Molecular basis for the assembly of the Vps5-Vps17 SNX-BAR proteins with Retromer

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ABSTRACT (175 words)

Retromer mediates endosomal retrieval of transmembrane proteins in all eukaryotes and was first discovered in yeast in complex with the Vps5 and Vps17 sorting nexins (SNXs). Cryoelectron tomography (cryoET) studies of Retromer–Vps5 revealed a pseudo-helical coat on membrane tubules where dimers of the Vps26 subunit bind Vps5 membrane-proximal domains. However, the Vps29 subunit is also required for Vps5–Vps17 association despite being far from the membrane. Here, we show that Vps5 binds both Vps29 and Vps35 subunits through its unstructured N-terminal domain. A Pro-Leu (PL) motif in Vps5 binds Vps29 and is required for association with Retromer on membrane tubules *in vitro*, and for the proper recycling of the Vps10 cargo in *Saccharomyces cerevisiae*. CryoET of Retromer tubules with Vps5–Vps17 heterodimers show a similar architecture to the coat with Vps5–Vps5 homodimers, however, the spatial relationship between Retromer units is highly restricted, likely due to more limited orientations for docking. These results provide new mechanistic insights into how Retromer and SNX-BAR association has evolved across species.

INTRODUCTION

Early endosomes are membrane bound cellular compartments that are essential for the sorting and maintenance of transmembrane cargo proteins and lipids. Transmembrane cargoes are delivered to the endosome from the cell surface of from intracellular organelles such as the Golgi and their trafficking is then controlled by a range of different peripheral membrane protein machineries. For the lysosomal degradative pathway, the cargoes are typically sorted by the Endosomal Sorting Complex Required for Transport (ESCRT) machinery, while recycling of cargoes to either the plasma membrane or the trans-Golgi network (TGN) is primarily driven by tubulovesicular carriers. The evolutionarily conserved Retromer complex is one of the key protein machineries controlling the cargo recycling and sorting at the endosome and is conserved from humans to the simplest single-celled eukaryotes[1-7]. Mutations in or altered expression of Retromer disrupt cellular homeostasis and have been strongly linked to a number of human diseases including Alzheimer's[8-11], Parkinson's disease[12-15] and more recently carcinogenesis[16].

Retromer is composed of the three vacuolar protein sorting (VPS) subunits Vps26, Vps29 and Vps35. It was first identified in the budding yeast *Saccharomyces cerevisiae* as a heteropentameric complex with Vps5 and Vps17 responsible for the retrieval of cargoes such as the carboxy peptidase Y (CPY) receptor Vps10 from endosomes to the Golgi through the formation of tubulovesicular structures[17-19]. The core Retromer trimer acts as a scaffold for sorting cargoes, and functions with adaptors from the sorting nexin (SNX) protein family, including Vps5 and Vps17 in yeast, which control membrane association and cargo recruitment. Vps5 and Vps17 possess a phox homology (PX) domain that defines the SNX family and typically associates with membrane phosphoinositide lipids. This is preceded by an unstructured N-terminal domain of more than 100 residues in length and a C-terminal bin/amphiphysin/rvs (BAR) domain that mediates homo and heterodimerization, oligomerisation and membrane tubulation. Although Retromer is conserved in higher metazoan species, the proposed SNX-BAR heterodimeric orthologues of SNX1/SNX2-SNX5/SNX6 are not stably associated with Retromer and can form a functionally distinct endosomal sorting complex (ESCPE-1)[20-24]. Although the human SNX-BAR proteins largely colocalise in with Retromer in endosomal retrieval domains, because they do not associate strongly with Retromer it remains under debate whether they interact directly in human cells as compared to their yeast counterparts[2,25-29]. An additional difference is that the SNX5/6 subunits of ESCPE-1 can specifically recognize transmembrane cargo proteins via an unusual PX domain, including the cation-independent mannose-6-phosphate receptor (CI-MPR) and neuropilin-1 (NRP1) [23,24,30]. In contrast, the Vps5-Vps17 proteins lack the unique PX domain structure and have so far not been identified to bind known cargo molecules. These studies highlight both similarities but also key differences between the Retromer and SNX-BAR machineries of higher and lower eukaryotes.

Structural studies are beginning to reveal some of the essential mechanistic features of Retromer assembly into a functional membrane coat. The cryoelectron tomography (CryoET)

structure of Retromer with a homodimer of Vps5 from the thermophilic yeast *Chaetomium thermophilum* revealed the formation of membrane tubules whereby the PX and BAR domains of Vps5 were aligned to the membrane surface, and arches of Retromer formed oligomeric structures atop the Vps5 layer[31]. Subsequent structures of tubules coated by Retromer with both the yeast and metazoan Snx3 adaptor protein revealed a mechanism whereby the Retromer Vps26 subunits associate with the adaptor to form a convex scaffold contacting the membrane thus substituting the SNX-BAR in this role while preserving the overall complex architecture. In both SNX3 and SNX-BAR complexes, Vps29 resides at arch apexes distal from the adaptors and the membrane surface[31,32]. Despite the insights provided by these cryoET snapshots of Retromer there remains an important discrepancy between the observed structural organisation and studies demonstrating a key role of Vps29 in SNX-BAR association *in vivo*. Studies of these proteins in *S. cerevisiae* have shown that the interaction of Retromer with the Vps5-Vps17 proteins requires the presence of Vps29[17,33,34], which in cryoET structures is far from contacting the membrane associated PX and BAR domains[31,32]. In contrast, Vps26 is dispensable for the interaction in co-immunoprecipitations[34] although it is the primary subunit associated with Vps5 once assembled on membranes[31,32]. The N-terminal intrinsically disordered domain of Vps5, which is not visible in cryoET reconstructions, is also required for interaction with Retromer[33]. These results thus raise the question of how to reconcile the contrasting structural and *in vivo* data.

In this work we have resolved this controversy, using, biochemistry, crystallography, cryoEM, cell biology and in vitro membrane reconstitution experiments to define the precise mechanism by which Retromer forms a high affinity complex with Vps5 (and the associated SNX-BAR protein Vps17) required for membrane recruitment, membrane tubule formation, and trafficking. Combining Alphafold predictions and crystallography we found that tight association between Vps5 and Retromer depends on two factors: the interaction of Vps29 with Vps5 via a PL motif and binding of a novel [K/R]Φ motif (Φ = hydrophobic residues) to the Vps29 and Vps35 interface. These sequences are unique to Vps5 homologues and are required for recruitment of Retromer by the functional Vps5-Vps17 heterodimer to form membrane tubule coats in vitro and for trafficking of vacuolar cargos in cells. This presents a mechanism of assembly of Retromer with SNX-BAR proteins first requiring a specific sequence in the disordered N-terminus of Vps5 for high affinity binding to the Vps35 and Vps29 subunits, which allows recruitment prior to the subsequent membrane-driven reorganization of the Retromer and SNX-BAR pentameric complex into the distinctive oligomeric coat.

RESULTS

The N-terminal unstructured region of Vps5 mediates high affinity interaction with Retromer

Vps5 from the thermophilic yeast *Chaetomium thermophilum* (ctVps5) consists of PX and BAR domains preceded by an intrinsically disordered region (IDR) of ~150 residues (**Figs. 1A and 1B**). ctVps5 expressed in bacteria can form a homodimer via its BAR domain that associates with the trimeric ctRetromer complex to form membrane tubules, even in the absence of the heterodimeric partner ctVps17[31]. We revisited this interaction using GST pulldown assays of ctVps5 including isolated domains of the protein. Full-length GST-ctVps5 binds strongly to both the ctRetromer trimer and the ctVps29–ctVps35 subcomplex in the absence of ctVps26 (**Fig.1C**), which is in agreement with previously published results that Vps26 is dispensable for binding in solution[34]. Further examination using various truncation constructs of GST-ctVps5 showed that the ctVps29–ctVps35 subcomplex did not significantly interact with the PX and BAR domains but bound robustly to the N-terminal IDR of ctVps5 (residues 1-150) (**Figs 1C and 1D**). Residues 51-100 of the ctVps5 IDR were required and sufficient for this interaction (**Fig. 1D**). We then carried out isothermal titration calorimetry (ITC) analysis to measure the binding affinity between ctRetromer and various truncation constructs of ctVps5. Consistent with our GST pull-down experiments, ctRetromer bound to ctVps5$_{51-100}$ with a K_d of 660 nM (**Fig. 1E; Table 1**) whereas other Vps5 fragments including the PX and BAR domains failed to interact with Retromer in solution.

According to the structure-based sequence alignment of Vps5, the N-terminal IDR is much more divergent across species than the PX and BAR domains (**Figs. 1F and S1A**). Interestingly, in the *Pezizomycotina* subdivision of fungi to which *C. thermophlilum* belongs, we identified a conserved dual PL-containing sequence motif (^{73}DPLGPL^{78} in ctVps5), within the N-terminal IDR region that binds Retromer (**Figs. 1F and S1A**). Notably, this sequence is reminiscent of the PL motifs previously shown to bind human Retromer subunit Vps29 such as in TBC1D5[35-37], and is identical to the Vps29-binding macrocyclic peptide RT-D3 (**Fig. S2**) that had been identified in our previous *in vitro* screens[38]. Vps5 sequences from other subdivisions including *Saccharomycotina* also contain a conserved DPL motif (**Figs. 1F, S1B and S1C**), whereas presumed orthologs in higher eukaryotic species such as human SNX1 and SNX2 do not possess conserved PL motifs in their unstructured N-terminal regions (**Figs. 1F and S3**). While ctRetromer bound the N-terminal domain of ctVps5 with a K_d of 720 nM in ITC experiments, human SNX1 and human Retromer showed no detectable association under the same conditions (**Fig. 1G**).

The PL-containing motif of Vps5 is required for Retromer association

The conservation of a PL-containing motif within the N-terminal IDR of yeast Vps5 led us to test if this sequence was essential for the association with Retromer. Using ITC, we found that the association of full-length ctVps5 and ctRetromer was perturbed by the addition of cyclic peptide RT-D3[38] (**Fig. 2A and S2; Table 1**). A mutant control peptide RT-D1 L7E that does not bind Vps29[38]

had no effect on this association (**Figs. 2A and S2; Table 1**). ctVps29 alone could bind ctVps5$_{51-100}$, but with a lower affinity (K_d of 1.6 μM) compared to the full ctRetromer complex (K_d of 660 nM; **Fig. 2B; Table 1**). In this instance, addition of the RT-D3 peptide completely blocks the binding. We also examined complex formation between ctRetromer and ctVps5 using mass photometry (**Fig. 2C**). On their own ctVps5 in physiological salt conditions (200 mM NaCl) has a mass consistent with homodimer formation (via the C-terminal BAR domain), while ctRetromer appears primarily as a 1:1:1 heterotrimer under the same conditions. When ctRetromer–ctVps5 are combined in a 1:1 ratio we see formation of a complex with a mass consistent with one copy of ctRetromer bound to the ctVps5 dimer. Decreasing the ionic strength (40 mM NaCl) resulted in an increased mass corresponding to two copies of ctRetromer bound to the ctVps5 dimer consistent with previous studies showing ctRetromer can form a dimer of trimers (**Fig. S4A and S4B**). In both cases, the complex was lost upon the addition of the cyclic peptide RT-D3 (**Fig. 2C**), indicating that the ctRetromer and ctVps5 complexes no longer associate due to competition with the PL motif binding groove.

Next, we examined the impact of the PL-motif interaction on membrane recruitment of the ctRetromer complex with ctVps5 by performing liposome-pelleting experiments in the presence of the cyclic peptide RT-D3 which blocks the PL-motif binding site, or with RT-L4 which binds to the interface of Vps35–Vps26 and is not expected to affect ctVps5 interaction[38] (**Figs. 2D and 2E**). In control experiments, ctVps5 itself only bound to Folch I membranes supplemented with the phosphoinositide PtdIns3*P* and this was not affected by either peptide. The ctRetromer complex was robustly recruited to PtdIns3*P*-containing liposomes in the presence of ctVps5 in agreement with previous results[31]. The cyclic peptide RT-L4 did not affect liposome recruitment of ctRetromer as expected, whereas RT-D3 significantly reduced the amount of ctRetromer that was bound. Thus, in agreement with the solution binding studies, the interaction of the ctVps5 PL motif with ctRetromer is important for assembly of the ctRetromer–ctVps5 complex on membranes.

Structural basis for Vps5 interaction with Retromer

To better understand how Vps5 and Retromer interact at the molecular level we first used Alphafold2-multimer[39,40] as implemented in ColabFold[41] to model the potential binding interfaces. The predicted alignment error (PAE) plots and interfacial pTM (iPTM) scores showed a strong correlation between ctRetromer and a highly specific N-terminal region of ctVps5 (**Figs. 3A and S5A**). Across multiple models, we identified a high-confidence binding sequence within the N-terminal IDR of ctVps5 that matched precisely with the region containing the PL motif identified in our biochemical experiments (**Figures 3B, 3C, S5B**). In all predicted models, this sequence adopts an extended conformation, with N-terminal residues forming direct contacts with both ctVps35 and ctVps29 near the interface of the two proteins, with the key ^{73}DPLG^{76} residues forming a short turn-conformation that docks into the conserved hydrophobic pocket of ctVps29 (**Figs. 3B and 3C**). But

unlike the PL motif binding pocket, the ctVps35 and ctVps29 interface responsible for binding to the N-terminal residues of ctVps5 is rather diverse across different yeast species. This suggests that the PL motif may act as the primary conserved binding region, while the ctVps35 and ctVps29 interface is likely to play a more supportive or specificity-defining role.

To test the prediction from Alphafold2, we next determined the structure of Vps29 in complex with a ctVps5 peptide containing the core ^{73}DPL^{75} motif. Initially, a peptide composed of residues ^{53}SRATPRRIVAKPKSLEAVEDDPLGPLGA^{80} containing both predicted ctVps35 and ctVps29-binding sequences was screened. Its binding affinity for ctRetromer (K_d of 740 nM) corresponded well to that of the full-length ctVps5 (K_d of 720 nM) (**Fig. 3D; Table 1**). Attempts to obtain crystals of ctRetromer bound to the ctVps5$_{53-80}$ peptide were unsuccessful. However, after screening a variety of constructs, crystals of mouse VPS29 (mVPS29) in complex with a shorter version of the ctVps5 peptide ^{72}DDPLGPLGA^{80} were obtained (**Figs 3E and S5C; Table 1**). The peptide-bound crystal structure was solved at 1.7 Å revealing two molecules in the asymmetric unit (**Fig. 3F and 3G; Table 2**). The ctVps5$_{71-80}$ peptide residues D71 to G79 were clearly delineated in the electron density map and confirm that it binds to the conserved hydrophobic groove of VPS29 consisting of Leu152, Tyr163, and Tyr165 (Leu159, Tyr170 and Tyr172 respectively in *C. thermophilum*)**.** Interestingly however, the ctVps5 peptide adopts a slightly different orientation and registration in the crystal structure compared to the AlphaFold2 predicted models (**Fig. 3H, 3I and 3J**). The sequence ^{73}D<u>PL</u>G^{76} is invariably predicted by AlphaFold2 to form the primary contact with the Vps29 binding pocket residues Leu159, Tyr170 and Tyr172 (using *C. thermophilum* numbering), while in the crystal structure the primary contact in both molecules in asymmetric unit is shifted to an adjacent PL motif formed by ctVps5 residues ^{76}G<u>PL</u>G^{79}. In the crystal structure, the ctVps5 Asp73 side chain interacts with Lys173 in mVPS29, promoting a second contact between ctVps5 Asp72 and mVPS29 Lys21 (**Fig. 3I and 3J**). In ctVps29 the equivalent to mVPS29 Lys173 is Ala185 which in AlphaFold2 predicted models contacts Val70 in ctVps5. We therefore propose that binding orientation in the crystal structure (binding mode B) is likely reflecting a weaker secondary conformation stabilised by Lys173 in mVPS29, while that predicted by AlphaFold2 is the primary mode for yeast protein interactions (binding mode A) (**Fig. 3J**).

High affinity interaction with Vps5 is due to avidity of binding both Vps29 and Vps35

According to our modelling, residues 53 to 71 of ctVps5 (upstream of the core PL motif) form an extended contact with the ctVps29–ctVps35 interface (**Figs. 4A and 4B**). This mechanism appears to be relatively conserved as AlphaFold2-derived models of the complex from various species including *S. cerevisiae* and *S. pombe* invariably predict that Vps5 forms a similar extended interaction with sequences binding both the Vps29–Vps35 interface and the PL-interacting surface of Vps29 (**Figs. 4A and S6A-S6C**). Unlike the conserved Vps5 PL motif however, the Vps5 residues that associate with the Vps29–Vps35 interface are relatively diverse across the different yeast Vps5

