Developmental Genetics of Secretory Vesicle Acidification During *Caenorhabditis elegans* Spermatogenesis

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ABSTRACT Secretory vesicles are used during spermatogenesis to deliver proteins to the cell surface. In *Caenorhabditis elegans*, secretory membranous organelles (MO) fuse with the plasma membrane to transform spermatids into fertilization-competent spermatozoa. We show that, like the acrosomal vesicle of mammalian sperm, MOs undergo acidification during development. Treatment of spermatids with the V-ATPase inhibitor bafilomycin blocks both MO acidification and formation of functional spermatozoa. There are several spermatogenesis-defective mutants that cause defects in MO morphogenesis, including *spe-5*. We determined that *spe-5*, which is on chromosome I, encodes one of two V-ATPase B paralogous subunits. The *spe-5* null mutant is viable but sterile because it forms arrested, multi-nucleate spermatocytes. Immunofluorescence with a *SPE-5*-specific monoclonal antibody shows that *SPE-5* expression begins in spermatocytes and is found in all subsequent stages of spermatogenesis. Most *SPE-5* is discarded into the residual body during spermatid budding, but a small amount remains in budded spermatids where it localizes to MOs as a discrete dot. The other V-ATPase B subunit is encoded by *vha-12*, which is located on the X chromosome. Usually, *spe-5* mutants are self-sterile in a wild-type *vha-12* background. However, an extrachromosomal transgene containing wild-type *vha-12* driven by its own promoter allows *spe-5* mutant hermaphrodites to produce progeny, indicating that *VHA-12* can at least partially substitute for *SPE-5*. Others have shown that the X chromosome is transcriptionally silent in the male germline, so expression of the autosomally located *spe-5* gene ensures that a V-ATPase B subunit is present during spermatogenesis.

VESICULAR organelles in eukaryotic cells frequently maintain an acidic pH (reviewed by Paroutis *et al.*

2004) that is created by the vacuolar H⁺-ATPase (V-ATPase). The V-ATPase is a large (~910-kDa) molecular machine that couples ATP hydrolysis to the movement of protons across biological membranes. The V-ATPase has a V₀-sector that creates the pore-for-proton translocation through the lipid bilayer and a V₁-sector, located in the cytoplasm, that is the site of ATP hydrolysis. Each V-ATPase holoenzyme is composed of 14 different subunits, some of which are present in multiple copies (reviewed by Toei *et al.* 2010). In yeast, there is one gene for each V-ATPase subunit, except for the “a” subunit, which is encoded by two genes (reviewed by Kane 2006). The physiological properties of the V-ATPase are in part determined by which of these two “a” subunits it contains (Kawasaki-Nishi *et al.* 2001). In humans and other animals, the “a” and other V-ATPase subunits are encoded by more than one gene (reviewed by Toei *et al.* 2010). Subunit diversity presumably allows the V-ATPase to be either

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customized for a specific function or utilized in a tissue-specific fashion.

In this article, we use pH-sensitive vital dyes and specific inhibitors to show that sperm-specific MOs use the V-ATPase to acidify their interior as spermatids mature. Pseudopodial extension during spermatid maturation into spermatozoa requires V-ATPase activity, suggesting a mechanistic connection between this process and MO acidification. We examined the microarray data for the 21 V-ATPase subunits encoded by *Caenorhabditis elegans* and found that there are two B-subunit paralogs with strikingly different patterns of expression (Reinke *et al.* 2000; Jiang *et al.* 2001). While the X chromosome-located *vha-12* gene showed somatic expression and the accompanying article in this issue by Ernstrom *et al.* (2012) shows the null phenotype of this gene to be lethal, the second gene encoding a B subunit showed upregulated expression during spermatogenesis. We used positional cloning techniques to show that the previously described *spe-5* gene, which is on chromosome I (Machaca and EHernault 1997), encodes this sperm-expressed B subunit. A null mutation in *spe-5* severely affects spermatogenesis, but it is not lethal, so we could examine the role of the V-ATPase during this process. *spe-5* mutants have defective MOs (Machaca and EHernault 1997) that do not get acidified during development, and immunofluorescence with specific antibodies revealed that *SPE-5* is located partly on MOs. Finally, placing *vha-12*, driven by its own promoter, in an extrachromosomal array allows this gene to partly rescue the self-sterility associated with *spe-5* null mutants, presumably due to *VHA-12* mis-expression during spermatogenesis. This suggests that *SPE-5* and *VHA-12* are functionally similar and that *spe-5* may have arisen to allow V-ATPase function during spermatogenesis when the X chromosome is transcriptionally quiescent (Kelly *et al.* 2002).

Materials and Methods

Strains, culture, and nomenclature

C. elegans var. Bristol (N2) (Brenner 1974) was the wild-type strain used for most experiments. Culturing, manipulation, and genetic analyses were performed as described (Brenner 1974) except as noted, and standard *C. elegans* nomenclature was employed (Horvitz *et al.* 1979). The following genetic markers were used: *dpy-5(e61)I* (Brenner 1974), *unc-38(x20)I* (Lewis *et al.* 1980), *mat-1(ax212)I* (Golden *et al.* 2000), *fer-1(b232)* (EHernault *et al.* 1988), *fem-3(q23gf)IV* (Barton *et al.* 1987), *fem-3(e1996)IV*, *spe-17(hcDf1)IV* (EHernault *et al.* 1993), *him-8(e1489)IV*, *him-5(e1490)V* (Hodgkin *et al.* 1979), *let-44(mg41)X*, and *lon-2(e678)X* (Brenner 1974). *spe-5* is located on chromosome I, and six alleles—*eb29*, *eb30*, *hc93*, *hc110*, *oz3*, and *oz46*—have been described previously (EHernault *et al.* 1988; Machaca and EHernault 1997). The *spe-5(ok1054)* deletion allele was provided by the *C. elegans* Knockout Consortium. Both balancer chromosomes *sDp2* (Howell

et al. 1987) and JK2739 *lin-6(e1466) dpy-5(e61) I/hT2[bli-4(e937) let-?(q782) qIs48](I;III)hT2[qIs48]/+ I; hT2/+ III* cover the left arm of chromosome I and include the genes *unc-38*, *spe-5*, and *dpy-5*. The balancer *mnDp31* (X;f) (Herman *et al.* 1979) covers both *let-44* and *lon-2*.

Genetic mapping and cloning of *spe-5*

Prior work placed *spe-5* between *unc-38* and *dpy-5* (Machaca and EHernault 1997). We mapped *spe-5* left of *mat-1* (see <http://www.WormBase.org>), which is located at -0.4 (Shakes *et al.* 2003) and relative to single nucleotide polymorphisms (SNPs) (Jakubowski and Kornfeld 1999). Primers (Supporting Information, Table S1) were used to PCR-amplify intervals between *unc-38* and *mat-1* from both the N2 and CB4856 Hawaiian (HI) strains (Hodgkin and Doniach 1997). These PCR products were sequenced and aligned to the complete N2 genomic sequence (Abbott *et al.* 1998) to identify SNPs (see Table S2) useful for mapping (Jakubowski and Kornfeld 1999). As summarized in Table S3, 20 recombinant worm strains were isolated from *unc-38(x20) spe-5(hc10) dpy-5(e61)/+* (HI) F₁ and used for SNP mapping.

