The VINE complex is an endosomal VPS9-domain GEF and SNX-BAR coat

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Abstract
Membrane trafficking pathways perform important roles in establishing and maintaining the endosomal network. Retrograde protein sorting from the endosome is promoted by conserved SNX-BAR-containing coat complexes including retromer which enrich cargo at tubular microdomains and generate transport carriers. In metazoans, retromer cooperates with VARP, a conserved VPS9-domain GEF, to direct an endosomal recycling pathway. The function of the yeast VARP homolog Vrl1 has been overlooked due to an inactivating mutation found in commonly studied strains. Here, we demonstrate that Vrl1 has features of a SNX-BAR coat protein and forms an obligate complex with Vin1, the paralog of the retromer SNX-BAR protein Vps5. Unique features in the Vin1 N-terminus allow Vrl1 to distinguish it from Vps5, thereby forming a complex that we have named VINE. The VINE complex occupies endosomal tubules and redistributes a conserved mannose 6-phosphate receptor-like protein from endosomes. We also find that membrane recruitment by Vin1 is essential for Vrl1 GEF activity, suggesting that VINE is a multifunctional coat complex that regulates trafficking and signaling events at the endosome.

Introduction
Transport of proteins and lipids at the endosome requires the concerted action of peripheral cargo-sorting complexes, Rab-family GTPases, membrane tethering complexes and soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) proteins (Barr and Lambright, 2010; Burd and Cullen, 2014; Jahn and Scheller, 2006; Numrich and Ungermann, 2014; Pfeffer, 2017; Stenmark, 2009; van Weering et al., 2010). Guanine nucleotide exchange factors (GEFs) belonging to the conserved VPS9 family are important regulators of endosomal function that activate endosomal Rab5-like GTPases (Carney et al., 2006; Delprato and Lambright, 2007). In yeast, the VPS9-domain GEFs Muk1 and Vps9 stimulate the endosomal Rabs Vps21, Ypt52 and Ypt53 to perform downstream functions including activating phosphoinositide 3-kinase (PI3K) to produce the anionic lipid species phosphatidylinositol 3-phosphate (PI3P; Christoforidis et al., 1999; Hama et al., 1999; Paulsel et al., 2013; Peplowska et al., 2007; Singer-Krüger et al., 1994). Together, PI3P and endosomal Rabs are important determinants of endosomal identity and are responsible for recruiting effectors, including coat proteins and vesicle tethers, to the endosomal membrane.

Sorting nexins (SNXs) are a conserved family of proteins that perform direct roles in endosomal trafficking by binding to transmembrane cargo proteins and enriching them into sorting domains (Carlton et al., 2005; Hong, 2019). SNXs localize to the endosome through conserved Phox homology (PX) domains that typically recognize PI3P (Cheever et al., 2001; Xu et al., 2001). A sub-family of SNXs known as SNX-BARs additionally contain a Bin/Amphiphysin/RVS (BAR) domain that mediates
dimerization with other BAR domain-containing proteins and imparts membrane binding/deforming properties (Frost et al., 2009; van Weering et al., 2012, 2010). These SNX-BAR dimers, which are capable of deforming cargo-rich membranes into sorting tubules, are emerging as important regulators of protein transport. The seven SNX-BAR proteins present in yeast include the conserved retromer subunits Vps5 and Vps17 (Horazdovsky et al., 1997), the SNX8 homolog Mvp1 (Suzuki et al., 2021), the SNX4 homolog Snx4 and its partners Snx41 and Atg20 (SNX7 and SNX30 in humans, respectively; Hetterma et al., 2003) and the Vps5 paralog Ykr078w. Retromer and Snx4 complexes promote cargo sorting from the endosome and vacuole (Arlt et al., 2015; Suzuki and Emr, 2018), while Mvp1 appears to function only at the endosome (Suzuki et al., 2021). There is no known sorting function for Ykr078w, which lacks a clear subcellular localization.

The best characterized of these SNX-BAR-containing complexes is the heteropentameric retromer complex, which is composed of the Vps26-Vps35-Vps29 trimer and the Vps5-Vps17 SNX-BAR dimer (Seaman et al., 1998). Retromer localizes to PI3P-rich membranes where it promotes the retrograde sorting of cargo including the well-characterized carboxypeptidase Y (CPY) receptor Vps10 (Burda et al., 2002; Seaman et al., 1998, 1997). In metazoans, the term retromer refers specifically to the VPS26-VPS35-VPS29 trimer which associates with a variety of adaptor proteins including SNXs to promote cargo sorting (Cullen and Korswagen, 2012; Gallon and Cullen, 2015). Mutations in VPS35 have been linked to neurodegenerative conditions including Parkinson’s disease and Alzheimer’s disease (Mohan and Mellick, 2017; Rahman and Morrison, 2019; Vilariño-Güell et al., 2011; Wen et al., 2011; Zimprich et al., 2011), establishing a connection between endosomal transport machinery and human neurological health.

We previously identified a physical association between yeast retromer and the VPS9-domain GEFs Vps9 and Muk1, whose activity is required to maintain endosomal pools of PI3P for retromer recruitment (Bean et al., 2015). We also identified a novel VPS9-domain protein, Vrl1, which is mutated and non-functional in strains previously used for trafficking studies. Vrl1 can function as the sole VPS9-domain GEF to stimulate production of endosomal PI3P. Notably, the human homolog of Vrl1, VARP, physically associates with retromer to drive an endosome-to-plasma membrane sorting pathway (Hesketh et al., 2014). Here, we show that Vrl1 is a member of the SNX-BAR protein family and that it specifically binds the Vps5 paralog Ykr078w/Vin1 to form what we now call the VINE complex. Our results suggest that VINE is both a VPS9-domain GEF and a SNX-BAR coat complex that may operate alongside the retromer, Mvp1 and Snx4 pathways.
Results

**Vrl1 is a predicted PX-BAR protein that interacts with conserved machinery at the endosome.**

Vrl1 and its human ortholog VARP share a VPS9 domain, as well as a conserved N-terminus and ankyrin repeat domain (AnkRD) that are not found in other yeast VPS9-domain GEFs (Herman et al., 2018; Figure 1A). Vrl1 also features a ~350 amino acid (aa) unannotated region downstream of the AnkRD that is not present in VARP. The protein fold recognition program Phyre2 (Kelley et al., 2015) identified a PX-BAR module with very high confidence (98.6%) in this region (aa 737-1089; Figure 1 S1A), and ab initio modeling of this region by AlphaFold2-powered ColabFold software (Mirdita et al., 2022) predicted a structure with striking similarity to the PX-BAR fold (Figure 1B, 1 S1B and 1 S1C). Because the predicted Vrl1 PX domain is missing key residues for PI3P binding (Figure 1 S1D), we refer to it as a “PX-like” domain. To our knowledge, Vrl1 is the first VPS9 domain-containing protein with predicted structural homology to the SNX-BAR family.

We found that Vrl1, when C-terminally tagged with the bright GFP variant Envy, was present at perivacuolar puncta that colocalize with the endosomal marker Did2-mRuby2, but not the Golgi protein Sec7-dsRed (56% and 2%, respectively, P < 0.0001; Figure 1C and 1D). These observations indicate that unlike other yeast VPS9-domain GEFs (Paulsel et al., 2013), Vrl1 constitutively localizes to endosomes. To identify endosomal partners of Vrl1, we performed a protein fragment complementation assay (PCA) based on a drug-resistant variant of the dihydrofolate reductase (DHFR) enzyme (Figure 1E; Michnick et al., 2010; Tarassov et al., 2008). Proximity between two proteins that are fused to complementary DHFR fragments reconstitutes enzyme activity and confers resistance to the inhibitor methotrexate. Full-length Vrl1, and a cytosolic fragment of Vrl1 lacking the AnkRD, PX-like and BAR domains (Vrl1\(^{1-465}\); Figure 1 S2), were expressed as DHFR\(^{Nt}\) fusions under the control of the constitutive *ADH1* promoter (*ADH1pr*). Z-scores were generated from the colony area ratio of full-length Vrl1 vs Vrl1\(^{1-465}\) (Figure 1F, Supplementary File 1). This identified the endosomal Rab GTPases Vps21 (Z = 18.7) and Ypt52 (Z = 7.6), and other conserved endosomal proteins including the retromer subunit Vps35 (Z = 13), the hydrolase receptors Vps10 (Z = 12.9) and Mr1 (Z = 7.7), and components of the Class C Core complex Vps11 (Z = 16.9) and Vps18 (Z = 12.2; Figure 1F). Functional enrichment analysis of Vrl1 interactors (Z > 2) highlighted relationships with other subunits of endosomal complexes including retromer and the CORVET complex (Figure 1G, Supplementary File 2; Ashburner et al., 2000; Gene Ontology Consortium, 2021). These results suggest that Vrl1 is an endosomal SNX-BAR-like protein that contacts both membrane tethering and trafficking machinery.