198 orthologues (**Figs. S1A-S1C**). Interestingly, the extended binding motif in all three representative
199 models contains multiple lysine or arginine sidechains followed by hydrophobic residues that we
200 refer to this as the [K/R]Φ motif (where Φ is any bulky hydrophobic sidechain) contacting residues
201 at the Vps29 and Vps35 interface (**Figs. 4B-4D**). Specifically, we found that Arg59 of ctVps5 (Arg152
202 and Arg168 of scVps5 or Lys59 of spVps5) forms electrostatic contacts with the conserved Glu763
203 of ctVps35 (Glu418 of scVps35 and Glu329 of scVps35) (**Figs. 4B-4D**). These sequences both
204 contribute to Retromer binding affinity, as the ctVps5$_{51-70}$ and ctVps5$_{61-80}$ constructs containing either
205 the [K/R]Φ or the PL motifs, respectively, bind very weakly to ctRetromer when compared to
206 ctVps5$_{51-100}$, which contains both motifs (**Fig. 4E; Table 1**). Similarly, a ctVps5$_{51-100}$ construct with
207 the PL motif at position 74 and 75 substituted by alanine resulted in much weaker binding to
208 ctRetromer, although it did not completely abolish the interaction (**Fig. 4F**). The Alphafold model and
209 biochemical studies thus show a role of both the [K/R]Φ and PL motifs in associating with Retromer.
210 In addition, we noted that AlphaFold2 models of *S. cerevisiae* and *S. pombe* Vps5 predicted
211 an additional extended peptide interaction with the rear-side of the Vps35 α-helical solenoid, which
212 is not seen in the *C. thermophilum* structures (**Figs. 4A, S7A and S7B**). This interaction involves
213 Leu and Phe sidechains of Vps5 binding the C-terminal end of Vps35, remarkably resembling the
214 LFa repeat motifs (Leu, Phe and acidic sidechains) in human FAM21 protein that, as we recently
215 showed, bind to the same region of human VPS35[42]. We speculate that Vps5 orthologues containing
216 these three sequences together will create an even higher binding affinity for Retromer due to
217 enhanced avidity.
218
219 **Assembly of Retromer with the Vps5–Vps17 heterodimeric complex on membrane tubules**
220 In yeast, Vps17 is a functional partner of Vps5, forming heterodimers via their C-terminal BAR
221 domains[17,33,43,44]. Vps17 also has a long N-terminal unstructured domain but does not have a
222 conserved PL motif that could be identified (**Fig. S8**). Using ITC the unstructured N-terminal region
223 of ctVps17 containing residues 1 to 163 did not show any observable binding to ctRetromer
224 compared to ctVps5$_{1-185}$ (**Fig. 5A**). In parallel experiments, pull-down of the GST-tagged ctVps5–
225 ctVps17 heterodimer showed strong binding to ctRetromer which was lost upon the addition of RT-
226 D3 (**Fig. 5B**). This suggests that the PL motif-containing N-terminal domain of ctVps5 is primarily
227 responsible for heteropentameric complex assembly without any significant contribution from
228 ctVps17.
229 The structure of ctRetromer on membrane tubules was previously revealed by cryoET, in
230 complex with ctVps5 in a membrane-associated homodimeric form[31,32]. In this complex there would
231 be two copies of the ctVps5 N-terminal domain available for ctRetromer binding and recruitment,
232 although the resolution of this structure did not allow visualisation of the ctVps5 N-terminus. We
233 therefore wondered if a similar membrane-assembled Retromer coat would be formed in the
234 presence of the functional Vps5–Vps17 heterodimer where only a single PL motif is available. To

address this, we first generated the pentameric complex composed of core ctRetomer, full-length ctVps5 and ctVps17$_{PX\text{-}BAR}$ (PX and BAR domains of ctVps17) (**Fig. 5C**). The truncated ctVps17$_{PX\text{-}BAR}$ complex was used because it was more stable than the full-length ctVps17 protein following purification, and as shown above, the N-terminal region of Vps17 is not involved in ctRetromer interaction (**Fig. 5A and 5B**). Negative stain electron microscopy of the ctRetromer–ctVps5–ctVp17 heteropentameric complex on Folch liposomes supplemented with PtdIns3*P* revealed extended membrane tubules (**Fig. S9A**). These had a diameter of ~40 nm and were indistinguishable from the tubules generated by the ctRetromer–ctVps5 complex (**Fig. S9B**). In cryoEM and cryoET imaging, these tubules also showed a distinctive protein coat (**Fig. 5D; Movie S1**) similar in appearance to those of ctRetromer–ctVps5[31,32], with visible Retromer arches. We collected a cryoET dataset and resolved the coat architecture by a subtomogram averaging (STA) in a subset of this data. We first picked all tubules for reference-free STA. The resulting cryoEM map poorly resolved the features in the z-direction of tomographic reconstructions. This is a common problem due to the lack of resolution in this direction in tomographic reconstructions and preferential orientation of Retromer arches in the coat. We circumvented this by focusing the further STA on well-ordered tubes, producing a 32 Å cryoEM map with isotropic resolution (**Fig. 5E; Fig. S9C and S9D**). Together with coordinates and orientations of subtomograms, this map allowed us to visualise the coat architecture and model Retromer subunits arrangement with rigid body fit. On tubules with well-ordered Retromer, the coat is formed by two rows of arches that wind around the membrane tubules (**Fig. 5D-5F**). This forms a pseudo-helical arrangement, lacking strict helical symmetry as it accommodates various membrane diameters and gaps in structure, and is a known feature of the Retromer coat[31,32]. However, here the packing of the coat involves a close abutment of Vps26 dimers in adjacent rows which form the membrane proximal attachment point for the extended Retromer arches. Unfortunately, the resolution does not allow positioning of the PX-BAR domains of Vps5 and Vps17 heterodimers, which are seen only as a dense protein layer on the membrane (**Fig. 5E-5F**). However, the cryoET data suggests that compared to when the PX-BAR layer is composed of the single Vps5 protein, where redundancy of docking points for Vps26 dimers results in multiple possible spatial arrangements between the dimers occurring in the same coat[31], the presence of the Vps5–Vps17 heterodimer presents a more restricted set of docking sites for the Vps26 dimers resulting in a coat with a single predominant organisation.

The PL motif is required for recruitment of Retromer to supported membrane tubules by *S. cerevisiae* Vps5

To examine the impact of the Vps5 N-terminus on the dynamics of pentameric coat formation we turned to a supported membrane tube (SMT) assay, which has been established for the complex from *S. cerevisiae*[43,45]. As described above, the scVps5 N-terminal domain is predicted to make similar but even more extensive contacts as the *C. thermophilum* protein with scVps29 and scVps35.

The *S. cerevisiae* pentameric complex was assembled from purified recombinant scRetromer carrying mRuby and scVps5–scVps17 heterodimer (SNX-BAR) tagged with GFP. Incubation of SMTs with these purified proteins allowed us to observe coat growth in real time (**Fig. 6**). The impact of the scVps5 PL motif on growth of the coat was tested by addition of the competing peptide RT-D3, or of the control peptide RT-D3 with a scrambled sequence (**Fig. S2**). In control experiments, SNX-BAR-GFP and scRetromer-mRuby were co-recruited to the tubes within a minute after their addition (**Fig. 6A; Movie S4**), and after 2 to 3 minutes strong fluorescence signals concentrated at multiple discrete sites, suggesting the formation of distinct oligomeric segments on the SMTs (**Fig. 6A, B; Movie S5**). The lipid tracer Cy5.5-PE showed a reciprocal loss of fluorescence at the sites of the oligomeric segments (**Fig. 6B-E**). In our previous analyses of such effects in this system, we interpret this loss of Cy5.5-PE as a constriction of the membrane tubes by the coat[43]. While the scrambled peptide-D3 did not affect coat formation and membrane constriction, the RT-D3 peptide that competes with the PL-motif binding to Vps29 strongly reduced scRetromer recruitment and prevented membrane constriction (**Fig. 6A-6E**). This underlines the importance of the Vps5 N-terminus, and the PL motif in particular, for Retromer interaction.

As a further test of specificity of the cyclic peptide competitor we exploited the fact that at higher concentrations (100 nM), the SNX-BAR complex alone suffices to form oligomeric clusters that constrict the SMTs[43]. Since the SNX-BAR complex did not carry a fluorescent label we used the reduction of lipid fluorescence, which results from coat formation and tube constriction, as an assay. Under these conditions, the cyclic peptide RT-D3 had no effect on coat formation and membrane constriction, supporting the notion that its effect is linked to scRetromer association (**Fig. 6F; Movie S6**). Together, these SMT assays confirm that the interaction between the Vps5 PL motif and scRetromer is a prerequisite for pentameric complex assembly and allows Retromer to promote coat formation and membrane constriction by the SNX-BAR proteins.

The interaction of Retromer with the Vps5 N-terminal domain is required for Vps10 recycling

Our data illustrates the importance of the Vps5 N-terminal unstructured domain and its conserved PL motif in pentameric complex assembly both in solution and on synthetic membrane tubes. Lastly, we tested the relevance of these same sequences for the Vps5-Retromer interaction using two assays: The first exploits the strong fragmentation of vacuoles that results from a loss of Vps5-Retromer association. In this case, the freed Retromer integrates into the endolysosomal membrane fission-promoting complex CROP[46]**.** The resulting excessive fission activity leads to a strong fragmentation of the vacuoles into clusters containing a very high number of small vesicles, which can be visualized after staining the vacuolar membrane with the vital dye FM4-64. A second indicator of Vps5-Retromer interaction is the sorting of Vps10, a well-characterized CPY receptor cargo, which is recycled from endosomes to the Golgi using both Retromer and SNX-BARs[18,19]. We generated *S.*

308 *cerevisiae VPS5* knockout cells (*vps5Δ*) expressing a C-terminal, genomic Vps10-mNeonGreen
309 fusion. *vps5*Δ cells were rescued with *vps5* alleles carrying mutations that should block interactions
310 of its N-terminal domain with Retromer (**Fig. 7A**). Yeast *vps5Δ* cells accumulated Vps10-
311 mNeonGreen on fragmented vacuoles labelled with the fluorophore FM4-64 (**Fig. 7B**). A clear
312 recovery of large vacuoles and a localization of Vps10-mNeonGreen on punctate structures was
313 detected when we re-introduced wildtype VPS5 into the knockout cells. In agreement with data
314 published previously we consider these Vps10-mNeonGreen puncta as Golgi elements[18,34,46-48].
315 Vps5 containing just PX and BAR domains (vps5Δ1-280) rescued neither the sorting defect nor the
316 excessive vacuolar fragmentation of *vps5Δ* cells and Vps10-mNeonGreen accumulated on the
317 vacuolar membranes. A single amino acid substitution in the core PL motif, Vps5(L196R), generated
318 a partial defect in Vps10 recycling and a partial rescue of vacuole fragmentation. The quadruple
319 mutant vps5(L121G/F122G/F161E/L196R), which alters residues in the three main sites of predicted
320 interaction - the PL motif, the [K/R]Φ motif, and the Vps35-binding LF sequences - showed a severe
321 Vps10 recycling sorting defect and strong vacuole fragmentation similar to *vps5Δ* cells. These results
322 corroborate the structural and biochemical data, and demonstrate the relevance of the conserved
323 PL and [K/R]Φ motifs of Vps5 for interacting and functioning with Vps29 *in vivo*.

DISCUSSION

The Retromer trafficking complex was originally discovered in yeast in the form of a pentameric complex with the SNX-BAR proteins Vps5 and Vps17. Recent structural studies of both Retromer from *C. thermophilum* and metazoan species have shown that the Retromer trimer of Vps35–Vps26–Vps29 forms an asymmetric and oligomeric arch-like structure through a series of heterotypic and homotypic interactions, including Vps35 homodimerization at the apex and Vps26 homodimers forming direct contacts with the membrane-adjacent SNX protein adaptors[31,32,49]. In the case of the SNX-BAR proteins, Retromer subunit Vps26 assembles atop the surface of the membrane-bound array of PX and BAR domains, with Vps29 located at the distal apex of the Vps35 subunit. However, this model could not account for previous studies in *S. cerevisiae* that have shown the interaction of Retromer with the Vps5–Vps17 proteins requires Vps29 but not Vps26, and also requires the disordered N-terminal domain of Vps5[17,33,34]. This had led to an alternate model for Retromer and SNX-BAR assembly[34].

Our *in vitro* and *in vivo* studies of Retromer from both *C. thermophilum* and *S. cerevisiae* reconcile and extend these models of Retromer and SNX-BAR assembly. Our data shows that Vps5 in diverse yeast species contains a conserved Vps29-binding PL motif in the N-terminal unstructured region that is required both for Retromer association in solution and for recruiting Retromer into a functional membrane-assembled complex. In addition, the high affinity binding of yeast Retromer with Vps5 is promoted by two other key interactions. The first involves a [K/R]Φ motif upstream of the PL motif that makes an extended contact with the interface between the Vps29 and Vps35 Retromer subunits. This is conserved across all yeast species we have analysed. A second involves binding of a Leu and Phe containing sequence in Vps5 to the conserved convex surface of the Vps35 α-helical solenoid. This is present in species such as *S. cerevisiae*, but absent in *C. thermophilum*, and the binding mechanism is very similar to the recently identified LFa motifs of FAM21 interacting with the human Retromer complex[42]. These findings are completely consistent with both the cryoET structures of membrane assembled Retromer and Vps5[31,32], as well as the cellular studies demonstrating the importance of Vps29 and Vps5 N-terminal regions in complex formation[17,33,34]. Although the membrane-assembled complex involves direct association of Vps26 with the PX and BAR domains as shown by cryoET, the highly extended N-terminal region of Vps5 is capable of high affinity engagement with Vps29 and the C-terminal segment of Vps35 and is essential for their recruitment (**Fig. 8**). As this work was being prepared, similar findings were reported by Shortill and colleagues, providing further verification of these results[50].

Although previous structural studies by cryoET employed a simplified complex containing a homodimer of Vps5[31,32], we show here that Vps5–Vps17 homodimers can assemble with Retromer into membrane coats with a similar morphology *in vitro*. In comparison to Vps5 we found that Vps17 by itself does not stably homodimerize and has only weak membrane binding and remodelling ability (**Figs S9A–S9C**). Phylogenetic analysis suggests that Vps17 is not present in some early eukaryotes

like Archaeplastida and Excavata where Retromer may instead form a complex with a Vps5 homodimer[51]. This raises the question of what role the Vps17 protein plays in Retromer-mediated transport, as it is not required for Retromer interaction and membrane assembly. One possibility is that Vps17 could allow the heteropentameric complex to differentiate between specific cargoes. This idea is at least partly supported by the finding that deletion of Vps17 affects the recycling of Vps10 but not the alternative Retromer cargo Ear1, while Vps5 is required for both[34].

The SNX-BAR family of proteins almost universally possess unstructured N-terminal regions of different lengths and sequences upstream of the structural PX and BAR domains required for membrane interaction and dimeric complex formation[52]. Unlike the PX and BAR domains, the functional importance of these disordered regions are only just starting to be uncovered. The N-terminus of SNX1 and SNX2, the human homologues of Vps5, contains multiple DLF motifs capable of binding to the Retromer cargo adaptor SNX27[53,54]. However, SNX1 and SNX2 appear to lack the PL motif found in yeast Vps5 and are unable to bind human Retromer under similar conditions where Vps5 binds yeast Retromer with high affinity. This explains why previous studies have reported either no association[20-22,55,56] (this study) or only transient association[25,27,28,57-59] between the human SNX-BAR proteins and Retromer complexes. Current data suggests that the metazoan SNX1 and SNX2 proteins most likely associate indirectly with Retromer, potentially through SNX27 to form a cargo handover complex[53,54], although the precise spatial and temporal sequence of events of this are unknown. In addition to SNX1 and SNX2, the yeast SNX-BAR protein Vin1 (also known as YKR078W/Vps501) has been reported to associate with the VARP homolog Vrl1 through its unstructured N-termini to form a novel endosomal transport carrier[60]. Although Vrl1 has a PX-like and a BAR domain, the interaction does not rely on the typical BAR domain dimerization. Instead, the unstructured N-terminus of Vin1 is the primary driver of the interaction. An unexpected role of the disordered N-terminus has also been reported in another yeast SNX-BAR protein Mvp1, a protein known to promote Vps1-mediated fission of Retromer – SNX-BAR tubules[61]. In this instance it was shown that the unstructured N-terminus of Mvp1 promotes the formation of autoinhibited tetrameric assembly[62]. Interestingly, several studies have also shown that some proteins, including BAR domain-containing proteins, can employ combinations of structured and disordered regions together to drive membrane remodelling through molecular crowding of the entropically disordered regions[63-65]. Whether the N-terminal regions of Vps5–Vps17 and their human counterparts play a similar role in promoting membrane bending remains to be tested.

In summary, the findings presented here uncover a previously suspected but molecularly uncharacterised high affinity interaction between Retromer and the N-terminal IDR of Vps5. Notably this involves several motifs that are generally conserved, but also show distinct plasticity in their precise sequences and mechanism of Retromer interaction. This suggests a conserved requirement for this interaction across yeast species, but that the interaction mechanism itself is evolutionarily malleable. This could help explain why in humans the SNX-BAR proteins have apparently lost this

398 Retromer-binding capacity but have instead evolved sequences in their N-terminal domains for
399 binding distinct but functionally related proteins such as SNX27[53,54]. Furthermore, the site in VPS29
400 that binds to PL-containing motifs has itself been adapted in the human protein to mediate
401 interactions with human specific proteins including the Rab7 GAP TBC1D5[35] and VPS35-related
402 protein VPS35L[5-7]. How such activities have evolved, and how they are regulated across species
403 will be an important question for the future. More generally, Retromer-targeting therapeutics are
404 currently being developed to bind specific Retromer interfaces to stabilise and chaperone its function
405 in endosomal homeostasis[38,66,67], and understanding the precise details of Retromer's scaffolding
406 functions will be essential for assessing the functional impacts of such compounds.

MATERIALS AND METHODS

Lipids

The following lipids were purchased from Avanti Polar Lipids (USA): Egg L-alpha-phosphatidylcholine (EPC); 1,2-dioleoyl-sn-glycero-3-phospho-L-serine sodium salt (DOPS); 1,2-dioleoyl-sn-glycero-3-phospho-(1'-myo-inositol-3'-phosphate) (PI3P); 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-N-(Cyanine 5.5) (Cy5.5 PE); 1-Oleoyl-2-[12-[(7-nitro-2-1,3-benzoxadiazol-4-yl)amino]dodecanoyl]-sn-Glycero-3-Phosphocholine (NBD-PC). 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC) with 1-palmitoyl-2-oleoyl phosphatidylethanolamine (POPE). Folch fraction I, brain extract from bovine brain was purchased from Sigma-Aldrich (USA). All lipids were dissolved in chloroform. Phosphatidylinositol phosphates were dissolved in chloroform/methanol/water (20:10:1). Texas red DHPE (Thermofisher cat. T1395MP) was purchased as a mixed isomer. The para isomer was separated by thin layer chromatography as previously described[45].

Molecular biology

For the expression of *C. thermophilum* Retromer in bacteria, full-length cDNA encoding Vps35, Vps26 and Vps29 were cloned into either His-tagged pET28a and GST-tagged pGEX4T-2 vectors as described previously[31]. For ctVps29, an addition PreScission protease recognition site was engineered in front of the gene for efficient cleavage of GST-tag during purification. In the case of mVps29, the long isoform consists of an additional 5 residues on the N-terminus (starting MAGHR) and was cloned into pGEX4T2 vector without any extra linker between the construct and the thrombin recognition site. We found that the GST-tag cleavage was successful in the long isoform Vps29 without the need of extra linker observed in short isoform Vps29 (starting MLVL). cDNA encoding full-length ctVps5 was cloned into His-tagged pET28a and GST-tagged pMCSG10 vectors. Full-length ctVps17 was cloned into pGEX4T-2 vector, while ctVps17 encoding the PX and BAR domains was cloned into pET28a vector. For the truncation constructs, the DNA sequence encoding ctVps5$_{1\text{-}50}$, ctVps5$_{51\text{-}70}$, ctVps5$_{61\text{-}80}$, ctVps5$_{51\text{-}100}$, ctVps5$_{101\text{-}150}$, ctVps5$_{1\text{-}185}$, ctVps5$_{PX\text{-}BAR}$, ctVps17$_{1\text{-}160}$, and ctVps17$_{527–643}$ were cloned into pGEX4T2 vector. Site-directed mutagenesis was performed to generate mutant constructs. All constructs were verified using DNA sequencing.

Cell culture, strains and plasmids

The *Saccharomyces cerevisiae* strains are derived from *SEY6210* (**Table S3**). Vps5 deletion and endogenous tagging of Vps10 was conducted based on the CRISPR-Cas9 system[68]. The vector co-expressing single guide RNA (sgRNA) and Cas9 enzyme was co-transformed into yeast cells with double-stranded oligonucleotides as the templates for homologous recombination (**Table S4**). For Vps5 deletion, the template contained 40-bp homology upstream and downstream of its coding sequence. Vps10^{mNeonGreen} was created by a template comprising its C-terminal region with mutated PAM sequence and fused mNeonGreen tag followed by 40-bp homology region after the stop codon.

Vam10 was cloned into pRS305 vector using Not1/Sma1 restriction sites to generate an integrative plasmid that was linearized, transformed and integrated ectopically into the auxotrophic selectable marker *LEU2* gene locus[69].

The *VPS*5 gene with its native promoter, C-terminally fused HA-tag and ADH1 terminator was cloned into pRS314 centromeric plasmid. A point mutation was introduced in the start codon of *VAM*10 (M-to-I) by site-directed mutagenesis, because *VAM10* overlaps with *VPS5*[70]. This exchange does not change the amino acyl residues encoded on the opposite strand for *VPS*5 but abolishes *VAM*10 expression. For the N-terminal deletion mutant of Vps5 (Δ1-280), residues 838-2025 of *VPS5* were amplified and subcloned into the *pRS314-VPS5-HA* plasmid described above using Nco1/Sma1 restriction sites. The plasmid bearing the PL motif mutation (L196R) was generated using site-directed mutagenesis in the *pRS314-VPS5-HA* plasmid. To abolish all three putative binding sites to Retromer, a synthetic gene fragment of *VPS5* (ordered from GenScript) containing containing the following mutations - start codon of *VAM*10 sequence(M-to-I), L196R, L121G, F122G, and F161E - was subcloned into the *pRS314-VPS5-HA* plasmid using Nco1/Stu1 restriction sites.