Sperm cytological analyses

Individual hermaphrodites were picked to separate plates and transferred daily, and progeny and oocytes were counted until the worms stopped laying (EHernault *et al.* 1988). Males that either were or were not *spe-5(ok1054)I* and either *him-5(e1490)V*, *dpy-5(e61)I* or *him-8(e1489)IV* were analyzed; these *him* and *dpy* mutations did not obviously affect sperm cytology, consistent with earlier observations (e.g., EHernault *et al.* 1988). Individual males were dissected in 5–10 μ l of 1 $\times$ sperm medium (SM) [50 mM HEPES (pH 7.8), 25 mM KCl, 45 mM NaCl, 1 mM MgSO₄, 5 mM CaCl₂] supplemented with 10 mM dextrose [SM (pH 7.8)/dextrose] (Machaca *et al.* 1996). Spermatids were activated to become spermatozoa *in vitro* with Pronase or triethanolamine (TEA) (Ward *et al.* 1983; EHernault and Roberts 1995). The vital dyes LysoSensor Yellow/Blue DND-160 or LysoSensor Blue DND-192 were used to stain MOs. These dyes were provided at 1 mM (in dimethyl sulfoxide) from the manufacturer (Molecular Probes, Eugene, OR), and they were diluted to 1–10 μ M into SM pH 7.8/dextrose just before use. The fungal toxin bafilomycin A1 (LC Laboratories, Woburn, MA) was prepared as a 25 mM stock in dimethyl sulfoxide and diluted to 25 μ M in SM (pH 7.8)/dextrose. Males were dissected in SM (pH 7.8)/dextrose containing a LysoSensor vital dye, either with or without bafilomycin and/or the TEA activator, and incubated in a moist chamber for 15 min, after which slides were sealed with silicone grease and viewed.

An Olympus BX60 equipped with either a Plan-Apo 1.4 numerical aperture (N.A.) $\times 60$ or a 1.35 N.A. $\times 100$ oil-immersion objective lenses or a Zeiss Axiophot compound microscope equipped with either a Plan-Apo 1.4 N.A. $\times 63$ or a 1.4 N.A. $\times 100$ oil-immersion objective lens was used to

view sperm. Images in Figures 2 and 7 were captured with either a DAGE 300T CCD (DAGE Instruments, Michigan City, IN) or a SensiCam digital camera (Cooke, Auburn Hills, MI) using either National Institutes of Health Image or Image-Pro PLUS (Media Cybernetics, Silver Spring, MD) together with VolumeScan 3.1 (VayTek, Fairfield, IA). Twelve-bit images from the SensiCam were converted to 8 bits in Image-Pro PLUS. In the case of Figures 3 and 8, Z-axis stacks of 18–23, 12-bit images were collected every 0.3 μ m using SlideBook software (Intelligent Imaging Innovations, Denver, CO). Resulting images were subject to a nearest-neighbor deconvolution algorithm and exported as 16-bit tif images. Images were compiled using Canvas 9 (ACD Systems, Miami, FL) or PhotoShop CS3 (Adobe Systems, San Jose, CA). The deconvolved images in Figure 8 are shown as individual slices for the red, green, and red/green/blue triple overlays and also as complete Z-axis stacks for the triple overlay.

Real-time PCR

Total RNA was isolated from *him-5(e1490)* males, *him-5(e1490)* hermaphrodites, *spe-5(ok1054) him-8(e1489)* males, *spe-5(ok1054) him-8(e1489)* hermaphrodites, *fem-3(q23gf)* hermaphrodites with a masculinized germline, and *fem-3(e1996 lf)* hermaphrodites with a feminized germline. For each genotype, 30 adult worms were picked into 10 μ l of a 1:10 dilution of worm lysis buffer [50 mM KCl, 10 mM Tris-Cl (pH 8.3), 2.5 mM MgCl₂, 0.45% NP-40, 0.45% Tween-20] and frozen in liquid N₂. The frozen worms were treated with Trizol/chloroform, RNase free glycogen was added as carrier, and RNA was precipitated with isopropanol (see http://www.pristionchus.org/wiki/index.php/RNA_preparation_from_few_worms). RNA was resuspended in RNase/DNase-free water. cDNA was synthesized using 200 ng of total RNA template with the iScript cDNA synthesis kit (Bio-Rad, Richmond, CA). Quantitative RT-PCR (qRT-PCR) was performed with 10 ng of total cDNA using the SsoFast EvaGreen Supermix (Bio-Rad) in a Bio-Rad CFX-96 real-time PCR system. The following primers were used to amplify cDNA levels: *spe-5* (prMH26 and prMH27), *vha-12* (prMH24 and prMH25), and *rpa-1* (prMH30 and prMH31) (see Table S1). The RNA sequences of *spe-5* and *vha-12* are >75% identical throughout most of their sequences (not shown), so primers were designed to unique regions that showed no cross hybridization between these two paralogous sequences. A dilution series of *him-5* hermaphrodite cDNA was used to calculate an arbitrary amount for each sample and primer set. The arbitrary amounts were normalized to the standard, which was the ubiquitously expressed mRNA (*rpa-1*) (Spieth *et al.* 1991), and the results were plotted as fold difference. RNA was isolated three independent times for worms of each genotype. The three RNA samples prepared for each worm genotype were subjected to qRT-PCR in triplicate (nine PCRs), and error bars representing standard error of the mean are shown.

Synthesis of cDNA and expression constructs

Total RNA was prepared from *fem-3(q23gf)* masculinized hermaphrodites (Barton *et al.* 1987) and used to create cDNA libraries according to published procedures (Gleason *et al.* 2006). The 5' end of the *spe-5* gene was amplified from the cDNA library by a nested PCR reaction using the Clontech SMART II primer and *spe-5* gene-specific reverse primers PDHLA, PDHLb, and PDHLA2 (Table S1). The 3' end of the *spe-5* gene was amplified by a nested PCR reaction using the Clontech CDS primer and two *spe-5* gene-specific forward primers, PDHra and PDHrb (Table S1).

Three *C. elegans* full-length cDNA constructs were used to create bacterial expression constructs for *spe-5*, *vha-12* (F20B6.2, a *spe-5* paralog), and W05B10.1 (histone 3.3) by previously described techniques (Gleason *et al.* 2006).

Construction of a vha-12 transgene

The plasmid pRW05 was created by cutting the recombinant cosmid C04E10 insert with *Apa*LI and subcloning the 6592-bp *Apa*LI fragment that included the *vha-12*-coding sequence (F20B6.2) into the Litmus 28 plasmid vector (New England Biolabs, Ipswich, MA). pRW05 has 3029 bp 5' to the *vha-12* start codon and 1933 bp 3' to the stop codon (1520 bp 3' from the end of the predicted transcript for *vha-12*). pRW05 (2 μ g/ml), pPD118.20 (10 μ g/ml; 1997 Fire Lab Vector Kit), a plasmid that contains the *myo-3* body-wall muscle promoter driving GFP, and carrier pBluescript II KS+ (76 μ g/ml; Stratagene) were microinjected into *let-44(mg41) lon-2(e678)X; mnDp31(X);f* hermaphrodites by standard techniques (Mello and Fire 1995). *Lon* animals that were GFP+ were selected to recover the *oxEx551* extrachromosomal transgenic array; *let-44(mg41)* proved to be an allele of *vha-12* (R. Weimer and E. Jorgensen, personal communication).

DNA sequencing

DNA sequencing was performed at either Iowa State University or the University of Michigan using standard ABI (Perkin-Elmer, Foster City, CA) automated fluorescent sequencing methods for PCR products. Genomic DNA was prepared from picked *spe-5* homozygous mutants (Barstead *et al.* 1991) and used to PCR-amplify the chromosome I Y110A7A.12 gene (Table S1). PCR products were pooled, purified using either GeneClean II (Bio 101, Carlsbad, CA) or the Qiaquick PCR purification kit (Qiagen, Valencia, CA), and sequenced to identify the mutant lesions.

