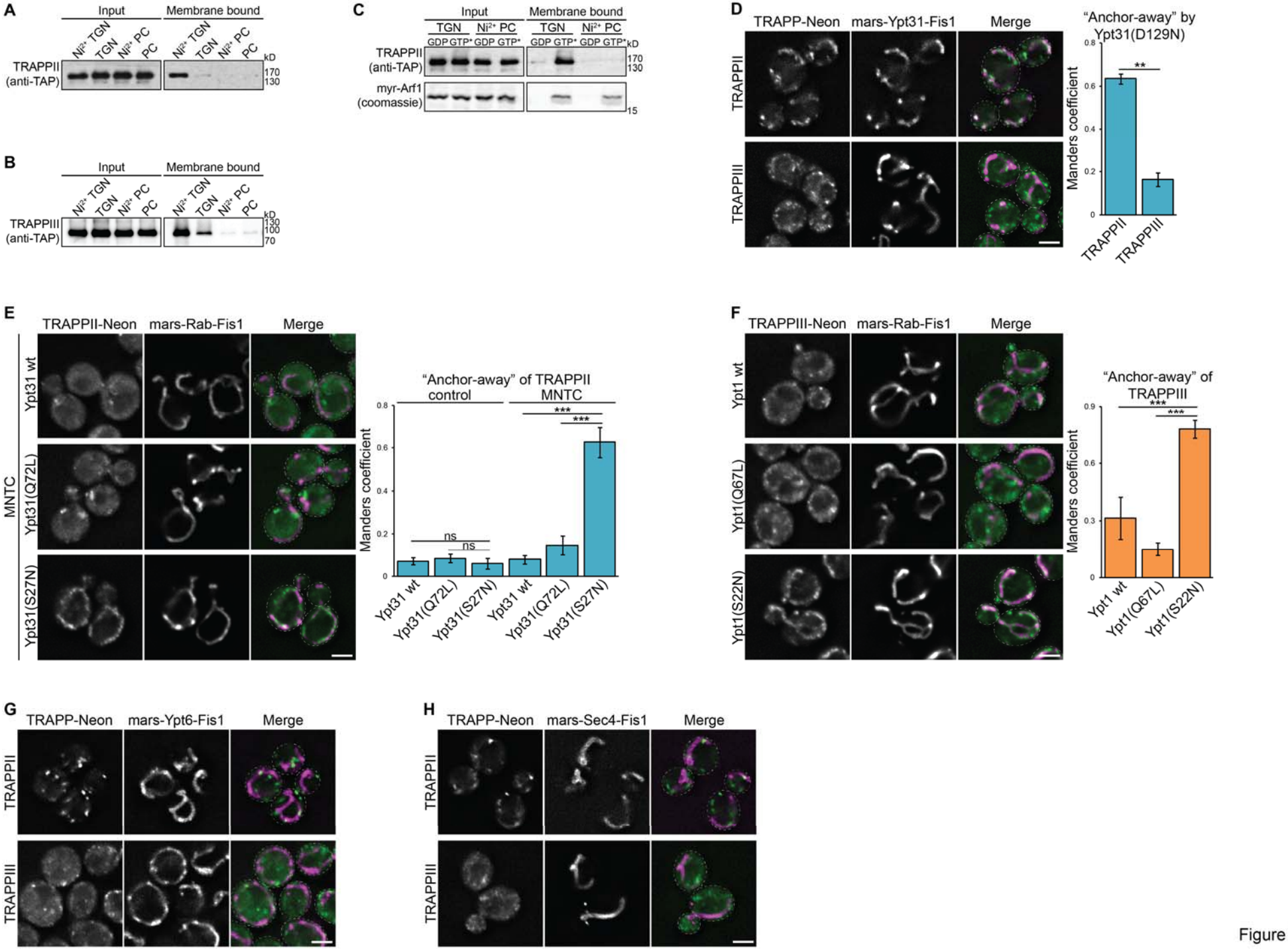
A
Input
Membrane bound
Ni2+ TGN
TGN
Ni2+ PC
PC
TRAPPII
(anti-TAP)
kD
170
130
B
TRAPPIII
(anti-TAP)
130
100
70
C
TGN
Ni2+ PC
GDP GTP*
myr-Arf1
(coomassie)
15
D
TRAPP-Neon
mars-Ypt31-Fis1
Merge
"Anchor-away" by
Ypt31(D129N)
Manders coefficient
**
E
TRAPPII-Neon
mars-Rab-Fis1
MNTC
Ypt31 wt
Ypt31(Q72L)
Ypt31(S27N)
"Anchor-away" of TRAPPII
control
MNTC
***
ns
F
TRAPPIII-Neon
Ypt1 wt
Ypt1(Q67L)
Ypt1(S22N)
"Anchor-away" of
TRAPPIII
G
TRAPP-Neon
mars-Ypt6-Fis1
H
mars-Sec4-Fis1