Live microscopy of yeast cells

Vacuoles were stained with FM4-64. Cells were grown in SD^{-URA} medium to an OD_{600} between 0.6 and 1.0. The culture was diluted to an OD_{600} of 0.4, and FM4-64 was added to a final concentration of 10 µM from a 10 mM stock in DMSO. Cells were labelled for 60 mins, washed three times in fresh media, and then incubated for another 60 mins. Cells were concentrated by a brief low-speed centrifugation and placed on a glass microscopy slide overlaid with a thin glass coverslip (#1.5). Z-stacks with a spacing of 0.3 µm were collected on a NIKON Ti2E Yokogawa spinning disk confocal microscope and maximum intensity z-projections were made.

Recombinant Protein Production in Bacteria

All the bacterial constructs were expressed in BL21(DE3) competent cells using standard isopropyl 1-thio-β-D-galactopyranoside (IPTG) induction or autoinduction method as described previously[38,71,72]. To obtain the Retromer trimer complex, the GST-tagged Vps29 and Vps35 co-expressed cell pellet were mixed with His-Vps26 and resuspended in lysis buffer containing 50 mM Tris-HCl pH 7.5, 200 mM NaCl, 5% glycerol, 50 µg/ml benzamidine and DNase I before passed through a Constant System TS-Series cell disruptor. Similarly, the GST-tagged ctVps5-ctVps17$_{PX-BAR}$ heterodimer were obtained using co-expression method as described above. For ctVps5 homodimer, the His-tagged construct was used for optimal expression yield. For the GST-tagged full-length ctVps17, approximately 100 g of cell pellet was collected and lysed for each purification due to the poor expression yield. For all constructs, the lysate was clarified by centrifugation and the supernatant was loaded onto either Talon® resin (Clonetech) or glutathione sepharose (GE healthcare) for initial purification. To obtain the correct stoichiometry ratio of the Retromer complex,

481 both Talon® resin and glutathione sepharose (GE healthcare) were applied. Removal of the GST-
482 tag from mVps29, ctVps29 and ctVps5 was performed using on-column cleavage overnight at 4°C.
483 The flow-through containing complex was further purified using size-exclusion chromatography
484 (SEC) equilibrated with a buffer containing 50 mM Tris-HCl pH 7.5, 200 mM NaCl, 2 mM β-ME. To
485 obtain ctRetromer–ctVps5 and the pentameric complexes, the purified ctRetromer was mixed with
486 either purified ctVps5 or ctVps5–ctVps17$_{PX\text{-}BAR}$ complex in 1 to 1 ratio for at least 1 h at 4°C. To avoid
487 the non-specific cleavage by the residual proteases such as thrombin, a tablet of protease inhibitor
488 cocktail was added into the complex mixture. To remove the unbound fractions, the complex mixture
489 was further purified using Superose 6 increase 10/300 GL column using the SEC buffer described
490 above.

491 Expression and purification of the GST-tag truncation constructs including hSNX1$_{1\text{-}139}$,
492 ctVps5$_{1\text{-}50}$, ctVps5$_{51\text{-}70}$, ctVps5$_{61\text{-}80}$, ctVps5$_{51\text{-}100}$, ctVps5$_{101\text{-}150}$, ctVps5$_{1\text{-}185}$, ctVps5$_{PX\text{-}BAR}$, and ctVps17$_{1\text{-}}$
493 $_{160}$ was performed using the same buffer and method described above. In brief, the clarified lysate
494 was loaded onto glutathione sepharose (GE healthcare) and eluted using elution buffer containing
495 50 mM Tris-HCl pH 7.5, 200 mM NaCl, 5% glycerol, 20 mM glutathione reduced and 50 µg/ml
496 benzamidine. The flow-through was further purified using SEC as the final purification step. To avoid
497 the degradation, the constructs containing N-terminal disordered parts of ctVps5 and ctVps17 were
498 used in fresh and experiments were finished within 3 days after purification.

499

500 **Recombinant Protein Production in Yeast**

501 TAP-tagged scRetromer and scSNX-BAR complexes were extracted from yeast as previously
502 described[44,73]. Briefly, a 50 mL preculture of cells was grown over night to saturation in YPGal
503 medium. The next day, two 1 L cultures in YPGal were inoculated with 15 mL of preculture and grown
504 for 20 h to late log phase (OD_{600} = 2 to 3). All following steps were performed at 4°C. Cells were
505 pelleted and washed with 1 pellet volume of cold RP buffer (Retromer purification buffer: 50 mM Tris
506 pH 8.0, 300 mM NaCl, 1 mM $MgCl_2$, 1 mM PMSF, Roche complete protease inhibitor). Pellets were
507 either processed immediately or flash-frozen in liquid nitrogen and stored at -80°C. For cell lysis, the
508 pellet was resuspended in one volume of RP buffer and passed through a French press (One shot
509 cell disruptor, Constant Systems LTD, Daventry, UK) at 2.2 Kpsi. 1 mg DNase I was added to the
510 lysate followed by a 20 min incubation on a rotating wheel. The lysate was precleared by
511 centrifugation for 30 min at 45’000 xg in a Beckman JLA 25.50 rotor and cleared by a 60 min
512 centrifugation at 150’000 xg in a Beckman Ti60 rotor. The cleared supernatant was passed through
513 a 0.2 µm filter and transferred to a 50 mL Falcon tube. 1 mL IgG bead suspension (GE Healthcare,
514 cat 17-0969-01) was washed three times with RP buffer and added to the supernatant. After 60 min
515 incubation on a rotating wheel, beads were spun down and washed 3 times with RP buffer. 250 µg
516 of purified HIS-TEV protease from *E. coli* was added to the beads. After 30 min incubation at 4°C,
517 beads were centrifuged, the supernatant containing purified Retromer subcomplex was collected

and concentrated on a 100 kDa cutoff column (Pierce™ Protein Concentrator PES, 100K MWCO). The concentrated protein fraction was re-diluted in RP buffer and reconcentrated 3 times. This final step allowed for removal of TEV protease and a high enrichment for intact complexes. Proteins were concentrated to ~2 mg/mL, aliquoted in 10 µL fractions and flash-frozen in liquid nitrogen. Proteins were stored at -80°C and used within 3 months. Thawed aliquots were used only once.

Isothermal Titration Calorimetry

The binding affinities between Retromer and various SNX-BAR constructs were determined using a Microcal PEAQ ITC (Malvern) at 25°C. All the proteins used for ITC experiments were prepared in buffer containing 50 mM Tris-HCl pH 7.5, 200 mM NaCl, 2 mM β-ME. To map the precise binding region between ctVps5 and ctRetromer, 300 µM of GST-tagged ctVps5 N-terminal truncation constructs including GST-ctVps5$_{51-50}$, GST-ctVps5$_{51-100}$, GST-ctVps5$_{101-150}$ and ctVps5$_{PX-BAR}$ was titrated into 10 µM ctRetromer. The effect of cyclic peptides on ctVps5 and ctRetromer interaction was performed by titrating 200 µM of full-length ctVps5 or GST-ctVps5$_{51-100}$ into 10 µM ctRetromer or 10 µM ctVps29 containing either 100 µM of RT-D3 or RT-D1 L7E mutant cyclic peptides. Under the same protein concentration, GST-ctVps5$_{51-100}$ containing PL74AA mutant was also titrated into ctRetromer or ctVps29 to confirm the effect of PL motif. To examine if the N-terminal region of ctVps17 also involved in binding with ctRetromer, 300 µM of GST-ctVps17$_{1-160}$ was titrated into 10 µM ctRetromer. In parallel, 300 µM of GST-ctVps5$_{1-185}$ was titrated into 10 µM ctRetromer or hVps29 for the comparison analysis. In all cases, the ITC titration sequence consisted of an initial 0.4 µl (not used in data processing) followed by 12 serial injections of 3.22 µl each with 180 sec intervals. The resulting titration data were integrated with Malvern software package by fitting and normalized data to a single-site binding model, yielding the thermodynamic parameters K_d, ΔH, ΔG and $-T\Delta S$ for all binding experiments. The stoichiometry was refined initially, and if the value was close to 1, then N was set to exactly 1.0 for calculation. Data presented are the mean of triplicate titrations for each experiment.

GST pull-down assays

For the GST pull-down assay, 1 nmol GST-tagged ctVps5, ctVps5–ctVps17$_{PX-BAR}$ heterodimer, ctVps5$_{1-50}$, ctVps5$_{51-100}$, ctVps5$_{1-150}$, and GST alone were mixed separately with 2 nmol of ctRetromer or ctDimer (ctVps29 - ctVps35 subcomplex). For the pull-down between GST-ctVps5 - ctVps17$_{PX-BAR}$ heterodimer and ctRetromer, either 8 molar excess of RT-D3 or equivalent volume of DMSO were added to examine the effect of PL motif on pentameric complex assembly. All reaction mixtures were incubated for 2 hr at 4°C before added to fresh glutathione Sepharose pre-equilibrated with buffer containing 50 mM Tris-HCl pH 7.5, 200 mM NaCl, 5% glycerol, 0.5 mM TCEP and 0.1% IGEPAL CA-630 for another 1 hour at 4°C. Beads were washed five times with the equilibration buffer and bounds proteins were analysed by SDS-PAGE gels.

555

556 **Liposome binding and tubulation assays with negative stain electron microscopy**

557 The control PC/PE liposome was prepared by mixing POPC with POPE in a 90:10 molar ratio. In
558 brief, 34.2 µl of POPC at 13.2 mM (10 mg/ml) was mixed with 3.6 µl of POPE at 13.9 mM (10mg/ml),
559 to a total volume of 500 µl chloroform in a mini round bottom flask. The lipid film was dried down on
560 the walls under N2 stream and left overnight in a vacuum desiccator. Similarly, the Folch I-PtdIns(3)*P*
561 lipid film was prepared by adding 50 µl of 10 mg/ml Folch fraction I with 47.9 µl of di-C16 PtdIns(3)*P*,
562 to a total volume of 500 µl chloroform. The lipid film was dried down in the same way as PCPE lipid
563 film. To form multilamellar vesicles (MLVs), the lipid film was hydrated with protein SEC buffer
564 containing 50 mM Tris-HCl pH 7.5, 200 mM NaCl, 2 mM β-ME for 30 min and then 10 cycles of rapid
565 freeze-thaw process. To form small unilamellar vesicle (SUVs), the MLVs were extruded 21 times
566 using 400 µm pore size membrane. The liposome pelleting assay was conducted in a total volume
567 of 60 µl comprising equal volume of 10 µM ctRetromer – ctVps5 complex, pentameric complex,
568 ctVps5 or ctVps17 alone and SUVs in the presence of DMSO, RT-D3 or RT-L4. The reaction mixture
569 was incubated at room temperature for 30 min followed by 60,000 rpm (rotor TLA100) for 35 min at
570 4°C. The supernatant and pelleted fractions were then carefully separated and analyzed by SDS-
571 PAGE.

572 Tubulation assays were performed similar to the protocol described previously[31]. In brief, 0.5
573 mg/ml final concentration of Folch I - PtdIns(3)*P* liposome was incubated with 4 to 5 µM of protein
574 or protein complex indicated including ctVps5, ctVps17, ctRetromer, ctRetromer–ctVps5,
575 ctRetromer–ctVps5–ctVps17 pentameric complex, hRetromer, SNX1 + RT-D1 or hRetromer–
576 SNX1+RT-D1 complex for 30 min at room temperature in protein SEC buffer before spotted on a
577 carbon/formvar-coated copper mesh grid. Sample on the grid was washed once with SEC buffer
578 followed by three times MilliQ water prior negative stained by 2% uranylacetate for 30 sec. The grid
579 was imaged and analyzed on a FEI Tecnai F30 G2 or JEOL JEM1011 transmission electron
580 microscope.

581

582 **Supported membrane tubes (SMTs)**

583 Supported membrane tubes were generated as described[45]. Briefly, glass coverslips were first
584 washed with 3 M NaOH for 5 min and rinsed with water before a 60 min treatment with piranha
585 solution (95% H_2SO_4 / 30% H_2O_2 3:2 v/v). Coverslips were rinsed with water and dried on a heat
586 block at 90°C. Coverslips were then silanized with 3-glycidyloxypropyltrimethoxysilane (Catalogue
587 no. 440167, Sigma) for 5 h under vacuum, rinsed with acetone and dried. Polyethylene glycol coating
588 was done by placing the coverslips in a beaker containing PEG400 (Sigma) at 90°C for 60 h.
589 Coverslips were washed with distilled water and stored for up to 2 months at room temperature in a
590 closed container.

To generate supported membrane tubes, lipids were mixed from 10 mg/mL stocks in a glass vial and diluted to a final concentration of 1 mg/mL in chloroform. The same lipid mix was used throughout this study (5% PI(3)P, 15% DOPS, 0.1% fluorescent lipid tracer (Cy5.5 PE or Texas red DHPE), 79.5% egg-PC). Lipids were then spotted (typically 1 µL, corresponding to about 1 nmol) on the coverslips and dried for 30 min under vacuum. The coverslip was mounted on an IBIDI 6-channel µ-slide (µ-Slide VI 0.4. IBIDI, catalog no: 80606). Lipids were hydrated for 15 min with buffer (PBS) and SMTs were generated by injecting PBS into the chamber using an Aladdin Single-Syringe Pump (World Precision Instruments, model n°. AL-1000) at a flow rate of 1,5 mL/min for 5 min. SMTs were left to stabilize without flow for 5 min before the start of the experiment. Protein stocks (typically 1-2 µM) were first diluted in PBS and then injected in the chamber at a flow rate of 80 µL per minute. Cyclic peptides were added to the proteins from a 30 mM stock in DMSO to a final concentration of 300 µM. the protein/peptide mix was incubated for 1 min at room temp (23°C) before being added to the SMTubes. Tubes were imaged with a NIKON Ti2 spinning disc confocal microscope equipped with a 100x 1.49 NA objective.

Crystallization and Data Collection

All crystals were grown by the hanging-drop vapor diffusion method at 20°C. For co-crystallization of ctVps5$_{71-80}$ peptide, 6-fold molar excess of ctVps5$_{71-80}$ peptide was added to the purified mVps29 protein to a final protein concentration of 11 mg/ml. Best quality crystals were obtained by mixing 1 µL of protein with 1 µL of well solution containing 40 mM KH_2PO_4, 16% PEG8000 and 5% glycerol at room temperature. Crystals grew after 13 days were cryoprotected in reservoir solution with additional 16% glycerol. X-ray diffraction data were carried out at the Australian Synchrotron MX2 beamline at 100 K.

Crystal structure Determination

Data collected from the Australian Synchrotron were indexed and integrated by AutoXDS and scaled using Aimless[74]. Both structures were solved by molecular replacement using Phaser[75] with native mVps29 structure as the initial model template. Both structures contain two copies of mVps29 in asymmetric unit with the electron density of peptide clearly visible. The refinement was performed using Phenix with inspection of resulting model in Coot guided by F_o–F_c difference maps[76]. Molprobity was used to evaluate the quality geometry of the refined structure[77]. Data collection and refinement statistics are summarized in **Table S1**. Molecular figures were generated using PyMOL and structural comparison was performed using DALI and Foldseek[78,79]. Sequence conservation was mapped on the structure with ConSurf server[80].

Cryo-electron tomography grid preparation and data acquisition

627 The ctRetromer – ctVps5 – ctVps17$_{PX\text{-}BAR}$ pentameric complex for CryoET data acquisition was
628 incubated at a final concentration of 6.5 μM purified complex with 0.6 mg/ml of freshly prepared
629 Folch I - PtdIns(3)*P* liposomes for 30 min at room temperature in SEC buffer. Prior to blotting, 10 nm
630 Protein A-gold fiducials were added to the protein-liposome tubulation mixture in 1:5 ratio. The
631 blotting was carried out by loading 3.2 μL of the reaction mixture on a glow-discharged holey carbon
632 grid (CF-2/1-3C, protochips) and plunge-frozen in liquid nitrogen cooled liquid ethane using a
633 Vitrobot (Mark IV, Thermo Fisher Scientific).
634 Imaging acquisition was performed on a 200 kV Talos Arctica (Thermo Fisher Scientific)
635 transmission electron microscope fitted with a Falcon 3EC direct electron detector (Thermo Fisher
636 Scientific) at the University of New South Wales, Australia. A total of 73 dose-symmetric tilt series
637 were collected with tilt range ± 60°, 3° angular increment and defoci between -3.5 μm and -5.0 μm.
638 The acquisition magnification was 45,000x with a pixel size of 3.24 Å. Total dose of each tilt series
639 was ranged from 120 - 130 e/A^2. Data collection parameters are summarized in Table S2.
640
641 **Tomogram reconstruction**
642 Assembly of tomograms from raw movies was done with IMOD 4.12.45 package[81]. Gain-reference
643 corrected movies from the microscope were aligned, summed and filtered according to the deposited
644 electron dose using aligframes (IMOD 4.12.45) to generate sorted tilt series used for tomogram
645 reconstruction. Per-tilt defocus estimation was done on non-dose weighted stacks in ctfplotter [82].
646 The stacks further aligned using fiducials-based, 2D-CTF corrected and used for weighted back
647 projection tomogram reconstruction by etomo package (IMOD 4.12.45). For visualisation only, 8-
648 times binned tomograms were processed with deep learning denoising and wedge restoration
649 package IsoNet [83].
650
651 **Subtomogram averaging**
652 Subtomogram alignment was done as previously[32] using the subTOM package
653 (https://github.com/DustinMorado/subTOM/releases/tag/v1.1.4), and custom and dynamo-
654 package[84] MATLAB scripts. Initial subtomogram positions were calculated by manual tracing of
655 coated tubules in bin4 tomograms using UCSF Chimera (1.16) and PickParticle plugin[85] and placing
656 subtomogram positions in the middle of the coat with orientation normal to the tube surface at
657 sampling frequency of 2 pixels (~30 Å). 29 coated tubules, including both well and poorly ordered
658 (see example in **Fig 5D**), were selected from 6 tomograms. The initial average was generated from
659 the seeded positions, and reference-free alignment with 2-fold symmetrisation using a cut-shell soft
660 shape masking the coat was iterated until the convergence with gradual refinement of angular and
661 translational search. The resulting cryoEM map contained arch-like structures that were smeared in
662 the narrow direction of arches. Inspection of tomogram positions in space (Chimera PlaceInSpace
663 plugin[85] indicated that this is due to failure of subtomograms to reliably lock on arches in z-direction

of tomograms on poorly ordered but not well-ordered tubules. Further STA was done only with ordered tubules after removing subtomogram duplicates converged to the same positions by distance threshold and using per tubule cross-correlation threshold to remove subprograms that lost membrane alignment. The cleaned dataset contained ~600 particles in 3 tubules split in odd-even datasets and refined independently in bin2 tomograms. The highest low-pass filter used in the STA was 38 Å, and FSC between the two half maps inside the alignment mask reached 32 Å at 0.143 (**Fig. S9D**). For the final map, the half maps were summed, sharpened with B-factor of -2500 and filtered to the measured resolution (**Fig. S9E).**

Model building

The PDB models of the ctRetromer arch (dimer of dimers of CtVps39/CtVps35, (7BLR) and dimer of ctVps26 (7BLQ)) were placed manually in densities and then fitted as ridged bodies using the sequential fit command in Chimera at resolution 32 Å. The initial placement of the arch PDB was decided by comparing the fit cc-values for its two orientations (0° and 180° rotated). The gap in cross-correlation scores was apparent for all four arch densities in the map (0.7 vs 0.9).

