Figure S1:

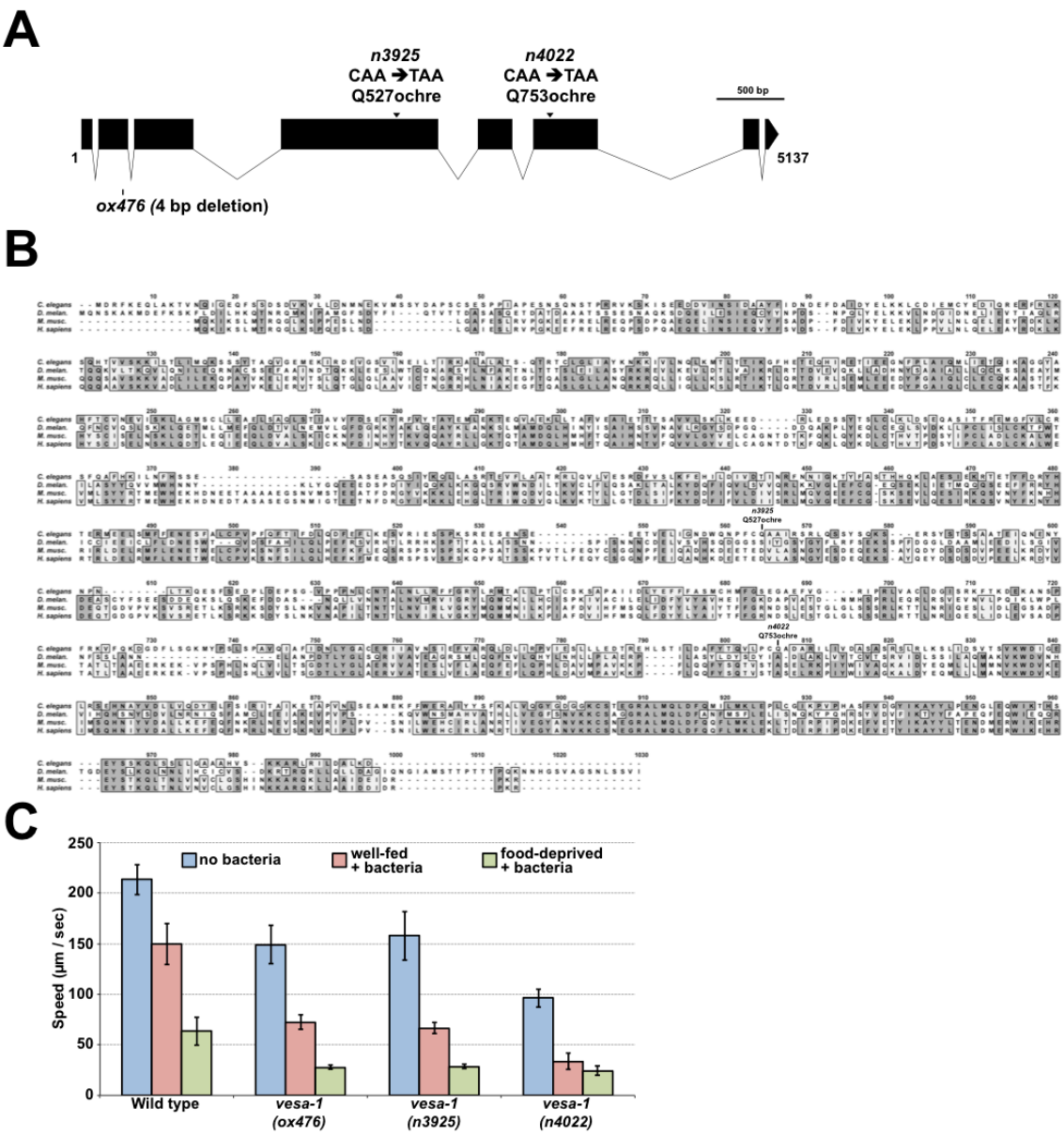

Figure S1 (related to Figure 1): (A) Schematic of *vps-50* exon-intron gene structure. *vps-50(ox476)* is a 4 bp deletion of nucleotides 304-307, *vps-50(n3925)* is a C-to-T mutation resulting in a Q527ochre change, and *n4022* is a C-to-T mutation resulting in a Q753ochre change. Translation of *vps-50* results in a 938 amino acid protein. Graph was generated using www.wormweb.org/exonintron. *n4022* and *n3925* are both nonsense alleles. (B) VPS-50 is conserved from worms to humans. Disrupted sites of *n3925* and *n4022* mutants are indicated. Sequence alignment of worm (*C. elegans*), fly (*D. melanogaster*), mouse (*M. musculus*) and human (*H. sapiens*) sequences is shown. Dark gray boxes mark identical residues, and light gray boxes mark similar residues. (C) Modulation of locomotion in response to the presence of food and past feeding experience. Speeds of *C. elegans* in the absence of food (blue bars), well-fed in the presence of food (red bars) and food-deprived returned to food (green bars). *vps-50(ox476)* and *vps-50(n3925)* similarly cause a weaker locomotion defect than that of *vps-50(n4022)* mutants. It is possible that *vps-50(ox476)* and *vps-50(n3925)* are incomplete loss-of-function mutants, while *vps-50(n4022)* might be a molecular null mutation: an uncloned shorter isoform of *vps-50* might still be present in *ox476* and *n3925* mutants. Alternatively, *ox476* and *n3925* might be null alleles, and *n4022* might be non-null but have a phenotype

stronger than that of null mutants. That *n4022* can be recued by the expression of *vps-50* shows that its stronger phenotype is not caused by a mutation in another gene.

Figure S2:

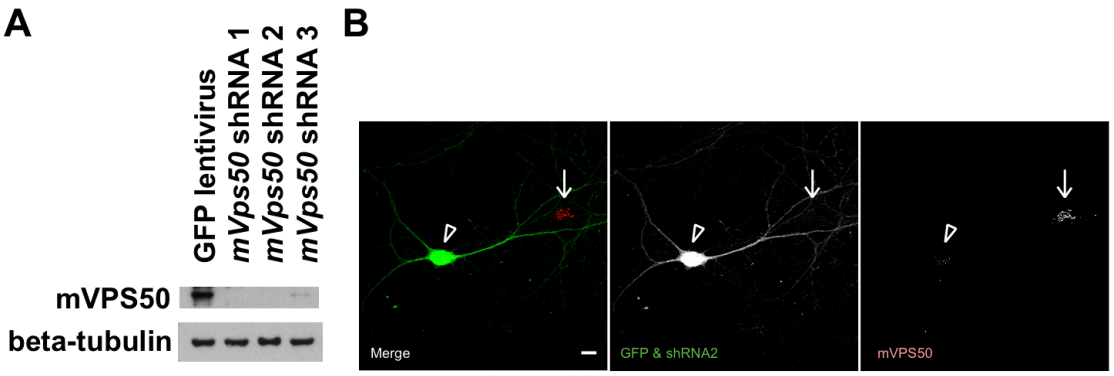

Figure S2 (related to Figure 3): (A) Immunoblotting analysis of mVPS50 using an antibody against the human VPS50 protein. Protein extracts from mouse primary cultured cortical neurons infected with GFP lentivirus (control) or shRNA lentiviruses against *mVps50* were analyzed by immunoblotting using an anti-VPS50 antibody. (B) Immunohistochemistry of mouse primary cultured cortical neurons infected with lentiviruses that coexpress GFP and an shRNA against *mVps50* using an anti-VPS50 antibody. Cells that express GFP do not express mVPS50 and *vice versa*. Arrowhead points to a GFP-positive cell (expressing the *mVps50* shRNA) in which mVPS50 signal is barely detectable. Arrow points to a GFP-negative cell (not expressing the *mVps50* shRNA) where mVPS50 is detected. Scale bar: 10 μm.

