V-ATPase $V_1$ sector is required for corpse clearance and neurotransmission in

*Caenorhabditis elegans*

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Abstract

The V-ATPase proton pump is composed of two sectors, the cytoplasmic $V_1$ sector that catalyzes ATP hydrolysis and the transmembrane $V_o$ sector responsible for proton translocation. The transmembrane $V_o$ complex directs the complex to different membranes, but also has been proposed to have roles independent of the $V_1$ sector. However, the roles of the $V_1$ sector have not been well characterized. In the nematode *C. elegans* there are two $V_1$ B-subunit genes; one of them, *vha-12*, is on the X-chromosome; whereas *spe-5* is on an autosome. *vha-12* is broadly expressed in adults, and homozygotes for a weak allele in *vha-12* are viable but are uncoordinated due to decreased neurotransmission. Analysis of a null mutation demonstrates, that *vha-12* is not required for oogenesis or spermatogenesis in the adult germline, but it is required maternally for early embryonic development. Zygotic expression begins during embryonic morphogenesis, and homozygous null mutants arrest at the two-fold stage. These mutant embryos exhibit a defect in the clearance of apoptotic cell corpses in *vha-12* null mutants. These observations indicate that the $V_1$ sector, in addition to $V_o$ sector, is required in exocytic and endocytic pathways.
Introduction

One role of vacuolar-type proton ATPases (V-ATPase) is to acidify organelles in secretory and endocytic pathways (Mellman et al., 1986; Futai et al., 2000; Forgac, 2007). The V-ATPase is composed of two sub-structures: a catalytic domain of eight different subunits called the V₁ sector, and a membrane anchored set of subunits called the Vₒ sector (Figure 1A). Both sectors are required for acidification of cellular organelles; however, genetic analysis suggests that the Vₒ domain may have an additional role in promoting membrane fusion (Peters et al., 2001; Hiesinger et al., 2005; Sun-Wada et al., 2006; Liégeois et al., 2006; Peri and Nüsslein-Volhard, 2008; Di Giovanni et al., 2010; Williamson et al., 2010; Strasser et al., 2011). To distinguish the roles of acidification versus potential additional roles of the V-ATPase, it is particularly important to identify the functions of the catalytic V₁ sector.

The differential expression and localization of specialized V₁ sector subunits suggests that the proton pump could be adopted for different purposes (Toei et al., 2010). For example, in mammals different B isoforms of the V₁ sector are localized to either the cell surface or to internal membranes. The B1 isoform is localized to the surface of specialized kidney, inner ear, and vas deferens epithelial cells where it pumps protons into the extracellular space, whereas the B2 subunit is typically associated with endosomes and pumps protons into the lumen of organelles (Brown et al., 2009). Consistent with the epithelial localization of B1 subunits, mutations in the B1 surface isoform are linked to renal tubule acidosis and deafness in humans (Karet et al., 1999; Stover et al., 2002; Hahn et al., 2003; Vargas-Poussou et al., 2006; Gil et al., 2007; Fuster et al., 2008).
However, a rigid B subunit specialization does not seem to be common in all animals. The mouse genome also encodes two B subunits. But unlike humans, mutation of the apical B1 subunit in mice does not result in deafness or kidney dysfunction (Finberg et al., 2005; Dou et al., 2003). The B2 subunit, normally localized to endosomes, can partially compensate for the loss of B1 subunits (Păunescu et al., 2007), suggesting that B-subunit specialization is not universal. Only one B-subunit gene is encoded in the *Drosophila* genome, suggesting that there is no specialization of subunits (Du et al., 2006). The fly VHA55 protein is localized on internal membranes as well as on the apical surface on the kidney-like Malpighian tubules, consistent with acidification of organelles and proton extrusion (Du et al., 2006). In contrast to B1 mutations in mice, mutations in *vha55* are larval lethal (Davies et al., 1996). Thus, mutations in B-subunit genes in mice and flies exhibit two phenotypic extremes: they are nearly wild-type or lethal.

The genome of the nematode *Caenorhabditis elegans* encodes two B-subunit genes, called *vha-12* and *spe-5*, as well as multiple genes for other V-ATPase subunits (Figure 1A). To investigate whether these B-subunit genes have specialized functions, we and the authors of the accompanying paper (Gleason et al., 2012) have cloned and characterized mutations in each of these loci. We isolated a null allele and a missense allele in the *vha-12* locus, which is on the X chromosome. The missense allele allowed us to examine the effects of impaired V₁ V-ATPase in adult cells. The VHA-12 B subunit is required for acidification of synaptic vesicles and the release of normal levels of neurotransmitter from adult neurons. VHA-12 is also required maternally for early embryogenesis, and is required zygotically during morphogenesis. We also show a previously unrecognized role of the catalytic B subunit in the clearance of apoptotic cell corpses in embryos. *vha-12* is thus required
broadly, whereas spe-5 is only required during spermiogenesis (Gleason et al., 2012).

Together these manuscripts suggest that B subunit diversity in C. elegans may have arisen to escape X-chromosome inactivation rather than to provide functional diversity for the V-ATPase proton pump.
Materials and methods

Strains and alleles

*Caenorhabditis elegans* strains were cultured using standard methods. The wild type is N2 Bristol (Brenner, 1974).

*Reference strains.* MT7907 *vha-12(n2915sd) X* is a viable mutation isolated in an EMS screen for jerky uncoordinated mutants and outcrossed to N2 four times. GR1029 *lon-2(e678) let-44(mg41) X; mnDp31* is a recessive X-linked embryonic lethal mutation. Briefly, *lon-2* males were mutagenized with EMS and crossed to *unc-20(e112) X* animals. Coordinated cross-progeny were individually screened for the absence of the *lon-2(e678)* X-linked marker in the second filial generation. The lethal mutation *mg41* was outcrossed seven times to N2 and balanced using the free duplication, *mnDp31*. CB189 *unc-32(e189) III* is a mutation in subunit *a* of the *V_0* sector (Pujol et al., 2001).

*Rescuing strains.* MT7907 *vha-12(n2915sd) X* was rescued by microinjection of DNA from single genes contained in the rescuing cosmid C04E10. The *vha-12* gene was amplified from genomic DNA using oligonucleotides oRW025 and oRW026 to generate a 5 kb PCR fragment (see below). Transformants were identified by co-injection of 20 ng/μL plasmid pEK1[lin-15(+)] into EG7341 *vha-12(n2915) lin-15(n765ts) X* (Clark et al., 1994). Two of two *lin-15(+) lines were rescued for the Vha-12 Unc phenotype (EG2371 *vha-12(n2915sd) lin-15(n765ts) X; oxEx397[vha-12(+); lin-15(+)]. The 5kb rescuing PCR fragment was subcloned to generate plasmid pRW05. pRW05 was microinjected into MT7907 *vha-12(n2915sd) to generate EG3250 *vha-12(n2915sd); oxEx552 [vha-12(+) pRW05 (2 ng/μL); Pmyo-3::GFP (10 ng/μL); Bluescript (76 ng/μL)]* for quantitative analyses of rescue. Embryonic lethality of *vha-12(mg41)* was rescued by injecting GR1029 with the *vha-12(+)
pRW05 plasmid to produce EG3249 lon-2(e678) vha-12(mg41) X; oxEx551[vha-12(+)]
pRW05 (2 ng/μL); Pmyo-3::GFP (10 ng/μL); Bluescript (76 ng/μL)]. To perform mosaic analysis we generated a strain that contained a rescuing vha-12(+) gene array that expresses GFP under the control of an early developmental promoter. The vha-12(+) rescuing plasmid was injected into GR1029 to generate strain EG7188 lon-2(e678) vha-12(mg41); oxEx1703[vha-12(+)] pRW05 (0.5 ng/μL); pCFJ420 Peft-3::GFP::HIS-58 (histone H2B) (10 ng/μL); Punc-122::GFP (60 ng/μL) coelomocyte marker; Promega DNA ladder (32.5 ng/μL)].