**Vrl1 and the Vps5 paralog Vin1 form the VINE complex**
Our DHFR screen identified a strong connection between Vrl1 and the uncharacterized SNX-BAR Ykr078w (Z = 9.5), the paralog of membrane-binding retromer subunit Vps5 (Byrne and Wolfe, 2005; Horazdovsky et al., 1997), which we have named “Vrl1-Interacting Sorting Nexin 1” or Vin1 (Figure 2A). Vin1 has a reported cytosolic distribution (Huh et al., 2003), which is surprising given that its paralog Vps5 localizes to endosomes in a PI3P-dependent manner (Burda et al., 2002) and that Vin1 interacts with PI3P in vitro (Yu and Lemmon, 2001). Increasing Vin1 levels did not alter its cytosolic distribution pattern which we observed in both endogenously expressed N- and C-terminally tagged strains (Figure 2 S1A and 2B). Since common laboratory *S. cerevisiae* strains carry the non-functional mutant *vrl1* allele (Bean et al., 2015), we wondered if complementing this mutation with a plasmid-expressed copy of *VRL1* featuring the corrected sequence (p*VRL1*) would affect the localization of Vin1. Indeed, we found that expression of *VRL1* caused a dramatic redistribution of Vin1-Envy from the cytosol to intracellular puncta (P < 0.0001; Figure 2B and 2C; Figure 2 S1A), and that over-expressing *VRL1* from the *ADH1pr* further increased the number of bright Vin1-Envy puncta (P < 0.0001; Figure 2 S1B and S1C). Deletion of *VIN1* prevented Vrl1-Envy from forming intracellular puncta (P < 0.001; Figure 2B and 2C), suggesting that the localization of Vrl1 and Vin1 is highly interdependent.

Vrl1 and Vin1 are also dependent on each other for stability, as the levels of triple hemagglutinin (3HA)-tagged Vrl1 and Vin1 were severely reduced in strains lacking *VIN1* or *VRL1* (17% of WT, P < 0.001 and 18% of WT, P < 0.01, respectively; Figure 2D and 2E). We found that Vin1-3HA strongly co-purified with Vrl1-Envy (64% recovery of Vin1; Figure 2F), and Vrl1-Envy and Vin1-mScarletI (-mScI) showed a high degree of colocalization at endosomal puncta (88%; Figure 2G), suggesting that these proteins form a complex.

Since both Vin1 and Vrl1 have predicted PX domains, we wondered if, like other SNX-BARs, they bind PI3P at endosomes. Vrl1 was displaced to the cytosol in a PI3K deletion mutant (*vps34Δ*, P < 0.01; Figure 2H and 2I). Since the Vrl1 PX domain is missing residues that are typically required to bind PI3P, this suggests the PX domain of Vin1 is important for endosomal recruitment. Indeed, a recent study demonstrated that Vin1, therein referred to as Vps501, binds to PI3P through an unconventional motif in its PX domain (Goyal et al., 2022). PI3K is activated by Rab5-like GTPases (Christoforidis et al., 1999), which in turn require VPS9-domain GEFs for their activity (Carney et al., 2006; Delprato and Lambright, 2007). We found that, in a *muk1Δvps9Δ* strain that lacks all other VPS9-domain GEFs, Vrl1 localization is dependent on a conserved catalytic residue in the VPS9 domain (Vrl1<sup>1037H</sup>; Bean et al., 2015; P < 0.001; Figure 2J and 2K), suggesting that Vrl1 may leverage its ability to stimulate endosomal PI3P production and promote its own membrane recruitment.
Taken together, our results suggest that Vrl1 and Vin1 form a novel complex that localizes to endosomes in a PI3P-dependent manner (Figure 2L). Since neither Vrl1 or Vin1 are stable or capable of membrane localization in the absence of the other, we reason that these proteins primarily exist as members of this complex which we have named the “VPS9 GEF-Interacting Sorting Nexin” or VINE complex.

**Vrl1 is predicted to form a BAR-BAR dimer with both Vin1 and Vps5**

SNX-BAR proteins interact via an extensive hydrophobic interface between the BAR domains (van Weering et al., 2012). ColabFold software (Mirdita et al., 2022) predicted that the PX-like and BAR domains of Vrl1 (aa 732-1090) bind to the PX and BAR domains of Vin1 (aa 110-585) to form a canonical SNX-BAR dimer (pTMscore = 0.75; Figure 3A and 3 S1A). To assess the accuracy of ColabFold in predicting specific BAR domain pairings, we systematically modelled pairwise homotypic and heterotypic interactions of all yeast SNX-BAR proteins (Figure 3B, Supplementary File 3). This accurately predicted the homodimerization of Mvp1 (Suzuki et al., 2021) and the heterodimerization of Vps5/Vps17 (Seaman and Williams, 2002). Neither Vrl1 nor Vin1 were predicted to form homodimers, though unexpectedly Vrl1 was predicted to pair equally well with both Vin1 and its paralog Vps5. By comparing plots of predicted alignment error (PAE) for different combinations of SNX-BARs we found that Vrl1 and Vps5 exhibit high confidence interactions with Vin1 and Vps17, respectively, whereas Vrl1 and Vps17 were predicted not to interact (Figure 3 S1B).

Given that Vrl1 was predicted to interact with both Vin1 and Vps5, we wondered if Vrl1 could functionally partner with Vps5 to form a novel retromer-like complex. First, we tested all known yeast SNX-BAR proteins for their ability to bind Vrl1 and found that Vin1 alone interacts with Vrl1 (Figure 3C). Vrl1 also failed to bind Vps5 when VIN1 was deleted from a strain that over-expresses Vrl1 to compensate for its instability in the vin1Δ mutant (Figure 3D; Figure 3 S2), suggesting that a possible Vrl1/Vps5 interaction was not overlooked due to competition from Vin1. Further, Vrl1 did not interact with any of the other retromer subunits (Figure 3 S3A), but this assay could fail to detect weak or transient interactions. Using functional readouts, we found that Vrl1 was unable to promote the endosomal localization of Vps10 (Figure 3 S3B) or Vps35 (Figure 3 S3C and S3D) in strains lacking Vps5 and/or Vps17, indicating that Vrl1 does not functionally pair with retromer SNX-BARs and that Vin1/Vrl1 cannot replace Vps5/17 to form a retromer-like complex. These results further suggest that Vrl1 has strong paralog specificity and that interactions beyond the BAR-BAR interface could be responsible for its specific recognition of Vin1.

**Vrl1 interacts with Vin1 primarily via the AnkRD**

To identify regions critical for Vrl1/Vin1 binding, we quantified the membrane recruitment of Vin1-mScarletI in cells expressing a series of Envy-tagged Vrl1 fragments (Figure 3E and 3F). We found that
the GEF-deficient mutant (Vrl1<sup>D373A</sup>; Bean et al., 2015) and the N-terminal truncation (Vrl1<sup>158-end</sup>) significantly recruited Vin1 (P < 0.0001 and P < 0.001, respectively; Figure 3G) despite the weaker punctate localization of the Vrl1<sup>158-end</sup> construct relative to WT, which could explain its reduced recruitment of Vin1. Deletion of C-terminal sequences (i.e. Vrl1<sup>1-465</sup>) blocked the membrane localization of both Vrl1 and Vin1. To test the role of the Vrl1 PX-like and BAR domains, we replaced this region with a localization module consisting of the PI3P-binding PX domain of Ypt35 fused to Envy which we refer to as “YPE” (Figure 3H). Strikingly, the resulting Vrl1(1-703)<sup>YPE</sup> chimera strongly recruited Vin1 to puncta (P < 0.0001; Figure 3G), suggesting that BAR-BAR interactions are dispensable for Vin1 recruitment. A further truncation that removed the AnkRD to create Vrl1(1-465)<sup>YPE</sup> localized to endosomes yet failed to recruit Vin1 (Figure 3F and 3G), indicating that the AnkRD contains a potent interacting interface for Vin1. In support of this idea, the Vrl1<sup>465-end</sup> fragment which contains the AnkRD, PX-like and BAR domains weakly localized and recruited a small but significant amount of Vin1 (P < 0.05) while the Vrl1<sup>700-end</sup> fragment containing only the PX-like and BAR domains did not localize or recruit Vin1 (Figure 3F and Figure 3G).

