METHODS

Bioinformatic screen. We searched the *C. elegans* proteome (WormBase release WS136) for all predicted ORFs containing the Asp-Pro-Phe (NPF) motif using the program myHits version 2.0 (myhits.isb-sib.ch). A total of 839 proteins were identified. These NPF-containing sequences were further selected for those containing multiple NPF tripeptides (57 proteins) or those followed by either aspartic acid or glutamic acid residues within 5 amino acids. We found 17 *C. elegans* NPF-containing proteins with varying degrees of D and/or E enrichment at these positions. *C. elegans* F58G6.1/Amphiphysin-like /Amphiphysin contained two NPF [D/E] sequences.

RNAi screen. Strain RT33, expressing GFP-RME-1 under *rme-1* promoter control in all somatic tissues, was examined by epifluorescence microscopy at × 630 magnification for abnormal RME-1 subcellular localization or recycling endosome morphology after RNAi of each of the 74 multi-NPF and NPF(D/E) candidates. RNAi clones were obtained from the Ahringer library[44] or were constructed by cloning cDNAs into RNAi vector L4440 and transforming HT115 cells. RNAi screening was performed by the feeding method as previously described[45]. Animals were scored as young adults in the P0 generation and after 4 more days of growth on fresh RNAi plates, animals of the next (F1) generation were also scored. All trials included a positive control (L4440-*rab-10*) and a negative control (L4440 empty vector). The screen was performed twice under identical experimental conditions.

Plasmid construction. Full-length AMPH-1 (AA1-869) was amplified from the *amph-1* expressed sequence tag (EST) clone yk1650f09 provided by Y. Kohara (National Institute of Genetics, Japan). This PCR product contained Gateway attB.1 and attB.2 sequence extensions and was introduced into the Gateway entry vector pDONR221 by a BP reaction according to the manufacturer's instructions (Invitrogen). The pDONR221 AMPH-1 (AA 1-869) construct was subjected to site-directed mutagenesis using the XL II Quik Change kit (Stratagene) to introduce the mutations creating the forms of AMPH-1 which have either or both NPF motifs mutated to NPA, thus creating cDNAs encoding AMPH-1(F309A), AMPH-1(F363A), and AMPH-1(F309A, F363A). Another form of full-length AMPH-1 with aspartic acid (D) residues 310–312 and 364–366 mutated to alanines (A) was similarly generated through site directed mutagenesis referred to in the text as AMPH-1(D310–312A, D364–366A). For yeast two hybrid experiments, OriGene DupLEXA yeast two hybrid system was used (OriGene Technologies). Bait vector pEG202 and target vector pJG4-5 (OriGene) were modified in house with the Gateway cassette (Invitrogen). RME-1 isoform D (amino acids 447–555) was cloned into the bait vector pEG202-Gateway [HIS3]. Prey constructs were made by introducing relevant regions of AMPH-1 amplified by PCR into a pDONR221 vector and later through a Gateway LR reaction into the vector pJG4-5-Gateway [TRP1]. Prey plasmids included only the central region of AMPH-1 (amino acids 230-394) lacking the N-terminal BAR domain and C-terminal SH3 domain. All constructs were transformed into yeast strain EGY48 [*MATα trp1 his3 ura3 leu2::6* LexAop-*LEU2*]. Origene pSH18-34 [*URA3*, 8 ops.-LacZ] was used as the reporter plasmid for all yeast two hybrid experiments. Transformations were performed as per manufacturer's instructions.

For GST pulldown experiments, a previously described vector, pcDNA3.1-2XHA_Gateway[17], was used to create pcDNA3.1-2XHA-AMPH-1(+), pcDNA3.1-2×HA-AMPH-1(F309A, F363A), pcDNA3.1-2×HA-AMPH-1(D310–312A, D364–366A) and pcDNA3.1-2×HA-Rme-1d (full length), for *in vitro* transcription and translation. The plasmid pcDNA3.1-2×HA-BIN1 isoform 10 was similarly generated via PCR amplification of a BIN1 isoform 10 cDNA plasmid (a gift from G. Prandergast, The Lankenau Institute for Medical Research, PA, USA). The Myc-EHD1 plasmid has been previously described[5]. pGEX-2T-RME-1d(442-576) has been previously described[17]. pGEX-2T-Eps15-EH domain 2 and pGEX-2T-Intersectin EH domains (a+b) were gifts from B. Kay (University of Illinois at Chicago, IL, USA). For construction of GST-tagged or tag-less versions of full-length *C. elegans* AMPH-1, AMPH-1(F309A, F363A) or RME-1 isoform d proteins, a PreScission protease recognition site, LeuGluValLeuPheGln/GlyPro, was introduced downstream of the GST tag for AMPH-1, AMPH-1(F309A, F363A) and RME-1d cDNA. The PCR amplified Gateway compatible constructs were introduced into pGEX-2T_Gateway to construct pGEX-2T-PreScission-AMPH-1, pGEX-2T-PreScission-AMPH-1(F309A, F363A) and pGEX-2T-PreScission-RME-1.

GFP-AMPH-1 driven by the *amph-1* promoter was made as follows: 1550 base pairs of *amph-1* promoter and ORF sequence was PCR amplified from *C. elegans* genomic DNA and cloned into a modified Gateway vector containing *C. elegans* GFP, vector pPD117.01 (original vector was from A. Fire, Stanford University School of Medicine, CA, USA). For the construction of C-terminally tagged GFP transgenes for AMPH-1(+) for expression in the worm intestine, Gateway destination vectors were used that contain the promoter region of the intestine-specific gene *vha-6* cloned into the *C. elegans* pPD117.01 vector, a Gateway cassette followed by a GFP coding sequences and then the *unc-119* gene of *C. briggsae*. An integrated transgenic line was selected for this study. For the construction of C-terminally tagged mCherry fusion transgenes for AMPH-1(+) or AMPH-1(F309A, F363A) for expression in the worm intestine, Gateway destination vectors were used that contain the promoter region of the intestine-specific gene *vha-6* cloned into the *C. elegans* pPD117.01 vector, a Gateway cassette followed by a mCherry coding sequence containing worm intron sequences to enhance expression and then the *unc-119* gene of *C. briggsae*. The vha-6p:SDPN-1–GFP expression plasmid was created by introducing the complete *sdpn-1* genomic coding region from start codon onwards but lacking a stop codon and introducing it upstream of the GFP coding region in the expression vector for C-terminal addition of GFP tag. The Dyn-1p-Dyn-1–GFP construct used to obtain DYN-1–GFP expressing transgenic strains was a kind gift from Z. Zhou (Baylor College of Medicine, Houston, TX, USA). All plasmids used in this study were sequenced and complete plasmid sequences are available on request.

Antibodies. Polyclonal anti-AMPH-1 antibodies were prepared using recombinant GST–AMPH-1(391-461). For affinity purification, the MBP-AMPH-1(391-461) antigen was coupled to a NHS-HiTrap column (GE Healthcare). Polyclonal antibodies purified from two rabbits detected a single band of the expected size (approximately 56 kDa) in western blots of wild-type N2 worm lysates. The corresponding band was absent from lysates of *amph-1(tm1060)* deletion mutants, confirming the specificity of the antibodies. Both antibodies displayed similar labelling patterns in immunofluorescence of dissected worm intestines and gonads. Of these we chose one, termed anti-AMPH-1 G2, for our studies. Other antibodies used in this study include affinity-purified polyclonal anti-RME-1 (ref. 1), monoclonal anti-RME-1 antibody clone 5G11, a gift from M. Nonet (Washington University School of Medicine, St. Louis, MO, USA; G. Gadwiger, S. Dour and M. L. Nonet (unpublished data), affinity-purified anti-EHD1 polyclonal antibody[3], mouse anti-BIN-1 antibody (Millipore, Clone 99D), monoclonal anti-actin (ICN Biomedicals, Inc., Clone C4), monoclonal anti-Actin for HeLa cell experiments (Abcam), monoclonal anti-HA antibody (Covance Research Products, Clone 16B12), polyclonal anti-GST antibody (Santa Cruz Biotechnologies, Clone Z-5). Goat anti- rabbit horseradish peroxidase (HRP) was obtained from Pierce (Thermo Fisher Scientific Inc.) and goat anti-mouse horseradish peroxidase (HRP) was obtained from Jackson ImmunoResearch Laboratories, Inc.