Figure S1

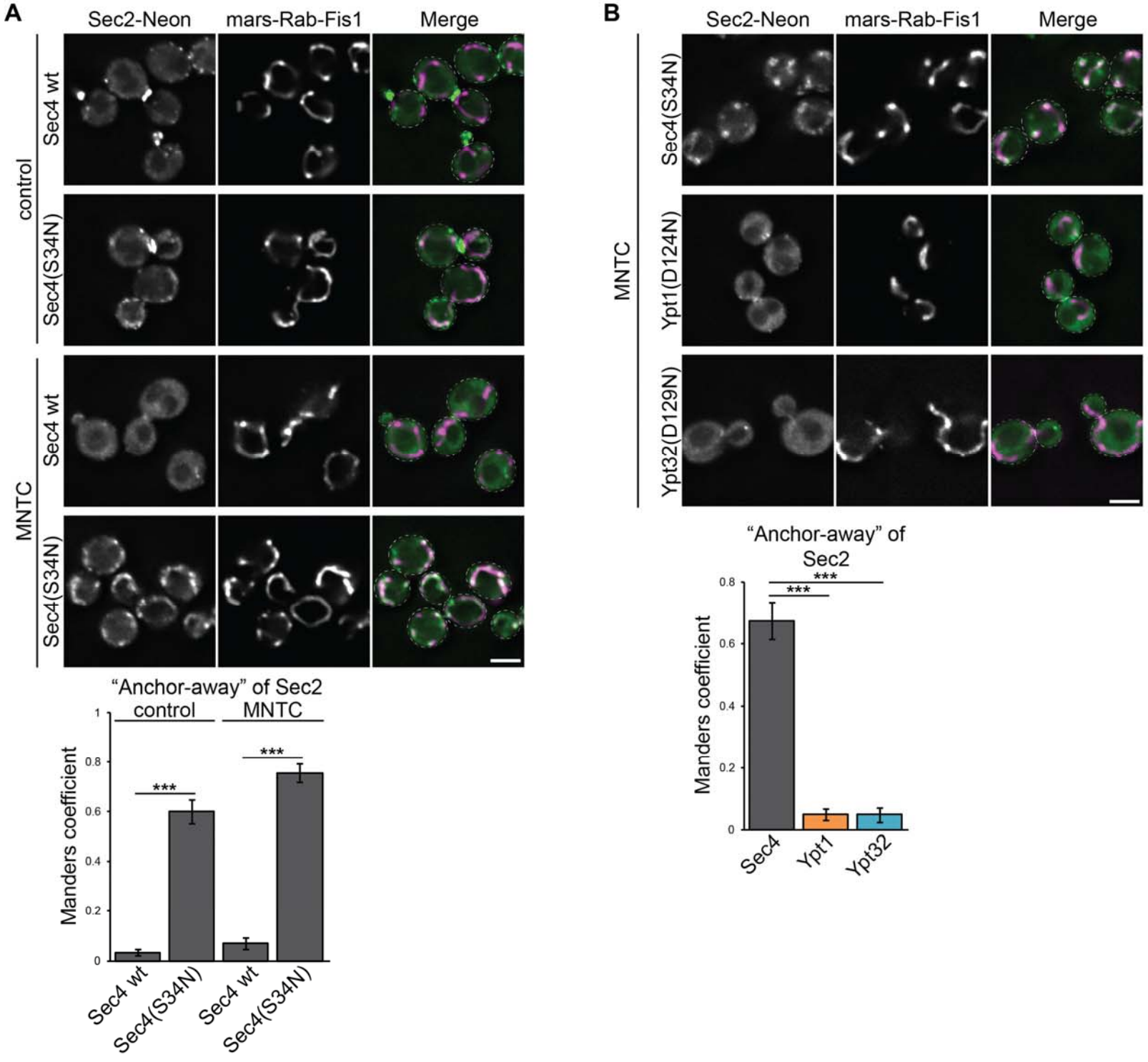
A
Sec2-Neon
mars-Rab-Fis1
Merge
control
Sec4 wt
Sec4(S34N)
MNTC
Sec4 wt
Sec4(S34N)
"Anchor-away" of Sec2
control
MNTC
Manders coefficient
***
***
Sec4 wt
Sec4(S34N)
Sec4 wt
Sec4(S34N)
B
Sec2-Neon
mars-Rab-Fis1
Merge
MNTC
Sec4(S34N)
Ypt1(D124N)
Ypt32(D129N)
"Anchor-away" of Sec2
Manders coefficient
***
***
Sec4
Ypt1
Ypt32

