Supplementary Figure Legends

Supplementary Figure 1. The apparent membrane thickening seen in Fig. 1a is an artifact of clustering of the negatively charged rhodamine-PE headgroup with His_6-tagged Ub-CFP. **a**, In the presence of unlabeled ESCRT-0, the uncharged membrane marker BODIPY-FL-C5-HPC does not co-cluster with Ub-CFP. **b**, Within Ub-CFP clusters, BODIPY-FL-C5-HPC fluorescence is not enriched above background. **c**, When unlabeled ESCRT-0 and unlabeled Ub are incorporated in amounts sufficient to produce large numbers of clusters, no BODIPY-FL-C5-HPC clustering is seen. Scale bar = 10 μm.

Supplementary Figure 2. ESCRT-I and II alone have no activity. **a**, ESCRT-I or **b**, ESCRT-II alone bind to GUVs but do not appreciably cluster Ub or deform the membrane. Scale bar = 10 μm.

Supplementary Figure 3. Vps20 has no effect on colocalization of Ub clusters and buds. The behavior of wild-type ESCRT-0 is the same in the presence of 15 nM Vps20 as in its absence (Fig. 4). Scale bar = 10 μm.

Supplementary Figure 4. Cargo is not required for bud formation. ESCRT-I and II were both present in the experiment shown, with ESCRT-I labeled. Ub was not present in the experiment. Scale bar = 10 μm.

Supplementary Figure 5. Physiological concentrations of full-length ESCRT-III subunits do not deform membranes. The experimental mixture contained 150 nM labeled Snf7, 40 nM unlabeled Vps20, and 90 nM unlabeled Vps24, all full-length. For comparison, ILVs are produced at the following superphysiological concentrations of ESCRT-III subunits: 600 nM full-length Snf7, 160 nM truncated activated Vps20, and 200 nM full length Vps24 (Ref. [14]). See Supplementary Table1 for the estimated concentrations present in yeast cells. Scale bar = 10 μm.

Supplementary Figure 6. FRAP of cargo without ESCRTs or with ESCRT-0, I, and II. **a**, In the absence of ESCRTs, cargo on the limiting membrane exchanges rapidly and completely. **b**, ESCRT-I and –II do not alter the proportion of cargo in ESCRT-0-Ub domains capable of exchange. **c**, ESCRT-0 has no effect on cargo confinement in buds. Scale bar = 10 μm.

Supplementary Figure 7. Permeability of buds and ILVs to bulk solution. Buds induced by ESCRT-I and II are connected to bulk solution (rows 2-4). All upstream ESCRTs, Vps20, and Snf7 induce a mixture of attached buds and detached ILVs (row 5). ILVs formed by all ESCRTs, including the downstream ESCRT-III subunit Vps24 which was omitted from other experiments, are detached from the limiting membrane and impermeable to bulk solution (row 6). To determine whether membrane buds were connected to the limiting membrane and accessible by the bulk solution, free Alexa-488 was added to the indicated reaction mixtures, yielding a final concentration of 2μM. Scale bar = 10 μm.

Supplementary Figure 8. Purification of ESCRT complexes. Coomassie-stained SDS gel showing the purified ESCRT complexes used in the study.