Yeast two-hybrid experiments. OriGene DupLEXA yeast two-hybrid system was used (OriGene Technologies, Inc.) according to manufacturer's instructions. Yeast strain EGY48 [MATα trp1 his3 ura3 leu2::6 LexAop-*LEU2*] was transformed with Origene plasmid pSH18-34 [*URA3*, 8 ops.-LacZ] as the reporter for all yeast two-hybrid experiments. Plasmid pEG202[*HIS3*] as an empty control or pEG202-RME-1(amino acid 447–555) were used as bait. Prey constructs included pJG4-5-Gateway [*TRP1*] as an empty vector control or pJG4-5-AMPH-1(230-394/+), pJG4-5-AMPH-1(230-394/F309A), pJG4-5-AMPH-1(230-394/F363A), and pJG4-5-AMPH-1(230-394/F309A,F363A). Transformations were performed as per manufacturer's instructions. 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-galactosidase activity was measured with the standard ONPG (o-nitrophenyl β -D-galactopyranoside) test[46]. β -galactosidase activity in Miller units was plotted as an average from assays performed in duplicate.

GST pulldown assays. N-terminally HA-tagged AMPH-1 protein, wild-type or mutant forms, was synthesized *in vitro* with the TNT coupled transcription-translation system (Promega) with DNA templates built from vector pcDNA3.1. The reaction was incubated at 30 °C for 90 min. Control glutathione S-transferase (GST) or the GST-RME-1d (AA 442–576) fusion protein containing the RME-1 EH domain, or EH domains from mouse mRme-1/EHD1, human Eps15 (EH2), or human Intersectin (EHa+b) were expressed in *Escherichia coli* BL21 cells. Cells were grown

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in 2×YTA media to an OD600 of 0.5, then induced with 1 mM IPTG and grown an additional 4 h at 30 °C. Bacterial pellets were lysed in 5 ml B-PER Bacterial Protein Extraction Reagent (Pierce (Thermo Fisher Scientific Inc.) with protease inhibitor cocktail (Roche Diagnostics GmbH). Lysed extracts were cleared by centrifugation, and GST-tagged proteins were affinity purified using Glutathione Sepharose 4B beads (GE Amersham Pharmacia). After incubation at 4 °C overnight, protein-bound beads were washed six times with cold STET buffer (10 mM Tris-HCl [pH 8.0], 150 mM NaCl, 1 mM EDTA, 0.1% Tween-20). For the binding reaction, *in vitro*-synthesized HA-tagged AMPH-1(+) or its variants (8 µl TNT mix diluted in 500 µl STET) was added to the beads and allowed to bind at 4 °C for 2 h. After six washes in STET, the proteins were eluted by boiling in 20 µl 2× sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS–PAGE) sample buffer. Eluted proteins were separated by 10% SDS–PAGE, transferred to nitrocellulose, and stained with either Ponceau S or anti-GST antibodies to detect over-expressed GST fusion proteins. The blot was subsequently probed with anti-AMPH-1 rabbit polyclonal antibody. In the reverse experiment, N-terminally HA-tagged RME-1(+) protein was synthesized *in vitro* with the TNT coupled transcription-translation system with DNA template pcDNA3.1 -2×HA-RME-1d. Control GST or GST-AMPH-1(+) fusion proteins were expressed in *Escherichia coli* Arctic express cells (Stratagene), and binding studies were performed as described above. Bound HA-RME-1 was detected with anti-HA monoclonal antibody. Equivalent loading of bait proteins was determined by Ponceau S staining of the blot. In the experiment performed with endogenous worm protein, worm lysate from wild type Bristol N2 strain was used as input for the GST pulldown experiment. N2 worms were grown on NGM-lite plates seeded with HB101 bacteria. Worms were washed off plates and gently suspended in ice cold M9 buffer. After several washes with M9 followed by lysis buffer (25mM Tris-HCl, pH 7.4, 150 mM sodium chloride, 1mM magnesium chloride, 1% Triton X-100) with protease inhibitor, the worm pellet was lysed in a precooled yeast bead beater using 5 mm Zirconia Silicon beads. The lysate was incubated on ice for 1 h. The lysate was precleared by incubation for 5 h with glutathione Sepharose 4B beads coated with control glutathione *S*-transferase (GST) protein. The precleared lysate was allowed to incubate for 1 h with control glutathione *S*-transferase (GST) or the GST–RME-1d (aa 442–576) fusion protein containing the RME-1 EH domain bound to Glutathione Sepharose 4B beads. The later steps of the pulldown were performed similar to other GST pulldowns described in this text.The pulldown was probed with rabbit anti-AMPH-1 antibody. Equivalent loading of bait proteins was determined by rabbit anti-GST probe of the blot.

Immunoblotting. AMPH-1 or RME-1 from worm lysates were detected by western blot analysis, as described[47]. Briefly, wild type N2 or *amph-1(tm1060)* synchronized worms were handpicked into a small volume of worm boil buffer (100 mM Tris pH 6.8, 8% SDS, 20 mM β-mercaptoethanol). After storage at -70 °C, the frozen pellet was lysed by boiling for 10 min in Laemmli buffer. Proteins were separated by SDS–PAGE and transferred to nitrocellulose. The anti-AMPH-1 polyclonal antibodies were incubated at a 1:5000 dilution with the blot overnight at 4 °C. HRP-conjugated goat anti-rabbit secondary antibody was used at a 1:10,000 dilution. The AMPH-1 polyclonal antibody recognizes a single band around 56kDa. For detecting BIN1, HeLa cells were either mock-treated or treated with *Bin-1*-RNAi for 72 h. After treatment, cells were collected and lysed for 15 min in buffer containing 25 mM Tris, pH 7.4, 150 mM NaCl, 0.5% Triton X-100 (w/v), and protease inhibitor cocktail (Roche). After removal of insoluble matter by centrifugation, the lysate supernatants were separated by 10% SDS–PAGE, and incubated with mouse anti-BIN1 (99D) and mouse anti-actin antibodies followed by an additional incubation with goat anti-mouse-HRP antibodies. The BIN1 antibody recognizes a doublet around 65–70 kDa in size. A non-specific low molecular weight band was also recognized. Enhanced chemiluminescence was used for detection.

Genetic analysis. 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[48]. All strains were maintained at 20 °C. A complete list of strains used in this study can be found under subheading 'Transgenic and mutant strains used in this study'. The *amph-1(tm1060)* mutant was obtained from the Japanese *C. elegans* knockout consortium (a gift from S. Mitani). The *amph-1(tm1060)* mutant was backcrossed 3× to wild-type strain N2 prior to further analysis and strain construction. Diagnostic PCR was used to confirm *amph-1* genotype in all constructed strains.

Immunofluorescence. Immunofluorescence on dissected intestine preparations was performed as previously described[1]. Briefly, dissected tissues were fixed in 1.25% Paraformaldehyde (Electron Microscopy Sciences) for 10 min at room temperature. Rabbit anti-AMPH-1 antibody G2 was used at 1:100 dilution overnight at 4 °C. Mouse monoclonal anti-RME-1 5G11 was used at 1:10 dilution. Secondary antibodies conjugated to Alexa-488 or Alexa-568 were purchased from Molecular Probes and were used at a dilution of 1:1000.

HeLa cells were grown on cover-glasses and fixed with 4% (v/v) paraformaldehyde in PBS. Fixed cells were incubated with primary antibodies prepared in staining solution [0.2% saponin (w/v) and 0.5% (w/v) BSA in PBS] for 1 h at room temperature. After washes in PBS, the cells were incubated with the appropriate fluorochrome-conjugated secondary antibody mixture in staining solution for 30 min at room temperature.

Transgenic worm strain construction. Low-copy integrated transgenic lines were obtained by the microparticle bombardment method[49]. The AMPH-1p:AMPH-1:GFP plasmid was co-bombarded with plasmid MMO16B encoding unc-119(+) so that transgenic lines could be established in an *unc-119(ed3)* mutant line. AMPH-1 rescue constructs were bombarded into an *amph-1(tm1060); unc-119(ed3)* strain. Both vha-6p-AMPH-1(+)-mCherry and vha-6p-AMPH-1(F309A, F363A)–mCherry showed equivalent expression levels in terms of pixel intensity of imaged live worms. A complete list of strains used in this study is provided under the subheading 'Transgenic and mutant strains used in this study'.

Mammalian cell RNAi. RNA interference (RNAi) duplexes (synthesized by Dharmacon) were transfected using Dharmafect (Dharmacon). Calibration experiments showed that 72 h of treatment was sufficient to significantly decrease BIN1 expression levels. The oligonucleotide sequence used for Bin1-knockdown was: cgggaaagatcgccagcaa. Efficacy of *Bin1* RNAi was confirmed by immunoblotting of HeLa cell lysates with anti-BIN1 antibodies. Efficient knockdown of up to 80 to 90% was achieved as observed by loss of BIN1-specific bands. Similar results were obtained upon utilizing another set of RNAi duplexes directed against Bin1 (Integrated DNA technologies) compared to scrambled oligonucleotide controls (data not shown).