Transcriptional reporter. EG2410 lin-15(n765ts) X; oxEx192 [Pvha-12::NLS::GFP; lin-15(+)].

Synaptic marker strains. Synaptic varicosities in a subset of motor neurons can be visualized in transgenic animals expressing the worm homolog of the synaptic vesicle protein synaptobrevin SNB-1 fused at its C-terminus to GFP (Jorgensen et al., 1995). The ‘wild-type’ synaptic marker strain was MT8247 lin-15(n765ts) nIs52[Punc-25::SNB-1::GFP; lin-15(+)] X. EG1956 vha-12(n2915sd) nls52 lin-15(n765ts) X was the ‘vha-12’ synaptic marker strain.

Axon commissure reporter strains. The unc-47 promoter drives GFP expression in GABA neurons (McIntire et al., 1997). Axon branching was analyzed by expressing cytoplasmic GFP in the GABA neurons using the unc-47 promoter in the ‘wild-type’ strain, EG1306 lin-15(n765ts) oxs12[Punc-47::GFP; lin-15(+)] X, and the ‘vha-12’ strain, EG1961 vha-12(n2915sd) lin-15(n765ts) oxs12 X.
**pHluorin strains.** Vesicle pH reporter constructs were similarly constructed as the synaptic marker reporter except GFP was substituted with the pH-sensitive version of GFP superecliptic pHluorin (Miesenböck et al., 1998; Sankaranarayanan et al., 2000). The X-ray integrated line was outcrossed five times to wild-type N2 animals to produce the “wild-type” pHluorin strain EG3440 oxls155 [Punc-25::snb-1::superecliptic pHluorin; lin-15(+)] IV; lin-15(n765ts) X. EG3478 oxls155 IV; vha-12(n2915sd) lin-15(n765ts) X; was the ‘vha-12’ pHluorin strain.

**Cell death strains.** MT1522 ced-3(n717) IV; EG6313 ced-3(n717) IV; lon-2(e678) let-44(mg41) X; mnDp31

**Molecular biology**

**Sequencing.** Sequencing templates were prepared from the PCR-amplified vha-12 gene from whole worm lysates. To prepare the lysates a single adult hermaphrodite or 5-10 embryos were added to 7.5 microliters of distilled water in a thin-walled PCR tube. Two microliters of 5x GC Phusion PCR buffer (Thermo Fisher Scientific) and 0.5 microliters of 20 mg/mL proteinase K (New England Biolabs, Ipswich, MA) were incubated at 65°C for one hour and at 95°C for 30 min. PCR was performed with high fidelity polymerase (Phusion, Thermo Fisher Scientific). PCR products were gel purified (Zymo Research, Irvine CA) and cloned into a vector and sequenced. To collect vha-12(mg41) embryos lacking the free duplication (mnDp31), a cohort of twenty embryos laid by vha-12(n2915) /lon-2(e678) vha-12(mg41) parents were transferred to a fresh plate. Embryos that did not hatch overnight were collected for vha-12(mg41) sequencing.
**Minimal rescuing fragment.** `vha-12(+)` was generated using the DNA polymerase cocktail Expand (Roche), wild-type genomic DNA and oligonucleotides oRW025 and oRW026. The PCR product was purified by gel extraction (Qiagen) before injection.

**Transcriptional reporter construct.** `Pvha-12::NLS::GFP` was generated by subcloning the PCR product amplified with oligonucleotides oRW075 and oRW077 into pPD95-67 (http://www.addgene.org/Fire_Lab), thus 3 kb of sequence upstream of the `vha-12` start codon was placed 5' to the nuclear signal sequence contained in pPD95-67.

**pHluorin construct.** Superecliptic pHluorin is a pH-sensitive version of the green fluorescent protein; at low pH the fluorescence of ecliptic versions of pHluorin is quenched (Miesenböck et al. 1998). Superecliptic contains additional mutations that shift the pKa from 7.07 to 7.18, and was described by Sankaranarayanan et al. (2000). Superecliptic pHluorin was amplified using Pfu DNA polymerase and oligonucleotides oRW109 and oRW110 and subcloned into plasmid pJL35 (Punc-47::SNB-1::GFP) to make the `Punc-47::SNB-1::superecliptic pHluorin` plasmid.

**Oligonucleotide sequences.** oRW25, ccatttccctgatattgtttctacc; oRW26, gagatatgctgaaaatagctagtgg; oRW075, ggatcctgatattgtttctacc; oRW077, gagctcattcctgaaaaattgc; oRW109, ccggtacccatgagtaaaggagaag; oRW110, gaattcttatttgtatagttcatcc. To generate overlapping `vha-12` genomic coding sequence fragments, the following oligonucleotides were used: oGE12 cgtatctatcaatgtatctgccac and oGE16 catgacgtcgatagcggagattc; oGE13 gaatggctgccgttgacgtc and oGE18 ctctgtagatggcagaggtgattc; oGE15 gaatctccgtatgcagctatcg and oGE20 cacgtggaagatacggagaag; oGE17 gatttggccacctacagag and oGE22 gttgatttccagtatcc.
Body-bending

Body-bending (thrashing) measurements were performed as described by Miller et al. (1996). All animals were assayed as one-day-old adults and body-bending events were captured with video imaging. Individuals were placed into a well of 96-well tissue-culture plate containing M9 liquid media. After an initial two-minute period, thrashing animals were imaged for three minutes. Images were acquired on a M2 Bio stereomicroscope (Kramer Scientific, Amesbury, MA) equipped with a Pulnix TM-200 CCD camera (Takeex, Inc). Analog video was digitized using in real time (Canopus® ADVC 100) and saved to a hard drive as an uncompressed Quicktime video file (Apple, Inc.). To allow accurate scoring of bend-bends the image sequence was manually advanced offline in Quicktime Player to count the number of mid-body bends.

Aldicarb assays

Aldicarb dose responses were measured for each genotype. Aldicarb was added to NGM petri plates seeded with bacterial food. Twenty to thirty one-day-old adult animals were placed on each assay plate. Paralysis — no locomotion even when prodded by a platinum wire probe — was scored by visual inspection six to eight hours later, and the fraction of paralyzed animals at each aldicarb dose was recorded. The genotype of the animals was masked to the observer during the paralysis scoring.

Levamisole assays

Levamisole (Sigma) was dissolved in S basal medium and added to weighed plates to give final concentrations spanning 0.1 mM to 3 mM. Twenty animals were placed on
each dose of levamisole, and animals were scored for paralysis two hours later. Genotypes were masked to the experimenter at the time the animals were scored.

**Body-length measurements**

For body-length measurements, stationary individuals on an agar plate seeded with OP50 bacteria were photographed on a stereomicroscope. Images were captured on a Pulnix (Takei, Inc.) TM-200 CCD camera and digitized (Canopus® ADVC 100) to a Macintosh computer. An ImageJ vector line tool was used to make body length measurements head to tail through the mid-body. Images were calibrated using a stage micrometer.

**Imaging anesthetized adult animals**

Fluorescence images were collected on a Zeiss LSM 5 PASCAL confocal Axioskop FS2 upright microscope. Whole adult animals were mounted on a 2% agarose pad prepared in physiological saline. A 6 µL drop of 1-2% 1-phenoxy 2-propanol was added to straighten animals, and a cover glass was placed on top. To prevent dehydration of the specimens, the edges of the cover glass were sealed with melted petrolatum. Paralyzed animals that rolled ventral-side up (as determined by viewing the vulva location with DIC optics) were imaged. Confocal settings (gain, offset, % power, scan speed, zoom, and pinhole diameter) were identical for quantitative comparison. Genotypes were masked during the acquisition and scoring of synaptic morphology and marker intensity.

