genesis 45:533–546 (2007)

ARTICLE

The DEAD Box RNA Helicase VBH-1 Is Required for Germ Cell Function in *C. elegans*

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Received 13 March 2007; Revised 13 June 2007; Accepted 20 June 2007

Summary: Vasa and Belle are conserved DEAD box RNA helicases required for germ cell function. Homologs of this group of proteins in several species, including mammals, are able to complement a mutation in yeast (DED1) suggesting that their function is highly conserved. It has been proposed that these proteins are required for mRNA translation regulation, but their specific mechanism of action is still unknown. Here we describe functions of VBH-1, a *C. elegans* protein closely related to Belle and Vasa. VBH-1 is expressed specifically in the *C. elegans* germline, where it is associated with P granules, the *C. elegans* germ plasm counterpart. *vbh-1(RNAi)* animals produce fewer offspring than wild type because of defects in oocyte and sperm production, and embryonic lethality. We also find that VBH-1 participates in the sperm/oocyte switch in the hermaphrodite gonad. We conclude that VBH-1 and its orthologs may perform conserved roles in fertility and development. genesis 45:533–546, 2007. © 2007 Wiley-Liss, Inc.

Key words: germline; DEAD box RNA helicase; sperm/oocyte switch

INTRODUCTION

Germ cells from many organisms possess specialized cytoplasm composed of electrondense granules that lack membrane and that contain mRNAs and proteins essential for germ line preservation and function. These granules are known as pole granules in *Drosophila melanogaster* and as P granules in *Caenorhabditis elegans*. Germline granules contain, among other proteins, several DEAD box RNA helicases that play important roles in germline development and function (Gruidl *et al.*, 1996; Johnstone *et al.*, 2005; Kuznicki *et al.*, 2000; Lasko and Ashburner, 1988; Navarro *et al.*, 2001). DEAD box RNA helicases belong to a conserved family of proteins, which unwind RNA and may facilitate RNA-protein interactions in an ATP-dependent manner (Andersen *et al.*, 2006; Linder, 2006). Many members of this family are involved in mRNA metabolism and processing including transcription, splicing, ribosome biogenesis, nuclear export, mRNA turnover, ribonucleoprotein-remodeling, and RNA interference (RNAi) (reviewed by Linder, 2006).

The *Drosophila* protein Vasa was one of the first germline DEAD box RNA helicases characterized (Hay *et al.*, 1988; Lasko and Ashburner, 1988). Vasa is expressed specifically in the germline, is conserved during evolution, is essential for *Drosophila*'s germline establishment during embryogenesis, and is indispensable for female fertility (Liang *et al.*, 1994; Schupbach and Wieschaus, 1986). Vasa interacts directly with translation initiation factor eIF5B, and this interaction is essential for its function (Johnstone and Lasko, 2004). Four DEAD box RNA helicases from *C. elegans*, known as GLH-1/-2/-3/-4, are closely related to Vasa and are redundantly essential for germ line development, and P granule structure (Gruidl *et al.*, 1996; Kawasaki *et al.*, 2004; Kuznicki *et al.*, 2000; Smith *et al.*, 2002). Vasa's mammalian homolog MVH is also germline-specific and essential for male fertility (Tanaka *et al.*, 2000). Germ cells from mice that do not express this protein fail to progress through

This article contains supplementary material available via the Internet at http://www.interscience.wiley.com/jpages/1526-954X/suppmat.

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Contract grant sponsors: NIH, UNAM-PAPIIT, CONACyT Mexico, L'OREAL/UNESCO and TWAS
Published online 14 September 2007 in Wiley InterScience (www.interscience.wiley.com).
DOI: 10.1002/dvg.20323

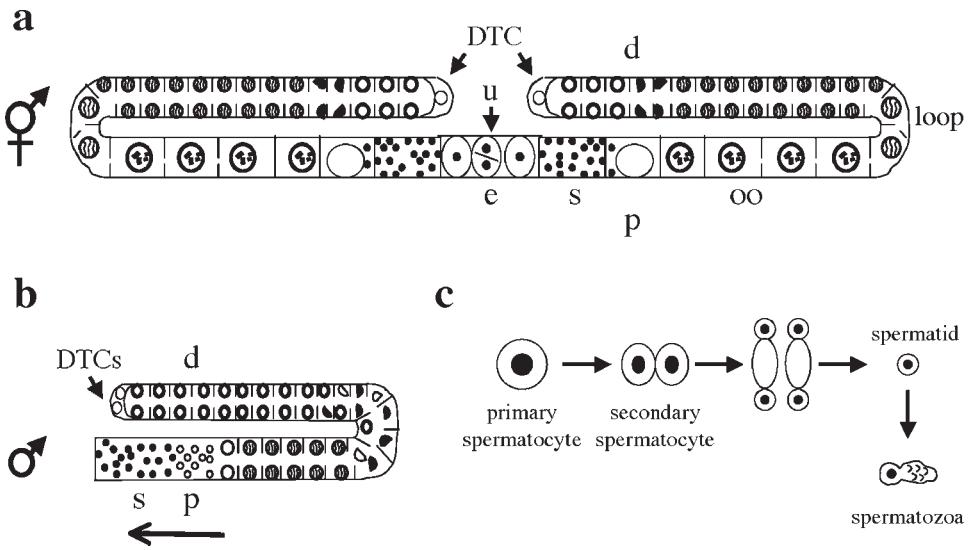


FIG. 1. Structure of the *C. elegans* hermaphrodite and male germline. (**a**) Diagram of the hermaphrodite gonad. The uterus connects two gonad arms. A somatic distal tip cell (DTC) maintains germ cell proliferation. During the L4 larval stage, the first 40 cells that enter meiosis become sperm that are stored in the spermatheca. Subsequently, only oocytes are formed. As germ cells move away from the DTC they enter meiotic prophase, thereafter germ cells progress from pachytene to diakinesis as they move proximally. Prior to fertilization, each oocyte undergoes maturation. Oocytes move through spermatheca, where they are fertilized and deposited in the uterus. (**b**) Diagram of the male gonad. The male gonad has a single gonad arm and produces sperm continuously. Two DTCs maintain germ cell proliferation. As germ cells approach the proximal gonad, they enter meiosis and sperm are formed. The arrow shows the direction in which spermatogenesis occurs. (**c**) Wild type spermatogenesis in *C. elegans*. Primary spermatocytes undergo two meiotic divisions to form secondary spermatocytes and finally spermatids. Spermatids bud away from a residual body, grow a pseudopod, and are activated to form mature spermatozoa. d = distal; p = proximal; oo = oocyte; s = spermatheca; u = uterus; e = embryos.

meiosis during spermatogenesis, and germ cells die by apoptosis.

Belle is a *Drosophila* protein that is closely related to Vasa. Belle plays an important role in female and male fertility, but is also essential for viability and growth (Johnstone *et al.*, 2005). The closest yeast-related protein to Belle is Ded1p. DED1 is an essential gene from *Saccharomyces cerevisae*, which participates in general translation initiation (Chuang *et al.*, 1997; de la Cruz *et al.*, 1997; Iost *et al.*, 1999). Two human DED1 homologs are known as DDX3Y (DBY) and DDX3X (DBX), which are located in the Y and X chromosomes, respectively. DDX3Y protein expression is restricted to the male germline, while DDX3X is expressed in all tissues. DDX3Y deletion leads to a significant reduction in germ cell number, and sometimes to a complete absence (Ditton *et al.*, 2004; Foresta *et al.*, 2000).

Although many DEAD box RNA helicases play important roles in the germline of several organisms, we still do not understand the mechanisms that these proteins use to accomplish their function. To understand the role of these proteins in the germline, we are using the nematode *C. elegans*, which is an excellent model organism for studying many biological processes. The *C. elegans* hermaphrodite gonad consists of two identical U-shaped tubes that are connected to the uterus (Fig. 1a). During the L4 larval stage, sperm is produced and stored in the spermatheca. In adulthood, only oocytes are produced (Hubbard and Greenstein, 2000; Schedl, 1997). Males, which only possess one gonad arm, start producing sperm in the L4 larval stage and continue throughout adult life (Fig. 1b).

Here we describe a *C. elegans* DEAD box RNA helicase that is closely related to Belle and also highly similar to Vasa, which we have called VBH-1 (Vasa and Belle-like helicase). VBH-1 is germline-specific and associates with P granules. Using RNA interference, we show that *vbh-1* is important for embryogenesis, spermatogenesis, and oocyte function. Half of the animals lacking VBH-1 are capable of producing few sperm while the other half are unable to initiate spermatogenesis. Fifty percent of *vbh-1(RNAi)* animals enter oogenesis earlier than wild type suggesting that VBH-1 participates in the sperm/oocyte switch in the *C. elegans* gonad.

RESULTS

VBH-1 Is a DEAD box RNA Helicase Similar to *Drosophila* Belle and Vasa

The best-characterized member of the DEAD box RNA helicase family is eIF4A, which functions to unwind mRNA to allow the beginning of translation (Linder, 2006). Looking for DEAD box RNA helicases that might have a specific function in the *C. elegans* germline, open reading frames Y54E10A.9 and Y71H2AM.19 were found to be similar to both the Vasa and the Belle subgroups of DEAD box RNA helicases (Fig. 2). A multiple sequence alignment was performed using protein sequences of the Vasa and Belle subgroups, and a phylogenetic tree

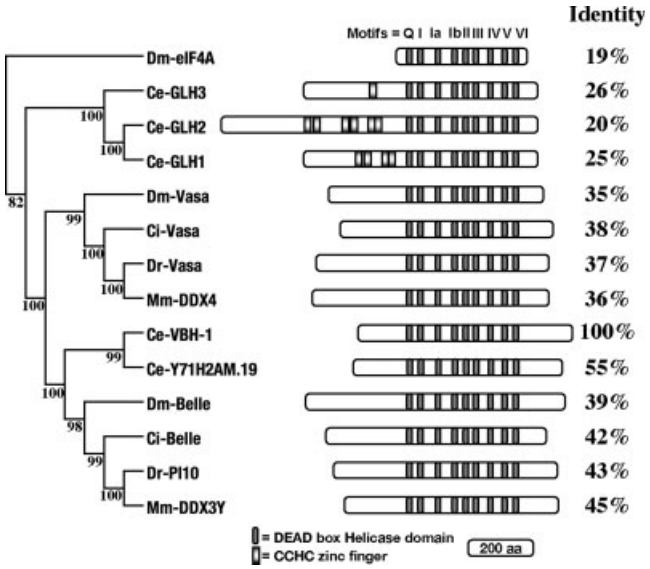


FIG. 2. VBH-1 is a DEAD box RNA helicase similar to the Vasa and Belle protein groups. A phylogenetic analysis and conserved domains in VBH-1 related proteins are shown. The DEAD box RNA helicase eIF4A (isoform A) from *Drosophila* was used as an outer group to construct the phylogenetic tree. Three main groups were formed in the tree; GLH-1/-2/-3 proteins from *C. elegans;* the Vasa, and the Belle groups, which also includes VBH-1 (Y54E10A.9). An identical tree was obtained when GLH-4 was included (not shown for simplicity). Numbers at the nodes are percentages of 1,000 bootstrap replicates in which that node was recovered. The schematic representation of the proteins (on the right side) shows the proportional size for each, and grey boxes indicate positions for the most conserved domains. Only the GLH-1/-2/-3 proteins have CCHC zinc finger domains. Percentage of identity was obtained comparing VBH-1 against each protein individually. Dm: *Drosophila melanogaster*; Ce: *C. elegans*; Ci: *Ciona intestinalis*; Dr: *Danio rerio* (zebrafish); Mm: *Mus musculus*.

was built using the translation initiation factor eIF4A as the outlying group. The GLH-1/-2/-3 proteins were included, which have been previously proposed to be Vasa homologs in *C. elegans* (Gruidl *et al.*, 1996; Roussell and Bennett, 1993; Smith *et al.*, 2002). Three main subgroups appeared in the tree (Fig. 2); a GLH-1/-2/-3 protein subgroup, a Vasa subgroup, and a Belle subgroup. Y54E10A.9 and Y71H2AM.19 proteins were clearly located in the Belle subgroup. An identical tree was obtained performing a protein alignment with the subregion encompassing motifs Q to VI from the same set of proteins (data not shown).