We then tested the Vrl1 truncation series (Figure 3E) for the ability to CoIP Vin1-3HA (Figure 3I), and found the Vrl1 constructs that strongly recruited Vin1 to puncta also showed physical interactions by CoIP. Taken together, the Vin1 recruitment assays, CoIPs and structural predictions suggest that the VINE complex assembles primarily through an interaction between Vin1 and the Vrl1 AnkRD, while a secondary interaction between the Vin1 and Vrl1 BAR domains may occur at the endosomal membrane.

**The Vrl1 AnkRD recognizes a small region of the disordered Vin1 N-terminus**

AnkRD interactions may explain how Vrl1 discriminates between Vin1 and its paralog Vps5. Vin1 and Vps5 have unstructured N-terminal regions preceding their respective PX domains (Vin1<sup>1-116</sup> and Vps5<sup>1-276</sup>; Figure 4A and 4 S1). When we expressed the N-terminal regions of Vin1 or Vps5 fused to mScarletI (Figure 4B and 4C), we observed strong recruitment of the Vin1 N-terminus by the Vrl1(1-703)<sup>YPE</sup> chimera, but not the YPE module alone (P < 0.0001; Figure 4D). In contrast, we detected no recruitment of the Vps5 N-terminus by any of our tested constructs suggesting that the N-terminal regions of the paralogous SNX-BARs dictate specificity for Vrl1.

We noticed that WT Vrl1-Envy recruited WT Vin1-mScI to colocalizing puncta (Figure 4C), but was unable to recruit the Vin1 N-terminus. The endogenous, untagged Vin1 may outcompete the Vin1 N-terminal fragment for recruitment by Vrl1, however this could not be tested directly because WT Vrl1 failed to localize when the Vin1 N-terminus was expressed in a vin1<sup>Δ</sup> strain (Figure 4 S2A and S2B). This observation indicates that the Vin1 PX and BAR domains also contribute to VINE assembly and membrane recruitment.
We generated an alignment from fungal orthologs of Vps5 and Vin1 (Figure 4 S3; Byrne and Wolfe, 2005) and identified three relatively conserved regions in the Vin1 N-terminus (Figure 4E). When each of these fragments was fused to mScarletI (Figure 4F), only region 3 (Vin1 aa 76-95) was recruited to puncta by Vrl1(1-703)YPephy in a vin1Δ strain (P < 0.05; Figure 4G). This suggests that the Vrl1 AnkRD distinguishes Vin1 from Vps5 through a short sequence in the unstructured Vin1 N-terminus.

ColabFold confidently predicted an interaction between Vrl1(1-703) and the minimal Vin1 fragment (Vin176-95; Figure 5A, 5B and 5 S1A). In this model, Vin176-95 binds Vrl1 at an interface between the VPS9 domain and AnkRD that is conserved in the Saccharomycetaceae family (Figure 5C). To identify other potential Vrl1-binding regions, we performed a prediction with the entire Vin1 N-terminal sequence and found that the exact Vin176-95 region that we identified in our subcellular recruitment assay (Figure 4F and 4G) was the only sequence predicted to associate with Vrl1 (Figure 5 S1B and 5 S1C). The Vin176-95 fragment contains a run of consecutive basic residues (Figure 5D). Interestingly, acidic and polar residues in the corresponding Vrl1 AnkRD interface were among the most conserved within Saccharomycetaceae (Figure 5D).

To assess the relative contribution of residues in the Vrl1 AnkRD site, we generated a series of stable Vrl1 mutants in the context of the Vrl1(1-703)YPephy chimera (Figure 5 S2). When five of the conserved acidic and polar residues were simultaneously substituted with alanine (EEDNE-5A), recruitment of the Vin1 N-terminus was lost (P < 0.0001; Figure 5E and 5F). In addition, swapping the charges of either E510 or D511 resulted in either complete or severe loss of recruitment, respectively (P < 0.0001; Figure 5E and 5F). These experiments validate the predicted interaction interface in the Vrl1 AnkRD and suggests that Vrl1 binds the Vin1 N-terminus through electrostatic interactions.

Vin1 regulates Vrl1 GEF activity via membrane localization

Disruption of the other VPS9-domain GEF proteins results in a severe temperature sensitivity phenotype and loss of endosomal PI3P (Paulsel et al., 2013; Singer-Krüger et al., 1994) that is rescued by Vrl1 in an activity-dependent manner (Bean et al., 2015). We found that deletion of VIN1, but not VPS5, prevented Vrl1 from rescuing the temperature sensitivity of the muk1Δvps9Δ strain (Figure 6A). We also found that localization of a fluorescent PI3P biosensor (Figure 6B) was restricted to the vacuolar membrane in muk1Δvps9Δvin1Δ cells expressing Vrl1 (Figure 6C), suggesting that VINE promotes the synthesis of endosomal PI3P only when fully assembled.

We previously found that Vrl1 recovers the PI3P-dependent endosomal localization of retromer in a muk1Δvps9Δ strain (Bean et al., 2015). By quantifying the localization of the endogenously tagged retromer subunit Vps26-GFP (Figure 6D), we reproduced this finding and found that deletion of VIN1
blocked rescue (P < 0.01; Figure 6E). We next tested if Vin1 was still required for Vrl1 activity when the PX-like and BAR domains of Vrl1 were replaced by the YPE endosomal anchor (Figure 3H) using Vps26-mScarletI localization as a readout (Figure 6F). We observed that Vrl1(1-465)YPE and Vrl1(1-703)YPE, but not WT Vrl1, fully rescued the endosomal localization of Vps26-mScarletI in a muk1Δvps9Δvin1Δ strain (P < 0.001; Figure 6G). These results suggest that Vin1 regulates the activity of Vrl1 by promoting its localization to endosomes.

The VINE complex exhibits characteristics of a SNX-BAR coat complex

We wondered if the VINE complex occupies endosomal membrane tubules as other SNX-BAR coat complexes do (Suzuki et al., 2021; van Weering et al., 2012; Zhang et al., 2021). To test this, we over-expressed both Vrl1 and GFP-Vin1 from the ADH1 and NOP1 promoters, respectively, and acquired images of GFP-Vin1 at 100ms intervals (Figure 7A). When compared to the endosomal marker Did2-mRuby2, we could observe GFP-Vin1 on tubular structures that eventually underwent scission and separated from the endosome (Figure 7A and 7B).

The budding of VINE-coated endosomal tubules suggests the VINE complex could transport cargo proteins from this organelle. We examined the localization of several candidate cargo proteins in the presence and absence of Vrl1 (Figure 7C), including the mannose 6-phosphate receptor (MPR) homolog Mr1, which had a strong DHFR interaction score with Vrl1 (Z = 7.7; Figure 1F), and two proteins that require other SNX-BARs for their transport (Seaman et al., 1998, 1997; Suzuki and Emr, 2018; Suzuki et al., 2021). We found that VRL1 expression had no significant effect on the localization of the retromer cargo Vps10 or the Snx4 cargo Atg27 but caused a significant decrease in the number of bright Mr11-GFP puncta per cell (61% decrease relative to vrl1, P < 0.05; Figure 7D). The bright Mr11 puncta in vrl1 cells colocalize with the endosomal markers Did2-Envy and GFP-Vps21 (69% and 48%, respectively), but not with the vacuolar marker GFP-Ypt7 (9.6%, Figure 7 S1A and S1B), suggesting that Vrl1 redistributes Mr11 from endosomes. Vrl1 GEF activity was not required for this effect (P < 0.05; Figure 7E and 7F), indicating that Vrl1 does not alter Mr11 distribution by influencing the local activity of endosomal Rabs. Correction of the vrl1 frameshift mutation using CRISPR-Cas9 gene editing technology restored the punctate localization of Vin1 (Figure 7 S1C) and caused a similar change in Mr11 localization (P < 0.01; Figure 7 S1D and S1E). This Vrl1-dependent change in Mr11 distribution suggests that the VINE complex directly or indirectly regulates the Mr11 intracellular trafficking itinerary.

The VINE complex could redistribute Mr11 by recognizing sequences in its cytosolic tail or bind another protein that interacts with the MPR-like lumenal domain of Mr11. A Vps10 mutant missing its cytoplasmic tail (Vps10ΔCt) lacks sorting signals and is transported to the vacuolar membrane and lumen (Bean et al., 2017; Cereghino et al., 1995; Cooper and Stevens, 1996). We hypothesized that if the Mr11...
tail contains a signal for VINE-mediated sorting, transplanting it onto Vps10ΔCt (Vps10ΔCt-Mrl1Ct; Figure 7G) would confer VINE-dependent effects. We found that expression of VRL1 did not alter the localization of WT Vps10 or Vps10ΔCt, the latter of which was targeted to the vacuolar membrane (Figure 7H). Vps10ΔCt-Mrl1Ct localized to perivacuolar puncta and the vacuolar membrane in vrl1 cells, indicating the presence of VINE-independent sorting signals in the Mrl1 tail (Figure 7H). VRL1 expression caused a small but significant decrease in the proportion of cells displaying punctate Vps10ΔCt-Mrl1Ct (14% decrease relative to vrl1, P < 0.001; Figure 7I), suggesting that the Mrl1 cytoplasmic tail is sufficient to confer VINE-mediated redistribution.

