Supplemental Data

A Novel Requirement for *C. elegans* Alix/ALX-1 in RME-1-Mediated Membrane Transport

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Supplemental Results

alx-1 Mutants Are Delayed in the Degradation of Membrane Proteins

Yeast Bro1p functions at the MVE with the ESCRT complex to downregulate membrane-associated receptors [S1]. Mammalian Alix has also been implicated in the multivesicular body pathway, interacting with ESCRT-I and -III [S2] and in vitro regulates the dynamics and function of the internal membranes of MVEs through interaction with the LBPA lipid [S3]. However, the degradation of EGF-Rs, a classic MVE-mediated transport event, was unaffected by the siRNA-mediated knockdown of Alix in HeLa cells [S4]. To test for defects in MVE-pathway-mediated degradation in *alx-1* mutant worms, we analyzed the degradation of CAV-1 protein in early *C. elegans* embryos. This analysis was facilitated by the use of transgenic animals expressing a well characterized CAV-1-GFP fusion protein [S5]. In normal zygotes, CAV-1-GFP is exocytosed in a nearly simultaneous wave during anaphase I of meiosis. CAV-1-GFP is then rapidly internalized and degraded through the standard clathrin pathway [S5]. In normal embryos, most CAV-1-GFP disappears by the two-cell stage, and none is visible by the four-cell stage (Figure S6A). In *alx-1* mutants, CAV-1-GFP degradation was significantly delayed, with abnormally high levels of internalized CAV-1-GFP remaining in two-cell and four-cell embryos (Figure S6C). Quantitative fluorescence measurements of CAV-1-GFP in two-cell embryos showed a 12-fold average increase in puncta number compared to the wild-type (Figure S6I). We observed a similar delay in the degradation of RME-2-GFP yolk receptors in *alx-1* mutant embryos (data not shown). These results indicate that ALX-1 is required for the efficient degradation of integral membrane proteins, consistent with a conserved role for this protein in MVE function.

We also examined the degradation of a lumenal cargo protein, YP170-GFP, in embryos [S6]. YP170 is a major yolk protein responsible for transporting lipids from the intestine to the oocyte [S7]. YP170 is secreted by the intestine and endocytosed by oocytes in the wild-type [S6, S8]. During embryogenesis, yolk stores are gradually depleted by lysosomal degradation, with some remaining in newly hatched embryos [S6, S8]. We did not find any change in the pattern or timing of YP170-GFP degradation in *alx-1* mutants (Figures S6E and S6G). These results suggest that ALX-1 is not required for the transport of lumenal endosome content to lysosomes for degradation.

Supplemental Experimental Procedures

General Methods and Strains

All *C. elegans* strains were derived originally from the wild-type Bristol strain N2. Worm cultures, genetic crosses, and other *C. elegans* husbandry were performed according to standard protocols [S9]. Strains expressing transgenes in the germline were grown at 25°C. Other strains were grown at 20°C. A complete list of strains used in this study can be found in Table S1.

RNAi was performed by the feeding method [S10]. Feeding constructs were prepared by polymerase chain reaction (PCR) from expressed sequence tag (EST) clones provided by Dr. Yuji Kohara (National Institute of Genetics, Japan), and subcloning into the RNAi vector L4440 followed [S10]. RNAi-treated F1 animals were used for the assay. Phenotypes were scored in adults.

Antibodies

The following antibodies were used in this study: mouse HA monoclonal antibody (16B12) (Covance Research Products [Berkeley, CA]), rabbit GST polyclonal antibody (Z-5) (Santa Cruz Biotechnologies [Santa Cruz, CA]), and mouse anti-human HLA-A,B,C monoclonal antibody (w6/32) (BioLegend [San Diego, CA]). Fluorescent-dye-conjugated secondary antibodies and Alexa568-Tf were purchased from Molecular Probes (Invitrogen [Carlsbad, CA]).

Yeast Two-Hybrid Analyses

The yeast two-hybrid screen was performed with the Gal4-based Proquest System (Invitrogen) according to manufacturer's instructions. The pDBLeu-RME-1(442–576) bait plasmid was transformed into yeast strain MaV203. MaV203 bearing the bait plasmid was amplified and transformed with the Vidal *C. elegans* library in prey vector PC86, essentially as described [S11, S12]. Clones (2.25 million) were screened on histidine dropout plates containing 20 mM 3-AT. Twenty-one positives were recovered that retested as positive in the His and β-gal assays after isolation and retransformation with fresh bait plasmid. Six of these 21 positive clones contained portions of *alx-1* complementary DNA (cDNA).

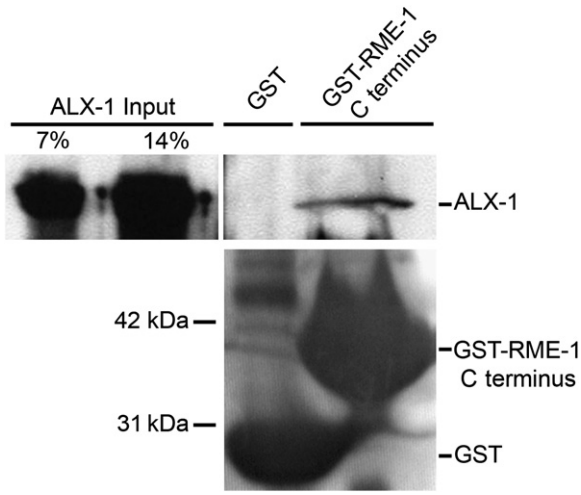

Figure S1. In Vitro Binding of RME-1 to ALX-1

Glutathione beads loaded with recombinant GST or GST-RME-1(442–576) were incubated with in vitro-expressed HA-ALX-1 and then washed so that unbound proteins could be removed. Bound proteins were eluted by laemmli sample buffer, separated by SDS-PAGE, and analyzed by western blotting assays with HA (top) and GST (bottom) antibodies. Input lanes contain in vitro-expressed HA-ALX-1 used for binding assays (7% and 14%).

