MIB-1 Is Required for Spermatogenesis and Facilitates LIN-12 and GLP-1 Activity in *Caenorhabditis elegans*

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ABSTRACT Covalent attachment of ubiquitin to substrate proteins changes their function or marks them for proteolysis, and the specificity of ubiquitin attachment is mediated by the numerous E3 ligases encoded by animals. Mind Bomb is an essential E3 ligase during Notch pathway signaling in insects and vertebrates. While *Caenorhabditis elegans* encodes a Mind Bomb homolog (*mib-1*), it has never been recovered in the extensive Notch suppressor/enhancer screens that have identified numerous pathway components. Here, we show that *C. elegans* *mib-1* null mutants have a spermatogenesis-defective phenotype that results in a heterogeneous mixture of arrested spermatocytes, defective spermatids, and motility-impaired spermatozoa. *mib-1* mutants also have chromosome segregation defects during meiosis, molecular null mutants are intrinsically temperature-sensitive, and many *mib-1* spermatids contain large amounts of tubulin. These phenotypic features are similar to the endogenous RNA interference (RNAi) mutants, but *mib-1* mutants do not affect RNAi. *MIB-1* protein is expressed throughout the germ line with peak expression in spermatocytes followed by segregation into the residual body during spermatid formation. *C. elegans* *mib-1* expression, while upregulated during spermatogenesis, also occurs somatically, including in vulva precursor cells. Here, we show that *mib-1* mutants suppress both *lin-12* and *glp-1* (*C. elegans* Notch) gain-of-function mutants, restoring anchor cell formation and a functional vulva to the former and partly restoring oocyte production to the latter. However, suppressed hermaphrodites are only observed when grown at 25°, and they are self-sterile. This probably explains why *mib-1* was not previously recovered as a Notch pathway component in suppressor/enhancer selection experiments.

KEYWORDS *C. elegans*; spermatogenesis; Mind Bomb; Notch signaling; ubiquitin E3 ligase; WormBook

THE protein composition of differentiating cells is dynamically managed by controlling cell-specific gene expression and protein post-translational modification, including protein degradation by proteolysis. Proteolysis and other forms of post-translational protein modification ensure that protein activity can be either altered or removed to change the physiological state of a cell. This process is especially

pronounced during spermatogenesis in animals, as mature spermatozoa discard and degrade many typical cellular constituents as they form [reviewed by Breucker *et al.* (1985)]. One major way eukaryotic cells degrade discarded proteins is via the ubiquitin system [reviewed by Hershko and Ciechanover (1998)].

Ubiquitin is a highly conserved 76-amino acid protein that is covalently attached to substrate proteins post-translationally, and ubiquitin attachment either alters a protein's function or targets it for degradation in the proteasome. Ubiquitin is first bound to an E1 ubiquitin-activating enzyme, and transferred to an E2-conjugating enzyme, which transfers it to an E3 ubiquitin ligase. The E3 ubiquitin ligase transfers ubiquitin to a substrate protein [reviewed by Hershko and Ciechanover (1998)]. In *Caenorhabditis elegans*, like many eukaryotes, there is one E1-activating enzyme, encoded by *uba-1*, and multiple E2s and E3s [22 and 180, respectively; reviewed by Kipreos (2005)]. Prior work revealed that a

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partial loss-of-function mutation in *uba-1*, which encodes the *C. elegans* E1-activating enzyme, resulted in cytologically normal-appearing sperm that were fertilization-defective (Kulkarni and Smith 2008). So far, no E2 enzymes that affect *C. elegans* spermatogenesis have been identified, and the anaphase promoting complex or cyclosome (APC/C) was the only known E3 enzyme active during spermatogenesis, but this meiosis component was also active during oogenesis (Golden *et al.* 2000). Most of the spermatozoa produced by mutants in either of two APC/C components (*emb-27* and *emb-30*) lack a nucleus but, nonetheless, are fertilization-competent (Sadler and Shakes 2000).

Mib is a really interesting new gene (Ring) finger (RF) E3 ubiquitin ligase [reviewed by Deshaies and Joazeiro (2009)] that was discovered as a modulator of Notch signaling activity [reviewed by Hori *et al.* (2013), Mašek and Andersson (2017), and Siebel and Lendahl (2017)]. Although Mib1 is widely expressed in adult tissues, null mutants exhibit lethality early in either *Drosophila* (Le Borgne *et al.* 2005), zebrafish (Itoh *et al.* 2003), or mouse (Barsi *et al.* 2005) development, making it challenging to study in adults. More recently, conditional knockouts, partial loss-of-function alleles, and RNA interference (RNAi) experiments have revealed that MIB-1 also participates in other signaling and cell differentiation pathways. For instance, Mib1 plays a role in Notch signaling during heart morphogenesis in humans and other vertebrates, since mutants exhibit cardiomyopathy (Luxán *et al.* 2013). Proteins that have not been directly implicated in Notch signaling have also been discovered to interact with Mib1. For instance, Mib1 ubiquitinates death-associated protein kinase-1 in gastrointestinal pre-secretory cells, and this ubiquitination is required for secretory cells to differentiate normally and avoid transitioning to a pre-cancerous state (Capoccia *et al.* 2013). Interferon production is induced in cultured human cells by viral infection and this does not occur when MIB1 expression is prevented via RNAi. In this case, viral infection activates MIB1-catalyzed ubiquitination of TRAF family member-Associated NF-Kappa-B activator (TANK)-binding kinase 1, resulting in the phosphorylation and activation of interferon regulatory transcription factor 3 (Li *et al.* 2011). Mib1 was also shown to function in *wnt*-mediated processes in both cultured mammalian cells and in *C. elegans* via RNAi (Berndt *et al.* 2011). Beyond its critical role in the initial formation of the nervous system, Mib1 also plays roles in modulating activities within differentiated nerves. Using small interfering RNA to Mib1 in cultured motor neurons or RNAi of *C. elegans mib-1* revealed that it participated in regulating proteins required for neuronal survival/function (Kwon *et al.* 2013).

In this paper, we show that a mutant previously known as *spe-16* encodes the E3 enzyme MIB-1, which is the *C. elegans* Mind Bomb (Mib) ortholog (Berndt *et al.* 2011). We characterized 10 different *mib-1* mutations, including molecular null mutants, and find that all show temperature-sensitive spermatogenesis defects that result in infertility. Mutants show a range of cytological defects, with some spermatocytes failing to divide and becoming multinucleate, while some

differentiate into spermatids that can lack chromosomes and/or fail to discard, and thus aberrantly retain, tubulin. Spermatids with less obvious cytological defects activate to form spermatozoa that are defective in both motility and fertilization.

Here, we also show that *mib-1* loss-of-function mutants can suppress phenotypic features found in the dominant *lin-12(n302)* and *glp-1(ar202gf)* mutants. For both cases, this suppression results in self-sterile hermaphrodites that lay unfertilized oocytes because they are spermatogenesis-defective, and this is only observed when worms are grown at 25°. Screens for genetic modifiers are usually done at the standard 20° growth temperature (Brenner 1974) and require self-fertility [reviewed by Greenwald and Kovall (2013)]. Consequently, it is not surprising that the numerous prior suppressor screens failed to identify *mib-1* as participating in either the *lin-12* or *glp-1* pathways.

Materials and Methods

Strains, culture, and nomenclature

We used standard techniques and terminology for *C. elegans*, and strains were derived from *C. elegans* variety Bristol (N2; Brenner 1974; Horvitz *et al.* 1979). The following mutants were used in this study: LGIII: *lin-12(n302gf)* (Ferguson and Horvitz 1985), *glp-1(ar202)* (Pepper *et al.* 2003), *glp-1(q231)* (Austin and Kimble 1987), *mib-1(hc54ts)* (Burke 1983; Shakes 1988; Hill *et al.* 2000), *unc-49(e382)*, and *dpy-18(e364)* (Brenner 1974); LG IV: *fem-3(q23)* (Barton *et al.* 1987) and *fem-1(hc17)* (Nelson *et al.* 1978); LGV: *him-5(e1490)* (Hodgkin *et al.* 1979); LG X: *syIs50[cdh-3::gfp]* (Pettitt *et al.* 1996; Inoue *et al.* 2002), *glo-1(zu391)* (Hermann *et al.* 2005), and VC40153, which has the *gk487236* mutation (Thompson *et al.* 2013) in F10D7.5, which is in the *C. elegans* neutralized ortholog. The balanced deficiency strain GE2180 *unc-32(e189) tDf7/qC1 dpy-19(e1259) glp-1(q339); him-3(e1147)* III was provided by R. Schnabel. Many of the strains used in this study were received from the *Caenorhabditis* Genetics Center (University of Minnesota, St. Paul, MN).

Origin of *mib-1* alleles

We treated homozygous *dpy-18(e364)* or *unc-49(e382) dpy-18(e364)* hermaphrodites with either 50 mM ethyl methane-sulfonate (EMS; Brenner 1974), 4.25 mM N-ethyl-N-nitroso urea (ENU), or 3.72 ENU (Anderson 1995) for 4 hr, after which they were mated at 20° in F1 noncomplementation screens. Permissively raised (reared at 20 or 16°) *mib-1(hc54ts); him-5* males (which are cross-fertile) were mated to either DpyUnc or Dpy mutagenized hermaphrodites, plates of F1 eggs were shifted to 25°, and individual non-Dpy or non-Dpy non-Unc F1 L4 hermaphrodites were picked to separate plates. Some mutagenized DpyUnc heterozygous hermaphrodites were mated to GE2180 *tDf7/qC1 dpy-19(e1259) glp-1(q339); him-3(e1147)* males at 20°, plates

of F1 eggs were shifted to 25°, and L4 hermaphrodites exhibiting a *Dpy* (which, like *mib-1*, fails to complement *tDf7*; Hill *et al.* 2000) non-*Unc* (which complements *tDf7*) phenotype were picked to separate plates. In both cases, plates were examined 1 day later for self-sterility, which was indicated by the presence of numerous unfertilized oocytes. Overall, 14,230 F1 hermaphrodites were screened by the above-discussed methods. For either mutant screen, we recovered self-sterile candidates, mated them to *N2* males, and recovered the new mutation at 20°, which is permissive for self-fertility. Mutants were all repeatedly outcrossed to *N2* and segregated away from *cis* morphological markers. After the gene was cloned, the *mib-1(eb154)* targeted deletion was created (see *Genetic transformation methods*, below). Like the canonical *mib-1(hc54)* mutant, all new *mib-1* mutants proved to be temperature-sensitive for the *Spe* phenotype, and they could be propagated as homozygotes at either 16 or 20°.