Transferrin (Tf) recycling assay. Tf-568 was purchased from Molecular Probes (Invitrogen). HeLa cells on plates were mock treated or treated with BIN-1-RNAi oligonucleotides. After 72 h, cells starved for 30 min in DMEM lacking serum (but containing 0.5% BSA) were either pulsed with 16.7 µg ml^{-1} transferrin-Alexa Fluor 568 (Tf-568; Invitrogen) for 5 min (Fig. 5a–d) and for 15 min (Fig. 5e) and fixed immediately ($t = 0$, uptake measurement), or then 'chased' in complete media for the indicated times prior to fixation. Images were acquired using a Zeiss LSM 5 Pascal confocal microscope (Carl Zeiss) by using a 63× 1.4 numerical aperture objective with appropriate filters. All micrographs shown are representative images from experiments that have been repeated at least three times. Quantification of mean fluorescence was done using LSM Pascal software. Bars represent the mean ± s.e.m. calculated from counting 70–80 cells each from three independent experiments. For Flow cytometry analysis, the remaining intracellular Tf-568 levels for 10,000 cells were determined at indicated time points after the start of chase. Error bars indicate s.e.m. from three independent experiments.

Immunoprecipitation protocol. HeLa cells were transfected with Myc–EHD1 or Myc–EHD1 and HA–BIN1. Cells were lysed for 1 h in buffer containing 25 mM Tris-HCl, pH 7.4, 125 mM NaCl, 1 mM MgCl2, 1% Brij98 (w/v), 0.25 mM AEBSF, 10 µM Leupeptin and 10 µM Aprotinin. Lysates were immunoprecipitated with goat anti-HA antibody-conjugated agarose beads (Bethyl Laboratories). After 14 h at 4 °C, immunoprecipitates were washed and eluted by 125 mM Tris (pH 6.8)/2% sodium dodecyl sulfate (SDS)/12% glycerol at 95°C. Immunoblotting was done with affinity-purified rabbit polyclonal peptide antibodies directed against human EHD1 (DLPPHLVPPSKRRHE) followed by donkey anti-rabbit HRP (Amersham Biosciences). To analyse levels of immunoprecipitated HA–Bin1, the blot was stripped for 3 min with 3 M solution of guanidinium isothiocyanate and reprobed with mouse anti-HA antibody (Covance) followed by goat anti-mouse horseradish peroxidase (HRP; Jackson ImmunoResearch Laboratories, Inc.)

Protein expression and purification of full-length tagless proteins. *C. elegans* AMPH-1, AMPH-1(F309A, F363A) and RME-1 full-length proteins were expressed as N-terminal GST-fusions followed by a PreScission cleavage site in *Escherichia coli* Arctic Express cells (Stratagene). Bacterial cultures in LB medium were induced

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at an OD_{600} of 0.5 with 1 mM IPTG, and grown overnight at 15°C. Bacteria were lysed in 50 mM HEPES (pH 7.5), 400 mM NaCl, 1 mM DTT, 1 mM Phenyl-methyl-sulfonyl fluoride (PMSF) (Sigma Aldrich, St. Louis, MO) using a microfluidizer M-110Y (Microfluidics). The bacterial lysate was centrifuged at 10,000*g* for 30 min in a Sorvall SS-34 rotor (Sorvall). The soluble supernatant fraction was centrifuged for another 40 min at 4 °C at 100,000*g* in a Beckman Ti-70 ultracentrifuge rotor (Beckman). The soluble extract was applied to a Glutathione sepharose 4B column (GE Amersham) equilibrated with lysis buffer. The column with bound protein was washed thoroughly with wash buffer (20 mM HEPES at pH 7.5, 300 mM NaCl, 1mM MgCl2, 1 mM DTT). To remove the GST tag, column-bound GST tagged protein was treated overnight at 4 °C with 225 μg PreScission protease (GE Amersham). The protein was eluted with several volumes of wash buffer. Eluted protein was re-applied to a Glutathione sepharose 4B column to bind any residual GST tag. The eluate containing purified tagless protein was analysed by SDS–PAGE followed by Coomassie staining to check the purity of the proteins and western blot with anti-RME-1 or anti-AMPH-1 antibodies. The proteins were extensively dialysed against 20 mM HEPES (pH 7.5), 300 mM NaCl, 1 mM MgCl2, 1 mM DTT and snap frozen in liquid Nitrogen and stored in aliquots at -80 °C. For use in all the biochemical experiments, the frozen aliquots were rapidly thawed, then spun at 20,000*g* at 4°C to remove any aggregated protein. Protein concentration was determined under denaturing conditions by absorbance at 280 nm[50].

Liposome preparation. Lipids were obtained in chloroform solution from Avanti Polar Lipids Inc. (Alabaster). For experiments utilizing PtdIns, PtdIns(4)P or PtdIns(4,5)P_2 lipids, the liposome composition comprised molar ratio of 80% Phosphatidylcholine (PtdChl), 10% Phosphatidylserine (PtdSer) and 10% of the respective phosphatidylinositol derivative. All glassware was thoroughly washed with chloroform (extra dry Chloroform, Acros Organics) and flushed with argon gas. Lipids were dried in a stream of argon gas. Residual chloroform was removed by vacuum over several hours or overnight. The dried lipid was resuspended at 1 mg ml^{-1} concentration in argon-purged liposome buffer (20 mM HEPES at pH 7.5, 150 mM NaCl, 1 mM MgCl2). 0.4 μm average diameter liposomes were formed by using 0.4 μm polycarbonate track-etched membrane filters (Whatman Ltd) with a Lipex Extruder, as per manufacturer's instructions (Northern Lipids Inc.).

Liposome binding. For liposome binding experiments, 1 μM final concentration of RME-1 or AMPH-1 proteins were incubated with 0.33 mg ml^{-1} final concentration of either 100% Phosphatidylserine (PtdSer), 100% Phosphatidylcholine (PtdChl) liposomes or 80% Phosphatidylcholine (PtdChl), 10% Phosphatidylserine (PtdSer) and 10% of either unphosphorylated phosphatidylinositol(PtdIns) or phosphatidylinositol-[4]- phosphate (PtdIns(4)P) or phosphatidylinositol-[4, 5]- bisphosphate (PtdIns(4,5)P_2) for 15 min at room temperature in the liposome buffer. The binding reaction was centrifuged at 90,000*g* in a TLA100.2 rotor (Beckman Coulter) at 25 °C for 15 min using an Optima TLX100 centrifuge (Beckman Coulter). Supernatants were precipitated with trichloro acetic acid (TCA) and washed. Supernatant precipitates and pellets from the same experiment were suspended in equal volumes of 2× Laemmli sample loading buffer and equal volumes of pellet versus supernatant were analysed by Coomassie gel staining.

Liposome tubulation. For liposomes tubulation experiments, 2.5 μM RME-1, AMPH-1 or AMPH-1(F309A, F363A) were incubated with 0.05 mg ml^{-1} final concentration of 100% Phosphatidylserine (PtdSer) liposomes in liposome buffer with 1 mM final concentration of either ATP-γ-S, or ADP (Sigma Aldrich). For another experiment, 2.5 μM RME-1 and/or AMPH-1 were incubated with 0.05 mg ml^{-1} final concentration of 80% Phosphatidylcholine (PtdChl), 10% Phosphatidylserine (PtdSer) and 10% Phosphatidylinositol-[4, 5]-bisphosphate (PtdIns(4,5)P_2) liposomes. The experiments were performed in the liposome buffer and in the presence of 1 mM final concentration of non-hydrolysable ATP analog ATPγS. For experiments to determine stoichiometry of protein action, 2.5 μM RME-1 andAMPH-1 or 2.5 μM RME-1 and 250 nM AMPH-1 were incubated with 0.05 mg ml^{-1} final concentration of 100% PtdSer liposomes and 1 mM ATP-γ-S. For experiments to determine the contribution of AMPH-1 NPF sequences to the tubulation reaction, experiments were performed as described in this section and using 2.5 μM RME-1 and/or AMPH-1(F309A, F363A) incubated with 0.05 mg ml^{-1} final concentration of 100%PtdSer liposomes and 1 mM ATP-γ-S. All samples were incubated on ice for 4 min and 12 min after the start of protein addition the samples were spotted on formvar carbon coated 300 mesh Copper grid. After negative staining with 1% uranyl acetate, electron microscopy was performed using a JEOL 1200 EX or JEOL 100 CX transmission electron microscope at 80 KV. Control experiments included no-liposome, protein incubations under identical experimental conditions, but with 2.5 μM each of GST alone, GST with RME-1 or GST with AMPH-1. Images were obtained at × 15,000 and × 50,000 magnification as indicated. For quantification of AMPH-1 tubulation in the presence and absence of RME-1 in an ADP nucleotide condition, micrographs were imaged at × 3,000, for large field in which tubules could be clearly distinguished. The electron micrographs were scanned using a CanoScan scanner. For the selected images included in Figs 6–8 and Supplementary Information, Fig. S7, the Adobe Photoshop CS Levels function was used to alter contrast to best represent the observed features. Quantification of liposome tubule length and diameter were performed using Adobe Photoshop CS Ruler function. Individual tubule lengths, diameters or inter striation distances were measured and converted to nm measures utilizing the measured length of the scale bar at that EM magnification. Mean values were calculated and plotted as graphs. Standard deviations were used for Y-error bars on graphs. Statistical analysis was performed utilizing a Student's paired *t*-test. Significance was determined at *P*-values less than 0.01 and is noted by asterisks on figures and in figure legends.