RNA interference of Y71H2AM.19 was found to result in pleiotropic defects and embryonic lethality (data not shown). In *Y71H2AM.19(RNAi)* animals, growth was arrested; some animals were unable to move properly, a few had a blistered cuticle, and some larval lethality was also observed. This phenotype had also been previously observed in a genome-wide RNAi screen (Sonnichsen *et al.*, 2005). *Y54E10A.9(RNAi)* animals showed reduced fertility and some embryo lethality, suggesting that this gene might play a role in the germline (see later). Because our interest was to study the function of these DEAD box RNA helicases in the germline, this study was focus exclusively on Y54E10A.9. Because of its similarity to Vasa and Belle proteins, Y54E10A.9 was named VBH-1 (Vasa, Belle-like helicase).

Although VBH-1 is very similar to the Belle subfamily several features distinguish it from the Belle subgroup members. VBH-1 lacks some of the conserved domains in the Belle subgroup that have been previously reported to be present in all members of this subfamily (Johnstone *et al.*, 2005). Glycine-rich domains are RNA binding sites typically present in the N-terminal or C-terminal regions from DEAD box RNA helicases. Whereas all Belle proteins have Glycine-rich domains in both the N-terminal and C-terminal regions, VBH-1 has only one Glycine-rich domain located near the C-terminus (data not shown). Vasa members also have only one Glycine-rich domain; however, it is at the N-terminus. These data suggests that VBH-1 is the *C. elegans* DEAD box RNA helicase most closely related to both Belle and Vasa.

VBH-1 Is Expressed Specifically in the Germline

The expression patterns of Vasa- and Belle-related proteins in other species predicted that VBH-1 would be expressed in the *C. elegans* germline, possibly specifically. To investigate whether the *vbh-1* mRNA is expressed in the germline, temperature sensitive mutants were analyzed in which production of oocytes, sperm, or both is impaired. At nonpermissive temperatures hermaphrodite animals from mutants *fem-1 (hc17)*, *fem-3(q20gf)*, and *glp-4(bn2)* have gonads that produce only oocytes, sperm or practically no germline, respectively (Barton *et al.*, 1987; Beanan and Strome, 1992; Kimble *et al.*, 1984). An abundant band of ~3.5 kb corresponding to the predicted *vbh-1* mRNA(s) was highly expressed in animals with feminized gonads (*fem-1*) (Fig. 3a). *vbh-1* was also detected at lower levels in wild type (N2) and masculinized (*fem-3*) animals (Fig. 3a). The *vbh-1* transcript was reduced significantly in *glp-4(bn2)* adult hermaphrodites, in which the germline is underproliferated (Fig. 3a) showing that *vbh-1* mRNA is germline enriched and is expressed during both spermatogenesis and oogenesis.

To determine the expression of VBH-1 protein, rabbit antisera was raised against a carboxyl-terminal VBH-1 peptide (see Materials and Methods). An affinity-purified antiserum identified several bands with molecular weights around 70 kDa on Western blot analysis (Fig. 3b,c). These bands were consistently absent in *vbh-1(RNAi)* animals indicating that the VBH-1 antibody is specific (Fig. 3c). Three alternative splicing forms have been reported for *vbh-1* (http://www.wormbase.org, release WS174, May 06, 2007), and their putative protein-predicted molecular weights are 72.6, 70.8, and 70.5 kDa. More than three protein bands were observed, which varied in their relative intensities among different protein extract preparations (Fig. 3b,c; data not shown) suggesting that VBH-1 is modified post-translationally. Western blot analysis using *glp-4(bn2)* mutant protein extracts (that possess virtually no germ line) showed

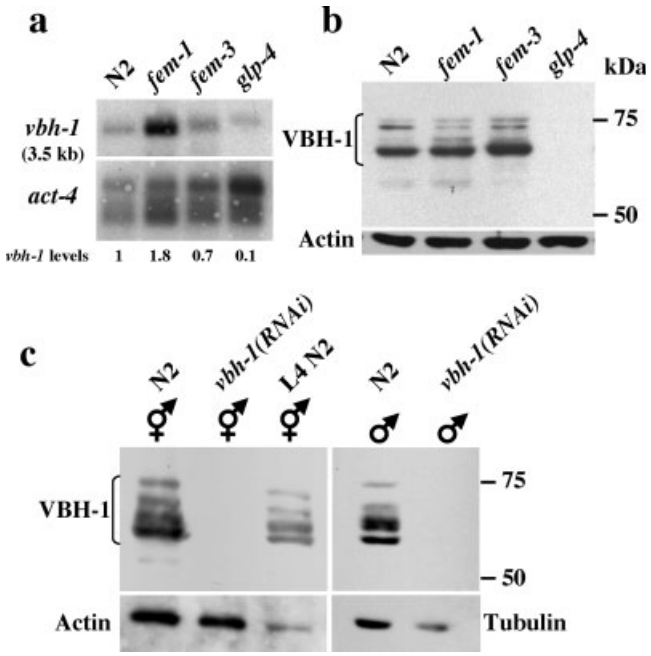


FIG. 3. *vbh-1* mRNA and protein expression. (**a**) Northern blot analysis using a *vbh-1* cDNA as a probe to test its expression in adult hermaphrodites from the following strains: N2 (wild type), *fem-1(hc17)* (produce only oocytes), *fem-3(q20gf)* (produce only sperm), and *glp-4(bn2)* (underproliferated germline). Expression levels were normalized to an actin gene and are indicated at the bottom. (**b**) Western blot from whole-animal protein extracts prepared from synchronized adult wild type, and indicated strains grown at restricted temperature. (**c**) Western blot from whole-animal protein extracts prepared from a population of 100 individuals of the indicated background. All blots were probed with rabbit VBH-1 antibodies. Either rabbit actin or mouse tubulin antibodies were used as loading control. Size markers are shown on the right.

that VBH-1 expression is germline-specific (Fig. 3b). In contrast, VBH-1 was abundant in *fem-1(hc17)* and *fem-3(q20gf)* animals that produce only oocytes or only sperm, respectively (Fig. 3b). Accumulation of any specific VBH-1 isoforms, in whole animal extracts from L4 hermaphrodites, feminized animals, masculinized animals or males, was not observed (Fig. 3b,c). These experiments suggest that the VBH-1 protein is germline-specific, and is expressed during both spermatogenesis and oogenesis.

VBH-1 Associates with P Granules

To determine the localization of the VBH-1 protein, full-length VBH-1 was fused with green fluorescence protein (GFP) under the control of the germline-specific *pie-1* promoter (Stitzel *et al.*, 2006). Transgenic animals were generated by biolistic transformation following a standard protocol (see Materials and Methods). Transgenic animals showed VBH-1:GFP localization in the cytoplasm of both somatic and germline blastomeres during embryogenesis (Fig. 4a–d) and in oocytes (Fig. 4i). VBH-1:GFP was also observed in foci within the germline and co-localized with the P granule component PGL-1 (Fig. 4e–l) (Kawasaki *et al.*, 1998). VBH-1 localization was not consistently observed in every P granule, particularly during oogenesis, and on occasion we observed that PGL-1 and VBH-1 overlap only partially (Fig. 4h,l). To corroborate these results, a specific VBH-1 antiserum was used in immunostaining assays. VBH-1 antibody staining showed the same localization in oocytes and embryos as observed with the VBH-1:GFP fusion (Fig. S1a, e, and i). To confirm VBH-1 antibody specificity, *vbh-1(RNAi)* gonads were stained, and showed an absence of protein expression (Fig. S2).

Extruded hermaphrodite and male gonads stained with the VBH-1 antibody showed high levels of VBH-1 expression (Fig. 5a–h). VBH-1 was observed throughout both the hermaphrodite and male gonad. In the male gonad, VBH-1 was observed in primary and secondary spermatocytes but not detected in mature sperm (Fig. 5g). VBH-1 was expressed in P granules throughout the gonad, and also diffusely in the cytoplasm. CGH-1, another DEAD box RNA helicase, is detected in P granules and also in cytoplasmic particles that may correspond to processing bodies (P bodies), structures in which many proteins involved in mRNA decay and translational control are concentrated (Boag *et al.*, 2005; Decker and Parker, 2006; Navarro and Blackwell, 2005; Navarro *et al.*, 2001). VBH-1, however, was not detected in the P-body-like particles (Fig. S1a–l).

VBH-1 Is Important for Fertility

To study VBH-1 function its expression was inhibited using RNAi by feeding following standard protocols (Timmons *et al.*, 2001). F1 animals grown in the presence of bacteria containing an empty plasmid (EP), as control, or full-length *vbh-1* dsRNA were studied. At 20°C, *vbh-1(RNAi)* hermaphrodites had reduced fertility (54.8% of offspring compared to wild type) (Table 1). At higher temperatures (24–26°C), fertility was further reduced in *vbh-1(RNAi)* animals, with only 12% as many viable offspring as wild type (Table 1). These results indicate that VBH-1 is important for hermaphrodite fertility.