Phenotypic analysis

All phenotypic analyses were performed on age-matched animals grown from embryos at 25° under tightly controlled conditions unless mentioned otherwise. Cells in the developing vulva cell region were identified and named as described previously (Sharma-Kishore *et al.* 1999). Light microscopy was performed as described previously (Gleason *et al.* 2012).

Hermaphrodite brood/oocyte counts and temperature-sensitive periods (TSPs) were performed as described previously (EHernault *et al.* 1988). Hermaphrodite self-sperm were visualized within the spermatheca by fixing for 2 min in −20° methanol, rehydrating in PBS-T (PBS with 0.1% Tween 20), and stained with DAPI (100 ng/ml) for 30 min in PBS-T. Sperm were isolated in Sperm Medium (SM) containing 10 mM glucose (Machaca *et al.* 1996), and *in vitro* activation with Pronase (EHernault and Roberts 1995) or Zn⁺⁺ (Liu *et al.* 2013) was as described previously.

Males were vitally stained with SYTO 17 as described (Hill and EHernault 2001) and used in mating experiments to *fem-1*; *glo-1* hermaphrodites. For cytological analyses, 5–10 males were picked into a 4–6 µl drop of SM/glucose containing either DAPI (50–150 ng/ml) or Hoechst 33258 (0.1–1.0 µg/ml), and either 3,3'-dihexyloxycarbocyanine iodide (0.1 µM) or SYTO 17 (1–3 µM; both dyes available from Thermo Fisher Scientific). Males were dissected with a 27-g hypodermic needle to release sperm into the dye solution, and preparations were sealed under a coverslip with silicone grease.

Worms, gonads, and males dissected to release sperm were freeze/cracked on dry ice and methanol fixed as described previously (Gleason *et al.* 2012) using poly-L-lysine-coated slides (Electron Microscopy Sciences). Hermaphrodites were mounted in Prolong Gold antifade reagent with DAPI (Life Technologies). For male gonad immunocytochemistry, a chicken primary antibody against GFP (catalog number GFP-1010; Aves Labs) was used at a dilution of 1:1000 (for whole gonads) or 1:2000 (for sperm), and the secondary antibody was an Alexa Fluor 594-conjugated Donkey Fab IgG Fragment

anti-Chicken IgY (Cat #703-586-155; Jackson ImmunoResearch) used at a dilution of 1:2000. Sperm released from hand-dissected males were stained for tubulin with the anti- α -tubulin monoclonal DM1A (Shakes *et al.* 2009) directly conjugated to Alexa Fluor 488 (16–232 EMD Millipore) and used at a 1:200 dilution. Membranous organelles (MOs) were stained with Texas-Red conjugated wheat germ agglutinin (WGA; Invitrogen, Carlsbad, CA; Molecular Probes, Eugene, OR) at a working concentration of 0.13 µg/ml.

General molecular biological methods

Standard genomic DNA, RNA, and cDNA methods were mostly as described previously (Kroft *et al.* 2005; Gleason *et al.* 2006). Candidate genes in the *mib-1* region were PCR amplified from genomic DNA and sequenced.

Northern blot analysis was carried out as described (Church and Gilbert 1984) using 20 µg of total RNA from *fem-3(q23gf)* hermaphrodites (Barton *et al.* 1987) that produce only sperm at 25° and *fem-1(hc17)* hermaphrodites (Nelson *et al.* 1978) that produce only oocytes at 25°. The blot was probed with an 810-bp *mib-1* genomic DNA probe made by PCR using oligonucleotides MR83 and MR84 (Supplemental Material, Table S2); this probe includes all of exons 6–9 and parts of exons 5 and 10 (see Figure S1). The blot was then stripped and reprobed with a 275-bp *rla-1* cDNA probe (Spieth *et al.* 1991) made by RT-PCR using oligonucleotides TK287 and TK288 (Table S2) as a loading control. Ethidium bromide staining of the gel before transfer also showed that the lanes were equally loaded (data not shown).

Genetic transformation methods

PCR of wild-type genomic DNA with primers MR100, MR101, MR102, and MR103 (Table S2) was used to create a transcriptional reporter (Boulin *et al.* 2006) that had 2154 bp of *C. elegans* genomic DNA 5' to the *mib-1* translational start ATG codon plus the first six codons of *mib-1* 5' to GFP coding sequence (Figure S1), and was followed by an *unc-54* 3'-UTR. GFP and *unc-54* 3'-UTR sequences were PCR amplified from pPD95.75 (Fire Lab Vector Kit 1995). The promoter fusion PCR products were injected into wild-type hermaphrodites at 10 ng/µl together with pRF4, which is a plasmid encoding the dominant marker *rol-6(su1006)* (Mello and Fire 1995). Stable lines were not recovered, so we evaluated the expression patterns in at least 20 L3–adult F1 transiently transfected hermaphrodites to generate Figure 9.

We also performed clustered regularly interspaced short palindromic repeats (CRISPR)-mediated genome engineering to insert a *gfp* coding sequence followed by three glycine codons at the 5' end of the *mib-1* coding sequence (Dickinson *et al.* 2013; Paix *et al.* 2014; Farboud and Meyer 2015). The Q5 Site-Directed Mutagenesis kit (New England Biolabs, Beverly, MA) was used with primers (HL45, HL46, HL56, or HL57; Table S2; Integrated DNA Technologies) to insert a 19-bp targeting sequence into the single guide RNA (sgRNA)/Cas9 expression plasmid pDD162 (gift from Bob Goldstein; Addgene plasmid # 47549; Dickinson *et al.* 2013). The *gfp::*

mib-1 homologous repair template (~2.5 kb) was made using a *mib-1* promoter gBlock1 [499-bp protospacer adjacent motif (PAM) mutated CCA to CGA; located at position -151 on Figure S6], GFP (864-bp PCR), and *mib-1* gBlock2 (1249-bp PAM mutated CGG to CTG; located at position 971 on Figure S6; Integrated DNA Technologies) that were assembled into the pBS vector using a Gibson Assembly Cloning Kit (New England Biolabs). The plasmid was digested with *Pst*I and *Sal*I, and the insert was gel purified (Zymo Research) to produce the repair template. The microinjection mix contained 75 ng/μl of repair template, 25 ng/μl Cas9 sgRNA targeting 5' of the *mib-1* ATG (initially created with either HL45 or HL56), 25 ng/μl Cas9 sgRNA targeting 3' of the *mib-1* ATG (initially created with either HL46 or HL57), and 20 ng/μl pCFJ104-*Pmyo-3::mCherry::unc-54* (gift from Erik Jorgensen; Addgene plasmid # 19328; Frøkjær-Jensen *et al.* 2008). Injected worms were recovered and initially grown at 16°, since all *mib-1* mutant hermaphrodites are self-fertile at this temperature. Microinjected N2 hermaphrodites (using sgRNAs derived from HL45 and HL46) resulted in strain SL1615, which was self-fertile when grown at 16° but self-sterile when grown at 25°. DNA sequence analyses revealed that this strain retained both NGG PAM sites, but deleted 1113 bp wholly within *mib-1*; this mutant allele was named *mib-1(eb154) III* (see Figure 7 and Figure S1). The second round of microinjections (using sgRNAs derived from HL56 and HL57) were performed on *mib-1(eb154)* hermaphrodites. These injections resulted in SL1618, which was a strain that was self-fertile at both 16 and 25° growth temperatures. DNA sequence analyses revealed that SL1618 had the *gfp* coding sequence and three glycine codons in-frame to a complete *mib-1* coding sequence, creating *mib-1(eb154) III* (Figure S6).

GST:: MIB-1 protein expression and purification

PCR was performed on the *mib-1* cDNA yk1216b06 template (Y. Kohara cDNA, see www.wormbase.org) with the 5' primer Fspe16C1SalI and either the 3' primer Rspe-16C1BglII (for full-length *mib-1* coding sequence) or Rspe16NRFBgII (for *mib-1* coding sequence lacking the two RF motifs; see Figure 7A, Figure S1, and Table S2). The resulting PCR products (which lacked a UTR sequence) were TA cloned into pCR2.1 (Invitrogen), sequence verified, and subcloned into pGEX-KK1 (a GST N-terminal fusion vector derived from pGEX-4T that was modified by K. Kaibuchi, Nagoya University, Japan). These expression vector plasmids were transformed into *Escherichia coli* BL21-Codon Plus (DE3)-RIL (Stratagene, La Jolla, CA) for protein production.

GST fusion protein expression was induced by adding 1 mM IPTG (isopropyl-β-D-thiogalactoside) to bacterial cultures that were subsequently incubated with shaking for 4 hr at room temperature (~21–22°). The bacteria were pelleted, resuspended in lysis buffer (50 mM Tris pH 7.4, 1 mM phenyl sulfonyl fluoride, and 1 mM EDTA), and lysed in a French press at 15,000 Psi, all at 0–4°. The resulting lysate was purified, bound to GST-Bind Resin, and eluted according to the manufacturer's instructions (Novagen). The Pierce Coomassie Plus

Protein Assay Kit (Thermo Fisher Scientific) was used to determine the concentration of the purified GST fusion proteins.