In cells with functional VINE, Mrl1 is prominently localized to the vacuole membrane but accumulates in endosomes in its absence. This is reminiscent of the vacuolar membrane protein Atg27, which follows an AP-3 dependent Golgi-vacuole-endosome recycling loop that relies on the sequential action of two sorting nexins: Snx4, which transports Atg27 from vacuoles to the endosome, and retromer, which controls its endosome-to-Golgi transport (Eising et al., 2022; Segarra et al., 2015; Suzuki and Emr, 2018). In cells lacking retromer, Atg27 accumulates at endosomes due to continued recycling by Snx4. We observed Mrl1-mScI primarily at the vacuolar membrane in a snx4Δ strain (Figure 7J), which coincided with a significant decrease in endosomal Mrl1-mScI (P < 0.0001; Figure 7K), suggesting that like Atg27, Mrl1 is transported from the vacuole to the endosome by Snx4-containing coat complexes. Because VINE is present on budding endosomal tubules and depletes Mrl1 from endosomes, we hypothesize that VINE is required for a subsequent endosome-to-Golgi retrograde transport step. Taken together, these results support a model where VINE enhances the recycling of the cargo protein Mrl1 at endosomes (Figure 7L).

Discussion

We have identified a novel endosomal SNX-BAR complex composed of the VPS9-domain GEF Vrl1 and the Vps5 paralog Vin1 which we have named the VINE complex. Our work suggests that VINE forms a novel endosomal coat with the potential to sort a unique set of cargo proteins that includes the mannose 6-phosphate receptor-like protein Mrl1.

Divergent N-terminal sequences in paralogous SNX-BARs specify complex formation

The function of the Vps5-related SNX-BAR protein Vin1 was not previously known. We found that in the absence of Vrl1, Vin1 is unstable and displaced to the cytosol suggesting that it functions solely as a member of the VINE complex. Vin1 and Vrl1 are both predicted to have PX-BAR domains and dimerize through a canonical BAR-BAR interface, yet this interaction is not the primary driver of Vrl1-Vin1 association. Instead, we found that a short sequence within the unstructured N-terminal extension of Vin1 binds specifically to the AnkRD of Vrl1, and this is necessary for selective incorporation into the VINE
complex. Our work suggests that VINE assembly requires two inputs: a strong interaction involving the Vin1 N-terminus and a weak interaction between the BAR domains that may occur primarily at the endosomal membrane.

The Vin1 paralog Vps5 also has an unstructured N-terminus that is critical for its assembly with the retromer complex and binds to a conserved patch on the Vps29 subunit (Collins et al., 2005; Seaman and Williams, 2002). Moreover, the interaction between Vps5 and Vps17 PX-BAR domains requires chemical crosslinkers to detect in detergent-solubilized lysates (Horazdovsky et al., 1997), suggesting the interaction is weak or mediated largely by hydrophobic contacts. This supports a model for retromer assembly that parallels that of the VINE complex, where the Vps5 N-terminus makes critical interactions with other retromer subunits and drives the assembly of the Vps5-Vps17 BAR-BAR dimer through an avidity effect.

Vin1 and Vps5 arose from a single ancestral gene during a whole-genome duplication (WGD) event (Byrne and Wolfe, 2005; Wolfe and Shields, 1997), and subsequently diverged to assume new roles in the VINE and retromer complexes, respectively. In pre-WGD species, the single ancestral form of Vps5 must partner with both Vrl1 and Vps17, which requires some promiscuity in BAR-BAR pairing. This is consistent with our structural modeling, which predicted a variety of pairings between the PX-BAR domains of Vps5, Vps17, Vrl1 and Vin1, while the PX-BAR protein Mvp1 was predicted to form only homodimers, consistent with in vivo observations (Suzuki et al., 2021).

Van Weering et al. (2012) have proposed a lock and key model to explain the specificity of SNX-BAR pairing. In this model, paired charges within the hydrophobic BAR-BAR interface enforce the specificity of BAR-BAR interactions, and loss of these charged residues results in more promiscuous BAR pairing. Promiscuous BAR-BAR coupling could explain why interactions mediated by the N-termini of Vin1 and Vps5 are needed to specify complex assembly.

New functions have been uncovered for the extended N-termini of other SNX-BAR proteins, suggesting these extended regions have previously unappreciated regulatory roles (Shortill et al., 2022). SNX1, which is the human homolog of Vps5 and Vin1, recognizes SNX5 (or its homolog SNX6) through BAR-BAR interactions based on lock-and-key charge pairing, and engages with other complexes, including SNX27 (Simonetti et al., 2022; Yong et al., 2021) and the retromer subunit VPS29 (Swarbrick et al., 2011), through its unstructured N-terminal domain. Thus, the N-termini of the SNX1/Vps5/Vin1 family of SNX-BAR proteins have diversified to bind different proteins and participate in different sorting complexes.
The VINE complex is both a SNX-BAR coat and a VPS9-domain GEF

The VINE complex is the first described SNX-BAR coat to possess a VPS9 domain-containing subunit. Retromer binds the VPS9-domain GEFs Vps9 and Muk1, which redundantly activate Rab5-like GTPases to stimulate PI3P production at endosomes (Bean et al., 2015). One benefit of wiring SNX-BARs to VPS9-domain GEFs could be to generate a local enrichment of PI3P that enhances SNX-BAR assembly. Indeed, we find that VINE localization requires its own GEF activity in a strain lacking other VPS9-domain GEFs.

The human Vrl1 homolog VARP also contains a VPS9 domain and associates with retromer (Hesketh et al., 2014), albeit through a distinct interaction involving a motif that is not present in Vrl1 (Crawley-Snowdon et al., 2020). This example of convergent evolution suggests that the linking of retromer to the activation of endosomal Rabs has an important and conserved role. VARP activates Rab21, which is related to Rab5 (Stenmark and Olkkonen, 2001) and interacts with PI3K in a proximity-based assay (del Olmo et al., 2019), though it has not yet been shown to stimulate PI3K activity. Because endosomal Rab GTPases also recruit a variety of effectors including the conserved tethering complexes Rabenosyn-5/Vac1 and CORVET (Cabrera et al., 2013; Christoforidis et al., 1999; Peplowska et al., 2007; Peterson et al., 1999) further work is required to clarify the conserved functional link between VPS9-domain GEFs and SNX-BAR sorting complexes.

VINE forms endosomal transport carriers and regulates cargo distribution

Our work suggests that VINE may also be a novel sorting complex that acts in a pathway-specific manner, thus joining the group of SNX-BAR complexes that promote independent sorting pathways from the endosome or vacuole in yeast (Ma and Burd, 2020; Suzuki et al., 2021). As VINE can be visualized at budding endosomal structures, we hypothesize that it recycles cargo from this organelle. We identified the MPR-related protein Mrl1 as a candidate VINE cargo. Although the function of Mrl1 is unclear, there is evidence that it works jointly with Vps10 to enhance the transport or maturation of some vacuolar proteases (Whyte and Munro, 2001). Restoring VINE function alters the steady state localization of Mrl1 and could modulate the rate at which it delivers proteins to the vacuole. Because Mrl1 contributes to protease delivery in a vrl1 mutant strain (Whyte and Munro, 2001), it is likely that other SNX-BAR complexes redundantly regulate Mrl1 sorting. Indeed, recent studies from our group and others have identified redundant cargo sorting roles of yeast SNX-BARs (Bean et al., 2017; Best et al., 2020; Suzuki et al., 2021).

Further work will be needed to determine if VINE binds directly to the Mrl1 cytosolic domain to direct its sorting as VARP does with VAMP7 (Crawley-Snowdon et al., 2020; Hesketh et al., 2014; Schäfer et al., 2012). VARP has additional domains not found in Vrl1 and it is not yet clear if the sorting functions of
VARP are conserved in Vrl1. It is instead possible that VINE promotes the redistribution of Mr1 indirectly, by regulating other processes such as those controlled by TORC1. Recent studies have placed functional pools of the TORC1 machinery at endosomes (Hatakeyama et al., 2018) and the TORC1-activating EGO/Ragulator complex is proximal to VINE based on our DHFR screening results. Vin1 was also recently reported to regulate TORC1 in strains carrying the vrl1 mutation (Goyal et al., 2022), suggesting a connection between VINE and TORC1. While a cargo-centric interpretation of our results is consistent with known functions of SNX-BAR complexes, a more detailed investigation is required to clarify the role of VINE and to understand metabolic implications of restoring VINE function.