Full-length *vbh-1* and Y71H2AM.19 cDNAs do not share regions of similarity that would be sufficient to trigger RNA cross-interference (Parrish *et al.*, 2000). However to exclude the possibility that *vbh-1* RNAi experiments might interfere with another DEAD box RNA helicase particularly Y71H2AM.19, the following control was performed. A ~400 bp cDNA fragment from the *vbh-1* region that showed the lowest homology with Y71H2AM.19, and was located outside of the conserved helicase domain was used to perform RNAi (Fig. S3). Full length VBH-1 and Y71H2AM.19 cDNAs shared 54% overall identity, but within this ~400 bp fragment their identity was only 45% (Fig. S3). RNAi performed using this *vbh-1* fragment showed the same phenotypes as the full-length cDNA, reduced fertility (60%), and some embryonic lethality (1.2%) (Table 1). In addition, *Y71H2AM.19(RNAi)* animals had a pleiotropic phenotype (data not shown and Sonnichsen *et al.*, 2005).

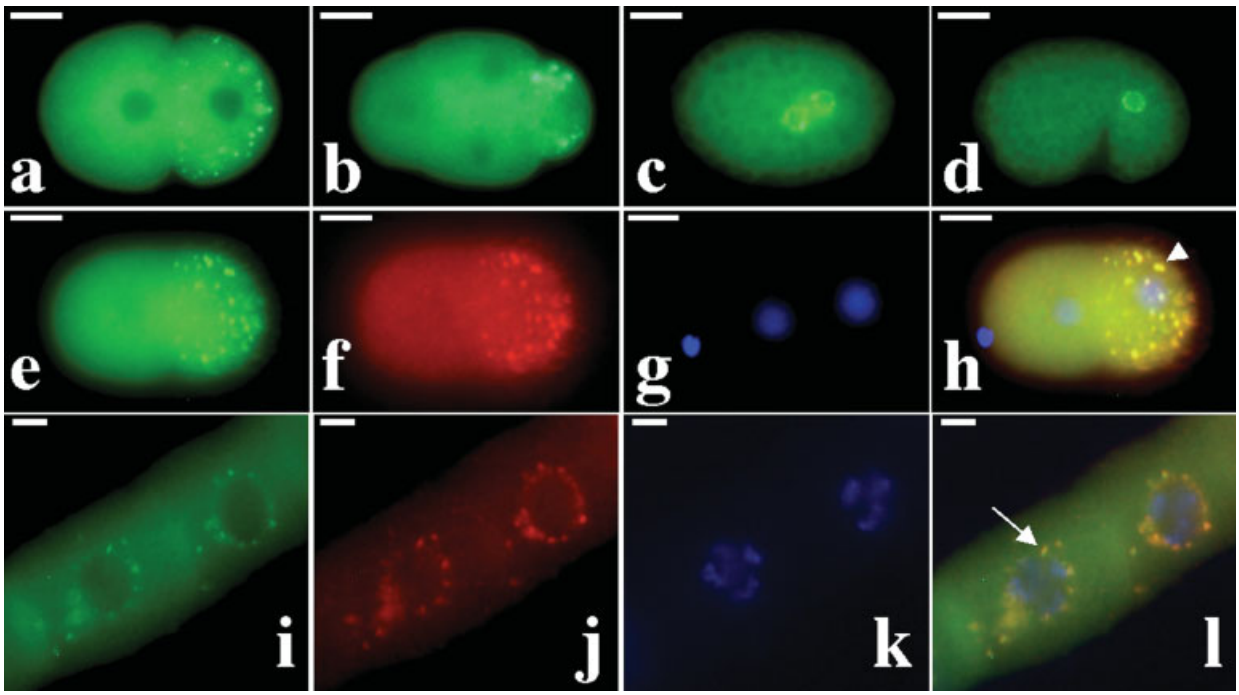
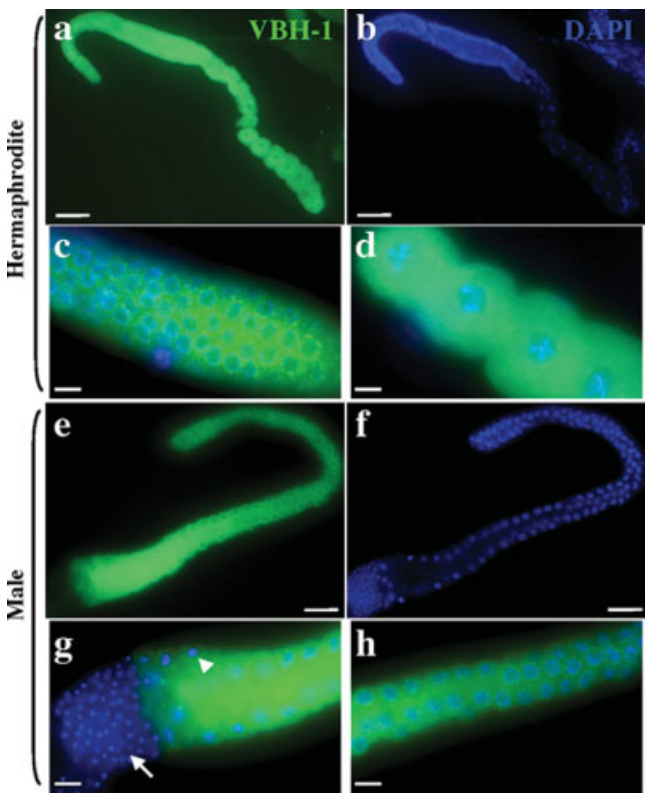


FIG. 4. VBH-1 protein localization in embryos and oocytes. Live embryos of 2-cell (**a**), 4-cell (**b**), ~100-cell (**c**) or 1.5-fold (**d**) stages expressing a GFP:VBH-1 fusion protein. GFP:VBH-1 localization in fixed late 1-cell embryo and oocytes (**e** and **i**) immunostained with PGL-1 antibody (**f** and **j**) and DAPI (**g** and **k**) and examined by fluorescence microscopy. (**h** and **l**) Merged images. VBH-1 and PGL-1 overlap in the majority of granules (yellow; arrowhead), but in some granules (particularly in oocytes), they overlap only partially (arrow). White bars represent 10 μm.



These data suggest that the RNA interference phenotype for *vbh-1* is specific to knocking down expression of this gene.

Using Nomarski microscopy many young *vbh-1(RNAi)* hermaphrodites were observed that had stacked oocytes in the proximal region of the gonad (6 and 41% at 20 and 25°C, respectively) (Table 2) (Fig. S4a,b). This phenotype is common in wild-type animals that have exhausted their sperm supply. Under these conditions, animals that are mated with wild-type males are able to resume oocyte maturation and fertility. To determine whether the reduced fertility and oocyte stacking seen in *vbh-1(RNAi)* animals derived from low sperm production, oocytes defects, or both, *vbh-*

FIG. 5. VBH-1 protein expression in adult hermaphrodite and male gonads. VBH-1 is expressed in the cytoplasm and P granules (perinuclear foci) throughout the hermaphrodite and male gonad. (**a–h**) Extruded gonads from 1-day-old wild type hermaphrodites (a–d) or males (e–h) were fixed and stained with affinity-purified rabbit VBH-1 antibody (a and e) and DAPI (b and f), then examined by fluorescence microscopy. (c and d) Details of merged images from the hermaphrodite gonad shown in different focal planes. (g and h) Details of merged images from the male gonad are shown in different focal planes. No VBH-1 staining was visible in the sperm (g). White arrow points toward mature sperm, and white arrowhead points toward primary spermatocytes. White bars represent 50 μm (a, b, e, and f) or 10 μm (c, d, g, and h).

Table 1
Reduced Fertility in vbh-1(RNAi) Hermaphrodites and Males

Genotype	Temperature	Total offspring	Embryo lethality	*n*
Hermaphodites				
N2 (EP)	20°C	100%	0	34
vbh-1(RNAi)	20°C	54.8%	2.1%	29
vbh-1(RNAi) (400 bp piece)	20°C	60%	1.2%	25
N2 (EP)	24°C	100%	0.1%	14
vbh-1(RNAi)	24°C	17.2%	1.6%	15
N2 (EP)	25°C	100%	0.1%	25
vbh-1(RNAi)	25°C	19.3%	5.3%	28
N2 (EP)	26°C	100%	2.1%	20
vbh-1(RNAi)	26°C	11.7%	23.3%	27
Hermaphrodites crossed with *him-8(e1489)* or wild type males				
N2(EP) × *him-8*	20°C	100%	0.1%	18
vbh-1(RNAi) × *him-8*	20°C	65.1%	1.2%	23
N2(EP) × N2	25°C	100%	0.1%	24
vbh-1(RNAi) × N2	25°C	35.7%	1.8%	23
fog-2(q71) hermaphrodites crossed with EP or *vbh-1(RNAi)* males				
fog-2(q71) × N2(EP)	25°C	100% (17/18)	0%	18
fog-2(q71) × *vbh-1(RNAi)*	25°C	52.5 (14/25)	1%	25

To study *vbh-1(RNAi)* animal fertility, hermaphrodites were individually selected as L4 specimens and then transferred to new plates every 24 h over the course of 3 days. Plates were scored for dead embryos and surviving offspring. Embryos not hatching within 24 h after being laid were considered dead. To test *vbh-1(RNAi)* hermaphrodite fertility, we crossed them with 3 *him-8(e1489)* or wild type males for 44 h and scored successful matings. Because males are not abundant within hermaphrodite progeny (1 every 300) in some experiments we used the *him-8(e1489)* strain, whose hermaphrodites produce 30% of male progeny. A successful mating was defined as production of male progeny. Progeny were scored as previously described. To test *vbh-1(RNAi)* male fertility, we crossed three animals with one *fog-2(q71)* hermaphrodite for 44 h. A successful mating was defined as production of embryos. Successful matings are indicated in parenthesis. EP = empty plasmid used as control.

Table 2
vbh-1(RNAi) Phenotype in Adult Animals

Genotype	Temperature	*n*	Wild type gonads	Gonads with stacked oocytes	Gonads with small germ cells
N2 (EP)	20°C	46	100%	0%	0%
vbh-1(RNAi)	20°C	78	88%	6%	6%
N2 (EP)	25°C	100	100%	0%	0%
vbh-1(RNAi)	25°C	137	21%	41%	38%

One-day old adults from the indicated background were anesthetized, mounted and observed using Nomarski microscopy to determine their phenotype. EP = empty plasmid used as control.

1(RNAi) hermaphrodites were mated with wild-type males. Mating increased the fertility of *vbh-1(RNAi)* hermaphrodites by only 16% compared to unmated animals (Table 1). Ovulation was restored in mated *vbh-1(RNAi)* animals, suggesting that the cause of oocyte accumulation was low sperm production (not shown). These animals continue producing fewer offspring than wild type, indicating that VBH-1 is necessary for both oocyte and sperm function (Table 1).