Ubiquitination assay

An autoubiquitination assay (Lorick *et al.* 1999) was performed in a 50 μl volume containing 50 mM Tris-Cl, pH 7.4, 0.5 mM MgCl₂, 0.2 mM ATP, 0.1 M DTT, 25 μM ZnCl₂, 10 mM creatine phosphate (Calbiochem, San Diego, CA), 5 unit/ml phosphocreatine kinase (Calbiochem), 30 μg ubiquitin (Sigma [Sigma Chemical], St. Louis, MO), 100 ng of E1 ubiquitin-activating enzyme (Calbiochem), 100 ng of Ubch5 E2 ubiquitin-conjugating enzyme (Boston Biochem), and either 40 ng E3 MIB-1 full-length GST fusion protein or control MIB-1 GST fusion protein without the two RFs. The reactions were carried out at 30° for 90 min, terminated by adding an equal volume of 2× SDS sample buffer (Laemmli 1970), and the products were subjected SDS-PAGE followed by western blot analysis with an anti-ubiquitin antibody (1:1000; Encore). We reprobed the blot with a monoclonal anti-GST antibody (1:1000; Sigma) to confirm that there was equal loading of fusion proteins.

Data availability

Any strains discussed in this paper are available on request or are already available from the *Caenorhabditis* Genetics Center. DNA primers used in this work are listed in Table S2. Unpublished genomic sequence encoding the *C. remanei*, *C. brenneri*, and *C. tropicalis* MIB-1 protein sequences was reanalyzed by visual inspection, and we determined new gene models based on their homology to the *C. elegans* MIB-1 sequence; these data are available from WormBase (www.wormbase.org) or by request. Supplemental materials (figures, tables, and movies) are available via Figshare: <https://doi.org/10.25386/genetics.5970946>.

Results

mib-1 mutants have a spermatogenesis-defective phenotype

Loss-of-function mutants were discovered in a gene that we initially named (spermatogenesis-defective) *spe-16*, which was later (see below) identified as *mib-1*. Canonical *mib-1(hc54)* hermaphrodites exhibit substantial self-fertility when propagated at 16° but profound self-sterility when grown at 25° (Table 1). *mib-1(hc54)* was placed onto the right arm of chromosome III by a combination of three-factor, four-factor, and deficiency mapping (data not shown; see www.wormbase.org). New EMS- and ENU-induced alleles were recovered by F1 noncomplementation screens to either *mib-1(hc54)* or *tDf7* (which deletes *mib-1*; see Figure S1 and Table S1). Eight mutants were recovered in these screens, plus we also evaluated two *mib-1* mutants (*gk936708* and *gk587388*; data not shown) recovered during the *C. elegans* million mutation project (Thompson *et al.* 2013), and all are temperature-sensitive sterile, like *mib-1(hc54)* (Table 1). Later, after *mib-1* was cloned, the *mib-1(eb154)* deletion null allele (see below) was created by CRISPR methods (see *Materials and Methods*) and, like all other *mib-1* mutants, it had a temperature-sensitive phenotype (Table 1).

Table 1 Quantitation of the *mib-1* self-sterile phenotype

Strain	16°				20°				25°			
	Progeny	Oocytes	Dead eggs	n =	Progeny	Dead eggs	% Hatch	n =	Progeny	Oocytes	Dead eggs	n =
Wild-type	413 ± 29	34 ± 11	1.9 ± 0.4	14	420 ± 22			11	293 ± 14	69 ± 19	1 ± 0.5	9
<i>mib-1(eb1s26)</i> ^a	413 ± 19			19	341 ± 16			8	124 ± 13			14
<i>mib-1(eb1s26)/+</i>	317 ± 31			12	420 ± 14			9	271 ± 17			12
<i>mib-1(hc54)</i>	217 ± 8	33 ± 5	1.6 ± 0.3	24					0.3 ± 0.1	103 ± 10	2 ± 0.3	26
<i>mib-1(eb33)</i>									0.8 ± 0.4			12
<i>mib-1(eb34)</i>									0.3 ± 0.2			16
<i>mib-1(eb35)</i>									3.9 ± 1.4			10
<i>mib-1(eb36)</i>									2.1 ± 1			10
<i>mib-1(eb37)</i>	157 ± 19	83 ± 21		8					1.8 ± 0.6			14
<i>mib-1(eb39)</i>	266 ± 15	140 ± 18	4 ± 2	20					2 ± 0.4			11
<i>mib-1(eb41)</i>									1.9 ± 1.3			10
<i>mib-1(eb42)</i>									2.1 ± 0.7			9
<i>mib-1(eb154)</i>	303 ± 11	11 ± 3	1 ± 0.3	20					2.1 ± 0.5	32 ± 8	0.5 ± 0.2	11
<i>mib-1(eb154)/+</i>									259 ± 13			12
<i>mib-1(eb154)/eb1s26</i>	327 ± 14			11	300 ± 35			8	245 ± 16			11
<i>mib-1(eb154)/(hc54)</i>	359 ± 12			10					1.7 ± 0.3			9
<i>glp-1(q231); him-5</i>					9 ± 2	143 ± 15	~6% ^b	15	0	0	0	9
<i>glp-1(q231) mib-1(eb37)</i>					16 ± 3	49 ± 7	~25% ^b	17	0	0	0	21
<i>dpy-18; him-5</i>												
<i>mib-1(eb37) dpy-18</i>					98 ± 9	11 ± 2	~90%	12	3.1 ± 0.8	115 ± 18	9 ± 2	14
<i>glp-1(ar202) dpy-18</i>									0.2 ± 0.1	0	< 0.1	37
<i>glp-1(ar202) mib-1(eb37)</i>									0	25 ± 5	0	29
<i>dpy-18</i>												

The number of progeny, oocytes, and dead eggs (± SEM) were determined for hermaphrodites at 16, 20, and 25° until they died. The number shown is the average per hermaphrodite for each mutant.

^a *eb1s26* is a clustered regularly interspaced short palindromic repeats-engineered gfp insertion that results in GFP fused to the N-terminal end of MIB-1.

^b The difference between these two percentages is significant; $P < 0.01$ by z test.

The time period during development when *mib-1(hc54)* is temperature-sensitive for fertility (the TSP) is between 30 and 45 hr of postembryonic development (Figure 1), which is during the L4 larval period when spermatogenesis occurs in wild-type hermaphrodites (Hirsh *et al.* 1976), like three other genes known to affect spermatogenesis (Ward and Miwa 1978; IHernault *et al.* 1988; Gleason *et al.* 2006). We also determined that the TSP for the *mib-1(eb154)* null mutant (Figure S2) is very similar to *mib-1(hc54)*, confirming that *mib-1* exhibits an intrinsically temperature-sensitive phenotype. The 10 *mib-1* mutants all have highly similar phenotypic characteristics and the data shown below from several different *mib-1* mutants are representative.

As discussed below, we observed small, abnormally located DNA masses in a number of *mib-1* mutant sperm (see Figure 4 and Figure 5), which suggests that one or more chromosomes are not properly incorporated into the *mib-1* sperm nucleus. Mutants that affect chromosome segregation can affect both autosomes and the sex (X) chromosome. Quantitating autosomal segregation defects is difficult due to associated lethality, but mutants affecting X chromosome segregation are viable and exhibit a High Incidence of Male (HIM) phenotype that elevates the number of males in the self-broods of hermaphrodites (Hodgkin *et al.* 1979). Full penetrance of Mib-1 phenotypic defects is only evident at a growth temperature of 25° (Table 1) when hermaphrodites have a highly penetrant self-sterile phenotype. However, ~90% of *mib-1* mutants show some self-fertility when grown

at 23°, suggesting partial penetrance of the Mib-1 phenotype at this growth temperature, as is the case for temperature-sensitive mutants in the endogenous (endo)-RNAi pathway (Gent *et al.* 2009). Under these growth conditions, N2 wild-type worms had a self-brood of 189 ± 21 ($n = 16$) and 0.3% (10/3022) of their progeny were male. In contrast, *mib-1(eb154)* hermaphrodites had a self-brood of 71 ± 5 ($n = 18$) and 1.2% (16/1285) of their progeny were male. These data show that *mib-1(eb154)* has a weak Him phenotype, where broods produced at a 23° growth temperature include significantly more males as compared to the wild-type control broods ($\chi^2_{[1]} = 12.54$; $P < 0.01$).

After oogenesis commences, the fertilization-related functions that are compromised during *mib-1* hermaphrodite development cannot be reversed by a shift to permissive (20° or lower) growth conditions; *mib-1* adult hermaphrodites are self-sterile for the rest of their life (Figure 1 and Figure S2, open circles). The first ovulation pushes spermatids into the spermatheca and, just prior to that point, the proximal gonad contains oocytes (o, in Figure 2, A and C) just distal to all hermaphrodite sperm. In wild-type worms, nearly all of these sperm in the proximal gonad are spermatids with a single haploid nucleus, many of which can be seen in DAPI-stained gonads (arrow, Figure 2, A and B). While *mib-1(eb154)* mutants also produce some spermatids with a single haploid nucleus (arrow, Figure 2, C and D), they also contain many large spermatocyte-like cells with multiple haploid nuclei (arrowhead, Figure 2, C and D). The profound self-sterility seen for all *mib-1* mutants (Table 1)

Figure 1 TSP curves for *mib-1(hc54)*. *mib-1(hc54)* hermaphrodites were shifted from permissive (16°) to restrictive (25°) temperature, or vice versa, at intervals throughout development to determine when the MIB-1 protein is required for fertility (the TSP). The mean number of progeny produced by 10 *mib-1(hc54)* hermaphrodites is plotted against their age at temperature shift, in 25° hr since hatch. Error bars are SEM. Data from upshifts (16–25°) and downshifts (25–16°) are plotted separately. Progeny counts plotted at 50 hr are control values from animals that were not shifted. The approximate positions of life cycle stages are indicated below the x-axis. L, larval stage; TSP, temperature-sensitive period.

suggests that very few of these sperm are fertilization-competent once they enter the spermatheca.

