RESEARCH ARTICLE

Figures and figure supplements

A PX-BAR protein Mvp1/SNX8 and a dynamin-like GTPase Vps1 drive endosomal recycling

Sho W Suzuki *et al*

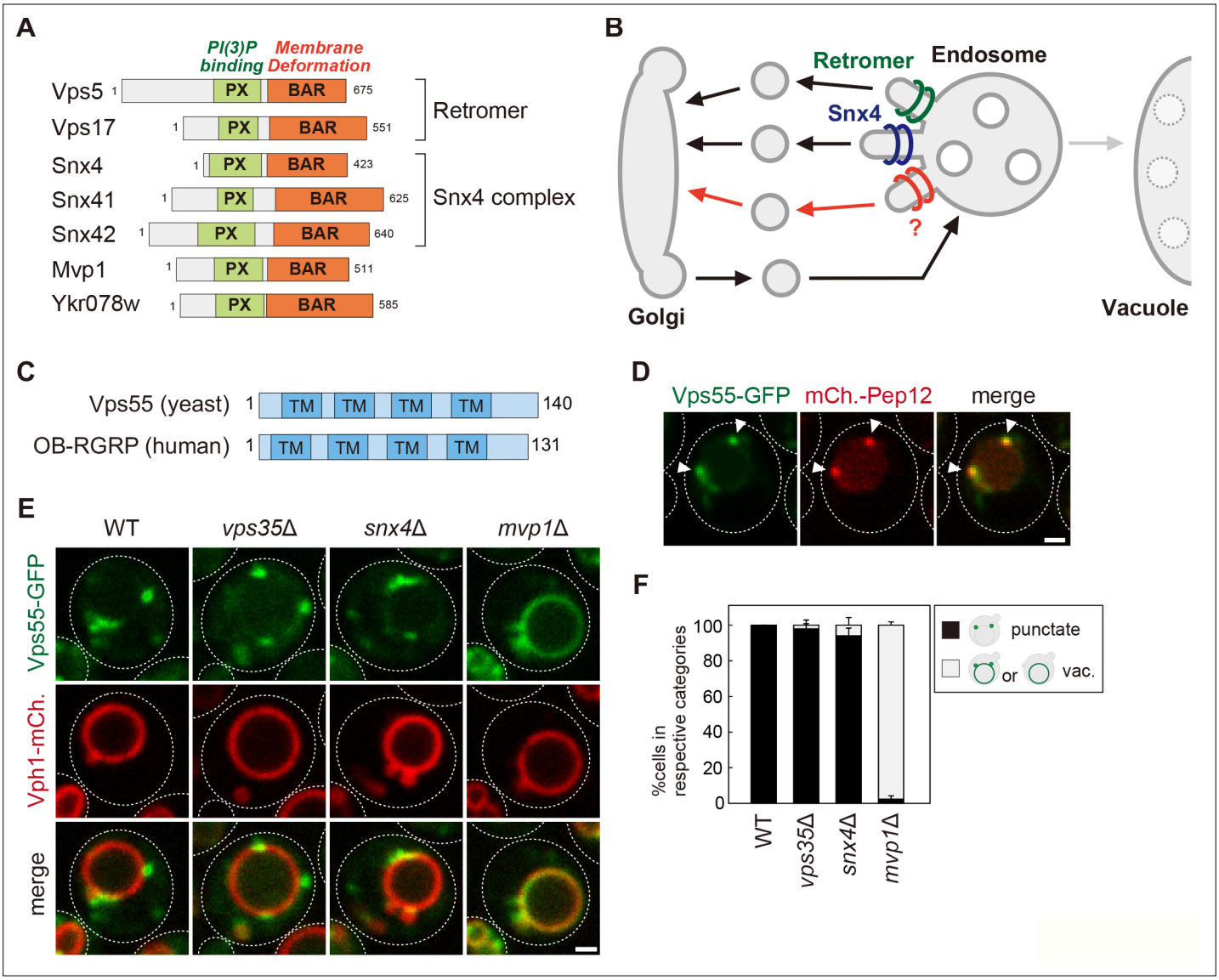

Figure 1. The endosomal localization of Vps55 requires Mvp1. (**A**) Schematic of SNX-BAR proteins in yeast. (**B**) Model of endosomal recycling pathways in yeast. (**C**) Schematic of Vps55 and OB-RGRP. (**D**) Vps55-GFP localization. The mCherry-Pep12 serves as an endosomal marker. (**E**) Vps55-GFP localization in wild-type (WT), *vps35*Δ (retromer mutant), *snx4*Δ (Snx4 complex mutant), and *mvp1*Δ. (**F**) Quantification of Vps55-GFP localization from three independent experiments. N = >30 cells. Scale bar: 1 μm.

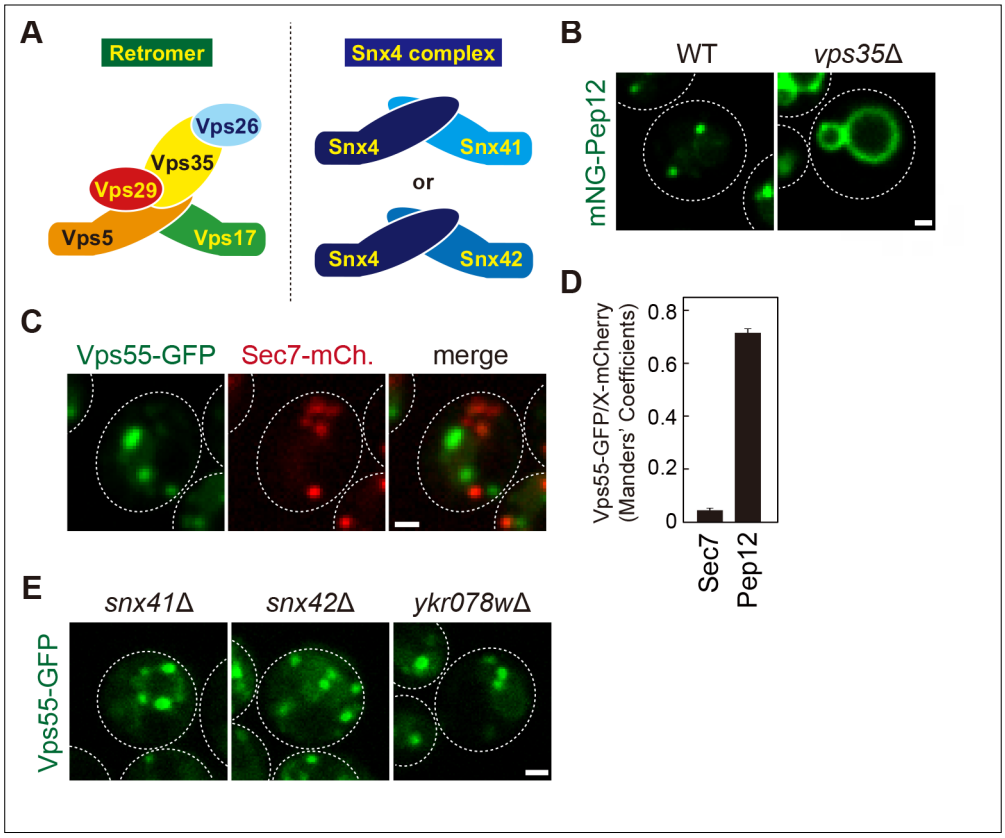

Figure 1—figure supplement 1. The localization of endosomal membrane proteins. (**A**) Schematic of retromer and Snx4 complexes. (**B**) The localization of mNeonGreen-Pep12 in wild-type (WT) and *vps35*Δ cells. (**C**) Vps55-GFP localization with Sec7-mCherry serving as a marker for the trans-Golgi. (**D**) Manders' coefficients of Vps55-GFP with Sec7-mCherry or mCherry-Pep12 from **C** and ***Figure 1D***. (**E**) Vps55-GFP localization in *snx41*Δ, *snx42*Δ, and *ykr078w*Δ cells. Scale bar: 1 μm.

