

Rab5-family guanine nucleotide exchange factors bind retromer and promote its recruitment to endosomes

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ABSTRACT The retromer complex facilitates the sorting of integral membrane proteins from the endosome to the late Golgi. In mammalian cells, the efficient recruitment of retromer to endosomes requires the lipid phosphatidylinositol 3-phosphate (PI3P) as well as Rab5 and Rab7 GTPases. However, in yeast, the role of Rabs in recruiting retromer to endosomes is less clear. We identified novel physical interactions between retromer and the *Saccharomyces cerevisiae* VPS9-domain Rab5-family guanine nucleotide exchange factors (GEFs) Muk1 and Vps9. Furthermore, we identified a new yeast VPS9 domain-containing protein, VARP-like 1 (Vrl1), which is related to the human VARP protein. All three VPS9 domain-containing proteins show localization to endosomes, and the presence of any one of them is necessary for the endosomal recruitment of retromer. We find that expression of an active VPS9-domain protein is required for correct localization of the phosphatidylinositol 3-kinase Vps34 and the production of endosomal PI3P. These results suggest that VPS9 GEFs promote retromer recruitment by establishing PI3P-enriched domains at the endosomal membrane. The interaction of retromer with distinct VPS9 GEFs could thus link GEF-dependent regulatory inputs to the temporal or spatial coordination of retromer assembly or function.

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INTRODUCTION

Retromer is a retrograde endosomal trafficking complex that facilitates recycling of integral membrane proteins to the late Golgi and the plasma membrane (Seaman, 2005, 2012; Bonifacino and Hurley, 2008; Attar and Cullen, 2010). It was first identified in yeast as a complex required to recycle the acid hydrolase receptor Vps10 and maintain the Golgi localization of Kex2 (Seaman et al., 1998). Since

then, it has been linked to many processes in higher organisms, including the endosome-to-Golgi recycling of the cation-independent mannose 6-phosphate receptor and the iron transporter DMT1 and the direct endosome-to-plasma membrane trafficking of the β 2-adrenergic receptor (Seaman, 2004; Tabuchi et al., 2010; Temkin et al., 2011). In addition, deficiencies in the retromer complex and its associated factors have been linked to Parkinson's (Vilariño-Güell et al., 2011; Zavodszky et al., 2014) and Alzheimer's diseases (Fjorback et al., 2012).

Yeast retromer is composed of a structural subcomplex containing the sorting nexins Vps5/Vps17 and a cargo-selective subcomplex (CSC) comprising Vps26/Vps29/Vps35 (Seaman et al., 1998). The sorting nexins bind phosphatidylinositol 3-phosphate (PI3P) at endosomes and deform the membrane (Burda et al., 2002), whereas the CSC recruits cargo into the retromer tubule (Nothwehr et al., 1999; Seaman, 2005). Although these two subcomplexes form a stable pentamer in yeast, the CSC and sorting nexins are not tightly associated in mammalian cells (McGough and Cullen, 2011). The mammalian CSC assembles with Vps5/Vps17 homologues and with

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Abbreviations used: CPY, carboxypeptidase Y; CSC, cargo-selective subcomplex (of retromer); Cub, C-terminal ubiquitin fragment; DSP, dithiobis(succinimidyl propionate); ESCRT, endosomal sorting complex required for transport; GEF, guanine nucleotide exchange factor; iMYTH, integral membrane yeast two-hybrid; Nub, N-terminal ubiquitin fragment; ORF, open reading frame; PI3K, phosphatidylinositol 3-kinase; PI3P, phosphatidylinositol 3-phosphate.

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other sorting nexins to form spatially distinct classes of retromer tubules that engage different cargo (Hartherink *et al.*, 2011). Whereas the sorting nexin SNX3 is enriched at early endosomes, retromer tubules formed by the Vps5 and Vps17 homologues SNX1/2 and SNX5/6, respectively (Wassmer *et al.*, 2009), are most abundant on endosomes undergoing the early-to-late transition (Rojas *et al.*, 2008; Cullen and Korswagen, 2012; van Weering *et al.*, 2012).

Rab GTPases are important for membrane identity, vesicle budding, and membrane fusion (Stenmark, 2009). Rabs are converted to their active, membrane-bound form by guanine nucleotide exchange factors (GEFs), which catalyze the exchange of GDP for GTP and are inactivated by GTPase-activating proteins, which increase the rate of GTP hydrolysis by the Rabs. As early endosomes mature into late endosomes, they undergo a Rab conversion in which active Rab7-family GTPases are recruited and Rab5-family GTPases are inactivated and extracted from the membrane (Rink *et al.*, 2005).

Both Rab5- and Rab7-family GTPases are implicated in the recruitment of retromer to endosomes in mammalian cells (Rojas *et al.*, 2008; Liu *et al.*, 2012). GTP-bound Rab5 does not bind retromer directly but instead recruits a complex containing VPS34, a phosphatidylinositol 3-kinase (PI3K; Christoforidis *et al.*, 1999). This catalyzes the production of PI3P, which is recognized by the Phox homology (PX) domains of the sorting nexins (Xu *et al.*, 2001; Yu and Lemmon, 2001; Cozier *et al.*, 2002). In contrast, direct binding of Rab7 to Vps35 is essential for the endosomal recruitment of mammalian CSC (Harrison *et al.*, 2014). The CSC also interacts with a number of regulatory factors, including the putative Rab7 GTPase-activating protein TBC1D5 (Seaman *et al.*, 2009; Harbour *et al.*, 2010), and retromer tubule formation is reported to be maximal at the time of Rab5-to-Rab7 conversion (Cullen and Korswagen, 2012; van Weering *et al.*, 2012). Thus retromer assembly at endosomes may be tightly coupled to the regulation of Rab activation.

In yeast, the regulation of retromer function by Rab proteins is less well understood. Vps35 binds to the yeast Rab7 homologue Ypt7, but although this interaction directs it to the vacuole, it is not required for recruitment to endosomes (Balderhaar *et al.*, 2010; Liu *et al.*, 2012). Moreover, many of the retromer-associated regulatory proteins identified in mammalian cells are not conserved in yeast. Thus the extent to which retromer assembly at endosomes is subject to Rab-dependent regulation is unknown.

Here we show that retromer physically interacts with the VPS9-domain GEFs Muk1 and Vps9 and that at least one of these must normally be present for retromer recruitment at endosomes. We further identify a new yeast VPS9-domain protein related to human VARP that is present in wild but not laboratory strains of *Saccharomyces cerevisiae*. All three proteins can act through Rab5-family GTPases and the PI3K Vps34 to localize retromer to endosomal membranes. This suggests that association with Rab5 family GEFs could provide a mechanism to regulate the location or extent of retromer assembly.

RESULTS

Retromer physically interacts with Rab5-family GEFs

Previous high-throughput mass spectrometry studies of yeast protein complexes suggested an interaction between several subunits of the retromer complex and the VPS9-domain GEF Muk1 (Krogan *et al.*, 2006; Babu *et al.*, 2012). This interaction was surprising, as VPS9-domain GEFs activate Rab5-family GTPases, whereas yeast retromer has been shown to interact only with the Rab7-like GTPase, Ypt7.