In *C. elegans* wild-type hermaphrodites, spermatozoa must remain localized in the spermatheca if they are going to successfully participate in fertilization. Oocytes are ovulated one at a time into the spermatheca, where they interact with and are fertilized by sperm. The resulting zygote pushes many of the remaining spermatozoa through the spermathecal valve into the uterus, and they crawl back into the spermatheca to await the next ovulation; this cycle repeats hundreds of times in wild-type worms (Ward and Carrel 1979; Nishimura and LHernault 2010). In young, wild-type hermaphrodites, highly condensed sperm nuclei are observed within the spermatheca or adjacent to the spermathecal valve (dotted circle in Figure 3A), but sperm are not present in the spermathecae of age-matched *mib-1(eb37)* hermaphrodites (dotted circle in Figure 3C). Consistent with its fertilization-defective phenotype (Table 1), unfertilized oocytes with endomitotic nuclei are observed in the uterus of *mib-1(eb37)* hermaphrodites (Figure 3, C and D, arrowheads). Unlike wild-type sperm, the defective sperm produced by *mib-1* hermaphrodites are incapable of maintaining their position in the spermatheca.

We also assessed the ability of male-derived *mib-1* sperm to participate in fertilization. For these analyses, either

Figure 2 The proximal gonad in wild-type and *mib-1(eb154)* hermaphrodites. Corresponding DIC (A and C) and DAPI staining (B and D) of N2 (wild-type control) (A and B) and *mib-1(eb154)* (C and D) dissected gonads showing the most proximal region. Each germ line had switched its identity from a testis to an ovary and the first oocytes are indicated (O) with accumulated sperm to their right (brackets). Arrows indicate spermatids with normal cytology and arrowheads indicate an aberrant spermatocyte containing four nuclei. In all figures, distal is left and proximal is right. The bar represents 20 μ m and applies to all individual panels.

mib-1(eb37); him-5 or *him-5* (control) males were vitally stained and mated to *fem-1(hc17); glo-1* females, which lack endogenous sperm (Nelson *et al.* 1978) and have low autofluorescence (Hermann *et al.* 2005). Subsequently, the inseminated fluorescent sperm were evaluated for fertilization competence (Hill and LHernault 2001). *him-5* (control) male-derived sperm always localize within *fem-1* spermathecae (dotted circle in Figure 3E), and these inseminations always yielded outcross progeny. In contrast, *mib-1(eb37); him-5* male-derived sperm were ejaculated into hermaphrodites during mating (data not shown), but they were never seen within *fem-1* spermathecae after mating (dotted circle in Figure 3F), and outcross progeny were never observed.

Figure 3 *mib-1* sperm localization defects in hermaphrodites. (A and B) The wild-type (WT) hermaphrodite spermatheca (outlined by a dotted line) contains numerous sperm that appear as small, DAPI-stained dots. WT worm is self-fertile, and developing embryos are observed within the uterus, some of which retain an intact eggshell so they fail to stain with DAPI (asterisks). (C and D) The *mib-1(eb37)* adult hermaphrodite spermatheca (outlined by a dotted line) does not contain sperm. While *mib-1* hermaphrodites produce sperm, they are fertilization- and motility-defective so that ovulated oocytes push them out of the spermatheca. Consequently, the uterus contains unfertilized oocytes that each have a large DAPI-stained mass because they have undergone endomitosis (arrowheads). (E and F) Spermathecae (outlined with dotted white lines) of adult *fem-1;glo-1* hermaphrodites after mating to males labeled with MitoTracker red (MT). Nomarski micrographs (right panels) correspond to fluorescence micrographs (left panels). (E) WT male-derived sperm were found within or near the spermatheca. (F) *mib-1(lf)* male-derived sperm were not observed within the spermatheca. In all images, anterior is left, ventral is down, and v marks the position of the vulva. The scale bar in (D) represents 30 μ m and applies (A–D). The scale bar in (F) represents 10 μ m and applies to (E and F).

Therefore, sperm from either *mib-1(eb37)* males or hermaphrodites cannot properly localize within the spermatheca and participate in fertilization. To gain a better understanding of the origin of *mib-1* spermatogenesis defects, subsequent studies were performed on males because they produce $\sim 10\times$ as many sperm as hermaphrodites, so much larger sample sizes are readily evaluated.

As for most animals [reviewed by EHernault (2006), Nishimura and EHernault (2010), Chu and Shakes (2013),

Demarco *et al.* (2014), and Mecklenburg and Hermann (2016)], *C. elegans* wild-type spermatogenesis is characterized by precise regulation of chromosomes during meiosis and coordination between nuclei and cytoplasm during cell division (Figure 4, A–E and Figure S3). A highly detailed analysis of meiosis during wild-type *C. elegans* spermatogenesis has been published (Shakes *et al.* 2009), and here we only show aspects so as to allow discussion of how *mib-1* is aberrant. Toward the end of prophase I, the chromosomes coalesce at the karyosome stage (Figure 4A), followed by diakinesis (Figure 4B and Figure S3A). The chromosomes then go through two orderly divisions (Figure 4, C–E and Figure S3, B–E) with well-formed meiotic spindles (green in Figure 4) that are arrayed to ensure placement of one diploid nucleus in each secondary spermatocyte (Figure 4, C and D) or one haploid nucleus in each spermatid (Figure 4E). This polarity of the meiotic spindle is closely mirrored by the segregation of mitochondria, so that roughly equal numbers of mitochondria are deposited into each wild-type spermatid, together with one nucleus (red, in Figure S3, F–J).

The cytology of spermatogenesis in *mib-1* mutants is quite variable. Many times, *mib-1(eb154)* males produce spermatocytes that appear to progress through meiosis and form spermatids. However, 20/54 of the randomly selected *mib-1(eb154)* spermatocytes we examined showed obvious abnormalities in the distribution of microtubules and/or nuclei. During prophase I, *mib-1(eb154)* chromosomes sometimes lag in their coalescence and accumulate off-center (Figure 4F, panel 3, yellow arrows). The position of the two meiotic spindles in secondary spermatocytes can appear relatively normal (lower cell in Figure 4G, panel 2), but spindles at odd angles relative to each other (Figure 4H, panel 2 and lower cell in Figure 4J, panel 2) or with unusual arrays of microtubules (Figure 4G, panel 2, upper cell and Figure 4I, panel 2) were also observed. In wild-type, the meiotic divisions of one primary spermatocyte produce two diploid secondary spermatocytes that become four haploid spermatids. For some *mib-1(eb154)* spermatocytes, these rules are sometimes violated and spermatocytes with three nuclei (Figure 4G, top cell) or where the two meiotic spindles are associated with vastly different amounts of DNA (Figure 4J, lower cell) have been observed (for reference, spermatids with haploid nuclei are indicated with arrowheads in Figure 4, G and K, panels numbered 1). Unlike wild-type, the *mib-1(eb154)* meiotic divisions are not necessarily coordinated with morphogenesis of spermatids (Figure 4, G and J, lower cell) and one consequence is that postmeiotic spermatocytes that contain four nuclei are observed (Figure 4K). We also examined the distribution of mitochondria during spermatogenesis in *mib-1(hc54)* and discovered that mitochondria can properly partition into spermatids that fail to receive a nucleus (Figure S3, K–T).

The successful completion of wild-type meiosis during spermatogenesis is followed by spermatids budding from the residual body (rb in Figure 5A). Budding involves a sorting process where spermatids retain mitochondria (Figure

Figure 4 Spermatogenesis in wild-type and *mib-1(eb154)* males. Spermatogenesis in *him-5* (wild-type control) (A–E) or *mib-1(eb154)* (F–K). DNA is DAPI-stained blue in panels numbered 1 and 2, and is white in panels numbered 3, while panels numbered 1 show differential interference contrast (DIC) images and panels numbered 2 show tubulin immunofluorescence (green). (A) Karyosome stage of a primary spermatocyte; (B) diakinesis of meiosis I showing the chromosomes moving toward the center of the cell; (C) metaphase I showing the chromosomes about to move toward the poles; (D) telophase I as the spermatocyte is about to transition to metaphase II; (E) anaphase II showing the four haploid nuclei; (F) four *mib-1* primary spermatocytes, each with a different chromosome distribution; (G) two *mib-1* secondary spermatocytes, the upper one with a tripartite meiotic spindle and the lower with two parallel spindles; (H) two connected *mib-1* secondary spermatocytes with well-formed meiotic spindles that are not parallel to each other; (I) two connected *mib-1* secondary spermatocytes with one chevron-shaped meiotic spindle; (J) two *mib-1* secondary spermatocytes where nuclear events are not properly coordinated with spermatid formation; and (K) a *mib-1* terminal spermatocyte containing four nu-

clei. Double-headed white arrows indicate the presumptive cell division planes, abnormally positioned DNA is indicated by yellow arrows, and white arrowheads indicate normal-appearing *mib-1* spermatids that coincidentally came to lie next to spermatocytes during specimen preparation. The scale bar represents 5 μ m and applies to all individual panels.

S3, E and J) and MOs that stain brightly with fluorescently tagged WGA (in red; Figure 5A, panel 3), but place nearly all tubulin into the residual body as one discrete array per nucleus (green in Figure 5A, panel 2), as described previously (Winter *et al.* 2017), except for what is in the centriole (arrowhead, Figure 5B, panel 2). The result is spermatids with a single, centrally placed nucleus surrounded by WGA-positive MOs (Figure 5B). *C. elegans* spermatids extend a single pseudopod and become spermatozoa through a process termed activation. Activation can be elicited by any one of a variety of treatments, including cell surface proteolysis by Pronase (Figure S4C; Ward *et al.* 1983) or exposure to micromolar Zn^{++} (Figure 5C, panel 1; Liu *et al.* 2013). In wild-type worms, once a pseudopod is extended, its position relative to the cell body remains fixed (Figure 5C, panel 1 and Figure S4, C and E).