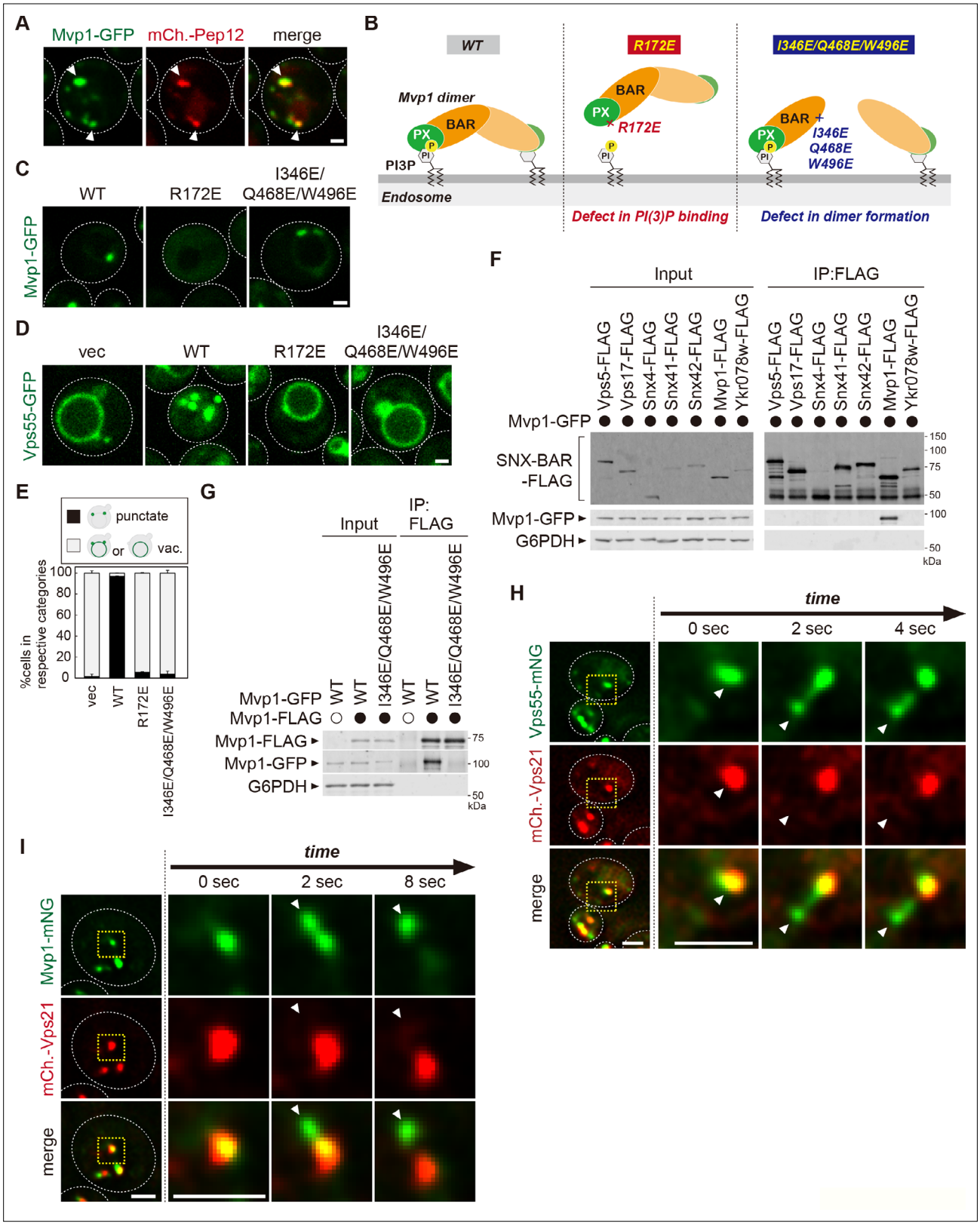

Figure 2. Mvp1 is an endosomal coat complex for Vps55 recycling. (**A**) Mvp1-GFP localization. The mCherry-Pep12 serves as an endosomal marker. (**B**) Schematic of Mvp1 mutants. (**C**) The localization of Mvp1-GFP mutants. (**D**) The localization of Vps55-GFP mutants. (**E**) Quantitation of Vps55-GFP localization of *mvp1* mutants from three independent experiments. N = >30 cells. (**F**) The binding of SNX-BAR proteins with Mvp1. FLAG-tagged SNX-BAR proteins were immunoprecipitated (IP) from cells expressing Mvp1-GFP, and the IP products were analyzed by immunoblotting using antibodies

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against FLAG, green fluorescent protein (GFP), and glucose-6-phosphate dehydrogenase (G6PDH). (**G**) The dimer formation of *mvp1* mutants. Mvp1-FLAG was immunoprecipitated from cells expressing Mvp1-GFP mutants, and the IP products were analyzed by immunoblotting using antibodies against FLAG, GFP, and G6PDH. (**H**) Live-cell imaging analysis of Vps55-mNeonGreen and mCherry-Vps21. (**I**) Live-cell imaging analysis of Mvp1-mNeonGreen and mCherry-Vps21. Scale bar: 1 μm.

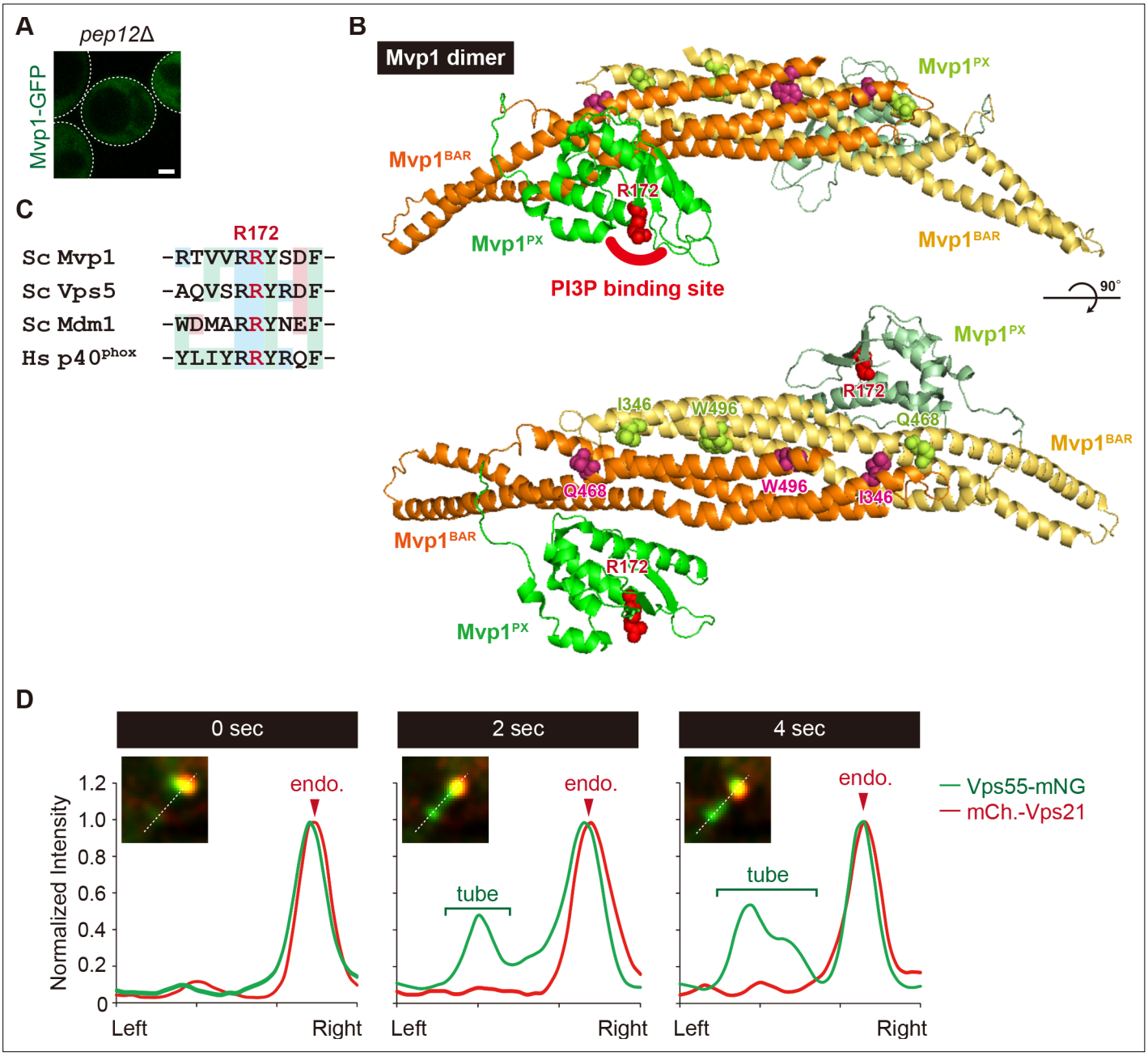

Figure 2—figure supplement 1. The analysis of Mvp1. (**A**) Mvp1-GFP localization in *pep12Δ* cells. Scale bar: 1 μm. (**B**) Mutation sites used in this study are shown on the cryo-electron microscopy (cryo-EM) structure of *Saccharomyces cerevisiae* Mvp1 (PDB ID code: 6Q0X). (**C**) Sequence comparison of the residue required for phosphatidylinositol 3-phosphate (PI3P) binding among Phox homology (PX) domain-containing proteins. (**D**) Line scan analysis performed for the region highlighted by the white line from ***Figure 2H***.

