZ-1 could also play a role in germ cell sex determination, either inhibiting spermatogenesis or promoting the switch to oogenesis.

DAZ-1 Can Bind to *fbf* mRNAs

We cloned several candidate mRNAs that could bind to DAZ-1 using a immunoprecipitation/subtractive PCR method described previously (Lee and Schedl, 2001). Briefly, a *daz-1(tj3)* homozygous strain was rescued by an integrated transgene *btl-2*, which expressed a FLAG-tagged DAZ-1 protein (DAZ-1-3xFLAG) (see *Materials and Methods*). The expression pattern of DAZ-1-3xFLAG in the *daz-1(tj3); btl-2* strain was confirmed to be identical with that of DAZ-1 in the wild-type strain (Figure 4A) (Maruyama *et al.*, 2005). DAZ-1-3xFLAG was immunoprecipitated with anti-FLAG antibody from a cell extract prepared from the *daz-1; btl-2* strain (Figure 4B), and coimmunoprecipitated mRNAs were purified and reverse transcribed into cDNA. Subtractive PCR was performed between this cDNA sample and a control sample prepared using anti-IgG antibody, to eliminate background precipitates. Candidates for a target of DAZ-1 thus identified included *fbf-1* and *fbf-2* mRNAs, which encode nearly identical translational repressors important for the sperm/oocyte and mitosis/meiosis decisions in germ cells (Zhang *et al.*, 1997; Crittenden *et al.*, 2002). We confirmed significant concentration of *fbf* mRNAs in the DAZ-1 immunoprecipitate by RT-PCR analysis, compared with *rpl-1* mRNA encoding ribosomal protein L1 as a control (Figure 4B). The 3' UTR of each *fbf* mRNA contains two (A/C/U)GUUC sequences and two U-rich tracts, which are known to be preferentially bound by some vertebrate DAZ proteins (Houston *et al.*, 1998; Venables *et al.*, 2001; Maegawa *et al.*, 2002) (Figure 4C). Related (A/C/U)GUnC sequences are also present in them. Biotin-labeled 3' UTRs of *fbf* mRNAs synthesized in vitro could effectively pull down the DAZ-1-3xFLAG protein from a worm extract (Figure 4D). In a mobility shift assay, DAZ-1 protein could bind to *fbf* 3' UTRs (Figure 4E). This binding was sequence dependent, because mutant forms of *fbf* 3' UTRs in which all the GUnC motifs were converted to their complementary sequences (CAN₃G) (Figure 4C) could not compete for the DAZ-1-RNA interaction as efficiently as the wild-type UTRs (Figure 4E).

Previous studies suggest that the activity of FBF is relatively low when spermatogenesis is initiated and that it is elevated at the transition from the L4 stage to the adult stage, when the sperm/oocyte switch is turned on. The *fbf* mRNAs show their highest expression in the L4 stage, and the FBF proteins are concentrated in the distal region of the adult gonad (Zhang *et al.*, 1997; Crittenden *et al.*, 2002). In immunoblotting, the level of the FBF proteins in L4 larvae was significantly decreased (21% decrease) in the *daz-1* mu-

Figure 5. Reduction of the FBF protein in L4 larvae of the *daz-1* mutant. FBF was detected by immunoblotting. One hundred and fifty hermaphrodites of the indicated genotype were collected at the L4 stage, boiled in SDS PAGE sample buffer, and loaded in one lane. Actin is shown as a loading control. The relative amount of FBF was quantified and indicated below each lane. (A) Wild type, *daz-1(tj3)*, *gld-3(ok308)*, and *daz-1(tj3) gld-3(ok308)* mutant at 20°C. (B) *fem-3(q20)* and *daz-1(tj3); fem-3(q20)* mutant at 25°C. (C) *gld-1(q485)*; mock RNAi treatment and *gld-1(q485); daz-1(RNAi)* at 25°C.

tant compared with that in the wild type (Figure 5A). A decrease of FBF proteins by the *daz-1* mutation was also evident in both *fem-3(q20)* and *gld-1(q485)* backgrounds (33 and 59% decrease, respectively), the former of which fixes germ cell fates to a spermatogenic state, and the latter, to a tumorigenic state, regardless of the *daz-1* activity (Karashima *et al.*, 2000) (Figure 5, B and C). In addition, the germline makeup does not seem to differ much between wild-type and the *daz-1* mutant at the L4 stage (our unpublished data). These results suggest that the DAZ-1 protein binds to *fbf* mRNAs to promote or maintain the expression of the FBF proteins, an idea consistent with the proposal that DAZ proteins in other organisms perform their function through the 3' UTR sequences of target mRNAs.