Besides stacking, no other oocyte defects were detected by Nomarski microscopy in *vbh-1(RNAi)* animals (Fig. 6a–d and S4b and c). Using DAPI staining some stacked *vbh-1(RNAi)* oocytes exhibited an Emo (endomitotic oocytes) phenotype (data not shown). An Emo phenotype is observed when unfertilized oocytes undergo multiple rounds of endomitotic DNA replication, resulting in aberrant polyploidy oocytes (Iwasaki *et al.*, 1996). This phenotype is characteristic of a defect in ovulation, as would be seen if sperm were absent or if the oocytes or sperm were not competent for fertilization. Emo oocytes were not observed in *vbh-1(RNAi)* animals that contained sperm or had been mated with wild type animals (data not shown), suggesting that the Emo phenotype observed in *vbh-1(RNAi)* animals is due to insufficient sperm production rather than oocyte or somatic defects.

VBH-1 is associated with P granule components, and is similar to Vasa and the GLH-1/-2/-3/-4 proteins, which are required for Polar or P granule structure, respectively (Breitwieser *et al.*, 1996; Kawasaki *et al.*, 1998). For this reason, the expression pattern of P granule components in *vbh-1(RNAi)* animals was studied to determine if their reduced fertility might derive from P granule abnormalities. No differences in the expression patterns of immunostained *vbh-1(RNAi)* hermaphrodite gonads with antibodies that recognize PGL-1 and GLH-1 were observed (Figs. S5 and S6). This suggested that VBH-1 is not required for P granule structure. Nevertheless, VBH-1 must play a role in oocyte function and/or development because a complete fertility rescue was not observed

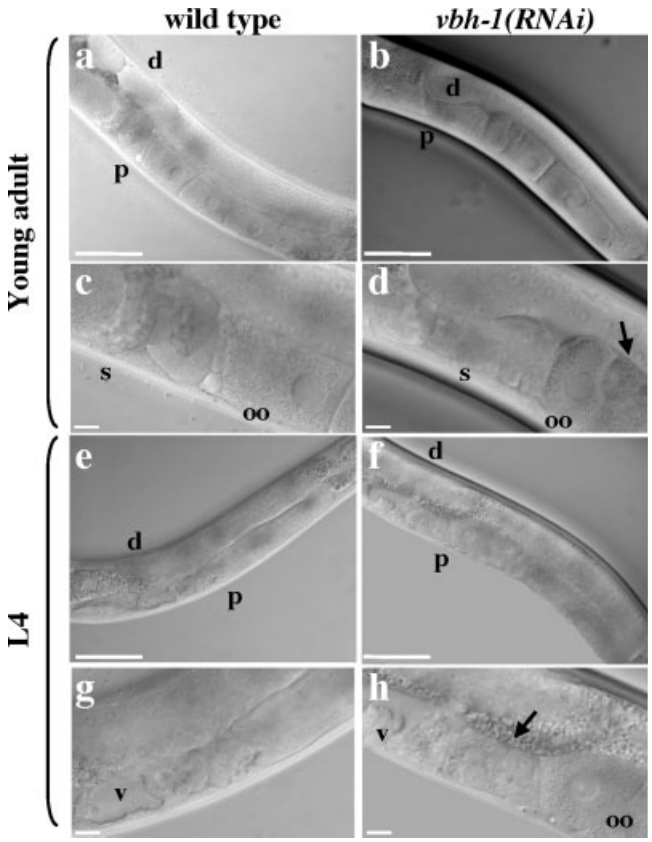


FIG. 6. *vbh-1(RNAi)* phenotypes observed by Nomarski microscopy. Young adult (**a–d**) and L4 larval stage (**e–h**) animals from wild type (empty plasmid as control) (a, c, e, and g) and *vbh-1(RNAi)* (b, d, f, and h) were grown at 25°C (c, d, g, and h). Details of gonads (a, b, e, and f; respectively) are shown. *vbh-1(RNAi)* animals had a defect in sperm production and the spermatheca is nearly empty (d) compared with the wild type (c). In panel d: A black arrow points toward a small germ cell, which presumably is a spermatocyte that did not complete spermatogenesis. (h) An oocyte in the gonad of an L4 animal can be observed, and several germ cells that may be spermatocytes that failed to progress through meiosis are shown (arrow) (note that the vulva is not yet developed, a characteristic of this stage). In wild type animals, oocytes are only produced during adulthood once spermatogenesis is completed. d = distal; p = proximal; s = spermatheca; oo = oocyte; v = vulva. White bars represent 50 μm (a, b, e, and f) or 10 μm (panels c, d, g, and h). Some pictures were modified using Photoshop CS (Adobe) to erase unused animals that appeared in the picture frame (f and h).

when *vbh-1(RNAi)* hermaphrodites were mated with wild type males (Table 1).

A low percentage of embryonic lethality was observed in *vbh-1(RNAi)* animals, (1.2 and 23.3% at 20 and 26°C) (Table 1). Embryonic lethality decreased after *vbh-1(RNAi)* hermaphrodites were mated with wild-type males. At 25°C, unmated animals generated 5.3% dead embryos; however, after mating this was reduced to 1.8% (Table 1). These results suggest that the embryonic lethality observed in *vbh-1(RNAi)* hermaphrodites is partially due to defects in sperm. However, *vbh-1* might play a role during embryogenesis because some embryonic lethality was still observed in the offspring of *vbh-1(RNAi)* hermaphrodites crossed with wild type males.

Table 3
vbh-1(RNAi) Hermaphrodites Produce Fewer Sperm Than Wild Type Animals

Genotype	Temperature	*n*	Gonads with no sperm	Gonads with sperm
N2 (EP)	25°C	20	0%	100% (132)
vbh-(RNAi)	25°C	15	53%	47% (56)
N2 (EP)	26°C	27	0%	100% (137)
vbh-1(RNAi)	26°C	43	49%	51% (84)

Gonads from young adult hermaphrodites of the indicated background were extruded, fixed and DAPI stained to visualized nuclei. Sperm was counted using fluorescence microscopy. The average spermatid number is indicated in parentheses. EP = empty plasmid used as control.

VBH-1 Is Important for Spermatogenesis

Fifty percent of *vbh-1(RNAi)* gonads lacked sperm or produced fewer sperm than wild type (50%), and some of these animals had small germ cells in the proximal gonad (6 and 38% at 20 and 23°C, respectively) (Fig. S4c and d and Fig. 6b–d) (Tables 2 and 3). As judged by size, these small germ cells appeared to be in the early stages of spermatogenesis, suggesting that a defect during this process could be the cause of low sperm production in these animals.

Under normal conditions the first 40 germ cells that enter meiosis differentiate into sperm during the L4 larval stage (Hubbard and Greenstein, 2000). Once sperm are formed, oogenesis begins and continues throughout adulthood (Fig. 1). To test whether any defects occur during spermatogenesis, *vbh-1(RNAi)* animals at L4 stage were studied. At 25°C, oocytes were already present in a small number of L4 *vbh-1(RNAi)* animals in whom spermatogenesis was not yet complete (4%) or in some cases, not even initiated (12%) (Table 4) (Fig. 6e–h). This phenotype was considerably more prominent at 26°C, at which nearly 50% of L4 animals had begun oogenesis without completed spermatogenesis (20%) or even initiated it (28%) (Table 4).

To confirm that oogenesis initiated prematurely in *vbh-1(RNAi)* animals gonads were stained with an oocyte marker, the yolk receptor RME-2. This protein is expressed from early oogenesis through oocyte maturation (Grant and Hirsh, 1999). Wild-type L4 hermaphrodites are not positive for RME-2 staining (Fig. 7a,b); however, L4 *vbh-1(RNAi)* gonads showed RME-2 staining even when no sperm were observed (Fig. 7c,d). Small germ cells observed in the proximal area of young adult *vbh-1(RNAi)* animals were not positive for RME-2 (Fig. 7e,g). These germ cells were positive for the sperm protein MSP, which is expressed in late stages of spermatogenesis and sperm, suggesting that these might be cells that failed to complete spermatogenesis (Fig. 7f). By DAPI staining, these germ cells appeared to be at early stages of meiosis (Fig. 7g). These data shows that VBH-1

Table 4
Production of Oocytes During the L4 Larval Stage in vbh-1(RNAi) Animals

Genotype	Temperature	*N*	Wild type gonads	Few sperm + oocytes	No sperm + oocytes
N2 (EP)	25°C	49	100%	0%	0%
vbh-1(RNAi)	25°C	70	84%	4%	12%
N2 (EP)	26°C	43	100%	0%	0%
vbh-1(RNAi)	26°C	29	52%	20%	28%

Gonads from L4 larval stage animals from the indicated backgrounds were dissected, fixed and stained using RME-2 antibody and DAPI to detect *vbh-1(RNAi)* gonads that have initiated oogenesis prematurely and have formed few or no sperm.