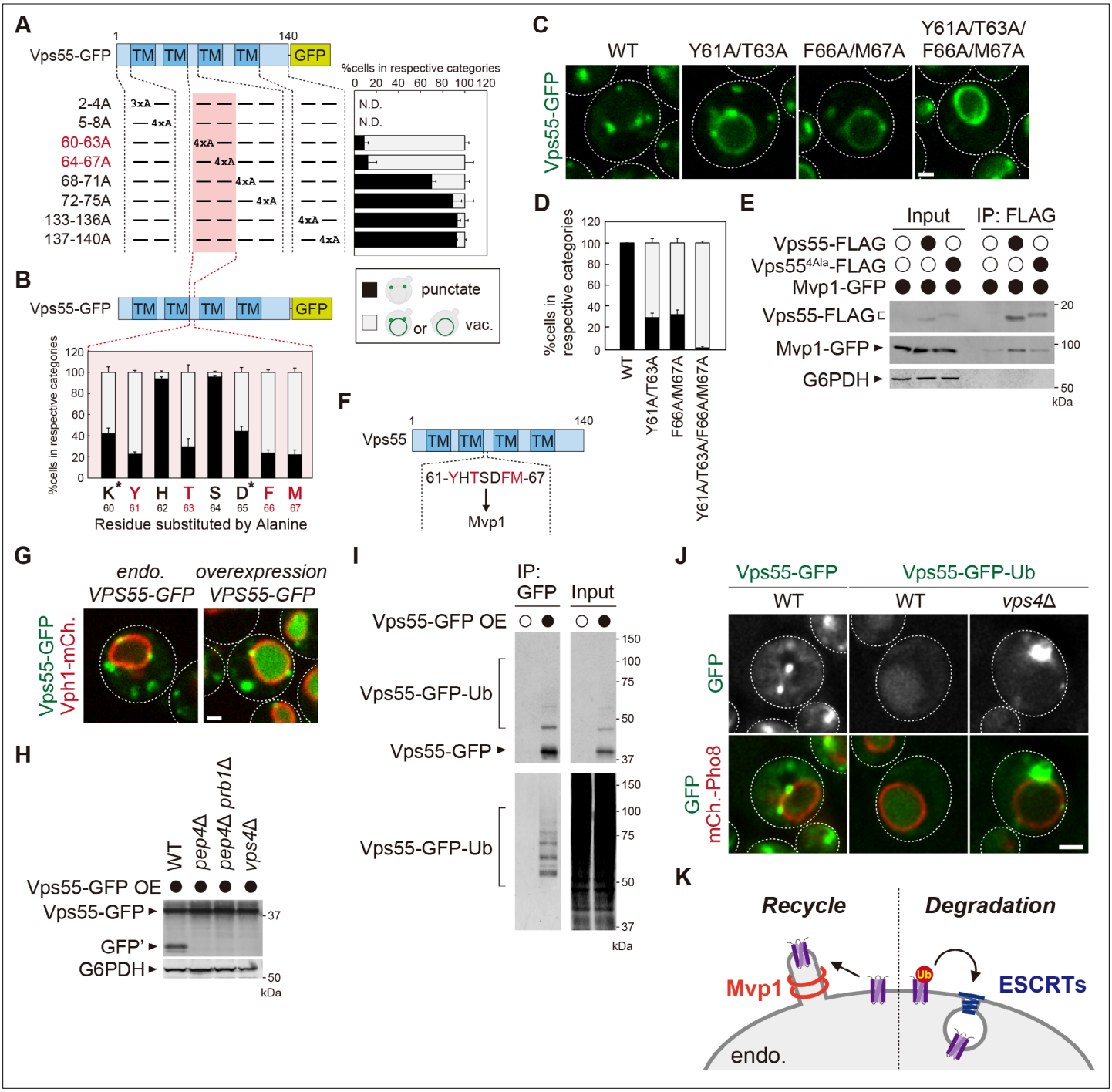

Figure 3. Mvp1 recognizes Vps55 through a specific sorting motif. (**A, B**, and **D**) Schematic of Vps55 mutational analysis and quantitation of Vps55-GFP mutant localization, from ***Figure 3—figure supplement 1A*** (**A**), ***Figure 3—figure supplement 1B*** (**B**), and ***Figure 3—figure supplement 1C*** (**D**). (**C**) The localization of Vps55-GFP mutants. (**E**) The Mvp1-Vps55 interaction in Vps55-FLAG mutants. Vps55-FLAG mutants were immunoprecipitated (IP) from cells expressing Mvp1-GFP, and the IP products were analyzed by immunoblotting using antibodies against FLAG, green fluorescent protein (GFP), and glucose-6-phosphate dehydrogenase (G6PDH). (**F**) Schematic of Vps55 and the residues facilitating its interaction with Mvp1. (**G**) The localization of overexpressed Vps55-GFP. (**H**) Vps55-GFP sorting in vacuolar hydrolases (*pep4Δ* and *pep4Δ prb1Δ*) and ESCRT (*vps4Δ*) mutants. Cell lysates expressing Vps55-GFP were analyzed by immunoblotting using antibodies against GFP and G6PDH. (**I**) The ubiquitination of overexpressed Vps55-GFP. Overexpressed Vps55-GFP was immunoprecipitated from yeast cells under denaturing conditions, and the IP products were analyzed by immunoblotting using antibodies against GFP and ubiquitin. (**J**) Vps55-GFP-Ub localization in ESCRT mutants. (**K**) Model of Vps55 recycling and degradation at the endosome. For all quantification shown in this figure, n = >30 cells from three independent experiments. Scale bar: 1 μm.

