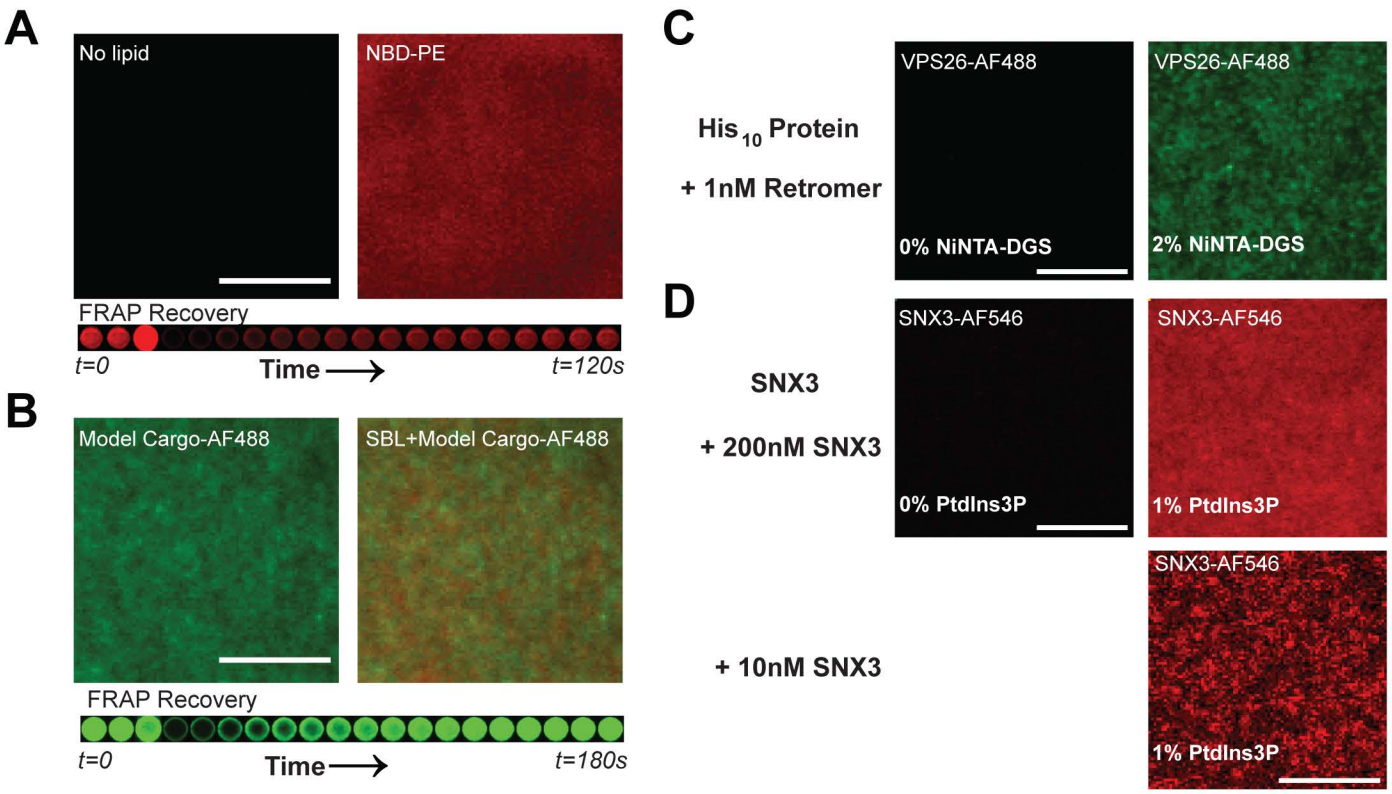

Figure S1. Evaluation of supported lipid bilayer quality and specificity of protein attachment. (A) A large field of view TIRF micrograph of a SLB containing NBD-PE (0.5 mol%) is shown. The NBD-PE exhibits a diffuse fluorescent signal (top), indicating that it is homogenously dispersed throughout the SLB. Fluorescence recovery after photobleaching (bottom) shows rapid recovery of NBD-PE fluorescence indicating free diffusion in the SLB. A lipid diffusion coefficient of 2.99 μm2/s is estimated from this recovery profile (41). (B) A large field of view TIRF micrograph of His-tagged, AF488-labeled model cargo protein adhered to a SLB is shown was attached to the SLB. Cargo protein was incubated with the SLB (solution concentration 40 nM) and then the SLB was washed and imaged. Attachment of the cargo protein is strictly dependent on the nickel lipid NiNTA-DGS. The results show that cargo peptide is distributed uniformly on the surface on a unilamellar SLB. Fluorescence recovery after photobleaching (bottom) shows that the cargo is mobile, with a diffusion coefficient of 0.8 $\mu m^2/s$. (C) Large field of view TIRF micrographs of Retromer, containing AF488-labeled His-tagged VPS26 (solution concentration 1nM), on SLBs not containing (left) or containing NiNTA-DGS lipid. The results show that association of His-tagged Retromer with the SLB requires NiNTA-DGS in the SLB. (D) Large field of view TIRF micrographs of AF546-labeled SNX3 attached to the SLB, directly labeled (AF546) SNX3 was incubated with SLBs not containing, or containing, 1% (mol/mol) PtdIns3P (solution concentration 200nM and 10nM). After washing, the SLBs were imaged. The results confirm that SNX3 with the SLB requires PtdIns3P. Scale bars are 10μm for all panels.