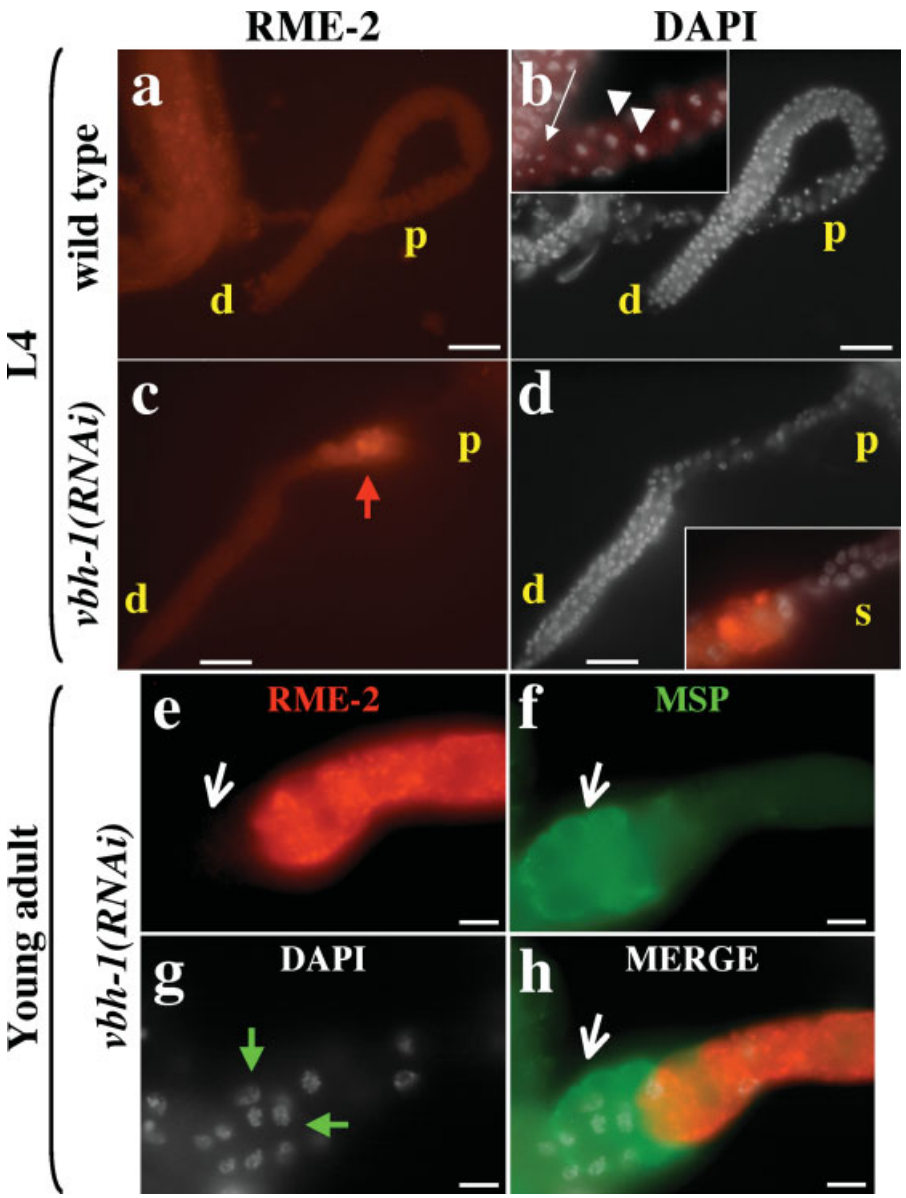


FIG. 7. Expression of the yolk receptor protein RME-2 and the sperm marker MSP in the gonad of wild type and *vbh-1(RNAi)* animals. Extruded gonads from wild type (empty plasmid as control) (**a** and **b**) and *vbh-1(RNAi)* (**c**–**h**) of L4 larva (a–d) or young adults (e–h) grown at 25°C were fixed and stained with a rabbit RME-2 antibody (a, c, and e), MSP antibody (f) or DAPI (b, d, and g). Red arrow (c) shows a group of germ cells that are positive for RME-2. RME-2 is expressed specifically in developing and mature oocytes. *vbh-1(RNAi)* hermaphrodites expressed RME-2 during the L4 larval stage (c and d), whereas wild type larvae did not (a and b). Panels b and d) Small boxes show details of merged images between RME-2 staining (red) and DAPI. The thin arrow points toward spermatids, arrowheads show spermatocytes. (h) Merged image between RME-2 (e), MSP (f) and DAPI staining (g). The white open arrow points toward a group of germ cells that are present between the spermatheca and oocytes, which are positive for the sperm marker MSP, and negative for the oocyte marker RME-2. Presumably these cells are spermatocytes that failed to progress through meiosis during spermatogenesis (green arrows in g). At 25°C, 11% ($n = 95$) of L4 *vbh-1(RNAi)* gonads were RME-2 positive, while at 26°C this phenotype increased to 37% ($n = 30$). d = distal; p = proximal; s = spermatheca. White bars represent 50 μM (a–d) or 10 μm (e–h).

is important during hermaphrodite development for the continued production of sperm, and for the inhibition of oogenesis, and that in its absence germ cells make the sperm/oocyte switch prematurely.

To test whether *vbh-1(RNAi)* low penetrance in germline functions might be due to functional redundancy between *vbh-1* and its closest homolog Y71H2AM.19 the following experiments were performed. To bypass the somatic defects seen with *Y71H2AM.19(RNAi)*, in which embryonic and larval development are abnormal, L1 larvae from the somatic RNAi resistant strain *rrf-1(ok589)* were used to perform feeding RNAi experi-

ments at 26°C (Miller *et al.*, 2003; Sijen *et al.*, 2001). *vbh-1(RNAi)* performed in *rrf-1(ok589)* showed the same phenotypes previously observed (data not shown). *rrf-1(ok589); Y71H2AM.19(RNAi)* animals were able to develop into adults that were completely sterile (data not shown) indicating that Y71H2AM.19 plays a role in germline function. Using Nomarski microscopy and RME-2 immunostaining of L4 larva, no premature sperm/oocyte switch was observed in these animals (data not shown). In contrast, an excess of sperm and small abnormal oocytes were observed in *Y71H2AM.19(RNAi)* gonads. Double RNAi in *vbh-1* and *Y71H2AM.19* produced completely sterile animals. A premature sperm/oocyte switch was observed in only 42% of these double RNAi animals (similar to *vbh-1(RNAi)* phenotype, Table 4), which also showed abnormal oocytes (data not shown). This premature sperm/oocyte switch appeared to compensate for excess sperm otherwise seen after *Y71H2AM.19(RNAi)*. These apparently opposing effects of *vbh-1* and *Y71H2AM.19* RNAi suggest that these genes are not functionally redundant.

To test whether VBH-1 plays a role in male spermatogenesis, *vbh-1(RNAi)* males were mated with *fog-2 (q71)* females. *fog-2(q71)* mutant animals are unable to specify male germ cells in hermaphrodites and therefore they produce only oocytes and become sterile; when mated with wild type or *fog-2* males, they are able to produce offspring (Schedl and Kimble, 1988). *fog-2(q71)* females mated with *vbh-1(RNAi)* males showed reduced fertility and produced only 52.5% as many viable offspring compared with wild-type males (Table 1). Also upon mating with *vbh-1(RNAi)* males, half of the *fog-2(q71)* animals failed to produce offspring (Table 1). These data suggest that VBH-1 is also important for spermatogenesis in males.

P granule structure was examined in the *vbh-1(RNAi)* male gonad and appeared normal, suggesting that aberrant P granules were not the cause of male infertility (Figs. S5 and S6). Mutants in genes that control the sperm/oocyte switch sometimes show a feminization of the soma (Schedl *et al.*, 1989). However, feminization of the *vbh-1(RNAi)* male germline was not observed under Nomarski microscopy or DAPI staining (not shown and Fig. 8b), suggesting that VBH-1 function might be restricted to the germline. By DAPI staining, *vbh-1(RNAi)* males produced normal numbers of spermatozoids, which did not present any evident defects (Fig. 8a–d). Late stages of spermatogenesis also appeared unaltered in *vbh-1(RNAi)* animals and their spermatozoids moved normally (Fig. 1c and Fig. 8e–l). Although *vbh-1(RNAi)* male gonads and sperm appeared normal, VBH-1 must play some role in male germline function because *vbh-1(RNAi)* males show reduced fertility.

DISCUSSION

Previous studies have shown that DEAD box RNA helicases, such as Vasa and Belle among others, are important for germline function in *Drosophila* and many other organisms (Hay *et al.*, 1988; Johnstone *et al.*, 2005; Lasko and Ashburner, 1988). Here we have shown that VBH-1, a DEAD box RNA helicase similar to both Vasa and Belle, is also required for germline function in *C. elegans*. VBH-1 is expressed specifically in the hermaphrodite and male germline. During early embryogenesis, VBH-1 is expressed in the cytoplasm of all blastomeres, but is also associated with P granules in germ cells. VBH-1 continues to be associated with P granules in the adult germline. Through RNAi studies, we found that VBH-1 is important for gamete formation and function, and embryogenesis. When maintained at 26°C, nearly 50% of *vbh-1(RNAi)* hermaphrodites were unable to produce sperm and in some cases the few germ cells that were able to enter spermatogenesis failed to progress through meiosis. Half of the *vbh-1(RNAi)* hermaphrodites made fewer sperm than wild type. *vbh-1(RNAi)* hermaphrodites also switched from spermatogenesis to oogenesis prematurely, suggesting that VBH-1 is important for the sperm/oocyte switch.

VBH-1 Is a Germline-specific DEAD box RNA Helicase

The *Drosophila* protein Vasa is a DEAD box RNA helicase that is conserved and expressed specifically in the germline of several organisms; therefore, it is commonly used as a germ cell marker. To date, the only known Vasa homologs in *C. elegans* have been the GLH-1/-2/-3/-4 proteins (Kuznicki *et al.*, 2000). These proteins are germline-specific and are necessary for gamete formation and important for maintaining P granule structure (Gruidl *et al.*, 1996; Kawasaki *et al.*, 1998; Kuznicki *et al.*, 2000; Smith *et al.*, 2002). It was confirmed by means of phylogenetic analysis that the GLH-1/-2/-3/-4 proteins are closely related with *Drosophila* Vasa. However, this group of proteins has a longer amino-terminus, and they also possess several CCHC zinc finger domains that are not present in Vasa proteins from other species (Kuznicki *et al.*, 2000).

Here we show that while *C. elegans* VBH-1 is closely related with Vasa (35% identity), it is more similar to Belle (39% identity). Despite their similarity, VBH-1 and Belle differ in some aspects: (1) VBH-1 lacks some putative domains that were previously described in the Belle group (Johnstone *et al.*, 2005), including a leucine-rich nuclear export signal that is functional in the An3 protein from *Xenopus* (Askjaer *et al.*, 1999), and a second domain of unknown function; (2) Belle has Glycine-rich regions in its amino- and carboxy-terminal regions, whereas VBH-1 only possesses one glycine-rich region near its carboxy-terminus. Glycine-rich domains may represent RNA binding sites, but also mediate protein-protein interactions (Cartegni *et al.*, 1996).

A second protein closely related to VBH-1 is present in *C. elegans* (Y71H2AM.19). RNAi studies have shown that lack of Y71H2AM.19 produce a pleiotropic effect and an embryonic lethal phenotype (data not shown and Sonnichsen *et al.*, 2005). This suggests that the func-