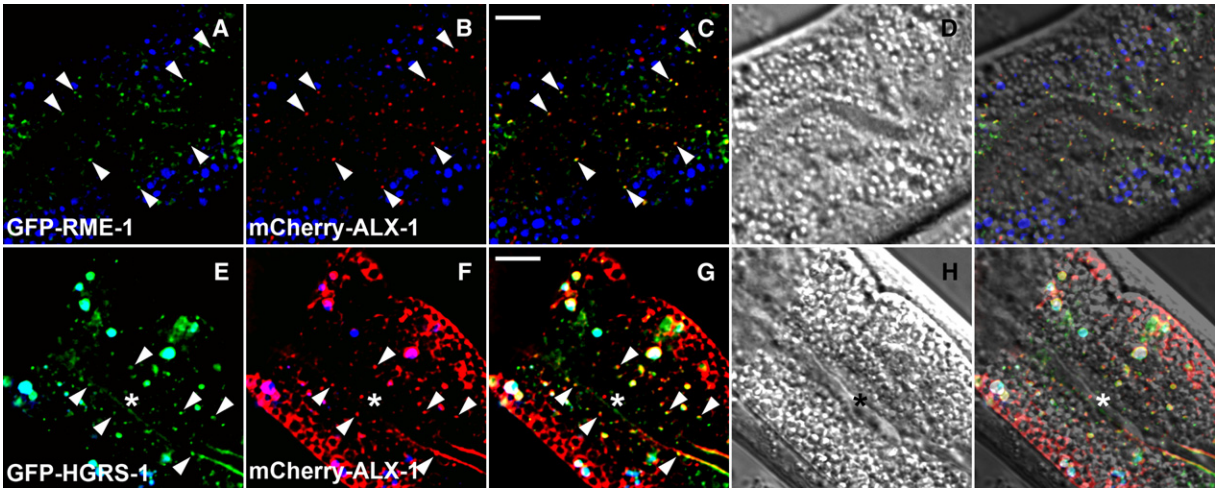

Figure S2. ALX-1 Associates with Two Types of Endosomes in the Intestine

(A–C) mCherry-ALX-1 colocalizes with GFP-RME-1 on basolateral recycling endosomes.

(D) Differential interference contrast (DIC) microscopy image shows basolateral focal plane of the intestine. Note that the depth of field is much greater for the DIC image than for the deconvolved epifluorescence. Arrowheads indicate endosomes labeled by both GFP-RME-1 and mCherry-ALX-1.

(E–G) mCherry-ALX-1 colocalizes with MVE marker GFP-HGRS-1 in the medial and apical cytoplasm.

(H) DIC image showing medial focal plane of the intestine.

Arrowheads indicate puncta labeled by both mCherry-ALX-1 and GFP-HGRS-1. Scale bars represent 10 μm.

The LexA-based DupLEX-A yeast two-hybrid system (OriGene Technologies [Rockville, MD]) was used for all subsequent analysis according to the manufacturer's instructions. pSH18-34 was used as reporter in all the yeast two-hybrid experiments. Constructs were introduced into the yeast strain EGY48 included in the system. To assess the expression of the *LEU2* reporter, transformants were selected on plates lacking leucine, histidine, tryptophan, and uracil, containing 2% galactose and 1% raffinose at 30°C for 3 days. β-gal activity was measured with the standard ONPG (o-nitrophenyl β-D-galactopyranoside) test [S13]. β-gal activity in Miller units was plotted as an average from assays performed in duplicate.

Protein Expression and Coprecipitation Assays

N-terminally HA-tagged ALX-1 protein was synthesized in vitro with the TNT coupled transcription-translation system (Promega) with DNA template pcDNA3.1-2xHA-ALX-1 (1.6 μg/each 50 μl reaction). The reaction cocktail was incubated at 30°C for 90 min. Control glutathione S-transferase (GST), or GST-RME-1d (aa 442–576) fusion protein, was expressed in *Escherichia coli* BL21 cells. Bacterial pellets were lysed in 3 ml B-PER Bacterial Protein Extraction Reagent (Pierce) with Complete Protease Inhibitor Cocktail Tablets (Roche). Extracts were cleared by centrifugation, and supernatants were incubated with glutathione Sepharose 4B beads (Amersham Pharmacia) at 4°C overnight. Beads were then washed six times with cold salt Tris EDTA triton (STET) buffer (EDTA: ethylenediamine tetraacetic acid) (10 mM Tris-HCl [pH 8.0], 150 mM NaCl, 1 mM EDTA, 0.1% Tween-20). In vitro-synthesized HA-tagged ALX-1 (10 μl TNT mix diluted in 500 μl STET) was added to the beads and allowed to bind at 4°C for 2 hr. After six additional washes in STET, the proteins were eluted by being boiled in 30 μl 2x sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer. Eluted proteins were separated on SDS-PAGE (10% polyacrylamide), blotted to nitrocellulose, and probed with anti-HA (16B12). Subsequently, the blots were stripped and reprobed with GST (Z-5) antibodies.