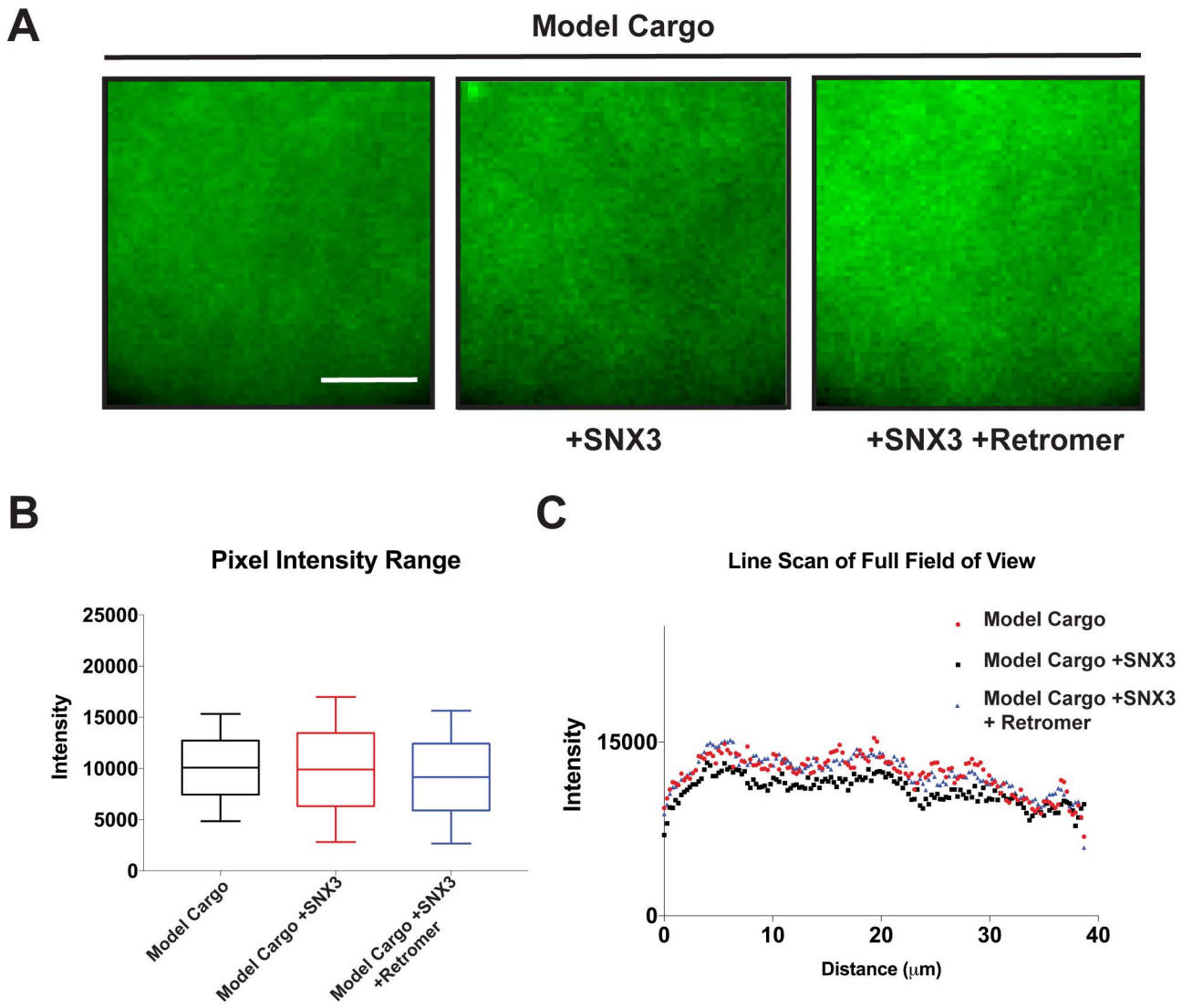

Figure S2. Characterization of Retromer, SNX3, and cargo distributions on a supported lipid bilayer. (A) Large field of view TIRF micrographs of His-tagged, AF488-labeled cargo protein on a SLB. Cargo protein (solution concentration of cargo 40 nM) was incubated with the SLB either alone (left), in the presence of unlabeled SNX3 (3μM), or Retromer (100 nM) and SNX3 (3μM). After washing, images of the SLBs were acquired. In all conditions labeled cargo is distributed uniformly on the surface of the SLB. Scale bar 5μm. (B) The pixel intensity range and mean of the model cargo in were determined using ImageJ for each micrograph and plotted. The pixel intensity range of the model cargo is shown after incubation with SNX3 and Retromer. The data indicate that SNX3, or SNX3 +Retromer do not cause large scale clustering of labeled cargo peptide on the SLB. (C) A representative line scan through the micrographs in (A) show a homogenous distribution of model cargo on the SLB that is unaffected by incubation with SNX3 or SNX3+Retromer.

Supplemental Table 1. Estimation statistics of replicate Retromer distributions

Condition	n	Mean	SD	SEM	95% Confidence Interval Mean	Difference (Δ) of the Mean	Compatibility Interval	p_mean	mean Δ of technical replicates	SD of technical replicates