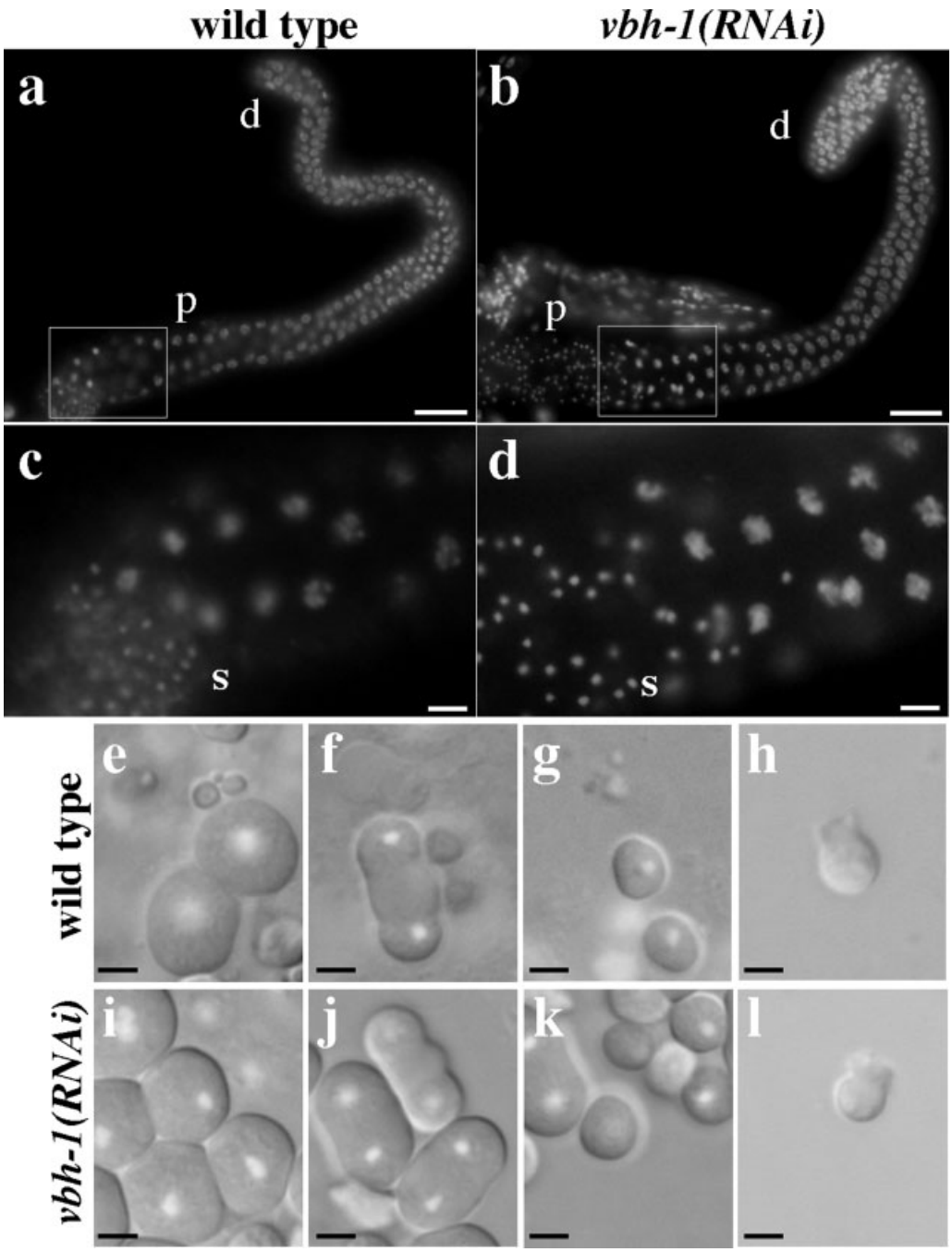


FIG. 8. Germ cell nuclear morphology in wild type and *vbh-1(RNAi)* males gonads. Extruded gonads from wild type (empty plasmid as control) (**a** and **c**) and *vbh-1(RNAi)* adult males (panels **b** and **d**) grown at 25°C were fixed and stained with DAPI. Panels c and d: A detail of the proximal gonad is shown. (**e–l**) Germ cells undergoing spermatogenesis were obtained from wild type (e–h) and *vbh-1(RNAi)* males (i–l), and stained with DAPI. Merged images from Nomarski and fluorescence microscopy are shown. (e and i) primary spermatocytes; (f and j) secondary spermatocytes, some of which were budding from the residual body; (g and k) spermatids; (h and l) spermatocytes that were activated using pronase following standard protocols. No evident nuclear defects are observed in *vbh-1(RNAi)* male gonads or during spermatogenesis. d = distal; p = proximal; s = spermatids. Scale bars represent 50 μm (a–d) or 10 μm (e–l).

tions of Y71H2AM.19, in contrast to VBH-1, might not be restricted to the germline. Although Y71H2AM.19 is also required in the germline, it was also observed that is not functionally redundant with *vbh-1*.

VBH-1 Plays a Role in the Germline

Our RNAi experiments indicated that *vbh-1* is important for gamete function and embryogenesis. *vbh-1(RNAi)* dead embryos did not arrest at a particular

stage, but were able to develop until late stages of embryogenesis (1.5- through 3-fold stage) (not shown). One of the causes of this embryonic lethality was defective sperm, because embryo viability slightly increased when *vbh-1(RNAi)* hermaphrodites were crossed with wild type males. In addition, *fog-2(q71)* hermaphrodites crossed with *vbh-1(RNAi)* males showed 1% embryo lethality. Embryonic lethality due to defective sperm has been previously observed in a *C. elegans* mutant in gene *puf-8*, which encodes a Pumilio homolog (Subramaniam and Seydoux, 2003). Although embryonic lethality was slightly restored when *vbh-1(RNAi)* hermaphrodites were crossed with wild type males, a small percentage of dead embryos was still observed (1.8%) suggesting that VBH-1 may also play a role during embryogenesis.

The strongest *vbh-1(RNAi)* phenotype was observed at high temperatures (25–26°C), as is seen with many germline genes (Gruidl *et al.*, 1996; Kawasaki *et al.*, 2004). The GLH-1/-2/-3/-4 proteins from *C. elegans* and Vasa from *Drosophila* are required to maintain P granule and polar granule structure, respectively (Breitwieser *et al.*, 1996; Kawasaki *et al.*, 1998). Although VBH-1 is very similar to these proteins, it is not required for the adult germline P granule structure. *vbh-1(RNAi)* oocytes also did not show an evident defect under Nomarski microscopy or DAPI staining, and no differences in physiological germ cell apoptosis were observed in *vbh-1(RNAi)* animals (data not shown). Despite these observations, VBH-1 must play a role in oocyte function because full recovery of *vbh-1(RNAi)* offspring production after mating *vbh-1(RNAi)* animals with wild-type males was not observed. In *Drosophila vasa* and *belle* null mutants, oogenesis initially proceeds normally, but later oocytes arrest and egg chambers degenerate, suggesting that these proteins might have related functions in the female germline (Johnstone *et al.*, 2005; Styhler *et al.*, 1998).

VBH-1 Has a Role in Spermatogenesis

Nearly half of *vbh-1(RNAi)* hermaphrodites were unable to form sperm while the remainder produced fewer sperm than wild type. Some *vbh-1(RNAi)* animals also presented products of early stages of spermatogenesis in the proximal gonad, suggesting that VBH-1 is important for completing spermatogenesis. Interestingly, although VBH-1 was not detected in the nucleus, this protein was recently isolated in a screen for proteins that associated with germ cell chromatin during spermatogenesis (Chu *et al.*, 2006), suggesting that VBH-1 might play a role during this process.

belle mutants are male sterile and their germ cells might have defects in chromosome segregation and cytokinesis (Johnstone *et al.*, 2005). Other members of the Belle family also play important roles in spermatogenesis. In humans, lack of DDX3Y results in sterility due to low sperm production and in some cases a complete absence of sperm has been observed (Ditton *et al.*, 2004; Foresta *et al.*, 2000). In *Drosophila*, Vasa is not required for spermatogenesis; nonetheless, MVH, the mouse Vasa homolog, is required for spermatogenesis (Tanaka *et al.*, 2000). Males homozygous for a mutation of Mvh produce no sperm; pre-meiotic germ cells cease differentiation before the zygotene stage and undergo apoptosis. These observations suggest that VBH-1 and its homologs are important for male germline function.

VBH-1 Is Important to Prevent an Early Switch to Oogenesis

Almost 50% of *vbh-1(RNAi)* hermaphrodites initiated oogenesis prematurely. The pathway that regulates the sperm/oocyte switch is well established in *C. elegans* (Ellis and Schedl, 2006; Kuwabara and Perry, 2001). One of the mechanisms that controls the sperm/oocyte switch is regulated at the mRNA level (Ellis and Schedl, 2006; Kuwabara and Perry, 2001; Puoti *et al.*, 2001). *tra-2* mRNA must be negatively regulated to allow hermaphrodite spermatogenesis; subsequently, *fem-3* mRNA is also negatively regulated to permit the switch to oogenesis (Goodwin *et al.*, 1993; Zhang *et al.*, 1997). The Belle homolog in yeast, Ded1p, is an essential protein that has been implicated in regulation of general translation initiation (Chuang *et al.*, 1997; de la Cruz *et al.*, 1997). Vasa interacts directly with translation factor eIF5B/dIF2 to promote mRNA translation, and this interaction is essential for many of its developmental functions and especially for accumulation of Gurken protein (Carrera *et al.*, 2000; Johnstone and Lasko, 2004). It has been shown that a proline residue within the carboxyl-terminal domain of Vasa is required to interact with eIF5B (Johnstone and Lasko, 2004). This proline is present in VBH-1 (not shown), consistent with the idea that it might be involved in regulating translation of a factor that controls the sperm/oocyte switch.

In summary, VBH-1 is expressed specifically in the *C. elegans* germline, is important for embryogenesis, gamete function and to prevent an early sperm/oocyte switch in the hermaphrodite gonad. VBH-1 also belongs to a conserved group of proteins whose role is important for germline function in several organisms.

MATERIALS AND METHODS

Strains

Maintenance and genetic manipulation of *C. elegans* were carried out according to standard procedures. *C. elegans* variety Bristol, strain N2, was used as the wild type. The following strains have been described previously: *fem-1(hc17)*, *fem-3(q20gf)*, *glp-4(bn2)*, *him-8(e1489)*, *fog-2(q71)* (Riddle *et al.*, 1997, or in the references therein), and *rrf-1(ok589)* (Sijen *et al.*, 2001).

Sequence Comparison

To build the phylogenetic tree, a multiple sequence protein alignment was made using the clustalw program and a Blosum 30 matrix (Henikoff and Henikoff, 1992). The resulting alignment was used to build the phyloge-

netic tree with the MacVector 7.2.3 software (Accelrycs Inc. CA) using the "neighbor-joining" method with uncorrected p-distance and random tie breaking. The tree's confidence was tested using bootstrapping analysis (1,000 replicates). As an outer group the eIF4A protein, isoform A from *Drosophila* was used (NP_723137). The GLH-1/-2/-3 proteins from *C. elegans* were also included (NP_491963, NP_491876 and NP_491681, respectively). The Vasa proteins from *Drosophila* (NP_723899); *Ciona intestinalis* (Ensembl ENSCINP00000028907); Zebrafish (NP_571132), and Mouse (Ensembl ENSMUSP00000096769). The VBH-1 protein from *C. elegans* isoform c (AAU20831), which is the largest, was used for the analysis. The *C. elegans* protein Y71H2AM.19 (NP_497615). The Belle proteins from *Drosophila* (NP_536783); *Ciona intestinalis* (Ensembl ENSCINP00000015656); Zebrafish (NP_571016), and Mouse (Ensembl ENSMUSP00000088729).