To validate the retromer–Muk1 interaction, we expressed Muk1-hemagglutinin (HA) from a *GAL1* promoter in strains containing

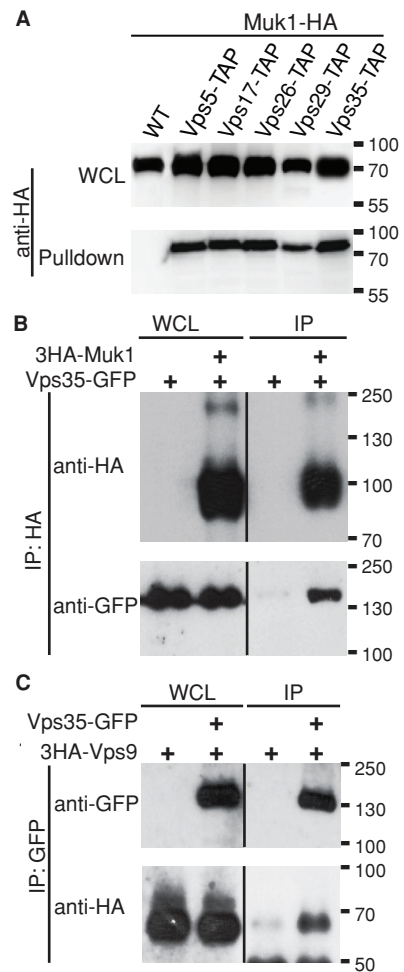


FIGURE 1: Retromer physically interacts with the Rab5-family GEFs Muk1 and Vps9. (A) TAP-tagged retromer subunits were pulled down using calmodulin beads from *GAL1pr-MUK1-HA* strains. Samples were resolved by 12% SDS-PAGE and detected by immunoblotting. Loading of lysates relative to the pull down was 1:25. (B) Endogenously tagged Vps35-GFP and *ADH1pr-3HA-Muk1* were cross-linked with 1.6 mg/ml DSP, 3HA-Muk1 was immunoprecipitated, and copurifying Vps35-GFP was probed by immunoblotting. Loading of lysates relative to immunoprecipitate (IP) was 1:1.6 (anti-HA) and 1:388 (anti-GFP). (C) Cells expressing Vps35-GFP and *ADH1pr-3HA-Vps9* were cross-linked and immunoprecipitated with anti-GFP. Copurification of 3HA-Vps9 was detected with anti-HA. Loading of lysates relative to IP was 1:1.6 (anti-GFP) and 1:388 (anti-HA).

different tandem affinity purification (TAP)-tagged retromer subunits and carried out pull downs using calmodulin resin under batch purification conditions similar to those used for mass spectrometry. This showed that all five retromer subunits were able to copurify Muk1 (Figure 1A).

To estimate the fraction of interacting proteins, we subsequently carried out small-scale coimmunoprecipitations in strains in which HA-Muk1 was expressed from the weaker *ADH1* promoter and the retromer subunit Vps35 was green fluorescent protein (GFP) tagged at its endogenous locus. Interactions between Muk1 and retromer were reproducibly detected in the presence of the crosslinker dithiobis(succinimidyl propionate) (DSP). However, purification of ~30% of cellular Muk1 coprecipitated <1% of the total pool of Vps35 (Figure 1B). The fact that interactions were detected only in the presence of the cross-linker and represented a minor fraction of the

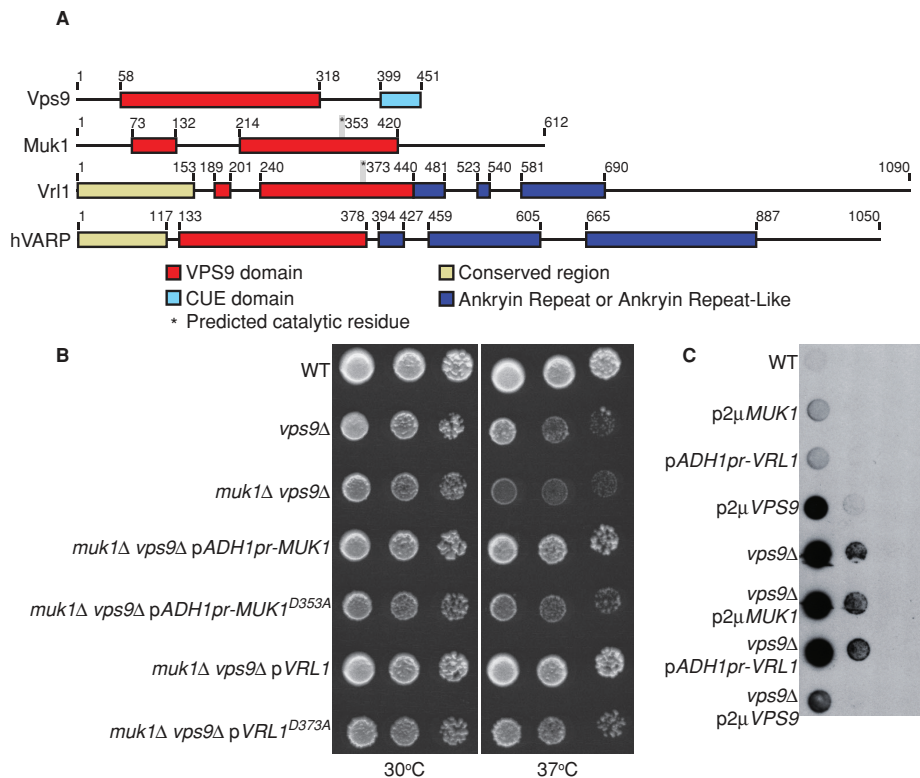


FIGURE 2: Vrl1 is a new yeast VPS9-domain protein. (A) Schematic of yeast VPS9 domain-containing proteins and human VARP based on Superfamily (Gough *et al.*, 2001) and ClustalW alignments. (B) Yeast were spotted in 10× dilution series and grown 2 d at 37°C to assess temperature sensitivity of the indicated strains. VRL1 was expressed from its endogenous promoter. (C) A colony overlay assay was used to assess carboxypeptidase Y (CPY) secretion. Cells spotted in 10× dilution series were overlaid with nitrocellulose and incubated for 16 h. The nitrocellulose was then immunoblotted with anti-CPY antibodies.

total Vps35 protein suggests that Vps35-Muk1 interactions are weak or disrupted on lysis and are likely to be substoichiometric. Furthermore, as retromer is a stable pentameric complex, we cannot exclude the possibility that Muk1 binds another subunit or interacts with retromer through a bridging protein.

Yeast express two known Rab5-family GEFs, Vps9 and Muk1, which share a catalytic VPS9 domain but have divergent C-termini (Carney *et al.*, 2006; Balderhaar *et al.*, 2010; Paulsel *et al.*, 2013). Retromer-Vps9 interactions were not previously identified by large-scale mass spectrometry. Nevertheless, we found that HA-Vps9, when expressed from the ADH1 promoter, reproducibly copurified with Vps35-GFP from DSP-treated cell lysates (Figure 1C). These results indicate that retromer binds more than one VPS9-domain GEF.

Identification of Vrl1, a third member of the yeast VPS9-domain family related to mammalian VARP

Muk1 and Vps9 are the only two VPS9-domain GEFs described in yeast to date. Of interest, the Superfamily database (Gough *et al.*, 2001), which identifies domains using hidden Markov models, reported a partial VPS9 domain at the N-terminus of the uncharacterized open reading frame (ORF) YML002w. In wild strains of *S. cerevisiae*, YML002w is continuous with an upstream ORF (YML003w) and is predicted to encode a single protein of 1090 amino acids with a full-length VPS9 domain. Comparison of sequences from wild and laboratory strains of *S. cerevisiae* suggests that a single thymine residue at chrXIII:264337 is deleted in commonly studied strains,

causing a frameshift and premature stop codon (Supplemental Figure S1A). Resequencing of the corresponding region in the BY4741 parental strain confirmed the presence of the mutation. Integration of a 3HA tag at the 5' end of the upstream ORF, YML003w, under control of the ADH1 promoter, showed that the frameshift produces a truncated protein of the predicted size (Supplemental Figure S1B). Thus it appears that wild yeast strains encode a third VPS9-domain protein that has been mutated in laboratory strains of *S. cerevisiae*.

