

Rab GTPase regulation of retromer-mediated cargo export during endosome maturation

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ABSTRACT The retromer complex, composed of sorting nexin subunits and a Vps26/Vps29/Vps35 trimer, mediates sorting of retrograde cargo from the endosome to the *trans*-Golgi network. The retromer trimer subcomplex is an effector of Rab7 (Ypt7 in yeast). Whereas endosome targeting of human retromer has been shown to require Rab7-GTP, targeting of yeast retromer to the endosome is independent of Ypt7-GTP and requires the Vps5 and Vps17 retromer sorting nexin subunits. An evolutionarily conserved amino acid segment within Vps35 is required for Ypt7/Rab7 recognition *in vivo* by both yeast and human retromer, establishing that Rab recognition is a conserved feature of this subunit. Recognition of Ypt7 by retromer is required for its function in retrograde sorting, and in yeast cells lacking the guanine nucleotide exchange factor for Ypt7, retrograde cargo accumulates in endosomes that are decorated with retromer, revealing an additional role for Rab recognition at the cargo export stage of the retromer functional cycle. In addition, yeast retromer trimer antagonizes Ypt7-regulated organelle tethering and fusion of endosomes/vacuoles via recognition of Ypt7. Thus retromer has dual roles in retrograde cargo export and in controlling the fusion dynamics of the late endovacuolar system.

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INTRODUCTION

Cargo in the endocytic system has two possible fates: it can be retained within maturing endosomes and delivered to lysosomes and degraded, or it can be exported to other organelles via the retrograde pathway. One major retrograde pathway is orchestrated by a multisubunit protein complex called retromer, which is proposed to constitute a membrane coat that packages cargo into carriers that bud and fission from endosomes (Seaman *et al.*, 1998). Retromer was discovered in budding yeast (*Saccharomyces cerevisiae*) based

on its role in endosome-to-Golgi retrieval of the Vps10 sorting receptor (Seaman *et al.*, 1997), and this function and its molecular components are generally conserved in evolutionarily divergent organisms (Attar and Cullen, 2010). Questions regarding the mechanisms of retromer function center on elucidating how retromer coordinates cargo recognition and packaging into tubules with tubule scission and transport to the *trans*-Golgi network (TGN).

Pioneering studies using yeast cells defined retromer as a complex of five subunits—Vps5, Vps17, Vps26, Vps29, and Vps35—based on genetic analysis of endosome-to-Golgi transport and copurification of these five proteins with antisera to any of the subunits (Seaman *et al.*, 1997). The retromer pentamer can be dissociated into two subcomplexes that have distinct functions. One subcomplex comprises Vps5 and Vps17 sorting nexins, each of which contains a Phox homology domain that binds phosphatidylinositol 3-phosphate, and a Bin-amphyphysin-Rvs (BAR) motif that mediates dimerization and is generally implicated in binding to high-curvature-membrane tubules (Seaman, 2005). In human cells there are at least four functional orthologues of yeast Vps5 and Vps17—Snx1, Snx2, Snx5, and Snx6, which dimerize in a combinatorial manner (Wassmer *et al.*, 2009). The Vps26, Vps29, and Vps35 retromer

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Abbreviations used: 5-FOA, 5-fluoroorotic acid; BAR, Bin-amphyphysin-Rvs; CI-MPR, cation-independent mannose 6-phosphate receptor; CSC, retromer cargo selective complex; DSP, dithiobis(succinimidyl) propionate; GFP, green fluorescent protein; GSH, glutathione; GST, glutathione S-transferase; TGN, *trans*-Golgi network; YFP, yellow fluorescent protein.

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subunits form an elongated trimer (Hierro *et al.*, 2007; Norwood *et al.*, 2011) that is implicated in cargo recognition and is therefore, termed the cargo selection complex (CSC; Nothwehr *et al.*, 1999, 2000; Arighi *et al.*, 2004). In addition, CSC recognizes GTP-loaded Rab7 (the orthologue of yeast Ypt7; Rojas *et al.*, 2008; Seaman *et al.*, 2009; Balderhaar *et al.*, 2010), indicating that it is an effector of Rab/Ypt7 signaling. In this study we address the role of the Rab7 GTPase cycle in retromer assembly and cargo export in yeast and human cells.

RESULTS

Distinct requirements for retromer CSC targeting

The yeast retromer sorting nexin subunits Vps5 and Vps17 are implicated in recruiting the CSC to endosome membranes (Seaman *et al.*, 1998; Seaman and Williams, 2002; Burda *et al.*, 2002). Consistent with this, Vps17-GFP and Vps5-GFP (not shown) localize to endosomes, as previously noted (Burda *et al.*, 2002; Strohlic *et al.*, 2007, 2008), which appear in fluorescence micrographs as puncta that are distributed throughout the cytoplasm, and steady-state endosome localization of Vps35–green fluorescent protein (GFP) and Vps26-GFP is abolished in *vps5Δ vps17Δ* double-mutant cells (Figure 1; Vps26-GFP not shown). Surprisingly, in these double-mutant cells, Vps35-GFP and Vps26-GFP localize robustly to the vacuole membrane (Figure 1; Vps26-GFP not shown). Vacuole localization of the CSC is not simply due to loss of Vps5 and Vps17, because close inspection of wild-type cells expressing mCherry-Ypt7, an established marker of the vacuole membrane, and Vps35-GFP reveals that Vps35-GFP (Figure 1) or FM4-64 (not shown) decorates the vacuole membrane in addition to endosomes. In addition, GFP-tagged forms of Vps35 and Vps26 are functional with respect to cargo sorting (Strohlic *et al.*, 2007, 2008) and sensitivity to hygromycin B (unpublished data), indicating that vacuole localization is not a consequence of functional inactivation. These data indicate that retromer CSC localizes to endosomes decorated with the Vps5 and Vps17 sorting nexins and also to the vacuole membrane via a mechanism that is independent of Vps5 and Vps17.

Because human and yeast retromer CSC are reported to bind Rab/Ypt7-GTP (Rojas *et al.*, 2008; Seaman *et al.*, 2009; Balderhaar *et al.*, 2010) and Ypt7 localizes predominantly to the vacuole membrane, we determined CSC localization in cells lacking Ypt7 (*ypt7Δ*; Figure 1). GFP-tagged forms of Vps35 and Vps26 (unpublished data) localize predominantly to punctate endosomes, with little to none present on larger, endosome-derived organelles that accumulate as a result of the loss of Ypt7. However, Vps35-GFP and Vps26-GFP localize to the cytosol in a triple-deletion strain that lacks Vps5, Vps17, and Ypt7 (*vps5Δ vps17Δ ypt7Δ*; Figure 1), indicating that Ypt7 can recruit CSC to organelle membranes independently of Vps5-Vps17. Furthermore, Vps35-GFP and Vps26-GFP (unpublished data) localize to punctate endosomes in cells that lack Ccz1 (*ccz1Δ*) or Mon1 (*mon1Δ*), which form a complex that catalyzes guanine nucleotide exchange on Ypt7 (Nordmann *et al.*, 2010), indicating that catalyzed GTP loading of Ypt7 *in vivo* is required for vacuole localization of CSC. Taken together, the data indicate that the yeast retromer CSC uses distinct mechanisms for targeting to organelle membranes; the Vps5 and Vps17 sorting nexins are required for recruitment to endosomes, and the GTP-loaded form of Ypt7 is required for vacuole localization.

