Supplemental Information

Supplemental Figure Legends

Figure S1: Purified proteins and localization of Sec7-GFP in mutant strains. (Related to Figure 1)

(A) 15% SDS-PAGE gel of 0.5 µg of each purified GTPase. The faster-migrating species in some of the preparations is likely due to proteolysis at the N-terminus, consistent with the disordered N-terminus of yeast Ypt31 (Ignatev et al., 2008).

(B) 8% SDS-PAGE gel of 1.5 µg of each purified Sec7 construct.

(C) anti-His immunoblot of purified GTPases.

(D) anti-His immunoblot of purified GEF constructs before and after TEV cleavage to remove the 6xHis tag (pre-TEV sample for Sec7ΔC+HDS1 not shown).

(E) Localization of an extra copy of GFP-Sec7 in yeast strains harboring the indicated gene deletions or temperature-sensitive mutations. Of the 20 strains tested, the mutants with at least partial cytoplasmic mis-localization are: *ypt1-3*, *arf1*Δ, *arl3*Δ, *sys1*Δ, *trs33*Δ, *trs85*Δ, *ypt6*Δ, and *gyp6*Δ. Certain strains exhibited a brighter GFP-Sec7 signal (*arl1*Δ*, arl3*Δ*, drs2*Δ*, gyp2*Δ*, gmh1*Δ*, sys1*Δ*, syt1*Δ) presumably due to upregulated expression. Although knockdown of Arl1 in metazoan cells results in mislocalization of BIG1/2 (Christis and Munro, 2012), GFP-Sec7 is not significantly mislocalized to the cytoplasm in *arl1*Δ yeast cells. This result was also observed previously (Setty et al., 2004).

(F) Co-localization analysis with Sys1-DsRed confirms that the GFP-Sec7 punctae correspond to Golgi compartments. Sys1 is a resident Golgi transmembrane protein that partially co-localizes with Sec7, but some DsRed signal is also visible in the cytoplasm (presumably due to partial proteolysis of the fusion protein) and the vacuole. The GFP channel in (F) and (G) is shown at different light levels compared to (E), for clarity of colocalization.

(G) Same as in (F), for GFP-tagged Sec7 alleles after 30 min at 37°C. Note that Sys1 appears to accumulate in enlarged structures after perturbation of Sec7 function, especially in the GFP-*sec7-1* strain.

Figure S2: In vitro assay data and controls. (Related to Figure 3)

(A) Example fluorescence trace of Arf1 activation by $Sec7_f$ in the presence of activated Ypt1. Insets, from left to right: Ypt1 activation by EDTA-triggered nucleotide exchange, $Sec7_f$ membrane recruitment and stimulation by activated Ypt1, and Arf1 activation by $Sec7_f$. Liposomes and buffer are present from the beginning of the trace.

(B) Arf1 activation rates by $Sec7_f$ in the presence of activated of Rab GTPases at various concentrations. n=3-5.

(C) Summary table of GEF activity assay results.

(D) Comparison of Arf1 activation by $Sec7_f$ in the presence of 100 nM activated Ypt31 or Ypt32. n=4.

(E) Arf1 activation by $Sec7_f$ in the presence of either 50 nM activated Ypt1, 50nM Ypt31, 50nM each of Ypt1 and Ypt31 (added simultaneously to the same population of liposomes), or 100nM of Ypt31. n=3.

(F) Arf1 activation by $Sec7_f$ in the presence of either 250 nM activated Arl1, 250nM Ypt1, 250 nM each of Arl1 and Ypt1 (added simultaneously to the same population of liposomes), 500 nM Arl1, or 500 nM Ypt1. n=3.

(G) Comparison of Arf1 activation by $Sec7_f$ or membrane-anchored his-$Sec7_f$. n=3.

In (B, D-G), error bars represent 95% CIs for the indicated n.

Figure S3: Fluorescence quantitation of time-lapse imaging and controls. (Related to Figure 4)

(A) Normalized fluorescence plotted versus time for the timelapses shown in Figure 4E.