**Mosaic analysis**
To isolate *vha-12* germline mosaics, 724 L4-stage hermaphrodites of the EG7188 strain *vha-12(mg41) lon-2(e678); oxEx1703*[vha-12(+); Pef3::GFP::HIS-58 (histone H2B); Punc-122::GFP coelomocyte marker] were cultured individually and screened beginning on day-1 of adulthood for an absence of *gfp(+) embryos*. Fourteen putative germline mosaics were isolated that produced broods exclusively of inviable embryos that arrested prior to approximately the 40-cell stage. Of a total of 560 embryos analyzed from the putative germline mosaics, all died as early embryos and exhibited a phenotype distinct from that of *vha-12(mg41)* homozygous embryos produced from oxEx1703- or mnDp31-transmitting parents or *vha-12(mg41)/+* heterozygotes. Because the *Pef3::GFP::histone* transgene is not expressed in the germ line or very early embryos, the conclusion that these fourteen animals are indeed germline mosaics is based on the following line of reasoning.

Transgenic extrachromosomal arrays are mitotically unstable, frequently being lost at rates of ~1/50-1/200 per cell division (Yochem *et al.*, 1998; Yochem and Herman, 2003). Were these fourteen animals not germline-loss mosaics, then there should be a distinct class of animals that produce broods of embryos that arrest late in embryogenesis, which was not observed. To test the possibility that the fourteen animals had suffered somatic losses that somehow interfered with the production of viable progeny, the animals were analyzed by fluorescent microscopy. All somatic tissues were observed to be *gfp(+), thus it was concluded that the fourteen animals were likely P4-loss mosaics. Twelve of the mosaics were mated individually to at least ten wild-type males, but no viable or *gfp(+) progeny* were detected. Based on these observations, it was concluded that *vha-12(+)* is both maternally required and sufficient for early embryogenesis, though the exact basis for the lethality will require further investigation.
**Analyzing synaptic varicosities in vivo**

For analysis of synapses, the synaptic varicosities in the ventral cord motor neurons were imaged. Maximum pixel intensity projections were generated from raw z-stacks, and the resulting projected images were analyzed in ImageJ by identifying peaks (>5x baseline). The spacing of synaptic varicosities was measured from peak-to-peak distances. To measure puncta intensity, a square box greater than the size of the average puncta (8x8 pixels) was placed around the puncta to determine its optical center of mass (Sankaranarayanan *et al.* 2000). A 4x4 pixel-box was then placed about this center of mass to measure its mean pixel intensity.

**4D imaging of embryonic development**

Under a standard *C. elegans* dissecting microscope, 2-4 cell stage embryos were collected by cutting open gravid adults in a shallow watch crystal. The embryos were transferred by mouth pipette to a four percent agarose pad. A number 1.5 cover glass was gently placed over the mounted embryos, a drop of distilled water was drawn across the pad-glass sandwich, and the edges were sealed with melted Vaseline.

Differential interference contrast, time lapse recordings of embryonic development were recorded on a Zeiss LSM 5 PASCAL confocal microscope equipped with a 63x, 1.4 N.A. plan-apochromat objective. Laser light served as the transmitted light source (488 nm at its lowest power setting). Images were captured on the photomultiplier tube. A stack of forty images up to 512 pixels x 512 pixels was collected in 0.5 μm-increments along the z-axis. Z-stacks were generated in five-minute intervals for twelve to sixteen hours. The scan speed was set to its fastest possible speed; it took approximately one minute to scan one stack.
This fast scan speed and minimal laser power setting were not phototoxic as determined by measuring the timing of developmental milestones (comma, 1 1/2-fold, two-fold, three-fold stage) of wild-type embryos.

Ten apoptotic cells, cell corpses, were tracked in each embryo. A cell corpse was identified as a raised disc-like cell approximately two micrometers in diameter. To measure cell corpse duration, the image stack was manually inspected to spot the first appearance of a corpse and the first interval in which the corpse disappeared from view. In each time interval the image stack was manually advanced along the z-axis to track the corpse in all focal planes. *vha-12(mg41)* embryos lacking the rescuing free-duplication were identified from a set of four to five embryos as the embryos that failed to complete embryonic development during the imaging period.

To image the onset of GFP expression from the *vha-12* promoter in embryos, embryos containing the reporter transgene were prepared and imaged in the same manner as described above. The only difference was that the green fluorescence was collected on a separate channel and photomultiplier tube was equipped with a 505-600 nm emission filter.

*Electron microscopy*

Embryos in a comma stage that do not carry the rescuing array were isolated based on the absence of fluorescence. The embryos were then frozen in the similar manner as previously described (McDonald *et al.*, 2010; Rostaing *et al.*, 2004). In short, a type B specimen carrier for a Baltec high-pressure freezer was coated with hexadecence and mounted onto a specimen holder with the flat side facing up. A single-slot TEM grid was
placed on top of the specimen carrier. Embryos along with bacteria were scooped using a paintbrush (#00) and gently placed in the slot of a grid. The specimen was then capped with another type B specimen carrier with the flat side facing the specimen. The specimen was then frozen and transferred into a cryovial containing 1% osmium and 1% glutaraldehyde in anhydrous acetone. Freeze-substitution was carried out in a Leica AFS 2 with the following program: 10 hours at -90°C, 5°C/hour to -20°C, 16 hours at -20°C, and 5°C/hour to room temperature. When the program ended, the fixatives were washed off with anhydrous acetone for 6 times with each wash separated by 15 minutes. The specimens were then infiltrated with Epon-Araldite in a stepwise fashion: 30% for 5 hours, 70% for 6 hours, 90% for over night, and 100% for 6 hours. Finally, plastic polymerization was carried out in a 60°C oven for 48 hours. 50nm thick sections were cut using a Leica UC 6, and 1000 contiguous sections were collected onto formvar coated grids. These sections were imaged using a Hitachi H7100 transmission electron microscope equipped with a GATAN Orius SC1000 camera.
Results

Viable and lethal mutations in the B subunit of the V-ATPase V1 sector

Mutants with defects in acetylcholine neurotransmission in *C. elegans* exhibit jerky uncoordinated locomotion (Brenner, 1974; Miller *et al.*, 1996). In a genetic screen for mutants with jerky locomotion we isolated a mutation, *n2915*, that mapped to a narrow interval on chromosome X between *lin-18* and *dpy-23* near the previously uncharacterized *let-44(mg41)* mutation (Figure 1). *let-44(mg41)* was isolated in an independent screen for recessive X-linked embryonic lethal mutations. The *mg41* mutation failed to complement the locomotion defect of the *n2915* mutation as well as other phenotypes associated with *n2915* mutants (see below). The gene mutated in these strains was identified by DNA microinjection rescue. Nine cosmid clones span the interval between *lin-18* and *dpy-23*. Injection of a single cosmid, C04E10, into *n2915* animals generated three independent lines carrying stable extrachromosomal arrays; two of these three lines rescued the *vha-12(n2915)* uncoordinated phenotype. Cosmid C04E10 was predicted to contain ten genes (Figure 1). Microinjection of a 5 kb PCR fragment containing the predicted gene F20B6.2 was sufficient to rescue both the uncoordinated phenotype of *n2915* (1 of 3 lines) and the lethality associated with *mg41* (2 of 3 lines). Analysis of the cDNAs from F20B6.2 identified a single reading frame and confirmed the predicted splice pattern (Figure 1). *vha-12* (*vacuolar H⁺-ATPase-12*) transcripts encode a 491 amino acid protein that shares a high level of sequence identity (71 to 84%) with other V-ATPase B-subunit genes (Figure S1). The *C. elegans* genome contains a second locus, called *spe-5* (Y110A7A.12), that encodes a V-ATPase B subunit. Because *spe-5* mutants are defective for spermatogenesis (see
(Gleason et al., 2012) accompanying paper) and vha-12 null mutants are lethal, these loci are not fully redundant and independent functions can be characterized.