We next tested whether yeast retromer subunits bind Ypt7-GTP (Figure 2A). Our attempts to produce yeast CSC by recombinant methods in *Escherichia coli* did not yield soluble complex, so extracts of soluble material from yeast cells expressing epitope-tagged retromer subunits were used for these experiments. Cell extracts

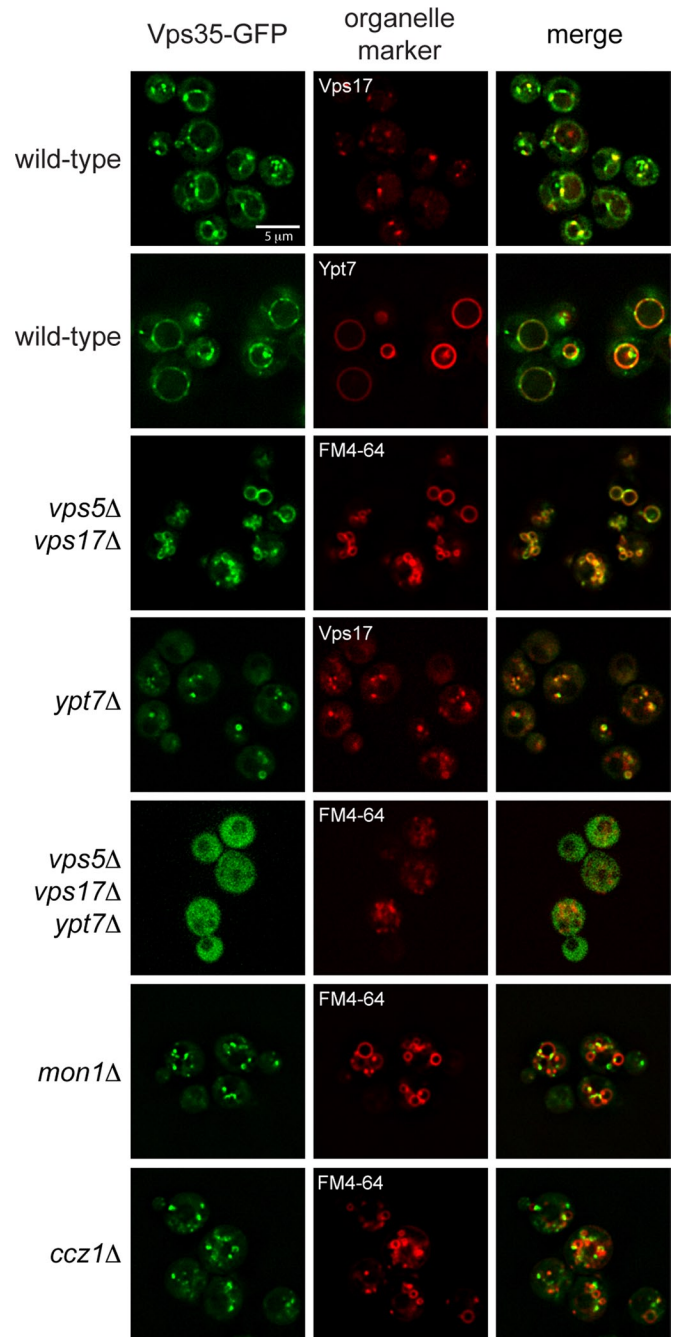


FIGURE 1: Localization of retromer CSC to endosomes requires the Vps5 and Vps17 sorting nexins, and localization to the vacuole membrane requires the Ypt7 Rab GTPase. Deconvolved fluorescence micrographs showing Vps35-GFP-labeled (left) and Vps17-mCherry-, mCherry-Ypt7-, or FM4-64-labeled cells (middle) of the indicated genotype are shown. Localization of Vps35-GFP is compared with that of mCherry-tagged Vps17 or Ypt7 in the top two rows. In the center column of all other rows, the fluorescent vital dye FM4-64 identifies the vacuole membrane. Right, merged images showing both fluorescent markers. Identical results to Vps35-GFP were observed with Vps26-GFP (unpublished data).

were incubated with immobilized glutathione S-transferase (GST)–Ypt7 or mutant forms of Ypt7 that are constitutively in the GTP- or GDP-bound conformation (Q68L or T22N, respectively), and similar constructs of Vps21, a yeast Rab5 orthologue, were included as a

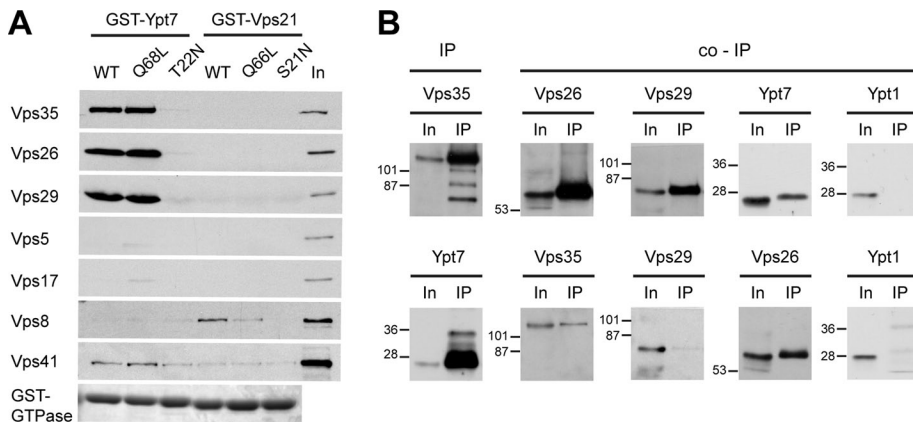


FIGURE 2: Retromer cargo-selection complex binds GTP-loaded Ypt7. (A) In vitro GST-Ypt7-binding assays. An extract generated from cells expressing each individual retromer subunit tagged on its C-terminus was incubated with immobilized GST-Ypt7, GST-Ypt7(Q68L) GTP-loaded mimic, or GST-Ypt7(T22N) GDP-loaded mimic. Bound proteins were eluted by denaturation, and retromer subunits in the eluates were detected by anti-HA immunoblotting. A parallel set of binding reactions using the early endosome Rab GTPase, Vps21, was included as a control for the specificity of the interactions. The Vps21(Q66L) and Vps21(S21N) mutants correspond to the GTP-bound and GDP-bound mimics, respectively. To control for functionality of the GST fusion proteins, binding reactions to tagged forms of Vps41, a previously documented Ypt7-binding protein, and to Vps8, a previously documented Vps21-binding protein, are shown. Note that each retromer CSC subunit is captured by Ypt7-GTP, but the Vps5 and Vps17 sorting nexin retromer subunits are not. (B) Retromer-Ypt7 cross-linking immunoprecipitation assays. Cells expressing epitope-tagged retromer CSC subunits (Vps35-HA, Vps26-FLAG, and Vps29-myc) were incubated with the bifunctional cross-linking agent DSP, detergent extracts were made, and then Vps35-HA (top) or Ypt7 (bottom) was immunopurified. Bound proteins were eluted by denaturation, and copurifying proteins were identified by immunoblotting with anti-FLAG, anti-myc, anti-Ypt7, or, as a control for specificity, anti-Ypt1 antisera. Top, a representative Vps35-HA immunoprecipitation assay. Bottom, a representative Ypt7 immunoprecipitation assay.

specificity control. GST-Ypt7 and GST-Ypt7(Q68L) beads efficiently capture the retromer CSC subunits (Vps26, Vps29, and Vps35), but, surprisingly, only trace amounts of the Vps5 and Vps17 are recovered, even though they are present in the starting extract in amounts roughly comparable to the other retromer subunits. (Retromer CSC capture by the native GST-Ypt7 fusion protein suggests that Ypt7 purifies from *E. coli* extract with bound GTP.) Failure of Vps5 and Vps17 to be captured is surprising because yeast retromer is considered to be a stable, pentameric complex containing Vps5 and Vps17 (Seaman, 2005). Capture of CSC subunits on GST-Ypt7 beads is very robust, exceeding the amount of (hemagglutinin [HA]-tagged) Vps41—a component of the HOPS Ypt7 effector complex (Seals et al., 2000)—that is captured. Retromer subunits are not captured by GST-Vps21, although these beads do capture a known Vps21 effector, Vps8, as expected based on a published report (Markgraf et al., 2009; Figure 2). These results indicate that yeast retromer CSC associates with GTP-bound Ypt7 and confirm results of similar assays that were published while the manuscript was being prepared (Balderhaar et al., 2010).

To determine whether Ypt7 and retromer CSC associate in vivo, we immunopurified Vps35-HA or Ypt7 from detergent cell extracts after chemical cross-linking with dithiobis succinimidyl propionate (DSP; to stabilize the bound complexes during detergent extraction) and probed the bound material with antibodies to Ypt7 or the Vps26 CSC subunit, respectively (Figure 2B). Ypt7 and Vps26-HA copurify with Vps35-HA, and, conversely, Vps35-HA and Vps26-HA copurify with Ypt7. Of importance, the Golgi Rab GTPase Ypt1 does not copurify with either Vps35-HA or Ypt7, demonstrating that copurification is specific.