Importantly, almost all studies on endosomal trafficking and signaling have been performed in strains that have a vrl1 mutation and lack the VINE complex, and we anticipate that other VINE-regulated cargoes may exist. Some proteins that are recycled by SNX-BAR-dependent pathways, such as phospholipid flippases, are themselves important for organelle function or signaling (Dalton et al., 2017; Liu et al., 2008). In the absence of functional VINE, such proteins may be missorted and/or have altered properties. Restoring VINE may therefore restore cellular processes and reveal new biology.

Materials and Methods

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**Yeast strains and plasmids**

Yeast strains and plasmids used in this study are described in Supplementary Files 4 and 5, respectively. Yeast strains were built in the BY4741 strain background using homologous recombination-based integration unless otherwise indicated. Gene deletions, promoter exchanges and tags were confirmed by
colony PCR and either western blot or fluorescence microscopy where possible. Plasmids were built by homologous recombination in yeast, recovered in *Escherichia coli* and confirmed by sequencing.

**Bioinformatic analysis of protein folding and sequence conservation**

Prediction of protein structure and binding interfaces was performed using Phyre2 (Kelley et al., 2015) and the ColabFold AlphaFold2 advanced server (Jumper et al., 2021; Mirdita et al., 2022) with default settings (https://colab.research.google.com/github/sokrypton/ColabFold/blob/main/beta/AlphaFold2_advanced.ipynb#scrollTo=bQe3KeyTcv0n). ColabFold complex prediction confidence is reported as a pTMscore, which is a predicted template modeling score (TM-score; Zhang and Skolnick, 2004) derived from the predicted alignment error (PAE; Jumper et al., 2021). Vrl1 amino acid sequences were from the *S. cerevisiae* strain RM11-1a. Orthologous sequences were obtained from the OrthoDB database (Kriventseva et al., 2019), aligned using the EMBL-EBI Multiple Sequence Comparison by Log-Expectation (MUSCLE) tool (https://www.ebi.ac.uk/Tools/msa/muscle) and presented using Jalview (http://www.jalview.org). Protein sequence conservation was mapped to predicted structure using ConSurf (https://consurf.tau.ac.il; Ashkenazy et al., 2016).

**DHFR protein fragment complementation assay and ontology analysis**

A MATα strain containing a plasmid that expresses the Vrl1-DHFR[1,2] (DHFR^Nt^) fusion from the ADH1 promoter, or the pADHpr-VRL1(1-465)-DHFR^Nt^ control, was crossed into a library of MATα strains (n = ~ 4,300) expressing proteins fused to DHFR[3] (DHFR^Ct^; Tarassov et al., 2008). Diploids were subjected to two rounds of double mutant selection followed by two rounds of selection on media containing 200 µg/ml methotrexate, in 1536 arrays. Manipulations were carried out using a BM3-BC pinning robot (S&P Robotics inc., Toronto, Canada). Colony area was analyzed using CellProfiler (Lamprecht et al., 2007) after 8 days at 30 °C. Z-scores were generated using median colony area from two technical replicates for each Vrl1-prey combination. Functional analysis of Vrl1 DHFR interactors (Z > 2) was performed using the Gene Ontology (Ashburner et al., 2000; Gene Ontology Consortium, 2021) GO Enrichment Analysis tool (Mi et al., 2019).

**Fluorescence microscopy and automated image analysis**

Yeast cells were diluted from overnight cultures in fresh synthetic dextrose-based media (SD) and incubated at 30 °C for ~4 hours or until they reached an optical density of ~ 0.4 - 0.7 OD_{600} unless otherwise indicated. Log phase yeast were transferred to concanavalin A-treated 96-well glass bottom plates (Eppendorf, Hamburg, Germany) and imaged using a DMi8 microscope (Leica Microsystems, Wetzlar, Germany) equipped with an ORCA-flash 4.0 digital camera (Hamamatsu Photonics, Shizuoka, Japan) and a high-contrast Plan-Apochromat 63x/1.30 Glyc CORR CS oil immersion lens (Leica...
Microsystems, Wetzlar, Germany). Image acquisition and processing was performed using the MetaMorph 7.8 software package (MDS Analytical Technologies, Sunnyvale, California). Yeast vacuoles were labelled with 100 µM CMAC (Setareh Biotech, San Jose, California) or 4 µM FM4-64 (Invitrogen, Waltham, Massachusetts) for 30 minutes at 30 °C. Dye-treated cells were washed once in SD media prior to imaging.

Linear intensity scale changes were uniformly applied to all images of a given fluorophore in an experimental set using MetaMorph 7.8 (MDS Analytical Technologies, Sunnyvale, California). For very dim or bright signals that could not be identically scaled, uniformly applied brightness settings are shown as insets and custom settings were used to show protein localization in the full-size image. Images were prepared for presentation using Photoshop CC 2020 (Adobe, San Jose, California) and Illustrator CC 2020 (Adobe, San Jose, California). Quantification was performed on unscaled raw images with scripted MetaMorph 7.8 journals (MDS Analytical Technologies, Sunnyvale, California). The Count Nuclei feature was used to filter out dead cells and identify live cells based on intensity about local background (IALB). The Granularity feature was used to identify puncta in a dead cell-masked intermediate image based on IALB. Masking functions were performed using the Arithmetic function with Logical AND.

**Coimmunoprecipitation, western blotting and spheroplasting**

For western blot-based stability assays, yeast cells were grown to log phase in SD media at 30 °C and 10 OD<sub>600</sub>/ml equivalents of cells were harvested and stored at -80 °C. Cells were thawed and lysed by vortexing in 100 µl of Thorner buffer (8 M Urea, 5% SDS, 40mM Tris-Cl (pH 6.4), 1% beta-mercaptoethanol and 0.4 mg/ml bromophenol blue) with ~100 µl of acid-washed glass beads/sample at 70 °C for 5 minutes. Lysates were centrifuged at 14,000 RPM for 30 seconds and separated on 8% SDS-PAGE gels followed by western blotting with mouse anti-HA (H9658, Clone HA-7, Sigma-Aldrich) or anti-PGK1 monoclonal antibodies (AB_2532235, 22C5D8, Invitrogen), and secondary polyclonal goat anti-mouse antibodies conjugated to horseradish peroxidase (115–035-146; Jackson ImmunoResearch Laboratories).

For CoIPs, yeast cells were grown to log phase in SD media at 30 °C and 75 OD<sub>600</sub>/ml equivalents of cells were incubated in 50 mM Tris-Cl with 10 mM DTT (pH 9.5) for 15 minutes at room temperature and digested in spheroplasting buffer (1.2 M sorbitol, 50 mM KH<sub>2</sub>PO<sub>4</sub>, 1 mM MgCl<sub>2</sub> and 250 µg/ml zymolase at pH 7.4) at 30 °C for 1 hour. Spheroplasts were washed twice with 1.2 M sorbitol, frozen at -80 °C, then incubated in 500 µl of lysis buffer (0.1% Tween-20, 50 mM HEPES, 1 mM EDTA, 50 mM NaCl, 1 mM PMSF and 1x fungal ProteaseArrest, pH 7.4) at room temperature for 10 minutes. 50 µl volumes of lysate were collected for each sample and mixed with 2x Laemmli buffer (4% SDS, 20% glycerol, 120 mM Tris-Cl (pH 6.8), 0.01g bromophenol blue and 10% beta-mercaptoethanol) for western analysis while...
remaining lysates were incubated with either a polyclonal rabbit anti-GFP (EU2, Eusera) or a polyclonal rabbit anti-HA antibody (ab9110, Abcam) at 4 °C for 1 hour. Antibody-treated samples were next incubated with Protein A Sepharose beads (Cytiva) at 4 °C for 1 hour. Beads were washed 3x in lysis buffer before being resuspended in 50 µl of Thorner buffer and heated at 80 °C for 5 minutes. Western blotting of proteins separated on 8% SDS-PAGE gels was carried out with monoclonal mouse anti-HA (H9658, Clone HA-7, Sigma-Aldrich), monoclonal mouse anti-HA (MMS-101R; Covance) or monoclonal mouse anti-GFP antibodies (11–814–460-001; Roche) prior to secondary antibody treatment with polyclonal goat anti-mouse conjugated to horseradish peroxidase (115–035-146; Jackson ImmunoResearch Laboratories). Blots were developed with Amersham ECL (GERPN2209, Cytiva) or Amersham ECL Prime (GERPN2232, Cytiva) chemiluminescent western blot detection reagents and exposed using Amersham Hyperfilm ECL (GE Healthcare). Densitometry of scanned films was performed using ImageJ (Schneider et al., 2012).