Figure S3:

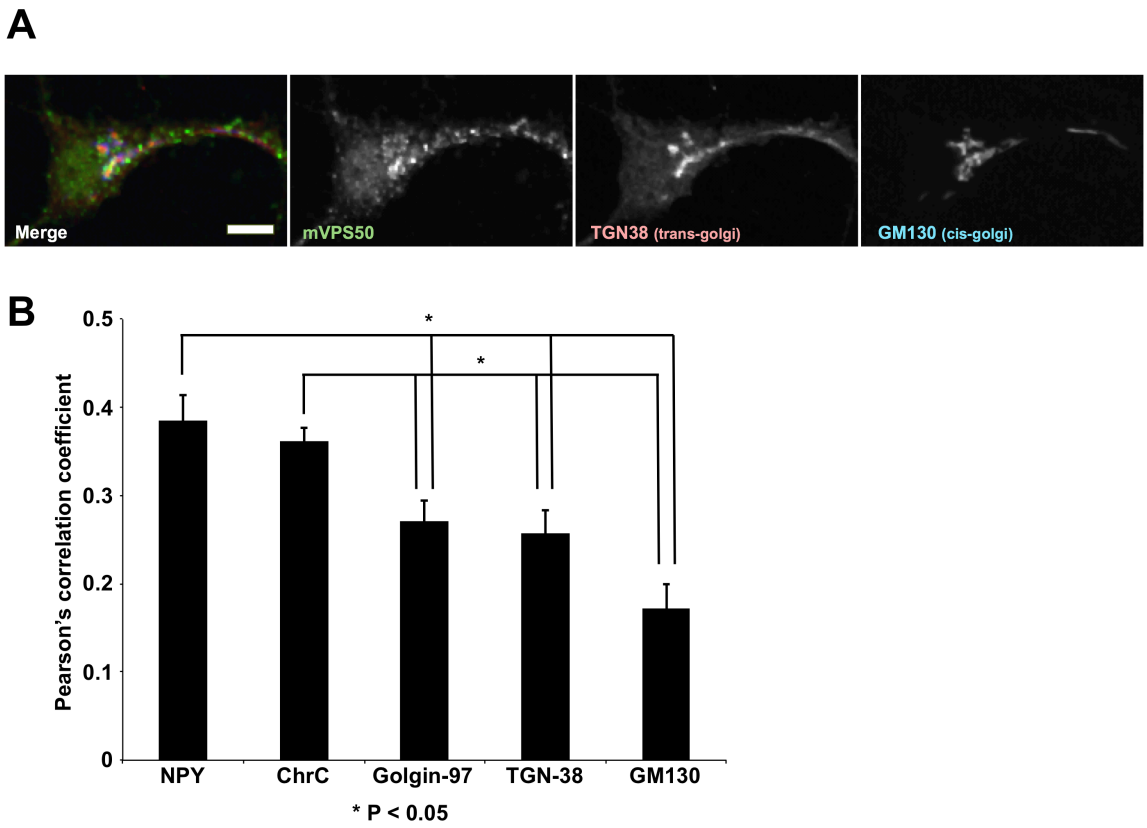

Figure S3 (related to Figure 4): (A) mVPS50 partially colocalized with the *trans*-Golgi marker TGN38 in mouse primary cultured cortical neurons. Endogenous mVPS50 and the *cis*-Golgi matrix protein GM130 and *trans*-Golgi protein TGN38 were detected by immunofluorescence. Scale bar: 5 µm. (B) Quantification of the colocalization between mVPS50 and Neuropeptide Y (NPY), Chromogranin C (ChrC), Golgin-97, TGN-38 or GM130. mVPS50 colocalizes more with neuropeptides NPY or ChrC than with *trans*-Golgi markers Golgin-97 or TGN-38, or with the *cis*-Golgi marker GM130. Significance was analyzed using Student's t test. Multiple comparisons were corrected using the Holm-Bonferroni method.

Figure S4:

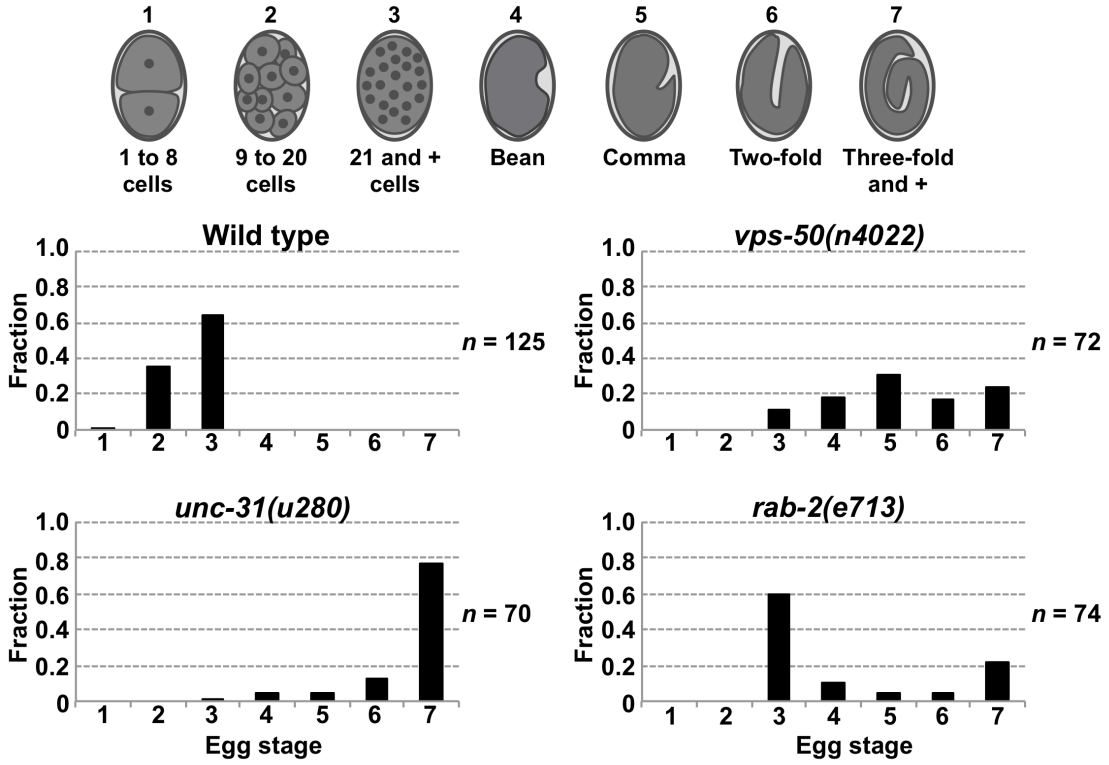

Figure S4 (related to Figure 5): Analyses of the *in utero* retention time of eggs, assayed by the distribution of the developmental stages of newly laid eggs. Like *vps-50* mutants, *unc-31* and *rab-2* mutant animals retain eggs *in utero* longer than do wild-type animals. (Wild type and *vps-50(n4022)* data are the same as in Figure 1D).

Figure S5:

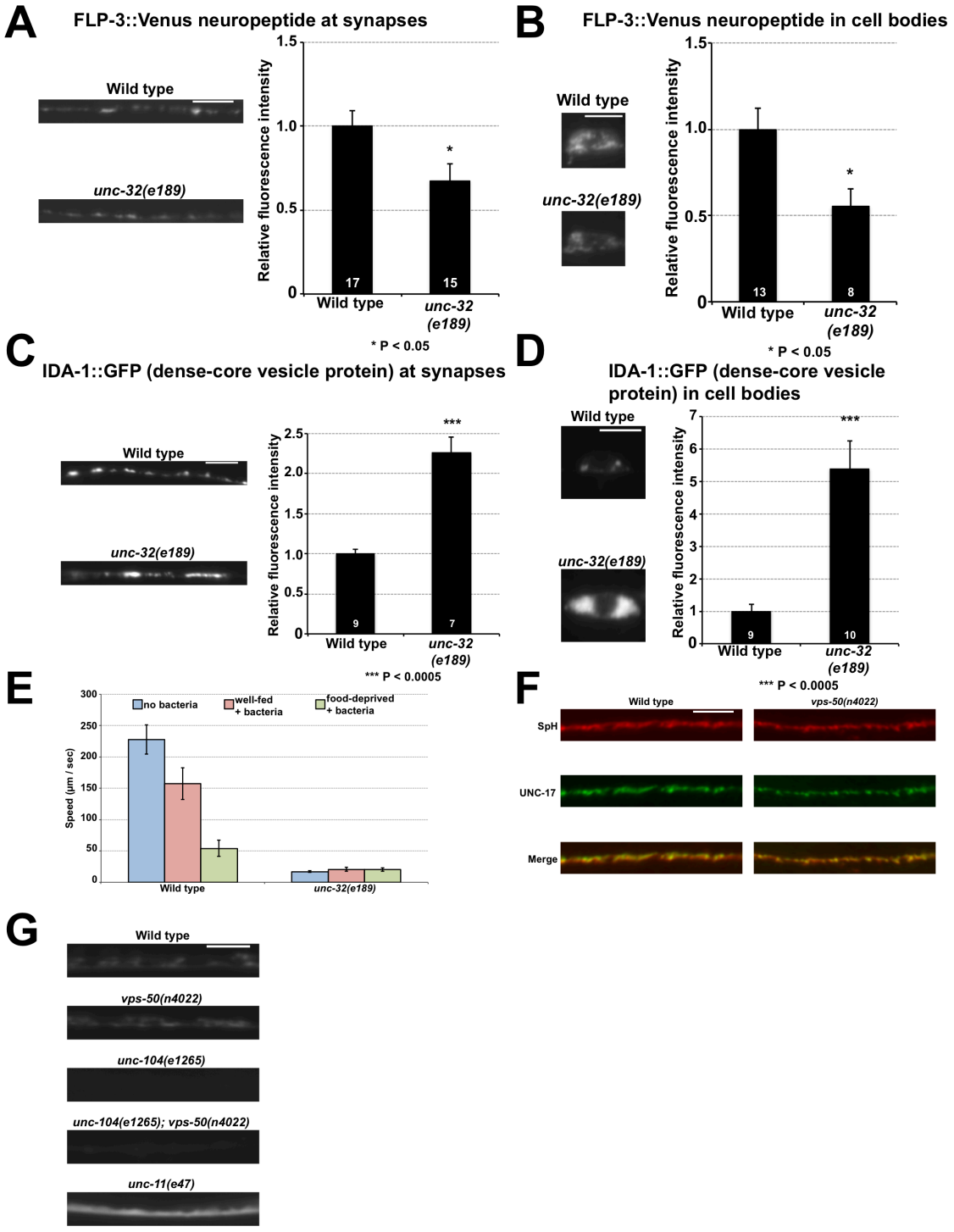

Figure S5 (related to Figure 6): Disruption of synaptic vesicle acidification leads to defects similar to those of *vps-50* mutants. Disruption of the V-ATPase complex subunit gene *unc-32* led to low levels of the FLP-3::Venus neuropeptide reporter (A) at synapses in the dorsal nerve cord and (B) in neuronal cell bodies in the ventral nerve cord. Representative fluorescence micrographs and quantification of FLP-3::Venus. Like *vps-50* mutants, *unc-32* mutants have increased levels of the dense-core vesicle marker

IDA-1::GFP (C) at synapses and (D) in neuronal cell bodies. Representative fluorescence micrographs (left) and quantification of the dense-core vesicle marker IDA-1::GFP (right) in the *C. elegans* dorsal nerve cord and cell bodies. Scale bars: 5 μm. Bar graphs, *n* values (number of animals) are indicated on bars, means ± SEMs. Significance was analyzed using Student's t test. (E) *unc-32* mutants have generally impaired locomotion. Modulation of locomotion in response to the presence of food and past feeding experience. Speeds in the absence of food (blue bars), well-fed in the presence of food (red bars) and food-deprived returned to food (green bars). (F) SpH localizes to synapses. SpH colocalizes with the vesicular acetylcholine transporter UNC-17 in both wild-type and *vps-50* mutant animals. SpH and endogenous UNC-17 localization were analyzed by immunohistochemistry. (G) Transport of SpH to dorsal cord synapses requires the molecular motor UNC-104/KIF1A in both wild-type and *vps-50* mutant animals. Unlike *vps-50* mutants, *unc-11*/AP180 mutants fail to recycle SpH, leading to its diffusion along the plasma membrane. Scale bars: 10 μm.