We next sought to elucidate how Ypt7 and retromer CSC associate. Our initial efforts focused on examining the role of Vps35 because it is proposed to function as a scaffold that binds cargo and other retromer-associated proteins (Seaman, 2005; Bonifacino and Hurley, 2008; Harbour et al., 2010). A second impetus emerged from a comparison of Vps35 amino acid sequences from evolutionarily divergent organisms (Figure 3A). This analysis shows that the binding site for Vps26, delineated by studies of human and yeast retromer assembly (Gokool et al., 2007; Hierro et al., 2007; Zhao et al., 2007; Norwood et al., 2011), is the most highly conserved portion of Vps35, with ~49% identity between yeast and human Vps35 sequences. Curiously, the region containing the Vps26-binding site appears to be conserved en bloc with an adjacent segment, spanning amino acids 162–292 (yeast Vps35 coordinates), but no function has been ascribed to this region. To test whether this region contributes to retromer targeting in vivo, we determined intracellular localization of carboxy-terminal Vps35 deletion constructs expressed from the *VPS35* locus as the only source of Vps35 in the cell; one truncation mutant contains only the minimal Vps26-binding region (amino acids 1–158; termed Vps35(N158)-GFP), and a second mutant contains the N-terminal 300 amino acids (termed Vps35(N300)-GFP), which encompasses the entire conserved N-terminal region (Figure 3B). Vps35(N300)-GFP localizes robustly to the vacuole membrane (and the cytosol), but the Vps35(N158)-GFP construct localizes solely to the cytosol (Figure 3B). The results suggest that the conserved region spanning amino acids 159–300 is necessary for targeting this truncated form of Vps35 to the vacuole membrane.

Conserved role of Vps35 in Rab recognition in vivo

On the basis of structural and amino acid sequence analyses of hVps35, Hierro et al. (2007) predicted that human Vps35 (hVps35) is composed of 17 two-helix repeats that form a solenoid. Yeast Vps35 (yVps35) appears to contain an insertion of one repeat between repeats 8 and 9 that is not present in human Vps35 (termed 8.5 in Figure 3A). In this model of Vps35 structure, the region spanning amino acids 159–292 comprises precisely three repeats, which we refer to as repeats 5–7. To probe the contribution of each of these regions to retromer targeting, we determined localization of a series of internal deletion mutants in which all three repeats (Vps35 Δ 5–7) or each individual repeat (Vps35 Δ 5, Vps35 Δ 6, or Vps35 Δ 7) were deleted and replaced with a short linker. Each mutant gene was expressed from the native *VPS35* locus as a C-terminal fusion to GFP or a 3xHA epitope tag and as the sole source of Vps35 for the cell. Because these repeats are adjacent to the Vps26-binding site and Vps35 is degraded if it does not associate with Vps26 (Reddy and Seaman, 2001; Restrepo et al., 2007), we first determined the steady-state levels of each mutant protein by immunoblotting of whole-cell extracts (Figure 3C). This analysis shows that the Vps35 Δ 6 and Vps35 Δ 7 mutant proteins are present in roughly equivalent amounts to wild-type Vps35, but both mutant proteins that lack

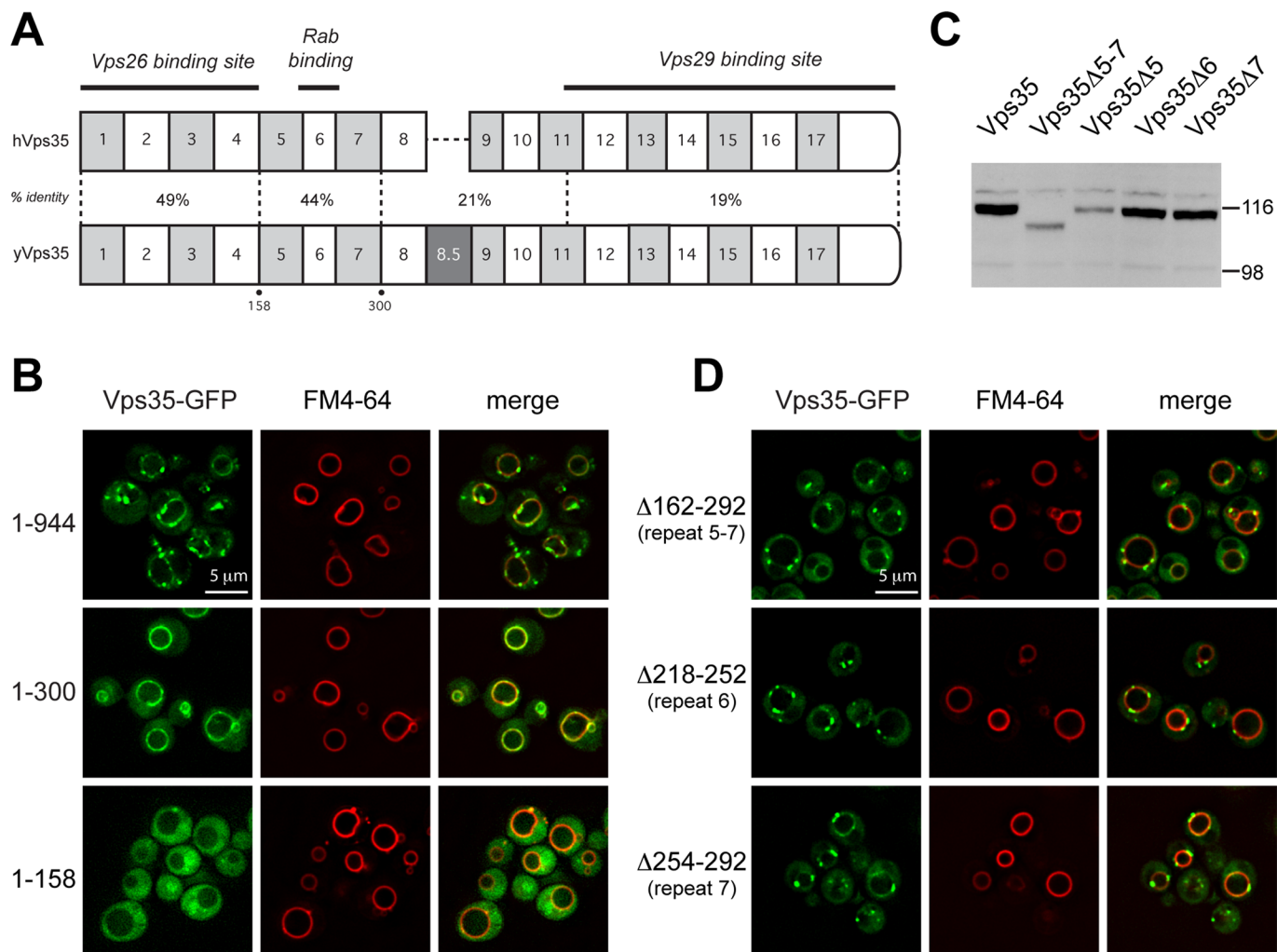


FIGURE 3: Identification of regions of Vps35 required for targeting to the vacuole membrane. (A) Comparison of human and yeast Vps35 amino acid sequence homology. A schematic diagram of the predicted and known helical repeats of Vps35 (Hierro *et al.*, 2007) is shown, with the minimal binding sites for human Vps26 and Vps29 indicated. The percentage identity between human and yeast sequences is indicated for the regions demarcated by the dashed lines. The positions of three predicted helical repeats (Hierro *et al.*, 2007) in the region spanning amino acids 159–300, (designated 5–7) are indicated. The black dots below the yeast Vps35 diagram indicate the positions of the last amino acid of the Vps35(N158)-GFP and Vps35(N300)-GFP truncation constructs. (B) Fluorescence microscopy analysis of intracellular localization of Vps35 truncation constructs. Localization of full-length Vps35-GFP (top) is compared with Vps35 truncation mutants expressing an in-frame fusion to GFP after codon 300 (1-300) or codon 158 (1-158). The brightness of the Vps35(1-158) image is increased ~1.5-fold because of the reduced amount of this protein. Middle, the same cells stained with FM4-64 to identify the vacuole membrane. (C) Anti-HA immunoblot of Vps35 internal deletion mutants. (D) Fluorescence microscopy analysis of intracellular localization of Vps35 internal deletion constructs. Vps35-GFP fusion proteins with deletions of the indicated amino acid segments are shown, with vacuoles identified (middle). The brightness of the Δ 5-7 image was increased ~1.5-fold because of the reduced amount of this protein. In B and D, one deconvolved image of z-stack showing the approximate center of the cells is shown for each channel; right, the merged images. All constructs were expressed from the endogenous *VPS35* locus as the sole source of Vps35 in the cell.

repeat 5 (Vps35 Δ 5 and Vps35 Δ 5-7), which is adjacent to the Vps26-binding region, were present in substantially reduced amounts. Coimmunoprecipitation analysis (unpublished data) indicates that substantially reduced amounts of Vps26 associate with Vps35 Δ 5 and Vps35 Δ 5-7, indicating that repeat 5 contributes to binding of Vps26; for this reason the Vps35 Δ 5-7 and Vps35 Δ 5 mutants were not characterized further. Fluorescence microscopy shows that Vps35 Δ 6-GFP localizes to endosomes, but vacuole localization appears to be entirely lost (Figure 3D). Vps35 Δ 7-GFP also localizes to endosomes, and its localization to the vacuole is substantially, although not completely, diminished. Thus the region of Vps35 span-

ning amino acids 218–252 (i.e., repeat 6) is most critical for targeting CSC to the vacuole, whereas amino acids 254–292 (i.e., repeat 7) make a minor contribution to vacuole localization.