Microscopy. *C. elegans* immunofluorescence images were obtained using an Axiovert 200M (Carl Zeiss MicroImaging Inc.) microscope equipped with a digital CCD camera (C4742-95-12ER; Hamamatsu Photonics). Metamorph software (Universal Imaging) was utilized for image acquisition and *z*-stacks of images were deconvoluted with AutoDeblur software (AutoQuant Imaging). Confocal imaging of *C. elegans* was performed with a Zeiss LSM510 Meta confocal microscope system (Carl Zeiss MicroImaging) with Argon 488 excitation and spectral finger printing to distinguish and separate out the worm's autofluorescence profile separate from the bona fide GFP signal. To observe live worms expressing transgenes, animals were mounted on agarose pads containing 100 mM tetramisole (MP Biomedicals) in M9 buffer. Quantification of fluorescence intensities, object area or object count was performed using Metamorph software (Universal Imaging, Downington). For HeLa cell experiments, all images were acquired using a Zeiss LSM 5 Pascal confocal microscope (Carl Zeiss), using a 63× objective with a numerical aperture of 1.4.

Statistical analysis. For all data sets the standard deviation value from data sets was utilized as Y-error bars on bar graphs plotted for mean value of the data. Data sets were subjected to Student's one-tailed paired *t*-test analysis. Data were considered significantly different if *P*-values were lower than 0.01. *P*-values for various experiments are included in the figure legends. In all experiments presented, *P*-values less than 0.01 were considered statistically significant and are marked by asterisks in the Figures.

Transgenic and mutant strains used in this study. *bIs46(Prme-1-RME-1-GFP)*; *pwIs722(Pvha6-SDPN-1-GFP)*; *dkIs8 (Pvha6-GFP-CHC-1*; ref. 18); *pwIs72(Pvha6-GFP-RAB-5*; ref. 19); *pwIs112(Pvha6-hTAC-GFP)*[19]; *pwIs717(Pvha6-hTfR-GFP)*; *pwIs87(Pvha6-GFP-RME-1*; ref. 17); *pwIs621(Pvha6-mCherry-RME-1*; ref. 17); *pwIs75(Psdpn-1-SDPN-1-GFP*; ref. 19); *pwEx116(Pamph-1-AMPH-1-GFP)*; *pwIs630(Pvha6-AMPH-1-GFP)*; *pwIs69(Pvha6-GFP-RAB-11*; ref. 19); *pwIs170(Pvha6-GFP-RAB-7*; ref. 19); *pwIs481(Pvha6-MANS-GFP*; ref. 19); *pwIs206(Pvha6-GFP-RAB-10*; ref. 1); *pwIs578(Pdyn-1-DYN-1-GFP)pwEx125(Pvha6-AMPH-1-mCherry)*; *pwEx126(Pvha6- AMPH-1(F309A, F363A)–mCherry)*; *rme-1(b1045)*; *amph-1(tm1060)* (provided by S. Mitani, Japanese National Bioresource Project for the Experimental Animal 'Nematode *C. elegans*').

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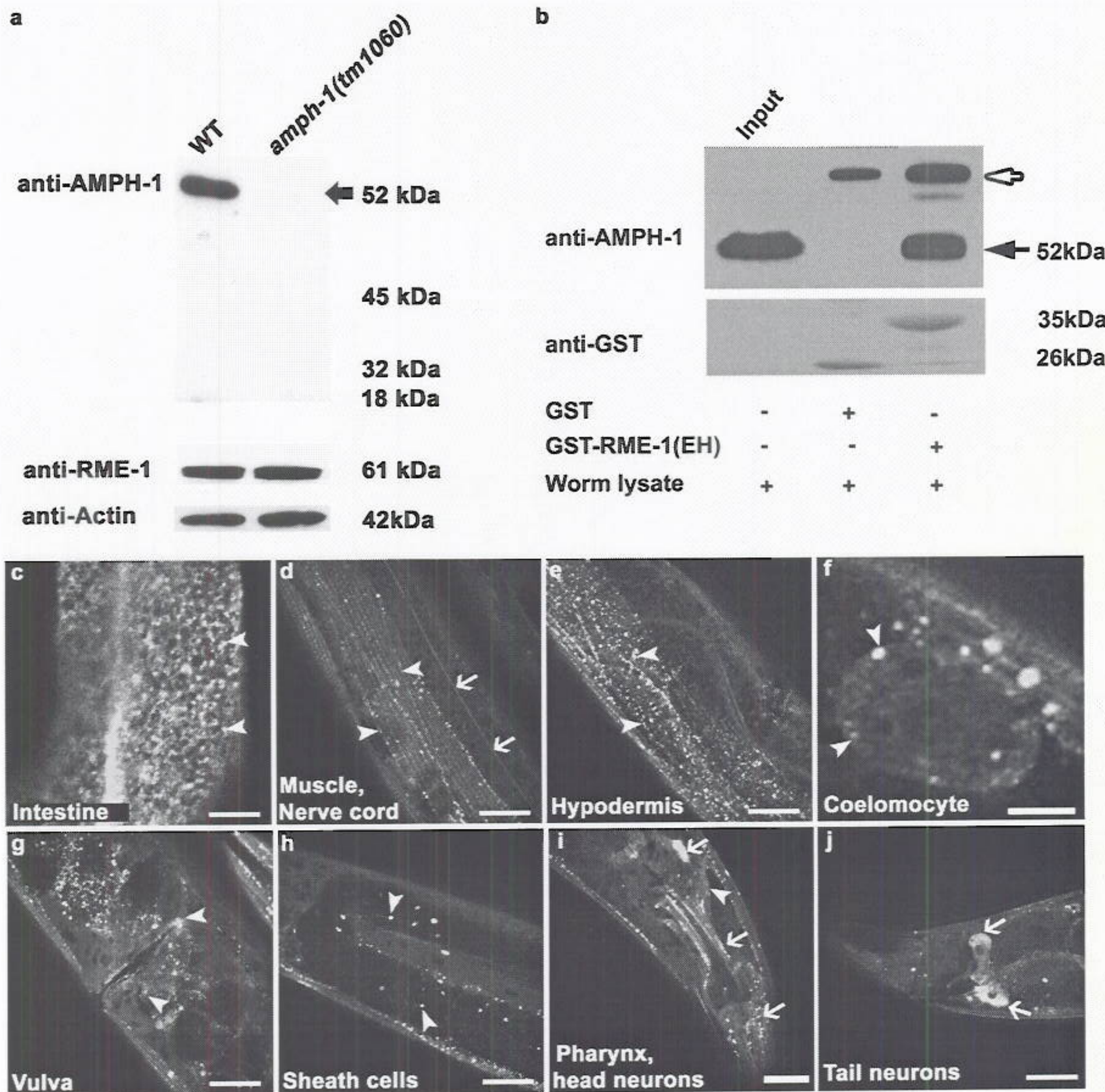

Figure S1 **(a)** A Western blot of total *C. elegans* proteins, 40 adult worms per lane, was probed with anti-AMPH-1 antibodies (upper panel). A single band of the expected size (52 kDa) was detected in wild-type strain N2. No AMPH-1 protein could be detected in the *amph-1(tm1060)* mutant indicating that it is a null allele. The same blot was re-probed with anti-RME-1 and anti-actin antibodies. **(b)** Glutathione beads loaded with recombinant GST or GST-RME- 1(442–576) were incubated with a lysate prepared from wild type worms and precleared by incubation with GST. Unbound proteins were washed away and bound proteins were eluted with Laemmli sample buffer, separated by SDS-PAGE, and analyzed by western blot with anti-AMPH-1 antibody. The AMPH-1 band observed in worms at 52 kDa was clearly pulled down by the GST-EH domain but not by GST alone. Additional non-specific bands were observed at approximately 90 kDa and are products of the GST pulldown itself judging from presence in both control and EH domain columns. Input lanes contain 40% of the worm lysate used in the binding assays. The lower panel shows equivalent loading of bait GST (26 kDa) or GST-RME- 1(442–576) (35 kDa) with anti-GST staining of the blot. **(c-j)** AMPH-1 is Broadly Expressed in *C. elegans*. The expression of a GFP-AMPH-1 transgene driven by the *amph-1* promoter is indicated and scale bars represent 10 μm except where specified. **(c)** 3-D projection of intestine basolateral cortex, arrowheads indicate cytoplasmic tubulovesicular structures. **(d)** Nerve cord (arrow) and body-wall muscles (arrow head). **(e)** Hypodermis contains cytoplasmic puncta (arrowheads) **(f)** Coelomocytes, cytoplasmic puncta (arrowheads). Scale bar represents 2.5 μm **(g)** Vulval muscle cells, cytoplasmic puncta. **(h)** Sheath cells, punctate structures. **(i)** The pharynx is indicated by an arrowhead and some head neurons and the nerve ring are indicated by arrows. **(j)** Tail neurons are depicted by arrowheads.