Northern Blot Analysis

To prepare RNA, the *fem-1(hc17)*, *fem-3(q20gf)*, *glp-4(bn2),* and N2 strains were grown at 15°C for 5 days. Embryos were extracted by bleaching and synchronized following standard procedures; then, after 3 days at 25°C the animals were harvested as adults. Poly(A)$^+$ RNA was prepared from total RNA using Oligotex mRNA kit (Qiagen). Poly(A)$^+$ RNA was fractionated in a formaldehyde gel, blotted onto Hybond-N membranes (Amersham), and then hybridized with ^{32}P-labeled probes. A full-length *vbh-1* cDNA probe (1.9 kb) was isolated digesting plasmid pSZ2 with EcoRI (see below). As loading control, a probe of a ~0.9 kb fragment of actin cDNA was made (gene *act-4*; wormbase ID number: M03F4.2) digesting a clone from the *C. elegans* ORF-RNAi library (Open Biosystems) with BglII and EcoRV. Expression levels were compared by phosphoimaging (Molecular Dynamics).

Transgene Construction

Gateway cloning (Invitrogen) was used to generate a VBH-1:GFP fusion protein. Full-length VBH-1 isoform B coding sequence was PCR-amplified from cDNA (yk624f9) and cloned into pDONR201. This construct was recombined into pID3.01B (for an N-terminal GFP fusion protein) (Stitzel *et al.*, 2006). The resulting plasmid contained the *pie-1* promoter, a fusion between GFP and *vbh-1* and the *pie-1* 3'UTR region. This construct was introduced into *C. elegans* by means of biolistic transformation (Praitis *et al.*, 2001).

Antibody Production and Immunofluorescent Staining

Two polyclonal rabbit antisera were raised (Cocalico Biologicals) against a peptide corresponding to VBH-1 isoform B residues 581–616 plus an amino terminal cysteine (NH_2-CSSLSSTSNSGGGGGFSGPRRGGFNSGMNR-COOH). These antisera (1 and 2) stained gonads similarly, and the corresponding preimmune sera each detected only a light diffuse background (not shown). No staining of either gonads or embryos was observed after affinity purified VBH-1 antisera had been depleted of specific reactivity by VBH-1 peptide (Sulfolink, Pierce). Affinity purified sera 1 and 2 were indistinguishable in their staining (not shown).

Staining with VBH-1 antibody was performed as described (Navarro *et al.*, 2001) but with fixing gonads or embryos for 20 min. Briefly, extruded gonads were frozen in liquid nitrogen and freeze-cracked, then fixed in 100% methanol at −20°C for 1 min, followed by treatment with 1× PBS, 0.08 M Hepes (pH 6.9), 1.6 mM $MgSO_4$, 0.8 mM EGTA, 3.7% paraformaldehyde for 20 min at room temperature. After washing and blocking, samples were incubated with a 1:1,000 dilution of an affinity purified rabbit VBH-1 antiserum. Antibody dilutions for reagents used in this work are described in parenthesis and methods for immunostaining are described in the following references: PGL-1 (1:10,000) (Kawasaki *et al.*, 1998), GLH-1 (1:1,000) (Kawasaki *et al.*, 2004), CGH-1 (1:100) (Navarro *et al.*, 2001), RME-2 (1:50) (Grant and Hirsh, 1999) and MSP (1:200) (Developmental Studies Hybridoma Bank) (Rose *et al.*, 1997). The following secondary antibodies were used; an alexa fluor 488 goat anti-rabbit IgG (H+L) at 1:50 (Molecular Probes); a Cy3-conjugated affinity purified goat anti-rabbit IgG (H+L) at 1:100 (Jackson); a Cy3-conjugated affinity purified donkey anti-rat IgG (H+L) (Jackson) at 1:100, and a fluorescein (FITC)-conjugated affinity purified goat anti-mouse IgG (H+L) (Jackson) at 1:100. A 1 ng/μl final concentration of DAPI was added during the final antibody incubation for DNA visualization. Images were obtained on a Nikon Eclipse E600 microscope equipped with an AxioCam digital camera (Zeiss) and were subsequently, processed in Photoshop CS (Adobe).

Western Blot Analysis

Total protein was isolated from the *fem-1(hc17)*, *fem-3(q20gf)*, *glp-4(bn2)* and N2 strains grown under restricted conditions (see the Northern blot analysis section). Adult animals were collected and washed four times with PBS buffer and finally resuspended in 0.5% TNTE buffer (50 mM Tris, 150 mM NaCl, 0.5% Triton, and 1 mM EDTA in the presence of the following protease and phosphatase inhibitors: 25 mM NaF; 1 mM NaPPi; 1 mM $NaVO_4$; 1 mM PMSF; 0.1 mg/ml trypsin inhibitor; 0.1 mg/ml pepstatin A; 0.1 mg/ml leupeptin; 0.1 mg/ml antipain; 0.1 mg/ml benzamidine, and 0.1 mg/ml beta-glicerophosfate). Animals were immediately frozen or sonicated in an UltraSonic Processor (Cole Parmer) to extract protein. Proteins were fractionated on a 7.5% polyacrylamide gel following standard procedures. Gel was blotted onto Immobilion-P transfer membranes (Millipore), and standard Western blot procedures were used. Affinity purified VBH-1 antibody was diluted 1:1,000 and incubated over night. A rabbit or mouse secondary antibody from Supersignal West Dura kit (Pierce) was utilized and reaction was revealed fol-

lowing manufacturer instructions. For Western blot analysis of *vbh-1(RNAi)* animals and its respective control, 100 adult animals grown at 25°C were collected and washed in PBS; PBS was later exchanged for loading buffer. To extract protein, samples were boiled for 5 min, spun for 1 min at 13,000 rpm, and supernatant was loaded into 7.5% polyacrylamide gels, this followed by Western blotting. As loading control, commercial antibodies against α-tubulin (1:500) (Sigma; T9026) or actin (1:500) (Santa Cruz Biotechnologies; sc-10731) were used.

RNAi Studies

To study *vbh-1* function, RNA interference by feeding (RNAi) was used (Timmons *et al.*, 2001). To clone *vbh-1* into the feeding vector (pPD129.36), the following primers were utilized: (1) 5′-GCGAATTCATGAACACACAATATTATGC-3′ and (2) 5′–CGGAATTCTTAAGCTTGTGGAGCTTGC-3′. The full-length *vbh-1* isoform b containing 1,922 base pairs of *vbh-1* was isolated from the *C. elegans* cDNA clone yk624f9 (courtesy of Dr. Yuji Kohara) by PCR. The *vbh-1* product was digested with EcoRI, cloned into the feeding vector, and the resulting plasmid was transformed into the *Escherichia coli* strain HT115 (pSZ2). A fragment containing the first ∼400 base pairs from *vbh-1* was also isolated from clone yk624f9 by PCR and cloned into the feeding vector as previously described (pSZ1). *vbh-1* fragment was clone using primer 1 (see above) and primer 3) 5′-CGGAATTCTCTCCATCACAGCCGGCCC-3′.

To make RNAi in gene Y71H2AM.19, a fragment of ∼1,600 bp was isolated by PCR using genomic DNA. This fragment was digested with enzymes SpeI and XhoI and cloned into the feeding vector. This fragment represents the first 400 bp of the Y71H2AM.19 coding region and is shown in Figure S3. Primers used were: (1) 5′-GACTAGTATGGAAAGTAACCAATCG-3′ and (2) 5′-CCGCTCGAGGGAAAGCTGTCCGGAG-3′.

To induce production of double stranded RNA in feeding bacteria, standard procedures were followed (Timmons *et al.*, 2001). Briefly, bacterial cultures were allowed to grow to optical density (OD) 0.6 at 37°C; after reaching this density, 0.4 mM IPTG was added to the cultures and incubation continued for 2 h at the same temperature. Bacteria were collected and resuspended (1 ml for each 10 ml of original culture) in LB containing antibiotics (50 μg/ml ampicillin and 12.5 μg/ml tetracycline), 0.4 mM IPTG, and 25% glycerol. One milliliter aliquots were immediately frozen in liquid nitrogen and stored at −70°C. When needed for experiments, aliquots were thawed, centrifuged, and the pellets were resuspended in 150 μl LB containing antibiotics and IPTG. Fifty or 10 ml of bacteria were seeded onto each 60- or 30-mm plate of NGM containing antibiotics, respectively. Plates were stored at 4°C for up to 1 week. To perform RNAi in two genes at the same time, 25 μl of each induced bacteria were mix, and seeded onto 60-mm plate of NGM containing antibiotics.

Spermatid Activation Assay

For spermatid activation, male gonads were dissected to liberate sperm in a sperm-medium salt buffer containing 200 μg/ml of pronase (SIGMA) and immediately observed under the microscope for pseudopod formation, which occurs within minutes (L'Hernault and Roberts, 1995).

ACKNOWLEDGMENTS

We are grateful to Sergio Zonszein and Marta Castro-Manrreza for technical support, and Dr. Margaret Brunner for editing this manuscript. We thank Dr. Geraldine Seydoux for reagents, advice, and critically reading the manuscript. We are grateful to Dr. Jennifer Schisa for kindly editing and critically reading the manuscript. We also would like to thank Yuji Kohara, Susan Strome, Caroline Spike, and Barth Grant for kindly providing reagents. Strains were provided by the *C. elegans Genetics Center*, which is supported by NIH's National Center for Genetics Resources. The MSP monoclonal antibody developed by David Greenstein was obtained from the Developmental Studies Hybridoma Bank.

LITERATURE CITED

Andersen CB, Ballut L, Johansen JS, Chamieh H, Nielsen KH, Oliveira CL, Pedersen JS, Seraphin B, Le Hir H, Andersen GR. 2006. Structure of the exon junction core complex with a trapped DEAD-box ATPase bound to RNA. Science 313:1968–1972.

Askjaer P, Bachi A, Wilm M, Bischoff FR, Weeks DL, Ogniewski V, Ohno M, Niehrs C, Kjems J, Mattaj IW, Fornerod M. 1999. RanGTP-regulated interactions of CRM1 with nucleoporins and a shuttling DEAD-box helicase. Mol Cell Biol 19:6276–6285.

Barton MK, Schedl TB, Kimble J. 1987. Gain-of-function mutations of fem-3, a sex-determination gene in *Caenorhabditis elegans*. Genetics 115:107–119.

Beanan MJ, Strome S. 1992. Characterization of a germ-line proliferation mutation in *C. elegans*. Development 116:755–766.

Boag PR, Nakamura A, Blackwell TK. 2005. A conserved RNA-protein complex component involved in physiological germline apoptosis regulation in *C. elegans*. Development 132:4975–4986.

Breitwieser W, Markussen FH, Horstmann H, Ephrussi A. 1996. Oskar protein interaction with vasa represents an essential step in polar granule assembly. Genes Dev 10:2179–2188.

Carrera P, Johnstone O, Nakamura A, Casanova J, Jackle H, Lasko P. 2000. Vasa mediates translation through interaction with a Drosophila yIF2 homolog. Mol Cell 5:181–187.