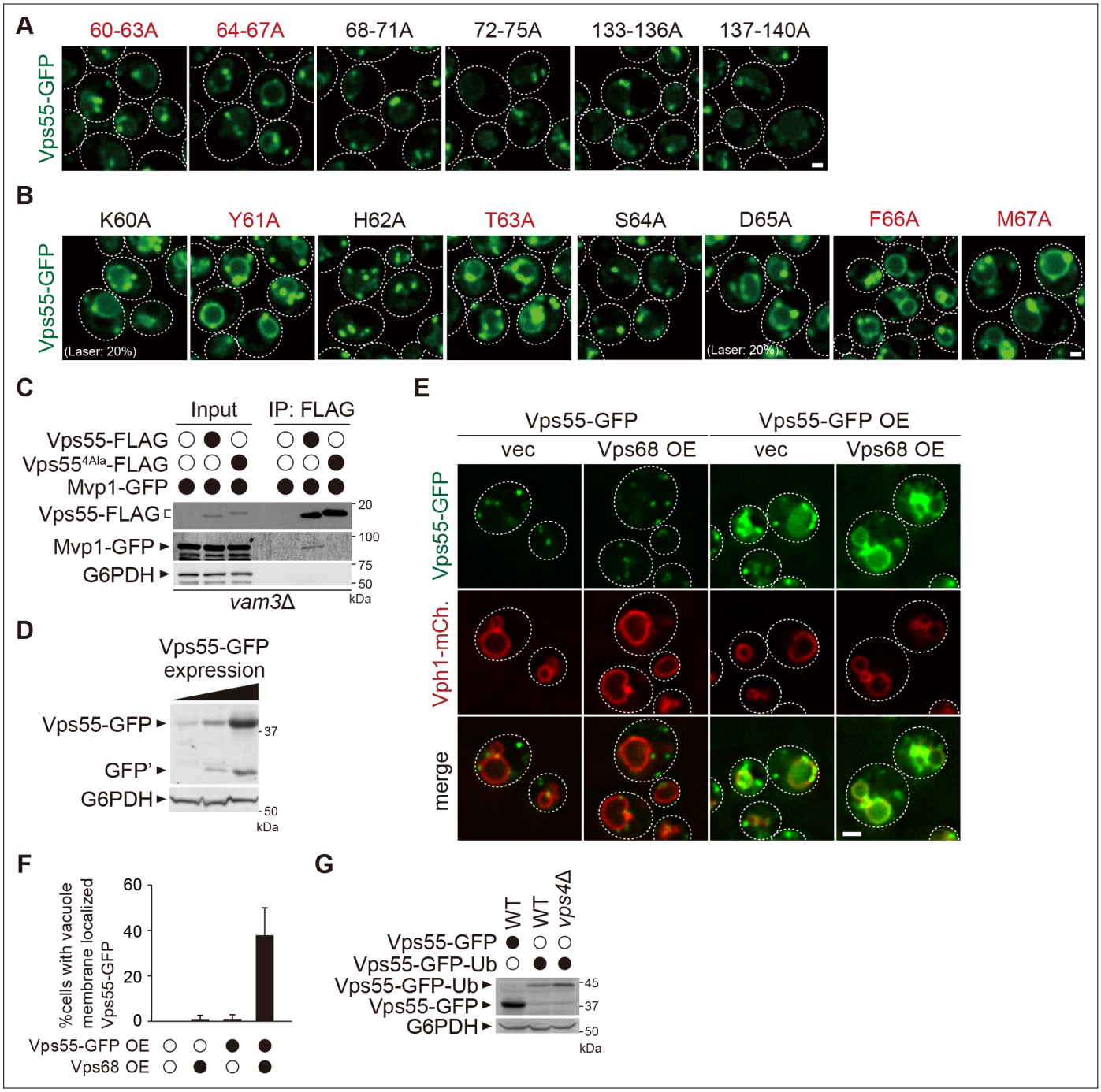

Figure 3—figure supplement 1. The analysis of Vps55. (**A and B**) Localization of Vps55-GFP mutants. In K60A and D65A mutants, the excitation laser intensity was lowered to 20%, because its expression was higher than other mutants. (**C**) The Mvp1-Vps55 interaction in *vam3*Δ cells. Vps55-FLAG mutants were immunoprecipitated (IP) from *vam3*Δ cells expressing Mvp1-GFP, and the IP products were analyzed by immunoblotting using antibodies against FLAG, green fluorescent protein (GFP), and glucose-6-phosphate dehydrogenase (G6PDH). (**D**) Immunoblotting of cells expressing increasing amounts of Vps55-GFP. (**E**) The localization of overexpressed Vps55-GFP and Vps68. (**F**) Quantification of Vps55-GFP localization from three independent experiments. N = >30 cells. (**G**) Cell lysates expressing Vps55-GFP or Vps55-GFP-Ub were analyzed by immunoblotting using antibodies against GFP and G6PDH. Scale bar: 1 μm.

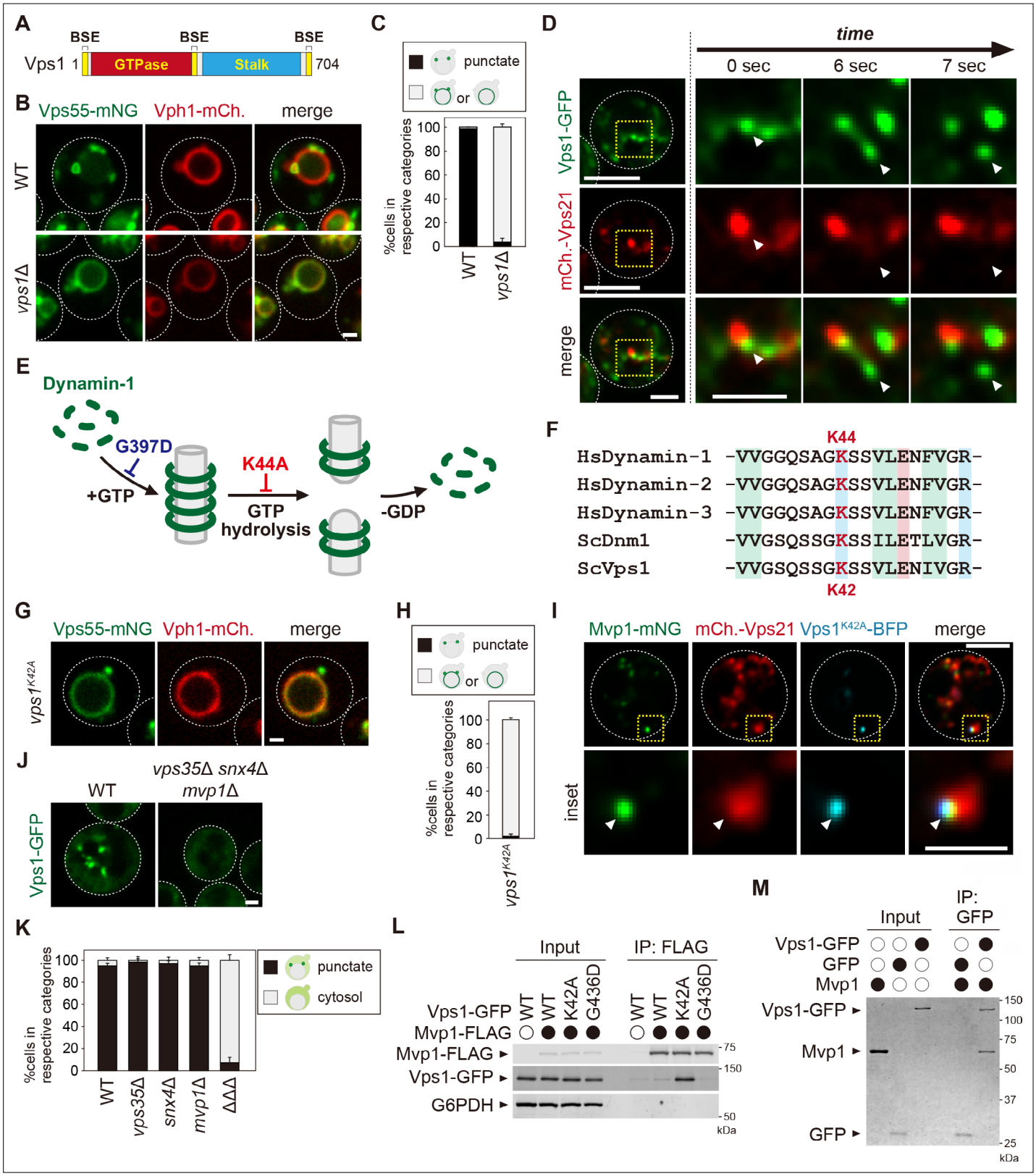

Figure 4. Mvp1 recruits dynamin-like GTPase Vps1 to catalyze membrane scission. (**A**) Schematic of Vps1. (**B**) Vps55-mNeonGreen localization in WT and *vps1*Δ cells. (**C**) Quantitation of Vps55-mNeonGreen localization, from **B**. (**D**) The live-cell imaging of Vps1-GFP. The mCherry-Vps21 serves as an endosomal marker. (**E**) Model of Dynamin-1-mediated membrane fission. (**F**) Sequence comparison of residues required for guanosine-5'-triphosphate (GTP) hydrolysis in *Homo sapiens* Dynamin-1, *H. sapiens* Dynamin-2, *H. sapiens* Dynamin-3, *Saccharomyces cerevisiae* Dnm1, and *S. cerevisiae* Vps1.

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(**G**) Vps55-mNeonGreen localization in *vps1*^{K42A} mutants. (**H**) Quantitation of Vps55-mNeonGreen localization, from **G**. (**I**) The localization of Mvp1-mNeonGreen, mCherry-Vps21, and Vps1^{K42A}-BFP. (**J**) Vps1-GFP localization in wild-type (WT) and *vps35Δ snx4Δ mvp1Δ* triple mutants. (**K**) Quantitation of Vps1-GFP localization, from **J** and ***Figure 4—figure supplement 1F***. (**L**) The Mvp1-Vps1 interaction. Mvp1-FLAG was immunoprecipitated (IP) from cells expressing Vps1-GFP mutants, and the IP products were analyzed using antibodies against FLAG, green fluorescent protein (GFP), and glucose-6-phosphate dehydrogenase (G6PDH). (**M**) In vitro binding assay between Mvp1 and Vps1-GFP. The proteins bound to anti-GFP magnetic beads were detected by Coomassie staining. For all quantifications shown in this figure, n = >30 cells from three independent experiments. Scale bar: 1 μm.