Sequencing the vha-12 locus from the n2915 and mg41 mutants identified the nucleotide changes associated with these mutations and confirmed the identity of vha-12 (Figure S1). vha-12(n2915) is a C to T transition in the third exon of F20B6.2 that results in the substitution of a highly conserved alanine at position 385 with a valine in the predicted protein (Figure S1). This allele is an antimorphic allele by genetic criteria, since it is semidominant and reduces V-ATPase function (see below). We generated a molecular model of VHA-12 by threading its sequence onto the crystal structure of the related archaeabacteria Methanosarcina A1 B subunit (Figure S2). The vha-12(n2915) mutation is near the catalytic site of the ATPase and in a region associated with a human kidney disease mutation (Figure S1). Alanine 385 is juxtaposed to a conserved glutamate (Glu149), a residue that is polymorphic, and possibly dysfunctional, in humans (human B1 Glu161Lys); B1 subunit proteins containing the Lys161 polymorphism are made but do not function in heterologous assays (Figure S2; Fuster et al., 2008). Alanine 385 is five residues downstream from a conserved and essential arginine residue (Arg380) (Vasilyeva et al., 2000; Nishi and Forgac, 2002). In the crystal structure of the related F-ATPase subunit α, this arginine (Arg373) rests in the catalytic pocket of the ATPase domain where it stabilizes the terminal phosphate group during ATP hydrolysis (Abrahams et al., 1994). Mutation of the arginine abolishes V-ATPase function in yeast, and the homologous residue in the human B1 subunit is linked to distal tubule renal acidosis (Liu et al., 1996; Vargas-Poussou et al., 2006). Given the propinquity of Ala385 to Arg380, it is likely that the n2915 lesion impairs ATP catalysis.
Sequence analysis of *vha-12*(mg41) arrested embryos identified two mutations within the gene: a C to T transition at nucleotide 506, and a C to A transversion at nucleotide 698. The latter mutation results in a missense mutation changing the non-conserved arginine 200 into a serine. The upstream mutation results in an amber stop at amino acid position 153, thereby truncating 70% of the protein (Figure S1). Genetic criteria indicate that this mutation is a null allele: First, *mg41* is recessive: *vha-12*(mg41) heterozygotes are indistinguishable from the wild type. Second, *mg41* behaves as a null in regard to the weaker allele: *vha-12*(n2915)/*vha-12*(mg41) animals exhibit a more severe phenotype than *vha-12*(n2915) homozygotes (Figure S3). Subunit B is a major constituent of V₃ sectors of the V-ATPase, and is required for full V₃ sector assembly (Kane, 2006). Because this nonsense mutation would delete important interface residues it is likely that *mg41* abolishes V₃ complex assembly and represents a complete loss V₃ functions. Thus, the *n2915* and *mg41* alleles provide reagents capable of characterizing the role of acidic organelles during development as well as in adult tissues in *C. elegans*.

**vha-12 is widely expressed in somatic cells**

Different V-ATPase subunit isoforms may be expressed in different subsets of tissues. To test whether *vha-12* is expressed in a specific cell type, we generated a transcriptional reporter in which the expression of GFP fused to a nuclear localization tag (SV40) was placed under the control of the *vha-12* promoter: 3 kb of DNA sequence preceding the predicted ATG start codon (Figure 2). This reporter construct was introduced via microinjection into the gonad to generate the stable extrachromosomal array, oxEx192. *vha-12* is broadly expressed in most, if not all, somatic cell-types in larvae.
and adults (Figure 2A). Robust GFP expression is observed in the H-shaped excretory cell, the excretory pore, intestine, and hypodermal cells (Figure 2B-F). GFP reporter expression is observed at low levels in muscle. Consistent with the uncoordinated phenotype, expression is observed in all neurons (Figure 2G).

**Neurotransmission is disrupted in *vha-12(n2915)* mutants**

The locomotory defect in the *vha-12(n2915)* mutants suggests a neuromuscular defect. Locomotion can be quantified by counting the number of body bends of an animal placed in a drop of liquid medium for a brief period (Miller *et al.*, 1996). Wild-type animals swim rapidly in liquid (221 ±14 bends per minute, n=3), whereas homozygous *vha-12(n2915)* animals swim slowly (62 ±7 bends per minute, n=3, p<0.0001) (Figure 3A). Compared to the wild type, heterozygous *vha-12(n2915)* animals had a statistically significant reduced swimming rate (172 ±11 bends per minute, n=3; P<0.01 two-tailed t-test) and thus *n2915* is a semidominant mutation. To determine if the neuromuscular defect was presynaptic, we measured the sensitivity of *vha-12(n2915)* mutant animals to drugs that affect neurotransmission. Acetylcholinesterase degrades acetylcholine after it has been released from neurons. The drug aldicarb inhibits acetylcholinesterase and the accumulation of acetylcholine causes animals to hypercontract; whereas animals with defects in acetylcholine release are resistant to aldicarb (Miller *et al.*, 1996). When wild-type animals were exposed to 0.7 mM aldicarb, 100% were paralyzed after two hours (Figure 3B). However, only 18% of *vha-12(n2915)* animals (±9%, n=5 assays) were paralyzed after a two-hour exposure to aldicarb. As a reference we analyzed aldicarb sensitivity of another strain defective in a V-ATPase subunit. The *unc-32(e189)* mutation
truncates a neural-specific isoform of the $V_o$ subunit (Pujol et al., 2001). Like $vha-12(n2915)$ mutant animals, $unc-32(e189)$ animals are viable as adults but uncoordinated. $unc-32(e189)$ mutants were also significantly aldicarb resistant with only 8.5% (±9%, n=5 assays) paralyzed after two hours exposure. The aldicarb resistance of the $vha-12(n2915)$ mutant animals suggests a role for the V-ATPase in neurotransmission.

Aldicarb resistance can be explained by either a decrease in acetylcholine release or decreased sensitivity of the muscle to acetylcholine. Muscle sensitivity can be determined by measuring the fraction of animals paralyzed by the acetylcholine receptor agonist levamisole. The sensitivity of $vha-12(n2915)$ to the postsynaptic acetylcholine receptor agonist, levamisole, was not detectably different from wild-type animals (Figure 3C). One hundred percent of wild-type animals were paralyzed by 1 mM levamisole, and 96% $vha-12(n2915)$ mutants were paralyzed by the same dose (±4%, n=5; p=0.35). The $V_o$ mutant $unc-32(e189)$ also exhibited comparable sensitivity to levamisole as wild-type animals. Taking the aldicarb resistance and levamisole sensitivity together, these pharmacological assays suggest $vha-12(n2915)$ mutant animals have a defect in neurotransmitter release.

We examined whether there were neuronal developmental defects in $vha-12(n2915)$ mutants by imaging axonal morphology and synaptic formation. No significant difference in the number of GABA commissures was found between wild-type and $vha-12(n2915)$ animals (Figure 3D,E; wild type: 16.4±1.1, n=7; $vha-12(n2915)$: 17.0±0.8, n=7, p=0.3 unpaired t-test). The distribution and expression levels of a transgenic synapse marker in ventral GABA motor neurons was also not significantly altered in $vha-12(n2915)$ mutants (2.87±0.58 varicosities per 10 µm, n=8 in wild-type vs. 2.79±0.62 varicosities per 10 µm,
n=9, p=0.8, Figure 3F,G). The normal distribution of synapses and axon morphology suggest that neuronal development in vha-12(n2915) mutants is not severely impaired.

We expected that synaptic vesicles would not be acidified in the V1 mutant, since neurotransmitter loading into vesicles relies on exchange of protons for neurotransmitter molecules. We examined whether synaptic vesicles were acidified by tagging the lumenal domain of synaptobrevin with superecliptic pHluorin, a pH-sensitive GFP reporter. Superecliptic pHluorin has the property that at low, acidic pH, its fluorescence is quenched. Superecliptic pHluorin has a reported pKa of 7.1, and is therefore particularly sensitive in a physiological pH range (Miesenböck et al., 1998; Sankaranarayanan et al., 2000). pHlourin puncta were on average three-fold brighter in vha-12(n2915) mutants compared to the wild type (Figure 4). Similar results were seen in the V0 subunit a mutant unc-32(e189).