Antisera preparation

We screened preimmune sera by immunoblot and immunofluorescence and identified rabbits that did not react with *C. elegans* proteins. Peptides CTNHRITALIQNYSTKPKLT and CGRNNETVN, respectively, corresponded to residues 23–40 and 211–218 of the SPE-5 polypeptide sequence (Figure 4), except for the underlined N-terminal cysteine. Peptide sequences were selected on the basis of antigenic probability (Hopp and Woods 1983) and low sequence identity to the *C. elegans* VHA-12 paralog. The N-terminal cysteine was

used to conjugate these peptides to keyhole limpet hemocyanin carrier protein. Each conjugated peptide was used to immunize two rabbits using standard protocols (Zymed Laboratories, South San Francisco, CA), and only the polyclonal antiserum R1 (from a rabbit immunized with the 23-40 peptide) proved specific. Affinity purification of SPE-5 antiserum R1 was performed as described previously (Gleason *et al.* 2006), and the glycine-eluted fraction was used for all experiments.

For monoclonal antibody production, the multiple antigen peptide (MAP) TEASEINLSDIKPIDVNTPTNHRITALIQ (residues 2–31) was synthesized (Tam 1989) and used to immunize mice. Tail bleeds were screened for MAP reactivity, the spleen from a reactive mouse was excised, and individual splenocytes were fused to multiple myeloma cells by polyethylene glycol-mediated fusion. Resulting hybridomas were screened with the MAP by limit dilution screening, and one reactive cell clone was recovered. This clone was grown in Dulbecco's modified Eagle's medium with hypoxanthine/aminopterin/thymine selection, and the culture supernatant, referred to below as the SPE-5 monoclonal antibody (Mab), was used in the experiments described in this article.

Immunoblots

The *C. elegans* SPE-5, VHA-12, and histone 3.3 (W05B10.1) fusion proteins were prepared from recombinant BL21 (DE3) Codon Plus RIPL *Escherichia coli* (Stratagene) that were induced with 1 mM IPTG at 25°. One milliliter of induced cultures was pelleted by centrifugation, resuspended in 100 µl 2× Laemmli sample buffer (Laemmli 1970), and boiled for 5 min, and 20-µl aliquots were loaded onto gels. Larger-scale SPE-5 fusion protein purification or sperm lysates and Western blotting were as described previously (Gleason *et al.* 2006). Western blots were probed with a monoclonal antibody to the c-MYC epitope [2 ng/ml; Clontech; used to confirm the authenticity of the SPE-5::Myc fusion protein (not shown)], SPE-5 R1 glycine affinity-purified primary antibody (5 or 50 µg/mL), or the SPE-5 Mab (1:1000) followed by a goat anti-rabbit (R1) or goat anti-mouse (c-MYC and SPE-5 Mab) IgG HRP-conjugated secondary antibody at 1:5000 (BioRad). ECL Plus (GE Healthcare) and BioMax MS Film (Kodak) were used for signal detection.

Immunocytochemistry

Sperm from male worms were prepared for immunohistochemistry by modifications to the freeze/crack methanol fixation method (Miller and Shakes 1995). Dissections of worms were performed on Colorfrost/Plus microscope slides (Fisher Scientific, Pittsburgh, PA) to facilitate adhesion. The SPE-5 Mab was incubated with samples overnight at a 1:1000 dilution for all immunofluorescent experiments described in this article. The secondary antibody was a goat anti-mouse IgG conjugated to horseradish peroxidase (Molecular Probes), and visualization was by 5 min of tyramine signal amplification (TSA) (Bobrow *et al.* 1989; Chao *et al.* 1996) with Alexa Fluor 488 conjugated to tyramine (Invitrogen).

Mouse monoclonal antibody 1CB4 (Okamoto and Thomson 1985; Arduengo *et al.* 1998) was used after completion of the SPE-5 Mab incubation/TSA enhancement step at 1:2000 to detect the fibrous body-membranous organelle (FB-MO) complexes. 1CB4 was visualized using a Texas Red-conjugated, affinity-purified goat anti-mouse IgG secondary antibody (Fab¹)₂ fragment at a final concentration of 0.75 µg/ml (Jackson ImmunoResearch Laboratories, West Grove, PA). Control experiments indicated that the labeled secondary (Fab¹)₂ fragment used to visualize 1CB4 localization did not also generate a detectable level of fluorescence due to cross-reaction with the SPE-5 Mab (data not shown). Resulting slides were mounted in ProLong Antifade (Molecular Probes), dried overnight, and viewed through appropriate filters in an Olympus BX60 microscope, and images were analyzed as described above (see *Sperm cytological analyses*).

Results

Secretory vesicles become acidified during *C. elegans* spermatogenesis

During *C. elegans* spermatogenesis, the unusual secretory vesicles FB-MOs play a major role in the accumulation and transport of macromolecules into developing spermatids (Figure 1; reviewed by LHernault 2009; Nishimura and LHernault 2010). The FB-MOs segregate into spermatids as they bud from the anucleate residual body (Figure 1C). As budding is completed, the membrane surrounding the FB retracts and the major sperm protein fibrous contents depolymerize into constituent dimers. The remaining portion of the FB-MO, now called the MO, localizes close to the plasma membrane (Figure 1D). Subsequently, the sessile spermatid activates to become a spermatozoon that crawls via its single pseudopod (Figure 1E), as is the case in many other nematodes (reviewed by Stewart and Roberts 2005). Successful participation of a spermatozoon in fertilization requires that MOs fuse with and insert new proteins into the plasma membrane. They also secrete their contents onto the cell surface, but the role of this process in sperm function is not yet clear (reviewed by LHernault 2009; Nishimura and LHernault 2010).

The secretory role shown by MOs (Figure 1E) suggests that they could be related to lysosomes or the acrosome (found in flagellated sperm), both of which can fuse with the cell surface (reviewed by Moreno and Alvarado 2006; Luzio *et al.* 2007). Both lysosomes and the acrosome can be stained with acidotropic vital dyes (Lin *et al.* 2001, 2003; Sun-Wada *et al.* 2002; Codelia *et al.* 2005), and we tried this approach to determine if FB-MO secretory vesicles maintain an acidic interior. We screened a variety of pH-sensitive fluorescent vital dyes and discovered three useful ones. LysoTracker Blue DND-192, LysoTracker Green DND-26, and LysoSensor Yellow/Blue (Y/B). These dyes fluoresce maximally under low-pH conditions (Molecular Probes/Invitrogen, Carlsbad, CA), and all showed similar staining patterns.

Figure 1 *C. elegans* spermatogenesis and its coordination with fibrous body-membranous organelle (FB-MO) morphogenesis. (A) Primary spermatocytes contain many nascent FB-MOs (in red) that each have a membranous organelle head (h) separated by a collar (c) from the body (b), which envelopes the growing fibrous body (FB). (B) Secondary spermatocytes frequently divide incompletely and contain full-sized FB-MOs. (C) Spermatids retain FB-MOs as they bud from the residual body and the FB disassembles. (D) Budded spermatids contain MOs that localize beneath the plasma membrane (pm) and have active V-ATPase (V_0 sector in orange and V_1 sector in green; not drawn to scale). (E) Maturation of spermatids into spermatozoa is associated with fusion of MOs with

the plasma membrane (pm) and extension of a motile pseudopod. The presence of the V_1 subunit in association with V_0 in spermatozoa has not yet been conclusively shown. Red lines lead from one representative FB-MO (A–C) or MO (D and E) in a cell to an expanded view of this structure (modified from L'Hernault 2006).

Developing wild-type sperm show a remarkable staining pattern after treatment with LysoSensor Y/B (Figure 2, A and B). Primary spermatocytes (1 in Figure 2B), secondary spermatocytes (2 in Figure 2B), and budding spermatids (3 in Figure 2B) all show a blue staining pattern that is both uniform and dim. Spermatids (4 in Figure 2A) that have separated from the residual body (rb) have bright blue dots superimposed on dim background staining (4 in Figure 2B). The blue dots observed in spermatids (left cell in Figure 2, C and D) disappear after they mature into spermatozoa (right cell in Figure 2, C and D). The size and position of the blue dots suggest that they are MO secretory vesicles (reviewed by L'Hernault 2009; Nishimura and L'Hernault 2010).