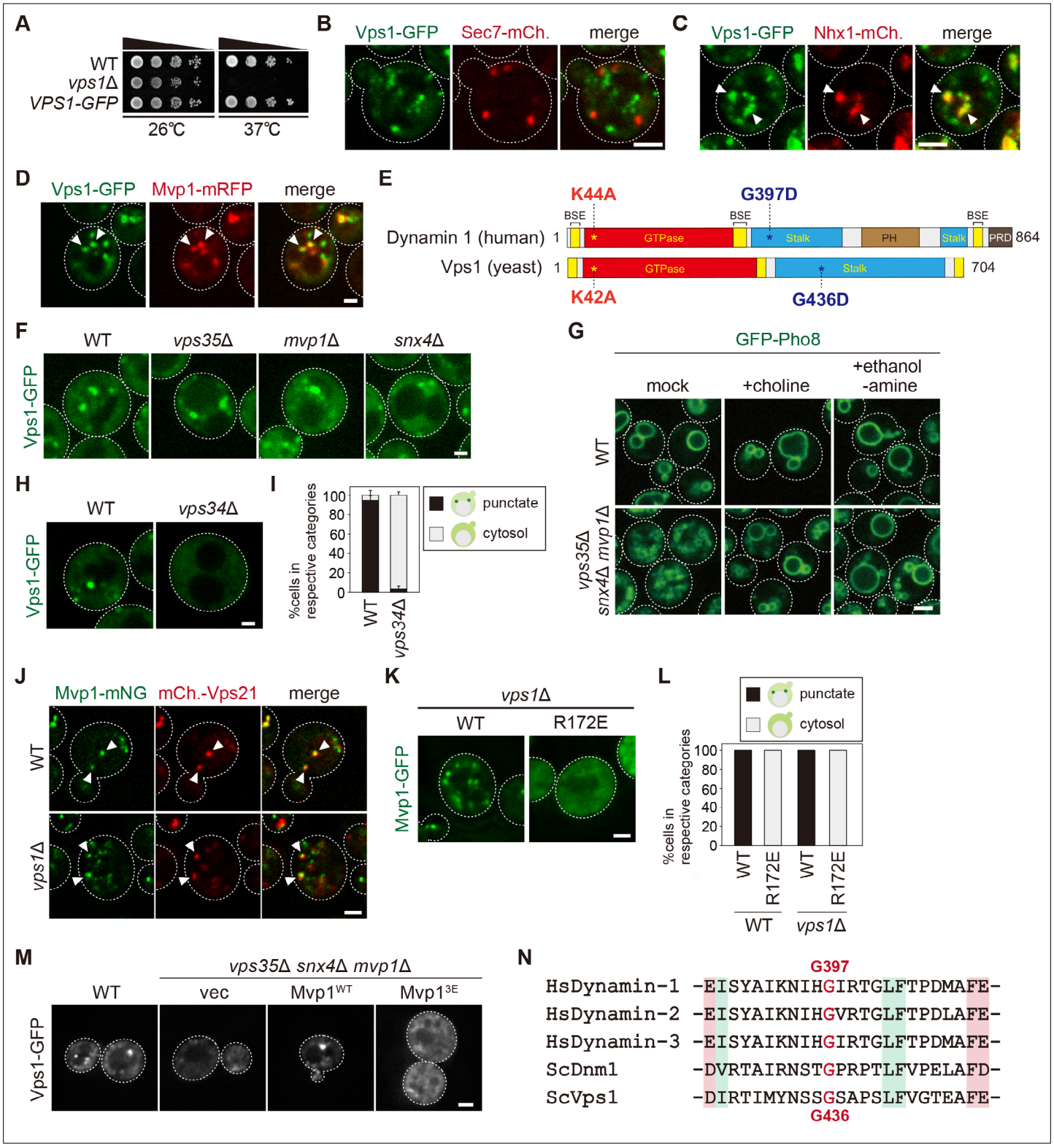

Figure 4—figure supplement 1. The analysis of Vps1. (**A**) Growth of Vps1-GFP-expressing cells. (**B, C, and D**) Vps1-GFP localization with Sec7-mCherry serving as a marker for the trans-Golgi (**B**), Nhx1-mCherry serving as a marker for the endosome (**C**), or Mvp1-mRFP (**D**). (**E**) Schematic of Dynamin-1 and Vps1 domains and mutated residues. (**F**) Vps1-GFP localization in wild-type (WT), *vps35Δ*, *snx4Δ*, and *mvp1Δ* cells. (**G**) Vacuole morphology in *vps35Δ*, *snx4Δ*, and *mvp1Δ* supplemented with 2 mM choline or 2 mM ethanolamine. (**H**) Vps1-GFP localization in WT or *vps34Δ* cells. (**I**) Quantification of Vps1-GFP localization from **H**. (**J**) Mvp1-mNeonGreen localization in WT and *vps1Δ* cells with mCherry-Vps21 serving as an endosomal marker. (**K**) Localization

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of Mvp1-GFP in *vps1*Δ cells. (**L**) Quantification of Mvp1-GFP localization from **K**. (**M**) Vps1-GFP localization in WT, *vps35*Δ, *snx4*Δ, and *mvp1*Δ cells expressing Mvp1 mutants. (**N**) Sequence comparison of residues required for the assembly of Vps1 in *Homo sapiens* Dynamin-1, *H. sapiens* Dynamin-2, *H. sapiens* Dynamin-3, *Saccharomyces cerevisiae* Dnm1, and *S. cerevisiae* Vps1. Scale bar: 1 μm.

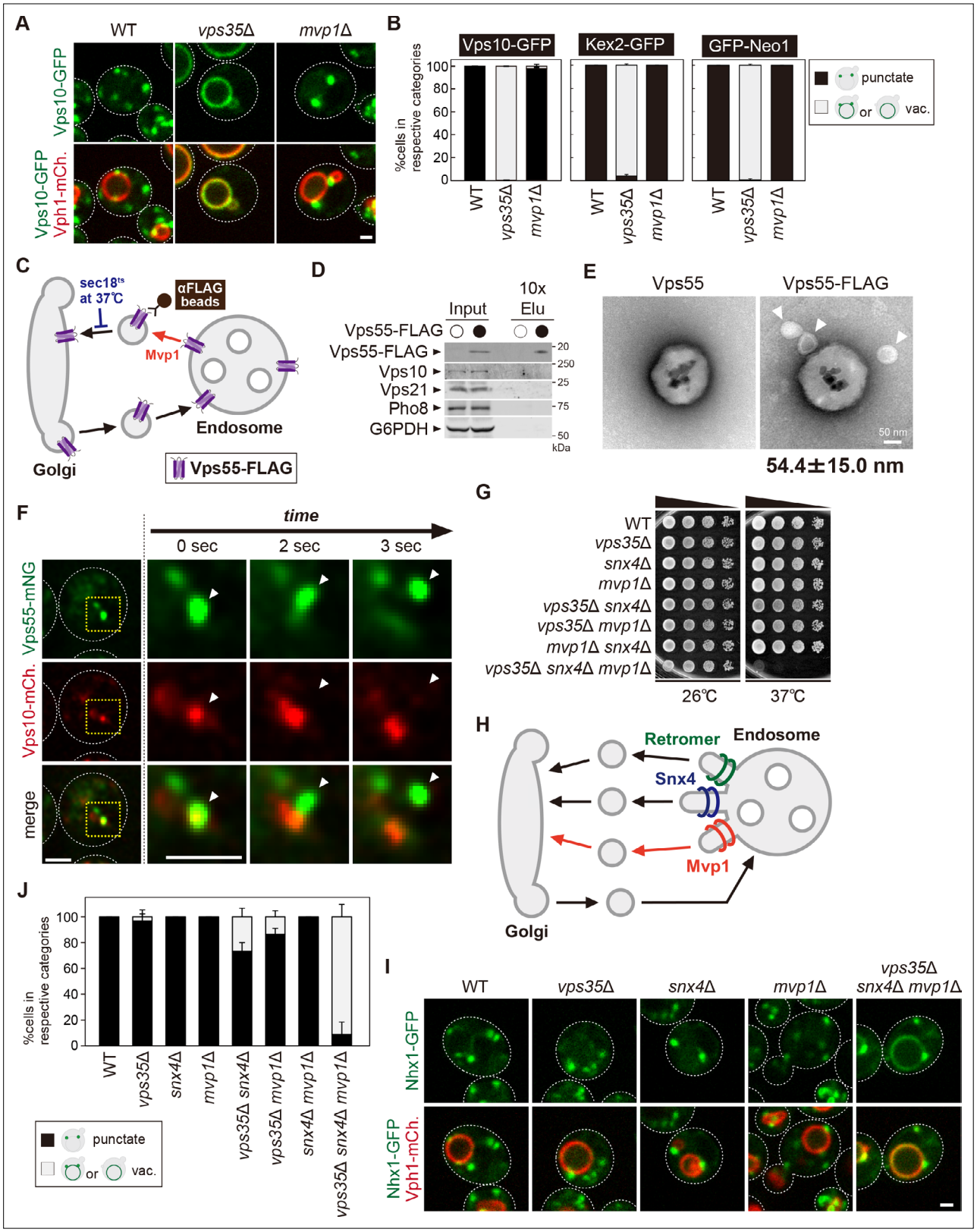

Figure 5. Mvp1 mainly mediates retromer-independent endosomal recycling. (**A**) Localization of Vps10-GFP in wild-type (WT), *vps35*Δ, and *mvp1*Δ cells. (**B**) Quantification of Vps10-GFP, Kex2-GFP, and GFP-Neo1 localization from **A** and ***Figure 5—figure supplement 1E,F***, respectively. N = >30 cells from three independent experiments. (**C**) Schematic for immunoisolation of Vps55-FLAG-containing structures. (**D**) The immunoisolation of Vps55-containing vesicles. Vps55-FLAG-containing structures were immunoisolated from *sec18*^{ts} mutants incubated at 37 °C for 1 hr, and the isolated structures were

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analyzed by immunblotting using antibodies against FLAG, Vps10 (retromer cargo), Vps21 (endosome), Pho8 (vacuole), and glucose-6-phosphate dehydrogenase (G6PDH) (cytoplasm). (**E**) Electron microscopy (EM) analysis of the isolated Vps55-FLAG-containing structures from **D**. (**F**) Live-cell imaging analysis of Vps55-mNeonGreen and Vps10-mCherry. (**G**) Cell growth in *vps35Δ snx4Δ mvp1Δ* triple mutants. Cells lacking Vps35 as well as Snx4 and Mvp1 were grown at 26°C and 37°C. (**H**) Model of retromer-, Snx4-, and Mvp1-mediated recycling. (**I**) Nhx1 localization in SNX-BAR mutants. (**J**) Quantitation of Nhx1-GFP localization, from **I** and ***Figure 5—figure supplement 1M***. N = >30 cells from three independent experiments. Scale bar: 1 μm.