Since the pH reporter was associated with the lumenal domain of a synaptic vesicle protein, if the protein is on the plasma membrane, the pH reporter will be exposed to the extracellular environment and would fluoresce more brightly. However, since synaptobrevin labeled with a non-pH sensitive GFP vesicle reporter was punctate rather than diffuse it is unlikely that synaptobrevin resides on the surface due to a defect in endocytosis. The simplest interpretation of the increased vesicular pHluorin fluorescence is that vha-12(n2915) mutant vesicles are less acidic than the wild type.

**Epidermal functions of V1**

In addition to a semidominant uncoordinated phenotype, vha-12(n2915) mutants exhibit a dominant dumpy phenotype, that can be quantified as a reduced body-length (Figure 5). Oddly, the shortened body-length phenotype was detected in heterozygotes but not
homozygotes. A dumpy phenotype was detected when \( n2915 \) allele was heterozygous with either the wild-type or \( mg41 \) allele (Figure 5). It was possible that the dominant dumpy phenotype could be caused by a linked mutation; however extensive outcrossing was unable to separate the uncoordinated and dumpy phenotypes, and the dumpy phenotype is fully rescued by the \( vha-12 \) transgene (\( oxEx552 \) in strain EG3250, Figure 5 column 3). The dumpy phenotype is often associated with defects in cuticle formation caused by impaired function of underlying epidermal cells (McMahon et al., 2003; Page and Johnstone, 2007), and is consistent with the expression of the \( vha-12 \) reporter in epidermal cells. Another epidermal defect was the high incidence of one day old adult \( vha-12(n2915) \) heterozygotes dragging recently molted cuticle (11/40 \( n2915/+ \) vs 0/20 of wild-type and 0/33 \( vha-12(n2915) \) homozygotes, Figure S4). Interestingly, reduced body-length is also observed in specific \( vha-5 \) mutants that preferentially disrupt cuticle formation (Liégeois et al., 2006); \( vha-5 \) encodes a \( C. \) elegans \( V_\circ \) \( \alpha \) \( \text{V-ATPase} \) subunit homolog expressed in the epidermis. These results suggest that epidermal function is not limited to \( V_\circ \)-specific roles, but may instead require the full activity of a \( V_1V_\circ - H^+\text{-ATPase} \) complex.

**\( vha-12 \) is required maternally for early embryonic development, not oogenesis**

Our transcriptional reporter did not indicate expression of \( vha-12 \) in the germline of adults. However, extrachromosomal arrays are usually silenced in the germline and lack of expression from a fluorescence reporter construct is not conclusive. In fact \( vha-12 \) is expressed during oogenesis based on SAGE analysis (Wang et al., 2009) and transcripts are observed in early embryos by in situ hybridization (Figure S5, Shin-i and Kohara, 2005).
Mosaic analysis further demonstrates that expression of vha-12 in the adult germline is required for proper development of embryos soon after fertilization. We rescued vha-12(mg41) mutants with a vha-12(+) extrachromosomal array containing a GFP marker (see Methods). We then screened for loss of the extrachromosomal array in the progenitor germ cells. From 724 hermaphrodites, fourteen germline-loss mosaics were recovered. Gonad and oocyte morphology was normal in mosaics that seem to have lost the rescuing copy of vha-12 in the P4 precursor cell to all germ cells (Figure 6A). Moreover, fertilized embryos were observed in the uterus, indicating that oocyte and sperm formation is normal in the absence of vha-12. These mosaics produce broods composed of only dead embryos (Figure 6B). The maternally mutant embryos arrest prior to the 40-cell stage, typically by the 28-cell stage; whereas zygotic mutants complete cell divisions during embryogenesis (Figure 6E). The maternally mutant embryos are grossly abnormal and there are examples of multinucleated cells (Figure 6B). Interestingly, embryos that fail to release extracellular components from cortical granules exhibit defects in cytokinesis and give rise to similar multinucleated cells (Bembenek et al., 2007; Sato et al., 2008). To determine if zygotic expression can rescue the early embryonic lethality, twelve germline mosaics were mated with wild-type males. No rescued progeny were observed in these matings suggesting that a wild-type copy of the vha-12 gene from a wild-type sperm cannot provide vha-12 function. The vha-12 gene is on the X chromosome and it is known that many genes on the paternal X chromosome are silenced during early embryogenesis (Bean et al., 2004). Thus vha-12(+) is expressed in the germ line and is required for early embryogenesis but not in the formation of germ cells.
**vha-12 is required zygotically for morphogenesis**

Zygotic expression begins mid-embryogenesis and appears to be expressed in all cells (Figure 6C,D). To examine the precise onset of zygotic vha-12 gene expression, we performed time-lapse microscopy on isolated embryos carrying our transcriptional reporter construct. One- to two- cell stage embryos were prepared for time lapse confocal imaging, monitoring both DIC for morphology and fluorescence for GFP. Fluorescence was not detected through the first 200 minutes of development; presumably maternally contributed VHA-12 suffices for development during the rapid cell divisions that mark this stage. At the end of gastrulation, approximately 200-220 minutes after the first cell cleavage, fluorescence driven by the vha-12 promoter first appears in embryos, particularly in cells along the midline. Robust fluorescence from the vha-12 reporter is observed in comma stage embryos. Thus, the first stages of *C. elegans* development occur without detectable levels of transcription from the vha-12 locus.

A requirement for zygotic expression appears during morphogenesis (Figure 6E), soon after transcription from the locus begins. Homozygous vha-12(*mg41*) progeny (vha-12(*m+z-*) from vha-12(*mg41+)/+ or vha-12(*mg41*); *Dp[vha-12(+)]* mothers) were analyzed using time-lapse video microscopy. In these experiments 1-2 cell stage embryos were dissected out of gravid hermaphrodite *mg41/mg41; mnDp3* adults, and each embryo was imaged until either they hatched or arrested. Approximately half of the embryos did not hatch, consistent with the reported loss of the *mnDp31* duplication. Of the embryos that did not hatch, 70% arrested at the 2-3 fold stage (450-520 min, 17/25 animals); 28% of the animals arrested earlier, at the lima bean stage (380 min 4/25), at the comma stage (395 min, 2/25), or at the 1.5-fold stage (430 min, 1/25). In this assay, one morphologically
abnormal embryo (1/25) hatched but arrested development shortly thereafter; these rare escapers are likely due to mosaicism, that is, the presence of the rescuing duplication in some cells. Thus, zygotic expression of the *vha-12* gene at the comma stage just proceeds its functional requirement during morphogenesis as determined from the lethal period.

**An apoptosis defect in strong *vha-12* mutants**

In the course of our video microscopy analyses of maternally-rescued *vha-12(mg41)* homozygous embryos, we observed a striking phenotype in the clearance of apoptotic cells (see supporting information movie files S1-S6). We tracked the persistence of apoptotic cell corpses with four-dimensional video recordings. In the course of normal *C. elegans* hermaphrodite embryogenesis 113 cells undergo programmed cell death (Sulston *et al.*, 1983). Using differential interference contrast optics, apoptotic *C. elegans* cells appear as raised disc-like structures about two micrometers in diameter (Conradt and Xue, 2005). Cell corpses are normally engulfed by neighboring cells and degraded. In the wild type, most cell corpses (73%, 16/30) were cleared within twenty minutes of their first appearance, consistent with previous studies (Yuan *et al.*, 1993), and no corpses persisted greater than sixty minutes (Figure 7). The average wild-type corpse duration was $18 \pm 2$ min ($n=30$). In the wild type, no cell corpses (0/15) remained longer than sixty minutes. By contrast, average corpse duration was $47 \pm 3$ min ($n=56$; $P<0.0001$ vs wild-type, Mann-Whitney Test). In *vha-12(mg41)* embryos 36% cell corpses (20/56) persisted sixty minutes or greater. To demonstrate that these cell deaths were due to apoptosis and not degenerative or necrotic cell death we blocked apoptosis using a *ced-3* mutation. CED-3 is the caspase that is required for apoptosis (Ellis and Horvitz, 1986; Yuan *et al.*, 1993). No
cell corpses were observed in a *ced-3(n717) vha-12(mg41)* double mutant. The life-span of *vha-12(mg41)* mutant embryos was not increased by suppressing programmed cell death; the double mutant embryos we observed arrested at the 2-fold stage.