Certain spermatogenesis-defective (*spe* or *fer*) mutants change the position and/or function of sperm secretory vesicles, and they were examined to help determine the identity of the above-described blue dots. The *fer-1* mutant makes MO secretory vesicles, but they do not fuse with the cell surface when a pseudopod is extended (Ward *et al.* 1981). Spermatids from a *fer-1* mutant (Figure 2, E and F, left cell) stain with LysoSensor Y/B in a dotted pattern (Figure 2F, left cell) that is similar to wild type (Figures 2D, left cell). However, unlike wild type (right cell in Figure 2D), *fer-1* spermatozoa continue to show cortical blue dots (right cell in Figure 2F), presumably because secretory vesicles do not fuse with the plasma membrane. Staining *spe-17* mutants with an antibody (1CB4) specific for MO secretory vesicles (Okamoto and Thomson 1985; Arduengo *et al.* 1998) reveals that they cluster opposite the nucleus in this mutant (Figure 2, H and K), rather than exhibiting the cortical location seen in wild-type spermatids (Figure 2, I and L). Like 1CB4 staining, the LysoSensor Y/B staining pattern in *spe-17* mutant spermatids was clumped to one side (Figure 2, G and J, arrows). These observations confirm that LysoSensor Y/B stains MO secretory vesicles in *C. elegans* spermatids. Taken together, these data indicate that MO secretory vesicles are acidified during development and that H^+ is released from these organelles during maturation of spermatids into spermatozoa.

Acidification of MO secretory vesicles and sperm activation require V-ATPase function

The above data indicate that secretory vesicles are acidified in a manner that is coordinated with spermatogenesis. We found that treating developing spermatids with bafilomycin A1, a specific inhibitor of the V-ATPase (Drose and Altendorf 1997), prevented LysoSensor Blue DND-192 staining of the MO secretory vesicles during *C. elegans* spermatogenesis (compare cortical dots in the Figure 3, B and D, control to their absence in Figure 3F, which is bafilomycin treated). We also attempted to activate spermatids into spermatozoa in the presence of bafilomycin. While control treatment with TEA (Ward *et al.* 1983) causes spermatids to extend a pseudopod and begin to move (Figure 3, I and J), bafilomycin treatment prevents completion of spermatid activation (Figure 3, G and H). Instead, bafilomycin-treated spermatids arrest at an intermediate stage (Shakes and Ward 1989) since they extend surface spikes that do not transition into a normal pseudopod (Figure 3G, arrows). As expected, no detectable MO staining was observed in TEA-treated spermatids that became spermatozoa in the absence of bafilomycin (Figure 3J) or were inhibited from maturing into spermatozoa by bafilomycin treatment (Figure 3H).

V-ATPase mutant that specifically affects spermatogenesis

The *C. elegans* genome encodes 21 V-ATPase subunits (see Figure 1A of the accompanying article in this issue by Ernstrom *et al.* 2012), and we examined their expression and RNA interference characteristics (see <http://www.wormbase.org/>, Jiang *et al.* 2001, and Reinke *et al.* 2000). Only the *Y110A7A.12* gene, which encodes a 502-amino-acid B subunit, showed both elevated expression during spermatogenesis and a viable (not dead) RNA interference (RNAi) phenotype when assayed by feeding; these are characteristics of many sperm-expressed genes (Kamath *et al.* 2003). *Y110A7A.12* is between *unc-38* (−0.65) and *mat-1* (−0.4) on chromosome I, and our mapping placed *spe-5* within this interval (see

Figure 2 The position and staining of MO secretory vesicles during *C. elegans* spermatogenesis. (A, C, E, G, H, and I) Nomarski DIC. (B, D, F, and J) LysoSensor Y/B fluorescent vital staining of MO secretory vesicles. (K and L) Indirect immunofluorescence of fixed cells with the 1CB4 antibody to reveal MO secretory vesicles. (A and B) Various cellular stages of spermatogenesis. (1) Primary spermatocyte in anaphase of meiosis I. (2) Secondary spermatocytes forming from a dividing primary spermatocyte. (3) Spermatids budding from residual bodies (rb). (4) Spermatids that have completed budding from the residual body. (C and D) A wild-type spermatid (left) and spermatozoon (right). (E and F) A *fer-1(b232)* spermatid (left) and spermatozoon (right). (G and H) A *spe-17(hcDf1)* spermatid (left) and spermatozoon (right). (I and J) Wild-type spermatids. (K and L) *spe-17(hcDf1)* spermatids. p, pseudopod of the spermatozoon. Arrows in D, F, J, and K point to MO secretory vesicles in fluorescent images or, in the case of G and H, their location in DIC images.

Materials and Methods) between SNP 64 (left) and SNP 79 (right) (Table S2 and Figure S1; detailed data available on request). Y110A7A.12 was amplified from the seven *spe-5* alleles, and each was found to have a unique mutation in this gene (Figure 4).

Three *spe-5* EMS-induced mutants each contain a missense mutation. The *spe-5(eb30)* g-to-a transition mutation changes codon 172 from Asp to Asn. The *spe-5(hc110)* g-to-a transition mutation changes codon 204 from Gly to Arg. The *spe-5(eb29)* c-to-t transition mutation changes codon 382 from Pro to Ser. Two of these missense mutations (*eb29* and *eb30*) caused nonconservative changes in residues that are identical in all cloned B subunits from yeast to mammals. *hc110* changes an amino acid that, although conserved among nematode sequences, is not conserved in other eukaryotes (Figure 4).

The *spe-5(oz3)* mutation arose in a *mut-3* genetic background (Collins *et al.* 1987), and this allele has a Tc1 insertion at position 1073 in exon 3 of the *spe-5* genomic sequence. This mutant has been extensively outcrossed to Bristol N2, and the Tc1 insertion in *spe-5(oz3)* is stably inherited in this genetic background. However, PCR of *spe-5(oz3)* genomic DNA with primers that flank the mutation site always show two bands, one where the ~1.6-kb Tc1 is inserted into *spe-5* and a second, smaller band of approxi-

mately the wild-type size (not shown). This second, smaller band contains a TATG footprint at the point where Tc1 was inserted. Presumably, Tc1 is imprecisely excised in somatic tissue but maintained stably in the germline, including during spermatogenesis, as would be expected for the Bristol strain (Emmons and Yesner 1984). When imprecise excision occurs, the *spe-5(oz3)* gene would encode 328 amino acids of SPE-5 plus 1 amino acid encoded by Tc1 sequence before termination at a Tc1-derived stop codon. The *spe-5(oz3)* mutant, with an intact Tc1 insertion, is predicted to encode the first 327 amino acids of SPE-5 plus 18 amino acids encoded by Tc1 sequence before termination at a stop codon derived from Tc1 sequence. Therefore, *spe-5(oz3)* mutants are predicted to produce a truncated SPE-5 protein that is ~65% the wild-type size, whether or not the Tc1 in *spe-5* is intact or imprecisely excised (Figure 4).

Two EMS-induced *spe-5* alleles (*hc93* and *oz46*) cause nonsense mutations. The *spe-5(hc93)* g-to-a transition mutation is located near the 3' end of the gene, where it changes codon 473 into an UGA opal stop codon. The *spe-5(oz46)* mutation is an 8-bp deletion that removes codons 319, 320, and the first two bases of codon 321. This results in 318 amino acids of the correct SPE-5 protein sequence followed by a frame-shift mutation that causes insertion of 6 novel amino acids before termination at a premature UGA opal

Figure 3 Bafilomycin A1 affects vital dye staining of MO secretory vesicles in *C. elegans* spermatids. (A, C, E, G, I, and K) Nomarski DIC. (B, D, F, H, J, and L) Lyso-Sensor Blue DND-192 fluorescent staining of MO secretory vesicles. (A, B, C, and D) Wild-type spermatids and spermatids budding from residual body (rb) under control (no drug) conditions. (E, F, G, and H) Wild-type spermatids treated with 25 μ M bafilomycin A1. (G, H, I, and J) Wild-type spermatids activated with 70 mM TEA (pH 7.8). Arrows in G point to fine processes extending from cells. (I) p, pseudopods on spermatozoa. (K and L) *spe-5(ok1054)* budding-arrested terminal spermatocytes.

stop codon; the missing 183-amino-acid region is almost identical in all sequenced V-ATPase B subunits (Figure 4).