(B) The same for timelapses shown in Figure 4D.

(C) Immunoblots to show expression levels of GFP-tagged Rab proteins relative to endogenous levels. The strain with GFP-Ypt31 integrated at the *YPT31* locus (CFY1805, which preserves the endogenous *YPT31* gene) was used to generate the data presented in Figure 4, Figure S3A, and Movie S2. G6PDH (Zwf1) serves as a loading control.

(D) Sec7 peak-to-peak times for strains with GFP-Ypt31 on a low expression plasmid, GFP-Ypt31 integrated at the *YPT31* locus, and GFP-Ypt32 integrated at the *URA3* locus. Error bars represent s.e.m for n=3 to 5.

(E) Sec7 peak-to-disappearance times are plotted indicating mean (26.6 seconds) and s.e.m. for 26 measurements.

(F) Average disappearance time versus average maximum fluorescence intensity for cargos.

Figure S4: Disruption of Ypt31/32 function results in a similar, but distinct, cargo-sorting phenotype compared to lowering Golgi Arf levels. (Related to Figure 4)

(A) Images showing Kex2-GFP overlapping significantly with the TGN (marked by Sec7-6xDsRed) in wild-type cells. Virtually all TGN localization of Kex2 is lost in both *ypt31-101 ypt32*Δ and *arf1*Δ cells (*S. cerevisiae* possesses two highly homologous Golgi-localized Arf proteins, Arf1 and Arf2. The *arf1*Δ strain expresses ~10% of Arf1/2 relative to wild-type cells (Stearns et al., 1990)). Imaging was performed at 26°C for all strains.

(B) GFP-Snc1 localization to the PM is diminished in *ypt31-101 ypt32*Δ cells, but not significantly mislocalized in *arf1*Δ cells.

(C) GFP-Tlg1 displays increased localization to the vacuole in *ypt31-101 ypt32*Δ. In *arf1*Δ cells, there is slight mislocalization of Tlg1 to the vacuole, but a significant portion remains TGN-localized.

(D) The significant portion of GFP-Tlg1 localizing to the TGN in *arf1*Δ cells allowed us to measure the sorting dynamics of GFP-Tlg1 in this mutant compared to wild-type cells. Error bars represent s.e.m. for n=5 (mutant) or n=4 (wild-type).

(E) Context for the main findings of this study: a schematic of the Golgi complex, showing major outgoing and incoming trafficking pathways (solid arrows), and the key GTPases. Blue-labeled GTPases regulate the tethering of incoming vesicles. Green-labeled GTPases regulate formation of outgoing vesicles. Magenta-labeled Arf1 represents the inactive form, which must be activated (dashed arrow) by its GEF, Sec7, at the TGN. Prior to this study, the Ypt31/32 Rab proteins had an unknown role in vesicle formation.

(F) The major finding of this study, that four GTPases differentially regulate Sec7. We propose that Sec7 is regulated by a two-stage process in which initial recruitment of Sec7 relies upon three GTPases, and subsequent activation requires the Ypt31/32 GTPase. In the absence of incoming traffic, we expect the signals from Ypt1 and Arl1 will be lower, thus dampening recruitment and activation of Sec7. It remains unknown how activation of Ypt31/32 is regulated.