Sequence comparisons suggest that this new VPS9-domain protein is conserved in most species and is related to human VARP (hVARP), a Rab21 GEF (Zhang *et al.*, 2006). Accordingly, we have named this ORF VRL1, for VARP-like1. hVARP is a multifunctional protein that binds the R-soluble N-ethylmaleimide-sensitive factor attachment protein receptor (R-SNARE) VAMP7 and is a key regulator of endosome-to-cell surface transport (Burgo *et al.*, 2009, 2012; Ohbayashi *et al.*, 2012). Whereas VARP GEF activity is essential for recycling to the cell surface in neurites, VARP has a separate role as a Rab32/38 effector in the trafficking of tyrosinase-related protein 1 to melanosomes, which does not require its GEF activity (Tamura *et al.*, 2011; Ohbayashi *et al.*, 2012). VARP and Vrl1 share extensive regions of conservation, including an N-terminal region not found in other GEFs, followed by the VPS9 domain and several ankryin repeats (Figure 2A and Supplemental Figure S2). However, the yeast protein lacks the second of two sets of ankryin repeats present in hVARP, and other hVARP features, including the Rab32/38-binding site and the VAMP7-interacting domain (Burgo *et al.*, 2009; Ohbayashi *et al.*, 2012; Schäfer *et al.*, 2012), are only partially conserved, suggesting that the proteins may not be functionally identical (Supplemental Figure S2C).

Vrl1, Muk1, and Vps9 have partially overlapping functions

Muk1 and Vps9 were previously shown to have redundant yet distinct functions. Muk1 overexpression rescues the temperature-sensitive (ts) growth phenotype of *vps9* cells but cannot replace Vps9's role in the late endosomal sorting of carboxypeptidase Y (CPY; Paulsel *et al.*, 2013). To determine whether Vrl1 can functionally substitute for Muk1 or Vps9, we cloned the genomic region that includes both YML003w and YML002w into a single-copy plasmid and inserted a nucleotide (T856) into YML003W to recreate the full-length ORF. We use VRL1 to refer to the full-length gene that results from the correction of the frameshift mutation; it is important to note that the laboratory yeast strains used here do not express functional VRL1. Introduction of a plasmid expressing VRL1 from its endogenous promoter fully restored growth of *muk1 vps9* strains at high temperatures, suggesting that it can replace some function of Muk1 and/or Vps9 (Figure 2B).

The VPS9 domain of Rabex-5 contains a single invariant residue, D313, which is required for catalytic activity (Delprato and Lambright, 2007). We mutated the corresponding aspartate residues in Muk1

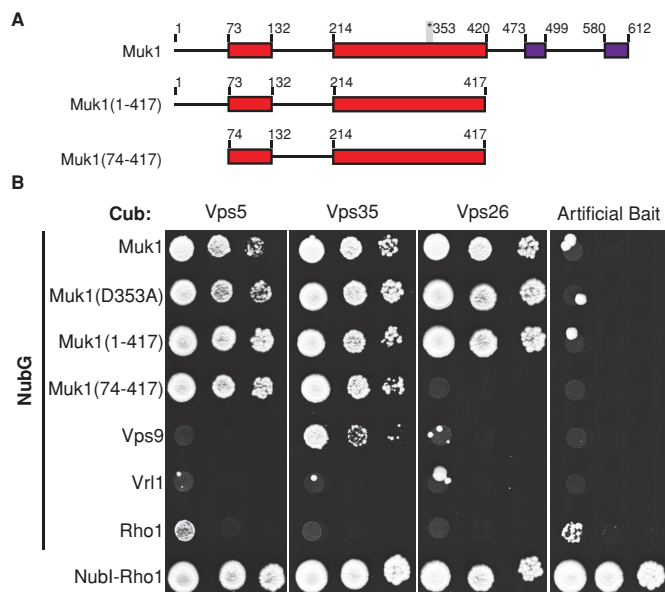


FIGURE 3: The VPS9 domain of Muk1 is sufficient for interaction with retromer subunits in the iMYTH assay. (A) Schematic of Muk1 truncations. The position of the invariant aspartate residue required for GEF activity and conserved C-terminal motifs are indicated. (B) Strains containing Cub-tagged retromer subunits and plasmids encoding Rab5-family GEFs tagged N-terminally with NubG were tested for activation of the *HIS3* reporter on selective medium lacking tryptophan, adenine, and histidine. NubG-tagged Rho1 acted as a negative control, whereas the Nubl tag, which binds tightly to Cub independently of other interactions, confirmed the expression of Cub fusions.

and Vrl1 and found that expression of Muk1^{D353A} or Vrl1^{D373A} failed to rescue the *muk1 vps9* ts growth defect, suggesting that suppression requires an active VPS9 domain (Figure 2B). However, overexpression of Vrl1 did not rescue the CPY secretion of a *vps9Δ* strain. In addition, high levels of Vrl1 induced mild CPY secretion in a wild-type strain similar to overexpression of Muk1 (Paulsel et al., 2013; Figure 2C). These data suggest that although Vrl1, Muk1, and Vps9 show some degree of redundancy, they are not functionally equivalent.

Because both known VPS9-domain GEFs interact with retromer, we tested Vrl1–retromer binding by coimmunoprecipitation. No physical interaction between *ADH1pr*-driven HA-Vrl1 and the retromer subunit Vps35-GFP was observed, although HA-Vrl1 was expressed at a significantly lower level than Muk1 or Vps9 (~5% of HA-Muk1 level; unpublished data), and thus any interaction may be below the detection threshold. Taken together, our data suggest that Vrl1 can partially substitute for Muk1/Vps9 but cannot replace all functions of these GEFs.

Retromer interacts with the VPS9 domain

Retromer physically interacts with both Muk1 and Vps9, and yet their sequence similarity outside the VPS9 domain is limited. To map the site of interaction, we used the integrated membrane yeast two-hybrid (iMYTH) system, which detects interactions of membrane-associated proteins at their normal organellar localization (Paumi et al., 2007; Snider et al., 2013). The N-terminal half of ubiquitin (NubG) was fused to full-length and truncated versions of Muk1 and coexpressed with retromer subunits fused to a ubiquitin C-terminus-transcription factor cassette (Figure 3A). Interactions that reconstitute ubiquitin result in the deubiquitinase-dependent release of the

transcription factor and the activation of reporter genes. Full-length Muk1 and a truncated version (amino acids [aa] 1–417) corresponding to the VPS9 domain interacted with three of the five retromer subunits, and this interaction was not affected by the D353A mutation predicted to block Muk1's GEF activity (Figure 3B). Vps9 also interacted with Vps35–C-terminal ubiquitin fragment (Cub) in this assay, although no interaction was observed with the remaining retromer subunits. We were unable to detect an interaction between Vrl1–N-terminal ubiquitin fragment (Nub) and any of the retromer Cubs; however, Vrl1–Nub was expressed at relatively low levels compared with the other Nub fusions (Supplemental Figure S3A). Moreover, interactions will not be detected if the Nub and Cub fusions are not positioned in close proximity in the complex. Our results suggest that retromer interacts, directly or indirectly, with the Muk1 VPS9 domain. This is the only domain shared by Muk1 and Vps9, suggesting that retromer might recognize a conserved feature of this domain.