SUPPLEMENTAL EXPERIMENTAL PROCEDURES

***C. elegans* strains:** *C. elegans* was cultured as described previously at room temperature (~22.5 °C) [S1]. The wild-type strain was Bristol N2. Mutants used were: LGI: *mod-5(n3314)*, *rab-2(e713)*, *unc-11(e47)*; LGII: *unc-104(e1265)*, *cat-2(n4547)*, *tph-1(n4622)*, *tdc-1(n3420)*; LGIII: *unc-32(e189)*, *vps-50(ox476)*, *vps-50(n3925)*, *vps-50(n4022)*, *unc-64(e246)*; LGIV: *unc-31(u280)*; LGV: *egl-3(n729)*; LGX: *lin-15AB(n765)*; Transgenes: *ceIs61(II)*[$P_{unc-129}$*::flp-3::Venus,* $P_{unc-129}$*::RFP:snb-1,* P_{ttx-3}*::RFP]*, *ceIs72*[$P_{unc-129}$*::ida-1::GFP,* P_{ttx-3}*::RFP]*, *nEx*[P_{unc-17}*::flp-3*$_{1-5}$*::mCherry::pHluorin*; P_{myo2}*::mCherry*], *nIs388*[P_{vps-50}*::vps-50::GFP; lin-15(+)]*, *nIs463*[P_{unc-17}*::SpH; lin-15(+)]*, *nIs468*[P_{vps-50}*::GFP; lin-15(+)]*, *nIs490*[P_{rab-3}*::vps-50::GFP; lin-15(+)]*, *nIs517*[P_{unc-17}*::vps-50::GFP; lin-15(+)]*, *nIs521*[P_{rab-3}*::mVps50::GFP; lin-15(+)]*, *nIs522*[P_{unc-47}*::vps-50::GFP; lin-15(+)]*, *nIs523*[P_{myo-3}*::vps-50::GFP; lin-15(+)]*, *nIs613*[P_{tax-2}*::vps-50::GFP; lin-15(+)]*, *nuIs183*[$P_{unc-129}$*::nlp-21::Venus*; P_{myo2}*::NLS::GFP*].

Isolation of *vps-50* mutants: To isolate mutants that behave as if they had been food-deprived even when well-fed, *mod-5(n3314)* animals were used as a sensitized background, because in the food-deprived state their locomotion is significantly slower than that of wild-type worms [S2]. *mod-5(n3314)* animals were mutagenized using EMS, and mutagenized parental (P_0) animals were transferred to Petri plates and grown at 20°C for four days. Adult F_1 progeny were bleached, and eggs were collected and allowed to hatch in S Basal medium in the absence of food to synchronize development. These F_2 progeny were grown to adulthood and assayed for behavioral responses to food. Specifically, animals were transferred to the center of a ring-shaped *E. coli* lawn, and the animals were allowed to move for 30 min. Animals that moved to the inner ring of the lawn and then stopped moving (i.e., could move but stopped moving after encountering *E. coli*) were picked and retested and then examined for coordinated locomotion in the absence of food to ensure that they were capable of coordinated locomotion and not simply paralyzed. *vps-50* mutations were outcrossed from the *mod-5(n3314)* background for further characterization.

Protein interaction assays: For the GST pull-down experiments, GST fusion proteins were purified as described previously [S3]. Worm protein extracts were prepared using a mortar and pestle cooled in liquid nitrogen. Eluted proteins were separated on 4-20% Mini-PROTEAN TGX precast gels (Bio-Rad) and stained with SYPRO Ruby (Invitrogen). Mass spectrometry was performed by the Swanson Biotechnology Center at MIT. For the yeast two-hybrid assay, *vps-50* was cloned in vector pGBKT7, giving a fusion with the Gal4p DNA-binding domain (DBD), and *vha-15* was cloned into vector pGADT7, giving a fusion with the Gal4p activation domain (AD) (Clontech). Plasmids were transformed in strain pJ69-4a, and interaction was assayed by growth in the absence of histidine.