Figure S1

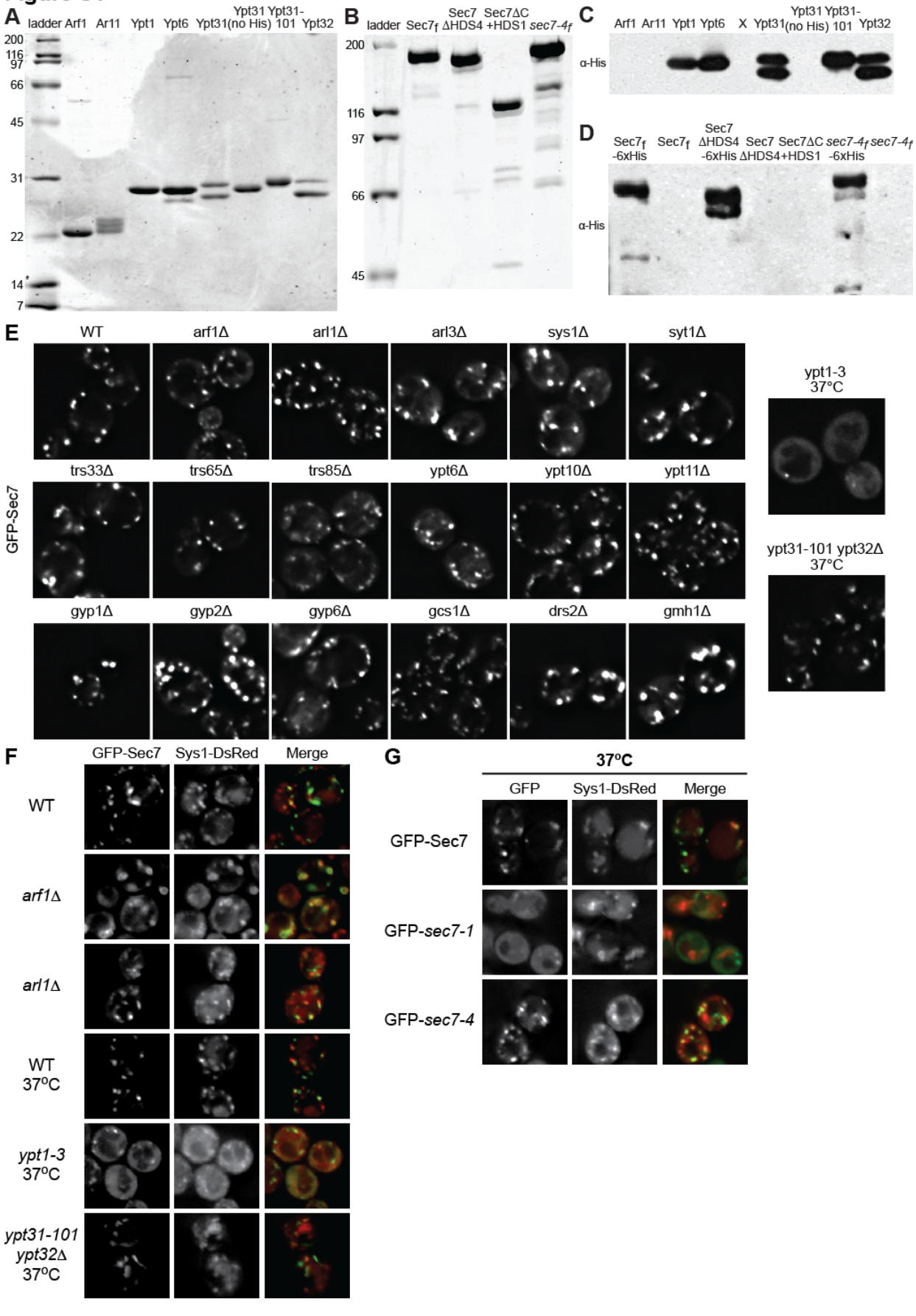
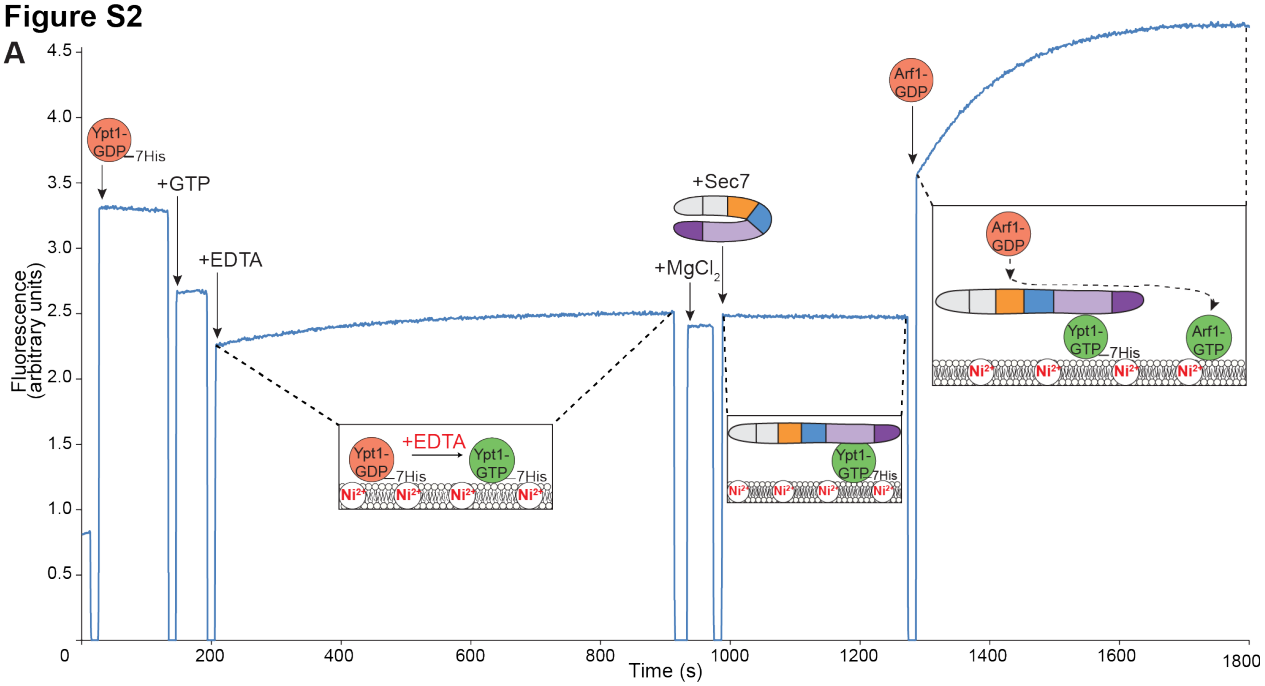