Cartegni L, Maconi M, Morandi E, Cobianchi F, Riva S, Biamonti G. 1996. hnRNP A1 selectively interacts through its Gly-rich domain with different RNA-binding proteins. J Mol Biol 259:337–348.

Chu DS, Liu H, Nix P, Wu TF, Ralston EJ, Yates JR, III, Meyer BJ. 2006. Sperm chromatin proteomics identifies evolutionarily conserved fertility factors. Nature 443:101–105.

Chuang RY, Weaver PL, Liu Z, Chang TH. 1997. Requirement of the DEAD-box protein ded1p for messenger RNA translation. Science 275:1468–1471.

de la Cruz J, Iost I, Kressler D, Linder P. 1997. The p20 and Ded1 proteins have antagonistic roles in eIF4E-dependent translation in *Saccharomyces cerevisiae*. Proc Natl Acad Sci USA 94:5201–5206.

Decker CJ, Parker R. 2006. CAR-1 and trailer hitch: Driving mRNP granule function at the ER? J Cell Biol 173:159–163.

Ditton HJ, Zimmer J, Kamp C, Rajpert-De Meyts E, Vogt PH. 2004. The AZFa gene DBY (DDX3Y) is widely transcribed but the protein is limited to the male germ cells by translation control. Hum Mol Genet 13:2333–2341.

Ellis R, Schedl T. 2006. Sex-determination in the germline. In: The *C. elegans* Research Community, editor. WormBook. p doi/10,1895/wormbook.1891,1821, http:/www.wormbook.org.

Foresta C, Ferlin A, Moro E. 2000. Deletion and expression analysis of AZFa genes on the human Y chromosome revealed a major role for DBY in male infertility. Hum Mol Genet 9:1161–1169.

Goodwin EB, Okkema PG, Evans TC, Kimble J. 1993. Translational regulation of tra-2 by its 3′ untranslated region controls sexual identity in *C. elegans*. Cell 75:329–339.

Grant B, Hirsh D. 1999. Receptor-mediated endocytosis in the *Caenorhabditis elegans* oocyte. Mol Biol Cell 10:4311–4326.

Gruidl ME, Smith PA, Kuznicki KA, McCrone JS, Kirchner J, Roussell DL, Strome S, Bennett KL. 1996. Multiple potential germ-line helicases are components of the germ-line-specific P granules of *Caenorhabditis elegans*. Proc Natl Acad Sci USA 93:13837–13842.

Hay B, Jan LY, Jan YN. 1988. A protein component of Drosophila polar granules is encoded by vasa and has extensive sequence similarity to ATP-dependent helicases. Cell 55:577–587.

Henikoff S, Henikoff JG. 1992. Amino acid substitution matrices from protein blocks. Proc Natl Acad Sci USA 89:10915–10919.

Hubbard EJ, Greenstein D. 2000. The *Caenorhabditis elegans* gonad: A test tube for cell and developmental biology. Dev Dyn 218:2–22.

Iost I, Dreyfus M, Linder P. 1999. Ded1p, a DEAD-box protein required for translation initiation in *Saccharomyces cerevisiae*, is an RNA helicase. J Biol Chem 274:17677–17683.

Iwasaki K, McCarter J, Francis R, Schedl T. 1996. emo-1, a *Caenorhabditis elegans* Sec61p gamma homologue, is required for oocyte development and ovulation. J Cell Biol 134:699–714.

Johnstone O, Deuring R, Bock R, Linder P, Fuller MT, Lasko P. 2005. Belle is a Drosophila DEAD-box protein required for viability and in the germ line. Dev Biol 277:92–101.

Johnstone O, Lasko P. 2004. Interaction with eIF5B is essential for vasa function during development. Development 131:4167–4178.

Kawasaki I, Amiri A, Fan Y, Meyer N, Dunkelbarger S, Motohashi T, Karashima T, Bossinger O, Strome S. 2004. The PGL family proteins associate with germ granules and function redundantly in *Caenorhabditis elegans* germline development. Genetics 167:645–661.

Kawasaki I, Shim YH, Kirchner J, Kaminker J, Wood WB, Strome S. 1998. PGL-1, a predicted RNA-binding component of germ granules, is essential for fertility in *C. elegans*. Cell 94:635–645.

Kimble J, Edgar L, Hirsh D. 1984. Specification of male development in *Caenorhabditis elegans*: The fem genes. Dev Biol 105:234–239.

Kuwabara PE, Perry MD. 2001. It ain't over till it's ova: Germline sex determination in *C. elegans*. Bioessays 23:596–604.

Kuznicki KA, Smith PA, Leung-Chiu WM, Estevez AO, Scott HC, Bennett KL. 2000. Combinatorial RNA interference indicates GLH-4 can compensate for GLH-1; these two P granule components are critical for fertility in *C. elegans*. Development 127:2907–2916.

L'Hernault SW, Roberts TM. 1995. Cell biology of nematode sperm. Methods Cell Biol 48:273–301.

Lasko PF, Ashburner M. 1988. The product of the Drosophila gene vasa is very similar to eukaryotic initiation factor-4A. Nature 335:611–617.

Liang L, Diehl-Jones W, Lasko P. 1994. Localization of vasa protein to the Drosophila pole plasm is independent of its RNA-binding and helicase activities. Development 120:1201–1211.

Linder P. 2006. Dead-box proteins: A family affair—Active and passive players in RNP-remodeling. Nucleic Acids Res 34:4168–4180.

Miller MA, Ruest PJ, Kosinski M, Hanks SK, Greenstein D. 2003. An Eph receptor sperm-sensing control mechanism for oocyte meiotic maturation in *Caenorhabditis elegans*. Genes Dev 17:187–200.

Navarro RE, Blackwell TK. 2005. Requirement for P granules and meiosis for accumulation of the germline RNA helicase CGH-1. Genesis 42:172–180.

Navarro RE, Shim EY, Kohara Y, Singson A, Blackwell TK. 2001. cgh-1, a conserved predicted RNA helicase required for gametogenesis and protection from physiological germline apoptosis in *C. elegans*. Development 128:3221–3232.

Parrish S, Fleenor J, Xu S, Mello C, Fire A. 2000. Functional anatomy of a dsRNA trigger: Differential requirement for the two trigger strands in RNA interference. Mol Cell 6:1077–1087.

Praitis V, Casey E, Collar D, Austin J. 2001. Creation of low-copy integrated transgenic lines in *Caenorhabditis elegans*. Genetics 157:1217–1226.

Puoti A, Pugnale P, Belfiore M, Schlappi AC, Saudan Z. 2001. RNA and sex determination in *Caenorhabditis elegans*. Post-transcriptional regulation of the sex-determining tra-2 and fem-3 mRNAs in the *Caenorhabditis elegans* hermaphrodite. EMBO Rep 2:899–904.

Riddle DL, Blumenthal T, Meyer BJ, Priess JR, editors. 1997. *C. elegans* II. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.

Rose KL, Winfrey VP, Hoffman LH, Hall DH, Furuta T, Greenstein D. 1997. The POU gene ceh-18 promotes gonadal sheath cell differentiation and function required for meiotic maturation and ovulation in *Caenorhabditis elegans*. Dev Biol 192:59–77.

Roussell DL, Bennett KL. 1993. glh-1, a germ-line putative RNA helicase from Caenorhabditis, has four zinc fingers. Proc Natl Acad Sci USA 90:9300–9304.

Schedl T. 1997. Developmental genetics of the germ line. In: Riddle DL, Blumenthal T, Meyer BJ, Priess JR, editors. *C. elegans* II. Cold Spring Harbor, NY: Cold Spring Harbor Press. pp 241–170.

Schedl T, Graham PL, Barton MK, Kimble J. 1989. Analysis of the role of tra-1 in germline sex determination in the nematode *Caenorhabditis elegans*. Genetics 123:755–769.

Schedl T, Kimble J. 1988. fog-2, a germ-line-specific sex determination gene required for hermaphrodite spermatogenesis in *Caenorhabditis elegans*. Genetics 119:43–61.

Schupbach T, Wieschaus E. 1986. Germline autonomy of maternal-effect mutations altering the embryonic body pattern of Drosophila. Dev Biol 113:443–448.

Sijen T, Fleenor J, Simmer F, Thijssen KL, Parrish S, Timmons L, Plasterk RH, Fire A. 2001. On the role of RNA amplification in dsRNA-triggered gene silencing. Cell 107:465–476.

Smith P, Leung-Chiu WM, Montgomery R, Orsborn A, Kuznicki K, Gressman-Coberly E, Mutapcic L, Bennett K. 2002. The GLH proteins, *Caenorhabditis elegans* P granule components, associate with CSN-5 and KGB-1, proteins necessary for fertility, and with ZYX-1, a predicted cytoskeletal protein. Dev Biol 251:333–347.

Sonnichsen B, Koski LB, Walsh A, Marschall P, Neumann B, Brehm M, Alleaume AM, Artelt J, Bettencourt P, Cassin E, Hewitson M, Holz C, Khan M, Lazik S, Martin C, Nitzsche B, Ruer M, Stamford J, Winzi M, Heinkel R, Roder M, Finell J, Hantsch H, Jones SJ, Jones M, Piano F, Gunsalus KC, Oegema K, Gonczy P, Coulson A, Hyman AA, Echeverri CJ. 2005. Full-genome RNAi profiling of early embryogenesis in *Caenorhabditis elegans*. Nature 434:462–469.

Stitzel ML, Pellettieri J, Seydoux G. 2006. The *C. elegans* DYRK kinase MBK-2 marks oocyte proteins for degradation in response to meiotic maturation. Curr Biol 16:56–62.

Styhler S, Nakamura A, Swan A, Suter B, Lasko P. 1998. vasa is required for GURKEN accumulation in the oocyte, and is involved in oocyte differentiation and germline cyst development. Development 125:1569–1578.

Subramaniam K, Seydoux G. 2003. Dedifferentiation of primary spermatocytes into germ cell tumors in *C. elegans* lacking the pumilio-like protein PUF-8. Curr Biol 13:134–139.

Tanaka SS, Toyooka Y, Akasu R, Katoh-Fukui Y, Nakahara Y, Suzuki R, Yokoyama M, Noce T. 2000. The mouse homolog of Drosophila vasa is required for the development of male germ cells. Genes Dev 14:841–853.

Timmons L, Court DL, Fire A. 2001. Ingestion of bacterially expressed dsRNAs can produce specific and potent genetic interference in *Caenorhabditis elegans*. Gene 263:103–112.

Zhang B, Gallegos M, Puoti A, Durkin E, Fields S, Kimble J, Wickens MP. 1997. A conserved RNA-binding protein that regulates sexual fates in the *C. elegans* hermaphrodite germ line. Nature 390:477–484.