Experimental Replicate 1									0.1775	0.401
T1	218	2.98	1.11	0.08	2.83 - 3.12	0.283	0.06 - 0.499	0.004		
T2	242	3.34	1.37	0.09	3.16 - 3.51	0.645	0.421 - 0.879	<0.001		
T3	216	2.38	1.16	0.08	2.22 - 2.53	-0.32	-0.532 - -0.109	0.002		
T4	208	2.79	1.11	0.08	2.64 - 2.94	0.102	-0.101 - 0.316	0.385		
T5 (reference)	224	2.69	1.16	0.08	2.54 - 2.84	-	-	1		
Experimental Replicate 2									-0.03525	0.604
T1	266	2.3	1.05	0.06	2.17 - 2.43	-0.396	-0.61 - -0.206	<0.001		
T2	281	2.19	1.36	0.08	2.03 - 2.35	-0.506	-0.737 - -0.282	<0.001		
T3	264	2.63	1.08	0.07	2.5 - 2.76	-0.065	-0.259 - 0.13	0.508		
T4	244	3.52	1.35	0.09	3.35 - 3.69	0.826	0.587 - 1.066	<0.001		
Experimental Replicate 3									0.089	0.371
T1	147	2.83	1.5	0.12	2.59 - 3.08	0.134	-0.139 - 0.397	0.295		
T2	170	3.31	2.15	0.17	2.98 - 3.64	0.62	0.266 - 0.993	<0.001		
T3	169	2.88	1.82	0.14	2.6 - 3.16	0.179	-0.148 - 0.484	0.214		
T4	140	2.59	1.8	0.15	2.29 - 2.9	-0.111	-0.447 - 0.218	0.518		
T5	172	2.32	1.86	0.14	2.04 - 2.6	-0.377	-0.678 - -0.067	0.009		
Experimental Replicate 4									-0.1705	0.605
1	171	2.59	1.36	0.1	2.38 - 2.79	-0.112	-0.371 - 0.154	0.416		
2	182	1.67	1.04	0.08	1.52 - 1.83	-1.024	-1.24 - -0.804	<0.001		
3	179	3.08	1.37	0.1	2.88 - 3.28	0.384	0.137 - 0.632	0.003		
4	162	2.77	1.32	0.1	2.56 - 2.97	0.07	-0.185 - 0.317	0.576		