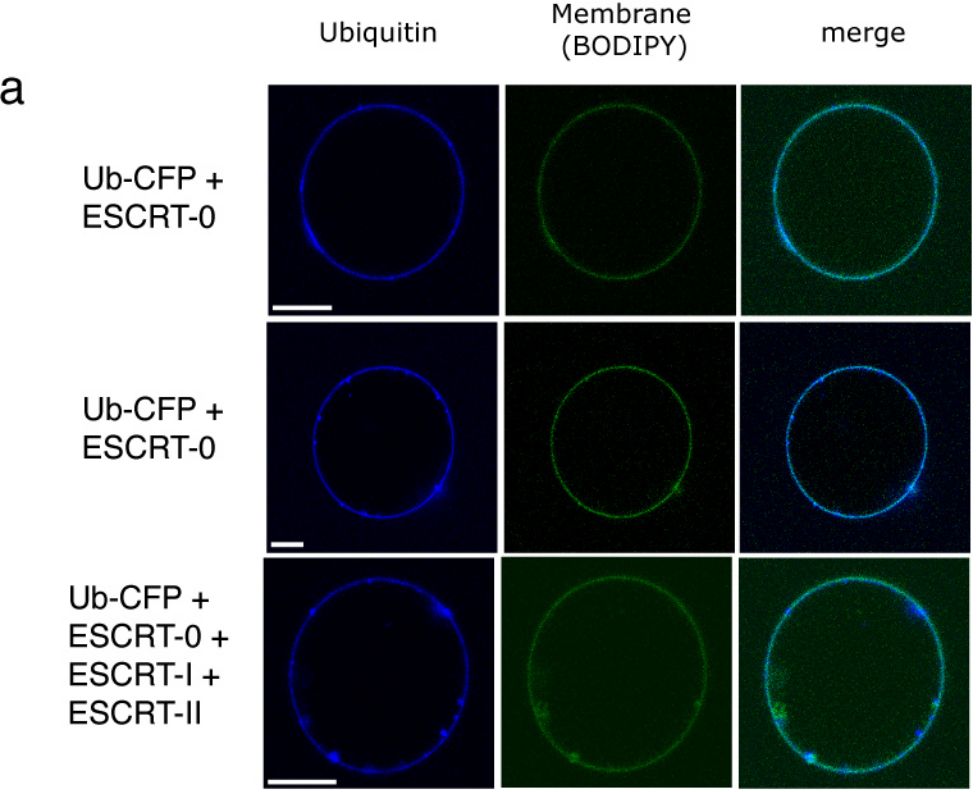
Supplementary Table 1. **Estimated cellular concentrations of ESCRT subunits**

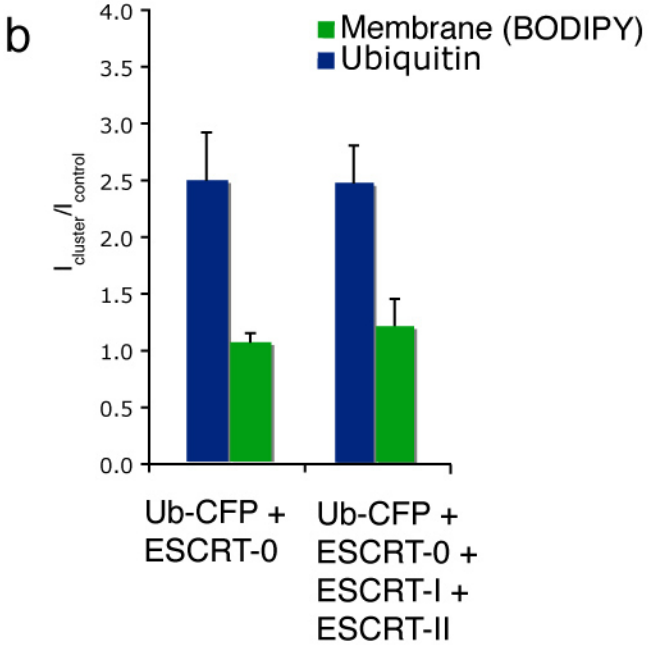
	No. copies Yeast GFP database	Concentration (nM)
Vps27	172	
Hse1	358	
Mean ESCRT-0	265	12
Vps23	1360	
Vps28	1420	
Mean ESCRT-I	1390	63
Vps22	1040	
Vps36	2470	
Mean ESCRT-II	1755	79
Vps20	937	42
Snf7	3270	147
Vps24	1890	85

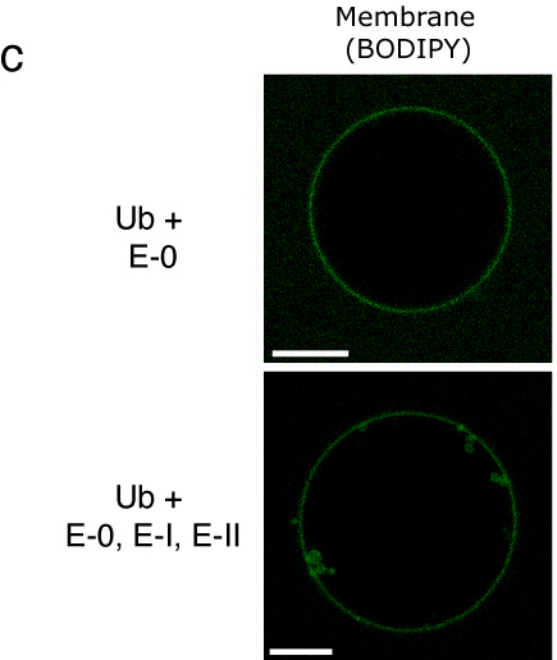
Values for number of copies are from the yeast GFP database [52]. Concentrations are based on an estimated mean volume of 37 fl per haploid yeast cell in exponential phase [53].