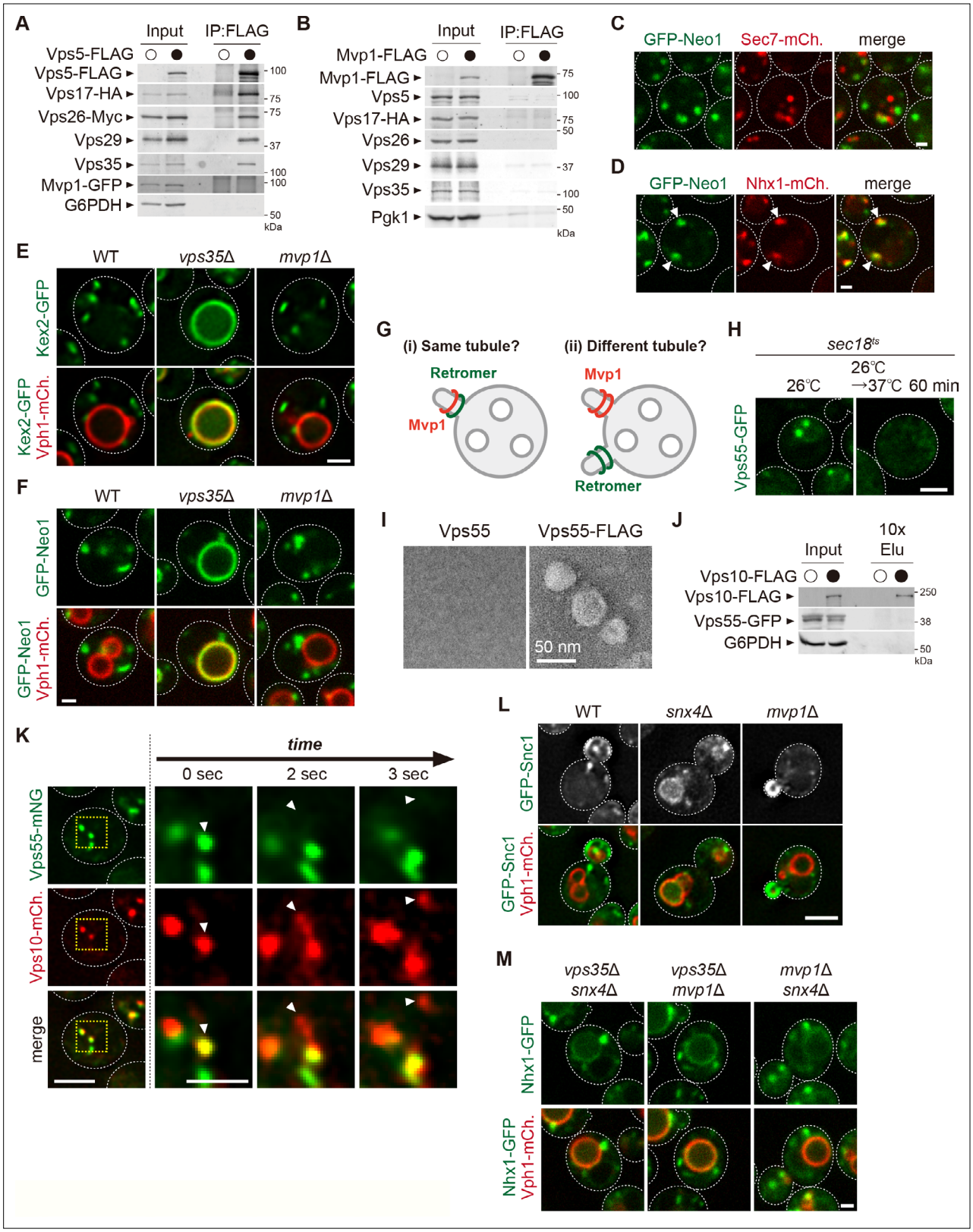

Figure 5—figure supplement 1. The analysis of the retromer pathway in Mvp1 mutants. (**A**) The association of Mvp1 with the retromer complex. Vps5-FLAG was immunoprecipitated (IP) from cells expressing Vps17-HA, Vps26-Myc, and Mvp1-GFP, and the IP products were analyzed by immunoblotting using antibodies against FLAG, hemagglutinin (HA), Myc, Vps29, Vps35, green fluorescent protein (GFP), and glucose-6-phosphate dehydrogenase (G6PDH). (**B**) Mvp1 binding to the retromer subunits. Mvp1-FLAG was immunoprecipitated from cells expressing Vps17-HA, and the IP products were

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analyzed by immunoblotting using antibodies against Vps5, HA, Vps26, Vps29, Vps35, and G6PDH. (**C and D**) Localization of GFP-Neo1 with Sec7-mCherry as a marker for the trans-Golgi (**C**) and Nhx1-mCherry as a marker for the endosome (**D**). (**E and F**) Localization of Kex2-GFP (**E**) and GFP-Neo1 (**F**) in wild-type (WT), *vps35*Δ, and *mvp1*Δ cells. (**G**) Hypothesis of Mvp1-mediated recycling. (**H**) Vps55-GFP localization in *sec18*^{ts} mutants at 26 °C or 37 °C for 60 min. (**I**) Electron microscopy (EM) analysis of the isolated Vps55-FLAG-containing structures. Vps55-FLAG-containing structures were immunoisolated from *sec18*^{ts} mutants incubated at 37 °C for 1 hr, and then eluated using FLAG peptides. Eluated products were analyzed by negative-stain EM. (**J**) Immunoisolation of Vps10-containing vesicles. Vps10-FLAG-containing structures were immunoisolated from *sec18*^{ts} mutants incubated at 37 °C for 1 hr, and the isolated structures were analyzed by immunblotting using antibodies against FLAG, GFP, and G6PDH. (**K**) Live-cell imaging of Vps55-mNeonGreen and Vps10-mCherry. (**L**) GFP-Snc1 localization in WT, *snx4*Δ, and *mvp1*Δ cells. (**M**) Nhx1 localization in *vps35*Δ *snx4*Δ, *vps35*Δ *mvp1*Δ, and *snx4*Δ *mvp1*Δ cells. Scale bar: 1 μm.