The *C. elegans* Knockout Consortium recovered the *spe-5(ok1054)* deletion mutation that removes the *spe-5* start codon, the first two exons and introns, and part of exon 3, so it is a candidate null allele. *spe-5* is the third gene in an $\sim$ 4.6-kb operon designated CEOP1216, and the *spe-5(ok1054)* deletion also removes the 72-bp 5' UTR, including the predicted acceptor for the SL2 spliced leader (that we empirically confirmed by RT-PCR) (Figure S2), plus another 100 bp of intergenic sequence. The second gene in this operon is *chp-1* and GeneFinder predicts that 57 bp separate the *spe-5(ok1054)* deletion from the predicted 3' end of the *chp-1* transcript. Like all other *spe-5* point and transposon mutants characterized previously (Machaca and LHernault 1997), *spe-5(ok1054)* causes a spermatogenesis-defective phenotype and no evident embryonic or somatic defects (data not shown). In contrast, RNAi knockdown of *chp-1* expression causes an embryonic lethal phenotype (e.g., Fraser *et al.* 2000; Sonnichsen *et al.* 2005), which suggests that the *ok1054* deletion affects only *spe-5*. These data indicate that the null phenotype of *spe-5* is likely to be spermatogenesis-defective without any obvious somatic defects. We examined *spe-5(ok1054)* mutants with pH-sensitive dyes and found, as expected, that there is no detectable staining of MOs (Figure 3L: compare to Figure 3D control).

spe-5 expression

We used a qRT-PCR approach to explore the relationship between spermatogenesis and mRNA expression of both *spe-5* (Figure 5A) and its paralog *vha-12* (Figure 5B). Three matched pairs of mRNA samples were evaluated for *spe-5*

and *vha-12* expression: males with and without *spe-5* function (Figure 5, gray bars), somatic “females” with either a male or a female germline (Figure 5, red bars), and post-spermatogenesis adult hermaphrodites with and without *spe-5* function (Figure 5, green bars). *spe-5* mRNA is dramatically upregulated in wild type males as compared to *spe-5* mutant males (Figure 5A, gray bars) and also in *fem-3* gain-of-function worms (*gf*; female soma, male germline; Figure 5A, left red bar) as compared to *fem-3* loss-of-function worms (*lf*; female soma, female germline; Figure 5A, right red bar). Adult wild-type hermaphrodites continue to show significant *spe-5* expression after they have completed spermatogenesis (Figure 5A, green bar), as do *fem-3* loss of function (*lf*; female soma, female germline) worms (Figure 5A, right red bar). These data indicate that, while *spe-5* expression is upregulated during spermatogenesis, it is also expressed in one or more other tissues.

Within each of the above-discussed matched pairs of mRNA samples, *vha-12* showed none of the expression biases observed for *spe-5*. However, *vha-12* expression was higher in worms with a somatic male phenotype (Figure 5B, gray bars) as compared to worms with a somatic female phenotype (Figure 5B, red and green bars), suggesting that one or more male somatic tissues contributed to this signal; males show significant anatomical differences from females in many tissues (Emmons 2005).

vha-12 can partially substitute for its *spe-5* paralog

Perhaps the small amount ($\sim$ 15%) of sequence differences between *SPE-5* and its *VHA-12* paralog help tailor the V-ATPase to play a sperm-specific role. To test this possibility, we examined if an extrachromosomal transgene containing

Figure 4 Alignment of V-ATPase B polypeptides from *C. elegans*, *C. briggsae*, *Drosophila melanogaster*, human, *Schizosaccharomyces pombe*, and *Saccharomyces cerevisiae*. Red boxes indicate identical residues among all shown B polypeptides. Black boxes show identity to the *C. elegans* sequence. Both the *spe-5(hc93)* point mutation and the *spe-5(oz46)* deletion mutation introduce premature nonsense mutations in *C. elegans* SPE-5, and they are indicated by asterisks (*). The amino acid change(s) in the missense *spe-5(eb30)*, *spe-5(hc110)*, and *spe-5(eb29)* mutants is indicated after an arrow. The position of a Tc1 insertion in the *spe-5(oz3)* mutant is indicated by a yellow triangle. Braces indicate the portion of the SPE-5 polypeptide deleted in the *spe-5(ok1054)* mutant; the deletion actually extends into the 5' noncoding region of the *spe-5* gene (not shown). Alignment was performed using the ClustalW2 program (Larkin *et al.* 2007). The Genbank accession number for *C. elegans* SPE-5 is NP_491518.1; for *C. briggsae* SPE-5, CAE65728.1; for *C. elegans* VHA-12, NP_508711.1; for *C. briggsae* VHA-12, CAE68535.1; for *Drosophila*, NP_476908.1; for human 1, NP_001683.2; for human 2, AAH30640.1; for *S. pombe*, NP_594623.1; and for *S. cerevisiae*, NP_009685.1.

Figure 5 qRT-PCR on total RNA. (A) *spe-5* mRNA levels. (B) *vha-12* mRNA levels. Gray bars represent mRNA levels from *him-5* male worms ("wt") and *spe-5(ok1054) him-8* males. Red bars indicate mRNA levels in *fem-3(q23gf)* masculinized hermaphrodites and *fem-3(e1996lf)* feminized hermaphrodites. Green bars show mRNA levels from *him-5* hermaphrodites ("wt") and *spe-5(ok1054) him-8* hermaphrodites. Three independent RNA samples were isolated and, for each of these three samples, qRT-PCR was performed in triplicate; error bars are the standard error of the mean. Matched colored bars represent data that were normalized relative to *rpa-1*, which is a ubiquitously expressed housekeeping gene (Spieth *et al.* 1991).

vha-12-coding sequence and its 5' and 3' flanking regulatory sequences (*oxEx551*; see *Materials and Methods*) could affect the self-sterility of *spe-5* strains. While *spe-5(hc93) dpy-5* mutant hermaphrodites are nearly self-sterile (mean brood size = 2 ± 2 , $n = 12$), *oxEx551/spe-5(hc93) dpy-5* have elevated self-fertility (mean brood size = 53 ± 11 , $n = 8$). This brood size is $\sim 36\%$ of the *dpy-5* control worms (mean brood size = 146 ± 24 , $n = 12$), and it indicates that *VHA-12* misexpression during spermatogenesis can partially replace the *SPE-5* that is missing in *spe-5* loss-of-function mutants. Like the germline expression of other transgenes (Kelly *et al.* 1997), this *vha-12*-containing transgene undergoes epigenetic silencing, and its effect on *spe-5*-associated self-sterility progressively diminished as the strain was propagated (data not shown). Nonetheless, this experiment shows that *vha-12* can, at least partially, substitute for *spe-5*.

***SPE-5* expression during spermatogenesis**

We tested our antibodies on bacterially expressed *SPE-5*, *VHA-12*, and a histone control to allow rigorous characterization of specificity. Immunoblots of gels containing these crude bacterial lysates reveal that both the mouse monoclonal (Figure 6A) and the R1 affinity-purified rabbit polyclonal antiserum (Figure 6B) were *SPE-5*-specific and showed no cross-reaction to either *VHA-12* or the histone control.