The delay in clearance of dying cells does not result from a general slowdown of embryonic development. We timed easily observable milestones in *vha-12(mg41)* embryos. The lima bean to comma stage took 48±3 min in wild-type and 49±10 min in *vha-12(mg41)* mutants; comma to 1.5-fold took 36 min for wild-type, and 29±5 min in *vha-12(mg41)*; 1.5-fold to 2-fold took 10±2 min for wild-type and 12±0.2 min for *vha-12(mg41)*. In the rare cases where a *vha-12(mg41)* embryo survived to a 3-fold stage, the *vha-12(mg41)* took 75 minutes to progress from the 2-fold to 3-fold stage, the wild-type took 70±2 minutes (n=3 wild-type and n=6 *vha-12(mg41)*). Based on these developmental timing observations, we conclude that although mutant embryos arrest prior to completing morphogenesis, the rate of morphogenesis leading up to arrest is not slowed down in *vha-12(mg41)* mutants.

The lingering of corpses is also not caused by a defect in cell engulfment by neighboring cells. To determine whether corpses can be engulfed in *vha-12(mg41)* mutants we analyzed comma-stage embryos using electron microscopy. Corpses with visible nuclei and mitochondria were observed inside three cells from among the ~60 cells examined (Figure 8A). The corpses were fully enveloped in the two cells that were reconstructed from serial sections, demonstrating that cells were capable of engulfing dying cells. Most cells also exhibited abnormal lysosomes that were filled with dense membranous matter (Figure 8B). Although there is not an absolute defect in engulfment, it is possible that corpse clearance is slowed either because the engulfing cell is not fully functional due to
the lack of a functional lysosome, or because the dying cell cannot undergo programmed cell death properly in the absence of acidified compartments.

**Discussion**

How important is acidification of organelles and secretion of protons by genetic criteria? By characterizing two mutations in the catalytic B subunit of the V-ATPase, we conclude that the B subunit, and by extension the entire V₁ complex, is required for normal cell proliferation, corpse clearance, morphogenesis, neurotransmission, and molting. In addition, given the high expression of *vha-12* in the excretory canal, it is likely that the V-ATPase is involved in secretion of protons in *C. elegans* as in the kidney of higher organisms. In short, VHA-12 is widely expressed and contributes to a variety of cellular functions. Our results confirm and extend observations of the role of V-ATPase in development and in the function of adult cells. Two issues bear further discussion: Are there differential functions for the *V₀* and *V₁* sectors? And are there differential functions provided by isoforms of particular subunits within these sectors?

**Sector diversity: V₀ versus V₁ functions**

The V-ATPase is required to acidify lysosomes and activate degradative enzymes. It is expected that both sectors will be required for acidification since the *V₁* sector forms the ATPase catalytic site and the *V₀* sector forms the pore that translocates protons across the membrane. In addition, it has been proposed that the *V₀* sector has additional functions independent of the *V₁* sector (Peters *et al.*, 2001). Specifically, the transmembrane *V₀* sector may mediate membrane fusion in the vacuole in yeast. To determine whether the *V₀* sector has additional functions, the phenotypes of *V₁* and *V₀* sector mutations must be
compared. If true, the $V_0$ sector mutants would exhibit additional phenotypes not observed in $V_1$ sector mutants. Unfortunately, mutants lacking $V_1$ sector functions have not been well-characterized.

Here we characterize the phenotype of a null mutation in the B subunit of the $V_1$ encoded by $vha-12$. First expression from the $vha-12$ gene begins when cell divisions are nearly complete (roughly 5 hours after the first cleavage). Most mutant embryos arrest three hours later during morphogenesis as the embryos elongate (2–3 fold stage 8 hours after the first cleavage). During embryogenesis 113 cells undergo programmed cell death (Sulston et al., 1983). Normally these dying cells are engulfed by neighboring cells and degraded in the phagocytic pathway (He et al., 2009). Specifically three things need to happen for an apoptotic corpse to be cleared: (1) the corpse needs to be engulfed, (2) the resulting phagosome has to fuse to a lysosome, and (3) acid-activated proteases in the lysosomes need to degrade the corpse. V-ATPases in different systems have been proposed to act in facilitating internalized material (Hurtado-Lorenzo et al., 2006; Dettmer et al., 2006), in organelle fusion steps (Peri and Nüsslein-Volhard, 2008; Williamson et al., 2010), and in lysosomal degradation competence (Lee J.H. et al., 2010). We sought to define at what steps the V-ATPase is involved in $C. elegans$ apoptotic corpse clearance. At the light microscopic level corpses in the $vha-12$ mutant take much longer to clear than in a wild-type background. Ultrastructural analysis of $vha-12$ mutants demonstrated that corpses are engulfed; serial reconstruction of two corpses demonstrated that the cell was completely engulfed by a neighboring cell. Engulfed corpses appeared to progress to a lysosome because the contents of a corpse were surrounded by a single membrane. The corpses had progressed to the lysosome but undegraded nuclei and mitochondria were clearly visible
inside the organelle, a phenotype that one might expect in an acidification defective mutant. Although we were not able to measure acidification of lysosomes in the arrested embryo, we were able to confirm a defect in acidification of synaptic vesicles in the weaker allele, confirming that the \( V_1 \) sector is required to pump protons into organelles. Moreover, lysosomes observed in electron micrographs were swollen and filled with membranes in most cells of mutant embryos, suggested that there are defects in lysosomal functions. These results suggest that a major role of the V-ATPase is to acidify the lysosome.

In two studies, mutations in the conserved a1 subfamily of \( V_o \) a subunits caused defects in the progression of phagocytic compartments, suggesting an additional function for the \( V_o \) sector. In zebrafish, apoptotic corpses were internalized but accumulated as immature phagosomes that did not fuse with the lysosome in animals where \( V_o \) a1 gene expression was knocked down by morpholinos (Peri and Nüsslein-Volhard, 2008). In *Drosophila*, autophagic vesicles accumulated in \( vha100-1 \) mutants (Williamson et al., 2010). In both of these studies the authors observed normal acidification of some organelles. These studies suggest that \( V_o \) has an acidification-independent function to promote vesicle trafficking and maturation of phagolysosomes. The assumption is that the \( V_1 \) sector is still functional and can acidify organelles in these mutants despite mutations in the \( V_o \) a1 isoform. One problem with the studies of \( V_o \) sector mutants is that it is not clear whether the phagosome was acidified and not some other compartment, and whether acidification was truly normal. Moreover, it is not clear how acidification takes place when the pore-forming a subunit expression is knocked-down (Peri and Nüsslein-Volhard, 2008) or is presumably absent (Williamson et al., 2010). One possibility is that there is enough residual function from the a1 subunit or from another partially redundant a subunit.
Alternatively, there may be V-ATPase independent mechanisms for acidification (Orlowski and Grinstein, 2007). Nevertheless, with the caveat that we lack kinetic information about phagosome maturation, our data aligns with the conclusion from these manuscripts: we did not detect a major defect in phago-lysosome maturation because presumably, the $V_1$ sector is absent in $vha-12$ mutants but the fuosgenic $V_o$ sector is present.