AlphaFold predictions

Structural predictions of Retromer and SNX-BAR proteins were performed using Alphafold2-multimer[39,40] implemented within the freely accessible ColabFold pipeline[41]. For each modeling experiment, ColabFold was executed using default settings where multiple sequence alignments were generated with MMseqs2. To map the exact binding regions between ctRetromer, ctVps5 and ctVps17, we first conducted the modelling using full-length proteins. The high-confidence binding region in Vps5 was then mapped and cross-checked with our biochemical data prior of generating a more "focused" model containing shorter sequences. The focused models including ctVps35$_{407\text{-}869}$ – ctVps29 – ctVps5$_{1\text{-}180}$ and ctVps35 – ctVps26 – ctVps17$_{527–643}$ complexes were performed in combination with AMBER energy minimization to optimize amino acid stereochemistry. In each run, we validated the quality of models using the predicted alignment errors (PAE), the prediction confidence measures including pLDDT and interfacial ipTM scores as well as the alignment of the multiple structures generated using pymol. Sequence conservation was mapped on the predicted structure with ConSurf server[80].

Mass photometry

Molecular mass measurement was performed using a Refeyn OneMP mass photometer (Refeyn Ltd) following manufacturer's instructions. Prior to the experiment, calibration was performed using protein standards with known molecular weight (i.e. 66, 132 and 480 kDa) in buffer containing 50 mM Tris-HCl pH 7.5, 200 mM NaCl, 2 mM β-ME. Next, 10 µl of indicated protein at a final protein concentration of 100 nM – 50 nM was loaded onto the mass photometer, and 1,000 frames were

701 recorded. For the molecular mass measurement at the low ionic strength condition, all proteins were
702 dialyzed into low salt buffer containing 50 mM Tris-HCl pH 7.5, 40 mM NaCl, 2 mM β-ME prior of
703 recording. The precise molecular mass was calculated using the calibration standards as reference.
704

705 **Data availability**

706 Crystal structure of hVps29 – ctVps5$_{71-80}$ peptide complex has been deposited at the Protein Data
707 Bank with accession codes (8FUD). All the relevant raw data related to this study is available from
708 the corresponding authors on request.
709

710 **Competing interests statement**

711 The authors declare that they have no competing financial interests.
712

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730 Initial Concept: BMC, NL, and KC.
731 Concept development: all authors.
732 Biochemistry, ITC, molecular modelling, structure determination and analysis: KC, NL, QG, and
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Writing – review & editing: all authors.

742 **FIGURE LEGENDS**

743 **Figure 1. Vps5 interacts directly with Retromer via its unstructured N-terminus.**

744 (**A**) Prediction of disordered sequences in ctVps5. Predicted regions of disorder were mapped using
745 the D2P2 web server[86]. (**B**) The predicted structure of ctVps5 from the Alphafold2 database[87]
746 coloured according to the per-residue confidence (pLDDT) score. The N-terminal IDR shows low
747 confidence metrics (represented as green and orange) suggesting a lack of secondary or tertiary
748 structure. (**C**) GST pull-down demonstrating the direct interactions between full-length GST-tagged
749 ctVps5 and either the full ctRetromer complex or the ctVps29-ctVps35 subcomplex. (**D**) GST pull-
750 down showing GST-ctVps5 unstructured N-terminal IDR (residues 1-150) is required and sufficient
751 to bind the ctVps29-ctVps35 subcomplex. (**E**) ITC measurements of binding between ctRetromer
752 and various truncation constructs of ctVps5. ctRetromer binds with high affinity to ctVps5$_{51-100}$. (**F**)
753 Sequence alignment of key regions of the Vps5 unstructured IDR showing the conserved PL motif
754 (highlighted in red square) across different yeast species but not in metazoan ortholog SNX1. *ct,*
755 *Chaetomium thermophilum; vd, Verticillium dahlia*; *cm, Cordyceps militaris; nc, Neurospora crassa;*
756 *mb, Metarhizium brunneum; Sc, Saccharomyces cerevisiae; zp,* Zygosaccharomyces parabailii*; pp,*
757 *Pichia_Pastoris; hb, Hyphopichia burtonii; Ag, Ashbya gossypii; h, Homo sapiens; m, Mus musculus;*
758 *r, Rattus norvegicus; d, Drosophila melanogaster; z, Danio rerio; ce, Caenorhabditis elegans.*
759 Extended sequence alignments are shown in **Fig. S1.** (**G**) ITC measurements of the binding between
760 ctRetromer and GST-ctVps5$_{1-185}$. Under the same conditions there is no detectable interaction
761 observed between hRetromer and GST-hSNX1$_{1-139}$. All ITC graphs show the integrated and
762 normalized data fit with a 1 to 1 binding ratio.

763

764 **Figure 2. The PL-containing motif of Vps5 is required for Retromer association.**

765 (**A**) ITC measurements of ctVps5 and (**B**) GST-ctVps5$_{51-100}$ with ctRetromer or ctVps29 in the
766 presence or absence of Vps29-binding macrocyclic peptides[38]. RT-D3 binds to Vps29 and inhibits
767 interactions with PL motif-containing proteins. RT-D1(L7E) is a mutant peptide that does not bind
768 Vps29 and acts as a negative control. (**C**) Mass photometry of ctVps5, ctRetromer and the ctVps5–
769 ctRetromer complex in the presence and absence of RT-D3 inhibitor. (**D**) Liposome-pelleting assays
770 of ctVps5 and ctRetromer–ctVps5 complex in the presence of indicated cyclic peptides. Unilamellar
771 vesicles were composed of either PC/PE control lipid, or Folch I lipids supplemented with PtdIns(3)*P*.
772 "S" and "P" indicates unbound supernatant and bound pellet respectively. **(E)** Quantitation of
773 Liposome-binding assay as shown in (**D**). Blue indicates the native sample containing DMSO, green
774 indicates sample in the presence of RT-D3 and orange indicates sample containing RT-L4 (which
775 binds to the Vps26–Vps35 interface and is not expected to interfere with Vps5 interaction). Data
776 represent the mean ± standard deviation of at least two independent experiments.

777

778 **Figure 3. Structural basis for Vps5 interaction with Retromer.**

779 **(A)** PAE plots showing the three top-ranked Alphafold2 predicted models of the trimeric ctVps35$_{407-}$
780 $_{869}$–ctVps29–ctVps5$_{1-180}$ subcomplex. **(B)** The top-ranked model of ctVps35–ctVps29 is shown in
781 ribbon diagram, with the bound region of ctVps5$_{1-180}$ of all three predictions overlaid. The close
782 overlap of the three predicted binding regions of ctVps5$_{1-180}$ suggests a confidently predicted
783 interaction. (**C**) As in (**B**) but with the predicted subcomplex model coloured according to the pLDDT
784 score. The conserved PL motif binding pocket (site 1) and the ctVps29–ctVps35 interface (site 2)
785 are indicated. The key ctVps5 residues involved in binding to the ctVps29–ctVps35 interface are
786 shown in further detail in the expanded box. **(D)** ITC measurements showing binding of ctRetromer
787 to a synthetic ctVps5$_{63-80}$ peptide. (**E**) ITC measurements showing binding of mVPS29 to a short
788 synthetic ctVps5$_{71-80}$ peptide. (**F**) Crystal structure of the mVPS29–ctVps5$_{71-80}$ peptide complex
789 showing two molecules in the asymmetric unit. For clarity, the ctVps5$_{71-80}$ peptide is shown in stick
790 mode. **(G)** Electron density of the ctVps5$_{71-80}$ peptide, corresponding to a simulated-annealing OMIT
791 Fo-Fc map contoured at 3σ. **(H)** Sequence conservation mapped onto the mVPS29 protein surface
792 highlights the PL motif binding surface. The ctVps5$_{71-80}$ peptide observed in the crystal structure is
793 shown in light orange, and the Alphafold2 prediction of same sequence bound to ctVps29 are shown
794 in stick representation for comparison. (**I**) Overlay of the mVPS29–ctVps5$_{71-80}$ crystal structure (light
795 purple) is aligned with the previously determined apo ctVps29 crystal structure[31] (pink; PDB ID:
796 5W8M) showing the key residues involved in binding. (**J**) Structural models showing how ctVps5
797 binds to ctVps29 through its dual PL motif binding mechanism. Left and right panels represent the
798 GPLG and DPLG binding mode respectively.
799
800 **Figure 4. High affinity interaction with Retromer involves multiple sequences within the Vps5**
801 **N-terminal domain.**
802 **(A)** Surface representation of the Vps29–Vps35–Vps5 Alphafold2-derived models from *C.*
803 *thermophilum*, *S. cerevisiae* and *S. pombe*. The key regions of Vps5 involved in binding are shown
804 as C_α ribbons. **(B)** Cartoon representation highlighting the key Lys, Arg and hydrophobic residues
805 upstream of ctVps5, **(C)** scVps5 and **(D)** spVps5 PL motifs involved in binding. For ctVps5, the green
806 and orange sticks represent the DPLG and GPLG binding mode respectively. (**E)** ITC measurements
807 showing the binding between ctRetromer and various truncation constructs of ctVps5 and **(F)**
808 ctVps5(P74A/L75A) (PL74AA mutant). GST-ctVps5$_{51-100}$ PL74AA mutant loss its binding affinity to
809 ctRetromer compared to the wild-type under the same conditions. All ITC graphs show the integrated
810 and normalized data fit with a 1 to 1 binding ratio.
811
812 **Figure 5. Assembly of Retromer with the Vps5–Vps17 heterodimeric complex on membrane**
813 **tubules.**
814 (**A**) ITC of ctRetromer with GST-ctVps17$^{1-163}$ or GST-ctVps5$^{1-185}$. Under the same conditions and
815 concentrations no interaction is detected with the Vps17 N-terminal domain. (**B**) GST pull-down

816 demonstrating the direct interaction between GST-ctVps5–ctVps17^{PX-BAR} and either ctRetromer or
817 the ctVps35–ctVps29 dimeric subcomplex. The interactions are blocked by adding inhibitory cyclic
818 peptide RT-D3. (**C**) Mass photometry of heteropentameric complex composed of full-length ctVps35,
819 ctVps26, ctVps26, ctVps5 and ctVps17^{PX-BAR}. The SDS-PAGE gel of the complex is shown on the
820 right. (**D**) Left panel shows a slice from a tomographic reconstitution showing examples of coated
821 tubules with well-ordered and poorly ordered Retromer complexes side by side. The right panel
822 shows the overlay of the tubule with a model that was generated by placing copies of the cryoEM
823 map at positions and orientations determined by STA. See also **Movies S2 and S3**. (**E**) Close up of
824 the tubule model shown in profile and cross-section. The coat is formed by the two chains of
825 Retromer arches (separate helical rows are clored in grey and caramel for clarity). These form
826 pseudo-helices winding around the membrane tubule. This arrangement lacks strict helical
827 symmetry as the pitch and units per turn adapts to the tubule diameter. (**F**). Molecular model
828 generated by rigid body fit of ctRetromer[38] to the cryoEM map. The specific arrangement of two
829 adjacent Vps26 dimers (in green) was not seen in the previous Retromer structure using Vps5
830 alone[38]. Note that the PX-BAR domains of the Vps5-Vps17 heterodimers are not resolved, and seen
831 only as a dense membrane-proximal layer.
832
833 **Figure 6. The PL motif of *S. cerevisiae* Vps5 is required for recruitment of Retromer to**
834 **supported membrane tubules.**
835 **(A)** Effect of RT-D3 and RT-D3 scrambled cyclic peptides on SNX-BAR-GFP and scRetromer-
836 mRuby recruitment on supported membrane tubes (SMTs). SMTs containing 5% PtdIns(3)*P* were
837 formed and incubated with 25 nM SNX-BAR-GFP and 50 nM scRetromer-mRuby while being
838 imaged by confocal microscopy. Frame rate: 0.1 Hz, cyclic peptide concentration: 300 μM. See also
839 **Movies S4 and S5**. Green: SNX-BAR-GFP, red Retromer-mRuby, blue: Cy5.5-PE. **(B)** Kymograph
840 of SMTs shown in **(A)** over the time course of the whole experiment (10 min). **(C)** Quantification of
841 the recruitment of SNX-BAR-GFP and Retromer-mRuby on the tubule. Quantification was done
842 using FIJI, measuring the mean fluorescence signal on the tubule over a 10 μM length (N = 14
843 tubules from 2 experiments). **(D)** SMTs after 10 min of incubation with 25 nM SNX-BAR-GFP, 50 nM
844 Retromer-mRuby, and 300 μM of RT-D3 or RT-D3 scrambled. **(E)** Line scan intensity plot of the
845 SMTs indicated by the white box in (**D**). **(F)** SMTs were incubated with 100 nM SNX-BAR and 300
846 μM of RT-D3 or RT-D3 scrambled and imaged by confocal microscopy at a frame rate of 0.5 Hz.
847 Red: Texas-Red DHPE. See also **Movie S6**.
848
849 **Figure 7. The interaction of Retromer with the Vps5 N-terminal domain is required for Vps10**
850 **recycling.**
851 (**A**) AlphaFold2 predicted structure of the scVps35–scVps29–scVps5 complex highlighting residues
852 targeted for mutagenesis. (**B**) Yeast cells (SEY6210) carrying a Vps5 deletion were complemented

853 with integrative plasmids expressing variants of Vps5 with Vam10 (because the *VAM10* gene is
854 embedded in *VPS5* and deleted with it). Vps10 was genomically tagged with a C-terminal
855 mNeonGreen fusion. These cells were logarithmically grown in SD^{-URA} medium, stained with FM4-
856 64 and analysed by confocal microscopy. Maximum intensity projections of z-stacks are shown.
857 Scale bar: 5 µm.
858
859 **Figure 8. Proposed model for the assembly of yeast Retromer with Vps5–Vps17 required for**
860 **vacuolar cargo recycling.**
861 Schematic diagram of the yeast Retromer–Vps5–Vps17 complex and its assembly on the
862 endosomal/vacuolar membrane. The sequences located in the flexible N-terminus of Vps5 include
863 PL and [K/R]ϕ motifs, and additional LF-containing sequences specific to the *Saccharomycotina*
864 subdivision binds to Vps29 and Vps35 sites at the apex of the Retromer arch. This high affinity
865 interaction is required for initial pentameric complex formation prior to subsequent assembly at the
866 membrane surface where Vps26 then forms homodimers on top of the BAR domains of Vps5–Vps17
867 heterodimers. Blocking the N-terminal Vps5 interaction disrupts Retromer assembly and prevents
868 recycling of cargos such as Vps10 resulting in their accumulation at the vacuole.
869
870

871 **SUPPLEMENTARY INFORMATION.**

872 **Figure S1. Sequence alignment of yeast and fungus Vps5.**

873 **(A)** Sequence alignment of Vps5 unstructured N-terminal domain across Pezizomycotina, **(B)**
874 Schizosaccharomycetes, and **(C)** Saccharomycotina subdivisions. *ct, Chaetomium thermophilum;*
875 *vd, Verticillium dahlia*; *cm, Cordyceps militaris; nc, Neurospora crassa; mb, Metarhizium brunneum;*
876 *sp, Saccharomyces pombe; ni, Neolecta irregularis; pj, Pneumocystis jirovecii; td, Taphrina*
877 *deformans; sj, Schizosaccharomyces japonicus*, *Sc, Saccharomyces cerevisiae; zp,*
878 Zygosaccharomyces parabailii*; pp, Pichia_Pastoris; hb, Hyphopichia burtonii; Ag, Ashbya gossypii.*

879

880 **Figure S2. Schematic diagram showing the three different cyclic peptides used in this study.**
881 Macrocyclic peptides used in this study and previously identified to bind Retromer[38]. The key DPL
882 motif sequence in RT-D3 is highlighted in red. RT-D3 binds to Vps29 and inhibits PL motif-containing
883 protein interactions. Control peptides are RT-D3 scramble and RT-D1(L7E) mutant which are both
884 unable to interact with Retromer due to loss of the core PL sequence.

885

886 **Figure S3. Sequence alignment of ctVps5 and SNX1 from higher eukaryotic species.**
887 *Ct, Chaetomium thermophilum; h, homo sapiens; m, Mus musculus; zf, Zebrafish; d, Danio rerio;*
888 *Ce, Caenorhabditis elegans.*

889

890 **Figure S4. ctVps5-ctRetromer complex formation in low ionic strength condition. (A)** Mass
891 photometry of ctRetromer, ctVps5 and the ctVps5–ctRetromer complex in buffer containing 40 mM
892 NaCl. **(B)** Schematic diagram showing the formation of 1 to 1 or 2 to 1 ctRetromer–ctVps5 dimer
893 ratio complex in solution depending on the ionic strength of the buffer.

894

895 **Figure S5. AlphaFold2 predictions of ctRetromer in complex with ctVps5 highlighting the**
896 **conservation of the PL motif mediated binding mechanism.**
897 **(A)** Predicted alignment Error (PAE) plots showing the three predicted models of ctVps26–ctVps35–
898 ctVps29–ctVps5 complex. **(B)** Superposition of the three representative models generated by
899 Alphafold2. Models were colored according to the pLDDT score. **(C)** ITC measurement showing
900 mVPS29 is capable of binding to GST-tagged ctVps5$_{1\text{-}185}$. ITC graph shows the integrated and
901 normalized data fit with a 1 to 1 binding ratio.

902

903 **Figure S6. AlphaFold2 predictions of Retromer in complex with Vps5 from two different yeast**
904 **species.**
905 **(A)** Right panel presents the PAE plots of the three predicted scRetromer–scVps5 models, **(B)**
906 scVps35$_{331\text{-}944}$–scVps29–scVps5$_{1\text{-}282}$ models, and **(C)** spVps35$_{379\text{-}836}$–spVps29–spVps5$_{1\text{-}199}$ models.