Plasmids and Transgenic Strains

The Proquest system two-hybrid bait plasmid pDBLeu-RME-1 contained a region of cDNA yk271a1, encoding RME-1 isoform D amino acids 442–576, cloned into the unique SalI-NotI sites. An equivalent *rme-1*(442-576) PCR product was introduced in frame into the BamHI and EcoRI sites of the pGEX-2T vector (GE Healthcare Life Sciences) for GST pulldown experiments. All OriGene system two-hybrid plasmids were generated as PCR products with Gateway attB.1 and attB.2 sequence extensions and were introduced into the Gateway entry vector pDONR221 by BP reaction. They were then transferred into the final destination vector with Gateway recombination cloning (Invitrogen) LR reaction. The bait vector pEG202 and target vector pJG4-5 (OriGene) were modified in house with the Gateway cassette (Invitrogen). RME-1 sequences were cloned into the bait vector pEG202-Gtwy. Target constructs were introduced into vector pJG4-5-Gtwy. Origene system two-hybrid bait plasmids contained the complete RME-1 isoform F C-terminal region (aa 447–555) or a slightly smaller region lacking the extreme C-terminal nine amino acids including the YPSL motif (aa 447–546, ΔYPSL). Prey plasmids encoded full-length ALX-1 isoform B (aa 1–882), ALX-1b lacking the C-terminal NPF tripeptide (precise deletion of aa 879–881), ALX-1b N-terminal fragment (aa 1–752), ALX-1b BRO1 domain (aa 1–365), the central fragment of ALX-1b (aa 365–752), or the C-terminal fragment (aa 753–882) amplified from *alx-1* cDNA clone yk2c8. All amplified regions were confirmed by DNA sequencing. The same pDONR221-ALX-1(aa 1–882) cDNA clone was transferred into an in-house-modified pcDNA3.1 (+) (Invitrogen) with 2xHA epitope tag and Gateway cassette (Invitrogen) for in vitro transcription and translation experiments.

For the creation of the GFP-ALX-1 plasmid driven by the *alx-1* promoter, 1.145 kb of *alx-1* promoter sequence was PCR amplified from *C. elegans* genomic DNA with primers containing XbaI and NotI restriction sites and cloned into the same sites in the *C. elegans* GFP vector pPD117.01 (gift from Andrew Fire). The entire *alx-1* gene body and 3′ untranslated region (UTR) (5.595 kb) was then PCR amplified with primers including NaeI and BspEI restriction sites and cloned into the same sites downstream of GFP. The GFP-ALX-1 plasmid (10 μg/ml) was coinjected with plasmid pRF4 encoding *rol-6(su1006)* so that transgenic lines could be established. Integrated transgenic lines were generated by standard gamma-irradiation methods [S14].

For the construction of GFP or mCherry fusion transgenes for expression in the worm intestine, two previously described Gateway destination vectors [S15] were used that contain the promoter region of the intestine-specific gene *vha-6* cloned into the *C. elegans* pPD117.01 vector, then GFP or mCherry coding sequences, a Gateway cassette (Invitrogen), and let-858 3′ UTR sequences, and then the unc-119 gene of *C. briggsae*. The sequences of *C. elegans hgrs-1(genomic), alx-1b (cDNA)* and *rme-1d (cDNA)* were cloned individually into entry vector pDONR221 by PCR and BP reaction and

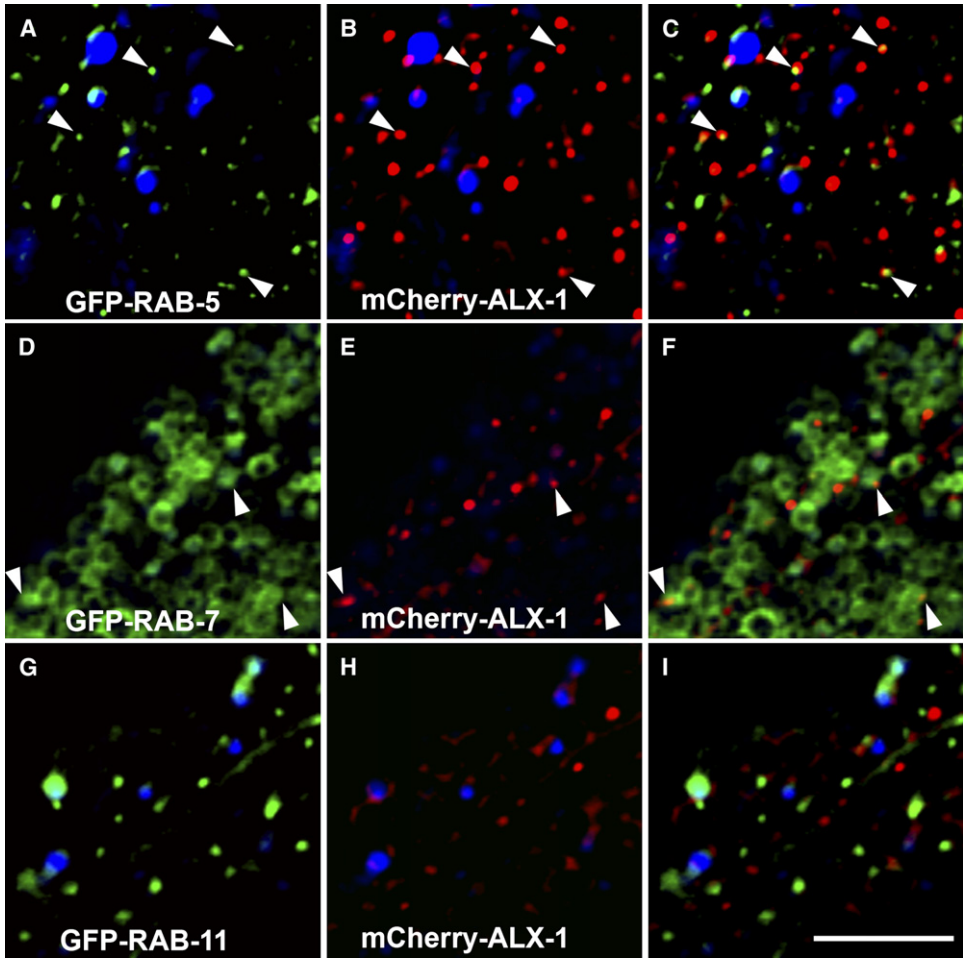

Figure S3. mCherry-ALX-1 Partially Colocalizes with an Early-Endosome Marker and a Late-Endosome Marker in the Intestine

(A–C) mCherry-ALX-1 partially colocalizes with GFP-RAB-5 on early endosomes. Arrowheads indicate basolateral puncta labeled by both GFP-RAB-5 and mCherry-ALX-1.