**Correction of genomic vrl1 mutation using CRISPR-Cas9**

A plasmid containing the Cas9 enzyme and a single guide RNA (sgRNA) targeting the VRL1-disrupting yml003w mutation was pre-cloned using small fragment golden gate assembly (Marillonnet and Grützner, 2020). BY4741 was co-transformed with linearized split URA3 marker Cas9-sgRNA(VRL1) plasmid and PCR product containing the corrected VRL1 sequence. Ura+ colonies were sequenced and an isolate with intact VRL1 was used for experiments.

**Statistical analysis of quantitative data**

Statistical tests were performed using GraphPad Prism 9.1.0 (GraphPad Software, San. Diego, California) as indicated in figure legends with the appropriate post-hoc tests. Normality of data was assumed but not formally tested and hypotheses were measured against a threshold of 95% confidence (or P < 0.05). Graphs were made in Microsoft Excel 2019 (Microsoft, Redmond, Washington). Column charts represent the average value from biological replicates while scatter points represent data from individual replicates and are coloured by replicate. Error bars report the standard error of the mean value.

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Conflict of Interest Statement

The authors declare that there are no conflicts of interest.

References


Stenmark H, Olkkonen VM. 2001. The Rab GTPase family. Genome Biol. 2(5), reviews3007.1


Swarbrick JD, Shaw DJ, Chhabra S, Ghai R, Valkov E, Norwood SJ, Seaman MNJ, Collins BM. 2011. VPS29 is not an active metallo-phosphatase but is a rigid scaffold required for retromer interaction with accessory proteins. PLoS ONE 6(5), e20420.


Figure Legends

Figure 1. Vr11 is a predicted PX-BAR protein that interacts with conserved machinery at the endosome. (A) Schematic of Vr11 and VARP domain architecture. (B) ColabFold predicts the Vr11 C-terminus has a SNX-BAR-like PX and BAR domain fold. (C) Vr11-Envy colocalizes with Did2-mRuby2-labeled endosomes, but not with the Sec7-dsRed Golgi marker. (D) Quantification of colocalization as the percentage of Vr11 puncta overlapping RFP puncta in C. Two-tailed equal variance t test; n = 3, cells/strain/replicate ≥ 1,395; **** = P < 0.0001. (E) Schematic of DHFR proximity screen methodology. (F) Z-score distribution of the ratio of colony areas from genome-wide DHFR screens of full-length and truncated Vr11 baits that localize to the endosome and cytosol, respectively. (G) Gene Ontology (GO) functional enrichment analysis of Vr11 DHFR interactors (Z-score > 2; http://geneontology.org). GO terms of the most specific hierarchical subclass with a fold enrichment value > 25 are presented as the negative base 10 log of the associated P-value from a Bonferroni-corrected binomial test of significance. Scale bars, 2 µm. Error bars report standard error of the mean (SEM). Enrich., enrichment. aa, amino acids.
Figure 2. Vrl1 and the Vps5 paralog Vin1 form the VINE complex. (A) Schematic of Ykr078w (Vin1) and its paralog Vps5. (B) Vin1-Envy and Vrl1-Envy require Vrl1 and Vin1, respectively, for localization to puncta. (C) Quantification of Vin1-Envy and Vrl1-Envy puncta per cell in B. Two tailed equal variance $t$ tests; $n = 3$, cells/strain/replicate $\geq 1,879$; *** = $P < 0.001$, **** = $P < 0.0001$. (D) Vrl1-3HA and Vin1-3HA require Vin1 and Vrl1, respectively, for protein stability by western blot. Pgk1 serves as a loading control. (E) Quantification of Vrl1-3HA and Vin1-3HA levels in D by densitometry. Two tailed Welch’s $t$ tests; $n = 3$, ** = $P < 0.01$, *** = $P < 0.001$. (F) Co-immunoprecipitation (CoIP) of Vin1-3HA with Vrl1-Envy suggests stable complex formation. (G) Vrl1-Envy colocalizes with Vin1-mScI at perivacuolar puncta. (H) Vrl1-Envy requires the PI3K catalytic subunit Vps34 for punctate localization. (I) Quantification of Vrl1-Envy puncta per cell in H. Two-tailed equal variance $t$ test; $n = 3$, cells/strain/replicate $\geq 897$; ** = $P < 0.01$. (J) Vrl1-Envy localization in the absence of VPS9-domain GEFs is dependent on the Vrl1 catalytic residue D373. (K) Quantification of Vrl1-Envy puncta per cell in J. One-way ANOVA with Tukey’s multiple comparison test; $n = 3$, cells/strain/replicate $\geq 1,705$; not significant, n.s. = $P > 0.05$, * = $P < 0.05$, *** = $P < 0.001$, **** = $P < 0.0001$. (L) Model of the Vin1 and Vrl1-containing VINE complex at endosomes. Scale bars, 2 µm. Error bars report SEM. Exp., Exposure.

Figure 3. Vrl1 interacts with Vin1 primarily through the AnkRD. (A) ColabFold-predicted physical interaction of Vrl1 and Vin1 BAR domains along the canonical BAR-BAR dimerization interface. pTMscore = 0.75. (B) Matrix of ColabFold-predicted BAR-BAR dimers for select yeast SNX-BARs. Hierarchical clustering was performed using an uncentered Pearson correlation with average linkage. (C) Vin1 is the only yeast SNX-BAR that interacts with over-expressed Vrl1-3HA by CoIP. (D) Vps5-3HA does not bind to Vrl1-Envy in a strain lacking Vin1. (E) Schematic of Envy-tagged Vrl1 truncations and chimeras in F. (F) The Vrl1 AnkRD is necessary to recruit Vin1-mScI to puncta. Images with very bright signals use custom settings to show protein localization; insets are scaled identically to other images in the same channel (see materials and methods for details). (G) Quantification of Vin1-mScI puncta per cell in F. One-way ANOVA with Dunnett’s multiple comparison test; $n = 3$, cells/strain/replicate $\geq 764$; not significant, n.s. = $P > 0.05$, * = $P < 0.05$, ** = $P < 0.01$, *** = $P < 0.001$, **** = $P < 0.0001$. (H) Diagram of chimeric Vrl1 fusion proteins that are artificially recruited to the endosomal system by the PX domain of sorting nexin Ypt35. (I) The Vrl1 AnkRD is necessary for physical interaction with Vin1-3HA by CoIP. Pgk1 serves as a loading control. Scale bars, 2 µm. Error bars report SEM. OE, over-expressed. YPE, Ypt35(PX)-Envy.

Figure 4. The Vrl1 AnkRD recognizes a small region of the Vin1 N-terminus. (A) Schematic of constructs used in C, D. Full-length Vps5 was not tested but is shown for comparison. (B) Diagram of chimeric Vrl1 recruitment assay used to test for interactions with the unstructured N-terminus of either
Vps5 (Vps5\textsuperscript{1-276}) or Vin1 (Vin1\textsuperscript{1-116}). (C) The AnkRD-containing Vrl1(1-703)	extsuperscript{YPE} chimera recruits the N-terminus of Vin1, but not Vps5. Insets are scaled to match other images in the same channel (see materials and methods for details). (D) Quantification of RFP puncta per cell in C. One-way ANOVA with Tukey’s multiple comparison test; \(n = 3\), cells/strain/replicate \(\geq 902\); not significant, n.s. = \(P > 0.05\), ** = \(P < 0.01\), *** = \(P < 0.001\), **** = \(P < 0.0001\). (E) Schematic of Vin1 N-terminal fragments used to map the Vrl1 recruitment site. (F) The AnkRD-containing Vrl1(1-703)	extsuperscript{YPE} chimera recruits a small fragment of the Vin1 N-terminus. Insets are scaled to match other images in the same channel. (G) Quantification of Vin1-mScI puncta per cell in F. Two-tailed equal variance \(t\) tests; \(n = 3\), cells/strain/replicate \(\geq 294\); not significant, n.s. = \(P > 0.05\), * = \(P < 0.05\), ** = \(P < 0.01\). Scale bars, 2 \(\mu m\). Error bars report SEM. OE, over-expressed. FL, full-length.