A Mutation in *gld-3* Partially Compensates *daz-1*

Because *daz-1* seemed to act positively on FBF, we examined whether factors that were known to antagonize FBF functionally could antagonize DAZ-1. GLD-3, a Bicaudal-C homologue, has been identified as a FBF-interacting protein. With regard to the sperm/oocyte switch, GLD-3 is supposed to suppress function of FBF directly and promote spermatogenesis (Eckmann *et al.*, 2002). Therefore, we asked whether a mutation in *gld-3* could alleviate the phenotype of the *daz-1* mutant (Figure 6).

Figure 6. Compensation of the *daz-1* mutation by the *gld-3* mutation. (A–D) DAPI-stained images of extruded gonads from adult hermaphrodites. (A) Wild-type *C. elegans*. (B) The *gld-3(ok308)* mutant. (C) The *daz-1(tj3)* mutant. (D) The *daz-1(tj3) gld-3(ok308)* double mutant. Arrows indicate germ nuclei at the diakinesis stage during oogenesis, and arrowheads indicate the distal end of each gonad. (E–H) Magnified images of the proximal regions of the gonads shown in A–D, respectively, double stained with OMA-2 antibody (red) and DAPI (green). Bar, 50 μ m.

Hermaphrodites of the *gld-3(ok308)* mutant are defective in spermatogenesis but produce mature oocytes. We constructed the *daz-1(tj3) gld-3(ok308)* double mutant. Seventy-three percent of the mutant hermaphrodites exhibited oocyte-like germ cells of the diakinesis stage (Figure 6D), which were rarely seen in the *daz-1(tj3)* single mutant (Figure 6C). A similar phenotype was observed in the *daz-1(tj3); gld-3(RNAi)* animals (our unpublished data). These oocyte-like cells accumulated OMA-2, a late-oogenesis marker that is required for oocyte maturation (Detwiler *et al.*, 2001), suggesting that they were closer to the mature oocytes (Figure 6H). This genetic interaction between *daz-1* and *gld-3* is consistent with the idea that DAZ-1 may up-regulate the FBF activity in the gametogenic pathway. One interpretation of this genetic interaction can be that the disruption of *gld-3* elevates the specific activity of FBF, which then compensates the decrease of FBF protein level caused by the *daz-1* mutation, because the level of FBF protein was lowered in the *daz-1 gld-3* double mutant as in the *daz-1* single mutant (Figure 5A). It should be noted, however, that this model needs further examination, because FBF, GLD-3, and DAZ-1 are all likely to have multiple downstream targets, and interactions among them can be complex. We also tested genetic interaction of *daz-1* with *gld-2*, whose protein product is proposed to promote meiosis with GLD-3 but rather independently of FBF (Wang *et al.*, 2002; Eckmann *et al.*, 2004). Two mutant alleles of *gld-2* examined (*q497* and *h292*) did not cause any recovery of oogenesis in the *daz-1* mutant (our unpublished data).

DISCUSSION

DAZ-1 in Germ Cell Sex Determination

We have demonstrated in this study that the *C. elegans daz-1* mutation can enhance the Mog phenotype of *fem-3(q20gf)*

significantly. This synthetic phenotype indicates that *daz-1* plays a role in the determination of germ cell sex. This role seems to be more prominent in a relative nematode, *C. briggsae*. Moreover, we have shown that the *C. elegans* DAZ-1 protein can bind to the 3' UTR sequences of *fbf* mRNAs and that the amount of FBF proteins in L4 larvae is decreased in the *daz-1* mutant. This reduction of FBF expression, although rather modest, seems to contribute to the sterility of the *daz-1* mutant, because an additional *gld-3* deletion, which should up-regulate FBF, could partially relieve the oogenic arrest in the *daz-1* mutant. The FBF proteins bind to the 3' UTR sequence of *fem-3* mRNA and negatively regulate the activity of *fem-3* posttranscriptionally to repress spermatogenesis. Thus, two contiguous steps of translational regulation, positive regulation of *fbf* mRNAs by DAZ-1 and negative regulation of *fem-3* mRNA by FBF, might ensure the faithful determination of germ cell sex.

Although our results are consistent with a hypothesis that the *daz-1* mutation causes up-regulation of *fem-3*, we have not yet shown that the activity of *fem-3* is indeed derepressed in the *daz-1* mutant. So far we have not succeeded either in producing antibodies specific enough to FEM-3 protein or in obtaining a transgenic line that expresses functional tagged FEM-3 protein. Another point to be noteworthy may be that, whereas the *gld-3(ok308)* mutation partially compensates the oogenic defect of the *daz-1* mutant, as shown in this study, a loss-of-function mutation in *fem-3*, *fem-3(e2006)* does not (Karashima *et al.*, 2000). This difference could be because DAZ-1 or FBF may have multiple downstream targets, as is often the case with RNA-binding proteins. A recent finding that the FBF proteins repress *fog-1* and *fog-3* in addition to *fem-3* seems consistent with this assumption (Thompson *et al.*, 2005). Efforts to identify additional targets of DAZ-1 and FBF will be required to answer this question.