References for supplementary information

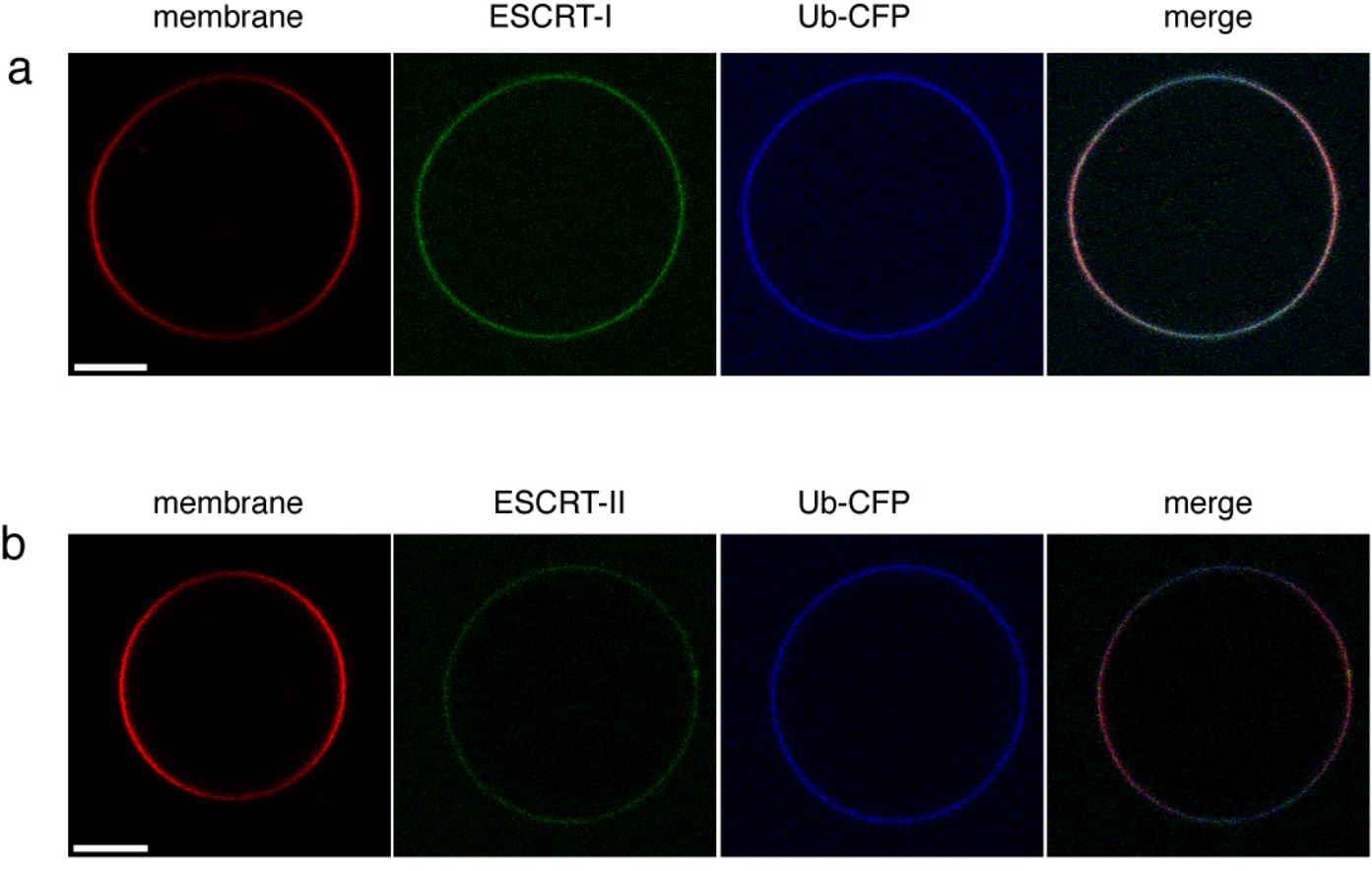
52. Huh, W. K. et al. Global analysis of protein localization in budding yeast. *Nature* **425**, 686-691 (2003).
53. Tyson, C. B., Lord, P. G. & Wheals, A. E. Dependency of size of Saccharomyces cerevisiase cells on growth rate. *J. Bacteriol.* **138**, 92-98 (1979).