We speculate that loss of vacuole localization of Vps35 Δ 6 is due to deficient association with Ypt7, and this was confirmed by two experiments. First, Vps35 Δ 6 is not captured from yeast cytosol by immobilized GST-Ypt7 under conditions in which full-length Vps35 is captured (Figure 4A). Second, Vps35 Δ 6 and Ypt7 do not coimmunoprecipitate from detergent cell extracts after chemical cross-linking (Figure 4B). Of importance, the Vps26 and Vps17 retromer subunits do copurify with Vps35 Δ 6, indicating that this mutant version of

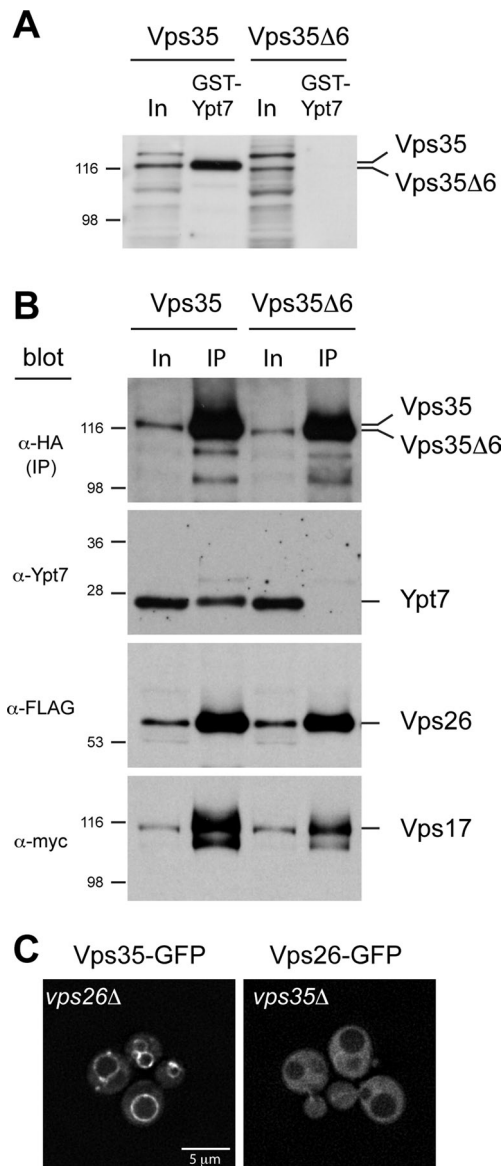


FIGURE 4: Identification of a region of Vps35 required for association with Ypt7. (A) Vps35 repeat 6 (amino acids 218–252) is required for binding to GST-Ypt7 *in vitro*. Extracts prepared from cells expressing wild-type Vps35 or the indicated internal deletions were probed for binding to immobilized GST-Ypt7 as described in the legend to Figure 2A. One percent of the volume of cell extract that was incubated with GST-Ypt7 is present in the Input lanes. (B) Vps35 repeat 6 is required for association with Ypt7 *in vivo*. Wild-type and Vps35Δ6 were immunopurified using anti-HA matrix from cells that were preincubated with DSP cross-linker, and the purified fractions (lanes labeled IP) were probed to detect Ypt7, Vps26, and Vps17. One percent of the volume of cell extract used to immunopurify Vps35 is present in the Input lanes. (C) Vps35-GFP localizes to the vacuole membrane independently of Vps26. The brightness of the Vps35-GFP image was increased ~1.5-fold to compensate for the reduced abundance of the protein in *vps26Δ* cells.

Vps35 assembles into the pentameric retromer complex, a result expected based on the endosome localization of Vps35Δ6. Taken together, the results indicate that recognition of Ypt7 is essential to target retromer CSC to the vacuole membrane, and they further suggest that Vps35 mediates association with Ypt7 via amino acids 218–292. In further support of a direct role for Vps35 in mediating

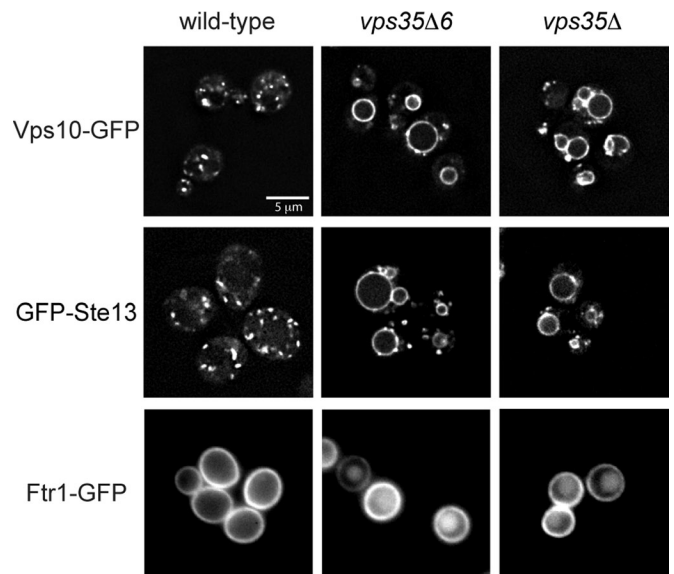


FIGURE 5: Vps35Δ6 mutant is defective for retromer-dependent sorting. Fluorescence microscopy was used to visualize the retromer-dependent cargoes Vps10-GFP, Ste13-GFP, and Ftr1-GFP in cells expressing wild-type Vps35, the Ypt7 interaction-defective Vps35Δ6 mutant, or *vps35Δ* deletion cells.

Ypt7-dependent targeting, Vps35-GFP decorates the vacuole membrane in *vps26Δ* cells (Figure 4C), even though it is unstable in the absence of Vps26 (Reddy and Seaman, 2001; Restrepo *et al.*, 2007). In contrast, Vps26-GFP localizes solely to the cytosol in *vps35Δ* cells (Figure 4C), indicating that Vps26 requires incorporation into the CSC to target it to membranes *in vivo*.

The interaction of human retromer with Rab7 is proposed to augment the roles of sorting nexins in recruiting retromer CSC to endosomes (Rojas *et al.*, 2008; Seaman *et al.*, 2009). In support of this, RNA interference-mediated depletion of Rab7 results in cytosolic localization of retromer CSC and lysosomal degradation of retromer-dependent cargoes (Rojas *et al.*, 2008; Seaman *et al.*, 2009). However, Rab7/Ypt7 is a master regulator of the late endocytic pathway, so these earlier studies could not specifically address the role of the Rab7–retromer interaction without considering secondary effects due to loss of other endosomal functions that are regulated by Rab7 (e.g., fusion reactions, organelle localization and motility). Results presented here indicate that Ypt7 is not required for endosome recruitment of retromer, and the construction of a Vps35 mutant that ablates recognition of Ypt7 while Ypt7 is still present in the cell afforded us the ability to probe for a Ypt7 requirement in retromer-dependent sorting pathways. In wild-type cells, two endosome-to-Golgi pathway cargoes—Vps10-GFP and GFP-Ste13—localize to punctate endosome and Golgi compartments with little to no detectable signal on the vacuole membrane, whereas in *vps35Δ* cells both of these cargoes localize to the vacuole membrane, as previously reported (Burda *et al.*, 2002). In cells expressing Vps35Δ6, each of these cargoes localizes to the vacuole membrane. Similarly, the endocytic recycling cargo Ftr1-GFP, which localizes solely to the plasma membrane of wild-type cells, accumulates in the lumen of the vacuole in Vps35Δ6-mutant cells, as observed in *vps35Δ* cells (Figure 5; Strohlic *et al.*, 2007). Missorting of Vps10 is unlikely to be due to loss of recognition by retromer, as mutations in Vps35 that affect Vps10 sorting map to repeats 11 and 12 at the opposite end of Vps35 (Nothwehr *et al.*, 1999, 2000). Moreover, Vps10 is recovered with immunopurified Vps35Δ6:HA protein (unpublished

