	Data collection #1 (March 2018)	Data collection #2 (October 2018)
Microscope	FEI Titan Krios (NRAMM Krios2)	FEI Titan Krios (NRAMM Krios3)
Cs	2.7mm	2.7mm
Voltage	300 keV	300keV
Detector	Gatan K2	Gatan K2
Magnification	105,000x	105,000x
Pixel size	1.096Å/pix	1.06Å/pix (rescaled to 1.096Å/pix with motioncorr2 script)
Dose rate	~8 $e^-/Å^2$/sec	~8 $e^-/Å^2$/sec
Total dose	69.34 $e^-/Å^2$	73.92 $e^-/Å^2$
Defocus range	0.7-2.6 μm	0.8-4.4 μm
Number of micrographs	1480	1299

Table S1. Data collection parameters, Related to Figure 1.

	heterotrimer	dimer	curved VPS35/ VPS35 sub-structure	chain interface I	flat VPS35/VPS35 sub-structure	tetramer	chain interface II
Total particles (autopicked)	439,646	439,646	439,646	439,646	439,646	439,646	443,117
Box size (Å)	252x252	395x395	180x180	395x395	180x180	329x329	482x482
Particles in 2D classification	29,771	31,202	34,156	76,927	78,994	6,126	15,234
Particles in final 3D model	26,369	31,022	32,435	75,790	69,195	6,015	13,782
Symmetry	C1	C1	C1	C2	C2	C1	C1
Map resolution (Masked FSC 0.143, RELION)	5.7 Å	9.3 Å	5.3 Å	6.9 Å	4.9 Å	25.5 Å	16.9 Å
B-factor	-212	-100*	-58	-189	-56	--	-212
EMDB accession code	EMD-21136	EMD-21117	EMD-21119	EMD-21116	EMD-21135	EMD-21101	EMD-21118
Refinement							
Program	PHENIX				PHENIX		
Number atoms	9793				7563		
Molprobity score	2.11				2.25		
Molprobity clash score	17.05				20.8		
RMSD from ideal							
Bond length (Å)	0.006				0.006		
Bond angles (°)	1.067				1.067		
Ramachandran plot							
Favored (%)	94.5				93.2		
Allowed (%)	5.5				6.8		
Outliers (%)	0.0				0.0		
Rotamer outliers (%)	0.55				1.3		
CC model-vs-map (masked)	0.70				0.64		
Map resolution (FSC 0.143/0.5)	5.8/7.0 Å				5.3/7.6 Å		
PDB ID	6VAC				6VAB		

Table S2. Data processing and refinement statistics. **Related to Figure 1.** Data processing statistics are reported for all structures and sub-structures. The heterotrimer and flat VPS35/VPS35 sub-structure underwent real space refinement in PHENIX. Asterisks mark user-imposed B-factors to minimize noise and optimize contrast (see STAR Methods for details).

Name	Genotype	Annotation	Source
PXYR1A	*MATα leu2-3,112 ura3-52 his3-Δ200 trp1-Δ901 suc2-Δ9 lys2-801 vps35Δ::KanMX6 vps26Δ::Hygro pRS315-VPS35 pRS416-VPS26*	WT	This study
PXYR1B	*MATα leu2-3,112 ura3-52 his3-Δ200 trp1-Δ901 suc2-Δ9 lys2-801 vps35Δ::KanMX6 vps26Δ::Hygro pRS315-VPS35 pRS416-VPS26*	WT	This study
PXYR1C	*MATα leu2-3,112 ura3-52 his3-Δ200 trp1-Δ901 suc2-Δ9 lys2-801 vps35Δ::KanMX6 vps26Δ::Hygro pRS315-VPS35 pRS416-VPS26*	WT	This study
PXYR3A	*MATα leu2-3,112 ura3-52 his3-Δ200 trp1-Δ901 suc2-Δ9 lys2-801 vps35Δ::KanMX6 vps26Δ::Hygro pRS315 pRS416-VPS26*	VPS35 Knockout	This study
PXYR3B	*MATα leu2-3,112 ura3-52 his3-Δ200 trp1-Δ901 suc2-Δ9 lys2-801 vps35Δ::KanMX6 vps26Δ::Hygro pRS315 pRS416-VPS26*	VPS35 Knockout	This study
PXYR3C	*MATα leu2-3,112 ura3-52 his3-Δ200 trp1-Δ901 suc2-Δ9 lys2-801 vps35Δ::KanMX6 vps26Δ::Hygro pRS315 pRS416-VPS26*	VPS35 Knockout	This study
PXYR4A	*MATα leu2-3,112 ura3-52 his3-Δ200 trp1-Δ901 suc2-Δ9 lys2-801 vps35Δ::KanMX6 vps26Δ::Hygro pRS315-vps35 3KE pRS416-VPS26*	VPS35 3KE mutant	This study
PXYR4B	*MATα leu2-3,112 ura3-52 his3-Δ200 trp1-Δ901 suc2-Δ9 lys2-801 vps35Δ::KanMX6 vps26Δ::Hygro pRS315-vps35 3KE pRS416-VPS26*	VPS35 3KE mutant	This study
PXYR4C	*MATα leu2-3,112 ura3-52 his3-Δ200 trp1-Δ901 suc2-Δ9 lys2-801 vps35Δ::KanMX6 vps26Δ::Hygro pRS315-vps35 3KE pRS416-VPS26*	VPS35 3KE mutant	This study
PXYR13A	*MATα leu2-3,112 ura3-52 his3-Δ200 trp1-Δ901 suc2-Δ9 lys2-801 vps35Δ::KanMX6 vps26Δ::Hygro pRS315-vps35 EED+3KE pRS416-VPS26*	VPS35 AAA3KE mutant	This study
PXYR13B	*MATα leu2-3,112 ura3-52 his3-Δ200 trp1-Δ901 suc2-Δ9 lys2-801 vps35Δ::KanMX6 vps26Δ::Hygro pRS315-vps35 EED+3KE pRS416-VPS26*	VPS35 AAA3KE mutant	This study
PXYR13C	*MATα leu2-3,112 ura3-52 his3-Δ200 trp1-Δ901 suc2-Δ9 lys2-801 vps35Δ::KanMX6 vps26Δ::Hygro pRS315-vps35 EED+3KE pRS416-VPS26*	VPS35 AAA3KE mutant	This study

Table S3. Yeast strains used in this study. Related to Figure 6.

	Wild type	3KE mutant	AAA3KE mutant
Number copies of retromer	2,117	2,161	2,280
Total number structures	1,693	1,878	2,250
Heterotrimers (%)	1,333 (78.7%)	1,621 (86.2%)	2,220 (98.7%)
Dimers (%)	328 (19.4%)	246 (13.1%)	30 (1.3%)
Tetramers (%)	32 (1.9%)	11 (0.6%)	0 (0%)

Table S4. Comparison of wild-type and electrostatic mutant retromer structures in negative stain. Related to Figure 5. Retromer heterotrimer copies and structures were counted across five representative micrographs each of wild-type and mutants. Distribution was calculated as percentage of total structures for each species. The 3KE mutant retains the ability to form some dimers *in vitro*, although the mutant shifts to favor heterotrimers. The AAA3KE mutant exists mostly as heterotrimer (>98%); we identified thirty potential dimers in this sample.