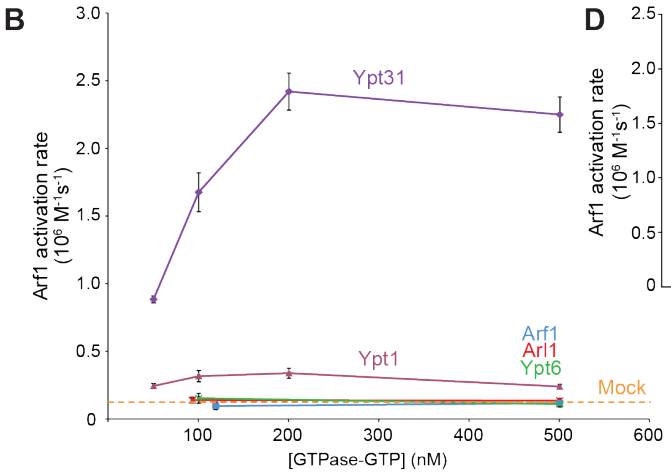
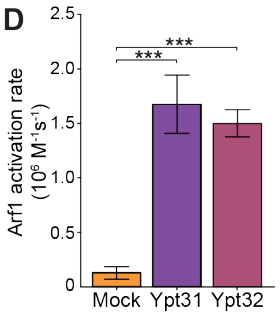
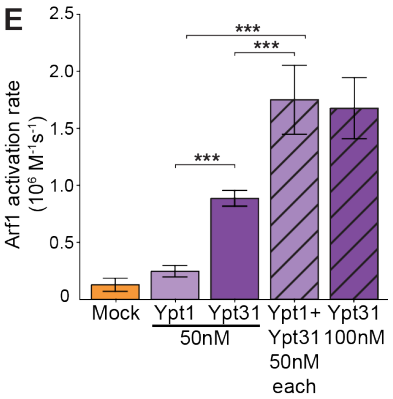