Figure S2

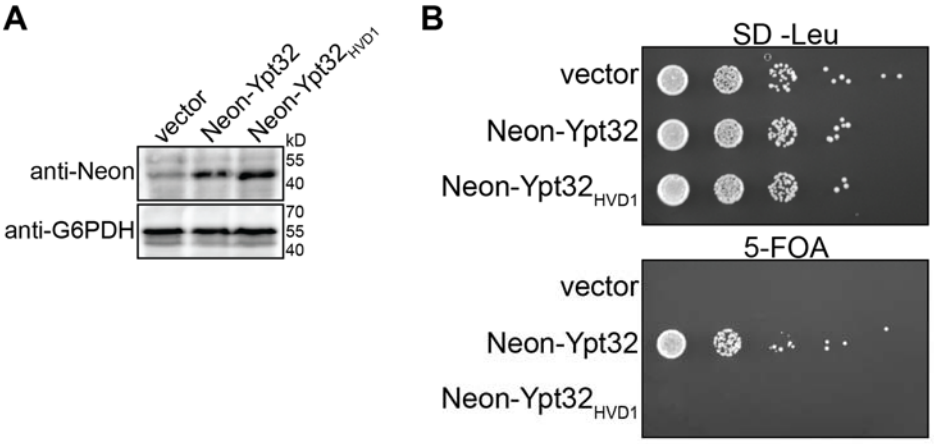
A
vector
Neon-Ypt32
Neon-Ypt32HVD1
kD
anti-Neon
55
40
70
anti-G6PDH
55
40
B
SD -Leu
vector
Neon-Ypt32
Neon-Ypt32HVD1
5-FOA
vector
Neon-Ypt32
Neon-Ypt32HVD1