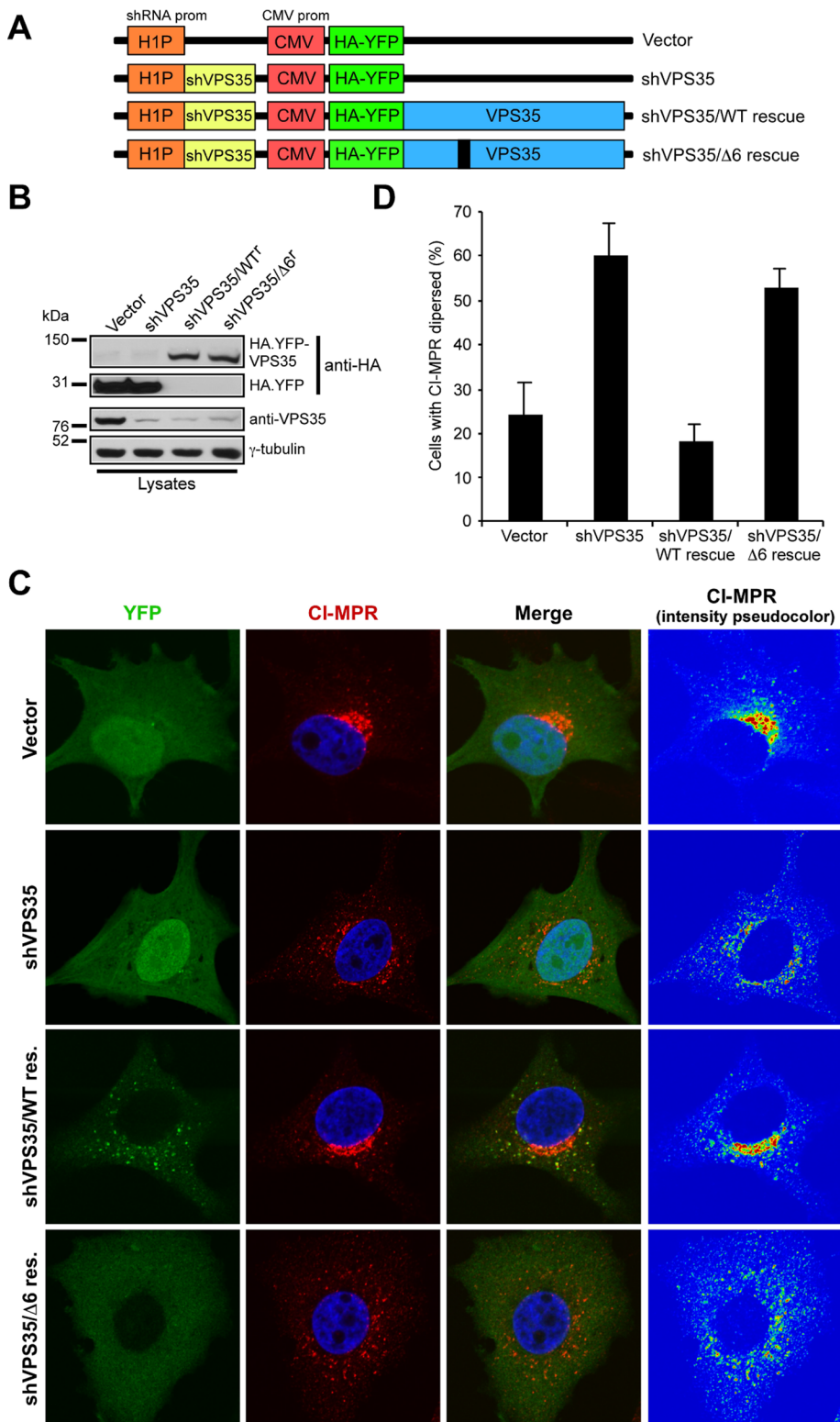


FIGURE 6: Repeat 6 of human Vps35 is required for retromer endosome localization and function. (A) Diagram of shRNA suppression/rescue constructs. (B) Immunoblot analysis of Vps35. Lysates from the indicated transfected HeLa cell cultures were probed with the indicated antisera. (C) Fluorescence analysis of YFP-Vps35 and CI-MPR localization. HeLa cells were transfected as indicated (green), fixed, and stained with anti-CI-MPR (red) and Hoechst 33342 (blue). Pseudocoloring of CI-MPR staining demonstrates pixel intensity. (D) Quantitative analysis of CI-MPR distribution. Transfectants were scored for either compact or dispersed CI-MPR. Bars, mean \pm SD from three independent experiments.

observations), indicating that repeat 6 is not involved in Vps10 recognition. These results indicate that recognition of Ypt7 by retromer is a general requirement for retromer function in yeast and that Ypt7 function is required after initial recruitment of retromer to the endosome membrane.

The region of yVps35 that is required for Ypt7-dependent targeting of CSC is highly conserved with hVps35 (hVps35), and we tested whether this region in hVps35 fulfils a similar functional requirement to that of yVps35. A short hairpin RNA-based knockdown/replacement strategy (Gomez and Billadeau, 2009) was used to deplete endogenous Vps35 and express YFP-tagged wild-type Vps35 or a mutant form lacking amino acids 237–252 (hVps35 Δ 6), which corresponds to the region of yVps35 that confers association with Ypt7 (Figure 6). Prior studies showed that depletion of GTP-bound Rab7 in HeLa cells results in cytoplasmic localization of CSC subunits (Rojas *et al.*, 2008; Seaman *et al.*, 2009), leading us to predict that hVps35 Δ 6 should localize to the cytosol, resulting in the loss of retromer sorting function. In cells depleted of endogenous Vps35, wild-type YFP-Vps35 localizes to punctate endosomes, but YFP-hVps35 Δ 6 localizes to the cytoplasm. The sorting function of hVps35 Δ 6 was determined by examining the distribution of the endogenous cation-independent mannose 6-phosphate receptor (CI-MPR; Figure 6, C and D). In this experimental system, depletion of Vps35 results in the steady-state redistribution of CI-MPR from the perinuclear region of the cell to peripheral endosomes in 60% of the cells assayed versus <18% of control cells that reexpress wild-type hVps35, indicative of a loss of retromer-dependent retrograde sorting (Gomez and Billadeau, 2009). Strikingly, 53% of cells expressing mutant hVps35 Δ 6 show dispersed CI-MPR, indicating that hVps35 Δ 6 is not functional in CI-MPR retrograde sorting. The steady-state levels of hVps35 and hVps35 Δ 6 are nearly equivalent, confirming that hVps35 Δ 6 is as stable as wild-type hVps35 (Figure 6B), just as is observed for the analogous yVps35 Δ 6 construct (Figure 3).

Retromer influences Ypt7-regulated endosome dynamics

A curious, unexplained phenotype of *vps5 Δ* - and *vps17 Δ* -mutant cells is that the vacuoles in these cells are fragmented and clustered, but the vacuoles in cells lacking any CSC retromer subunit are similar in size and copy number to wild-type cells (Raymond *et al.*, 1992; Figure 7A). Because Vps5 and Vps17

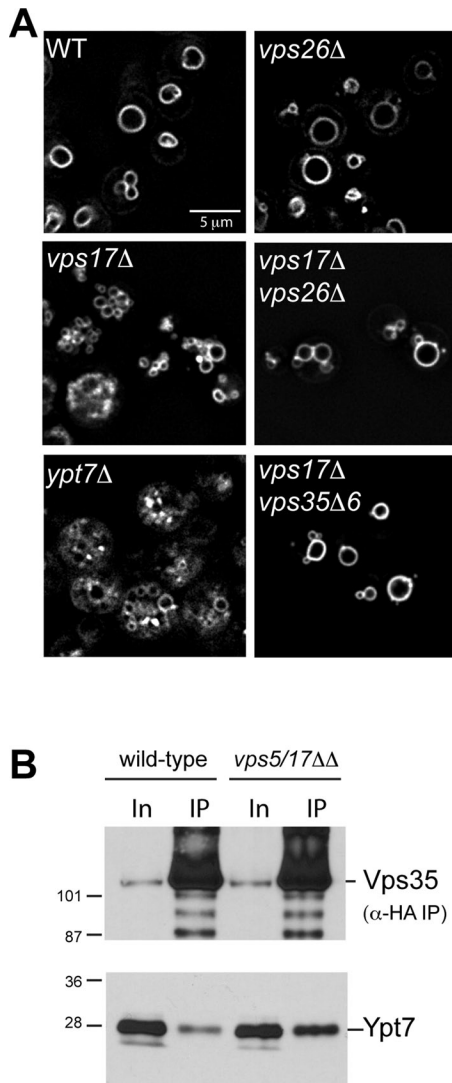


FIGURE 7: Defective vacuole morphology of *vps17Δ* cells is rescued by deletion of the retromer CSC subunit Vps26 and by deletion of the region of Vps35 required for recognition of Ypt7. (A) Vacuoles were visualized in the indicated strains by staining with FM4-64. (B) Enhanced association of Ypt7 and retromer CSC in cells lacking the Vps5 and Vps17 sorting nexins. Vps35:HA was immunopurified from DSP cross-linked cell extracts, and copurifying Ypt7 was detected as described in the legend to Figure 3.