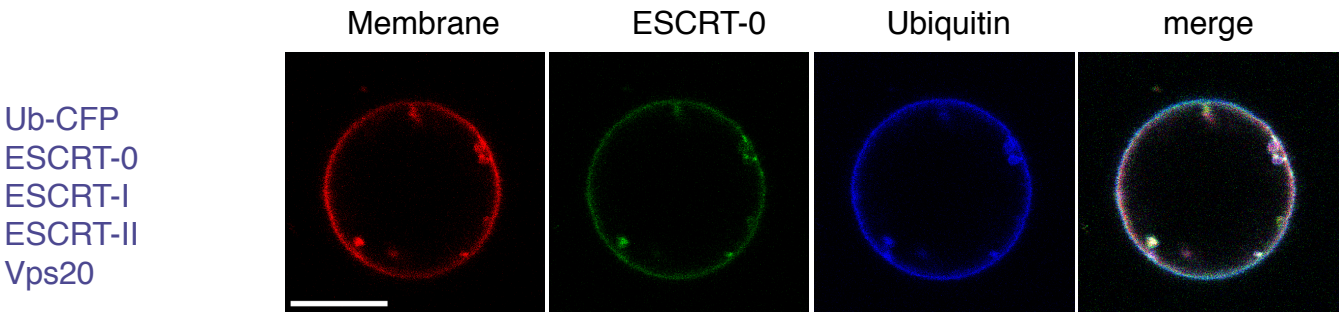




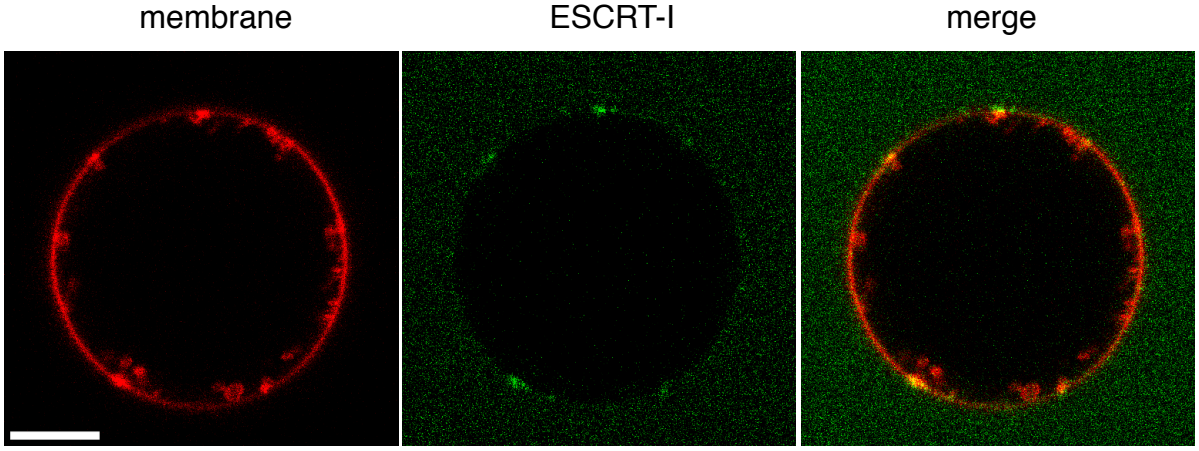
Supplementary Figure 1



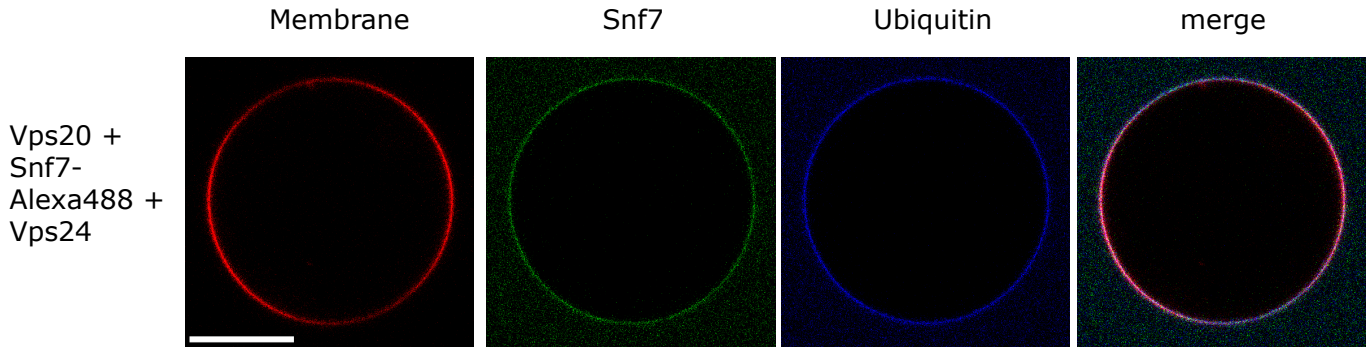
Supplementary Figure 2



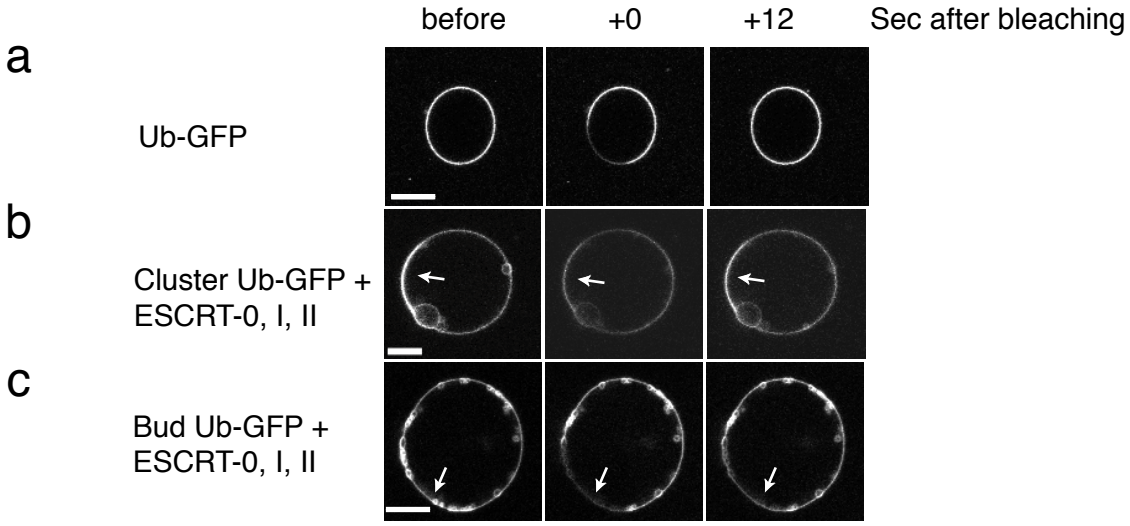