Fluorophore bleaching data for four independent preparations (experimental replicates) of Retromer, were collected by single particle TIRFM. For each preparation of Retromer, technical replicate data sets were acquired from independent supported lipid bilayers. Estimation statistics was used to assess reproducibility of replicates using the means of the distributions to compare effect size (25). Compatibility interval is the range of values most compatible with the raw data. Note that in all experiments, the variation of the means of the distributions correspond to less than one Retromer complex per cluster.

Supplemental Table 2. Difference Analysis of Retromer Oligomer Distributions

Condition	n	Mean	SD	SEM	95% Confidence Interval Mean	Difference (Δ) of the Mean	Compatibility Interval	p_mean
Retromer Only	3655	2.71	1.45	0.02	2.66 - 2.76	-	-	1
Retromer-RRS	3294	2.53	1.48	0.03	2.48 - 2.58	-0.18	-0.248 - -0.11	<0.001
Retromer +WASHC2C-5								
Retromer Only	798	2.79	1.87	0.07	2.66 - 2.92	-	-	1
Retromer +1.5pM WASHC2C-5	850	2.57	2.11	0.07	2.43 - 2.72	-0.217	-0.403	0.021
Retromer +3.75pM WASHC2C-5	791	3.21	2.12	0.08	3.06 - 3.36	0.422	0.228	<0.001
Retromer +15pM WASHC2C-5	751	3.48	2.13	0.08	3.33 - 3.64	0.697	0.498	<0.001
Retromer+75pM WASHC2C-5	706	3.08	2	0.08	2.93 - 3.23	0.298	0.103	0.005
Retromer +1nM WASHC2C-5	415	2.09	1.69	0.08	1.93 - 2.25	-0.698	-0.9	<0.001
Retromer + WASHC2C-21								
Retromer Only	2163	2.74	1.28	0.03	2.69 - 2.8	-	-	1
Retromer +1.5pM WASHC2C-21	2516	2	1.18	0.02	1.95 - 2.04	-0.744	-0.807	<0.001
Retromer +3.75pM WASHC2C-21	2512	2.33	1.29	0.03	2.28 - 2.38	-0.411	-0.481	<0.001
Retromer +15pM WASHC2C-21	2468	2.27	1.31	0.03	2.22 - 2.32	-0.473	-0.542	<0.001
Retromer +75pM WASHC2C-21	2187	2.45	1.36	0.03	2.39 - 2.5	-0.293	-0.371	<0.001
Retromer +1nM WASHC2C-21	2033	2.28	1.19	0.03	2.23 - 2.34	-0.458	-0.532	<0.001
Retromer-RRS + WASHC2C-21								
Retromer-RRS	3294	2.53	1.48	0.03	2.48 - 2.58	-	-	1
Retromer-RRS +1.5pMWASHC2C-21	1749	2.42	1.95	0.05	2.33 - 2.51	-0.11	-0.214	0.019
Retromer-RRS +75pMWASHC2C-21	2173	2.44	1.9	0.04	2.36 - 2.52	-0.087	-0.183	0.06

Single particle TIRFM was used to collect Retromer and Retromer-RRS fluorophore bleaching data in the absence and presence of varying amounts of WASHC2C proteins. Estimation statistics was used to compare effect sizes between the means of the distributions (25). Note that in all cases examined, the differences of the means between control and WASHC2C distributions is less than one Retromer complex per cluster.