These antisera were then characterized on immunoblots of whole-worm lysates prepared from somatically female

animals with germlines that produced only oocytes (*fem-1lf*) or only sperm (*fem-3gf*). *SPE-5* expression apparently occurs at a very low level or is somehow partly masked, so long exposures (~ 20 min) were required to visualize it in whole-worm lysates, where a band appears in *fem-3gf* worms that contain sperm but is missing in *fem-1lf* worms that never contain sperm (data not shown). The *SPE-5* protein is more prominent and easily detected (exposure times of < 1 min) in purified sperm ($> 90\%$ spermatids) that were isolated from *fem-3gf* worms (Figure 6C, arrow). The deduced protein sequence of *SPE-5* indicates that the molecular weight (MW) should be ~ 56 kDa, and the epitope-tagged recombinant clone expressed in bacteria is expected to encode a protein that is ~ 63 kDa. However, we consistently observed that both the bacterially expressed (with an apparent MW of ~ 83 kDa) and the worm-encoded forms (with an apparent MW of ~ 68 kDa) of *SPE-5* show a significantly higher-than-expected molecular weight when subjected to SDS gel electrophoresis, perhaps due to anomalous detergent binding as has been observed for a wide variety of other proteins (e.g., Rath *et al.* 2009). Nonetheless, these data suggest that *SPE-5* expression occurs during spermatogenesis.

***SPE-5* localization in the testes and sperm**

We next subjected male gonads from (wild-type-like) *him-8* control and *spe-5(ok1054) him-8* to immunofluorescence with the anti-*SPE-5* antisera. The R1 affinity-purified rabbit polyclonal *SPE-5* antiserum yielded only background staining

Figure 6 Characterization of SPE-5-specific antibodies on Western blots. (A and B) Blots of gels that were each loaded with crude bacterial lysates prepared from cells induced to express SPE-5 (1), VHA-12 (2), or histone 3.3 (3) (W05B10.1) pDual fusion proteins. Blot in A was probed with a monoclonal antibody raised to a SPE-5-derived peptide. Blots in B and C were probed with an affinity-purified polyclonal antiserum raised to a SPE-5-derived peptide. (C) Blot of a gel that was loaded with affinity-purified SPE-5 fusion protein (1) and 1.6×10^7 purified sperm (2). For each blot, the position of the SPE-5 fusion protein is indicated by an asterisk. Short lines to the left of each panel mark the positions of protein standards. The molecular weight standards to the left of A (in kDa) apply to A and B, while C has different standards that are indicated to the left of that panel.

that persisted in *spe-5(ok1054)* mutant gonads (not shown); it was judged not suitable for immunofluorescence. However, the mouse monoclonal antibody showed that SPE-5 expression was coincident with spermatocytes, where it was distributed throughout the cytoplasm and excluded from the nucleus (Figure 7C). A faint mottled pattern was observed in the cytoplasm, suggestive of punctate staining. *spe-5(ok1054) him-8* gonads lack spermatocyte staining with the mouse monoclonal antibody beyond a faint non-specific background (Figure 7F). Neither the control nor the *spe-5(ok1054)* male gonads had significant staining with the anti-SPE-5 monoclonal antibody in their respective distal gonad arms (d in Figure 7: compare A to C and D to F), which is where germ-cell proliferation occurs (reviewed by Kimble and Crittenden 2005). These data suggest that significant SPE-5 expression occurs during spermatogenesis in the male germline and that *spe-5(ok1054)* is a molecular null mutant, as would be expected for this deletion mutation.

Subcellular localization of SPE-5 was also performed on sperm released from dissected males. For these studies, the SPE-5 distribution was examined in cells co-stained with the 1CB4 antibody (Okamoto and Thomson 1985), which visu-

alizes the MO secretory vesicles and their precursors during spermatogenesis (Arduengo *et al.* 1998). As spermatocytes bud from the proximal end of the rachis (as in Figure 7, A–C), they enter meiosis I (Figure 8A). During meiosis I, SPE-5 appears distributed throughout the cytoplasm, some of it in discrete dots (Figure 8, A and B, arrows). The second meiotic division occurs just before spermatid formation (Figure 8C), and the resulting haploid nuclei segregate into spermatids that bud from the residual body (RB in Figure 8D). MO secretory vesicles segregate with budding spermatids and are not observed within the residual body as judged by 1CB4 staining (red in Figure 8D) and as shown previously (Arduengo *et al.* 1998; reviewed by LHernault 2009; Nishimura and LHernault 2010). In contrast, SPE-5 staining (green in Figure 8D) is observed in both spermatids and the residual body (RB in Figure 8D), and much of the staining in both regions is in discrete dots. Budded spermatids have distinct MOs just below their plasma membrane (arrows at red spheres in Figure 8E). Most of the SPE-5 signal is also localized near the plasma membrane, much of it as discrete dots (green in Figure 8E) that are adjacent to MOs. In certain favorable regions (Figure 8E, arrows), it appears that there is one green dot per MO. These observations indicate that SPE-5 is located near the MO secretory vesicles of spermatids, consistent with mediating MO acidification. As expected, no SPE-5 signal above the low nonspecific background was detected in terminally arrested *spe-5(ok1054)* spermatocytes (Figure 8F).

Discussion

Much prior work has shown that *C. elegans* spermatogenesis requires the proper morphogenesis and function of its MO secretory vesicles. While many mutants that affect the ultrastructure and cellular position of MO secretory vesicles are known (reviewed by LHernault 2006, 2009), little is known about how the affected proteins alter cell physiology. In this article, we show that morphogenesis of MO secretory vesicles is associated with a physiological transition when this compartment becomes acidified. This acidification is apparently synchronous and occurs coincident with spermatid budding from the residual body, which is when each FB-MO matures into a MO secretory vesicle (Figure 1, D and E). This acidification requires the V-ATPase, which is composed of a V_0 sector that forms a pore in the membrane and a V_1 sector that hydrolyzes ATP to provide the electromotive force for proton transport through V_0 (reviewed by, *e.g.*, Toei *et al.* 2010). *spe-5* mutants do not show this MO acidification. In addition, blockage of V-ATPase activity with bafilomycin A1 in wild-type spermatids prevents both this acidification and spermatid activation into spermatozoa.

Spermatid budding places all ribosomes into the residual body (reviewed by LHernault 2009), so the V-ATPase activity that we observe in MOs cannot be due to *de novo* protein synthesis in the spermatid. One common way to regulate the V-ATPase is to maintain V_1 in a cytoplasmic pool and to

Figure 7 Testes localization of SPE-5 expression. Male-derived *him-8* control (A–C) and *spe-5(ok1054); him-8* (D–F) dissected testes were viewed by DIC (A and D), after DAPI staining to visualize DNA (blue in B and E) or after SPE-5 Mab staining that was enhanced by tyramine signal amplification (green in C and F).

regulate its association with V_0 , as was first shown in *Maduca sexta* (Sumner *et al.* 1995) and is now realized to be a widespread phenomenon (reviewed by Beyenbach and Wieczorek 2006). Much of the observed SPE-5 immunofluorescent signal during spermatogenesis prior to spermatid budding does not obviously localize with FB-MOs (Figure 8, A–C). In contrast, most SPE-5 is near the plasma membrane in spermatids, suggesting that any V_1 not destined to associate with MOs is discarded in the residual body. One SPE-5 dot is observed next to certain MOs, so it is possible that there is either only one V-ATPase molecule per MO or, perhaps, multiple V-ATPase molecules clustered at one location. However, there are multiple ~ 800 -nm MOs in each 5- to 6- μ m spermatid, so establishing that each MO has only one V-ATPase dot is beyond the resolution of our light microscopy equipment.