907 Next to the PAE plot is the superposition of the three top ranked predicted complexes colored
908 according to the pLDDT score.
909
910 **Figure S7. Extended Vps35 binding sites observed in AlphaFold2 models of Retromer in**
911 **complex with Vps5 from *S. cerevisiae* and *S. pombe*.**
912 **(A)** Representative (top-ranked) predicted model of scVps35$_{331-944}$–scVps29–scVps5$_{1-282}$ complex,
913 and **(B)** spVps35$_{379-836}$–spVps29–spVps5$_{1-199}$ complex. In both cases only the C-terminal region of
914 Vps35 and the key residues of Vps5 involved in binding are shown.
915
916 **Figure S8. Sequence alignment of Vps17 across the Pezizomycotina subdivision**.
917 The N-terminal disordered region of Vps17 is highly divergent in sequence compared to the PX and
918 BAR domains. *ct, Chaetomium thermophilum; vd, Verticillium dahlia*; *cm, Cordyceps militaris; nc,*
919 *Neurospora crassa; mb, Metarhizium brunneum.*
920
921 **Figure S9. Analysis of Retromer tubules by negative stain EM and cryoEM.**
922 (**A**) Example micrographs of negatively stained Folch I - PtdIns(3)*P* liposomes incubated with
923 ctRetromer–ctVps5 complex (left) or ctRetromer–ctVps5–ctVps17PX-BAR complex (right). (**B**)
924 Histogram showing the diameter of tubules generated by ctRetromer–ctVps5 or ctRetromer–ctVps5–
925 ctVps17PX-BAR complexes binned as percentage of total number of tubules. (**C**) FSC of two
926 independently processed half maps. (**D**) Orthoslices through the final sharpened and filtered to EM
927 map, sharpened with B -2500. (**E**) Mass photometry of full-length ctVps17. (**F**) Liposome-pelleting
928 assays of ctVps17. Unilamellar vesicles were composed of either PC/PE control lipid, or Folch I lipids
929 supplemented with PtdIns(3)*P*. “S” and “P” indicates unbound supernatant and bound pellet
930 respectively. (**G**) Example micrographs of negatively stained Folch I - PtdIns(3)*P* liposome, extruded
931 to 400 nm diameter and incubated with either ctVps5 or ctVps17.
932
933
934 **Movie S1 (related to Fig. 5F).** CryoET image of ctRetromer–Vps5–Vps17 coated membrane
935 tubules.
936
937 **Movie S2 (related to Fig. 5G).** Reconstructed tomogram of ctRetromer–Vps5–Vps17 coated tubule
938 shown along the tubule axis.
939
940 **Movie S3 (related to Fig. 5G).** Reconstructed tomogram of ctRetromer–Vps5–Vps17 coated tubule
941 shown from the side.
942

943 **Movie S4 (related to Fig. 6A).** Recruitment of scSNX-BAR-GFP and scRetromer-mRuby on
944 supported membrane tubes in the presence of RT-D3 scrambled.
945
946 **Movie S5 (related to Fig. 6A).** Recruitment of scSNX-BAR-GFP and scRetromer-mRuby on
947 supported membrane tubes in the presence of RT-D3
948
949 **Movie S6 (related to Fig. 6F).** Supported membrane tubes with 100 nM scSNX-BARs and and 300
950 μM of the cyclic peptide RT-D3 or RT-D3 scrambled.
951

952 **Table 1: Thermodynamic parameters for the binding of Retromer and Vps5/SNX1 by ITC.**

953

	K_d (μM)	ΔH (kcal/mol)	ΔG (kcal/mol)	$-T\Delta S$ (kcal/mol)
ctRetromer				
ctVps5	0.72 ± 0.01	-8.06 ± 0.76	-8.39 ± 0.01	-1.03 ± 0.24
GST-ctVps5$_{1-185}$	0.72 ± 0.02	-3.24 ± 0.80	-8.38 ± 0.01	-5.16 ± 0.81
GST-ctVps5$_{1-50}$	39.70 ± 8.60	-2.61 ± 0.34	-6.01 ± 0.13	-3.41 ± 0.47
GST-ctVps5$_{51-70}$	No binding detected			
GST-ctVps5$_{61-80}$	60.07 ± 12.24	-7.22 ± 1.94	-5.76 ± 0.12	1.45 ± 2.07
GST-ctVps5$_{51-100}$	0.66 ± 0.01	-4.10 ± 0.6	-8.44 ± 0.01	-4.31 ± 0.54
GST-ctVps5$_{51-100}$ PL74AA	5.18 ± 0.14	-1.74 ± 0.32	-7.21 ± 0.01	-5.48 ± 0.30
GST-ctVps5$_{101-150}$	No binding detected			
ctVps5$_{PX-BAR}$	No binding detected			
ctVps5$_{53-80}$ peptide	0.74 ± 0.07	-2.94 ± 0.71	-8.38 ± 0.01	-5.44 ± 0.77
ctVsp5$_{71-80}$ peptide	No binding detected			
GST-ctVps17$_{1-163}$	No binding detected			
ctRetromer + RT-D3				
ctVps5	9.07 ± 0.63	-10.95± 0.35	-6.88 ± 0.04	4.09 ± 0.37
ctRetromer + RT-D1 L7E				
ctVps5	0.72 ± 0.02	-7.58 ± 0.09	-8.37 ±0.05	-0.81 ± 0.12
ctVps29				
ctVps5$_{51-100}$	1.60 ± 0.03	-2.16 ± 0.60	-7.87 ± 0.06	-5.75 ± 0.61
hRetromer				
mSNX1	No binding detected			
mVps29				
ctVps5$_{1-185}$	4.88 ± 1.34	-1.04 ± 0.39	-7.18 ± 0.04	-5.98 ± 0.57
ctVsp5$_{71-80}$ peptide	53.60 ± 10.90	-8.00 ± 1.50	-5.90 ± 0.10	-2.10 ± 1.30

954

955

956

957 **Table S1. Summary of crystallographic structure determination statistics**

Data collection statistics	mVps29 – ctVps5$_{71-80}$
PDB ID	8FUD
Space group	C121
Resolution (Å)	42.46 – 1.68
	(1.71 – 1.68)
a, b, c (Å)	54.05, 68.60, 211.14
α, β, γ (°)	90.00, 90.00, 90.00
Total observations	332,485 (15,268)
Unique reflections	44,804 (2,057)
Completeness (%)	98.9 (91.4)
R_{merge}^{+}	0.043 (0.301)
R_{pim*}	0.017 (0.116)
CC1/2	0.999 (0.972)
$<I/\sigma(I)>$	18.6 (4.0)
Multiplicity	7.4 (7.4)
Molecule/asym	2
Refinement statistics	
R_{work}/R_{free} (%) ¶#	17.7/20.3
No. protein atoms	3189
Waters	230
Wilson B (Å^2)	21.74
Average B (Å^2)^	27.4
Protein	26.3
peptide	39.9
Water	35.5
rmsd bonds (Å)	0.007
rmsd angles (°)	1.068
Ramachandran plot:	
Favored/outliers (%)	98.4/1.1

958 Values in parentheses refer to the highest resolution shell. $^{+}R_{merge} = \Sigma|I - <I>| / \Sigma<I>$, where I is the
959 intensity of each individual reflection. $^{*}R_{pim}$ indicates all I^{+} & I^{-}. $^{¶}R_{work} = \Sigma h|F_o - F_c| / \Sigma_l| F_o|$, where F_o
960 *and* F_c are the observed and calculated structure-factor amplitudes for each reflection *h.* $^{\#}R_{free}$ was
961 calculated with 10% of the diffraction data selected randomly and excluded from refinement.
962 ^Calculated using Baverage.

963

964

965 **Table S2: CryoET data collection and processing statistics**

Data collection (units)	ctRetromer – ctVp5 – ctVps17$_{PX\text{-}BAR}$
Magnification	45,000
Detector	Falcon 3EC
Voltage (keV)	200
Electron exposure (e/ Å^2)	120 - 130
Defocus range (µm)	-3.5 to -5.0
Pixel size (Å)	3.24
Energy filter	Yes
Slit width (eV)	20
Tilt range (min/max, step)	-60 ° /+60 °, 3 °
Tilt scheme	Dose-symmetrical
Symmetry imposed	No

966

967 **Table S3: Yeast strains used in this study**

Strain	Genotype
SEY6210	Δvps5 Vam10-V5::LEU2 Vps10^{mNeonGreen}
SEY6210	Δvps5 Vam10-V5::LEU2 Vps10^{mNeonGreen} , plasmid pRS314-VPS5-HA::TRP1
SEY6210	Δvps5 Vam10-V5::LEU2 Vps10^{mNeonGreen} , plasmid pRS314-VPS5$^{\Delta 1\text{-}280}$-HA::TRP1
SEY6210	Δvps5 Vam10-V5::LEU2 Vps10^{mNeonGreen} , plasmid pRS314-VPS5^{L196R}-HA::TRP1
SEY6210	Δvps5 Vam10-V5::LEU2 Vps10^{mNeonGreen}, plasmid pRS314-VPS5$^{L196R\ L121G\ F122G\ F161E}$-HA::TRP1

968

969

970

971

972 **Table S4: Primers used in the yeast study**

Primers	Sequence 5' -> 3'
RC71_Fw guide Vps5	*ATCACTGCCCAAGTACAACGGTTTTAGAG*
RC72_Rv guide Vps5	*CTAGCTCTAAAACCGTTGTACTTGGGCAGTGATACGT*
RC75_ FwVps5del	*GGGATTTTATAAACTTTCATACATCCTGCAATAACAAGCCATGATCTGTATACAATAACTTTATGTGTCACGTTCCTCAG*
RC76_ R Vps5 del	*CTGAGGAACGTGACACATAAAGTTATTGTATACAGATCATGGCTTGTTATTGCAGGATGTATGAAAGTTTATAAAATCCC*
RC77_F Vps5 chk up	*GTCCGCTAAGAACAACTAAG*
RC78_R Vps5 chk dw	*GGATATTGAAATGCTTCTGAGC*
RC57_Pr Vam10- F-NotI	*tctaaGCGGCCGCCGCCTGCTTACCTGTGC*
RC63_Vam10-R-Sma1	*aatctCCCGGGAATATCTACAAATGAAGAAAGAAAAGAC*
RC64_pCM189-V5-CYC1-Sma1-F	*aatctCCCGGGGGCAAGCCCATCCCCAAC*
RC65_pCM189-V5-CYC1 (HindIII)-R	*CATGATTACGCCAAGCTTGGCC*
RC66_Kpn1-F-Vps5 Pr	*catctGGTACCCTGTACTGAAGTAGTCAGGAATC*
RC67_Sma1-R-Vps5	*aatctCCCGGGAAGATTGGTTTGGTAGAATGTCTC*
RC68_Sma1-F-HA-ADH1 Tr	*aatctCCCGGGggtagtgctggaagtgca*
RC69_mNG-HA-ADH1 Tr-R-Spe1	*caggcACTAGTccctgttatccctagcgg*
RC70_N-ter Del_Vps5 -Nco1F	*aatctCCATGGctTTCAAAGTTGAAGTTAAAGAC*
RC79_Vam10 start Codon Mut-F	*TCACCAAATACTTCAAAAAGtATCTCTGGAAGAAGATCCGG*
RC80_Vam10 start Codon Mut-R	*CCGGATCTTCTTCCAGAGATaCTTTTTGAAGTATTTGGTGA*
RC81_Vps5 Mut-L196R-F	*CACCCTTTACTGATCCCagaAAGAAAGCTGAAAAAGAG*
RC82_Vps5 Mut-L196R-R	*CTCTTTTTCAGCTTTCTTtctGGGATCAGTAAAGGGTG*

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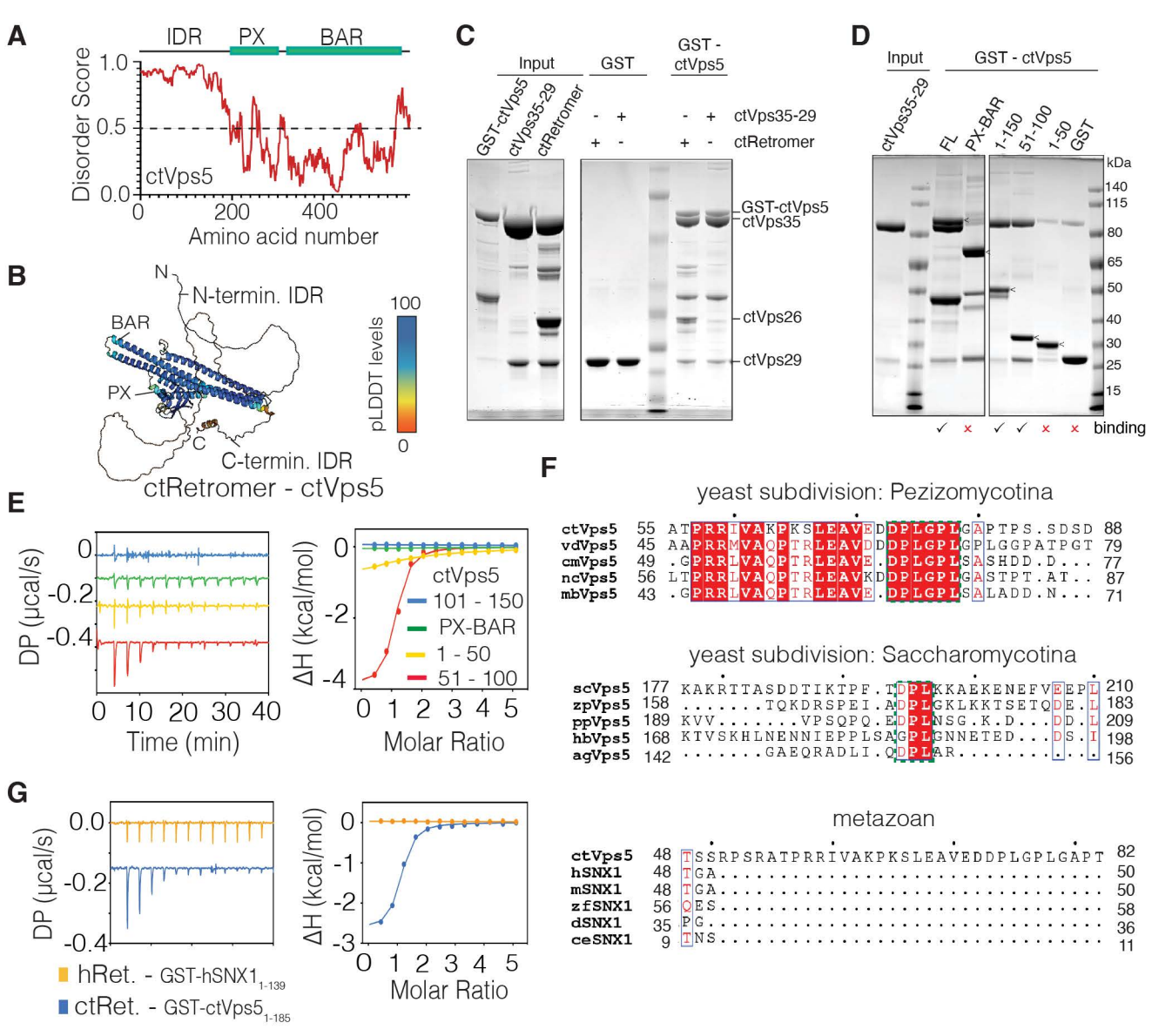


Figure 1. Vps5 interacts directly with Retromer via its unstructured N-terminus.
(A) Prediction of disordered sequences in ctVps5. Predicted regions of disorder were mapped using the D2P2 web server78. (B) The predicted structure of ctVps5 from the Alphafold2 database79 coloured according to the per-residue confidence (pLDDT) score. The N-terminal IDR shows low confidence metrics (represented as green and orange) suggesting a lack of secondary or tertiary structure. (C) GST pull-down demonstrating the direct interactions between full-length GST-tagged ctVps5 and either the full ctRetromer complex or the ctVps29-ctVps35 subcomplex. (D) GST pull-down showing GST-ctVps5 unstructured N-terminal IDR (residues 1-150) is required and sufficient to bind the ctVps29-ctVps35 subcomplex. (E) ITC measurements of binding between ctRetromer and various truncation constructs of ctVps5. ctRetromer binds with high affinity to ctVps551-100. (F) Sequence alignment of key regions of the Vps5 unstructured IDR showing the conserved PL motif (highlighted in red square) across different yeast species but not in metazoan ortholog SNX1. ct, Chaetomium thermophilum; vd, Verticillium dahlia; cm, Cordyceps militaris; nc, Neurospora crassa; mb, Metarhizium brunneum; Sc, Saccharomyces cerevisiae; zp, Zygosaccharomyces parabailii; pp, Pichia_Pastoris; hb, Hyphopichia burtonii; Ag, Ashbya gossypii; h, Homo sapiens; m, Mus musculus; r, Rattus norvegicus; d, Drosophila melanogaster; z, Danio rerio; ce, Caenorhabditis elegans. Extended sequence alignments are shown in Fig. S1. (G) ITC measurements of the binding between ctRetromer and GST-ctVps51-185. Under the same conditions there is no detectable interaction observed between hRetromer and GST-hSNX11-139. All ITC graphs show the integrated and normalized data fit with a 1 to 1 binding ratio.

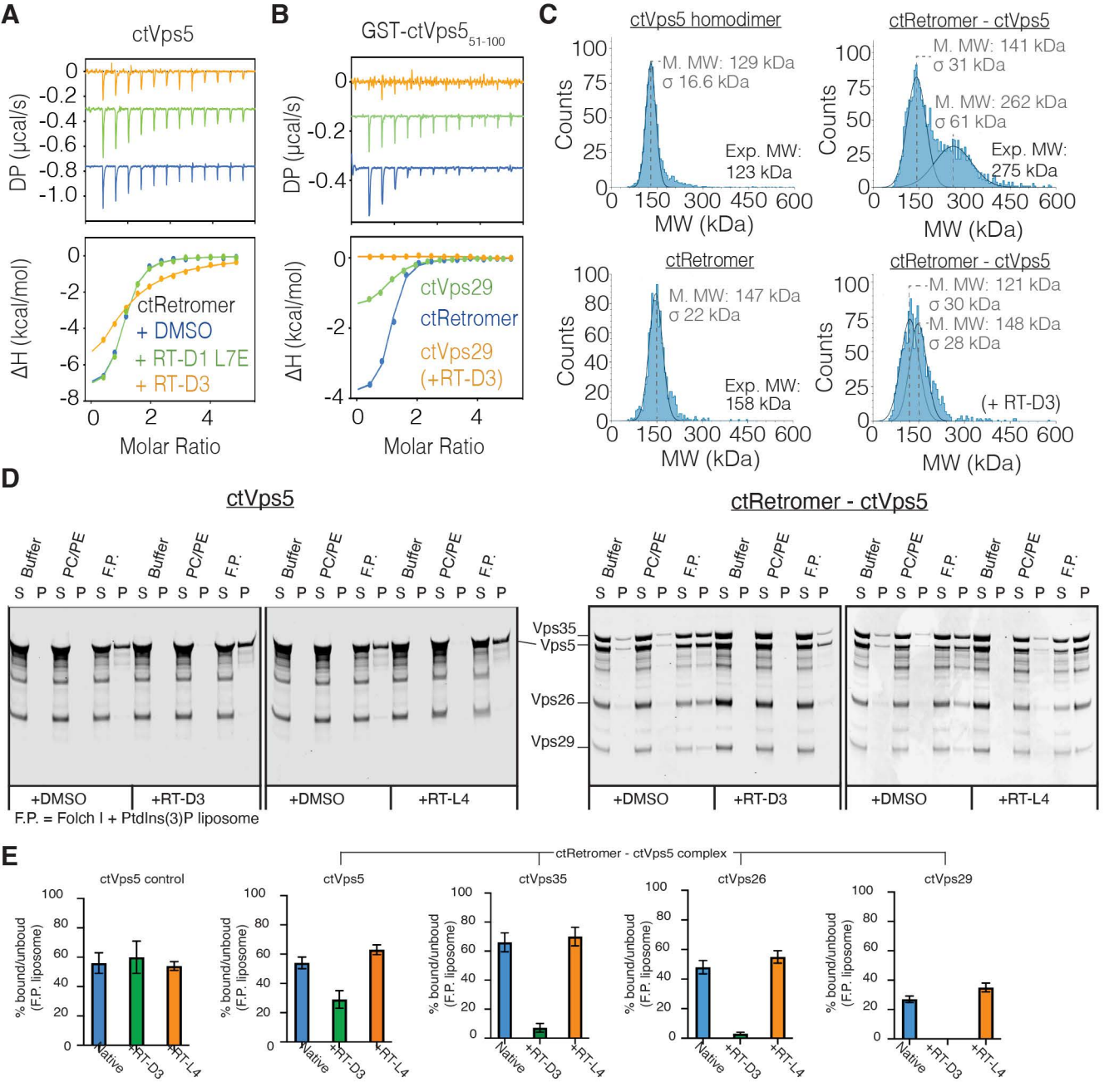


Figure 2. The PL-containing motif of Vps5 is required for Retromer association.
(A) ITC measurements of ctVps5 and (B) GST-ctVps551-100 with ctRetromer or ctVps29 in the presence or absence of Vps29-binding macrocyclic peptides38. RT-D3 binds to Vps29 and inhibits interactions with PL motif-containing proteins. RT-D1(L7E) is a mutant peptide that does not bind Vps29 and acts as a negative control. (C) Mass photometry of ctVps5, ctRetromer and the ctVps5–ctRetromer complex in the presence and absence of RT-D3 inhibitor. (D) Liposome-pelleting assays of ctVps5 and ctRetromer–ctVps5 complex in the presence of indicated cyclic peptides. Unilamellar vesicles were composed of either PC/PE control lipid, or Folch I lipids supplemented with PtdIns(3)P. "S" and "P" indicates unbound supernatant and bound pellet respectively. (E) Quantitation of Liposome-binding assay as shown in (D). Blue indicates the native sample containing DMSO, green indicates sample in the presence of RT-D3 and orange indicates sample containing RT-L4 (which binds to the Vps26–Vps35 interface and is not expected to interfere with Vps5 interaction). Data represent the mean ± standard deviation of at least two independent experiments.