Functional Conservation and Divergence of the Metazoan DAZ Family

It has been suggested that the principal molecular function of the DAZ family proteins is promotion of mRNA translation. *Drosophila* *Boule* has been proposed to translationally control *twine*, which encodes a meiosis-specific version of Cdc25 protein phosphatase. Mutants defective in *boule* or *twine* are both blocked in male meiosis, and overexpression of *twine* in the *boule* mutant can partially suppress the meiotic defect (Maines and Wasserman, 1999). Zebrafish *zDazl* up-regulates translation of its targets through their 3' UTR sequences (Maegawa *et al.*, 2002). Our hypothesis that *C. elegans* DAZ-1 binds to *fbf* mRNAs to up-regulate FBF expression seems to be in agreement with these previous observations.

Previous studies have revealed two aspects of gametogenesis in which the DAZ family proteins function, one during meiosis and the other before the commitment to meiosis. The former is the progression of meiosis under the regulation of a conserved cell cycle regulator Cdc25 protein phosphatase. *Drosophila* *boule* regulates *twine*, as mentioned above, and mouse *Dazl* binds to the mRNA for Cdc25C in vivo (Venables *et al.*, 2001). Our preliminary analysis, however, has shown that transcripts of none of the three *C. elegans* *cdc25* homologues (*cdc-25.1*, *cdc-25.2*, and *cdc-25.3*) (Ashcroft *et al.*, 1998) can be coimmunoprecipitated with DAZ-1 (our unpublished data). Thus, although *C. elegans* DAZ-1 clearly has a role in meiosis, it may be unrelated to *cdc25*.

The latter aspect is the maintenance and differentiation of PGCs. Depletion of *Xenopus* *Xdazl* mRNA causes a defect in the differentiation of primordial germ cells (Houston and King, 2000). *Dazl* knockout mice are sterile in both female and male, because they lack gametes due to a blockage in meiotic prophase I (Ruggiu *et al.*, 1997; Saunders *et al.*, 2003). As shown in this study, *C. elegans* DAZ-1 seems to act in the regulatory pathway that executes two critical decisions in germ cells: the mitosis/meiosis decision and the sperm/oocyte decision. It will be interesting to investigate whether this DAZ-1 function is homologous to any extent with the aforementioned functions of *Xenopus* and mouse DAZ family proteins.

DAZ Homologues in *Caenorhabditis* Nematodes

Several lines of phylogenetic analysis suggest that *C. briggsae* and *C. remanei* are more closely related to each other than to *C. elegans* (Fitch *et al.*, 1995; Haag and Kimble, 2000; Chen *et al.*, 2001; Rudel and Kimble, 2001). Given this assumption, the ancient DAZ protein in the *Caenorhabditis* nematode must have possessed a long C-terminal region, which has been lost in *C. briggsae* during evolution. It is of interest whether this change in the protein structure is related to the phenotypic difference seen between depletion of *daz-1* and *Cb-daz-1*. It is also presumable, however, that the apparent divergence of the roles of the DAZ family proteins may depend more on the cellular contexts in which they are situated than on their molecular functions, because a wide range of interspecies complementation is possible among them. *Xenopus* *Xdazl* and human *BOULE* can substitute *Drosophila* *boule*, and human DAZ and DAZL can rescue the *Dazl* knockout mouse to some extent (Houston *et al.*, 1998; Slee *et al.*, 1999; Vogel *et al.*, 2002; Xu *et al.*, 2003). Further analysis of the biochemical properties of *C. elegans* DAZ-1 may clarify these interesting questions.

ACKNOWLEDGMENTS

We thank Min-Ho Lee and Tim Schedl for kind advice on the RT-PCR methods and helpful discussion and Kaoru Saigo for helpful comments on protein alignment. Antibodies against OMA-2 were generous gifts from Rueyling Lin (UT Southwestern Medical Center, Dallas, TX). Some strains used in this work were provided by the *Caenorhabditis* Genetics Center funded by the National Institutes of Health National Center for Research Resources. This work was supported by a Grant-in-Aid for Specially Promoted Research from the Ministry of Education, Culture, Sports, Science and Technology (to M. Y.) and a Grant-in-Aid for Young Scientists (B) from the Japan Society for the Promotion of Science (to T. K.).

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