Supplementary Figure 3



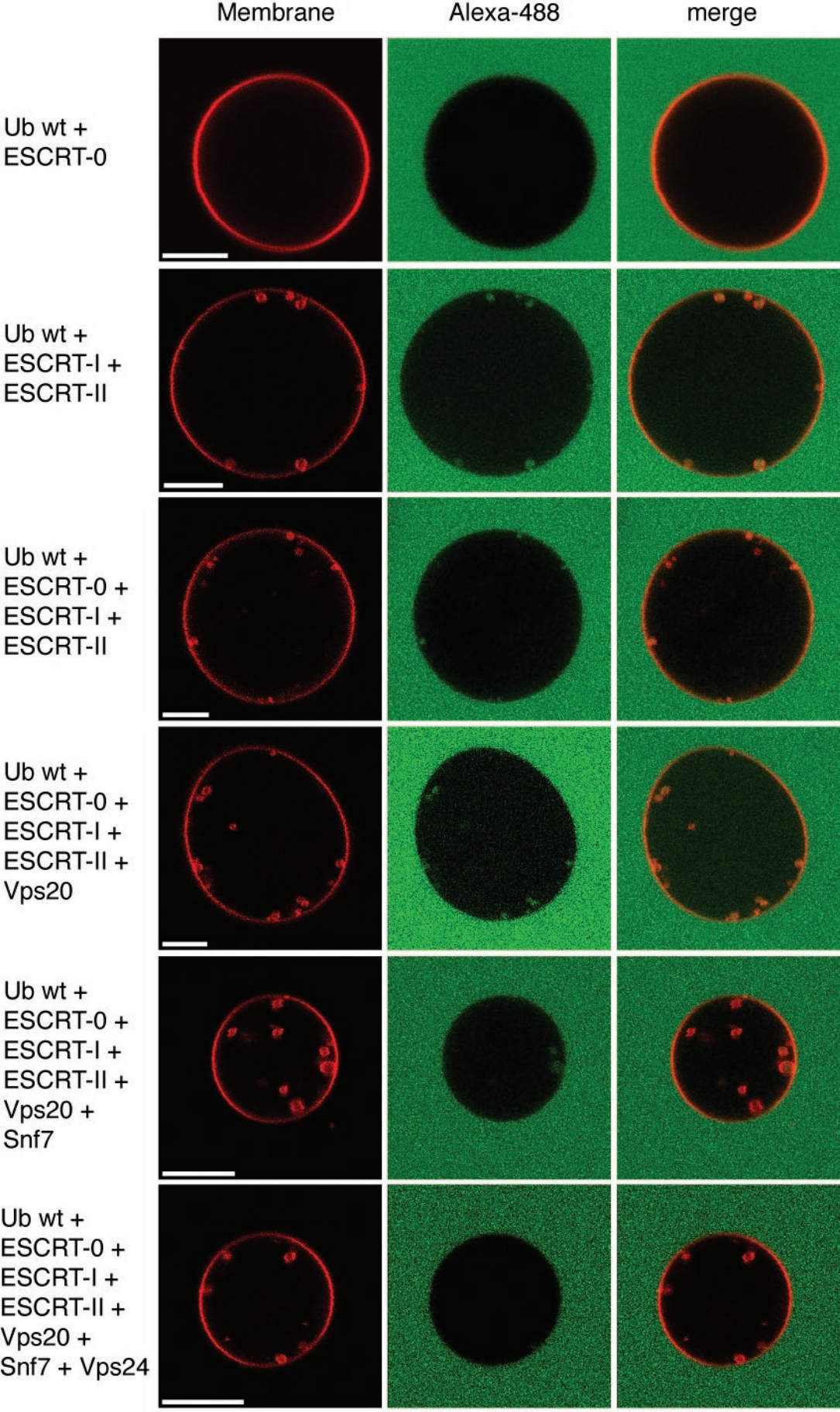
Supplementary Figure 4



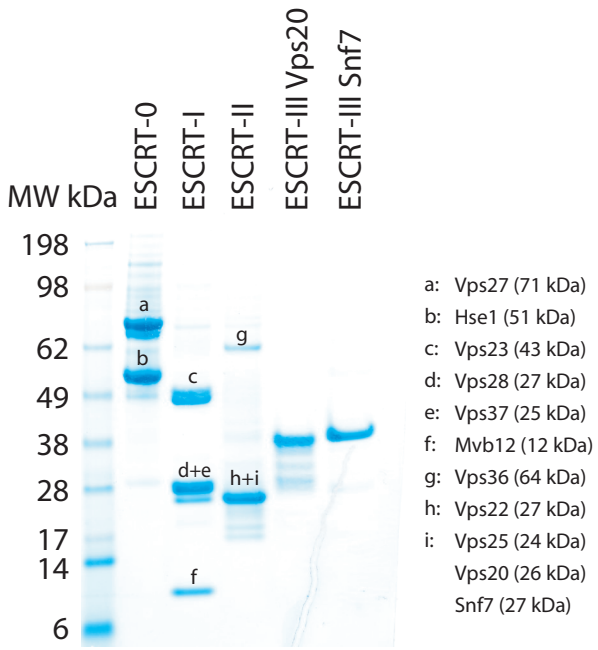
Supplementary Figure 5



Supplementary Figure 6



Supplementary Figure 7



a: Vps27 (71 kDa)
b: Hse1 (51 kDa)
c: Vps23 (43 kDa)
d: Vps28 (27 kDa)
e: Vps37 (25 kDa)
f: Mvb12 (12 kDa)
g: Vps36 (64 kDa)
h: Vps22 (27 kDa)
i: Vps25 (24 kDa)
Vps20 (26 kDa)
Snf7 (27 kDa)

Supplementary Figure 8