While many *mib-1(eb154)* spermatocytes complete meiosis and form spermatids, they are frequently abnormal in

obvious ways (Figure 5, D–H). We examined 231 wild-type spermatids/spermatozoa and found that 230 had a single nucleus and only one had two nuclei. None of these 231 wild-type spermatids/spermatozoa had more tubulin than one, faint dot, presumably associated with the centriole (arrowhead, Figure 5B, panel 2; the centriole is not always detected by immunofluorescence). In contrast, 45/355 (12.7%) of *mib-1(eb154)* spermatids/spermatozoa lacked a nucleus (*, Figure 5, D and G) and 13/355 (3.7%) had two nuclei (**, Figure 5D). These nuclei tended to be off-center, and there sometimes were additional DNA masses in cells (yellow arrows, Figure 5, G and H). Examination of vitally stained *mib-1(hc54)* dividing spermatocytes shows how spermatids can form without a nucleus (Figure S3, O and T). Like WGA-stained MOs (Figure 5, D–H), mitochondria (Figure S3, O and T) are usually segregated to spermatids and this can occur in the absence of proper nuclear segregation. We also found that 174/355 (49%) *mib-1(eb154)*

Figure 5 Wild-type and *mib-1(eb154)* male-derived spermatids and spermatozoa. (A–C) *him-5* and (D–H) *mib-1; him-5* sperm. All panels show DAPI-stained DNA (blue) while panels numbered 1 show differential interference contrast (DIC) images, those numbered 2 show tubulin immunofluorescence (green), and those numbered 3 show sperm-specific membranous organelles revealed by fluorescent-wheat germ agglutinin (WGA) staining (red). Pseudopods or pseudopod-like structures are indicated by white arrows, tubulin accumulations in sperm are indicated by white arrowheads, and abnormally small amounts of DNA are indicated by yellow arrows. Cells that are bracketed either have no nucleus (indicated by *) or two nuclei (indicated by **). The spermatozoa shown in (C and F–H) were activated with $ZnCl_2$. The scale bar represents 5 μm and applies to all individual panels. rb, residual body.

spermatids/spermatozoa retained more tubulin than was observed for wild-type worms, sometimes as a wreath around the nucleus (arrowhead, Figure 5E, panel 2) or in other cellular distributions (arrowheads, Figure 5, F, G, and H, panels numbered 2). When *mib-1(eb154)* spermatids are activated with Zn^{++} (white arrows, Figure 5, F, G, and H, panels numbered 1) or *mib-1(eb37)* spermatids are activated with Pronase (Figure S4D), abnormal pseudopods are extended (compare to controls in Figure 5C, panel 1 and Figure S4C).

Pronase-activated, live *mib-1(eb37); him-5* and *him-5* control spermatozoa were also examined using time-lapse recordings. *him-5* control spermatozoa have a single pseudopod extending from a cell body location that does not change over time (Figure S4E and Movie S1). While a *mib-1(eb37)* sperm might extend a single, short pseudopod exhibiting defective motility (Movie S2), some repeatedly extend and retract a short pseudopod (Figure S4F, arrowheads in 54s panel, from Movie S3) or extend multiple pseudopods (Movie S4), something

never seen for the *him-5* spermatozoa. In conclusion, *mib-1* mutants show a wide array of phenotypic defects during spermatogenesis that are summarized in Figure 6.

***mib-1* loss-of-function mutations affect a ubiquitin E3 ligase**

The gene causing the *Spe* phenotype described above mapped between the *tra-1* and *dpy-18* genes on chromosome III. Repeated attempts to create transgenic worms via germ line transformation with clones that span this interval were unsuccessful (data not shown), so we sequenced candidate genes. This approach revealed a unique single-base pair mutation in Y47D3A.22, which is *mib-1*, for each of nine EMS- or ENU-induced mutants (Figure 7A, Figure S1, and Table S1). Subsequently, we used CRISPR gene editing (Dickinson *et al.* 2013; Paix *et al.* 2014; Farboud and Meyer 2015) to generate *mib-1(eb154)*, which is a 1113-bp deletion that removes much of the promoter sequence, the first two exons, and part

Figure 6 Summary of the mutant spermatogenesis defects seen in *mib-1* mutants. (A) The pathway of wild-type spermatogenesis and (B) the variable terminal stages at which *mib-1* mutants arrest spermatogenesis. Near wild-type cytology can be observed for at least part of spermatogenesis in *mib-1* mutants after which cytology becomes abnormal, as indicated by horizontal arrows pointing right. Sometimes, a terminally arrested spermatocyte is observed where four nuclei (filled black circles) share a common cytoplasm. Errors in meiosis result in *mib-1* spermatids and spermatozoa that have 0, 1, or 2 condensed masses of DNA, and such DNA masses are usually placed off-center in the cell. These DNA masses can be approximately the same size as that found in wild-type spermatids, which are always haploid, or they can be much smaller in size (*). Some spermatids/spermatozoa cells can have one small and one large DNA mass (data not shown). Pseudopod morphology in *mib-1* is much more variable than what is observed in wild-type and, generally, it is smaller than what is seen in wild-type. Additionally, while the position of the pseudopod is stable in wild-type (lower left drawing), it is unstable and can change rapidly in *mib-1* spermatozoa (indicated at the bottom right of the figure). rb, residual body.

of exon 3 (Figure 7A, Figure S1, and Table S1); it has phenotypic features that are similar to those exhibited by the single-base pair *mib-1* mutants. Relative to the housekeeping gene *rla-1*, steady-state *mib-1* mRNA expression is higher when *C. elegans* are engaged in spermatogenesis, but it is also expressed in feminized animals that are not engaged in spermatogenesis (Figure 7B). The *mib-1* locus is predicted to encode a 765-amino acid protein containing multiple ankyrin repeat motifs and two C-terminal RING-type zinc finger domains (Figure 7A).

The relative positions of the 10 analyzed *mib-1* mutants are shown in Figure 7A and Figure S1. The *mib-1(eb34)* mutation alters the start methionine codon so that it would encode isoleucine. Two *mib-1* missense mutants, *eb36* and *eb41*, alter amino acids that are conserved among *Caenorhabditis* species (Figure S5), but the affected regions are of unknown functional significance. Two *mib-1* mutants, *eb37* and *eb39*, respectively mutate a splice donor for intron 7 or a splice acceptor for intron 8 (Figure S1), and both mutants were analyzed by RT-PCR. These analyses revealed that *mib-1(eb37)* mutants use a downstream cryptic splice acceptor that causes a frameshift and the encoding of nine novel amino acids before a premature stop codon. *mib-1(eb39)* mutants use a cryptic upstream splice acceptor to introduce eight novel amino acids into an otherwise normal MIB-1 protein sequence. The *mib-1(hc54)*, *mib-1(eb35)*, and *mib-1(eb42)* mutants affect MIB-1 ankyrin repeats, while *mib-1(eb33)* affects the second RING domain (Figure 7A, Figure S1, and Table S1); all are conserved (but not identical in the cases of *hc54* and *eb35*) in four other *Caenorhabditis* species (Figure S5). Ankyrin repeats facilitate protein–protein interactions and often occur in tandem or multiple arrays, as is seen for MIB-1 (Li *et al.* 2006), and RING-type zinc finger domains characterize a class of E3 ubiquitin ligases (Figure 7A; Lorick *et al.* 1999). RF E3 ubiquitin ligases accept ubiquitin from a donor E2 ligase to their RF, and they subsequently transfer ubiquitin to one or more substrates that either show modified activity or are targeted for proteolytic degradation. When MIB-1 biochemical activity is examined *in vitro*, it can self-conjugate to ubiquitin (Figure 7C; prominent ~260 kDa ubiquitin-positive band in left lane of left panel), and this depends on the presence of both an E2 donor and the MIB-1 RFs, confirming that it can function as an E3 ligase.

MIB-1 is expressed during spermatogenesis

The intrinsically temperature-sensitive nature of the *mib-1*-associated *Spe* phenotype facilitated the creation of a gene that expressed functional GFP::MIB-1. The *mib-1(eb154)* mutant (Figure S1) was transformed with a CRISPR construct composed of the missing *mib-1* genomic DNA sequence plus a 5' *gfp* sequence. Hermaphrodites were microinjected at the permissive 16° growth temperature and screened for elevated self-fertility at 25°, which resulted in *mib-1(ebIs26)* due to homologous gene replacement (Figure S6). *mib-1(ebIs26)* hermaphrodites show self-fertility that is ~42% wild-type when grown at 25° and ~81% wild-type when grown at 20°, but are indistinguishable from the wild-type control when grown at 16°. We compared brood sizes of *mib-1(ebIs26/+)*, *mib-1(ebIs26/mib-1(eb154))*, *mib-1(ebIs26)/ebIs26* and the wild type control. All genotypes showed a reduced brood size when grown at 25° as compared to 16° or 20°, but the most affected were the *mib-1(ebIs26)* homozygotes (Table 1).