A
ladder Arf1 Ar11 Ypt1 Ypt6 Ypt31 Ypt31(no His) Ypt31-101 Ypt32
200
116
97
66
45
31
22
14
7
B
ladder Sec7f Sec7 ΔHDS4 Sec7ΔC +HDS1 sec7-4f
200
116
97
66
45
C
Arf1 Ar11 Ypt1 Ypt6 X Ypt31 Ypt31(no His) Ypt31-101 Ypt32
α-His
D
Sec7f -6xHis Sec7f Sec7 ΔHDS4 -6xHis Sec7 ΔHDS4 Sec7ΔC +HDS1 sec7-4f -6xHis sec7-4f
α-His
E
WT arf1Δ arl1Δ arl3Δ sys1Δ syt1Δ
trs33Δ trs65Δ trs85Δ ypt6Δ ypt10Δ ypt11Δ
gyp1Δ gyp2Δ gyp6Δ gcs1Δ drs2Δ gmh1Δ
GFP-Sec7
ypt1-3 37°C
ypt31-101 ypt32Δ 37°C
F
GFP-Sec7 Sys1-DsRed Merge
WT
arf1Δ
arl1Δ
WT 37°C
ypt1-3 37°C
ypt31-101 ypt32Δ 37°C
G
37°C
GFP Sys1-DsRed Merge
GFP-Sec7
GFP-sec7-1
GFP-sec7-4