**Figure 5. The Vrl1 AnkRD associates with the Vin1 N-terminus through electrostatic interactions.**

(A) Schematic of query sequences used to predict the interact between Vrl1 and the Vin1 N-terminus. Modelled regions are shown as completely opaque. (B) ColabFold-predicted interaction between the Vrl1 AnkRD and a minimal fragment of the Vin1 N-terminus (Vin1\textsuperscript{76-95}; pTMscore = 0.73). (C) Vrl1 sequence conservation within family Saccharomycetaceae determined by ConSurf and mapped to a surface model that was predicted by ColabFold. Strong sequence conservation can be seen at the predicted Vin1\textsuperscript{76-95} interacting site and near the catalytic D373 residue. (D) Top: Vin1\textsuperscript{76-95} is predicted to associate with Vrl1 through a run of basic residues. Bottom: Acidic and polar residues in the predicted Vin1-associating Vrl1 AnkRD site are among the most conserved within family Saccharomycetaceae. (E) Mutation of acidic and polar residues in the Vrl1 AnkRD reduces recruitment of the Vin1 N-terminus by the Vrl1(1-703)	extsuperscript{YPE} chimera. (F) Quantification of Vin1\textsuperscript{1-116}-mScI puncta per cell in E. One-way ANOVA with Tukey’s multiple comparison test; \(n = 3\), cells/strain/replicate \(\geq 863\); not significant, n.s. = \(P > 0.05\), * = \(P < 0.05\), *** = \(P < 0.001\), **** = \(P < 0.0001\). Scale bars, 2 \(\mu m\). Error bars report SEM. OE, over-expressed. Nt, N-terminus.

**Figure 6. Vin1 controls Vrl1 GEF activity via membrane localization.** (A) Deletion of VIN1, but not VPS5, prevents Vrl1 from rescuing the temperature sensitivity of a strain lacking other VPS9-domain GEFs. (B) Schematic of PI3P-binding fluorescent biosensor. (C) Deletion of VIN1 prevents Vrl1 from stimulating endosomal PI3P production in a strain lacking other VPS9-domain GEFs. (D) Deletion of VIN1 prevents Vrl1 from rescuing Vps26-GFP localization in a strain lacking other VPS9-domain GEFs. (E) Quantification of Vps26-GFP puncta per cell in D. One-way ANOVA with Tukey’s multiple comparison test; \(n = 3\), cells/strain/replicate \(\geq 1,503\); not significant, n.s. = \(P > 0.05\), * = \(P < 0.05\), ** = \(P < 0.01\), *** = \(P < 0.001\). (F) Vin1 is dispensable for Vrl1 activity when fragments containing the N-terminus and VPS9 domain are artificially recruited by a YPE endosomal anchor. Insets are scaled to
match other images in the same channel (see materials and methods for details). (G) Quantification of Vps26-mScI puncta per cell in F. One-way ANOVA with Tukey’s multiple comparison test; \( n = 3 \), cells/strain/replicate \( \geq 750 \); not significant, n.s. = \( P > 0.05 \), *** = \( P < 0.001 \), **** = \( P < 0.0001 \). Scale bars, 2 µm. Error bars report SEM. OE, over-expressed.

Figure 7. The VINE complex exhibits characteristics of a membrane sorting complex. (A) Time-lapse imaging of cells over-expressing GFP-Vin1 and Vrl1 show tubules emanating from Did2-labeled endosomes. Images were uniformly enlarged using a bicubic expansion function to show detail. Solid arrowheads mark a tubule, open arrowhead marks a scission event. (B) Normalized intensity line scan analysis performed on images from A along the yellow dotted line. (C) Punctate localization of GFP-tagged Mrll, but not other endosomal recycling cargo, is decreased in cells expressing VRL1. (D) Quantification of GFP-tagged puncta in WT and \( \textit{vrl1} \) strains in C. Two tailed Welch’s \( t \) tests; \( n = 3 \), cells/strain/replicate \( \geq 902 \); not significant, n.s. = \( P > 0.05 \), * = \( P < 0.05 \). (E) Mutation of the D373 residue required for VPS9 GEF activity does not prevent Vrl1 from redistributing Mrll. (F) Quantification of Mrll-mScI puncta per cell in E. One-way ANOVA with Tukey’s multiple comparison test; \( n = 3 \), cells/strain/replicate \( \geq 1,788 \); not significant, n.s. = \( P > 0.05 \), * = \( P < 0.05 \). (G) Schematic of Vps10 cytosolic tail mutant and Mrll cytosolic tail chimera tested for VINE-mediated sorting in H, I. (H) The Mrll cytosolic tail is sufficient to confer VINE-mediated redistribution. (I) Percent of cells showing punctate localization of indicated GFP-tagged constructs in H. Blind scoring of GFP signal was conducted manually. One-way ANOVA with Tukey’s multiple comparison test; \( n = 3 \), cells/strain/replicate \( \geq 237 \); not significant, n.s. = \( P > 0.05 \), *** = \( P < 0.001 \), **** = \( P < 0.0001 \). (J) Mrll-mScI puncta are reduced in a \( \textit{snx4} \Delta \) strain. (K) Quantification of Mrll-mScI puncta per cell in J. One-way ANOVA with Tukey’s multiple comparison test; \( n = 3 \), cells/strain/replicate \( \geq 1,036 \); not significant, n.s. = \( P > 0.05 \), * = \( P < 0.05 \), ** = \( P < 0.01 \), *** = \( P < 0.001 \), **** = \( P < 0.0001 \). (L) Model for VINE activity and redistribution of Mrll. VINE promotes its own recruitment to endosomes through a positive feedback loop involving Vrl1 GEF activity and local PI3P production. VINE-coated tubules then recycle cargo, such as Mrll, from endosomes. VINE may target Mrll to the Golgi for subsequent delivery to the vacuolar membrane by the AP-3 complex. Mrll is then returned to the endosome by Snx4-containing complexes. See text for details. Scale bars, 2 µm. Error bars report SEM. OE, over-expressed. TM, transmembrane.

Supplementary Figure and File Legends

Figure 1 S1. The Vrl1 PX-like domain is missing key PI3P-binding residues. (A) Results from Phyre2 analysis of Vrl1 sequences (Intensive mode, http://www.sbg.bio.ic.ac.uk/phyre2). (B) ColabFold-
predicted Vrl1 C-terminus with the predicted local-difference distance test (pLDDT; Jumper et al., 2021; Mirdita et al., 2022) scores mapped to each residue. (C) ColabFold-generated predicted alignment error (PAE; Jumper et al., 2021; Mirdita et al., 2022) plot for the Vrl1 C-terminus demonstrates a high confidence folding prediction. (D) Sequence alignment of Vrl1 C-terminus (aa 737-1089) with yeast and human PX domain-containing proteins. The canonical PI3P-binding “RRY” motif is absent in Vrl1 (highlighted with red dots above alignment).

Figure 1 S2. The Vrl1 N-terminus and VPS9 domain are not sufficient to localize to puncta. The Vrl1 N-terminus (aa 1-465) does not localize to puncta when expressed from the endogenous VRL1 promoter (VRL1pr) or the strong ADH1pr and instead accumulates in the cytosol. Scale bars, 2 µm.

Figure 2 S1. Vrl1 is indispensable for Vin1 puncta localization. (A) N-terminally tagged Vin1 requires Vrl1 for localization to puncta at three different expression levels. By relative fluorescence, the NOP1 promoter (NOP1pr) is stronger than the VIN1pr and the TEF2pr is stronger than the NOP1pr. Insets are scaled to match other images in the same channel (see materials and methods for details). (B) Over-expression of Vrl1 from the ADH1pr increases the average number of bright Vin1-Envy puncta per cell. (C) Quantification of Vrl1-Envy puncta per cell in B. One-way ANOVA with Tukey’s multiple comparison test; n = 3, cells/strain/replicate ≥ 1,495; *** = P < 0.001, **** = P < 0.0001. Scale bars, 2 µm. Error bars report SEM.

Figure 3 S1. Confidence measures of yeast SNX-BAR dimer predictions. (A) ColabFold-predicted physical interaction of Vrl1 and Vin1 BAR domains with the predicted local-difference distance test (pLDDT; Jumper et al., 2021; Mirdita et al., 2022) scores mapped to each residue. (B) ColabFold-generated predicted alignment error (PAE; Jumper et al., 2021; Mirdita et al., 2022) plots for yeast SNX-BAR pairs demonstrate confidence of inter-chain contacts, indicating the relative likelihood of functional pairings. On-diagonal boxes with low PAE values reflect confidently predicted intramolecular interactions while off-diagonal boxes with low PAE values reflect confidently predicted intermolecular interactions between two separate polypeptide sequences. aa, amino acids.

Figure 3 S2. Over-expression of VRL1 restores protein levels in a vin1Δ mutant. Vrl1 protein levels are restored in a vin1Δ mutant by western blot when VRL1 is over-expressed on the ADH1pr. Pgk1 serves as a loading control.