(D–F) mCherry-ALX-1 occasionally appears as puncta on or near the rim of GFP-RAB-7-labeled late-endosomal rings. Arrowheads indicate puncta labeled by mCherry-ALX-1 on or near GFP-RAB-7 rings.

(G–I) ALX-1 and RAB-11 label different endosome types. In each image, autofluorescent lysosomes can be seen in all three channels with the strongest signal in blue, whereas GFP appears only in the green channel and mCherry only in the red channel. Signals observed in the green or red channels that do not overlap with signals in the blue channel are considered bona fide GFP or mCherry signals, respectively. The scale bar represents 10 μm.

then transferred into intestinal expression vectors by Gateway recombination cloning LR reaction to generate N-terminal fusions. The *sdpn-1* expression plasmid was created by PCR amplification and Gateway cloning of the complete *sdpn-1a* genomic region (7.870 kb), lacking a stop codon and including the predicted *sdpn-1* promoter region (4.259 kb), into vector pPD117.01 modified with a Gateway cassette inserted at the Asp718I site just upstream of the GFP coding region. Complete plasmid sequences are available on request. Low-copy integrated transgenic lines were obtained by the microparticle bombardment method [S16].

Microscopy and Image Analysis

Live worms were mounted on 2% agarose pads with 10 mM levamisol as described previously [S17]. Fluorescence images were obtained with an Axiovert 200M (Carl Zeiss MicroImaging [Oberkochen, Germany]) microscope equipped with a digital CCD camera (C4742–12ER, Hamamatsu Photonics [Hamamatsu, Japan]), captured with Metamorph software (Universal Imaging [West Chester, PA]), and then deconvolved with AutoDeblur software (AutoQuant Imaging [Watervliet, NY]). Images taken in the DAPI channel were used for the identification of broad-spectrum intestinal autofluorescence caused by lipofuscin-positive lysosomes [S18]. To obtain images of GFP fluorescence without interference from autofluorescence, we used the spectral finger printing feature of a Zeiss LSM510 Meta confocal microscope system (Carl Zeiss MicroImaging) with argon 488 excitation [S15]. Quantification of images was performed with Metamorph software (Universal Imaging). Most GFP versus mCherry colocalization analysis was performed on L3 larvae generated as F1 crossprogeny of GFP-marker males crossed to mCherry-marker hermaphrodites [S15].

Cell Culture, Transfections, and Pulse-Chase Analysis

All tissue culture supplies were from GIBCO (Invitrogen). HeLa cells were a kind gift from Dr. J. Donaldson and were grown in Dulbecco's modified Eagle's medium (DMEM) containing 5% fetal bovine serum (FBS) and 100 U/ml penicillin-streptomycin.

MHC class I uptake and recycling in HeLa cells was done essentially as previously described [S19]. HeLa cells were seeded onto glass coverslips at a density of 10,000 cells per well, in 24-well plates, and grown for 24 hr before they were transfected with 0.5 μg of plasmid DNA (EGFP, FLAG-Alix(467–869), mRFP1, or mRFP1 cotransfected with FLAG-Alix(467–869) with jetPEI reagent (Polyplus-transfection [San Marcos, CA]). All experiments were done 24 hr after transfection. Cotransfection efficiency in control experiments

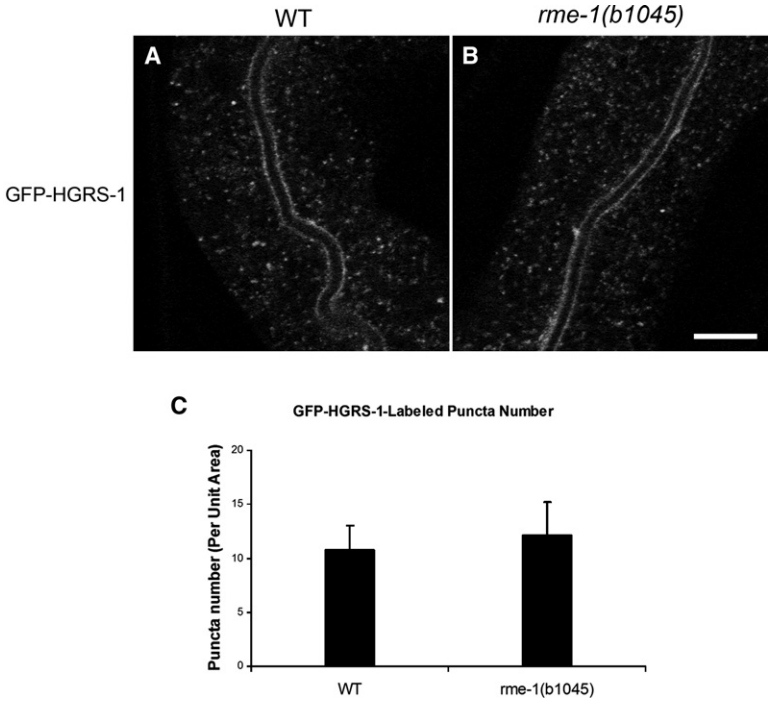

Figure S4. No change in GFP-HGRS-1-Labeled Puncta Number in *rme-1* Mutants

(A and B) Confocal images in wild-type (A) and *rme-1(b1045)* mutant (B) intestinal cells expressing MVE marker GFP-HGRS-1. The scale bar represents 10 μm.