are not known to participate in fusion reactions involving the vacuole, this phenotype suggests that the equilibrium of the vacuole fusion system is perturbed in *vps5Δ*- and *vps17Δ*-mutant cells but not in mutants lacking CSC components. Ypt7 is a central regulator of this system via interactions with effectors, including the HOPS and CORVET tethering factors that promote fusion of endosomes and vacuoles (Seals *et al.*, 2000; Peplowska *et al.*, 2007). Loss of Ypt7 function results in the accumulation of endosomes and fragmented vacuoles, similar in nature to the phenotypes of *vps5Δ* and *vps17Δ* cells (Nothwehr and Hindes, 1997; Seeley *et al.*, 2002). Our results showing that retromer CSC binds independently to the Vps5/17 subcomplex and to Ypt7 suggest a possible explanation for the fragmented vacuole phenotype of *vps5Δ* and *vps17Δ* cells; in the absence of the Vps5/17 sorting nexins, CSC interferes with Ypt7 regulation of endosome and vacuole-tethering factors. The results of three experiments support this idea (Figure 7). Deletion of *VPS26*

(*vps26Δ*), which is required for Ypt7 recognition by CSC *in vitro* (Balderhaar *et al.*, 2010), rescues the morphology of vacuoles when combined with a deletion of *VPS17* (*vps17Δ vps26Δ*) (Figure 7A). Second, the vacuole fragmentation phenotype of *vps17Δ* and *vps5Δ* (unpublished data) cells is remedied by expression of the Vps35Δ6 mutant that does not recognize Ypt7 (Figure 7A). Finally, coimmunoprecipitation analysis shows a 2.2-fold increase in the amount of CSC that is associated with Ypt7 in *vps5Δ vps17Δ* cells (Figure 7B). These data indicate that retromer CSC is essential for the defective vacuole morphology of *vps5Δ*- and *vps17Δ*-mutant cells and correlate this with increased Ypt7-retromer association.

Activation of Ypt7 regulates retromer-mediated cargo export

Because human retromer CSC fails to be recruited to the endosome when Rab7-GTP is depleted (Rojas *et al.*, 2008; Seaman *et al.*, 2009), it is not possible to test for a possible contribution of Rab regulation downstream of initial membrane targeting. However, this can be addressed using yeast CSC because the Vps5 and Vps17 sorting nexins recruit CSC to the endosome independently of Ypt7. To do so, we examined localization of Vps10 in cells lacking *MON1* (*mon1Δ*; Figure 8), encoding a subunit of the Ypt7 guanine nucleotide exchange factor (GEF; Nordmann *et al.*, 2010). These cells were previously demonstrated to secrete vacuolar proteins that are sorted by Vps10 (Bonangelino *et al.*, 2002; Wang *et al.*, 2002), suggesting that Vps10 function is (indirectly) compromised. Of importance, because native (i.e., not mutant) retromer subunits are expressed by these cells, this approach complements our earlier analyses of Vps35 mutants by eliminating concern that the Vps35 mutants might be functionally compromised in an unidentified manner. To establish benchmarks for this approach, we first examined Vps10-GFP in wild-type and *vps35Δ* cells and calculated Pearson's *r* for Vps10-GFP and FM4-64 as a quantitative measure of Vps10 localization (Figure 8, A and C). As expected, in wild-type cells Vps10-GFP localizes to punctate compartments (Golgi and endosome), with essentially no signal observed on the vacuole membrane, as indicated by a correspondingly low *r* value for Vps10-GFP and FM4-64 ($r = 0.28$). In contrast, in *vps35Δ* cells Vps10-GFP localizes to the limiting membranes of late endosomes and vacuoles, and there is a correspondingly high correlation with FM4-64 to the same extent as in wild-type cells ($r = 0.30$ vs. 0.28 ; $p = 0.17$). Although this result indicates that loss of Mon1-catalyzed activation of Ypt7 does not result in delivery of Vps10-GFP to the vacuole compartment, the appearance of Vps10-GFP is clearly distinct from that in wild-type cells, being larger and brighter (Figure 8, A and B). To investigate the basis of this difference, we determined the fluorescence correlation of Vps10-mCherry and Vps35-GFP in wild-type and *mon1Δ* cells (Figure 8, B and C) and find that the correlation between Vps10-mCherry and Vps35-GFP fluorescence signals in *mon1Δ* is significantly increased compared with wild-type cells ($r = 0.70$ vs. 0.50 ; $p = 0.003$). This result suggests that Vps10 accumulates in endosome that are decorated with Vps35 in *mon1Δ* cells and that accumulation requires retromer.

DISCUSSION

Previous studies showed that the human and yeast CSC trimer recognizes Rab/Ypt7 (Rojas *et al.*, 2008; Seaman *et al.*, 2009; Balderhaar *et al.*, 2010), although the basis of Rab recognition was not elucidated. On the basis of the following key results presented in this study, we conclude that recognition of Rab/Ypt7-GTP is mediated by a small segment of the Vps35 retromer subunit:

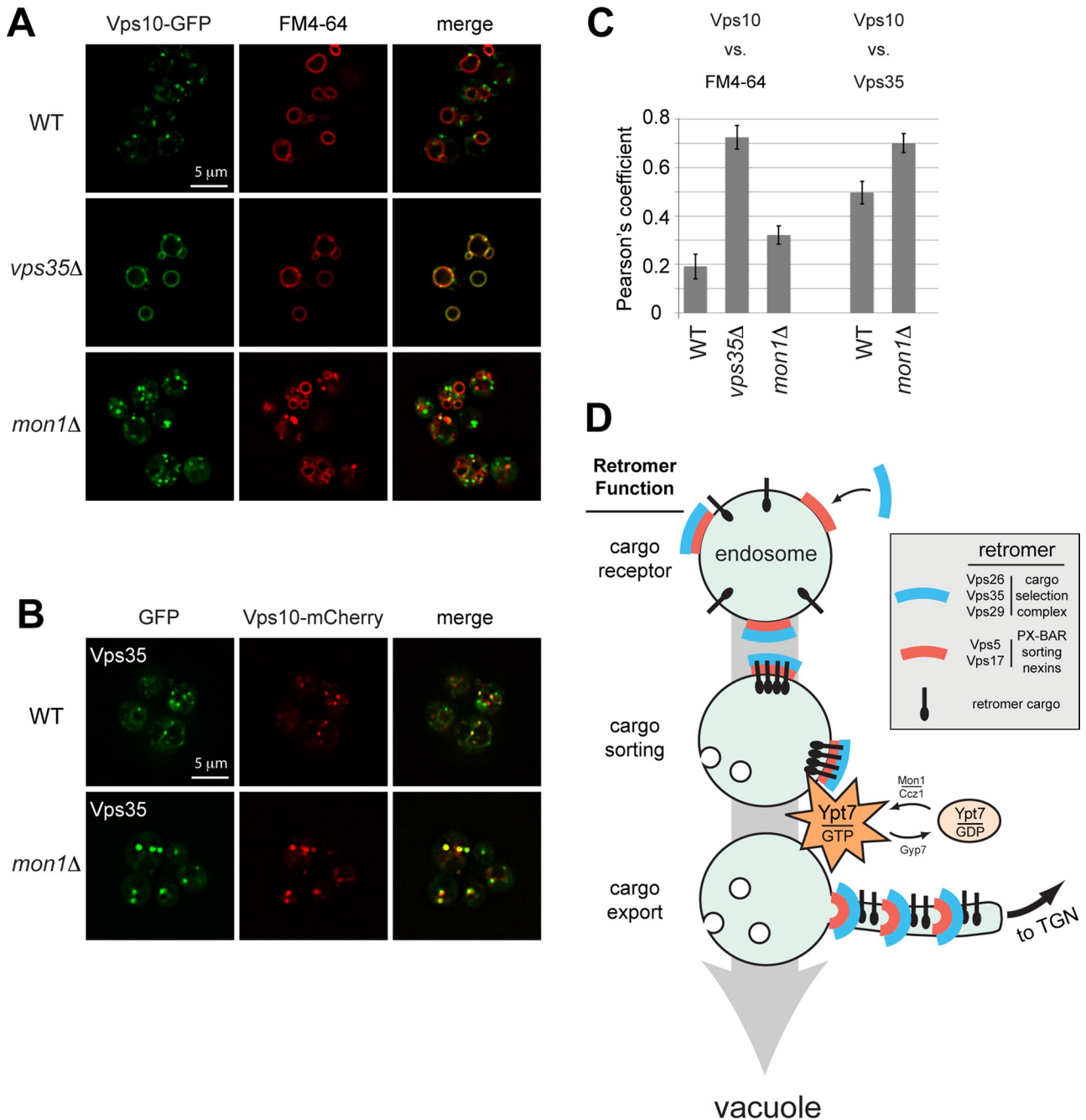


FIGURE 8: Analysis of Vps10 retromer cargo distribution in Ypt7 GEF mutant cells. (A) Vps10 localization to the vacuole membrane in wild-type, *vps35Δ*, and *mon1Δ* cells. Vps10-GFP is compared with vacuole membranes identified by FM4-64 fluorescence. (B) Colocalization analysis of Vps10 and Vps35 retromer CSC subunit. Vps10-mCherry is compared with Vps35-GFP. (C) Correlation analyses of Vps10 distribution. Pearson's *r* was calculated for at least four image stacks containing a minimum total of 50 cells. The standard errors of the means are indicated. (D) Summary and model of proposed role of Ypt7 regulation of retromer. In yeast, the Vps5/17 sorting nexin subcomplex recruits the Vps26/29/35 subcomplex to the endosome, where it functions as a retrograde cargo receptor. On the endosome, the Ypt7 GEF, Mon1/Ccz1, activates Ypt7, which is recognized by CSC. The association of CSC and Ypt7 promotes export of cargo from the endosome via tubular/vesicular domains decorated by retromer.

1) Localization of Vps35 to the vacuole membrane does not require Vps26. 2) Targeting of yeast CSC to the vacuole membrane, as well as that of human Vps35 to the endosome, is ablated by deletion of a 35-amino acid conserved element of Vps35. 3) Association of retromer CSC with Ypt7 *in vivo* is ablated by the Vps35Δ6 mutation. 4) *In vitro* capture of retromer CSC to immobilized GST-Ypt7 is ablated by the Vps35Δ6 mutation. Although the

N-terminal ~300 amino acids of Vps35, which includes the Vps26-binding site, appear to be evolutionarily conserved *en bloc*, the results of our systematic deletion analysis show that deletion of repeats between the Vps26- and Vps29-binding sites result in stable expression of mutant forms of CSC that assemble with other retromer subunits, lending support to the predicted modular nature of Vps35. However, given the close proximity of the

Vps26- and Rab-Ypt7-binding sites, we do not rule out that Vps26 may also contribute to Rab recognition, either indirectly, by influencing Vps35 structure, or directly, by participating in recognition of Rab. In fact, the link between retromer and Rab7 was first suggested by the discovery that *Entamoeba histolytica* Vps26 possesses a unique C-terminal extension that confers binding to Rab7 (Nakada-Tsukui *et al.*, 2005), so the association of Vps26 and Rab/Ypt7 with the N-terminal region of Vps35 is a conserved functional feature of CSC structure.

Previous studies implicated Rab7 in recruitment of retromer CSC to the endosome in cultured human cells (Rojas *et al.*, 2008; Seaman *et al.*, 2009) and led to the proposal that Rab7 augments the role of membrane-bound sorting nexins and recognition of cargo in endosome recruitment of CSC (Rojas *et al.*, 2008; Seaman *et al.*, 2009). In yeast cells, superphysiological expression of Ypt7 results in massive recruitment of Vps26-GFP to the vacuole membrane (Balderhaar *et al.*, 2010), and our data show that a physiological level of Ypt7 is sufficient to confer vacuole localization of CSC. Of importance, the data also indicate that CSC is recruited to the vacuole membrane via a mechanism that is independent of all of the known retromer-associated sorting nexins, and on this basis we conclude that CSC has a function(s) that is independent of sorting nexins. Given the role of retromer in retrograde sorting, this localization raises the possibility that it may function in a retrograde pathway that originates from the vacuole; however, retromer membrane-remodeling subunits (i.e., Vps5 or Vps17) do not detectably localize to the vacuole membrane, so it is unclear how a CSC decorated transport vesicle/tubule might be generated. A second possibility, suggested by the results of the epistasis experiments with regard to vacuole morphology (Figure 7), is that vacuolar CSC modulates Ypt7-regulated tethering and fusion of endosomes with the vacuole and/or vacuole homotypic fusion. In this SNX-independent context, more retromer CSC is associated with Ypt7, and this correlates with a fragmented vacuole phenotype that is characteristic of *vps5Δ* and *vps17Δ* mutants and resembles loss of function of the Ypt7 GTPase module. We suggest that this reveals a contribution by retromer to endosome maturation through the coordination of retrograde export with Ypt7-dependent tethering and fusion reactions. Intriguingly, a regulator of Rab7 signaling—the GTPase activating protein TBC1D5—is recruited to endosomes by binding the Vps29 CSC subunit, where it acts to terminate signaling by Rab7 (Seaman *et al.*, 2009). Thus down-regulation of Rab/Ypt7 signaling appears to be a conserved functional aspect of CSC function, but this is accomplished through different mechanisms in yeast and human cells. A physiological function of such regulation may be to coordinately regulate retromer-mediated export of membrane from the endolysosomal system and vacuole–vacuole fusion to modulate vacuole surface area to volume ratio of these organelles.

The increase in the correlation of cargo (Vps10) and Vps35 fluorescence signals that we observe for *mon1Δ* cells (Figure 8C) indicates that cargo is inefficiently exported from endosomes in the absence of Ypt7-GTP, even though retromer and cargo localize to these endosomes. This leads us to suggest that the Rab/Ypt GTPase cycle regulates retromer after recognition of cargo in a stage required to export cargo from the endosome. How might Ypt7/Rab7 regulate this aspect of retromer function? That association of yeast retromer CSC with the Vps5/17 sorting nexins appears to be mutually exclusive (Figure 2; Balderhaar *et al.*, 2010) suggests two possibilities: first, recognition of SNX-BAR subunits and Rab on the endosome follows an ordered pathway, in which Vps5/17 mediates initial endosome recruitment of CSC, which then hands off to Ypt7. Alternatively, CSC may form complexes with Vps5/17 or Ypt7 indepen-

dently. We note that recent genetic evidence from studies with metazoan cells indicates that sorting of one particular retromer cargo (Wntless protein) does not require the SNX-BAR proteins, although it does require the Snx3 sorting nexin (Harterink *et al.*, 2011), indicating that metazoan CSC-containing complexes have multiple compositions. Nonetheless, we favor the first model, in which the Rab GTPase cycle regulates a structural rearrangement or dissociation of retromer subcomplexes that promotes the formation of transport carriers (Figure 8D). Regardless of the distinctions between these models, the conserved, dual functions of Ypt7/Rab7 in promoting retromer-mediated export of cargo from the endosome and delivery of endosome traffic to the vacuole via tethering reactions indicate that Rab7 constitutes a principal axis of regulation by which late stages of endosome maturation are coordinated with retrograde cargo sorting.

MATERIALS AND METHODS

Yeast strains and growth conditions

All yeast strains were constructed in the BY4742 background (*MATα his3-1, leu2-0, met15-0, and ura3-0*). Unless otherwise indicated, all yeast strains were constructed by integration using recombination of gene-targeted, PCR-generated DNAs using the method of Longtine *et al.* (1998), which ensures expression from the native loci. The majority of mutant strains used here were derived from the European *Saccharomyces cerevisiae* Archive for Functional Analysis (Institute for Molecular Biosciences, Johann Wolfgang Goethe-University Frankfurt, Frankfurt, Germany) KANMX deletion collection (Open Biosystems, Thermo Biosystems, Huntsville, AL). Double mutants were made by replacement of the complete reading frame of the second gene with the *HIS3MX6* or *URA3* cassette and in the BY4742 background (*MATα his3-1, leu2-0, lys2-0, and ura3-0*). For making the triple mutant *vps5Δ vps17Δ ypt7Δ*, random spore analysis was performed with a *VPS5 vps5Δ::NATMX VPS17 vps17Δ::HISMX YPT7 ypt7Δ::KANMX* diploid. Deletion of each gene was confirmed by PCR amplification of the deleted loci. All strains were grown in standard synthetic complete medium lacking nutrients required to maintain selection for auxotrophic markers (Sherman *et al.*, 1979). Ftr1p-GFP-expressing cells were cultured at 30°C to OD₆₀₀ ≈ 1.0 in growth medium containing 50 μM of the iron chelator bathophenanthrolinedisulfonic acid.