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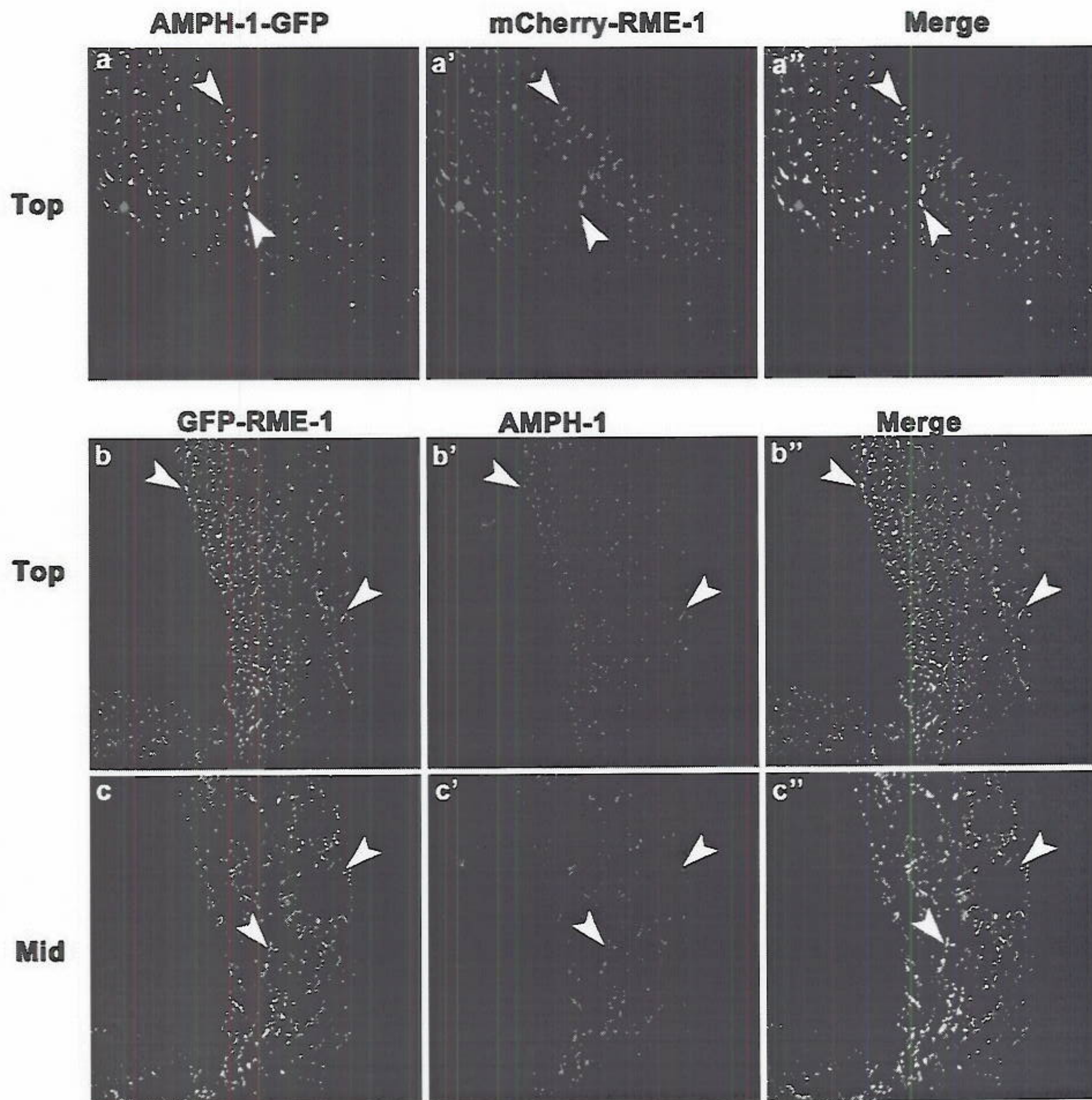

Figure S2 AMPH-1 Localizes to the Recycling Endosome in the *C. elegans* intestine. **(a-a")** AMPH-1-GFP co-localizes with mCherry-RME-1 on the recycling endosome in live transgenic worms. Intestinal autofluorescent gut granules appear in the blue channel. **(b-b")** Endogenous AMPH-1(red) co-localizes with the recycling endosome marker GFP-RME-1(green) in the basolateral intestine. **(c-c")** Endogenous AMPH-1(red) co-localizes with GFP-RME-1(green) on weakly labeled sub-apical structures. Scale bars represent 10 μm

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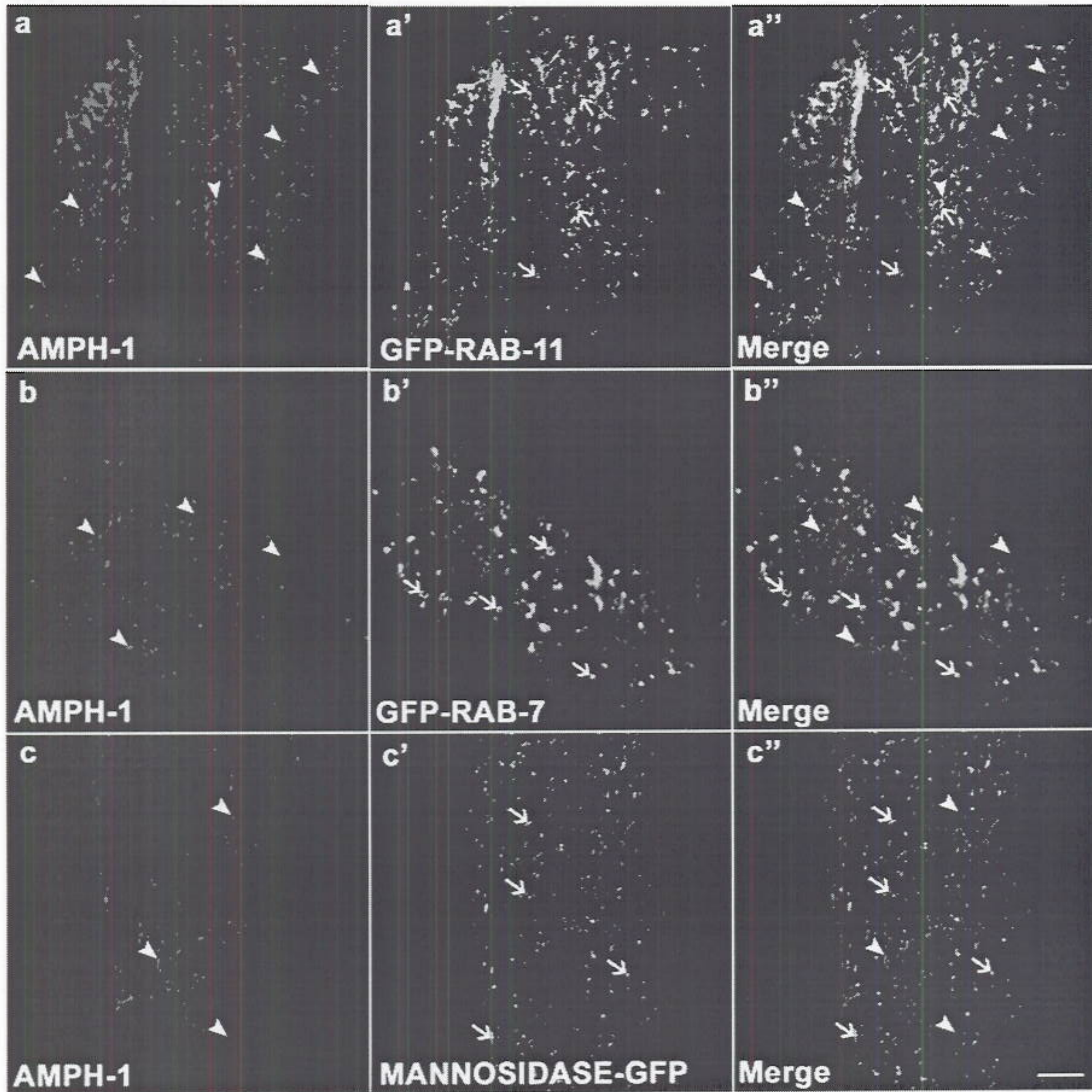