On the other hand, another study finds that fully assembled V-ATPases are required for autophagosome maturation and lysosomal degradation competence (Lee, J.H. et al., 2010). If the $V_o$ is not targeted to the lysosome neither are $V_1$ subunits. Lee et al. (2010) found that presenilin is required for a posttranslational modification of a $V_o$ a subunit that targets the subunit to the lysosome. In the absence of presenilin, autophagosomes accumulate, and of those autophagosomes that do fuse to lysosomes, autophagocyteosed material was not degraded. It must be emphasized that we observe a similar phenotype with the loss of the $V_1$ sector in $vha-12(mg41)$ mutants. Together these data suggest that loss of either the $V_o$ or $V_1$ sectors results in the same phenotype, probably due to a defect in acidification as well as a defect in maturation. We are left with contradictory conclusions.

In hindsight, there are two problems in these studies. First, it is not clear whether the a subunit mutants represent a complete loss of $V_o$ sector function, given that there are multiple isoforms of a subunits in all eukaryotic genomes analyzed. Second, the comparisons described above are between mutants in different organisms. In the future studies must be performed comparing V-ATPase $V_1$ and $V_o$ sector mutants in the same organism in which direct measurements of organelle pH are made with measures of organelle fusion in parallel.
**$V_1$ diversity: VHA-12 versus SPE-5**

Why do organisms encode a diversity of individual subunits for the V-ATPase in their genomes? The $V_o$ sector subunit $a$ in particular shows a large amount of diversity. For example, in *C. elegans* there are four genes encoding the $a$ subunit with multiple alternative splice forms (Figure 1A, Pujol *et al.*, 2001; Lee, S.K., *et al.*, 2010). Mammals also encode four $a$ subunits and yeast encode two $a$ subunits (Toei *et al.*, 2010). $V_1$ sector subunits can also be diverse, for example mammals encode two $V_1$ B subunits, two C subunits, two E subunits, and three G subunits. *C. elegans* encodes two B subunits, VHA-12 and SPE-5, and two H subunits. The existence of multiple subunit isoforms may tune V-ATPase activity or confer additional functions independent of proton pumping. This is clearly the case for different subunit $a$ $V_o$ isoforms in yeast; proton coupling to ATPase hydrolysis is weaker in the Golgi-specific isoform than the vacuole-specific isoform (Kawasaki-Nishi, S, and Bowers, T. *et al.* 2001; Kawasaki-Nishi, S., Nishi, T., and Forgac, M., 2001). Also, as discussed above synaptic vesicle $V_o$ subunit $a1$ isoform may also have a fusogenic role distinct from its function in pumping protons (Morel *et al.*, 2003; Hiesinger *et al.*, 2005; DiGiovanni *et al.*, 2010).

Do $V_1$ subunit isoforms similarly tune V-ATPases or confer additional functions? Or is there another explanation for subunit diversity? In mammals, the B1 isoform secretes protons into the lumen of excretory ducts, and the B2 subunit acidifies organelles (Brown *et al.*, 2009). Because B2 can partially substitute for B1 function in kidneys (Păunescu *et al.*, 2007), and B2 isoforms are found on the surface osteoclasts (Lee *et al.*, 1996), it is not clear whether the presence of either isoform changes the activity of the V-ATPase. Although *C. elegans* contains two B subunit paralogs, our functional and expression pattern analyses
suggests that VHA-12 is required in essentially every somatic *C. elegans* cell. By contrast, SPE-5 seems to be largely limited to a requirement in sperm (Gleason et al., 2012). It is possible that SPE-5 confers specialized attributes or regulation to the V₁ sector in sperm. However, VHA-12 can at least partially compensate for loss of SPE-5 (Gleason *et al.*, 2012).

An alternative reason for the duplication of B subunit genes is not to modify V-ATPase function by incorporating different subunit variants, but rather simply to escape epigenetic chromosome inactivation mechanisms. The *vha-12* gene is on the X-chromosome and the *spe-5* gene is on an autosome (Gleason *et al.*, 2012). The X-chromosome exhibits complex expression patterns in the germline. The X-chromosome is largely silenced in the distal gonad in the female germline (Reinke *et al.*, 2000; Kelly *et al.*, 2002). However, the female germline and oocytes do not appear to possess acidified organelles, so *vha-12* expression in the mitotic germ cells might not be required (Kostich *et al.*, 2000). On the other hand, we found that *vha-12* function is required in the female germline for viability during the very early divisions of the embryo. This requirement is fully consistent with the activation of expression from the X-chromosome in late pachytene of oocytes (Reinke *et al.*, 2000; Kelly *et al.*, 2002). *vha-12* is expressed during oogenesis (Wang *et al.*, 2009) and these maternally expressed transcripts are observed in early embryos (Figure S5, Shin-i and Kohara, 2005). Although oocytes appear not to possess acidic compartments, acidified compartments are required soon after fertilization to degrade paternal mitochondria (Sato and Sato, 2011; Al Rawi *et al.*, 2011; Zhou *et al.*, 2011). Thus, portions of the X-chromosome, including *vha-12*, must be reactivated during meiosis despite chromosome condensation in pachytene to prepare for proliferative stages of embryogenesis.
The X-chromosome does not reactivate in the male germline. The X-chromosome is inactivated during gonadogenesis in hermaphrodites and males (Reinke et al., 2000; Kelly et al., 2002) and X-chromosomes derived from males may remain silenced even after fertilization (Bean et al., 2004). Consistent with this pattern of silencing, sperm-enriched genes are nearly completely absent from the X-chromosome (Reinke et al., 2000). By contrast, the membranous organelles of spermatocytes must be acidified to generate functional sperm. As proposed by Gleason et al., (2012), the presence of spe-5 on an autosome might circumvent the effects of X-chromosome inactivation of during spermatogenesis. In fact there are only two \( V_1 \) subunits in the \textit{C. elegans} genome that have multiple subunit isoforms: subunit B and subunit H, and each of these subunits has one isoform on the X-chromosome. Thus, it is possible that there are not specialized \( V_1 \) sectors, but rather they all function identically; gene duplication is simply a means to ensure expression in the face of X-chromosome silencing.
Figure Legends

**Figure 1. Molecular analysis of the vha-12 locus.** (A) *C. elegans* V-ATPase. The *C. elegans* genome contains homologs of each of the fourteen unique vertebrate subunits of the V-ATPase (Lee S.K. *et al.*, 2010) (http://www.wormbase.org, release WS224, 03-Apr-2011). The V sector is shaded orange, and the V sector is shaded blue. Four subunits have multiple isoforms; the V subunits B and H have two isoforms, the $V_o a$ subunit has four isoforms, and the V c subunit has three isoforms. (B) *vha-12* cloning. Genetic map, top: Mapping and rescue of the *n2915* and *mg41* mutations. The *n2915* mutation was mapped between *lin-18* and *dpy-23* loci. Physical map, middle: Cosmid C04E10 rescued the *n2915* locomotion defect and contains the coding region for 10 genes. A 5 kilobase PCR amplification of F20B6.2 was sufficient to completely rescue *vha-12(n2915)* mutants. Bottom: Sequencing the corresponding region in *vha-12(n2915)* adults and in arrested *vha-12(mg41)* mutant embryos identified the nucleotide changes corresponding to nonsense (stop) and missense (stars) mutations.

**Figure 2. vha-12 is expressed broadly in somatic tissues.** GFP expression driven by the wild-type *vha-12* promoter. Three kb of DNA upstream of the *vha-12* translation initiation codon were fused in frame to GFP (strain EG2410). Top: (A) Image of an L3 larva showing strong *vha-12* promoter activity in the excretory cell and the intestine. (B) Confocal section of an adult head showing *vha-12* promoter activity in pharynx, neurons, and hypoderm. (C) Confocal section of adult head showing intense *vha-12* expression in excretory duct. (D) Confocal section of an adult tail showing expression of
neurons in the tail ganglia and tail hypodermis. Plane of section is coronal. (E) Most, if not all, cells in the comma stage embryo show strong vha-12 promoter activity. (F) Ventral hypoderm, muscle and motor neurons (mn) of the ventral nerve cord. (G) The nervous system expresses vha-12. The transgenic strain was fed GFP dsRNA to eliminate expression in non-neuronal tissue; the nervous system is insensitive to RNA interference.