Muk1, Vps9, and Vrl1 can localize to endosomes

Both Muk1 and Vps9 have a predominantly cytosolic distribution in wild-type cells (Paulsel et al., 2013). Vps9 is believed to be recruited to ubiquitinated cargo via its C-terminal CUE domain (Carney et al., 2006), which is not present in Muk1. Accordingly, Vps9, but not Muk1, localizes to the aberrant endosome of endosomal sorting complex required for transport (ESCRT) mutants (Paulsel et al., 2013), where ubiquitinated proteins accumulate. Overexpression of the tagged ESCRT-III subunit Snf7–red fluorescent protein (RFP) disrupts ESCRT function and causes an endosomal maturation defect (Froissard et al., 2007). We confirmed that GFP-Vps9 colocalizes with Snf7-RFP at endosomes (21% of cells, SE = 6.5%; Figure 4A). Of interest, when expressed from a plasmid under its endogenous promoter, GFP-Muk1 could be detected at Snf7-RFP–marked endosomes in some cells (5%, SE = 1.5%; Figure 4A). Furthermore, GFP-Vrl1 formed puncta that colocalized with Snf7-RFP puncta in 16% (SE = 3.7%) of cells (Figure 4A). Taken together, these results suggest that all three VPS9-domain proteins are able to associate with endosomes to some extent.

When GFP-Muk1 was expressed from the stronger *ADH1pr* promoter, bright puncta were seen in 37% (SE = 4.8%) of cells, and these overlapped with the endosomal markers FM4-64 and Snf7-RFP and with the retromer subunit Vps26-RFP (Figure 4B). Two regions in the Muk1 C-terminal domain, corresponding to residues 473–499 and 580–612, are highly conserved in different fungal species (Figure 4C). A truncation that removes the last 37 aa of Muk1 (GFP-Nt-Muk1) did not alter protein stability (Supplemental Figure S2B), and yet it abolished puncta formation (Figure 4D), suggesting that this region contributes to Muk1's localization. However, the Muk1 C-terminus alone (aa 503–612), although expressed at similar levels to the full-length GFP-Muk1, did not form puncta, indicating the Muk1 C-terminal domain is necessary but not sufficient for endosomal localization (Figure 4D). The localization determinant may not be correctly folded in the truncated protein. Alternatively, membrane localization of Muk1 may require multiple interacting domains.

To test whether retromer is involved in recruiting the Rab5 GEFs to endosomes, we deleted retromer subunits *VPS26* or *VPS35* from the strains expressing *ADH1pr*-driven GFP-Muk1 or GFP-Vps9. Both GEFs were found in puncta that colocalized with Snf7-RFP in the retromer deletion strains (Figure 4E), suggesting that Muk1 and Vps9 localize to endosomes independent of their interactions with retromer.

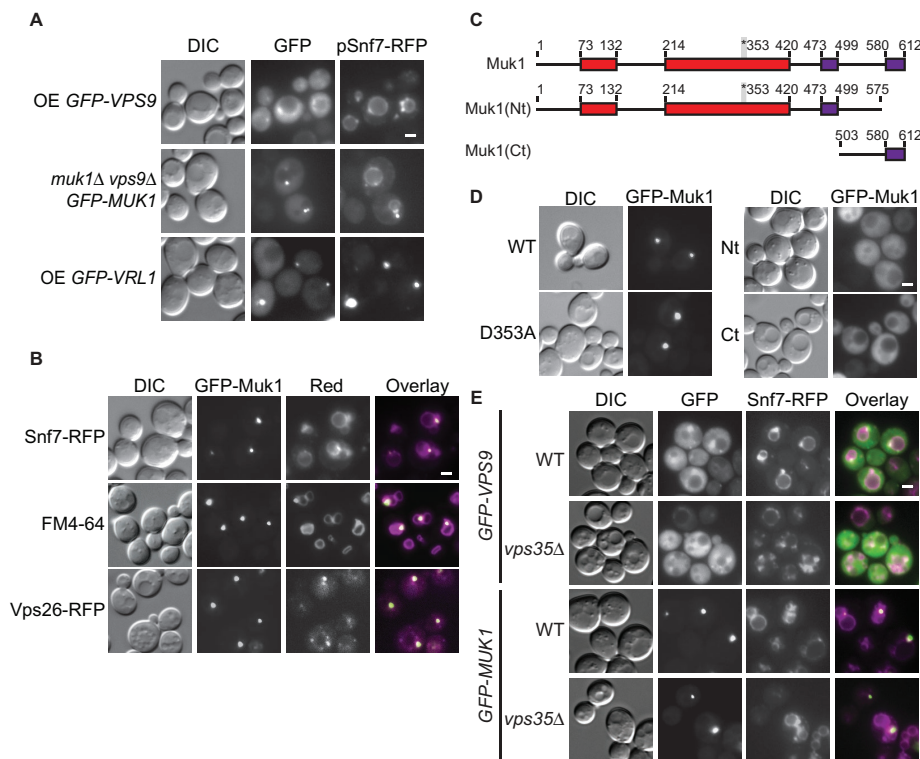


FIGURE 4: The yeast VPS9-domain proteins Muk1, Vps9, and Vrl1 localize to endosomes. (A) *MUK1pr-GFP-Muk1*, *ADH1pr-GFP-Vps9*, and *ADH1pr-GFP-Vrl1* localize to Snf7-RFP-marked late endosomes by fluorescence microscopy. (B) Fluorescence microscopy of *ADH1pr-GFP-Muk1* with Snf7-RFP, the lipophilic dye FM4-64, and the tagged retromer subunit RFP-Vps26. (C) Schematic of Muk1 truncations used in D, showing conserved C-terminal motifs identified by alignments (purple). (D) Fluorescence microscopy of full-length or mutated *ADH1pr-GFP-Muk1*. (E) Deletion of *VPS35* does not disrupt the endosomal localization of overexpressed GFP-Vps9 or GFP-Muk1. Scale bars, 2 μ m; OE, overexpressed.

Retromer localization to endosomes is impaired by loss of Rab5 GEFs

We showed that retromer interacts with two different VPS9-domain GEFs and that these GEFs can localize to endosomes. To test whether the GEFs are required for retromer recruitment, we deleted them singly and in combination (Figure 5A). Vps26-GFP localized to puncta in wild-type cells and *muk1* and *vps9* single mutants, although punctate localization was reduced in the *vps9* strain. Strikingly, deletion of both *MUK1* and *VPS9* greatly reduced localization of Vps26-GFP and Vps5-GFP to endosomes. This was complemented by expression of either *VPS9* or *MUK1* from single-copy plasmids (Figure 5, A and B). Strikingly, the *MUK1*^{D353A} GEF mutant failed to restore Vps26-GFP localization, demonstrating the importance of Muk1 GEF activity in vivo. Similarly, expression of Vrl1, but not Vrl1^{D373A}, from its endogenous promoter also restored retromer localization to *muk1 vps9* mutant cells. Thus any of the three active VPS9-domain proteins is sufficient to recruit retromer to endosomes.