Figure S3 AMPH-1 is not enriched on early or late endosomes. **(a-a")** Endogenous AMPH-1(red) partially co-localizes with the recycling endosome marker GFP-RAB-11(green). Arrowheads indicate structures labeled by AMPH-1 and arrows indicate structures labeled by GFP-RAB-11. **(b-b")** Endogenous AMPH-1(red) does not colocalize with the late endosome marker GFP-RAB-7 (green). Arrowheads indicate structures labeled by AMPH-1 and arrows indicate structures labeled by GFP-RAB-7. **(c-c")** Endogenous AMPH-1(red) does not colocalize with the golgi stack marker Mannosidase-GFP (green). Arrowheads indicate structures labeled by AMPH-1 and arrows indicate structures labeled by Mannosidase-GFP. Scale bars represent 10 μm

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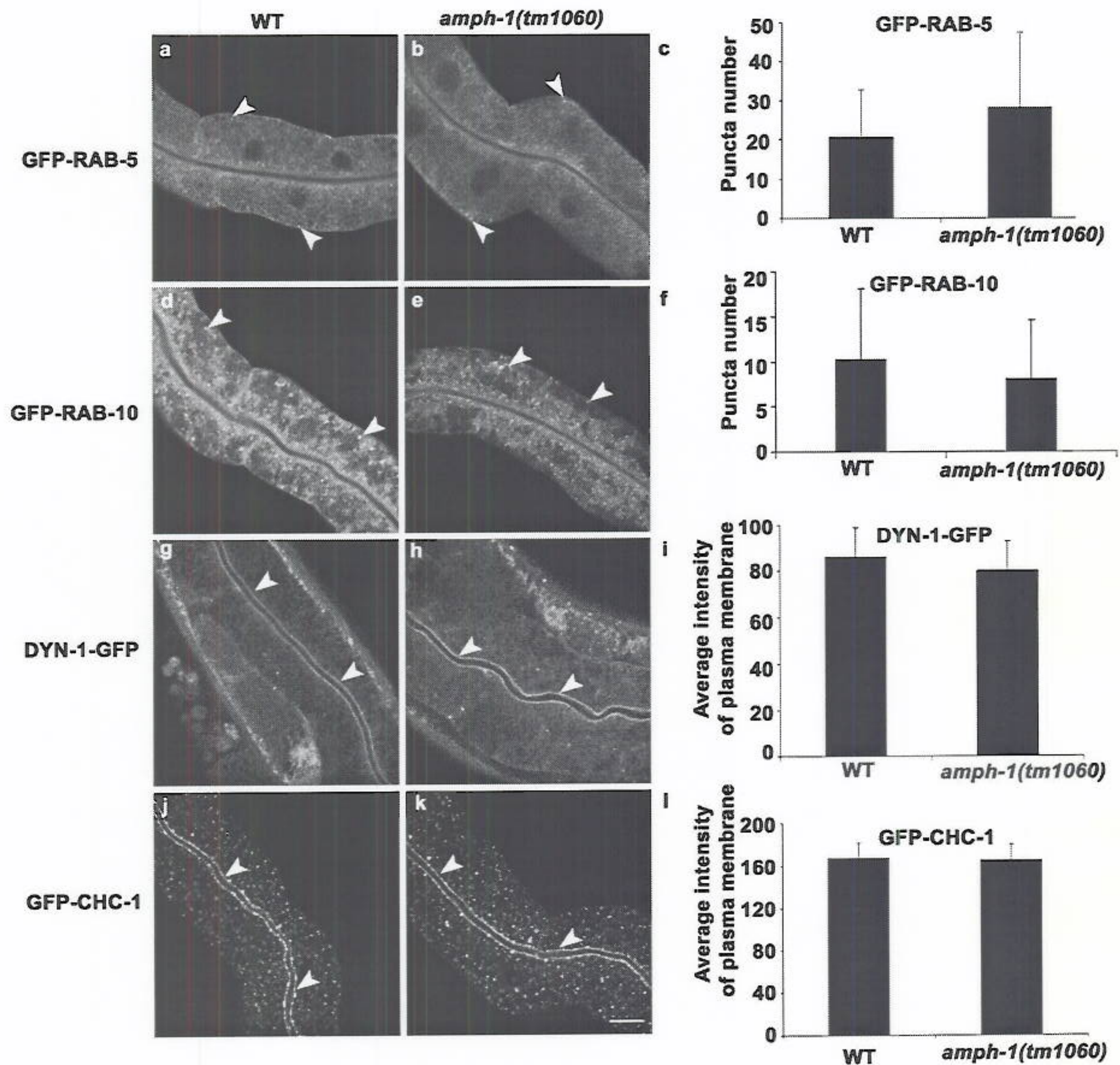

Figure S4 Early endosome morphology is normal in *amph-1* mutants. **(a-c)** The early endosome population marked by GFP-RAB-5 is not affected in *amph-1(tm1060)* mutants compared to wild-type animals. General appearance and distribution of early endosomes appears normal in mutants. **(c)** Quantification of GFP-RAB-5 labeled early endosomes in the *amph-1* mutant in terms of number of puncta of GFP-RAB-5. One-tailed Student's T-test, p=0.09696. **(d-f)** The early endosome population marked by GFP-RAB-10 is not affected in *amph-1(tm1060)* mutants compared to wild-type animals. **(f)** Quantification of GFP-RAB-10 labeled early endosomes in the *amph-1* mutant in terms of number of puncta. One-tailed Student's T-test, p=0.133643. **(g-i)** *C. elegans* Dynamin DYN-1-GFP is not affected in *amph-1(tm1060)* mutants compared to wild-type animals. The apical plasma membrane labeling of DYN-1 was unaffected in *amph-1* mutants. **(i)** Quantification of DYN-1-GFP apical plasma membrane labeling with data plotted as average arbitrary units of pixel intensity. One-tailed Student's T-test, p=0.282319. **(j-l)** Clathrin heavy chain GFP-CHC-1 is not altered in the intestine of *amph-1(tm1060)* mutants. **(l)** Quantification of GFP-CHC-1 apical plasma membrane labeling with data plotted as average arbitrary units of pixel intensity. One-tailed Student's T-test, p=0.125286. Error bars represent ±standard deviation from the mean value (n = 30 each, six animals of each genotype sampled in five different regions of each intestine). Scale bars represent 10 μm