The above data indicate that the placement of GFP at the N terminal end of MIB-1 in *mib-1(ebIs26)* resulted in a strain that was self-fertile. Although it had altered self-fertility as

Figure 7 *mib-1* protein, gene expression, and protein function. (A) Structure of the *C. elegans* MIB-1 protein, location of *mib-1* mutations, and a comparison to Mind Bomb proteins from other animals. (B) *mib-1* differential northern blot. A 2.4-kb band was detected in both strains but was more abundant in male RNA. The ubiquitously expressed *rla-1* was the loading control. (C) MIB-1 ubiquitin ligase *in vitro* activity assay. Purified GST-fusions of full-length MIB-1 (MIB-1) or a truncated MIB-1 that lacks the RING zinc finger domains (MIB no RF) were incubated in a reaction mixture in the presence (+) or absence (−) of the E2 ubiquitin-conjugating enzyme Ubch5a. Reaction mixtures were separated by SDS-PAGE and blotted. Ubiquitinated proteins and free ubiquitin were detected with an anti-ubiquitin antibody (left). MIB-1 self-ubiquitination only occurred in the presence of E2 and required the MIB-1 RING zinc finger domain. An anti-GST antibody (right) was used to confirm equal loading of fusion proteins. RING, ; RF, RING finger.

compared to wild-type, we utilized *mib-1*(*ebIs26*) to gain insight into the localization of MIB-1 during spermatogenesis because repeated attempts to generate a specific antibody to the natural MIB-1 protein were not successful (data not shown). Since we could not detect a GFP signal in living *mib-1*(*ebIs26*) animals, we used an antibody directed against GFP for indirect immunofluorescence. Examination of the entire germ line from *mib-1*(*ebIs26*) males by immunofluorescence revealed that GFP::MIB-1 expression occurs throughout the germ line and peaks in spermatocytes (meiotic region; Figure 8, A and B). The somatic gonad, including the distal tip cell, and spermatids (dtc and circle, respectively, in Figure 8, A and B) all have very little/no detectable GFP::MIB-1. Examination of sperm from dissected *mib-1*(*ebIs26*) males reveals that all detectable GFP::MIB-1 concentrates in the residual body as spermatids form (Figure 8, E and F) and that mature sperm lack detectable GFP::MIB-1 (data not shown). These data indicate that MIB-1 expression occurs in germ cells and peaks during spermatocyte stages in the testes.

mib-1 is expressed in vulval tissue and participates in *lin-12* signaling

C. elegans MIB-1 is homologous to the C-terminal half of Mind Bomb (Mib), an E3 ubiquitin ligase first described as a Notch

pathway component in the zebrafish *Danio rerio* (Figure 7A; Itoh *et al.* 2003). While *mib-1* expression is not confined to spermatogenesis (Figure 7B), no expression of the GFP::MIB from *mib-1*(*ebIs26*) was detected in vulval tissue where Notch signaling is known to be active so we took a promoter fusion approach, characterizing multiple lines that showed a bright GFP signal but failed to stabilize (see *Materials and Methods*). A diffuse signal is first observed during the third larval stage in the presumptive vulva region (L3; Figure 9, A and B). The signal reaches peak expression in D cells, with lesser expression in A and B cells, during the fourth larval stage (L4; Figure 9, C and D), and expression in A, B, and D cells persists in the adult vulval region (Figure 9, E and F). So, like transgenic reporters of the *C. elegans* delta/serrate/lag-2 (DSL) gene *lag-2*, *mib-1* is expressed within the developing vulva (Chen and Greenwald 2004).

In wild-type vulva development, two initially equivalent anchor cell (AC)/ventral uterine precursor cell (VU) precursor cells interact and, when one becomes the AC, the other becomes a VU (Kimble and Hirsh 1979). In wild-type worms, the AC expresses the *syIs50*[*cdh-3::gfp*] transgenic reporter (Figure 9, G and H; Pettitt *et al.* 1996; Inoue *et al.* 2002; Karp and Greenwald 2003). In *lin-12* gain-of-function (*gf*)

Figure 8 MIB-1 expression in the germ line. The male gonad (A and B) and sperm (E and F) from worms expressing GFP fused to the MIB-1 N terminal end was compared to the male gonad (C and D) and sperm (G and H) from non-transgenic control worms. Images are paired DIC (A, C, E, and G) and indirect immunofluorescence against GFP (B, D, F, and H). Germ line regions where pachytene (p), karyosome (k), and spermatocytes are located in the divisions of meiosis (d) are indicated. DAPI staining of DNA appears in blue and anti-GFP staining appears in red. rb, residual body; dtc, distal tip cell. The scale bar in (C) represents 20 μm and applies to (A–D); the scale bar in (G) represents 5 μm and applies to (E–H).

mutants, both AC/VU precursor cells adopt a VU fate (Greenwald *et al.* 1983) and no expression of the *syIs50[cdh-3::gfp]* AC reporter is observed (Figure 9, I and J). *lin-12(n302gf)* hermaphrodites are fertilization-competent, lack a vulva, and are egg-laying defective (Egl; Table 2; Ferguson and Horvitz 1985). Approximately 18–20% of double *lin-12(n302gf) mib-1(lf)* mutants express the *syIs50[cdh-3::gfp]* transgene (Figure 9, K and L) and lay eggs through a normal vulva (Table 2); such hermaphrodites can also mate and produce cross progeny (data not shown). Suppression of *lin-12(n302gf)* by *mib-1(lf)* was temperature-dependent, and not observed when *lin-12(n302gf) mib-1(lf)* double mutants were examined at a 20° or lower growth temperature (data not shown).

***mib-1* affects *glp-1* signaling**

C. elegans has another Notch paralog, **GLP-1**, and *glp-1* null mutants have germ line stem cells that all precociously com-

plete meiosis and differentiate as ~15 sperm; such mutants never make oocytes and, consequently, are self-sterile (Austin and Kimble 1987). The *glp-1(q231)* partial loss-of-function mutant has been used to recover suppressor mutants affecting genes in this Notch signaling pathway (Maine and Kimble 1993). We created a *glp-1(q231) mib-1(eb37)* double mutant that showed no suppression of the *Glp* phenotype at a 25° growth temperature (Table 1), and these double mutants had a *Glp* germ line (data not shown). However, we did find that *mib-1(eb37)* significantly suppressed the embryonic lethal phenotype exhibited by *glp-1(q231)* mutants when they were grown at 20° (Table 1); at this growth temperature, this double mutant likely has partial gene function for both *glp-1* (Safdar *et al.* 2016) and *mib-1*. The *glp-1(q231) mib-1(eb37)* double mutant grown at 20° also shows a reduced number of dead eggs (Table 1), presumably because many, but not all, sperm produced

Figure 9 *mib-1* expression and genetic activity in vulva cells. Paired Nomarski (left, A, C, E, G, I, and K) and corresponding fluorescence micrographs (right, B, D, F, H, J, and L) of the hermaphrodite vulva region are shown. (A–F) Expression of a *mib-1::gfp* transcriptional fusion from transgenic lines that did not stabilize. (A and B) *mib-1::gfp* is expressed in unidentified primary- and secondary-fated vulva cells during the wild-type (wt) hermaphrodite larval (L)3 stage; (C and D) in L4 and (E and F) adult-staged hermaphrodites, toroid vulva cells expressing *mib-1::gfp* are identified and cell positions indicated by red letters. (G–L) Expression of a *cdh-3::gfp* transgene is observed when an anchor cell (ac) is present. (G and H) In wt (*lin-12*+) hermaphrodites, the ac is located within the developing somatic gonad, above 1°-fated vulval cells; (I and J) *lin-12(n302gf)* animals do not have an ac and are unable to form a vulva; and (K and L) some *lin-12(n302gf) mib-1(lf)* double mutants have an ac and can form a vulva. The scale bar in (K) represents 10 μ m and applies to all panels.

by *mib-1* mutants are defective at this growth temperature (data not shown).

Genes that interact with other *glp-1* mutations have also been identified as suppressors (Dunn *et al.* 2010), and this includes the unusual *glp-1(ar202ts)* gain-of-function mutant (Pepper *et al.* 2003). When shifted from 16° to 25° as L1-stage larvae, on average, young *glp-1(ar202)* hermaphrodites produce <1 oocyte that is fertilized and either dies or hatches (Table 1). Older *glp-1(ar202)* hermaphrodites exhibit a proximal germ line tumor (Pro; Figure 10, A and B) and no evidence of oocytes. In contrast, the *glp-1(ar202) mib-1(eb37)* double mutant lays ~25 oocytes (Table 1) because this Pro phenotype is partially suppressed (Figure 10, E and F), while the *mib-1(eb37)* single mutant has wild-type-like

germ line morphology (Figure 10, C and D) and lays ~115 oocytes (Table 1) on the growth plate. Collectively, these data indicate that MIB-1 can affect GLP-1 activity in the *C. elegans* germ line and during embryogenesis.

Discussion

Prior studies in vertebrates and *Drosophila* have mainly focused on the role of Mind Bomb during Notch signaling and neuronal function. Although *mib-1* is widely expressed in adult animals, Mind Bomb null mutants die prior to becoming adults so analyses in the adult are challenging (*e.g.*, Schier *et al.* 1996; Lai *et al.* 2005; Koo *et al.* 2007). Informatics approaches suggested that a Mib-1 homolog is encoded by

Table 2 Suppression of the *lin-12gf* phenotype by *mib-1*

Genotype	% ac positive	n =	% nonEgl	n =
Wild-type (control)	100	69	100	100
<i>mib-1(eb36)</i>	100	73	100	67
<i>mib-1(eb37)</i>	100	88	100	76
<i>lin-12(n302gf)^a</i>	0	65	0	48
<i>lin-12(n302gf) mib-1(eb36)^a</i>	17.9	134	18.2	96
<i>lin-12(n302gf) mib-1(eb37)^a</i>	19.8	106	15.2	96

ac, anchor cell; nonEgl, non egg-laying defective.

^a Also contains *dpy-18(e364)*.

the *C. elegans* genome (Berndt *et al.* 2011); however, no prior work has reported characterization of *bona fide* *mib-1* mutants, despite their recovery during the Million Mutation project (Thompson *et al.* 2013). The role of *mib-1* during the *C. elegans* life cycle has been unclear because most phenotypic analyses are performed at a growth temperature of 20°, and here we show that *mib-1* loss-of-function/null mutant hermaphrodites are temperature-sensitive and only show self-sterility and other defects when worms are grown at 25°. This self-sterility is due to a failure of spermatogenesis and, despite prior work showing that *mib-1* is highly expressed in mammalian testes (Choe *et al.* 2007), this is the first clear link between MIB-1 and sperm function in any organism.