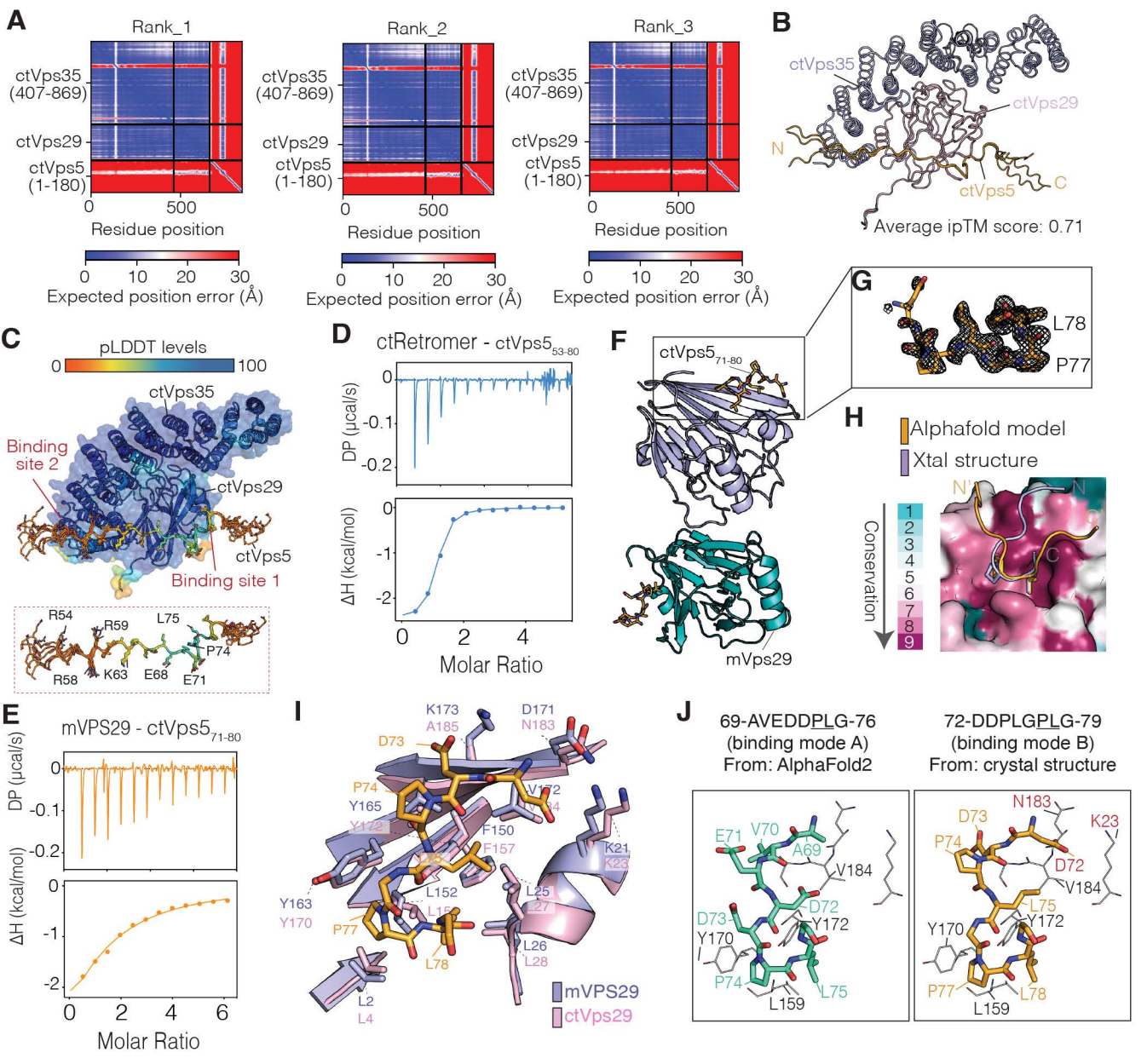


Figure 3. Structural basis for Vps5 interaction with Retromer.
(A) PAE plots showing the three top-ranked Alphafold2 predicted models of the trimeric ctVps35407-869–ctVps29–ctVps51-180 subcomplex. (B) The top-ranked model of ctVps35–ctVps29 is shown in ribbon diagram, with the bound region of ctVps51-180 of all three predictions overlaid. The close overlap of the three predicted binding regions of ctVps51-180 suggests a confidently predicted interaction. (C) As in (B) but with the predicted subcomplex model coloured according to the pLDDT score. The conserved PL motif binding pocket (site 1) and the ctVps29–ctVps35 interface (site 2) are indicated. The key ctVps5 residues involved in binding to the ctVps29–ctVps35 interface are shown in further detail in the expanded box. (D) ITC measurements showing binding of ctRetromer to a synthetic ctVps563-80 peptide. (E) ITC measurements showing binding of mVPS29 to a short synthetic ctVps571-80 peptide. (F) Crystal structure of the mVPS29–ctVps571-80 peptide complex showing two molecules in the asymmetric unit. For clarity, the ctVps571-80 peptide is shown in stick mode. (G) Electron density of the ctVps571-80 peptide, corresponding to a simulated-annealing OMIT Fo-Fc map contoured at 3σ. (H) Sequence conservation mapped onto the mVPS29 protein surface highlights the PL motif binding surface. The ctVps571-80 peptide observed in the crystal structure is shown in light orange, and the Alphafold2 prediction of same sequence bound to ctVps29 are shown in stick representation for comparison. (I) Overlay of the mVPS29–ctVps571-80 crystal structure (light purple) is aligned with the previously determined apo ctVps29 crystal structure31 (pink; PDB ID: 5W8M) showing the key residues involved in binding. (J) Structural models showing how ctVps5 binds to ctVps29 through its dual PL motif binding mechanism. Left and right panels represent the GPLG and DPLG binding mode respectively.

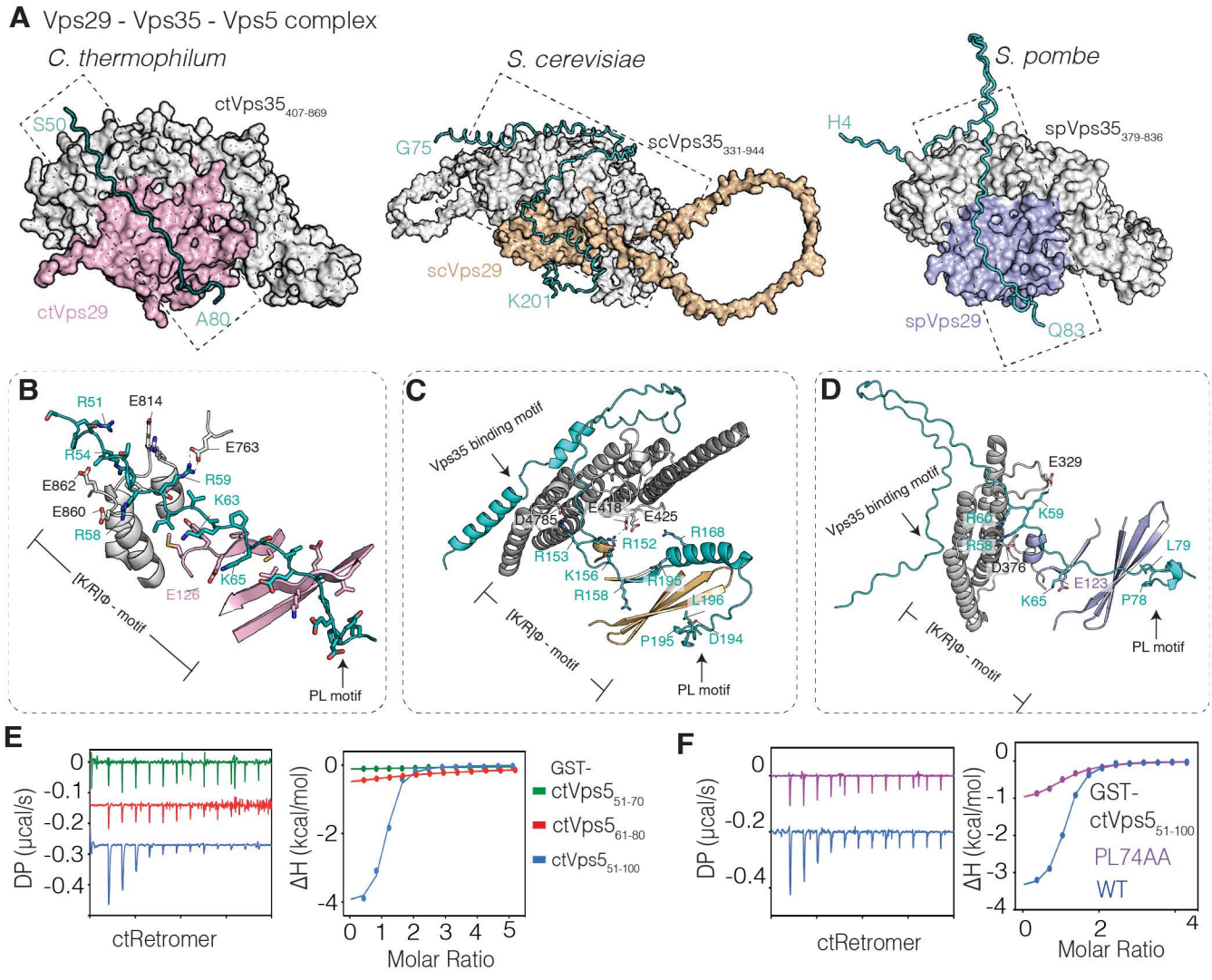


Figure 4. High affinity interaction with Retromer involves multiple sequences within the Vps5 N-terminal domain.
(A) Surface representation of the Vps29–Vps35–Vps5 Alphafold2-derived models from C. thermophilum, S. cerevisiae and S. pombe. The key regions of Vps5 involved in binding are shown as Cα ribbons. (B) Cartoon representation highlighting the key Lys, Arg and hydrophobic residues upstream of ctVps5, (C) scVps5 and (D) spVps5 PL motifs involved in binding. For ctVps5, the green and orange sticks represent the DPLG and GPLG binding mode respectively. (E) ITC measurements showing the binding between ctRetromer and various truncation constructs of ctVps5 and (F) ctVps5(P74A/L75A) (PL74AA mutant). GST-ctVps551-100 PL74AA mutant loss its binding affinity to ctRetromer compared to the wild-type under the same conditions. All ITC graphs show the integrated and normalized data fit with a 1 to 1 binding ratio.

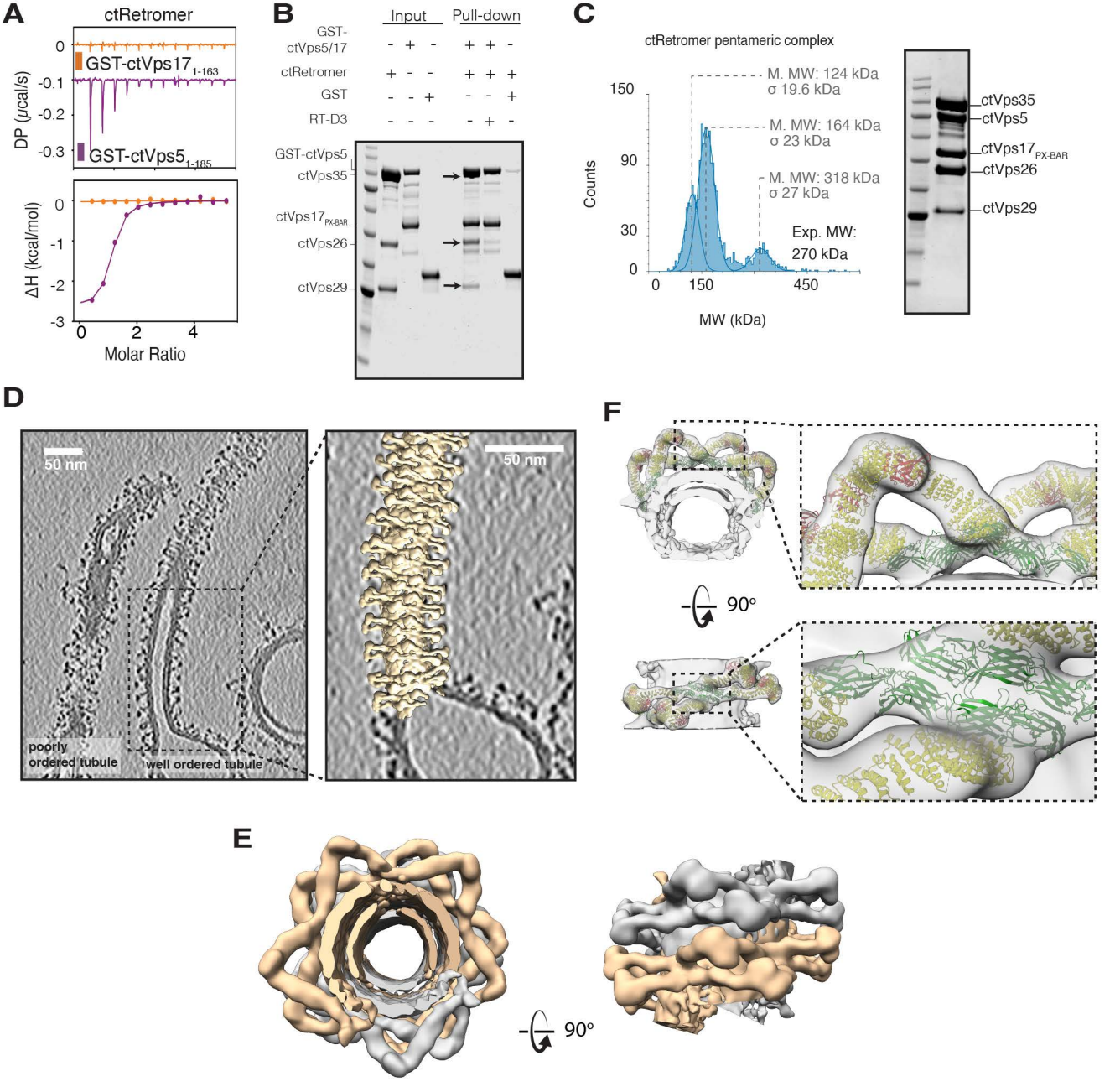


Figure 5. Assembly of Retromer with the Vps5–Vps17 heterodimeric complex on membrane tubules.
(A) ITC of ctRetromer with GST-ctVps171-163 or GST-ctVps51-185. Under the same conditions and concentrations no interaction is detected with the Vps17 N-terminal domain. (B) GST pull-down demonstrating the direct interaction between GST-ctVps5–ct-Vps17PX-BAR and either ctRetromer or the ctVps35–ctVps29 dimeric subcomplex. The interactions are blocked by adding inhibitory cyclic peptide RT-D3. (C) Mass photometry of heteropentameric complex composed of full-length ctVps35, ctVps26, ctVps26, ctVps5 and ctVps17PX-BAR. The SDS-PAGE gel of the complex is shown on the right. (D) Left panel shows a slice from a tomographic reconstitution showing examples of coated tubules with well-ordered and poorly ordered Retromer complexes side by side. The right panel shows the overlay of the tubule with a model that was generated by placing copies of the cryoEM map at positions and orientations determined by STA. See also Movies S2 and S3. (E) Close up of the tubule model shown in profile and cross-section. The coat is formed by the two chains of Retromer arches (separate helical rows are clored in grey and caramel for clarity). These form pseudo-helices winding around the membrane tubule. This arrangement lacks strict helical symmetry as the pitch and units per turn adapts to the tubule diameter. (F). Molecular model generated by rigid body fit of ctRetromer38 to the cryoEM map. The specific arrangement of two adjacent Vps26 dimers (in green) was not seen in the previous Retromer structure using Vps5 alone38. Note that the PX-BAR domains of the Vps5-Vps17 heterodimers are not resolved, and seen only as a dense membrane-proximal layer.