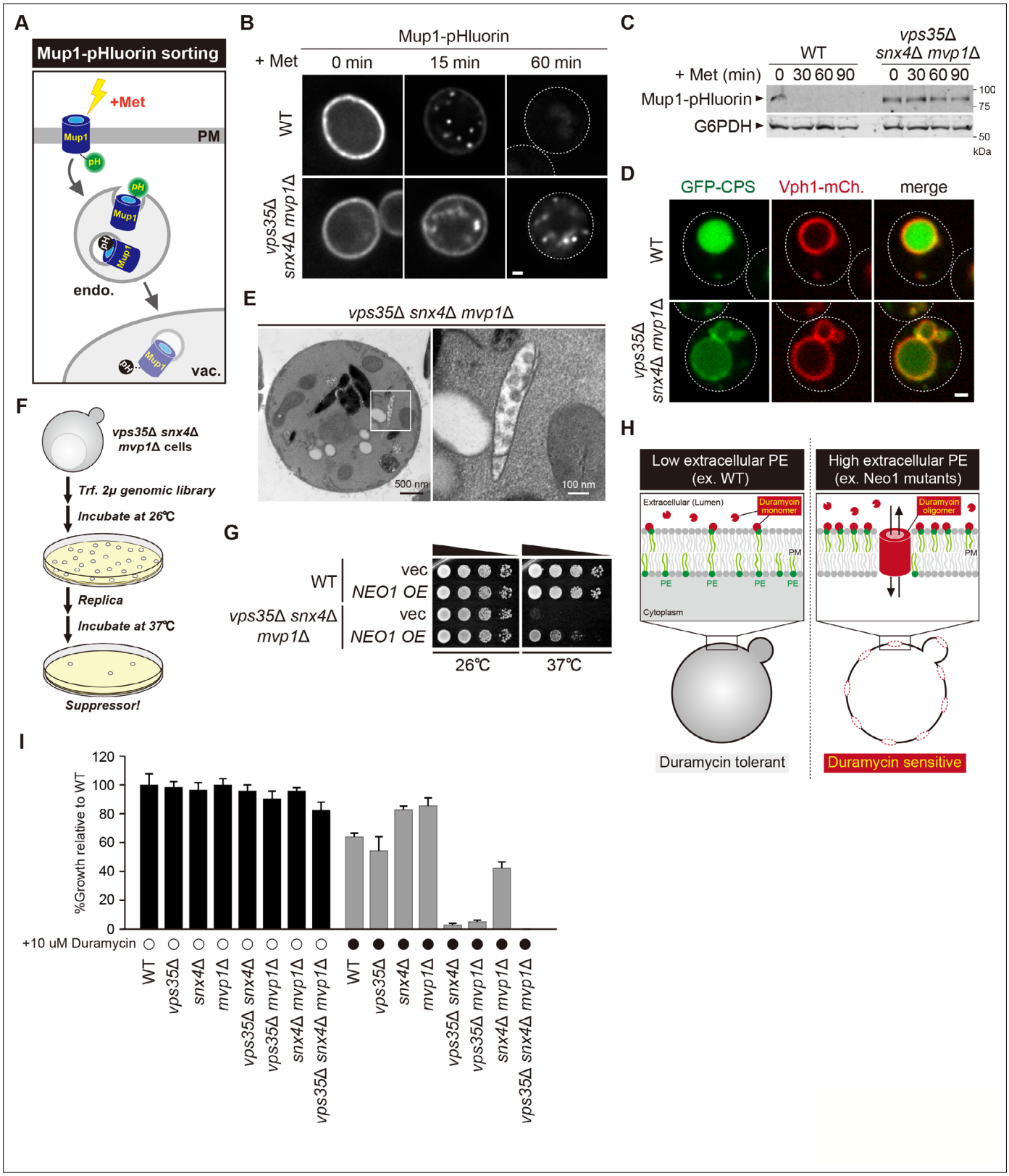

Figure 6. Retromer, Snx4, and Mvp1 complexes are required for proper function of the endosome. (**A**) Schematic of Mup1-pHluorin sorting. (**B**) Mup1-pHluorin localization in wild-type (WT) and *vps35Δ snx4Δ mvp1Δ* cells. Cells expressing Mup1-pHluorin were grown to mid-log phase and stimulated with 20 μg/ml methionine. Scale bar: 1 μm. (**C**) Mup1-pHlourin processing in WT and *vps35Δ snx4Δ mvp1Δ* cells. Mup1 sorting was stimulated as in **B**. (**D**) GFP-CPS sorting in WT and *vps35Δ snx4Δ mvp1Δ* cells. (**E**) Thin-section electron microscopy (EM) of an endosome in *vps35Δ snx4Δ mvp1Δ*

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cells. (**F**) Schematic of screening for multicopy suppressors of *vps35Δ snx4Δ mvp1Δ* triple mutants. (**G**) Growth of *vps35Δ snx4Δ mvp1Δ* triple mutants overexpressing Neo1. (**H**) Schematic of duramycin assay to evaluate extracellular phosphatidylethanolamine (PE). (**I**) Growth of recycling mutants in the presence of duramycin.

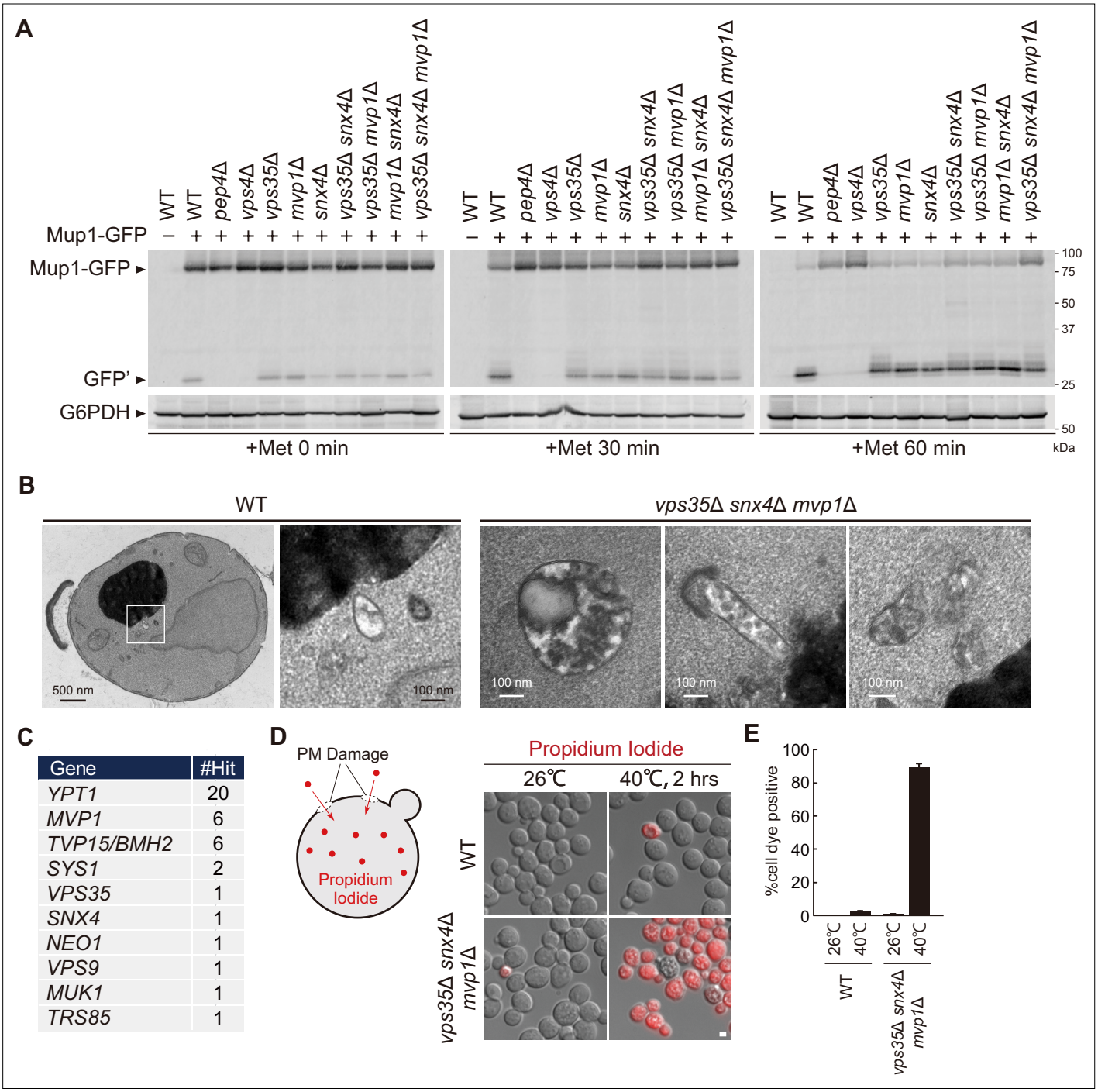

Figure 6—figure supplement 1. The analysis of triple recycling pathway mutants. (**A**) Mup1-GFP sorting in SNX-BAR mutants. Mup1 sorting was stimulated as in ***Figure 6B***. (**B**) Thin-section electron microscopy (EM) of endosomes in wild-type (WT) and *vps35Δ snx4Δ mvp1Δ* cells. (**C**) Summary of identified multicopy suppressors from ***Figure 6F***. (**D**) Schematic for the plasma membrane (PM) integrity assay for WT and *vps35Δ snx4Δ mvp1Δ* triple mutants. (**E**) Quantitation of propidium iodide-stained cells from **D**.