Prior work showed that a partial loss-of-function mutant of the sole ubiquitin-activating enzyme, *uba-1*, resulted in fertilization defects with no obvious defects in sperm cytology or motility (Kulkarni and Smith 2008). This is in contrast to *mib-1* mutants, which show a diverse spectrum of spermatogenesis defects, affecting both cytology and motility. In this regard, null *mib-1* mutants show several phenotypic similarities to the endo-RNAi pathway null mutants, *rff-3*, *eri-1*, *eri-3*, *eri-5*, and the *alg-3*; *alg-4* double mutant [*alg-3* and *alg-4* are redundant; reviewed by Billi *et al.* (2014)]. In fact, mutations in at least two genes, *fer-3* (Ward *et al.* 1981) and *fer-15* (Roberts and Ward 1982), that had been classified in the *Spe/Fer* phenotypic group, later proved to be alleles of *eri-3* and *rff-3*, respectively (Conine *et al.* 2013). All mutant lesions, including deletion null alleles, in the *mib-1* and endo-RNAi genes are temperature-sensitive because the processes affected by these genes are intrinsically temperature-sensitive and only seen at a ~25° growth temperature. Loss-of-function in any of these genes causes sterility because spermatogenesis is defective, but all have apparently normal oogenesis. Sometimes, meiosis in the testis is uncoupled from cell division, resulting in multinucleate, terminal spermatocytes. Many spermatocytes manage to divide and form spermatids, but some lack a nucleus and ~40–50% show a significant accumulation of tubulin in excess of the centriole that is, usually, the only detectable tubulin in wild-type spermatids. Those spermatids that activate to form spermatozoa can show abnormalities in pseudopod extension, including spike formation rather than a normal pseudopod. Both *mib-1* and the endo-RNAi mutant hermaphrodites have a *Him* phenotype (between 1 and 2% male progeny; ~10–20× greater than wild-type)

when grown at 23°, where all are somewhat self-fertile. However, there are two significant differences between the endo-RNAi and *mib-1* mutants. First, while all endo-RNAi mutants produce some spermatozoa that successfully fertilize oocytes, the resulting embryos die, while the rare *mib-1* sperm that fertilizes an oocyte becomes a zygote that usually develops normally. Second, some genes, including *unc-73*, are RNAi-resistant in wild-type worms but they become RNAi-sensitive in any of the endo-RNAi mutants; *unc-73* was not RNAi-sensitive in *mib-1* mutants (data not shown). The endo-RNAi pathway is thought to affect spermatogenesis by regulating the expression of genes via gene-specific 26G RNAs, and *mib-1* is one of the genes for which a 26G RNA has been found (Conine *et al.* 2010). Perhaps endo-RNAi mutants are *mib-1* null mutants because they lack *mib-1*-specific 26G RNA, thus explaining why *mib-1* and the endo-RNAi mutants share phenotypic features. However, this is not the case because we used RT-PCR to show that *mib-1* expression in *eri-1(mg366lf)* mutants does not differ from that seen in wild-type worms (data not shown).

In addition to the endo-RNAi mutants, there are many other mutants that share at least some phenotypic similarities with *mib-1*. For instance, spermatogenesis in a diverse collection of mutants is characterized by meiosis that loses the normal coordination with cellular differentiation. This can manifest itself in one of two obvious ways. First, the APC/C mutants *emb-27* and *emb-30* produce large numbers of anucleate sperm (97 and 81%, respectively; Sadler and Shakes 2000), which is seen for a smaller number (~13%) of *mib-1* sperm. One distinct difference is that the vast majority of *mib-1* sperm are not able to fertilize oocytes, and that when they rarely do the resulting embryo is usually viable, while these APC/C mutants produce sperm that are fertilization-competent, but the resulting embryo always dies (Sadler and Shakes 2000). Phenotypic defects similar to those seen in the APC/C mutants were subsequently found for the topoisomerase-2 (*top-2*) mutants (Jaramillo-Lambert *et al.* 2016).

Some *mib-1* spermatocytes arrest with four nuclei sharing a common cytoplasm, forming what has previously been named a terminal spermatocyte (l'Hernault and Arduengo 1992). In wild-type worms, spermatids retain the nucleus, mitochondria, and fibrous body (FB)-MOs as they separate from the residual body, and this requires both the actin and microtubule cytoskeleton. As budding is completed, all microtubules (other than the centriole), actin, and myosin (plus other cellular components) are placed into the residual body (Ward 1986). Recent work showed that the microtubules become disassociated from both the chromosomes and centrosomes, but retain γ -tubulin, as they reorganize into what develops as the residual body. This likely depends on a normally formed meiotic spindle being located in the right place at the proper time prior to residual body formation (Winter *et al.* 2017). Terminal spermatocytes are also observed in *spe-4* and *spe-5* mutants that have defects in proteins associated with FB-MOs, but these mutant spermatocytes also show abnormal distribution of tubulin (l'Hernault and Arduengo 1992; Machaca and l'Hernault 1997; Arduengo *et al.* 1998;

Figure 10 *mib-1* interaction with *glp-1* in the adult hermaphrodite germ line. Paired DAPI fluorescence (A, C, and E) and corresponding Nomarski micrographs (B, D, and F) of the hermaphrodite germ line on either side of the vulva (v) are shown. (A and B) The *glp-1(ar202)* mutant shows proximal

Gleason *et al.* 2012). Perhaps the temporal and spatial abnormalities of the meiotic spindle that are sometimes seen in *mib-1* (e.g., Figure 4, G–J), *spe-4*, and *spe-5* mutants prevents spermatocytes from properly polarizing and forming a residual body.

While the majority of *mib-1* spermatids have one nucleus, it tends to be eccentrically placed. This phenotypic defect is also observed in a number of other mutants that affect spermatogenesis in diverse ways, including those affecting endo-RNAi [reviewed by Billi *et al.* (2014)], *top-2*, (Jaramillo-Lambert *et al.* 2016), *spe-10*, and *spe-17* (Shakes and Ward 1989; Gleason *et al.* 2006). While the endo-RNAi and *top-2* mutants probably have direct effects in the nucleus on either gene expression or chromosome function/replication, *spe-10* and *spe-17* mutants affect the morphogenesis/function of FB-MOs (Shakes and Ward 1989; Gleason *et al.* 2006). How any of these diverse phenotypic defects are associated with the eccentric nuclear phenotype seen in any of these mutants is currently unclear.

Wild-type spermatids extend a single pseudopod only once, and its position relative to the cell body is fixed. In contrast, *mib-1* mutant spermatids extend a pseudopod that is frequently smaller than wild-type and can be unstable, changing its position relative to the cell body. Aspects of this phenotype are shown by *fer-1* mutants, which are unable to fuse their MOs with the cell surface and have a pseudopod that shows chaotic movement (Ward *et al.* 1981; Washington and Ward 2006). Motility of *mib-1* spermatozoa is severely impaired in nearly all cases, preventing all but the occasionally successful fertilization (Table 1). When such a fertilization event occurs at 25°, the resulting embryo usually grows up to become a spermatogenesis-defective adult with no obvious additional phenotypic features (data not shown). This indicates that *mib-1* loss-of-function does not exhibit any maternal effects on embryogenesis.

C. elegans mib-1(lf) mutants do not have any obvious Notch signaling defects when Notch is wild-type. However, analyses in genetically sensitized backgrounds revealed that *C. elegans MIB-1* plays a temperature-dependent, nonessential role in three well-defined Notch-mediated processes: LIN-12-mediated specification of the AC fate (Seydoux and Greenwald 1989), GLP-1 control of the meiosis/mitosis decision in the germ line (Austin and Kimble 1987), and GLP-1 control of cell-cell interactions during embryogenesis [reviewed by Priess (2005)]. For *lin-12*, this role was revealed because *mib-1(lf)* can suppress the *lin-12(n302gf)* vulvaless phenotype so that suppressed animals can form a

mitotic proliferation (Pro) near the vulva and no evident oocytes or embryos; (C and D) the *mib-1(eb37)* mutant produces oocytes (o) and an occasional embryo (e) because self-sterility is incompletely penetrant; (E and F) the *glp-1(ar202) mib-1(eb37)* double mutant shows suppression of the Pro phenotype and oocytes (o) are evident. All strains also included *dpy-18(e364)*. The scale bar in (F) represents 25 μ m and applies to all panels.

functional vulva that restores their ability to lay eggs. For *glp-1*, *mib-1(lf)* can suppress the *glp-1(ar202gf)* proximal mitotic (Pro) phenotype and the embryonic lethal phenotype of *glp-1(q231)* when propagated at 20°. While *lin-12(n302gf)* *mib-1(lf)*, *glp-1(ar202gf)* *mib-1(lf)*, and *glp-1(lf)* *mib-1(lf)* double mutants all show incomplete penetrance (respectively, 20, 83, and ~19% of individual worms show detectable suppression), this is typically the case for genetic suppression of either of these Notch paralogs (Tax *et al.* 1997; Katic *et al.* 2005; Katic and Greenwald 2006; Dunn *et al.* 2010; Safdar *et al.* 2016). In two of the cases, MIB-1 expression is detectable in the region (vulva precursor cells and proximal germ line) where the Notch paralog-associated phenotype is evident.