The need for an intact VPS9 domain suggests that active Rabs are required for efficient retromer recruitment to endosomes. There are three Rab5-family GTPases in yeast: Vps21, Ypt52, and Ypt53. Ypt53 is normally expressed at very low levels and is activated only under conditions of stress (Nickerson et al., 2012). We found that loss of *VPS21* alone caused a slight reduction in retromer recruitment (Figure 5A), consistent with other observations (Balderhaar et al., 2010), but deletion of both *VPS21* and *YPT52* strongly reduced retromer recruitment. This was not complemented

by expression of *VRL1*, suggesting that Vrl1 acts upstream of the Rab5 GTPases, consistent with its role as a putative GEF. Together the results suggest the GEFs redundantly activate different Rab5 GTPases, and this in turn is needed for retromer's localization to endosomes.

Rab5 GEFs are required for normal PI3P localization

Overexpression of active Rabs can often overcome loss of their respective GEFs (Siniosoglou et al., 2000; Lynch-Day et al., 2010). Therefore we expressed different forms of Vps21 from high-copy plasmids to see whether this would rescue retromer recruitment (Figure 6, A and B). Surprisingly, expression of the constitutively active Vps21Q66L in wild-type (WT) and *muk1 vps9* strains stimulated recruitment of retromer to the vacuolar membrane but not to endosomes.

Because retromer recruitment to early endosomes requires PI3P, its vacuolar localization might result from loss of endosomal PI3P or from elevated levels of PI3P at the vacuole in these mutant strains. Indeed, the PI3P-binding biosensor GFP-FYVE showed endosomal localization in WT strains but prominent vacuole rim staining in a *muk1 vps9* strain (Figure 6C), similar to that of a *vps21 ypt52* strain (Nickerson et al., 2012). This vacuolar pool of PI3P could originate at endosomes and accumulate at the vacuole due to a disruption in its turnover or metabolism. Alternatively, PI3P could be produced at the vacuolar membrane by a vacuole-localized pool of the PI3K Vps34 (Burda et al., 2002). We examined Vps34-GFP localization in each of the mutants and found it was strongly mislocalized to the vacuole in *muk1 vps9* mutants and partially mislocalized in a *vps9* mutant (Figure 6D). VPS34 is recruited by Rab5 GTPases in mammals (Christoforidis et al., 1999), and others have shown that, in yeast, constitutively active forms of Vps21, which cannot be extracted from membranes by Rab guanine dissociation inhibitors, are transported to the vacuolar membrane (Markgraf et al., 2009). Thus, overexpressed Vps21Q66L may activate Vps34 at the vacuole of *muk1 vps9* strains, resulting in high levels of PI3P that drive retromer recruitment to the vacuolar membrane.

These results suggest that VPS9-domain GEFs are critical for maintaining an endosomal pool of Rab5-family GTPases that in turn recruit and activate the PI3K Vps34. The partial mislocalization of Vps34 in *vps9* but not *muk1* mutants suggests that the three VPS9-domain proteins do not contribute equally to this process. Nevertheless, expression of Vrl1, but not the VPS9-domain mutant Vrl1^{D373A}, was found to rescue Vps34 localization (Figure 6D) and restore endosomal pools of PI3P in *muk1 vps9* strains (Figure 6C). Taken together, these results suggest that Vps9-domain GEFs play a key role in promoting the enrichment of PI3P at endosomes.

DISCUSSION

Here we identify two VPS9-domain Rab5-family GEFs as new retromer-associated proteins and show that their activity is important

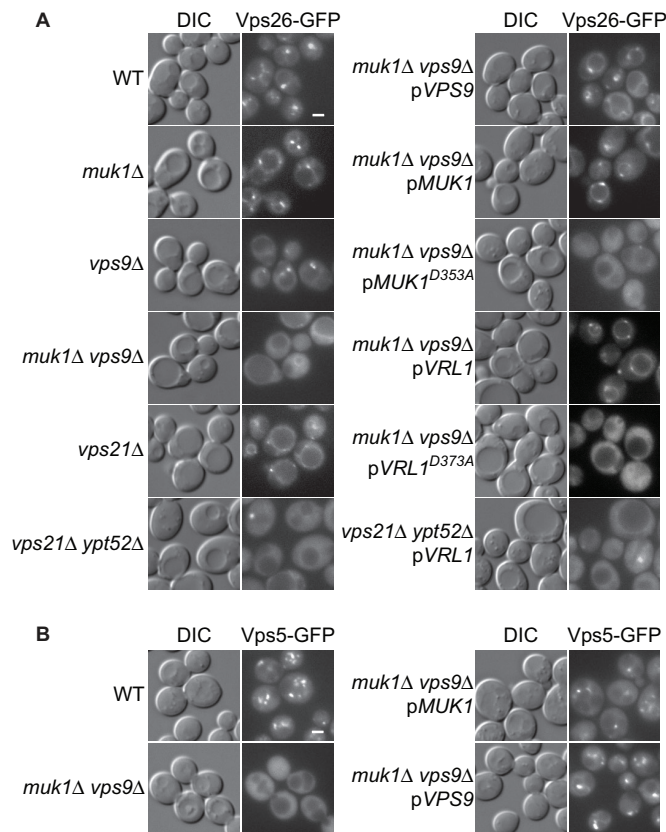


FIGURE 5: Rab5-family GEFs and GTPases are required for endosomal recruitment of retromer. (A) Fluorescence microscopy of Vps26-GFP in strains lacking Rab5-family GEFs and Rab5-family GTPases. Muk1 and Vrl1 are expressed from endogenous promoters. Muk1^{D353A} and Vrl1^{D373A} are predicted to be catalytically inactive. (B) Microscopy of Vps5-GFP in strains with deletions of Rab5-family GEFs. Scale bars, 2 μ m.

for the recruitment of retromer to endosomal membranes. Although many of the retromer accessory factors that have been identified in higher cells are absent in yeast (Harbour *et al.*, 2010; Seaman, 2012), VPS9-domain proteins constitute a broadly conserved family and may contribute to fundamental aspects of retromer assembly or function present in all eukaryotic cells.

Our coimmunoprecipitation experiments showed clear interactions between retromer and two different VPS9-domain GEFs, Muk1 and Vps9. Of interest, the interaction was mapped to the VPS9 domain of Muk1, suggesting that the binding of retromer to the VPS9 domain might regulate its GEF activity. Indeed, the human homologue of Vps9, Rabex-5, is autoinhibited by a conserved helix C-terminal to the VPS9 domain, and this is overcome by binding of the Rab5 effector Rabaptin-5 (Delprato and Lambright, 2007). Because Rabaptin-5 helps recruit Rabex-5 to endosomes, this creates a positive feedback loop that results in robust Rab5 activation (Horiuchi *et al.*, 1997; Zhang *et al.*, 2014). It is not known whether Muk1 is subject to autoinhibition or whether retromer binding could similarly affect GEF activity. However, the interaction with retromer is expected to increase the local concentration of the GEF, which would thus serve to enhance Rab5 activation at sites of retromer tubule formation.

Yeast retromer does not bind directly to Rab5-family GTPases (Liu *et al.*, 2012). Instead, we found that Rab5-family GTPases and an active VPS9-domain GEF are required for correct localization of

the PI3K Vps34 at endosomes and production of the endosomal pool of PI3P. Although Rab5 directly recruits the human homologue of Vps34, hVPS34, a similar relationship between yeast Rab5-family GTPases and Vps34 has not previously been reported (Christoforidis *et al.*, 1999). Because the structural subcomplex of retromer binds PI3P through the PX domains of Vps5/17 (Burda *et al.*, 2002), the loss of endosomal PI3P provides a likely explanation for the retromer localization defect in strains lacking active VPS9-domain GEFs.