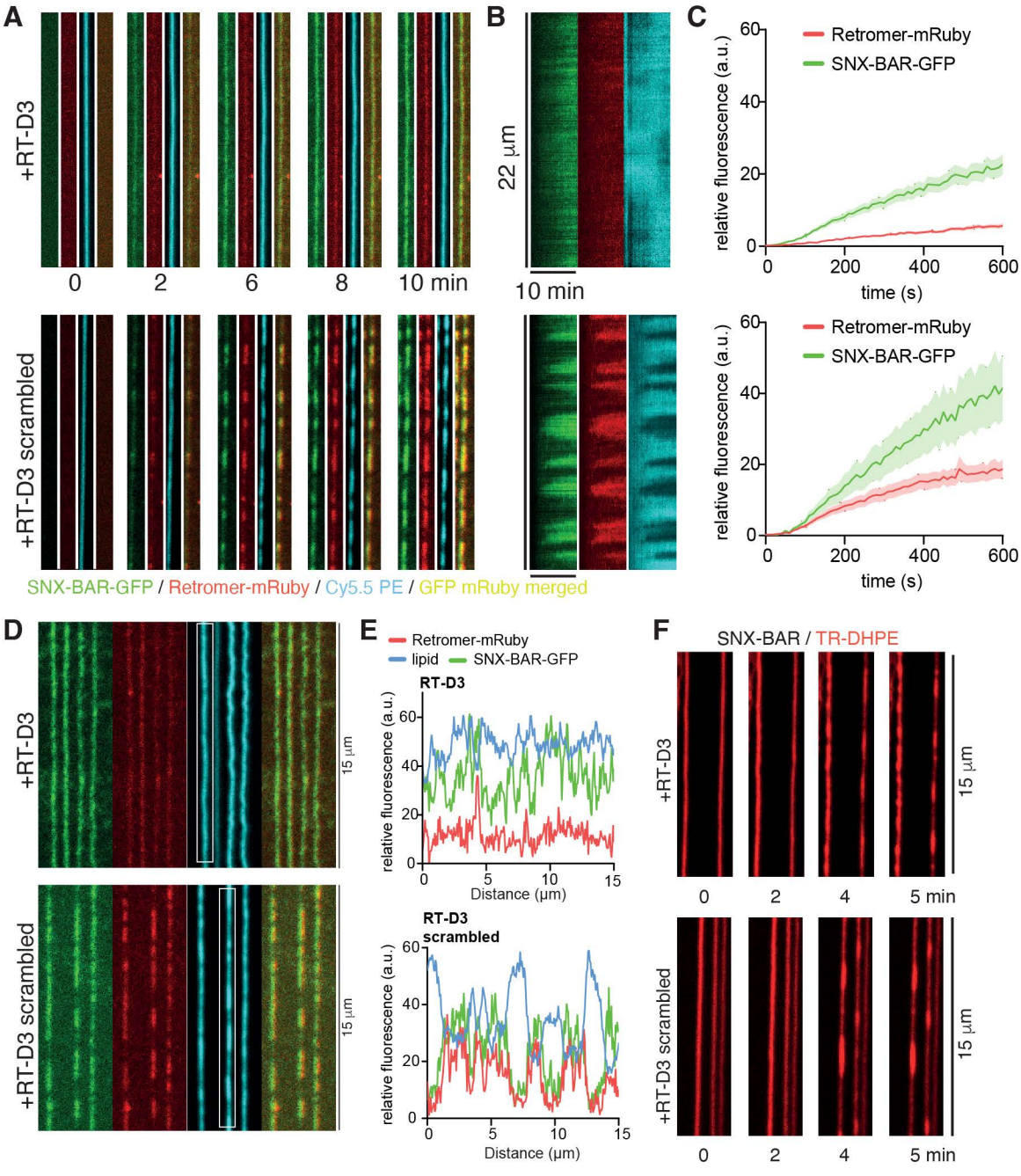


Figure 6. The PL motif of S. cerevisiae Vps5 is required for recruitment of Retromer to supported membrane tubules.
(A) Effect of RT-D3 and RT-D3 scrambled cyclic peptides on SNX-BAR-GFP and scRetromer-mRuby recruitment on supported membrane tubes (SMTs). SMTs containing 5% PtdIns(3)P were formed and incubated with 25 nM SNX-BAR-GFP and 50 nM scRetromer-mRuby while being imaged by confocal microscopy. Frame rate: 0.1 Hz, cyclic peptide concentration: 300 µM. See also Movies S4 and S5. Green: SNX-BAR-GFP, red Retromer-mRuby, blue: Cy5.5-PE. (B) Kymograph of SMTs shown in (A) over the time course of the whole experiment (10 min). (C) Quantification of the recruitment of SNX-BAR-GFP and Retromer-mRuby on the tubule. Quantification was done using FIJI, measuring the mean fluorescence signal on the tubule over a 10 µM length (N = 14 tubules from 2 experiments). (D) SMTs after 10 min of incubation with 25 nM SNX-BAR-GFP, 50 nM Retromer-mRuby, and 300 µM of RT-D3 or RT-D3 scrambled. (E) Line scan intensity plot of the SMTs indicated by the white box in (D). (F) SMTs were incubated with 100 nM SNX-BAR and 300 µM of RT-D3 or RT-D3 scrambled and imaged by confocal microscopy at a frame rate of 0.5 Hz. Red: Texas-Red DHPE. See also Movie S6.

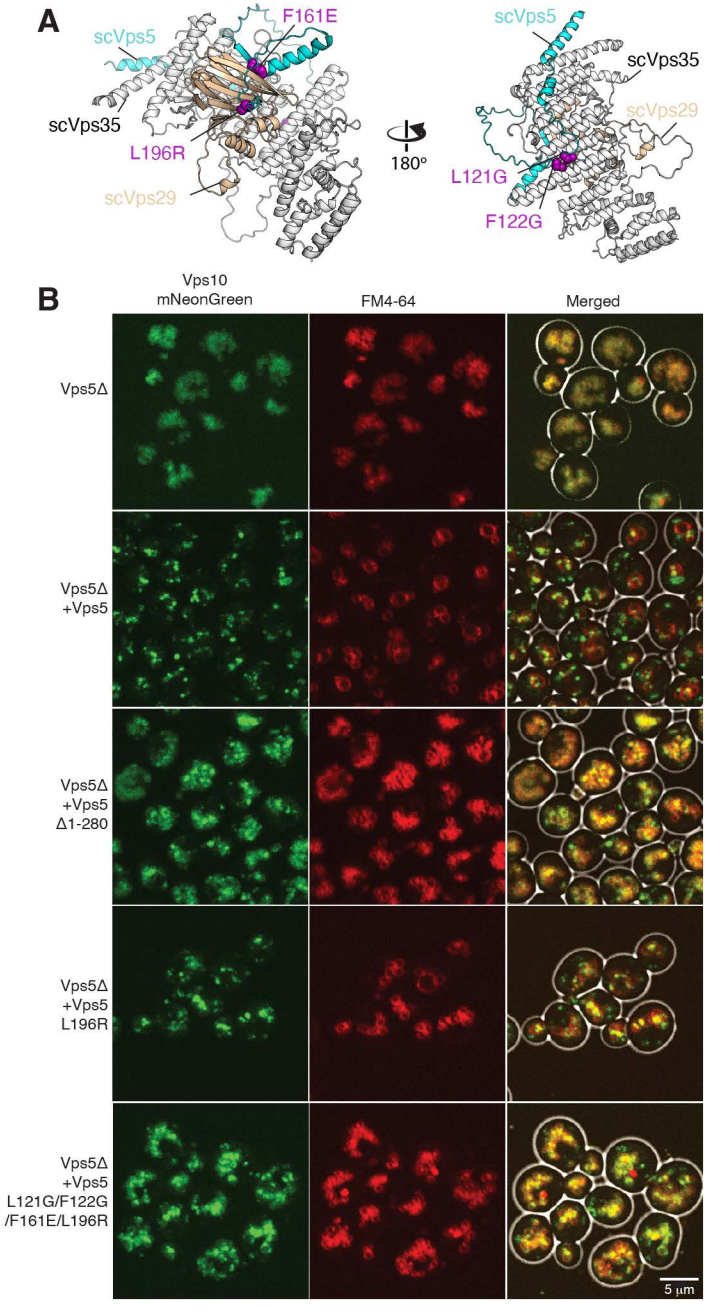


Figure 7. The interaction of Retromer with the Vps5 N-terminal domain is required for Vps10 recycling.
(A) AlphaFold2 predicted structure of the scVps35–scVps29–scVps5 complex highlighting residues targeted for mutagenesis. (B) Yeast cells (SEY6210) carrying a Vps5 deletion were complemented with integrative plasmids expressing variants of Vps5 with Vam10 (because the VAM10 gene is embedded in VPS5 and deleted with it). Vps10 was genomically tagged with a C-terminal mNeonGreen fusion. These cells were logarithmically grown in SD-URA medium, stained with FM4-64 and analysed by confocal microscopy. Maximum intensity projections of z-stacks are shown. Scale bar: 5 µm.

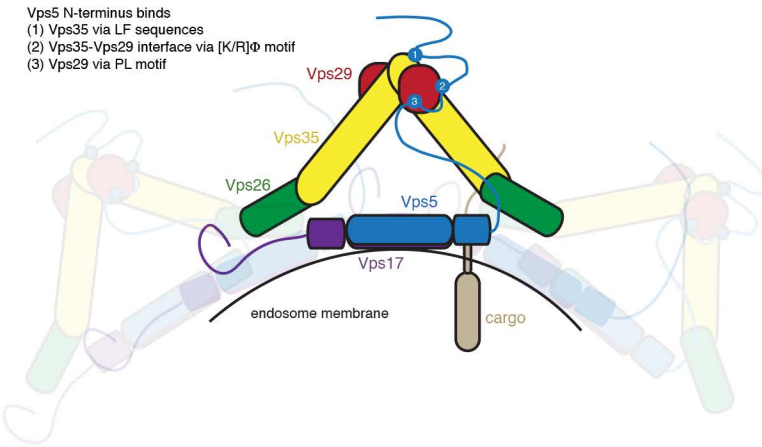
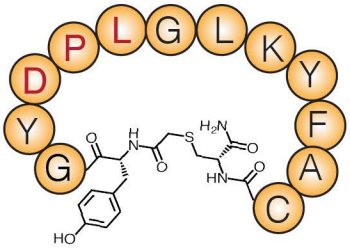
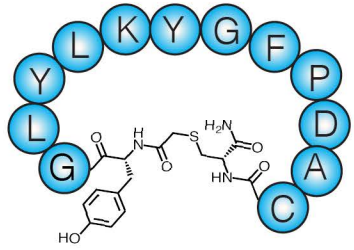


Figure 8. Proposed model for the assembly of yeast Retromer with Vps5–Vps17 required for vacuolar cargo recycling.
Schematic diagram of the yeast Retromer–Vps5–Vps17 complex and its assembly on the endosomal/vacuolar membrane. The sequences located in the flexible N-terminus of Vps5 include PL and [K/R]⊠ motifs, and additional LF-containing sequences specific to the Saccharomycotina subdivision binds to Vps29 and Vps35 sites at the apex of the Retromer arch. This high affinity interaction is required for initial pentameric complex formation prior to subsequent assembly at the membrane surface where Vps26 then forms homodimers on top of the BAR domains of Vps5–Vps17 heterodimers. Blocking the N-terminal Vps5 interaction disrupts Retromer assembly and prevents recycling of cargos such as Vps10 resulting in their accumulation at the vacuole.

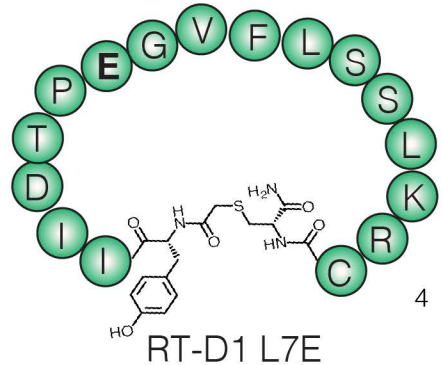
Figure S1. Sequence alignment of yeast and fungus Vps5.
(A) Sequence alignment of Vps5 unstructured N-terminal domain across Pezizomycotina, (B) Schizosaccharomycetes, and (C) Saccharomycotina subdivisions. ct, Chaetomium thermophilum; vd, Verticillium dahlia; cm, Cordyceps militaris; nc, Neurospora crassa; mb, Metarhizium brunneum; sp, Saccharomyces pombe; ni, Neolecta irregularis; pj, Pneumocystis jirovecii; td, Taphrina deformans; sj, Schizosaccharomyces japonicus, Sc, Saccharomyces cerevisiae; zp, Zygosaccharomyces parabailii; pp, Pichia_Pastoris; hb, Hyphopichia burtonii; Ag, Ashbya gossypii.



RT-D3



RT-D3 scramble



RT-D1 L7E

4

Figure S2. Schematic diagram showing the three different cyclic peptides used in this study.
Macrocyclic peptides used in this study and previously identified to bind Retromer38. The key DPL motif sequence in RT-D3 is highlighted in red. RT-D3 binds to Vps29 and inhibits PL motif-containing protein interactions. Control peptides are RT-D3 scramble and RT-D1(L7E) mutant which are both unable to interact with Retromer due to loss of the core PL sequence.

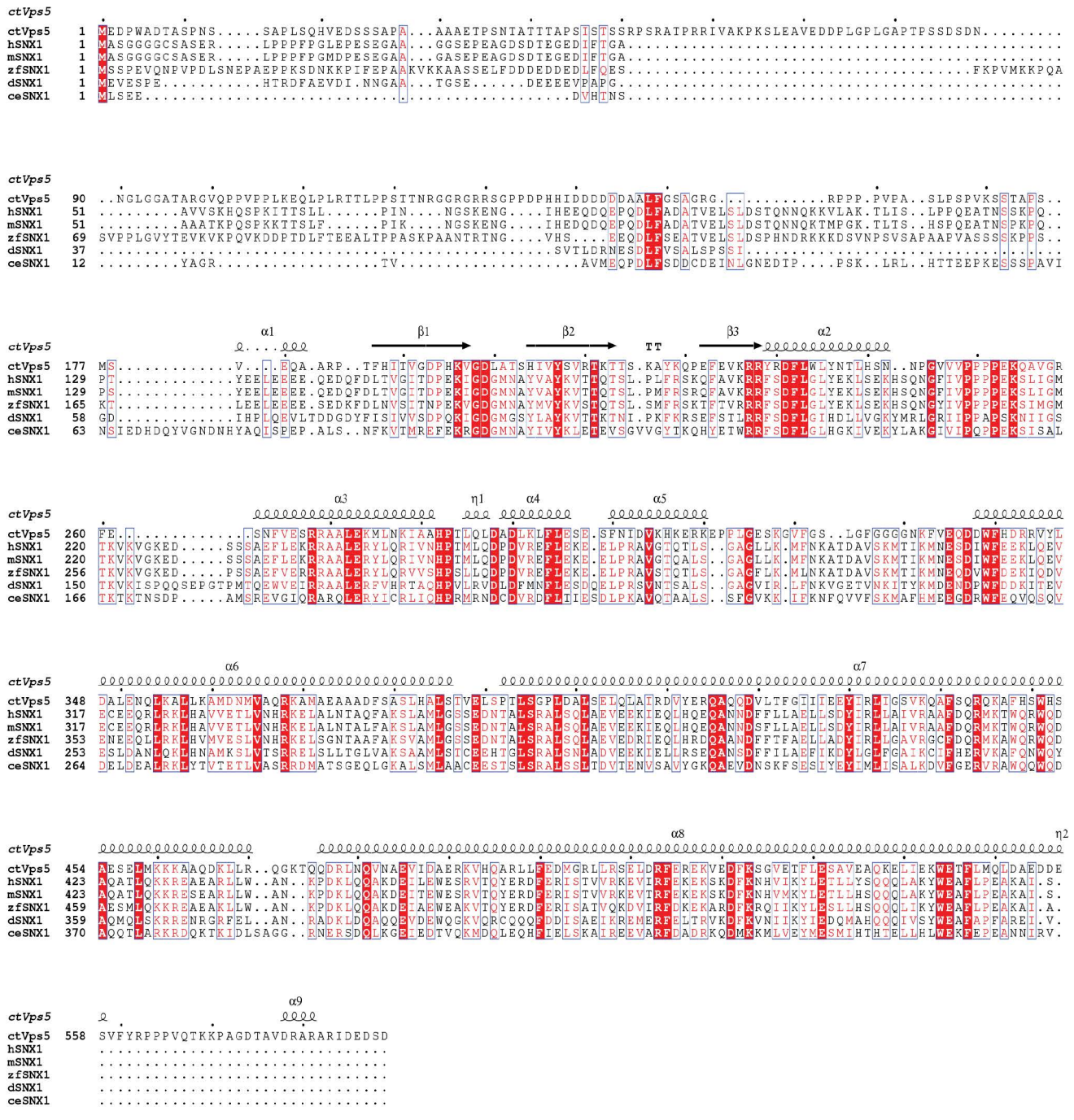


Figure S3. Sequence alignment of ctVps5 and SNX1 from higher eukaryotic species.
Ct, Chaetomium thermophilum; h, homo sapiens; m, Mus musculus; zf, Zebrafish; d, Danio rerio; Ce, Caenorhabditis elegans.

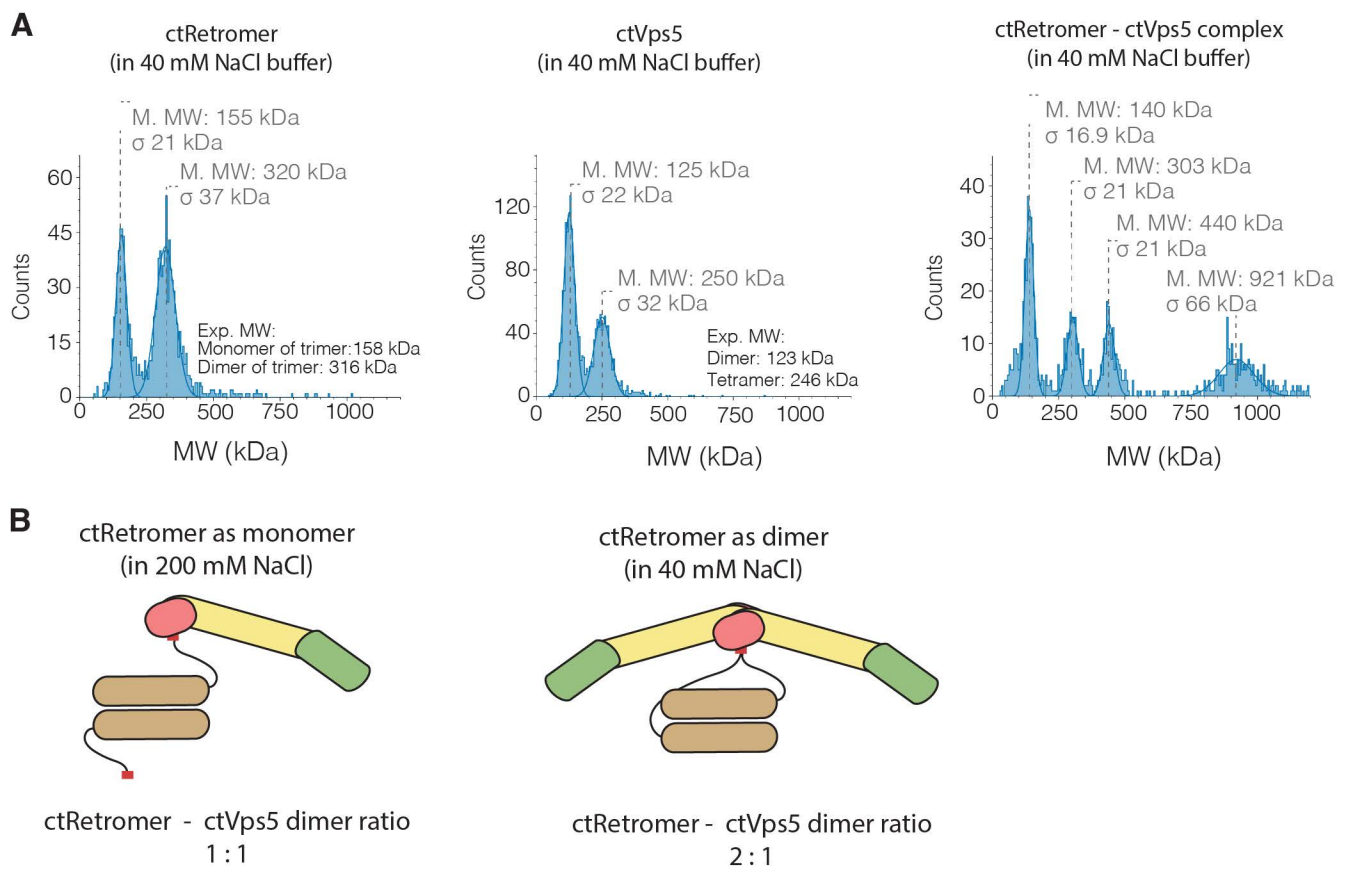


Figure S4. ctVps5-ctRetromer complex formation in low ionic strength condition. (A) Mass photometry of ctRetromer, ctVps5 and the ctVps5–ctRetromer complex in buffer containing 40 mM NaCl. (B) Schematic diagram showing the formation of 1 to 1 or 2 to 1 ctRetromer–ctVps5 dimer ratio complex in solution depending on the ionic strength of the buffer.