Figure S2





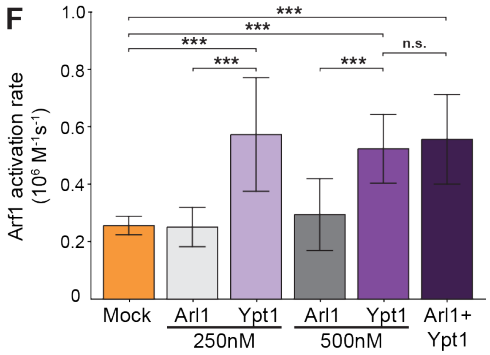




C

Summary of Sec7 GEF activity assays
GTPase at 500 nM unless otherwise indicated

Construct	Experiment	Result
Sec7f	Stimulation by Arf1	none
	Stimulation by Arl1	none
	Stimulation by Ypt1	2-fold
	Stimulation by Ypt6	none
	Stimulation by Ypt31	20-fold at 500 nM
		13-fold at 100 nM
	Stimulation by Ypt32	12-fold at 100 nM
	Stimulation by ypt31-101	2-fold
	Stimulation by soluble Ypt31	none
Sec7ΔHDS4	Increase in activity relative to Sec7f	5-fold
	Stimulation by Arf1	3.5-fold
	Stimulation by Arl1	2.5-fold
	Stimulation by Ypt1	none
	Stimulation by Ypt31	7-fold
Sec7ΔC+HDS1	Increase in activity relative to Sec7f	4-fold
	Stimulation by Arf1	3-fold
	Stimulation by Arl1	2-fold
	Stimulation by Ypt1	none
	Stimulation by Ypt31	none
6xHis-Sec7f	Increase in activity relative to Sec7f	1.5-fold
	Stimulation by Ypt1	2-fold
	Stimulation by Ypt31	10-fold