Figure S3

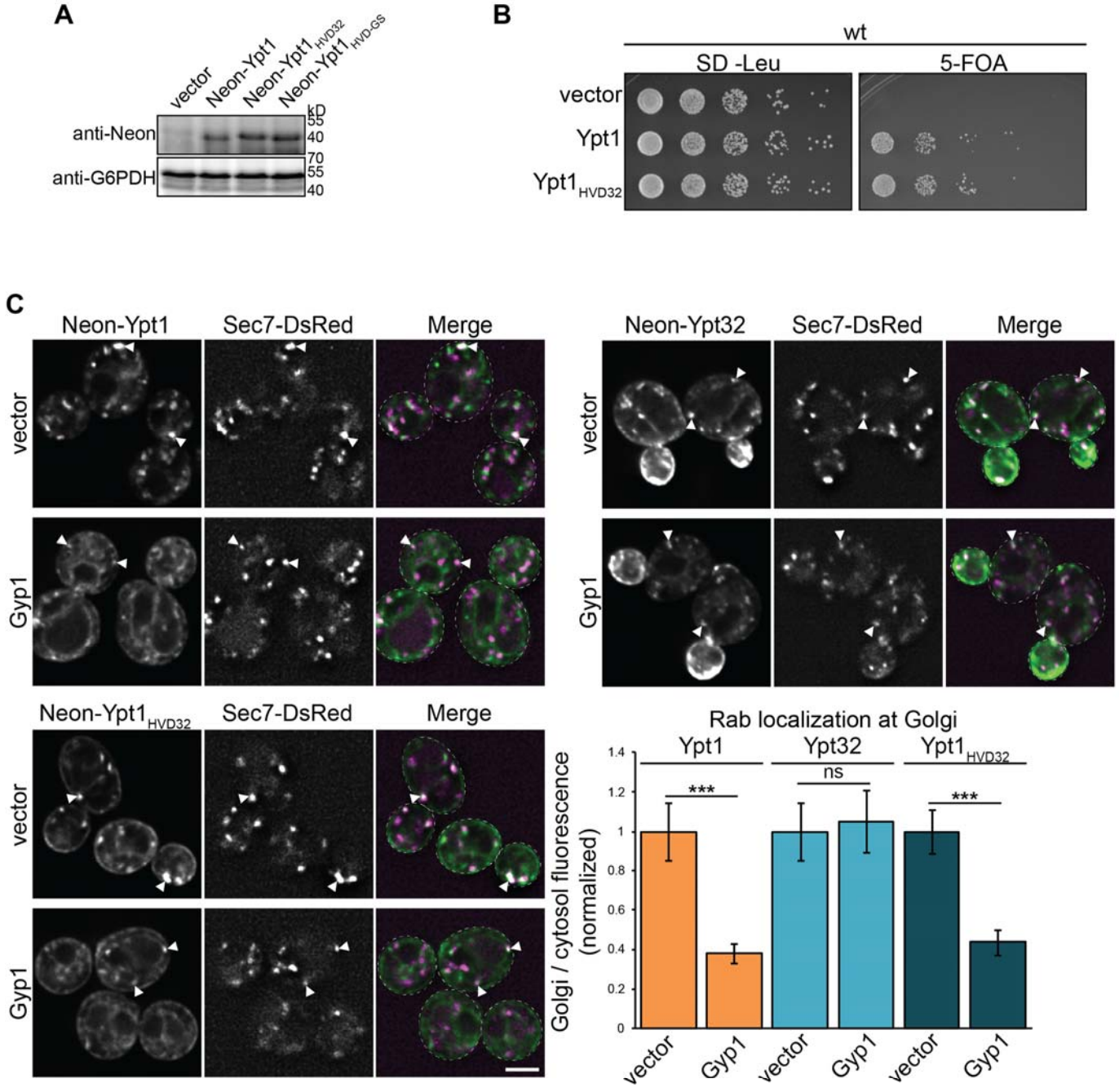
A
vector
Neon-Ypt1
Neon-Ypt1HVD32
Neon-Ypt1HVD-GS
kD
anti-Neon
55
40
70
anti-G6PDH
55
40
B
wt
SD -Leu
5-FOA
vector
Ypt1
Ypt1HVD32
C
Neon-Ypt1
Sec7-DsRed
Merge
vector
Gyp1
Neon-Ypt32
Sec7-DsRed
Merge
vector
Gyp1
Neon-Ypt1HVD32
Sec7-DsRed
Merge
vector
Gyp1
Rab localization at Golgi
Ypt1
Ypt32
Ypt1HVD32
***
ns
***
Golgi / cytosol fluorescence (normalized)
1.4
1.2
1
0.8
0.6
0.4
0.2
0
vector
Gyp1
vector
Gyp1
vector
Gyp1