Strains expressing mutant Vps35 proteins with internal deletions were constructed by a multistep PCR-based method as follows. First, a *URA3-KANMX* DNA cassette was amplified by PCR using primers containing 40 base pairs upstream and downstream of the deletion points, and this DNA was used to transform a strain with a wild-type *VPS35* locus containing a 3xHA epitope tag immediately upstream of the stop codon. Integration of this DNA by homologous recombination disrupts the *VPS35* open reading frame by removing the DNA between the endpoints (indicated in the *Results* section) of the PCR-generated DNA and inserting the *URA3-KANMX* cassette. Next, for each internal deletion a two-step PCR was used to construct DNA containing 40 base pairs upstream and downstream of the internal deletion points with a Gly-Ser-Gly-Ser-Gly-Ser linker in between. This DNA was used to transform the strains containing the disrupted *VPS35::URA3-KANMX* strains constructed in the first step. Homologous recombination of this DNA at the disrupted *VPS35* locus results in loss of the *URA3-KANMX*. To obtain such colonies, transformed strains were plated on nonselective yeast extract/peptone/dextrose medium and grown for 1 d, and these plates were then replica plated to medium containing 5-fluoroorotic acid (5-FOA) to counterselect Ura⁺ cells. Colonies resistant to 5-FOA

were then screened for loss of resistance to G418. To confirm loss of the *URA3-KANMX* cassette and deletion of the intervening *VPS35* sequence, PCR was used to amplify across the deletion points, and colonies yielding the predicted size products were retained. Anti-HA immunoblotting was used to identify colonies in which expression of the C-terminal tagged Vps35 mutant protein was restored. In addition, the PCR products that amplified across each deletion point were sequenced to further confirm the expected deletion points. A set of deletion strains in which the 3xHA tags were replaced with GFP was constructed using standard methods (Petracek and Longtine, 2002).

Antibodies

The following mouse monoclonal antibodies were used in this study: 9E10 anti-myc (1:10,000; Applied Biological Materials, Richmond, Canada), anti-HA (1:1000; Covance, Berkeley, CA), and anti-FLAG M2 (1:2000; Stratagene, Santa Clara, CA). A previously described rabbit polyclonal antibody to Ypt7p was used (1:1000). Horseradish peroxidase (HRP)-conjugated secondary anti-mouse and anti-rabbit immunoglobulin G were purchased from GE Healthcare (Piscataway, NJ). Monoclonal antibodies against Cl-MPR (1:500; AbD Serotec, Raleigh, NC), γ -tubulin (1:5000; Sigma-Aldrich, St. Louis, MO), and anti-HA-HRP (1:3000; Roche, Indianapolis, IN) were used. An antiserum against hVps35 (1:3000) was generated by immunizing rabbits with GST-hVps35 fusion protein (amino acids 461–796; Cocalico Biologicals, Reamstown, PA).

GST-Rab protein expression and pulldown

Vectors expressing different forms of Ypt7 (wild type, Q68L, T22N) and Vps21 (wild type, Q66L, S21N) were gifts from C. Ungermann (University of Osnabruck, Osnabruck, Germany). These plasmids were transformed into *E. coli* BL21 (DE3; Novagen, EMD4Biosciences, Gibbstown, NJ), and expression was induced by the addition of 1 mM isopropyl- β -D-thiogalactoside at 37°C for 3 h. Cell pellets were resuspended in lysis buffer (phosphate-buffered saline [PBS]; 1 mM KH_2PO_4 , 10 mM Na_2HPO_4 , 137 mM NaCl, and 2.7 mM KCl, pH 7.4) + 1 \times Complete Mini Protease Inhibitor Cocktail [Roche]. Cells were lysed by freeze-thaw and sonication, followed by centrifugation (20,000 \times g for 20 min) to produce clarified lysates. The GST-GTPase fusion proteins were bound to equilibrated GSH beads (GE Healthcare).

Extracts of yeast cells expressing different retromer subunits in lysis buffer (100 mM KCl, 10 mM MgCl_2 , 40 mM Tris, pH 7.5) were generated by disruption of cells by vortexing in the presence of glass beads. Cell debris was removed by centrifugation at 10,000 \times g for 15 min at 4°C. Supernatants (0.1 ml) were incubated with the beads for 2 h at 4°C on a rotating wheel. Beads were then gently collected by centrifugation and washed five times with binding buffer. Proteins were eluted by the addition of sample buffer and were separated by 10% SDS-PAGE, followed by Western blotting with anti-HA antibodies.

Immunopurification

The *in vivo* cross-linking procedure is based on a published method (Hetteema *et al.*, 2003). In brief, 50 OD₆₀₀ of the indicated strains were grown to log phase (OD₆₀₀ \approx 1.0) in synthetic media, harvested, washed once with water, and converted to spheroplasts. Spheroplasts were resuspended in 1 ml of cross-linking buffer (25 mM potassium phosphate, 0.2 M sorbitol, and 1 \times EDTA-free protease inhibitor cocktail, pH 7.4), and 2 mM DSP (Pierce Chemical Co., Rockford, IL) prepared freshly in dimethyl sulfoxide. Cross-linking reactions were incubated for 30 min at 4°C. Tris 25 mM, pH 7.5,

was added for 15 min to quench the reaction, and then 1% Triton X-100 was added to solubilize the material. Tubes were spun at 13,000 \times g for 10 min to remove insoluble debris, and the cross-linked cell lysate was incubated with anti-HA affinity matrix (Roche) and allowed to rock overnight at 4°C. Beads were gently collected by centrifugation and washed five times with 1 ml of wash buffer (1 \times PBS and 1% Triton X-100). Bound proteins were eluted, and cross-links were cleaved with 2 \times sample buffer containing 20 mM dithiothreitol and were identified after SDS-PAGE by immunoblotting.

Microscopy

Yeast cells from cultures grown to OD₆₀₀ \approx 0.8 were mounted in growth medium, and three-dimensional image stacks were collected at 0.5- μm z-increments on a DeltaVision workstation (Applied Precision, Issaquah, WA) based on an inverted microscope (IX-70; Olympus, Tokyo, Japan) using a 100 \times /1.4 numerical aperture oil immersion lens. Images were captured at 24°C with a 12-bit charge-coupled device camera (CoolSnap HQ; Photometrics, Tucson, AZ) and deconvolved using the iterative-constrained algorithm and the measured point spread function. Fluorescence microscopy of Ftr1p-GFP constructs was done using a microscope (Eclipse E800; Nikon, Melville, NY) fitted with a cooled, high-resolution charge-coupled device camera (model C4742-95; Hamamatsu, Hamamatsu, Japan). Images were acquired using Phase 3 Imaging software (Phase 3 Imaging Systems, Glen Mills, PA). To visualize vacuoles (Vida and Emr, 1995), cells were incubated at 30°C with the lipophilic dye FM4-64 (16 nM) for 15 min, washed with medium, and further incubated in medium lacking FM4-64 for 60 min. For HeLa cell immunofluorescence studies, cells were grown directly on coverslips, fixed in 4% paraformaldehyde, and stained as previously described (Gomez and Billadeau, 2009). Images were acquired using an LSM-710 laser scanning confocal microscope using the 100 \times /1.46 numerical aperture oil objective and analyzed using the LSM software package Zen2008 (Carl Zeiss, Jena, Germany).

Images of yeast cells were scaled and false colored using ImageJ, version 1.38 (National Institutes of Health, Bethesda, MD). In all figures, one image of the z-stack from the approximate center of the cells is shown. Pearson's correlation analyses of deconvolved z-stacks were done using Volocity Visualization software, version 5 (PerkinElmer-Cetus, Waltham, MA). Two-tailed Mann-Whitney statistical analysis of *r* values was done using XLSTAT software (Addinsoft, New York, NY).

Experiments with cultured human cells

The pCMS3.H1p/HA-YFP reexpression system and the shVps35 targeting sequence (CAGAGCAGATTAACAAACA) were previously described (Gomez and Billadeau, 2009). hVps35 was cloned from Jurkat T-cell cDNA library, and the $\Delta 6$ and shVps35-resistant (CtGAaCAaATcAAtAAACA) versions of hVps35 were generated using QuikChange Site-Directed Mutagenesis (Stratagene). For quantification of Cl-MPR dispersal, >100 cells for each transfected cell population in at least three independent experiments were blindly scored as previously described (Gomez and Billadeau, 2009).

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