Mind Bomb was first described in *Drosophila* and zebrafish as regulating Notch ligand endocytosis, and *mib(lf)* mutants have reduced Notch signaling (Lai *et al.* 2001; Itoh *et al.* 2003; Chen and Casey Corliss 2004). In wild-type *C. elegans* hermaphrodites, the Notch ligand LAG-2 regulates both the LIN-12-mediated AC/VU decision during vulva formation and the GLP-1-mediated mitosis/meiosis decision during germ line formation (Tax *et al.* 1994). These L3–L4-stage phenomena are challenging to study in *lag-2* null mutants because they are L1 larval lethal, but other approaches have revealed the role of LAG-2 (Tax *et al.* 1997). The *lin-12(n302gf)* mutant is vulvaless and, when a double mutant with the rare *lag-2(n1255)* semidominant mutant is created, it restores vulva formation to ~95% of the hermaphrodites. Reducing LAG-2 activity by creating a *lin-12(n302gf)*; *lag-2(n1255)*/*lag-2* null strain reduces the degree of suppression by about one half. These data suggest that the suppression of *lin-12(n302gf)* we observe in *mib-1(lf)* mutants is probably not due to reduced LAG-2 activity (Tax *et al.* 1997; Katic *et al.* 2005). LAG-2 is also a ligand for GLP-1 in the *C. elegans* germ line, but its major expression is in the distal tip cell (Henderson *et al.* 1994), where we did not see any MIB-1 expression. Furthermore, *glp-1(ar202gf)*; *lag-2(q420ts)* conditional *lag-2lf* double mutants show an enhanced Pro phenotype (Pepper *et al.* 2003), which is opposite from the suppressed Pro phenotype we see in *glp-1(ar202gf)* *mib-1(lf)* double mutants. This suggests that suppression of the *glp-1(ar202gf)* Pro phenotype observed in *mib-1(lf)* mutants occurs by a mechanism other than downregulating LAG-2 function. The GLP-1 ligands APX-1 and ARG-1 are present in the proximal germ line and form what has been termed a “latent” niche. This “latent” niche does not affect wild-type worms because signaling-competent GLP-1 is not present in proximally located germ line cells. However, Pro mutants, such as *glp-1(ar202gf)*, encode a mutant GLP-1 that retains its capability to respond to ligand signal in the proximal germ line. Reducing APX-1, ARG-1, or especially both suppresses the *glp-1(ar202gf)* Pro phenotype (McGovern *et al.* 2009). If wild-type MIB-1 acts to degrade APX-1 and/or ARG-1, one would expect *glp-1(ar202gf)* *mib-1(lf)* double mutants (which have no MIB-1 activity) to have an enhanced, not suppressed, Pro phenotype. Complicating any interpretation

of Notch suppression is the fact that *mib-1(lf)* also suppresses *glp-1(lf)* in the embryo, but only under special conditions (20° growth) and not when the *glp-1* and *mib-1 lf* phenotypes are most severe (25° growth conditions); this is similar to what has been previously found for the UBR-5 ubiquitin ligase (Safdar *et al.* 2016). Collectively, these data indicate that *mib-1(lf)* likely suppresses *lin-12(gf)* and *glp-1(gf)* by some mechanism other than ligand degradation. The nature of the observed suppression suggests that MIB-1 acts either by degrading an unidentified negatively acting Notch pathway component or by facilitating signaling via a novel mechanism if acting through ligand degradation. Additional studies will be needed to determine whether these three examples of MIB-1-mediated Notch suppression occur via the same or different pathway component(s).

Screening for suppressor mutants of either the *lin-12* or *glp-1* mutant phenotypes has allowed the identification of many proteins that up- or downregulate one or both of these Notch signaling proteins [reviewed by Greenwald and Kovall (2013)]. For two genes, loss-of-function suppressor mutations were recovered that regulate Notch via ubiquitin E3 ligase activity. The first gene, *sel-10*, was identified as a suppressor of the partial loss-of-function *lin-12(n676n930)* mutant (Sundaram and Greenwald 1993). Subsequent work showed that SEL-10 is a CDC4 family F-box protein that is a component of a complex that has ubiquitin E3 ligase activity (Hubbard *et al.* 1997). The *sel-10* suppressors enhance the *lin-12(n379)* dominant, partially penetrant egg-laying defective phenotype, indicating that wild-type SEL-10 negatively regulates LIN-12/Notch signaling, presumably via ubiquitin-mediated negative regulation (Sundaram and Greenwald 1993; Hubbard *et al.* 1997). The second gene, *sog-1*, was identified as a suppressor of *glp-1(q224ts)* or *glp-1(q231ts)* loss-of-function mutations grown under semipermissive conditions (Maine and Kimble 1989, 1993). This gene was subsequently shown to encode a homologous to the E6-AP carboxyl terminus (HECT)-type ubiquitin E3 ligase homologous to UBR-5 (Safdar *et al.* 2016). *ubr-5*, like *mib-1*, has been evaluated in a *glp-1(ar202)* gain-of-function background. While *mib-1* loss-of-function mutations suppress the germ line phenotype caused by *glp-1(ar202)*, *ubr-5* loss-of-function mutations enhance *glp-1(ar202)*-associated tumor formation. So, the wild-type functions of the SEL-10 and UBR-5 ubiquitin E3 ligases are to downregulate Notch signaling, acting synergistically (Safdar *et al.* 2016), while the wild-type MIB-1 ubiquitin E3 ligase somehow facilitates Notch-mediated signaling.

Structure–function studies in *Drosophila* and zebrafish revealed that Mib/HERC2 and ZZ zinc finger domains in MIB-1 are essential for interaction with DSL ligands (Figure 7A; Itoh *et al.* 2003; Chen and Casey Corliss 2004; McMillan *et al.* 2015). *C. elegans* MIB-1 lacks these conserved domains, as do the highly homologous MIB-1’s (63% identical and 86% similar; Figure S5) encoded by four other *Caenorhabditis* species (data not shown). So, while genetic interactions indicate that MIB-1 participates in LIN-12/GLP-1 signaling, how, or if,

MIB-1 interacts with the *C. elegans* DSL protein LAG-2 is unresolved. While the Notch/LIN-12/GLP-1 pathway is made up of conserved components, there are prior cases where a component exhibited a protein sequence that deviated substantially from the consensus. For instance, Mastermind was first discovered in *Drosophila* (Nüsslein-Volhard *et al.* 1984; Smoller *et al.* 1990) and is readily detected by homology searches in vertebrates, but not in *C. elegans* (Petcherski and Kimble 2000a). The *C. elegans* Mastermind ortholog was eventually defined based on its interaction with LIN-12 and LAG-1 (the *C. elegans* ortholog of *Drosophila* suppressor of hairless) in yeast two-hybrid transcriptional activation assays (Petcherski and Kimble 2000b), genetic experiments (Doyle *et al.* 2000), and cocrystals analyzed by X-ray diffraction (Wilson and Kovall 2006).

The *lin-12(n302gf)* mutant has a vulvaless phenotype that is not affected by growth temperature. This vulvaless phenotype can be partially suppressed in *lin-12(n302gf) mib-1* double mutants, but only when the double mutant is grown at 25°. *mib-1* has not previously been recovered in extensive *lin-12* or *glp-1* suppressor selections, but these experiments either required suppressor activity at 20° or self-fertility at 25°, neither of which are true for *mib-1* (Tax *et al.* 1997; Katic *et al.* 2005; Dunn *et al.* 2010). Perhaps MIB-1 helps mediate heat-sensitive phenomena, such as the heat-shock response [reviewed by van Oosten-Hawle and Morimoto (2014)], to ensure that defective proteins are properly degraded. Alternatively, it could be a positive factor that activates a regulatory kinase, as has been observed for the innate immune response (Li *et al.* 2011). Prior work in other species has revealed that aspects of Notch signaling can be temperature-sensitive. One well-studied example is the *Drosophila rumi* mutant, which exhibits a temperature-sensitive Notch phenotype (Acar *et al.* 2008). The *rumi* gene encodes a polysaccharide O-glucose transferase that modifies Notch EGF domains, and the glycosylation events mediated by Rumi activity seem to stabilize them in a manner that is only essential at elevated growth temperatures (Leonardi *et al.* 2011).

In other species, Mib and Neuralized have redundant roles as E3 ubiquitin ligases during Notch signaling (Lai *et al.* 2005; Pitsouli and Delidakis 2005). However, while both facilitate ligand endocytosis, only *neuralized*, not *mib*, promotes ligand degradation (Deblandre *et al.* 2001; Lai *et al.* 2001). Although the expression pattern of the closest *C. elegans* *neuralized* homolog, F10D7.5, is not known, RNAi experiments report that it has an essential phenotype, including sterility (see www.wormbase.org). We have created a double *mib-1(lf); F10D7.5(gk487236)* *neuralized* mutant and, as far as we can tell, it does not differ from the single *mib-1(eb154)* mutant at either the 16 or 25° growth temperature (data not shown). These data suggest that, if there is a relationship between MIB-1 and Neuralized in *C. elegans*, it is not associated with an effect on viability or otherwise obvious.

In *C. elegans*, MIB-1 has been found to have roles in two prior RNAi reduction-of-function studies. *C. elegans smn-1*

mutants exhibit neuronal degeneration and a lowered rate of pharyngeal pumping. *mib-1* RNAi in *smn-1* mutants showed a partial restoration of the pharyngeal pumping rate, suggesting that MIB-1 targets SMN-1 for proteosomal degradation as occurs in cultured cells (Kwon *et al.* 2013). MIB-1 also seems to act together with LIN-18, which is a receptor tyrosine kinase active during WNT signaling in vulval precursor cells (Inoue *et al.* 2004). A bivulva phenotype was observed in ~34% of *lin-18(lf)* mutants, and this frequency was elevated to ~45% when *mib-1* RNAi was performed in *lin-18(lf)* mutant worms (Berndt *et al.* 2011). These authors also found a bivulva phenotype in ~24% of N2 worms subjected to *mib-1* RNAi, which is surprising as we have never observed this phenotype in any of the *mib-1* mutants characterized in our study. Perhaps this prior RNAi work unintentionally targeted both *mib-1* plus one or more additional genes involved in vulva morphogenesis.

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