(C) Quantification of endosome number in wild-type animals and *rme-1* mutants as visualized by GFP-HGRS-1. Error bars represent standard deviations from the mean (n = 18 each, six animals of each genotype sampled in three different regions of each intestine).

was determined after the cells were fixed for 15 min in 3.7% (v/v) paraformaldehyde in phosphate-buffered saline (PBS) at room temperature, and permeabilization of the cells with 0.1%(v/v) Triton X-100 for 15 min at room temperature followed. After cells were blocked for 30 min in 3% (w/v) bovine serum albumin (BSA), the cells were labeled with anti-FLAG primary antibody for 1 hr at room

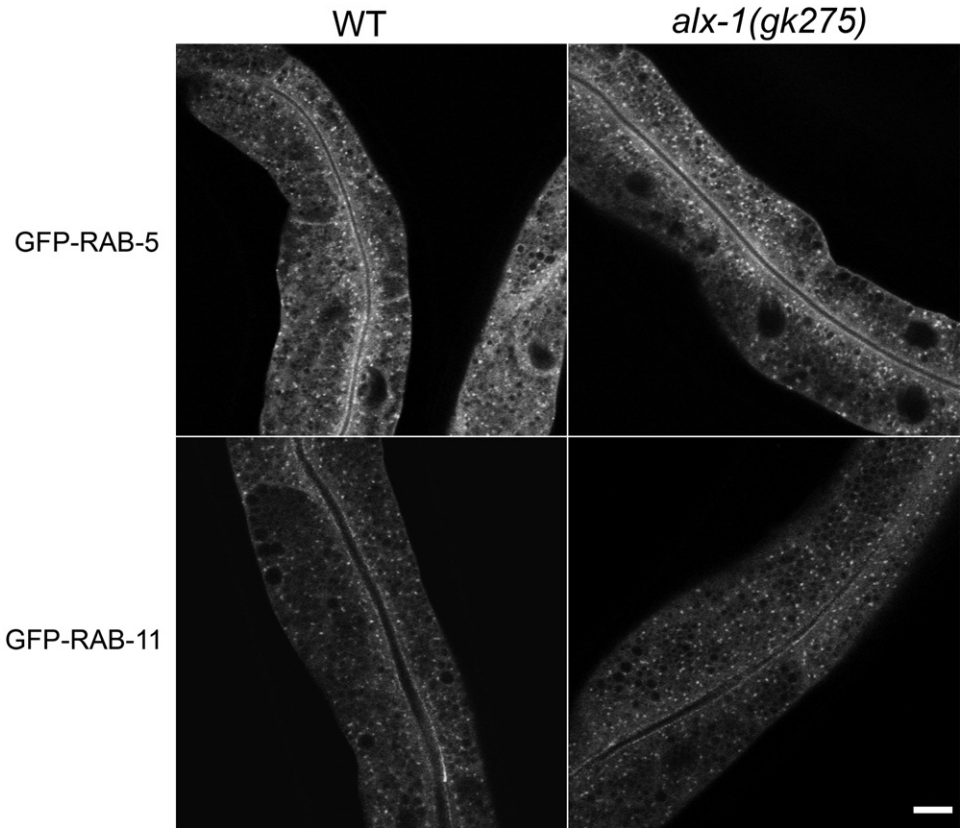

Figure S5. Additional Analysis of GFP-Tagged Endosome Markers and Endocytic Cargo Markers in *alx-1* Mutants

Confocal images of wild-type and mutant animals showing GFP-labeled structures (as indicated). GFP-RAB-5 labels early endosomes in the intestine; GFP-RAB-11 is a marker for apical recycling endosomes and TGN. The scale bar represents 10 μm.