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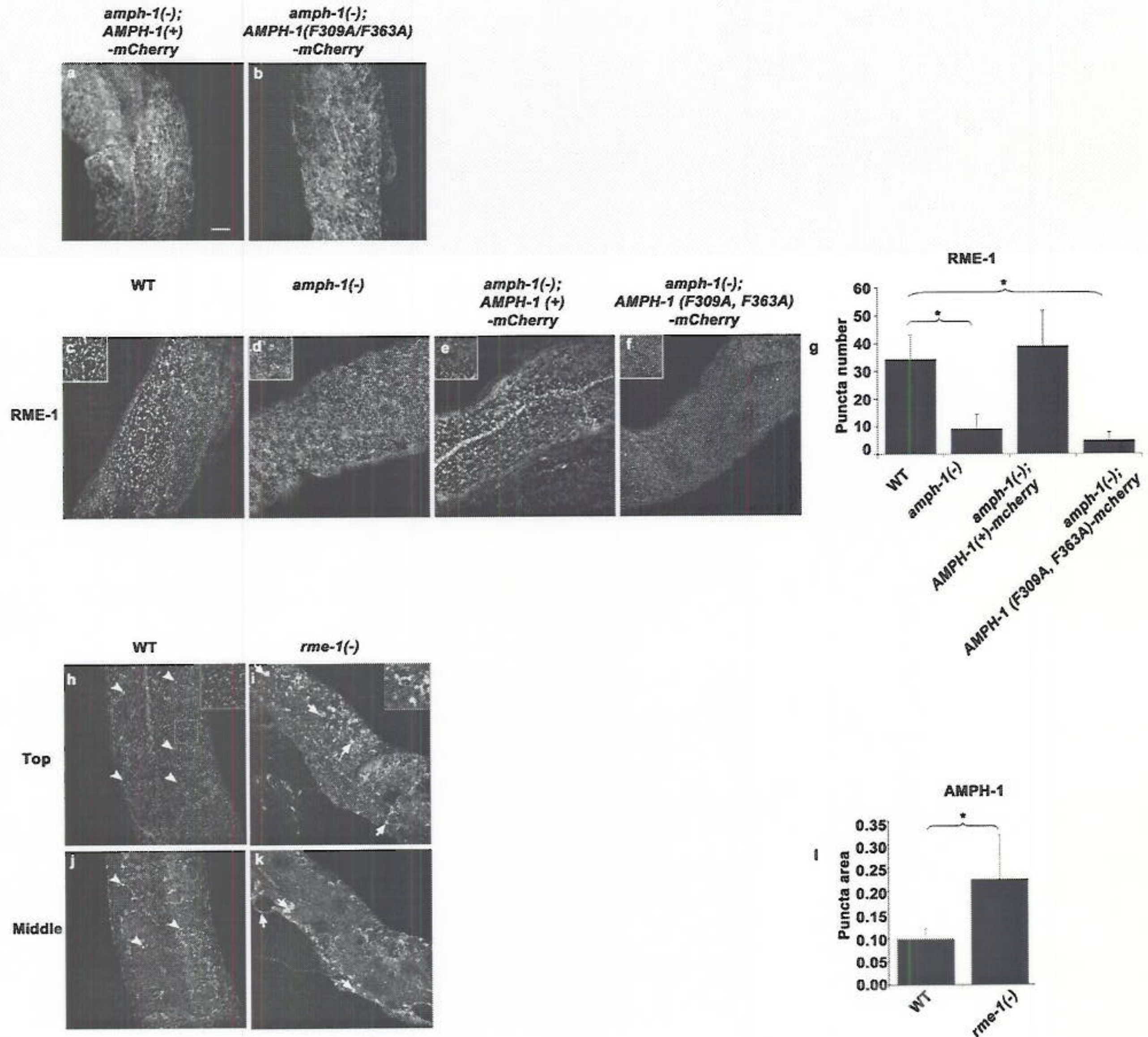

Figure S5 AMPH-1 function in endocytic recycling requires interaction with RME-1. **(a-b)**AMPH-1(+) and interaction deficient AMPH-1(F309A, F363A) were expressed as mCherry fusions in amph-*1(tm1060)* mutant intestines. **(c-g)** AMPH-1(+) but not AMPH-1 (F309A, F363A) could restore the tubulovesicular character of anti-RME-1 staining in dissected amph-*1(tm1060)* intestines. This could indicate a loss of RME-1 recruitment to the tubular recycling endosome. **(g)** Quantification of the RME-1 labeled structures in worms with respect to average number of labeled structures per unit area. The asterisk indicates a significant difference in the one-tailed Student's T-test, p value for WT compared against *amph-1(tm1060)* = $1.0X10^{-37}$ and for WT compared with *amph-1(tm1060); AMPH-1(F309A, F363 A)-mCherry*=$4.7X10^{-42}$. **(h-l)** AMPH-1 localization is abnormal in *rme-1* mutants. In a *rme-1(0)* mutant, endogenous AMPH-1 was found in abnormally enlarged structures. **(h-i)** Confocal micrographs visualizing endogenous AMPH-1 in the top basolateral plane of dissected worm intestines in WT and *rme-1(b1045)* mutants. **(j-k)** Confocal micrographs visualizing endogenous AMPH-1 in the middle plane of the worm intestine in WT and *rme-1(b1045)* mutants. **(l)** Quantification of AMPH-1 labeled structures in WT and *rme-1(0)* animals with respect to puncta area. Error bars represent ±standard deviation from the mean (n = 30 each, six animals of each genotype sampled in five different regions of each intestine). The asterisk indicates a significant difference in the one-tailed Student's t- test, p-value = $2.5X10^{-9}$. Scale bars represent 10 μm.

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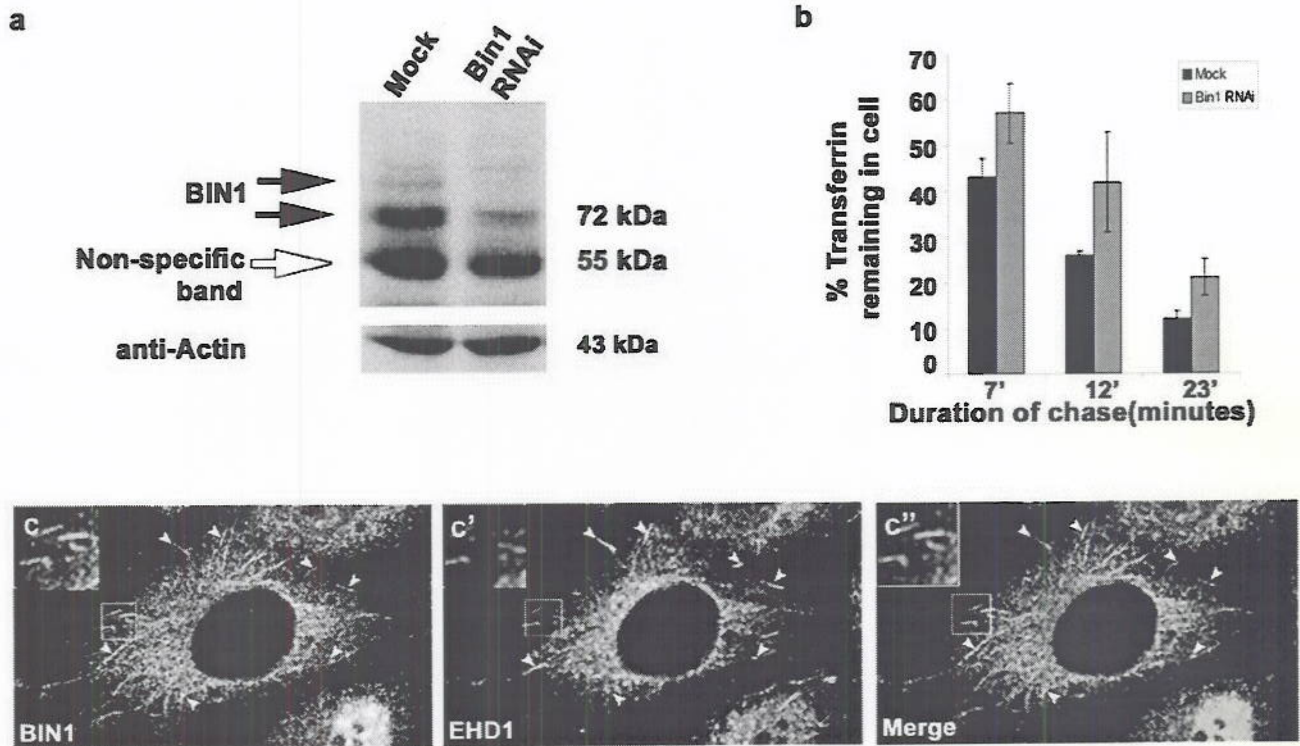

Figure S6 Bin1/ Amph2 functions in endocytic recycling in HeLa cells. **(a)** Western analysis HeLa cell lysates following mock treatment or Bin1 RNAi treatment. Note that all Bin1 splice forms detected (a doublet around 60-75 kDa) (closed arrows) were diminished upon Bin1 knockdown. The antibody also detects a non-specific band of lower molecular weight that is not affected by Bin1 RNAi(open arrow). The knockdown was approximately 80-90% efficient. The lower panel shows equivalent loading of proteins on the same blot, as determined by immunoblotting for actin. **(b)** Average total cell fluorescence was measured by fluorescence activated cell sorting analysis (FACS) of either mock treated or Bin1 RNAi treated HeLa cells, after Alexa-488 transferrin uptake followed by chase in complete medium for the indicated times. **(c)**Endogenous Bin1 and mRme-1/EHD1 colocalize on intracellular tubules. Untransfected HeLa cells were visualized by confocal microscopy after immunostaining with monoclonal anti-Bin1 antibody **(c)** and affinity-purified polyclonal rabbit anti-EHD1 **(c')** antibodies, followed by Alexa Fluor 546-conjugated anti-mouse and Alexa Fluor 488 anti-rabbit secondary antibodies. The merged image showing colocalization of Bin1 with EHD1 positive tubules is shown in panel **c''**. Arrowheads indicate structures positive for Bin1 and EHD1.