The endosomal localization of yeast VPS9-domain GEFs does not depend on retromer, and thus a physical interaction between retromer and the GEF may not be absolutely required for endosomal PI3P production and retromer recruitment. Instead, we propose that retromer–GEF binding enhances the rate or extent of retromer assembly through a positive feedback loop like that described for Rabaptin-5 (Horiuchi *et al.*, 1997; Figure 7A). In this model, the initial recruitment of retromer to PI3P-labeled endosomes allows it to bind and concentrate Muk1 and/or Vps9, which then recruit and activate Rab5-like GTPases in the vicinity of the forming retromer tubule. This, in turn, stimulates the recruitment and activation of PI3K, increasing local PI3P production and promoting further retromer assembly. Interactions with distinct VPS9-domain GEFs could conceivably enhance retromer assembly at different endosomal compartments or in response to different stimuli. A similar model has been proposed to explain the action of the *Salmonella* effector protein SopB, which causes overactivation of Rab5, promoting PI3P production that drives the formation of extensive Snx3 and Snx1-coated tubules at *Salmonella*-containing vacuoles (Braun *et al.*, 2010).

Discovery of a new yeast VPS9-domain protein

As this article was being prepared, it was reported that the human VPS9-domain GEF hVARP interacts with retromer and that this interaction is responsible for the normal trafficking of GLUT1 from endosomes to the plasma membrane (Hesketh *et al.*, 2014). This, together with the work presented here, raises the possibility that retromer interacts with a variety of VPS9-domain GEFs and that retromer–GEF interactions may contribute to protein trafficking in yeast and mammals. The protein Vrl1, which is present in wild strains of *S. cerevisiae* but mutated in common lab strains, has many similarities to hVARP yet exhibits key differences. hVARP binds the retromer subunit VPS29 through two conserved cysteine-rich motifs, and this interaction recruits hVARP to endosomal tubules. These cysteine-rich motifs are not present in Vrl1, and we found that Vrl1 was not fully dependent on retromer for localization to endosomes. Although our coimmunoprecipitation and iMYTH experiments did not identify an interaction between retromer and Vrl1, such an interaction may be undetectable by these methods due to the relatively low level of Vrl1 expression.

A key role of hVARP in the trafficking of GLUT1 is to recruit the R-SNARE VAMP7 into retromer-derived vesicles and thus enable their fusion with the plasma membrane (Hesketh *et al.*, 2014). hVARP has a VAMP7-interacting domain (Burgo *et al.*, 2009; Ohbayashi *et al.*, 2012; Schäfer *et al.*, 2012), which is conserved in Vrl1 homologues from many fungal species, although it is less well conserved in *S. cerevisiae*. A retromer-mediated recycling pathway from endosomes to the plasma membrane has not been reported in yeast, but the laboratory strains used in most trafficking studies lack functional Vrl1. Thus it is intriguing to speculate that Vrl1, like hVARP, repurposes an R-SNARE to regulate a yet-undiscovered recycling pathway missing in lab strains but present in other yeast species.

The role of hVARP in VAMP7 transport suggests an alternative model for the function of retromer–GEF interactions: GEFs bind

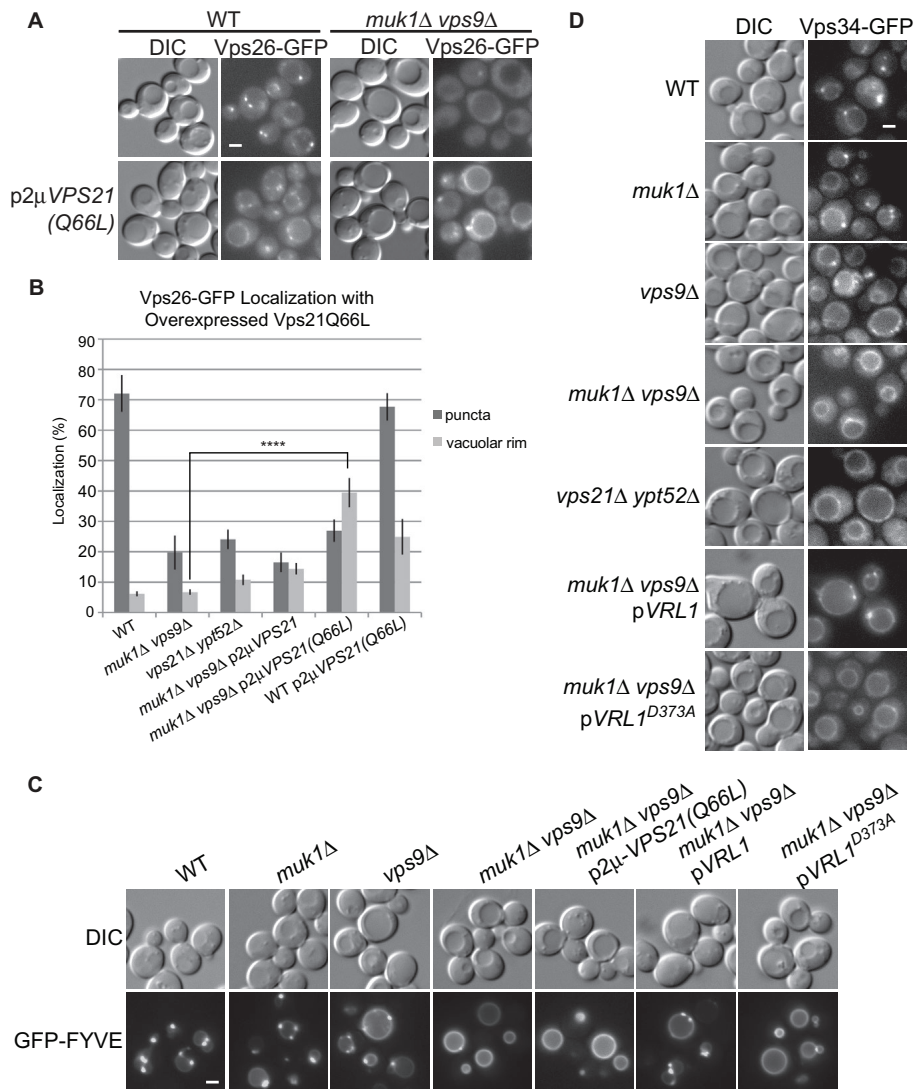


FIGURE 6: Rab5-family GEFs are needed for PI3P production at endosomes, and this cannot be bypassed by expressing the constitutively active Rab5-family GTPase Vps21(Q66L). (A) Fluorescence microscopy shows that Vps26-GFP is mislocalized to the vacuolar rim when Vps21(Q66L) is expressed, even in the absence of Rab5-family GEFs. (B) Quantification of Vps26-GFP fluorescence microscopy. Images of Vps26-GFP localization from four independent experiments were manually scored for localization to the vacuolar rim or puncta ($N \geq 140$ /strain per experiment). Unpaired one-way analysis of variance: $p < 0.0001$ overall; **** $p < 0.0001$. (C) Fluorescence microscopy of GFP-FYVE, a biomarker for PI3P, in strains with deletions of Rab5-family GEFs. (D) Vps34-GFP localization to puncta is dependent on expression of Rab5-family GEFs and GTPases. Vrl1 is expressed from the endogenous promoter in C and D. Scale bars, 2 μ m.

retromer to recruit specific cargo proteins or accessory factors to forming retromer tubules (Figure 7B). In fact, Vps9 has a ubiquitin-binding CUE domain that is important for the normal progression of ubiquitinated cargo through endosomes (Davies *et al.*, 2003; Donaldson *et al.*, 2003). Although Muk1 lacks a CUE domain or other recognizable motifs, it has conserved regions that could mediate interactions with additional factors that influence the composition or targeting of endosome-derived vesicles.