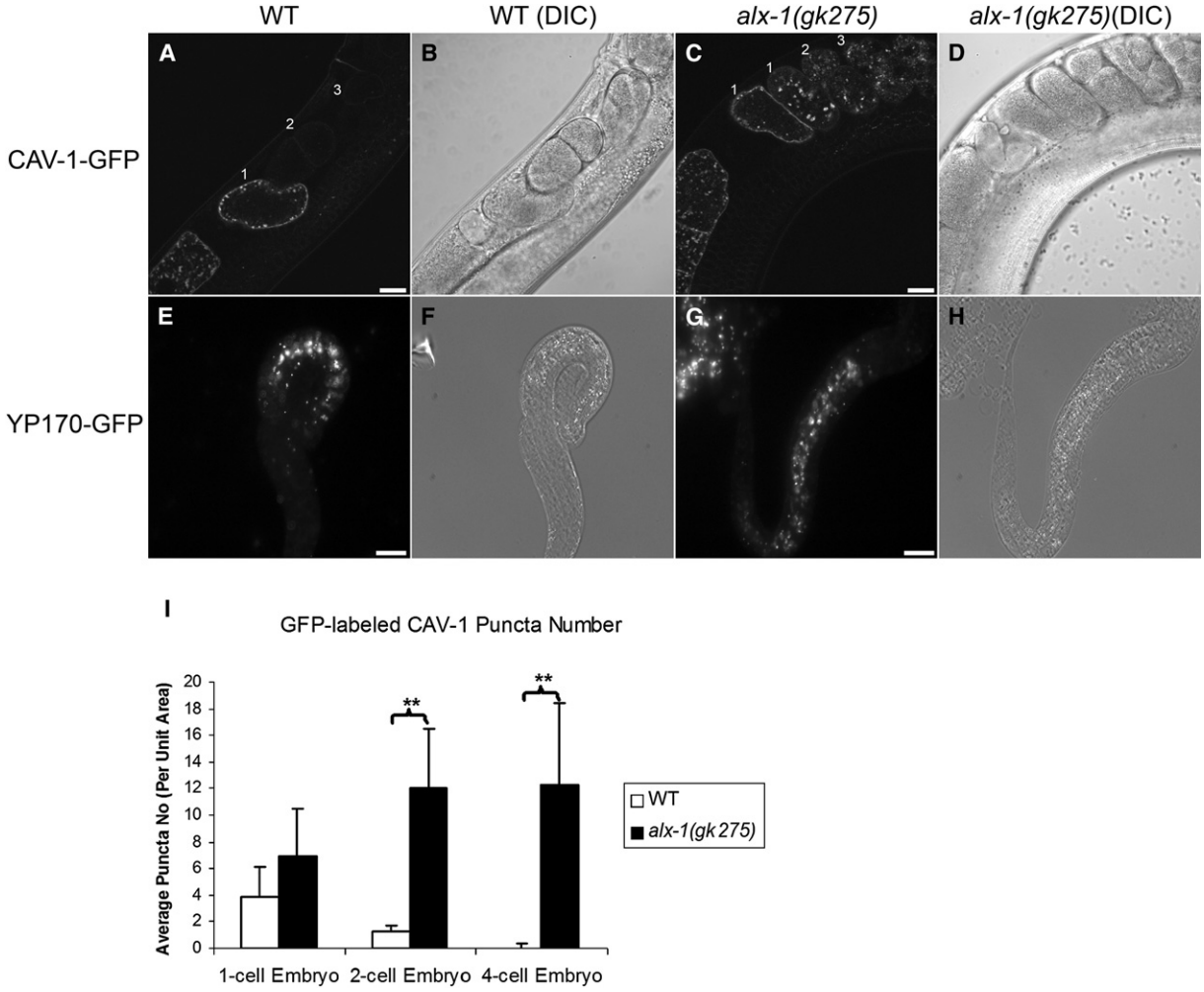

Figure S6. *alx-1* Mutants Are Delayed in the Degradation of Membrane Proteins

CAV-1-GFP is normally exocytosed in a nearly simultaneous wave during anaphase of the first embryonic meiosis. CAV-1-GFP is then rapidly endocytosed and degraded through the standard clathrin pathway.

(A and B) In wild-type embryos, CAV-1-GFP is degraded by the two-cell stage, with virtually no visible CAV-1-GFP-labeled structures remaining by the four-cell stage.

(C and D) Degradation of internalized CAV-1-GFP is severely delayed in *alx-1* mutants. CAV-1-GFP punctae remain in *alx-1* mutant two-cell and four-cell embryos. One-cell, two-cell, and four-cell embryos are indicated by numbers 1, 2, and 3, respectively.

(E–H) No change in the pattern or timing of lumenal cargo protein YP170-GFP degradation was found in *alx-1* mutant embryos. Remaining levels of undegraded YP170-GFP in 3-fold stage embryos (G and H) was similar to that of wild-type embryos (E and F) at the same stage.

(I) Quantification of CAV-1-GFP punctae. Asterisks indicate a significant difference in the one-tailed Student's t test ($p < 0.01$).

Scale bars represent 10 μm.

temperature, and incubation with secondary antibody for 1 hr at room temperature followed. Cells expressing mRFP and FLAG-Alix (467–869) were counted in three random fields. Ninety-nine percent of cells expressing mRFP also expressed FLAG-Alix (467–869), and 68% of cells expressing FLAG-Alix (467–869) also expressed mRFP1. MHC class I and transferrin uptake and recycling assays were done as previously described [S19].

For the analysis of MHC class I uptake and recycling, cells were incubated on ice for 30 min with 50 μg/ml of W6/32 antibody so that antibody binding to endogenous MHC class I could be allowed. Cells were then washed with ice-cold PBS so that unbound antibody could be removed and incubated for 30 min at 37°C in the presence of 1 mM LatrunculinA so that the uptake and accumulation of MHC class I in recycling endosomes could be allowed. The remaining surface-bound antibody was removed by the incubation of the cells for 40 s in stripping buffer (0.5% acetic acid, 0.5 M NaCl [pH 3.0]), and two washes with PBS and two washes with DMEM followed. A set of cells were fixed for 15 min at room temperature in 3.7% paraformaldehyde in PBS (representing MHC class I uptake), whereas another set of cells were transferred to full supplemented DMEM, for 30 min at 37°C, so that MHC class I recycling could be allowed, and then fixed as above. The internalized or recycled MHC class I antibody was revealed by the incubation of cells with Alexa-488 conjugated anti-mouse secondary antibody in the presence (total pool) or absence (surface pool) of 0.2% saponin, respectively.

For the analysis of Alexa568-Transferrin (Alexa568-Tf) uptake and recycling, cells were serum starved for 30 min at 37°C in DMEM containing 0.5% (w/v) BSA. Then 5 μg/ml Alexa 568-Tf was internalized for 30 min at 37°C. At the end of the internalization, cells were stripped of surface-bound Tf as described above, and one set of cells were fixed (representing Tf uptake), whereas other set of cells was incubated in complete medium for 30 min followed by fixation (representing Tf recycling).