When spermatids become spermatozoa (Figure 1, D and E), many MO secretory vesicles fuses with the plasma membrane, and a single pseudopod is extended (Figure 1E) (reviewed by LHernault 2009; Nishimura and LHernault 2010). Prior work has shown that the bulk pH of the cytoplasm rises ~ 0.5 pH unit during spermiogenesis induced by TEA treatment (Ward *et al.* 1983), and resulting spermatozoa are fertilization-competent during artificial insemination, indicating that TEA treatment does not adversely affect sperm physiology (LaMunyon and Ward 1994). We found that spermatids do not form a normal pseudopod after TEA treatment when they are simultaneously treated with the V-ATPase inhibitor bafilomycin A1. Instead, spermatid activation arrests at what is normally an intermediate stage, characterized by extension of fine spike-like processes (Shakes and Ward 1989). This suggests that the V-ATPase participates in the bulk cytoplasmic pH rise that occurs during spermiogenesis (Ward *et al.* 1983) and that this process is somehow mechanistically linked to pseudopodial extension.

Topologically, the V-ATPase V_1 sector (green, Figure 1D) must be oriented in the spermatid cytoplasm so that protons move through V_0 (orange, Figure 1D) in the MO membrane to acidify the MO interior. Our vital dye data indicate that fusion of the MO with the plasma membrane, when a spermatid (Figure 2D) matures into a spermatozoon (Figure 2E), releases protons within the MO interior to the extracel-

lular space. The fused MOs are permanently opened to the extracellular space (Figure 1E), and the V-ATPase would likely be oriented so it could pump additional protons out of the cytoplasm, assuming that V_1 (green, Figure 1D) remains associated with V_0 (orange, Figure 1D) at this stage.

Before SPE-5 was known to be a V-ATPase subunit, electron microscopy revealed that FB-MO morphogenesis was severely disrupted in *spe-5* mutants, starting in spermatocytes (for wild type, see Figure 1, A and B) (Machaca and LHernault 1997). *spe-5* mutant spermatocytes do not usually divide to form spermatids, which might be a direct consequence of a nonfunctional or mispositioned V-ATPase in FB-MOs. However, mutants affecting either the cell cycle or other diverse biochemical processes during sperm morphogenesis all show defects in both MO position and spermatid formation, so this failure of *spe-5* mutants to form spermatids is not an unusual defect seen only in this V-ATPase mutant (reviewed by LHernault 2009). Furthermore, our immunocytochemistry shows that most of the SPE-5 seen in spermatocytes is discarded into the residual body during spermatid budding. This discarded SPE-5 is over and above that needed in MOs for them to become acidified in budded spermatids. We currently do not know what, if anything, the SPE-5 not seen associated with MOs is doing in spermatocytes.

The null phenotype of *spe-5* mutants is severe disruption of spermatogenesis, but an exclusive role for SPE-5 during spermatogenesis is not consistent with several observations. First, our qRT-PCR results (Figure 5) indicate that the *spe-5*-encoded mRNA is present in “female” animals that are not engaged in spermatogenesis. This indicates that *spe-5* is expressed in somatic tissues and/or oocytes. Studies published by others show that mutations and/or RNAi of a variety of V-ATPase-encoding genes, including both *spe-5* and *vha-12*, partially suppresses the necrotic cell death of touch neurons found in *mec-4gf* dominant mutants (Syntichaki *et al.* 2005). In addition, RNAi of *spe-5* lengthened life span by 23% (Curran and Ruvkun 2007) and caused decreased survival following exposure of worms to hypertonic stress (Choe and Strange 2008). Collectively, these data indicate that *spe-5* is expressed in a variety of somatic tissues and, under the right conditions, is required in those tissues.

Figure 8 SPE-5 localization during spermatogenesis. Sperm cytology was visualized by DIC overlaid by DAPI staining of nuclei (blue on gray panels) or indirect immunofluorescence with either antibody 1CB4 that recognizes FB-MOs (Texas Red; red panels) or SPE-5 Mab enhanced by tyramine signal amplification (Alexa 488; green panels). For each field of cells, a z-series of fluorescent image slices in the DAPI, Texas Red, and Alexa 488 channels was collected, and each resulting wide-field image was deconvolved. For each slice, DAPI, red, and green channels were overlaid to create a triple-labeled slice. All triple-labeled slices for each field of cells were also stacked to create a through-field projection image stack (far right column of images). (A, B, C, and E) *dpy-5 him-5* control (23 plane slices, slice #16 from the bottom shown). (D) *him-8* control (21 plane slices, slice #16 from the bottom shown). (F) *spe-5(ok1054) him-8* (18 plane slices, slice #10 shown). Differentiation stages: (A) primary spermatocytes, (B and C) secondary spermatocytes, (D) budding spermatids, (E) budded spermatids, and (F) *spe-5* terminally arrested spermatocytes. Arrows indicate SPE-5 accumulation in dots. For D and E, arrows point to SPE-5 accumulation that is close to MOs. RB, residual body. Scale bar: 5 μ m for each row of images.

Many prior studies indicate that the B subunit is absolutely required for V-ATPase function (e.g., reviewed by Marshansky and Futai 2008). We showed that a high-copy-number extrachromosomal array containing the *vha-12* promoter and coding sequence allows (normally self-sterile) *spe-5* mutants to produce small broods. This indicates that, under the right circumstances, *VHA-12* can (at least partly) replace *SPE-5* and leads one to question why there are two paralogs in *C. elegans*. The probable explanation is that the *vha-12* gene normally resides on the X chromosome and

transcription from the X chromosome is usually silenced during spermatogenesis (Kelly *et al.* 2002). Placing *vha-12* on an extrachromosomal transgene allows it to escape X chromosome silencing and be expressed in the testes. The very weak self-fertility of all *spe-5* mutants, including the *spe-5(ok1054)* null mutant, might be due to occasional incomplete *vha-12* transcriptional repression. If true, *vha-12* misexpression could allow phenotypic suppression of the *spe-5* self-sterile phenotype, at least to a limited degree; we are currently exploring this possibility further.

Caenorhabditis briggsae also encodes two V-ATPase B subunits and, like *C. elegans*, the *spe-5* ortholog is on chromosome I and the *vha-12* ortholog is on the X chromosome (Stein *et al.* 2003; Hillier *et al.* 2007). For each nematode species, we used bootstrap methods to create a phylogenetic tree and derive an ancestral consensus sequence (Figure S3A). *SPE-5* had more amino acid changes than did *VHA-12* when compared to the ancestral sequence, and these results were true for both species (Figure S3, A and C). Like *C. elegans*, the *C. briggsae vha-12* ortholog is not part of an operon. The *C. briggsae spe-5* ortholog is the third gene in an operon that shows the same syntenic relationship to *pas-3* and *chp-1* as exists in the equivalent *C. elegans* operon (CEOP1216; see above). Since organizing nematode genes into operons is a derived condition not seen in most animal groups (Blumenthal 2005), this suggests that gene duplication and localization of *spe-5* within an operon on chromosome I preceded the reproductive isolation of *C. elegans* from *C. briggsae* 80–110 million years ago (Hillier *et al.* 2007). The relatively more rapid evolution of *SPE-5* as compared to *VHA-12* might indicate that this V-ATPase B subunit is becoming specialized for some as-yet-unknown role.

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Note added in proof: See Ernstrom *et al.* 2012 (pp. 461–475) in this issue for a related work.

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Supporting Information

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Developmental Genetics of Secretory Vesicle Acidification During *Caenorhabditis* *elegans* Spermatogenesis

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Figure S1 A genetic and molecular representation of the *spe-5* region on chromosome I. The small black bars are cosmids, and the relative location of each of the five characterized SNPs is indicated. The exploded view of part of *Y110A7A* shows the predicted genes in the region, and the *spe-5* (*tag-300*) and *mat-1* positions are indicated. The image of the predicted genes is courtesy of WormBase (www.wormbase.org).