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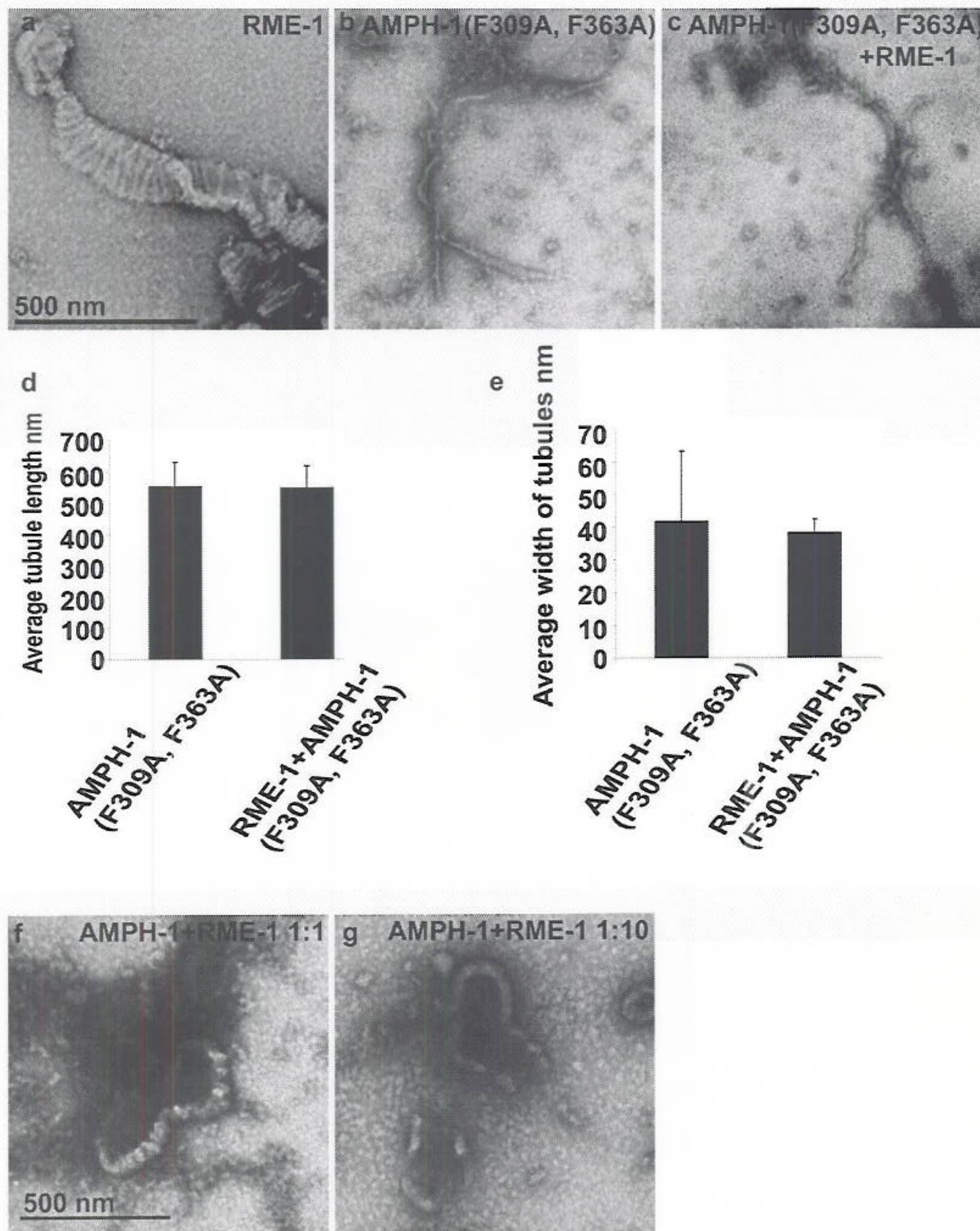

Figure S7 Membrane tubulation by AMPH-1(F309A, F363A) and RME-1 *in vitro.* **(a-c)** Electron micrographs of negatively stained 100% Phosphatidylserine (PtdSer) liposomes used at 0.05 mg/ml in the presence of 1mM ATP-γ-S with 2.5 μM proteins, RME-1 **(a)**, AMPH-1(F309A, F363A) **(b)**, or equimolar quantities of AMPH-1(F309A, F363A) and RME-1 **(c)**.**(d)** Quantification of tubule widths. For each experimental condition, width was measured for every tubule on each of 10 electron micrographs. For tubules demonstrating uneven width, an average measurement was made after taking measures at several representative points along the tubule. In the one-tailed Student's T-test for tubule width : AMPH-1(F309A, F363A) vs RME-1 + AMPH-1(F309A, F363A) p value= 0.30. **(e)** Quantification of tubule lengths. The length from the edge of the liposome body to end of tubule was measured for every tubule on each of 10 electron micrographs per experimental condition. In the one-tailed Student's T-test for tubule length : AMPH-1(F309A, F363A) vs RME-1 + AMPH-1(F309A, F363A) p value= 0.494524. Error bars represent ± standard deviation from the mean. **(f-g)** Relatively low AMPH-1 concentration can produce the qualitative difference in hybrid AMPH-1 and RME-1 tubules. Electron micrographs of negatively stained 100% Phosphatidylserine (PtdSer) liposomes used at 0.05 mg/ml in the presence of 1mM ATP-γ-S with **(f)** equimolar quantities (2.5 μM) of proteins RME-1 and AMPH-1 or **(g)** RME-1 (2.5 μM) with a 10-fold lower concentration of AMPH-1(250 nM).

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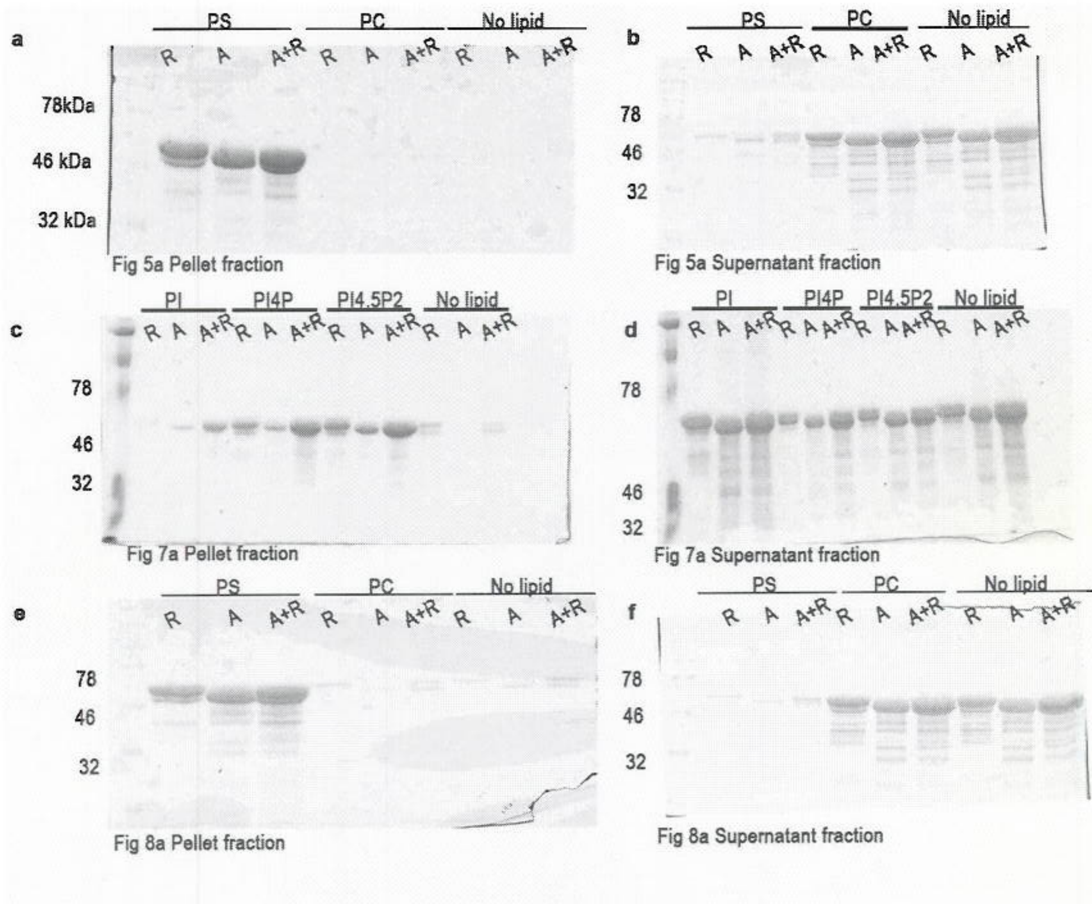

Figure S8 Full scans

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