Images of at least 100 cells per trial from random fields on each coverslip were captured with a Zeiss F-fluor 40×/1.3 oil objective. Fluorescence intensities in HeLa cells were measured by the thresholding and outlining of whole individual cells, and then the determination of integrated fluorescence intensities, which were then

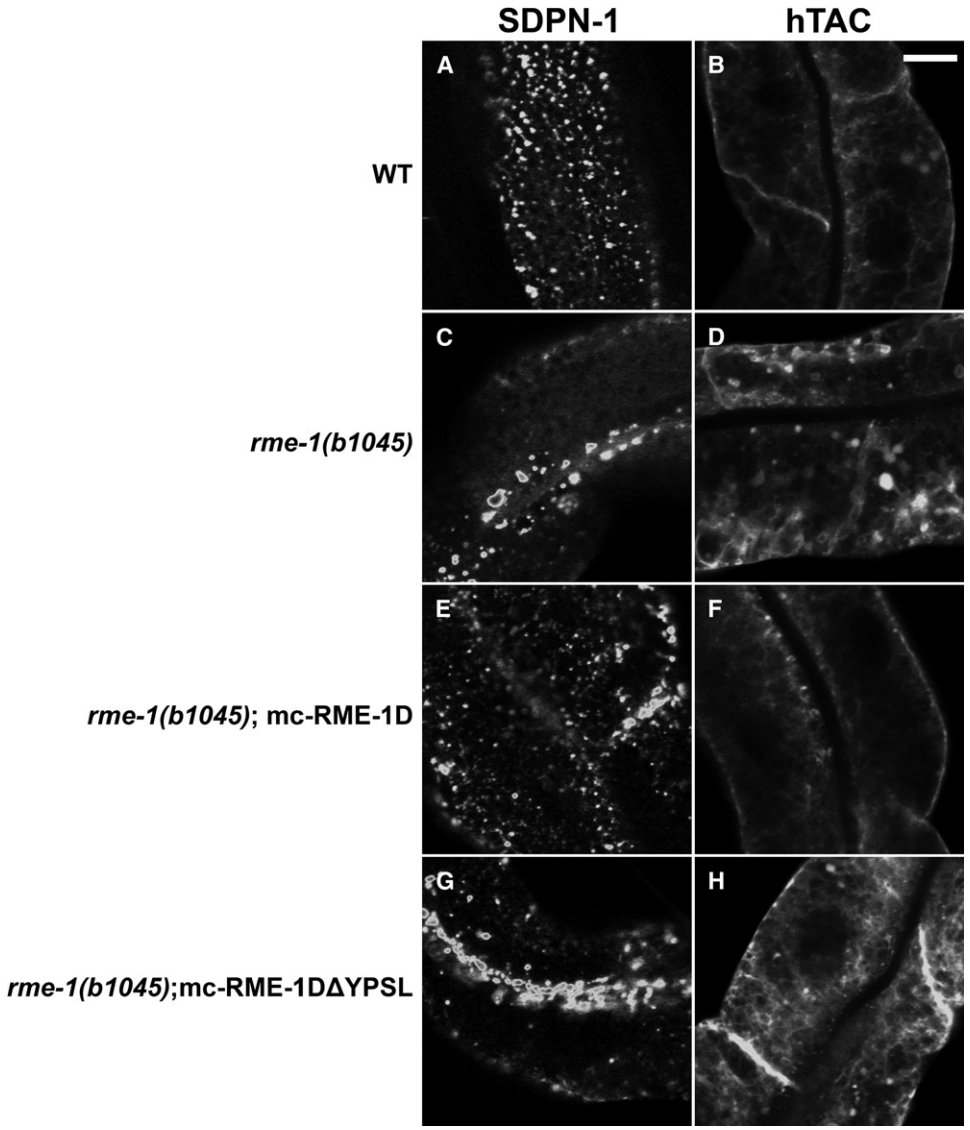

Figure S7. mCherry-RME-1 Lacking the YPSL Tail Cannot Rescue Certain *rme-1*-Mutant-Associated Phenotypes

(A, C, E, and G) Confocal images of SDPN-1-GFP-labeled endosomes (top focal plane near the intestinal basal membrane) in animals of the following genotypes: wild-type, *rme-1(b1045)* mutant, *rme-1(b1045)*; mCherry-RME-1, and *rme-1(b1045)*;mCherry-RME-1ΔYPSL. Note the rescue of SDPN-1-GFP localization by the expression of full-length mCherry-RME-1 and the lack of rescue by mCherry-RME-1ΔYPSL.

(B, D, F, and H) Confocal images of hTAC-GFP-labeled structures (middle focal plane showing the intestine in cross section) in animals of the following genotypes: wild-type, *rme-1(b1045)* mutant, *rme-1(b1045)*; mCherry-RME-1, and *rme-1(b1045)*;mCherry-RME-1ΔYPSL. Note the rescue of hTac-GFP localization by the expression of full-length mCherry-RME-1 and the lack of rescue by mCherry-RME-1ΔYPSL. The scale bar represents 10 μm.

normalized to cell area. Average recycled MHC class I surface values (30 min–0 min) and average MHC class I total values (0 min) were expressed as a ratio relative to control cells. Each bar in Figures 5J and 5K represents average values from four experiments ± the standard deviation (SD). The average internalized Alexa568-Tf was expressed as a ratio relative to control (control set to 100%) ± SD from three experiments. Average recycled Alexa568-Tf was expressed as a percentage of internalized Alexa568-Tf ± SD from three experiments.