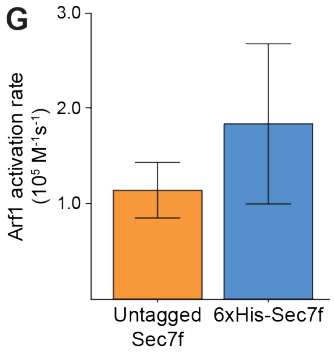
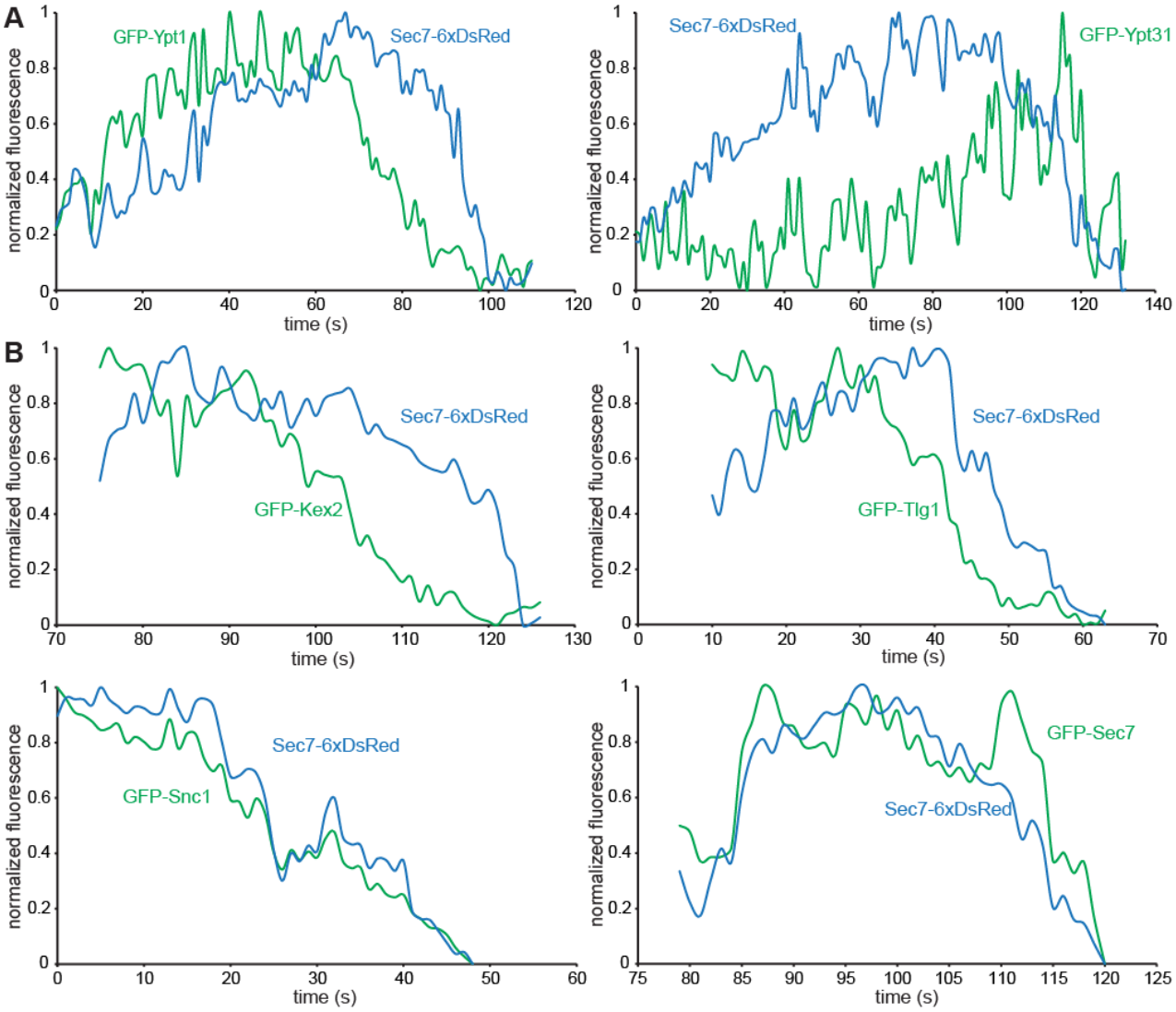
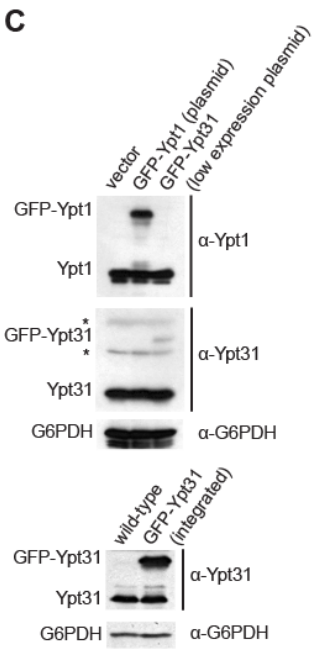
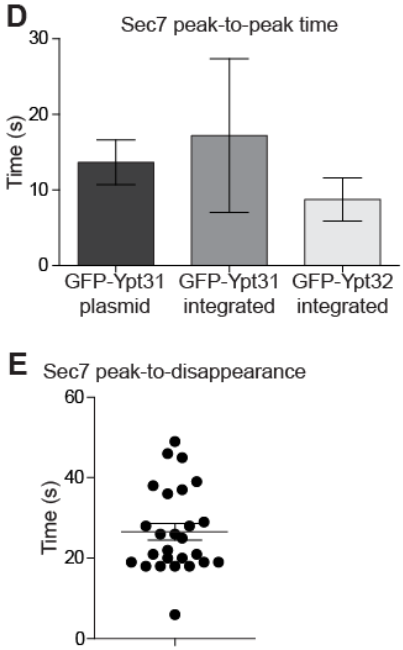