**Figure 3. Neuronal function is impaired in vha-12(n2915) mutants.** vha-12(n2915) animals are uncoordinated. (A) Thrashing. vha-12(n2915) body-bending rate in liquid media was severely reduced compared to wild-type animals and the mg41 mutation failed to complement the bending defect. The bending rate of vha-12(n2915) heterozygotes was less than wild-type but greater than vha-12(n2915) homozygotes. (B) Aldicarb sensitivity. vha-12(n2915) mutants were strongly resistant to the paralyzing effects of aldicarb. (C) Levamisole sensitivity. Homozygous vha-12(n2915) mutants were slightly resistant to the paralyzing effects of the postsynaptic acetylcholine receptor agonist levamisole. Values are mean±S.E.M. of five independent experiments; unc-32(e189) is a neuronal mutation in the a subunit of the V_o domain of the V-ATPase (Pujol et al., 2001). (D) GABA motor neuron axonal morphology is not perturbed. Representative images of animals expressing GFP driven by the GABA-specific unc-47 promoter in wild-type (oxIs12). Scale bar is 0.1 mm. (E) No significant difference in axon number from GABA neurons was detected between wild-type and vha-12(n2915) animals (n=3). (F) Left, the spacing of synaptic puncta was approximately three puncta per ten microns in wild-type and vha-12(n2915) animals (2.87±0.58 n=8 wild-type
animals and $2.79\pm0.62$ n=9 vha-12(n2915) animals mean±SD). Right, quantification of intensity of synaptobrevin-GFP staining puncta. These puncta represent synaptic varicosities. (G) vha-12(n2915) synaptic morphology was normal and expressed normal quantities of the synaptic vesicle (synaptobrevin) GFP fusion protein. Scale bar is ten micrometers.

**Figure 4. Synaptic vesicle acidification is defective in V-ATPase mutants.** Imaging of the ventral nerve cord of adult hermaphrodites expressing the pH-sensitive GFP reporter (pHlourin) targeted to the lumenal domain of synaptobrevin. Fluorescence from pHluorin-tagged synaptic vesicles is increased at both $V_1$ and $V_0$ V-ATPase mutant synaptic varicosities (for example red arrow head). Wild-type ventral cord motor neuron (top), $V_1$ sector mutant vha-12(n2915) motor neuron (middle), $V_0$ sector mutant unc-32(e189) (bottom). Box and whisker plots (right) show the average synaptic fluorescence from individual animals comparing V-ATPase mutant synaptic puncta to matched wild-type controls. Synaptic vha-12(n2915) puncta compared to the wild type (average 665±210 fluorescence units, n=10 for wild-type; 2494±334 fluorescence units, n=10 for vha-12(n2915), $P<0.0001$ two-tailed t-test). Fluorescence at synaptic puncta in unc-32(e189) mutants was increased relative to a separate set of wild-type animals imaged in parallel (average 1060±155 fluorescence units, n=12 for wild-type, 2939±123 fluorescence units, n=13 for unc-32(e189) $P<0.0001$ two-tailed t-test). Median is middle line and box defines the 25th and 75th percentiles. The upper and lower ends of the box whiskers are the ninetieth and tenth percentiles respectively. Increased fluorescence intensity is consistent with more alkaline vesicles. Scale bar is ten micrometers. Figure
insets are 2x magnified regions of interest.

**Figure 5. Reduced body length in vha-12(n2915) heterozygotes.** Animals heterozygous for the vha-12(n2915) mutation were shorter than wild-type animals (WT 0.97±0.05 mm, n=5; vha-12(n2915)/+ 0.76±0.02 mm, n=5; P<0.0001 two-tailed t-test vs WT; and n2915/mg41 0.81±0.07 mm n=9; P=0.001 vs WT). All animals were scored as one day old adults. Asterisks indicate statistically significant different values from wild-type.

**Figure 6. vha-12 is required for embryonic development**

(A) Normal somatic and germline morphology is observed in vha-12(mg41) mosaics in which the rescuing array was lost in the P4 germline progenitor cell. The distal gonad, uterus (ut), spermatheca, sperm (sp), oocytes (numbered relative to spermatheca, -1,-2,-3), and ovulation are normal. Inviable embryos are located in the uterus. Scale bar 50 μm. (B) Loss of maternal vha-12(+) expression results in early embryonic arrest. vha-12(mg41) maternal(-) embryos produced from vha-12(mg41) germline clones from EG7188 vha-12(mg41); oxEx1703 hermaphrodites. Multinucleated cells were frequently observed. Animals arrested with less than 40 cells (560 embryos from germline clones). Scale bar, 20 μm. (C) and (D) Expression of GFP driven by the vha-12 promoter is not detected in early embryos (approximately between 0 and 200 minutes after the first cell cleavage). Images from a movie of embryonic development from a Pvha-12::GFP transgenic strain. In each row a transmitted light image of the embryo, at a single plane (C), is shown along with the projected image acquired from the GFP channel (D). Scale bar 20 μm. (E)
vha-12(mg41) homozygotes arrest between two- and three-fold. Three panels from a time-lapse movie taken in a single focal plane of vha-12(mg41) lon-2(e678) embryos (arrows) produced from a vha-12(mg41) lon-2(e678); mnDp31 hermaphrodite. See also supplemental movie file S6.

**Figure 7. Apoptotic cell corpses persist in vha-12(mg41) mutants.**

(A) Representative images from movies in wild-type and vha-12(mg41) embryos. The insets are 2x-scaled images of the apoptotic cell being tracked in each image; the nuclei of dying cells have a characteristic raised button appearance in DIC. Scale bar is ten micrometers. (B) Scatter plot indicating the duration of individual cell corpses; each point represents the duration of a single tracked corpse. Ten apoptotic cell corpses, were tracked in 3-6 embryos. Most wild-type cell corpses are cleared by twenty minutes. Most vha-12(mg41) cell corpses require more than thirty minutes to clear, if at all. Arrows indicate the time at which tracking was stopped Presumably these corpses persisted longer than shown.

**Figure 8. Cell corpses are engulfed in vha-12(mg41) mutants.** Electron micrographs of cells in a comma-stage embryos in vha-12(mg41). vha-12(mg41) embryos were recognized as segregants from the rescued strain EG7188 that lacked GFP expression from the Peft-3:GFP construct which marked the rescuing array. (A) A cell corpse can be observed in an engulfing cell. A condensed nucleus (‘DNA’) and a mitochondria from the corpse are undegraded and surrounded by the single membrane of a lysosome.
Reconstruction of this cell from serial electron micrographs demonstrated that the corpse was completely surrounded by the engulfing cell. (B) Most cells in the vha-12(mg41) mutant embryos contained swollen lysosomes that contained many undegraded vesicles.

LITERATURE CITED


Strasser, B., J. Iwaszkiewicz, O. Michielin, and A. Mayer, 2011 The V-ATPase proteolipid cylinder promotes the lipid-mixing stage of SNARE-dependent fusion of yeast vacuoles. EMBO J **30**: 4126-4141.


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Figure 2 Ernstom et al.
Figure 3 Ernststrom et al.

A

Body-bends/minute

Wild-type
n2915
n2915
mg41+\nmg41

P<0.01

B

Percent Paralyzed

Aldicarb (mM)

Wild-type
vha-12(n2915)
unc-32(e189)

C

Percent Paralyzed

Levamisole (mM)

Wild-type
vha-12(n2915)
unc-32(e189)
unc-74(ox78)

D

Punc-47::GFP

E

\# GABA commissures

F

Puncia / 10 microns

Mean puncta intensity

G

wild-type; Punc-25:snb-1::GFP

vha-12(n2915); Punc-25:snb-1::GFP
Figure 5  Ernststrom et al.
Figure 6  Ernstrom et al.
Figure 7. Ernstrom et al.

A

Wild-type

vha-12(mg41)

B

Cell Corpse Duration (min)

Wild-type

vha-12(mg41)
Figure 8. Ernstrom et al.