Subcellular fractionation: All buffers contained a protease inhibitor cocktail (Roche), and all procedures were performed on ice or at 4 °C. Subcellular fractionation was performed as described [S4, S5]. The cortexes collected from 2-month old male C57BL/6 mice or mouse primary cultured neurons were homogenized in 0.32 M sucrose and 4 mM HEPES (pH 7.4) and centrifuged at 1,000 g for 10 min to remove the nuclear pellet and debris. The supernatant (S1) was centrifuged at 10,000 g for 10 min to obtain a crude synaptosomal fraction (P2). The supernatant, cytosolic and microsomal fraction (S2) was centrifuged at 165,000 g for 2 hours to obtain the cytosolic supernatant (S3) and the microsomal fraction (P3). The crude synaptosomal fraction (P2) was lysed by hypo-osmotic shock and centrifuged at 25,000 g for 20 min to obtain the synaptosomal membrane fraction (LP1). The resulting supernatant synaptic vesicular and cytosolic fraction (LS1) was centrifuged at 165,000 g for 2 hours to obtain the synaptic vesicular membrane fraction (LP2) and the supernatant fraction (LS2).
Isolation of synaptic and dense-core vesicle fractions was performed using sucrose density gradient centrifugation. The synaptic vesicle fraction (LS1) was prepared from the cortex of adult male mice as described above and layered onto a sucrose gradient (0.6 M – 1.6 M) and centrifuged at 100,000 g for 2 hours at 4 °C. The 12 fractions were collected from the top and analyzed by western blotting.
Antibodies used were: mVPS50/CCDC132 (rabbit, Sigma), PSD-95 (mouse, NeuroMab), GluN1 (mouse, BD Biosciences), beta tubulin (rabbit, Abcam), beta actin (mouse, Sigma), clathrin heavy chain (mouse, BD Biosciences), Chromogranin C (mouse, Abcam), synaptophysin (rabbit, Invitrogen and mouse, Sigma), V-ATPase V1 A subunit (rabbit, Proteintech), V-ATPase V1 B1/2 subunit (mouse, Santa Cruz) V-ATPase V1 H subunit (mouse, Santa Cruz). The secondary antibodies were: goat anti-mouse or anti-rabbit conjugated with HRP (Pierce and Jackson Immunoresearch).

Immunoblotting of *C. elegans* proteins: Proteins were extracted by heating animals in 1X loading buffer at 95 °C for 20 min. The soluble fraction was separated on an AnyKD Mini-PROTEAN TGX precast gel (Bio-Rad) and transferred to Hybond-ECL nitrocellulose (GE Healthcare). Membranes were incubated first with a monoclonal mouse anti-GFP antibody (1:1,000) (Roche) and second with goat anti-mouse-HRP antibody (1:10,000) (Sigma) for detection of FLP-3::Venus. Membranes were stripped and blotted with a polyclonal rabbit anti-RFP antibody (1:1,000) (Clontech) and second with goat anti-rabbit-HRP antibody (1:60,000) (Sigma) for detection of mCherry::SNB-1.

Construction of the lentiviral *mVps50* shRNA vector: The lentiviral shRNA plasmid pLL 3.7 (gift from Carlos Lois, University of Massachusetts, Worcester, MA) was modified to enhance neuronal expression of GFP or mCherry by replacing the CMV promoter with the human synapsin 1 promoter [S6, S7]. The following oligonucleotides encoding short hairpin RNAs (shRNAs) were inserted under the U6 promoter between HpaI and XhoI sites.

shRNA1:

tGCTTCCTCCTGTTCTCAATCTcgaaAGATTGAGAACAGGAGGAAGCttttttc

tcgagaaaaaaGCTTCCTCCTGTTCTCAATCTttcgAGATTGAGAACAGGAGGAAGCa

shRNA2:

tggCTATTACTTGTATGCCATATAcgaaTATATGGCATACAAGTAATAGttttttc

tcgagaaaaaaCTATTACTTGTATGCCATATAttcgTATATGGCATACAAGTAATAGcca

shRNA3:

tggATAGCATTGAACAAGTCTATTcgaaAATAGACTTGTTCAATGCTATttttttc

tcgagaaaaaaATAGCATTGAACAAGTCTATTttcgAATAGACTTGTTCAATGCTATcca

All three shRNAs were validated for *mVps50* knockdown efficiency, and shRNA1 and shRNA2 were used for further experiments.

Lentivirus Production: Lentiviruses were produced as previously described [S8]. Briefly, human embryonic kidney 293T (HEK 293T) cells were transfected using Lipofectamine 2000 (Invitrogen) with lentiviral shRNA plasmid, pCMV-dR8.91 plasmid and pCMV-VSV-G plasmid at 20, 15, and 10 µg of DNA per 15 cm plate. 48 hours after transfection, culture medium was collected and centrifuged at 2,000 g for 10 min. Supernatants were filtered through a 0.45-µm filter and centrifuged at 83,000 g for 1.5 hours, and the resulting pellets were resuspended in PBS. The titer of lentivirus was between 5×10^4 and 1×10^5 infectious units per µL.

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