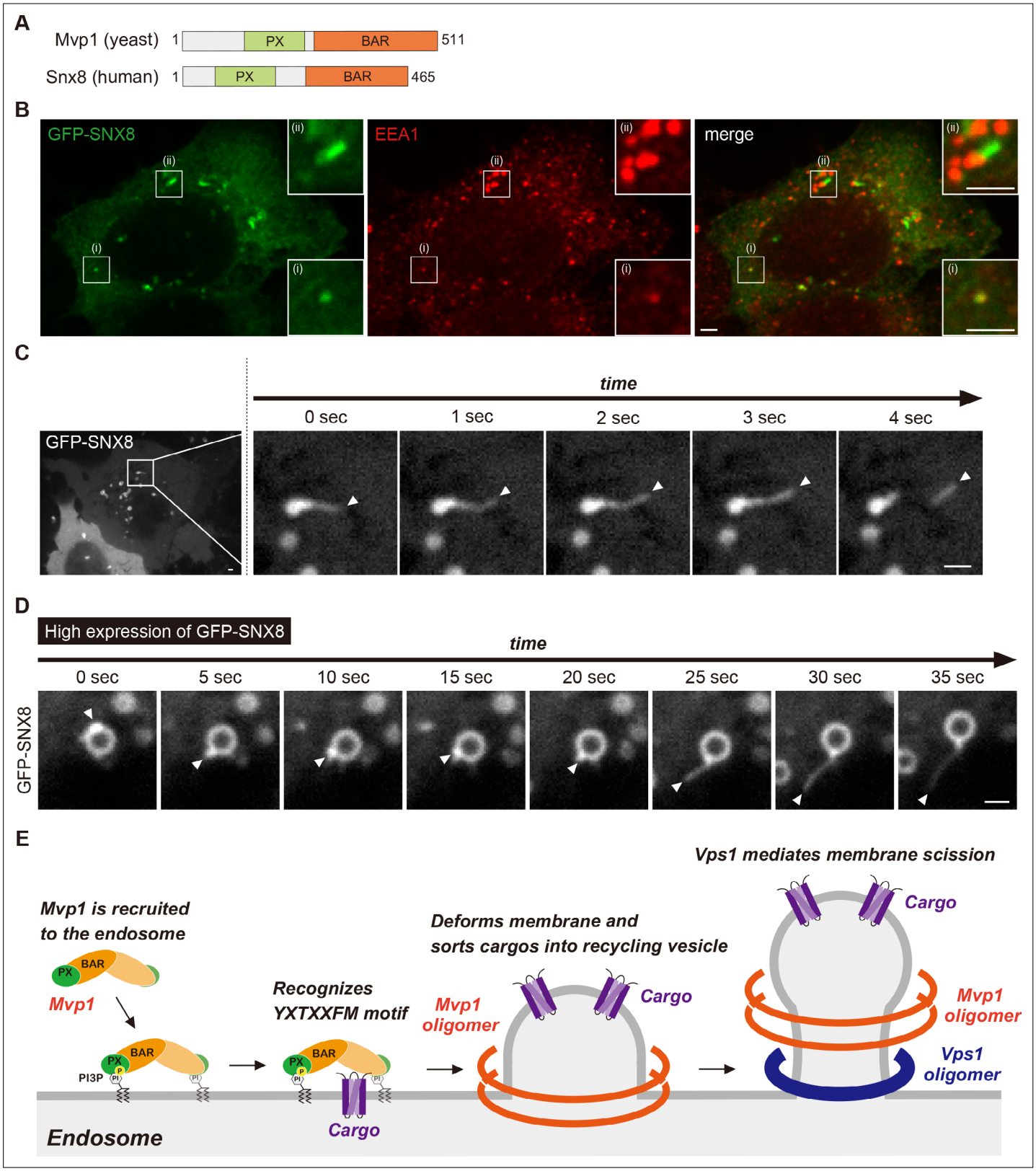

Figure 7. Mvp1-mediated endosomal recycling is evolutionarily conserved. (**A**) Schematic of Mvp1 and Snx8. (**B**) Immunofluorescence of GFP-SNX8-expressing Hela cells, with EEA1 serving as an endosomal marker. (**C**) Live-cell imaging of GFP-SNX8. (**D**) Live-cell imaging of highly expressed GFP-SNX8. (**E**) Model of Mvp1-mediated endosomal recycling. Scale bar: 1 μm.

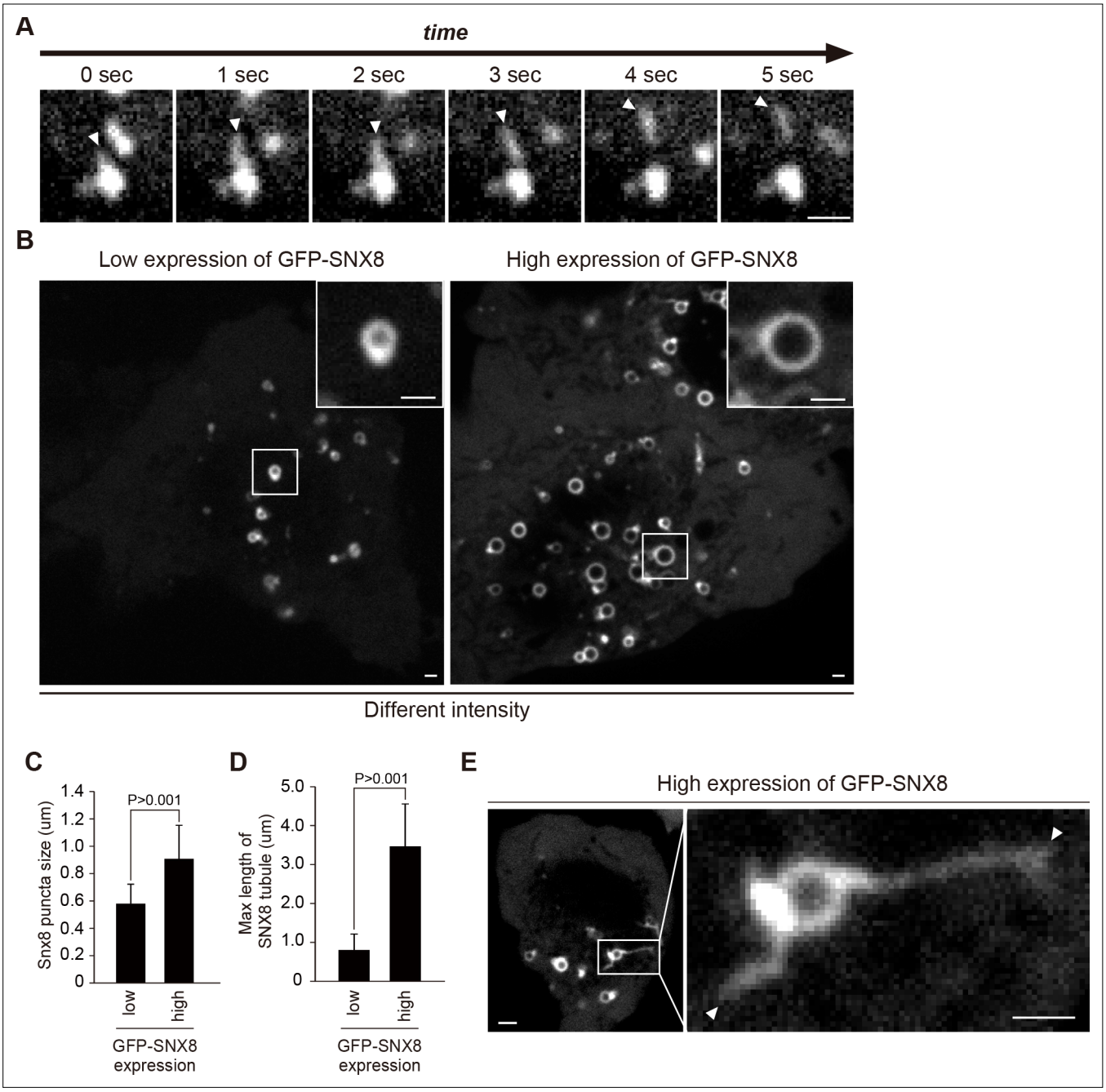

Figure 7—figure supplement 1. The analysis of GFP-SNX8 tubule structure. (**A**) Live-cell imaging of GFP-SNX8. (**B**) GFP-SNX8 localization of Hela cells expressing GFP-SNX8 at low or high levels. (**C and D**) Quantification of SNX8 puncta size (**C**) or max length of SNX8 tubule (**D**) from **B**. (**E**) GFP-SNX8 tubule structures in GFP-SNX8 highly expressing cells. Scale bar: 1 μm.

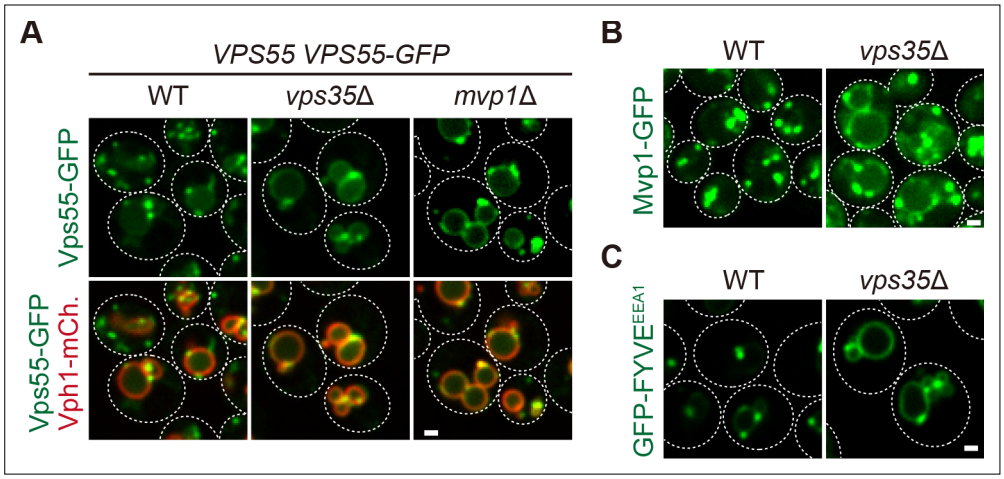

Figure 7—figure supplement 2. Highly expressed Vps55 is mislocalized in the retromer mutants. (**A**) Vps55-GFP localization in cells expressing both Vps55 and Vps55-GFP. (**B**) Mvp1-GFP localization in wild-type (WT) and *vps35*Δ cells. (**C**) GFP-2xFYVE localization in WT and *vps35*Δ cells. Scale bar: 1 μm.