The 5' End of the *spe-5* Transcript

SL2/ -72
GGTTTAAACCCAGTTACTCAAGGTCATTACCGTACACGTGAATGCAACGCCA
ATATTTTCAAAATTTGTTGACAAGTTTTCATTATTCAAAATATG
/translation start

Figure S2 The 5' end of the *spe-5* transcript was found to be trans-spliced to SL2.

A.

B.

Nematode Ancestor Consensus B Subunit Sequence Determined by Bootstrap
(461 amino acid region)

YTTKPRLTYQ TVSGVNGPLV ILNDVKFPQF NEIVKITLPD GSVRSGQVLE
ISKNKAVVQV FEGTSGIDAK NTTCEFTGDI LRTPVSEDML GRIFNGSGKP
IDKGPPVLAE DFLDINGQPI NPWSRIYPEE MIQTGISAID VMNSIARGQK
IPIFSAAGLP HNEIAAQIVR QGGLVPHEQT NFAIVFAAMG VNMETARFFK
QDFEENGSM NVCLFLNLAN DPTIERIITP RIALTAAEFL AYQCSKHVLV
VLTDMSYAE ALREVSARE EVPGRRGFP YMYTDLATY ERAGRVEGRD
GSITQIPILT MPNDDITHPI PDLTGYITEG QIYVDRQLHN RLIYPPINVL
PSLSRLMKSA IGEGMTREDH SDVSNQLYAC YAIGKDVQAM KAVVGEEALS
SDDLLEYFL GKFEKNFISQ GHYENRTIFE SLDIGWELLR IFPREMLKRI
PESILDKYYP R

C.

Results from Aligning the Consensus to Nematode B Subunit Sequences

Nematode B subunit	# amino acid changes
<i>C. elegans</i> SPE-5	62/461
<i>C. briggsae</i> SPE-5	51/461
<i>C. elegans</i> VHA-12	19/461
<i>C. briggsae</i> VHA-12	18/461

Figure S3 A, Inference of the ancestral nematode B subunit sequence. The amino acid sequences of *C. elegans* SPE-5 and eight other orthologous and paralogous proteins from six species have been aligned first by using CLASTAL W (see Figure 4; LARKIN *et al.* 2007) and further modified visually. Then, the amino acids corresponding to residues 1-32, 208-215, and 499-501 of *C. elegans* SPE-5 were excluded from the analyses because of the ambiguity introduced by insertions/deletions. The phylogenetic tree of the nine sequences has been constructed by applying the neighbor-joining (NJ) method (SAITOU and NEI 1987) to the 461 amino acid residues common to the nine amino acid sequences. Based on this tree topology, we inferred the amino acid sequences at all interior nodes using the computer program, PAML 4 (YANG 2007). B, The 461 amino acid nematode ancestor sequence (arrow in Figure S3A). C, Results from aligning the 461 amino acid nematode ancestral sequence to extant B subunit protein sequences from *C. elegans* and *C. briggsae*.

Table 1 PCR Primers

Primer Name	Sequence (5' to 3')	Use
42L	CAGTTCCGAAAATGTTATGGT	SNP 41, left
41R	CGCCCGTTTTAGTCGTTAGTC	SNP 41, right
53L	GGAATGCCTGTTTGCCTATGT	SNP 53, left
53R	AAGTTTCCGTTGATTTTATTCCT	SNP 53, right
55L	TTGGCGAAAAGAACTAAATC	SNP 55, left
55R	CGCCTCGAGAAAGACTGC	SNP 55, right
64L	ATCATATGCGCAAACACGGTAGGT	SNP 64, left
64R	ATAGAAAGAACGCGAAGATGTGAC	SNP 64, right
79L	CCTCTGGGACGACGATGACC	SNP 79, left
79R	TGACGATGGAGTGATACGGAAAGA	SNP 79, right
Y12CL	TGAAAAGGAGCAAGACG	Amplification of the entire Y110A7A.12 ORF
Y12CR	TTCCAAAATTCGCAAGAG	
PDHLa	CACACGACCAGCTCTTCATAGATCG	Y110A7A.12 5' cDNA primers
PDHLb	AGCAGAGATCTCTCTCAGTGCTTCAGC	
PDHLa2	CCTCAGTGATGTATCCAGTGAGATCGGG	
PDHRa	CACCACGTATTGCTCTCACTGCAGCC	Y110A7A.12 3' cDNA primers
PDHRb	GAAGCACTGAGAGAGATCTCTGCTGC	
prMH26	TGTCAACACTCCAATCACCAA	<i>spe-5</i> real time PCR primers
prMH27	GGTCCATTGACTCCGAAAAC	
prMH24	CAACGGACAGCCAATCAAC	<i>vha-12</i> real time PCR primers
prMH25	GGAAGACCAGAGGCAGAGAA	
prMH30	TTGGTCACGCATTCACTTTC	<i>rpa-1</i> real time PCR primers
prMH31	TGCTCAACTTCACGTTTGATTT	

Table S2 The SNPs used to map *spe-5*

SNP	DNA Clone	Type of Polymorphism	DNA Sequence Position	Comments
41	F54C1 (cosmid)	N2: ATTTT G AAATA HI: ATTTT A AAATA	Chromosome I 5,013,424	The HI SNP creates a <i>Dra I</i> restriction site (5'-TTT^AAA-3')
53	C46H11 (cosmid)	N2: TCCCT _ CTCTC HI: TCCCT T CTCTC	Chromosome I 1 bp insertion after 5,027,007	The N2 SNP creates a <i>Mnl I</i> restriction site (5'-CCTC(N) ₇ ^3')
55	C46H11 (cosmid)	N2: CTGCA A TGTTG HI: CTGCAG T GTGTTG	Chromosome I 5,058,843	The HI SNP creates a <i>Pst I</i> restriction site (5'-CTGCA^G-3')
64	C30F8 (cosmid)	N2: TCCTT C TTTTT HI: TCCTT T TTTTT	Chromosome I 5,096,273	No restriction sites are created by either SNP; must be typed by sequencing
79	Y110A7A (YAC)	N2: GATTG T CTTGG HI: GATTG C CTTGG	Chromosome I 5,126,047	The HI SNP creates a <i>Sty I</i> restriction site. (5'-C^CAAGG-3' or 5'- C^CTTGG-3')

Table S3 Single Nucleotide Polymorphisms Near the *spe-5* Gene

Strain	Recomb type	SNP41 Genotype	SNP53 Genotype	SNP55 Genotype	SNP64 Genotype	SNP79 Genotype
SL816	Unc nonSpe	HI	HI	-	-	-
SL817	Unc nonSpe	HI	HI	-	-	-
SL835	Unc nonSpe	HI	HI	-	-	-
SL836	Unc nonSpe	HI	HI	-	-	-
SL837	Unc nonSpe	HI	HI	-	-	-
SL851	Unc nonSpe	HI	HI	-	-	-
SL852	Unc nonSpe	HI	HI	-	-	-
SL858	Unc nonSpe	N2	N2	N2	HI	-
SL866	Unc nonSpe	N2	N2	HI	HI	-
SL821	Dpy nonSpe	HI	HI	-	HI	HI
SL838	Dpy nonSpe	HI	HI	-	HI	HI
SL853	Dpy nonSpe	HI	HI	-	HI	HI
SL854	Dpy nonSpe	HI	HI	-	HI	HI
SL855	Dpy nonSpe	HI	HI	-	HI	HI
SL856	Dpy nonSpe	HI	HI	-	HI	N2
SL857	Dpy nonSpe	HI	HI	-	HI	HI
SL872	Unc Spe	N2	N2	-	-	-
SL873	Unc Spe	N2	N2	-	-	-
SL870	Dpy Spe	-	-	-	-	-
SL871	Dpy Spe	-	-	-	-	-

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