It is important to note that the two models presented in Figure 7 are not mutually exclusive. The retromer-GEF interaction could enhance retromer assembly by promoting local Rab5 activation and PI3P production while at the same time recruiting specific cargo or accessory factors. By providing a means of reinforcing local recruit-

ment, this could explain why retromer coats subdomains of endosomes (Seaman *et al.*, 1998; Temkin *et al.*, 2011). Moreover, the positive feedback model could address puzzling aspects of retromer biology in higher cells. Because the mammalian CSC does not bind tightly to the PI3P-binding sorting nexins, it is unclear what prevents the sorting nexins from driving the formation of empty tubules devoid of cargo (Cullen and Korswagen, 2012). If recruitment of hVAPB by the VPS29 subunit of the CSC also increases local PI3P production, this could enhance coassembly with the sorting nexin subcomplex and link membrane deformation to cargo recruitment.

The VPS9 family

There are three VPS9-domain proteins in yeast and at least nine in humans (Carney *et al.*, 2006). Although the yeast VPS9-domain proteins share some overlapping functions, our results suggest they have unique functions that are as yet uncharacterized. Some mammalian Vps9-domain GEFs can preferentially activate a subset of Rab5-family GTPases (Delprato and Lambright, 2007) or contain domains that confer distinct localizations (Balaji *et al.*, 2012). Although in vitro activity assays suggest that yeast VPS9-domain GEFs do not have differential specificity for Rab GTPases (Singer-Krüger *et al.*, 1994; Cabrera *et al.*, 2013; Paulsel *et al.*, 2013), we and others have found that they show differential recruitment to endosomes. Further studies will be needed to determine whether the yeast GEFs localize to distinct endosome subpopulations or associate with membranes only in response to specific regulatory inputs. It will also be important to determine whether the retromer binding is a general feature of VPS9-domain GEFs in humans and to what extent the interaction serves to reinforce retromer recruitment or select other cargo.

MATERIALS AND METHODS

Yeast strains and plasmids

A list of strains and plasmids used can be found in Supplemental Tables S1 and S2. With the exception of the strains to be described here, all strains from this study were made by homologous recombination as described (Longtine *et al.*, 1998; Janke *et al.*, 2004; Sheff and Thorn, 2004). The iMYTH strains were made via integration of the L2 Cub cassette as previously described (Snider *et al.*, 2013). VPS34 and VPS5 were tagged with a bright GFP variant, GFP+ (Scholz *et al.*, 2000), by amplifying and transforming GFP+::NAT from pLC1318, a gift from R. Rachubinsky (University of Alberta).

Plasmids were made by homologous recombination in yeast (Scholz *et al.*, 2000), rescued in *Escherichia coli*, and confirmed by sequencing. To make pGFP-FYVE(EEA1)::LEU2 (pBB21), pGFP-FYVE(EEA1)::TRP1 (#36096; Addgene, Cambridge, MA) was cut

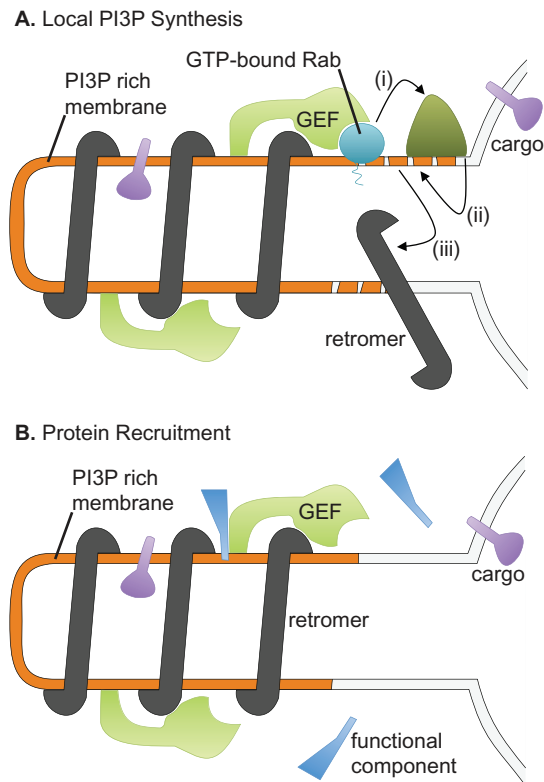


FIGURE 7: Models for the function of the interaction between retromer and Rab5-family GEFs at endosomes. (A) In the first model, the GEFs concentrate Rab5-family GTPases at the retromer tubules. The GTPases recruit the PI3K Vps34 (i), which locally increases the concentration of PI3P (ii), leading to further retromer recruitment (iii). (B) In the second model, GEFs physically recruit specific factors to the tubules.

with *Bsu361* and cotransformed with a *LEU2* PCR fragment containing flanking homology to *TRP1* 5' and 3' regions. *pNubG-HA-MUK1 FL* (pAO470), *pNubG-HA-MUK1 1-417* (pAO538), and *pNubG-HA-MUK1 74-417* (pAO542) were made by cotransforming *MUK1* gene regions amplified from yeast genomic DNA together with *SmaI*-digested pPR3-N MYTH prey vector, as described previously (Snider *et al.*, 2013). The *Muk1 D353A* mutant (pAO531) was made via site-directed mutagenesis of *pNubG-HA-MUK1 FL* (pAO470) construct following the QuikChange II protocol (Agilent, Santa Clara, CA). *pNubG-HA-VPS9* (pBB9) and *pNubG-HA-VRL1* (pBB24) were made by *EcoRI*-HF/*Clal* digestion of pPR3-N and its cotransformation with the respective genes as previously described (Snider *et al.*, 2013).

Other *MUK1* plasmids made in this study were based on pRS416 (*URA3 CEN*) as follows. We amplified 972 base pairs of the 5' untranslated region of *MUK1*, referred to as *MUK1pr*, using primers with homology to pRS416 and two-thirds of a 3HA tag. A second product containing *MUK1* was generated using primers with homology to two-thirds of the 3HA tag and a downstream pRS416 sequence. The two products were cotransformed with *KpnI*/*SacI*-digested pRS416 to generate *pMUK1pr-3HA-MUK1* (pMD120). pMD120 was *AatII*/*BamHI* digested and cotransformed with a GFP PCR product with ends homologous to 5' and 3' of the 3HA tag to make *pMUK1pr-GFP-MUK1* (pMD121). The pMD121 *Muk1* promoter was *KpnI*/*BamHI* digested, and the cut plasmid was cotransformed with an *ADH1* promoter PCR product that had homology outside of the cut region, forming *pADHpr-GFP-MUK1*

(pMT1). Both pMD120 and pMT1 were cut with *HindIII*/*NruI* and cotransformed with PCR products containing *MUK1-D353A* with terminal homology outside the cut sites to generate *pMUK1pr-3HA-MUK1-D353A* (pBB12) and *pADH1pr-GFP-MUK1-D353A* (pBB14). pMD120 and pBB12 were cut with *KpnI* and transformed with an *ADH1* PCR product with flanking *MUK1* homology to form *pADH1pr-3HA-MUK1* (pBB33) and *pADH1pr-3HA-MUK1-D353A* (pBB34). *pADH1pr-GFP-MUK1(1-575)* (pBB15) was made by cutting out base pairs 544–1800 of *MUK1* from pMT1 using *BglII* and cotransforming the cut plasmid with a PCR product containing base pairs 1–1725 of *MUK1* with flanking homology to the plasmid. *pADH1pr-GFP-MUK1(503-612)* (pMT2) was made by excising the N-terminus of *MUK1* in pMT1 with *Clal*/*HindIII* and cotransforming the plasmid with a hybridized oligo with homology to GFP and *MUK1* after base pair 1506.