Figure S3


A
GFP-Ypt1
Sec7-6xDsRed
normalized fluorescence
time (s)
Sec7-6xDsRed
GFP-Ypt31
B
Sec7-6xDsRed
GFP-Kex2
GFP-Tlg1
GFP-Snc1
GFP-Sec7


C
vector
GFP-Ypt1 (plasmid)
GFP-Ypt31 (low expression plasmid)
GFP-Ypt1
Ypt1
α-Ypt1
GFP-Ypt31
Ypt31
α-Ypt31
G6PDH
α-G6PDH
wild-type
GFP-Ypt31 (integrated)


D
Sec7 peak-to-peak time
Time (s)
GFP-Ypt31 plasmid
GFP-Ypt31 integrated
GFP-Ypt32 integrated
E
Sec7 peak-to-disappearance
Time (s)

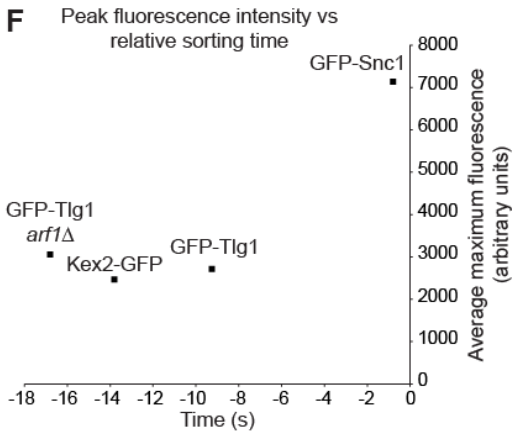
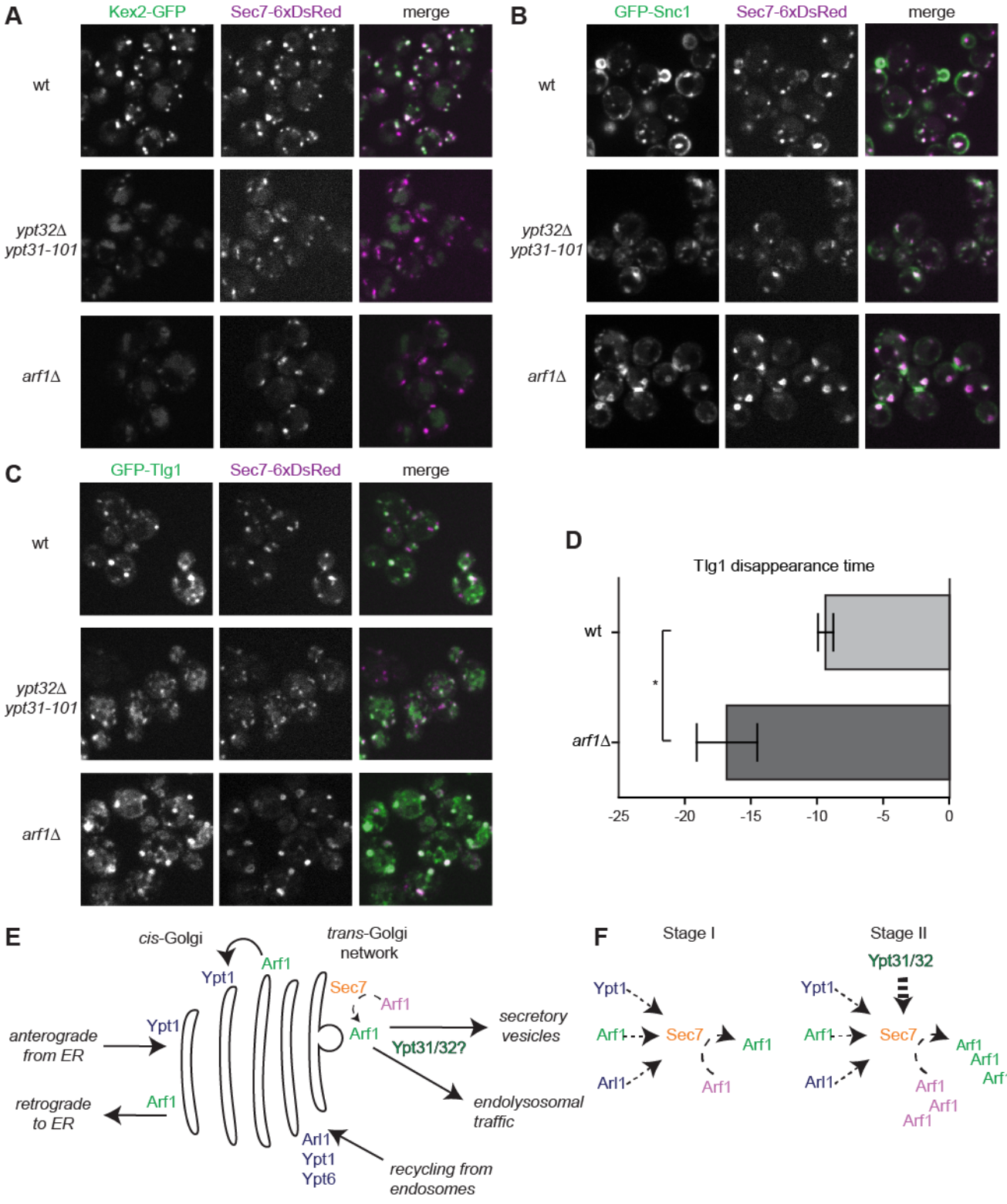

F
Peak fluorescence intensity vs relative sorting time
GFP-Snc1
GFP-Tlg1 arf1Δ
Kex2-GFP
GFP-Tlg1
Time (s)
Average maximum fluorescence (arbitrary units)

Figure S4


A
Kex2-GFP
Sec7-6xDsRed
merge
wt
ypt32Δ ypt31-101
arf1Δ
B
GFP-Snc1
Sec7-6xDsRed
merge
wt
ypt32Δ ypt31-101
arf1Δ
C
GFP-Tlg1
Sec7-6xDsRed
merge
wt
ypt32Δ ypt31-101
arf1Δ
D
Tlg1 disappearance time
wt
arf1Δ
*
-25
-20
-15
-10
-5
0
E
cis-Golgi
trans-Golgi network
Ypt1
Arf1
Sec7
Arf1
anterograde from ER
Ypt1
Arf1
secretory vesicles
Ypt31/32?
retrograde to ER
Arf1
endolysosomal traffic
Arl1
Ypt1
Ypt6
recycling from endosomes
F
Stage I
Ypt1
Arf1
Sec7
Arf1
Arl1
Arf1
Stage II
Ypt31/32
Ypt1
Arf1
Sec7
Arf1
Arf1
Arf1
Arl1
Arf1
Arf1
Arf1