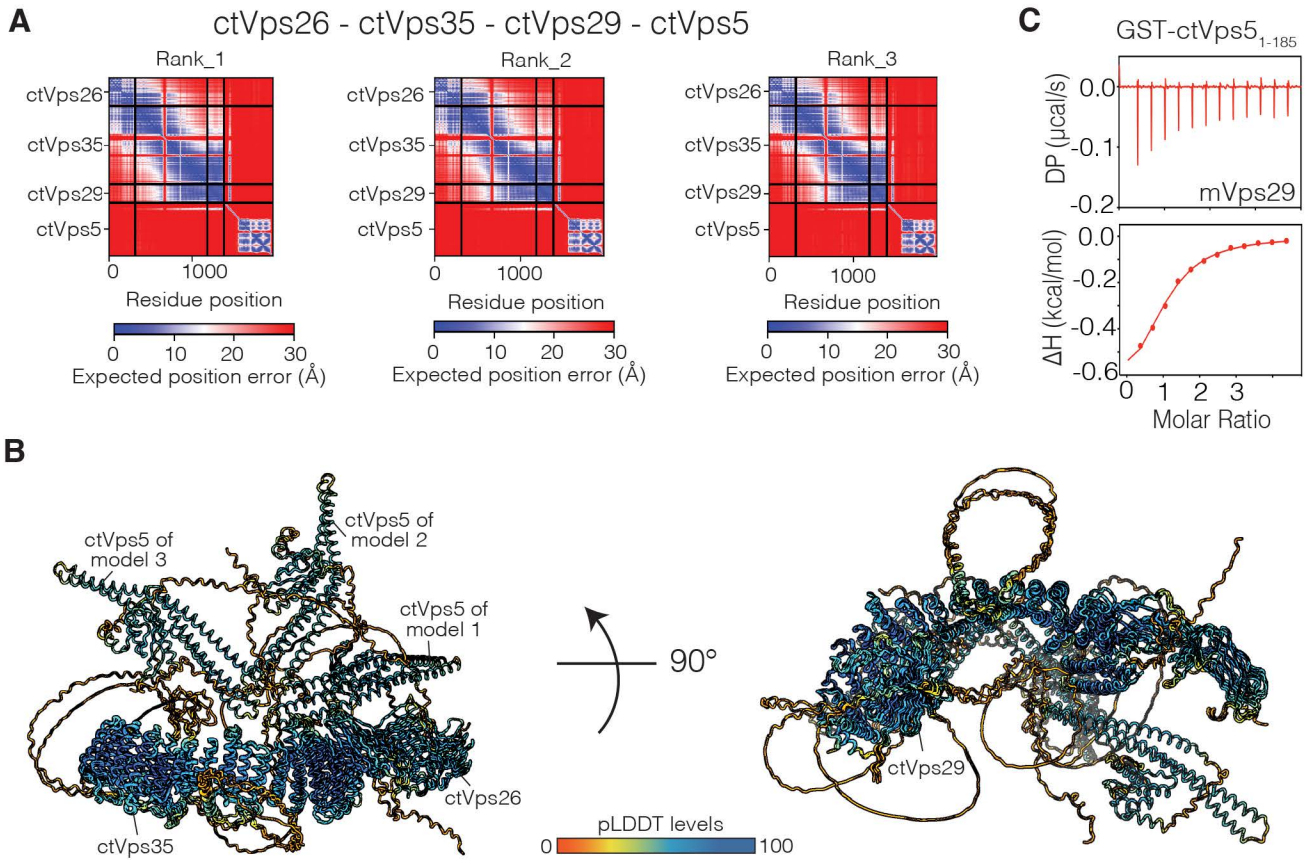


Figure S5. AlphaFold2 predictions of ctRetromer in complex with ctVps5 highlighting the conservation of the PL motif mediated binding mechanism.

(A) Predicted alignment Error (PAE) plots showing the three predicted models of ctVps26–ctVps35–ctVps29–ctVps5 complex. (B) Superposition of the three representative models generated by Alphafold2. Models were colored according to the pLDDT score. (C) ITC measurement showing mVPS29 is capable of binding to GST-tagged ctVps51-185. ITC graph shows the integrated and normalized data fit with a 1 to 1 binding ratio.

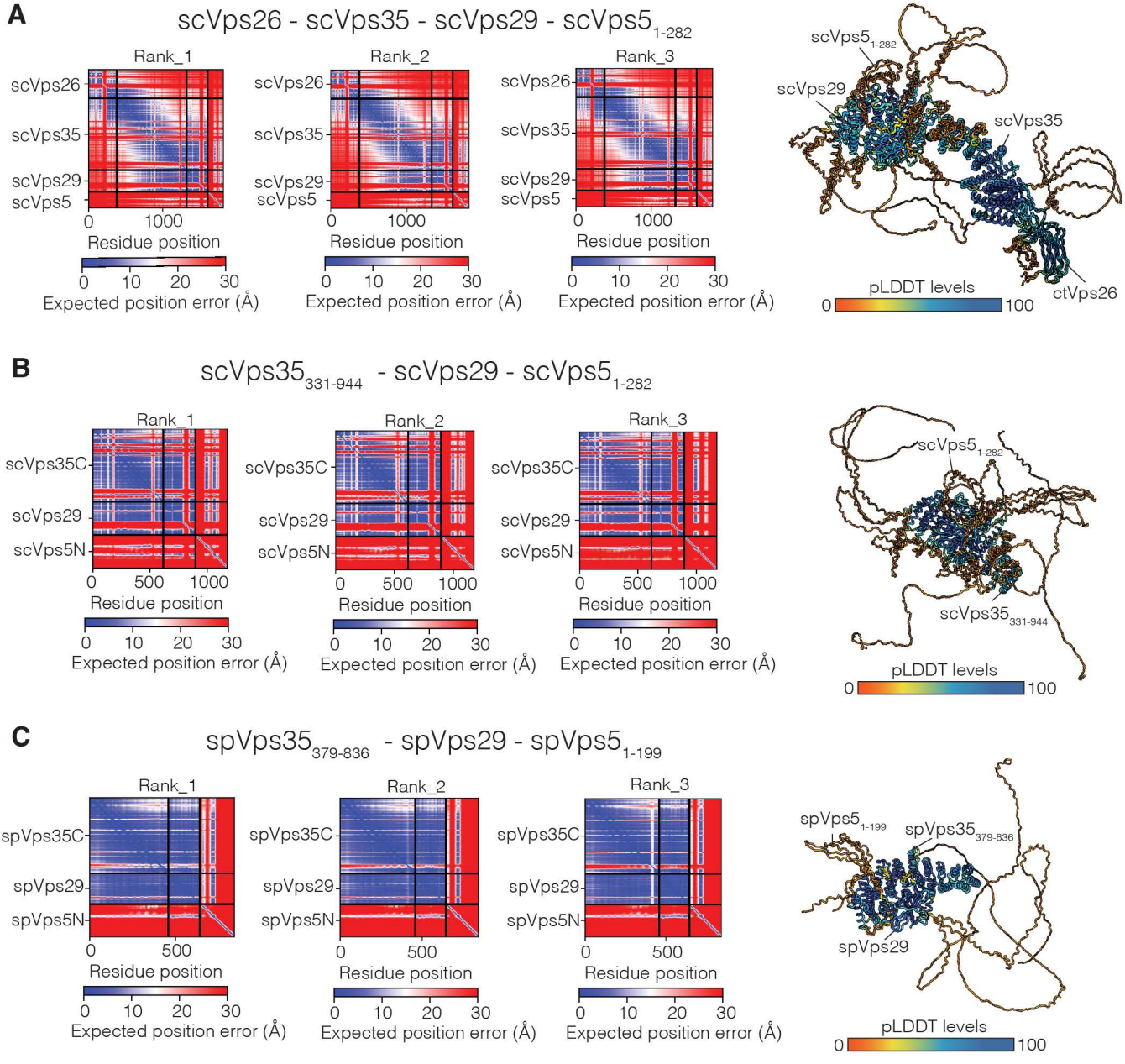


Figure S6. AlphaFold2 predictions of Retromer in complex with Vps5 from two different yeast species.
(A) Right panel presents the PAE plots of the three predicted scRetromer–scVps5 models, (B) scVps35331-944–scVps29–scVps51-282 models, and (C) spVps35379-836–spVps29–spVps51-199 models. Next to the PAE plot is the superposition of the three top ranked predicted complexes colored according to the pLDDT score.

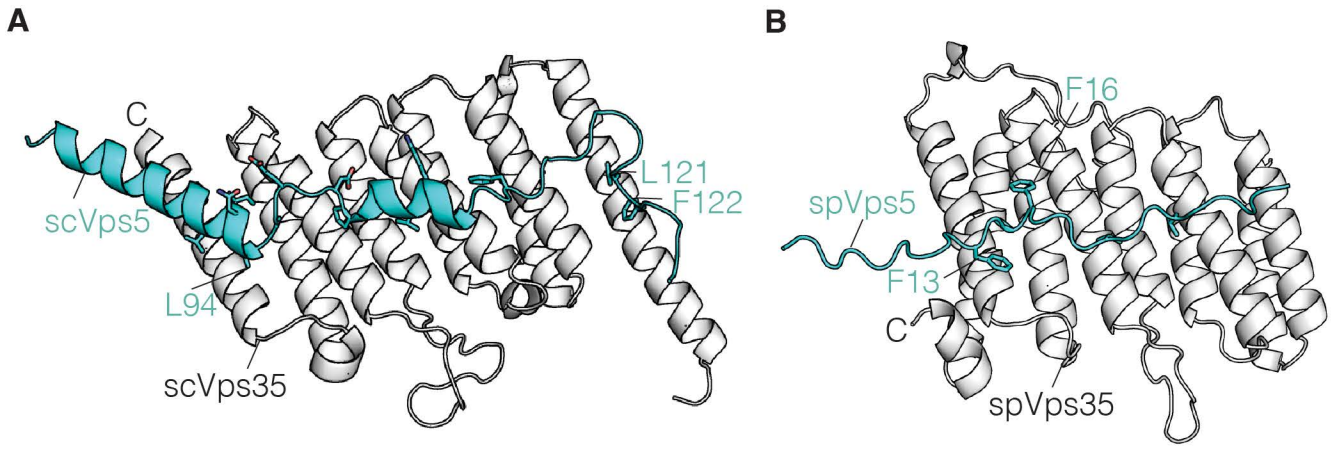


Figure S7. Extended Vps35 binding sites observed in AlphaFold2 models of Retromer in complex with Vps5 from S. cerevisiae and S. pombe.
(A) Representative (top-ranked) predicted model of scVps35331-944–scVps29–scVps51-282 complex, and (B) spVps35379-836–spVps29–spVps51-199 complex. In both cases only the C-terminal region of Vps35 and the key residues of Vps5 involved in binding are shown.

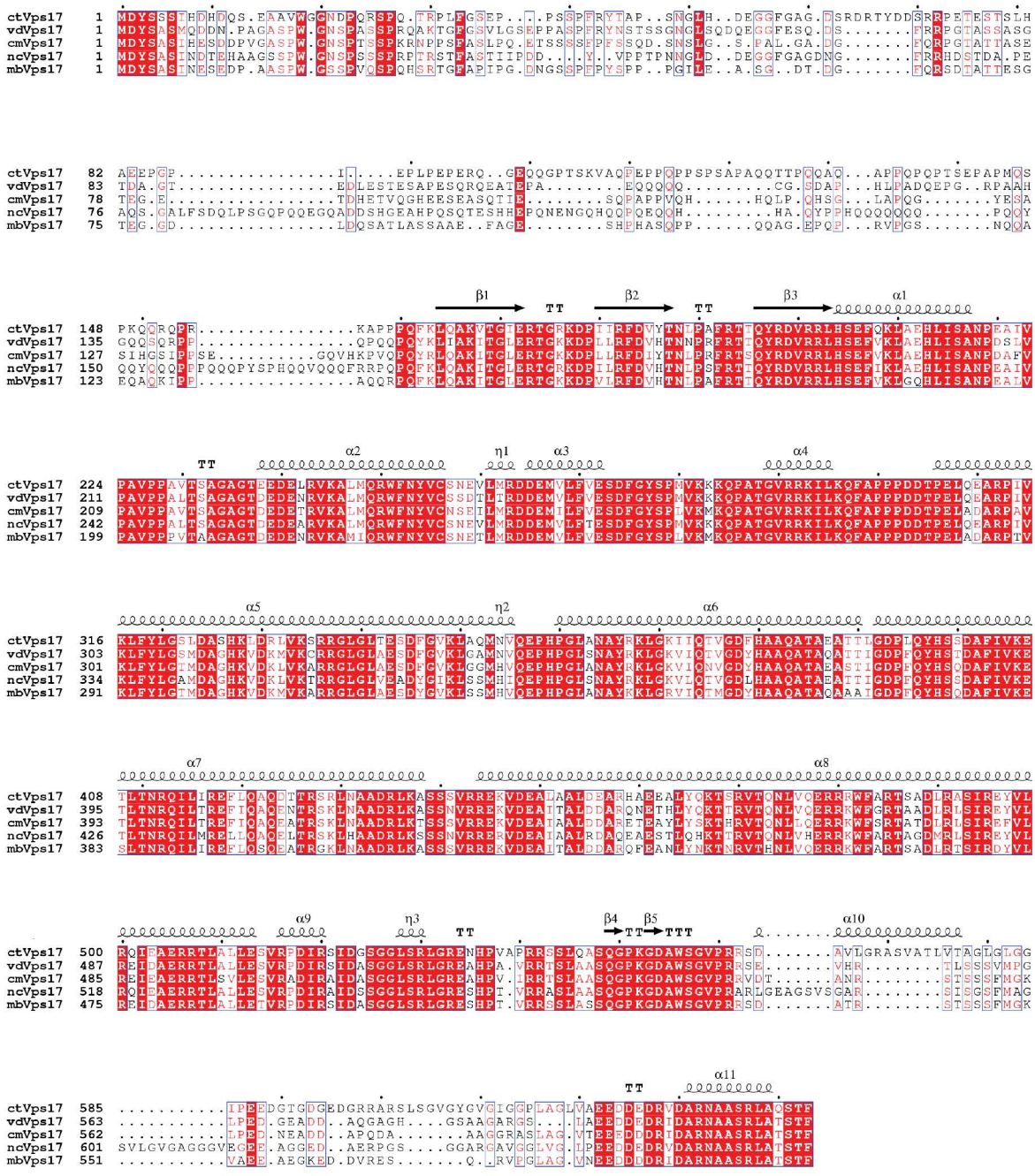


Figure S8. Sequence alignment of Vps17 across the Pezizomycotina subdivision.
The N-terminal disordered region of Vps17 is highly divergent in sequence compared to the PX and BAR domains. ct, Chaetomium thermophilum; vd, Verticillium dahlia; cm, Cordyceps militaris; nc, Neurospora crassa; mb, Metarhizium brunneum.

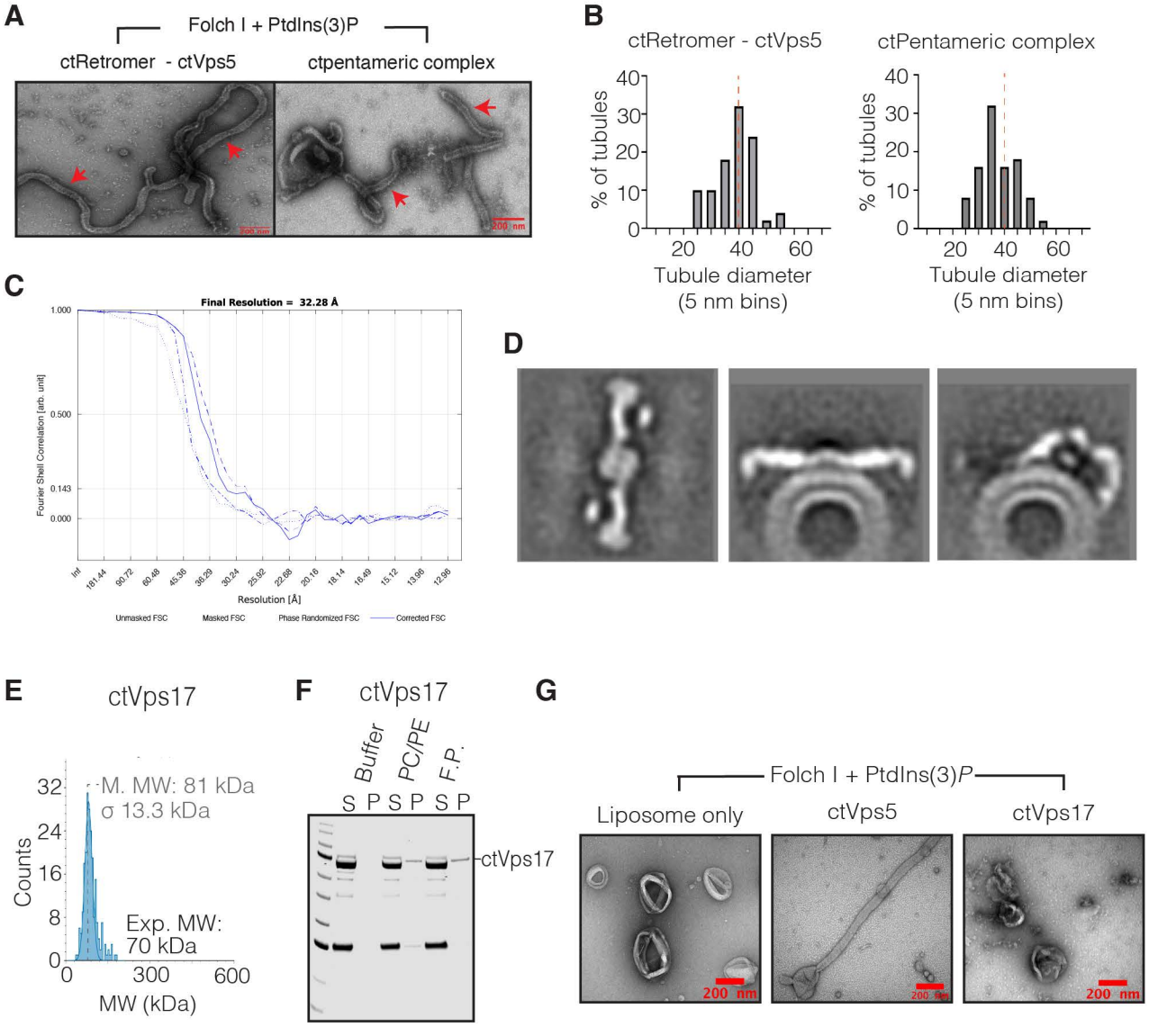


Figure S9. Analysis of Retromer tubules by negative stain EM and cryoEM.
(A) Example micrographs of negatively stained Folch I - PtdIns(3)P liposomes incubated with ctRetromer–ctVps5 complex (left) or ctRetromer–ctVps5–ctVps17PX-BAR complex (right). (B) Histogram showing the diameter of tubules generated by ctRetromer–ctVps5 or ctRetromer–ctVps5–ctVps17PX-BAR complexes binned as percentage of total number of tubules. (C) FSC of two independently processed half maps. (D) Orthoslices throgh the final sharpened and filtered to EM map, sharpened with B -2500. (E) Mass photometry of full-length ctVps17. (F) Liposome-pelleting assays of ctVps17. Unilamellar vesicles were composed of either PC/PE control lipid, or Folch I lipids supplemented with PtdIns(3)P. “S” and “P” indicates unbound supernatant and bound pellet respectively. (G) Example micrographs of negatively stained Folch I - PtdIns(3)P liposome, extruded to 400 nm diameter and incubated with either ctVps5 or ctVps17.