VRL1 plasmids were based on *pADH1pr-GFP-MUK1* (pMT1). Genomic DNA was used as a template for *VRL1*, and primers were used to correct the thymine deletion in *YML003W* of the parental yeast strain. The DNA upstream of the deletion was amplified with the reverse oligo (5' ATATTTATATTTTTCAGTGTCTACTTCGTGGCCTTTGAAATGTGTAGTAAGCCTAGACCA), and the region downstream was amplified with (5' TGGTCTAGGCTTACTACACATTTCA AAGGCCACGAAGTAGACACTGAAAAATATAAATAT). The reverse oligo was used to amplify upstream *YML003W* with homology to either the *ADH1* promoter or GFP of pMT1, and the forward oligo was used to amplify downstream *YML003W* and *YML002W* with homology to pMT1 after *MUK1*. The two sets of PCR products were cotransformed with *HindIII*-cut pMT1 to make plasmids *pADH1pr-VRL1* (pBB23) and *pADH1pr-GFP-VRL1* (pBB22). The GFP tag of pBB22 was cut with *HpaI* and cotransformed with an oligo containing 3HA and flanking homology to make *pADH1pr-3HA-VRL1* (pBB25). Expression of N-terminally HA- or GFP-tagged forms of *VRL1* resulted in a protein of the expected size. The *VRL1* promoter was substituted for the *ADH1* promoter by cutting pBB25 with *SphI* and cotransforming with a PCR product containing 366 base pairs upstream of *YML003w* and flanking homology to form *pVRL1pr-3HA-VRL1* (pBB25). pBB25 was cut with *MscI* and *BglII* and cotransformed with two overlapping PCR products containing *VRL1* with the D373A mutation, forming *pVRL1pr-3HA-VRL1D373A* (pBB32).

Growth and colony overlay assays

For the concentration-limiting growth assays, 4 μ l of 1 OD₆₀₀/ml log-phase yeast was spotted onto yeast extract/peptone/dextrose (YPD) medium in 10 \times serial dilutions and imaged using a CanoScan 4400F scanner after 2 d of growth at the indicated temperatures. For the colony overlay assay, yeast (containing pRS415 and pRS416 as required) were spotted onto synthetic amino acid dropout medium lacking histidine and uracil and overlaid with a nitrocellulose membrane. After 16 h, the membrane was blotted with mouse anti-carboxypeptidase Y (A6428; Molecular Probes, Carlsbad, CA) and then goat anti-mouse conjugated to horseradish peroxidase (HRP; 115035146; Jackson ImmunoResearch, West Grove, PA). The blot was developed with the enhanced chemiluminescent West Pico (34077; Pierce, Rockford, IL) and exposed to Amersham Hyperfilm (28906839; GE Healthcare, Little Chalfont, United Kingdom).

Western blotting and coimmunoprecipitation

For coimmunoprecipitation from batch cultures, 50-ml samples of the appropriate strains were grown in YPD medium to mid log phase, washed, and transferred to galactose medium for 1 h. Cells were harvested, resuspended in an equal volume of IPLB buffer

(20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid-KOH, pH 7.4, 150 mM KOAc, 2 mM Mg(Ac)₂, 1 mM ethylene glycol tetraacetic acid, 10% glycerol, protease inhibitor cocktail, and 1% Triton), and disrupted by glass beads for 5 min. Cell lysates were then cleared by centrifugation at 1500 × g for 3 min. Half of the cleared lysate was incubated with 50 μl of calmodulin beads for 2 h and washed with 10 volumes of fresh IPLB. Then 10 μl of loading buffer (5% SDS, 50 mM Tris, pH 6.8, 0.4 mg/ml bromophenol blue, and 1% β-mercaptoethanol) was added to 20 μl of beads, and eluates were resolved on 12% SDS-PAGE gels. Gels were then transferred to nitrocellulose membrane using the iBlot transfer system following the manufacturer's instructions (Life Technologies, Carlsbad, CA). Membranes were probed using anti-HA (sc-805; Santa Cruz Biotechnology, Dallas, TX) or anti-TAP (#A01435; GenScript, Piscataway, NJ) rabbit primary antibodies and HRP-tagged goat anti-rabbit secondary antibody (#31462; Pierce) and visualized using a Kodak image station 4000.

Western lysates were prepared from log-phase cells by bead bashing, freezing, and resuspension in Thorner buffer (8 M urea, 5% SDS, 50 mM Tris, pH 6.8, 0.4 mg/ml bromophenol blue, and 1% β-mercaptoethanol). Lysates were heated to 70°C, and the equivalent of 0.5 OD₆₀₀ of cells was loaded into 10% SDS-PAGE gels. In cross-linking coimmunoprecipitation experiments, fresh spheroplasts were prepared by digesting cell walls with Zymolyase (SK1204911; MJS BioLynx, Brockville, Canada). We cross-linked 20 OD₆₀₀ of spheroplasts with 1.6 mg/ml DSP and lysed them as described (Čopič *et al.*, 2007), except that we used 1% *n*-dodecyl-β-D-maltoside in the lysis buffer instead of Triton X-100. The lysates were incubated at 4°C with rabbit anti-HA (sc-805; Santa Cruz Biotechnology) or rabbit anti-GFP (A6455; Molecular Probes), followed by protein A-Sepharose beads (17-5280-04; GE Healthcare). The beads were washed, and proteins were eluted by heating at 95°C for 5 min. Samples were loaded into 10% SDS-PAGE gels. For both Western blots and coimmunoprecipitations, proteins were transferred overnight to nitrocellulose membranes and blotted with either mouse anti-GFP (11814460001; Roche, Basel, Switzerland) or mouse anti-HA (MMS-101R; Covance, Princeton, NJ). Probing with secondary antibodies and exposure were done as in the colony overlay assay.

IMYTH

Log-phase yeast were serially diluted by a factor of 10 from 1 OD₆₀₀, and 4 μl was spotted onto synthetic dextrose dropout plates lacking tryptophan, adenine, and histidine. Tryptophan selected for Nub plasmids and adenine and histidine for an interaction between the Cub bait and Nub prey constructs (Snider *et al.*, 2013). Yeast were grown at 37°C for 4 d and then imaged using a CanoScan 4400F scanner.

Fluorescence microscopy

Log-phase yeast were imaged in minimal selective medium at room temperature with a Plan-Apochromat 100×/1.40 numerical aperture oil immersion objective lens on an Axioplan 2 fluorescence microscope (Carl Zeiss, Jena, Germany). Images were taken with a CoolSNAP camera (Roper Scientific; Tucson, AZ) using MetaMorph 7.7 software (MDS Analytical Technologies, Sunnyvale, CA) and adjusted using MetaMorph and Photoshop CS5 (Adobe, San Jose, CA). Exposure times varied from 100 ms to 3 s based on the protein tagged with GFP or RFP but were kept the same within a given experiment. When the FM4-64 (T-3166; Life Technologies) lipophilic dye was used, cells were incubated with the dye for 1 h in minimal medium, washed once, and then grown another hour in minimal medium before imaging. Cellular features were quantified by manually scoring images.

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