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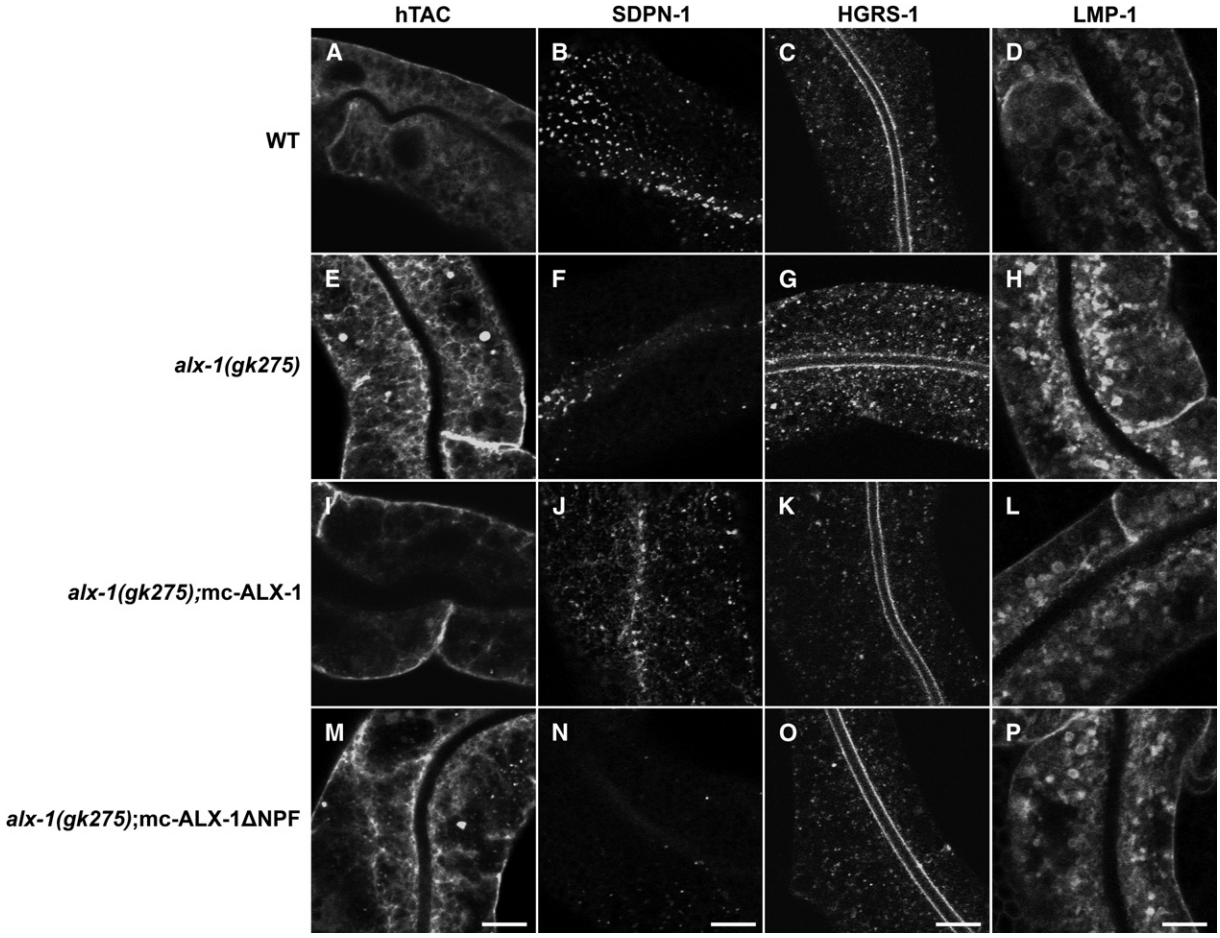

Figure S8. mCherry-ALX-1 Lacking the Carboxy-Terminal NPF Sequence Can Rescue MVE- and/or Late-Endosome-Associated *alx-1* Mutant Defects but Not Recycling-Endosome-Associated Defects

(A, E, I, and M) Confocal images of hTAC-GFP-labeled structures (middle focal plane showing the intestine in cross section) in animals of the following genotypes: wild-type, *alx-1(gk275)* mutant, *alx-1(gk275)*;mCherry-ALX-1, and *alx-1(gk275)*;mCherry-ALX-1ΔNPF.
(B, F, J, and N) Confocal images of SDPN-1-GFP-labeled structures (top focal plane near the intestinal basal membrane) in animals of the following genotypes: wild-type, *alx-1(gk275)* mutant, *alx-1(gk275)*;mCherry-ALX-1, and *alx-1(gk275)*;mCherry-ALX-1ΔNPF.
(C, G, K, and O) Confocal images of GFP-HGRS-1-labeled structures (middle focal plane showing the intestine in cross section) in animals of the following genotypes: wild-type, *alx-1(gk275)* mutant, *alx-1(gk275)*;mCherry-ALX-1, and *alx-1(gk275)*;mCherry-ALX-1ΔNPF.
(D, H, L, and P) Confocal images of LMP-1-GFP-labeled structures (middle focal plane showing the intestine in cross section) in animals of the following genotypes: wild-type, *alx-1(gk275)* mutant, *alx-1(gk275)*;mCherry-ALX-1, and *alx-1(gk275)*;mCherry-ALX-1ΔNPF.
Scale bars represent 10 μm.

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Table S1. Transgenic and Mutant Strain List Used in This Study

Strains
pwIs1(palx-1::GFP::ALX-1)
pwIs112(pvha6::hTAC::GFP) [S15]
pwIs170(pvha6::GFP::RAB-7) [S15]
pwIs206(pvha6::GFP::RAB-10) [S15]
pwIs281(ppie1::CAV-1::GFP) [S5]
pwIs116(prme-2::RME-2::GFP) [S20]
pwIs50(plmp-1::LMP-1::GFP) [S21]
pwIs518(pvha6::GFP::HGRS-1)
pwIs521(pvha6::mCherry::ALX-1)
pwIs522(pvha6::mCherry::ALX-1)
pwIs524(pvha6::GFP::ALX-1)
pwIs590(pvha6::mCherry::ALX-1ΔNPF)
pwIs621(pvha6::mCherry::RME-1)
pwIs626(pvha6::mCherry::RME-1ΔYPSL)
pwIs69(pvha6::GFP::RAB-11) [S15]
pwIs72(pvha6::GFP::RAB-5) [S15]
pwIs76(psdpn-1::SDPN-1::GFP)
pwIs87(pvha6::GFP::rme-1) [S15]
pwIs90(pvha6::hTfR::GFP) [S15]
rme-1(b1045) [S22]
alx-1(gk275) (From the *C. elegans* Gene Knockout Consortium)

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