Figure 3 S3. Vrl1 does not form a novel retromer-like complex. (A) Vrl1 does not interact strongly with subunits of retromer by CoIP. (B) Expression of VRL1 does not rescue loss of endosomal Vps10-Envy in a vps5Δ strain. (C) Expression of VRL1 does not rescue loss of endosomal Vps35-GFP or vacuolar morphology defects in retromer SNX-BAR deletion strains. (D) Quantification of Vps35-GFP
puncta per cell in *C*. One-way ANOVA with Tukey’s multiple comparison test; *n* = 3, cells/strain/replicate ≥ 1,243; not significant, n.s. = *P* > 0.05, *** = *P* < 0.001. Scale bars, 2 µm. Error bars report SEM. OE, over-expressed.

**Figure 4 S1. The N-terminal regions of Vin1 and Vps5 are predicted to be disordered.** A lack of off-diagonal signal in the ColabFold-generated predicted alignment error (PAE; Jumper et al., 2021; Mirdita et al., 2022) plots of Vin1 and Vps5 indicates a shared lack of structure in the N-terminus of either protein. On-diagonal signal for the PX and BAR domains provides a point of comparison for how structured domains appear in prediction PAE plots.

**Figure 4 S2. The Vin1 PX-BAR region is indispensable for Vrl1 localization.** (A) The Vin1 N-terminus is not sufficient to localize over-expressed Vrl1-Envy to membranes in a *VIN1* deletion strain. The Vrl1(1-703)YPE chimera recruits the Vin1 N-terminal prey construct to puncta in strains lacking the chromosomal copy of *VIN1*. (B) Quantification of RFP puncta per cell. One-way ANOVA with Tukey’s multiple comparison test; *n* = 3, cells/strain/replicate ≥ 1,068; not significant, n.s. = *P* > 0.05, ** = *P* < 0.01, *** = *P* < 0.001, **** = *P* < 0.0001. Scale bars, 2 µm. Error bars report SEM.

**Figure 4 S3. The Vin1 N-terminus has three conserved regions in fungal homologs.** Sequence alignment of Vin1 and Vps5 orthologs collected using the Yeast Gene Order Browser ([http://ygob.ucd.ie/](http://ygob.ucd.ie/)). Three conserved regions were selected for expression as mScI-tagged fragments: Vin11-29 (Region 1), Vin141-78 (Region 2) and Vin176-95 (Region 3).

**Figure 5 S1. Confidence measures of Vrl1 AnkRD-Vin1 N-terminus binding predictions.** (A) ColabFold-predicted interaction between the Vrl1 AnkRD and a minimal fragment of the Vin1 N-terminus (Vin176-95; pTMscore = 0.73) with pLDDT scores mapped to each residue of Vin1 (left) or Vrl1 (right). In each case, the interacting protein partner is shown in grey. (B) ColabFold-generated predicted alignment error (PAE; Jumper et al., 2021; Mirdita et al., 2022) plot for Vrl1(1-703) and the Vin1 N-terminus (Vin11-116) indicates an intermolecular interaction in the off-diagonal boxes. (C) A plot of predicted local-difference distance test (pLDDT; Jumper et al., 2021; Mirdita et al., 2022) scores for five different models of Vrl1(1-703) and Vin11-116 indicates that the minimal region of Vin1 that was recruited by the Vrl1(1-703)YPE chimera, Vin176-95, is the sole confidently predicted region. aa, amino acids. Nt, N-terminus.

**Figure 5 S2. Vrl1(1-703)YPE chimeras with AnkRD mutations are stably expressed.** AnkRD-containing Vrl1(1-703)YPE chimeras are stable by western blot when mutations are introduced to sites that are predicted to interact with the Vin1 N-terminus. Pgk1 serves as a loading control.
Figure 7 S1. Vrl1 redistributes Mr11 puncta from endosomes. (A) Mr11-mScI puncta colocalize with Did2-Envy and sfGFP-Vps21-labeled endosomes, but not with sfGFP-Ypt7 puncta that label vacuolar sites. Insets are scaled to match other images in the same channel (see materials and methods for details). (B) Quantification of colocalization as the percentage of Mr11 puncta overlapping GFP puncta in A. One-way ANOVA with Tukey’s multiple comparison test; $n = 3$, cells/strain/replicate $\geq 2,286$; ** = $P < 0.01$, **** = $P < 0.0001$. (C) Localization of Vin1-mScI in BY4741 (vrl1) and an isogenic strain with the vrl1 mutation corrected at the endogenous VRL1 locus using CRISPR-Cas9 gene editing technology. Vin1-mScI localizes to puncta in a CRISPR-corrected VRL1 strain. (D) Mr11-mScI is redistributed from puncta in the CRISPR-corrected VRL1 strain. (E) Quantification of large, bright Mr11-mScI puncta per cell in D. Two tailed equal variance $t$ test; $n = 3$, cells/strain/replicate $\geq 1,183$; ** = $P < 0.01$. Scale bars, 2 $\mu$m. Error bars report SEM.

Supplementary File 1. Vrl1 DHFR Interactors. List of Z-scores from the Vrl1 DHFR screen.

Supplementary File 2. Vrl1 DHFR Ontology Enrichment. List of enriched ontology terms for Vrl1 DHFR interactors ($Z > 2$).


Supplementary File 4. List of Saccharomyces cerevisiae strains used in this study.

Supplementary File 5. List of plasmids used in this study.

Figure 1 – Source Data 1. Data associated with Figure 1D.

Figure 1 – Source Data 2. Data associated with Figure 1G.

Figure 2 – Source Data 1. Data associated with Figure 2C.

Figure 2 – Source Data 2. Data associated with Figure 2E.

Figure 2 – Source Data 3. Data associated with Figure 2G.

Figure 2 – Source Data 4. Data associated with Figure 2I.

Figure 2 – Source Data 5. Data associated with Figure 2K.

Figure 2 – Source Data 6. Uncropped blot data associated with Figure 2D.

Figure 2 – Source Data 7. Uncropped blot data associated with Figure 2F.

Figure 2 S1 – Source Data 1. Data associated with Figure 2 S1C.
Figure 3 – Source Data 1. Data associated with Figure 3B.

Figure 3 – Source Data 2. Data associated with Figure 3G.

Figure 3 – Source Data 3. Uncropped blot data associated with Figure 3C.

Figure 3 – Source Data 4. Uncropped blot data associated with Figure 3D.

Figure 3 – Source Data 5. Uncropped blot data associated with Figure 3I.

Figure 3 S2 – Source Data 1. Uncropped blot data associated with Figure 3 S2.

Figure 3 S3 – Source Data 1. Data associated with Figure 3 S3D.

Figure 3 S3 – Source Data 2. Uncropped blot data associated with Figure 3 S3A.

Figure 4 – Source Data 1. Data associated with Figure 4D.

Figure 4 – Source Data 2. Data associated with Figure 4G.

Figure 4 S2 – Source Data 1. Data associated with Figure 4 S2B.

Figure 5 – Source Data 1. Data associated with Figure 5F.

Figure 5 S2 – Source Data 1. Uncropped blot data associated with Figure 5 S2.

Figure 6 – Source Data 1. Data associated with Figure 6E.

Figure 6 – Source Data 2. Data associated with Figure 6G.

Figure 7 – Source Data 1. Data associated with Figure 7B.

Figure 7 – Source Data 2. Data associated with Figure 7D.

Figure 7 – Source Data 3. Data associated with Figure 7F.

Figure 7 – Source Data 4. Data associated with Figure 7I.

Figure 7 – Source Data 5. Data associated with Figure 7K.

Figure 7 S1 – Source Data 1. Data associated with Figure 7 S1B.

Figure 7 S1 – Source Data 2. Data associated with Figure 7 S1E.
**Figure 1**, Shortill et al., 2022
### Domain Template Information

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### Per-Residue Model Confidence (pLDDT)

- **Very low (<50)**
- **Low (50-70)**
- **Confident (70-90)**
- **Very high (>90)**

### Figure 1 S1, Shortill et al., 2022
Figure 1 S2, Shortill et al., 2022
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Figure 3 S2, Shortill et al., 2022
Figure 3 S3, Shortill et al., 2022
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Figure 4 S1, Shortill et al., 2022
Figure 4 S2, Shortill et al., 2022
Figure 4 S3, Shortill et al., 2022
Figure 5, Shortill et al., 2022
Figure 5 S1, Shortill et al., 2022
Figure 5 S2, Shortill et al., 2022
**Figure 6,** Shortill et al., 2022
Figure 7, Shortill et al., 2022
Figure 7 S1, Shortill et al., 2022