Figure S4

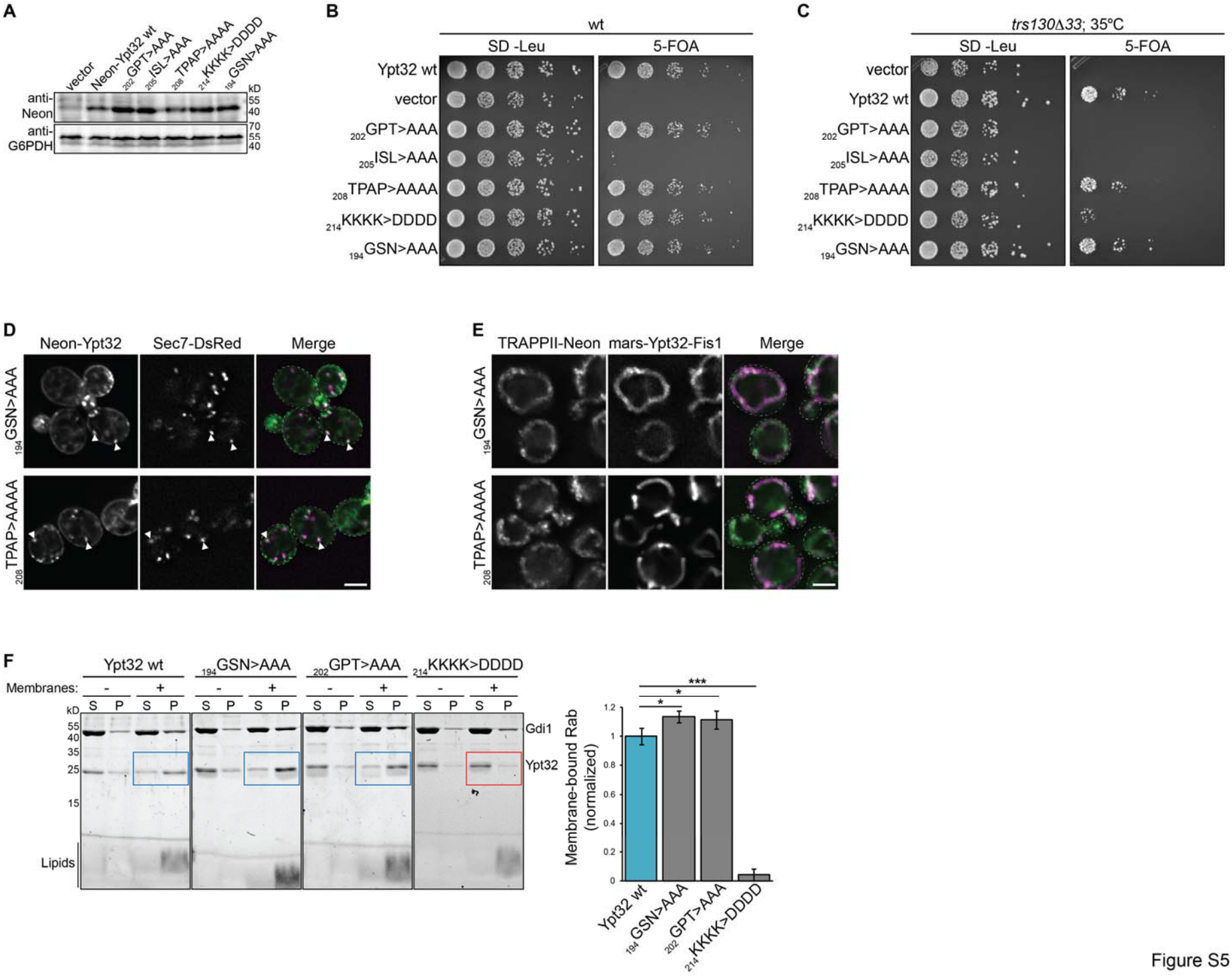
A
vector
Neon-Ypt32 wt
202GPT>AAA
205ISL>AAA
208TPAP>AAAA
214KKKK>DDDD
194GSN>AAA
kD
anti-Neon
anti-G6PDH
55
40
70
55
40
B
wt
SD -Leu
5-FOA
Ypt32 wt
vector
202GPT>AAA
205ISL>AAA
208TPAP>AAAA
214KKKK>DDDD
194GSN>AAA
C
trs130Δ33; 35°C
SD -Leu
5-FOA
vector
Ypt32 wt
202GPT>AAA
205ISL>AAA
208TPAP>AAAA
214KKKK>DDDD
194GSN>AAA
D
Neon-Ypt32
Sec7-DsRed
Merge
194GSN>AAA
208TPAP>AAAA
E
TRAPPII-Neon
mars-Ypt32-Fis1
Merge
194GSN>AAA
208TPAP>AAAA
F
Ypt32 wt
194GSN>AAA
202GPT>AAA
214KKKK>DDDD
Membranes:
-
+
S
P
kD
55
40
35
25
15
Gdi1
Ypt32
Lipids
Membrane-bound Rab (normalized)
1.2
1
0.8
0.6
0.4
0.2
0
***
*
*
Ypt32 wt
194GSN>AAA
202GPT>AAA
214KKKK>DDDD

Figure S5

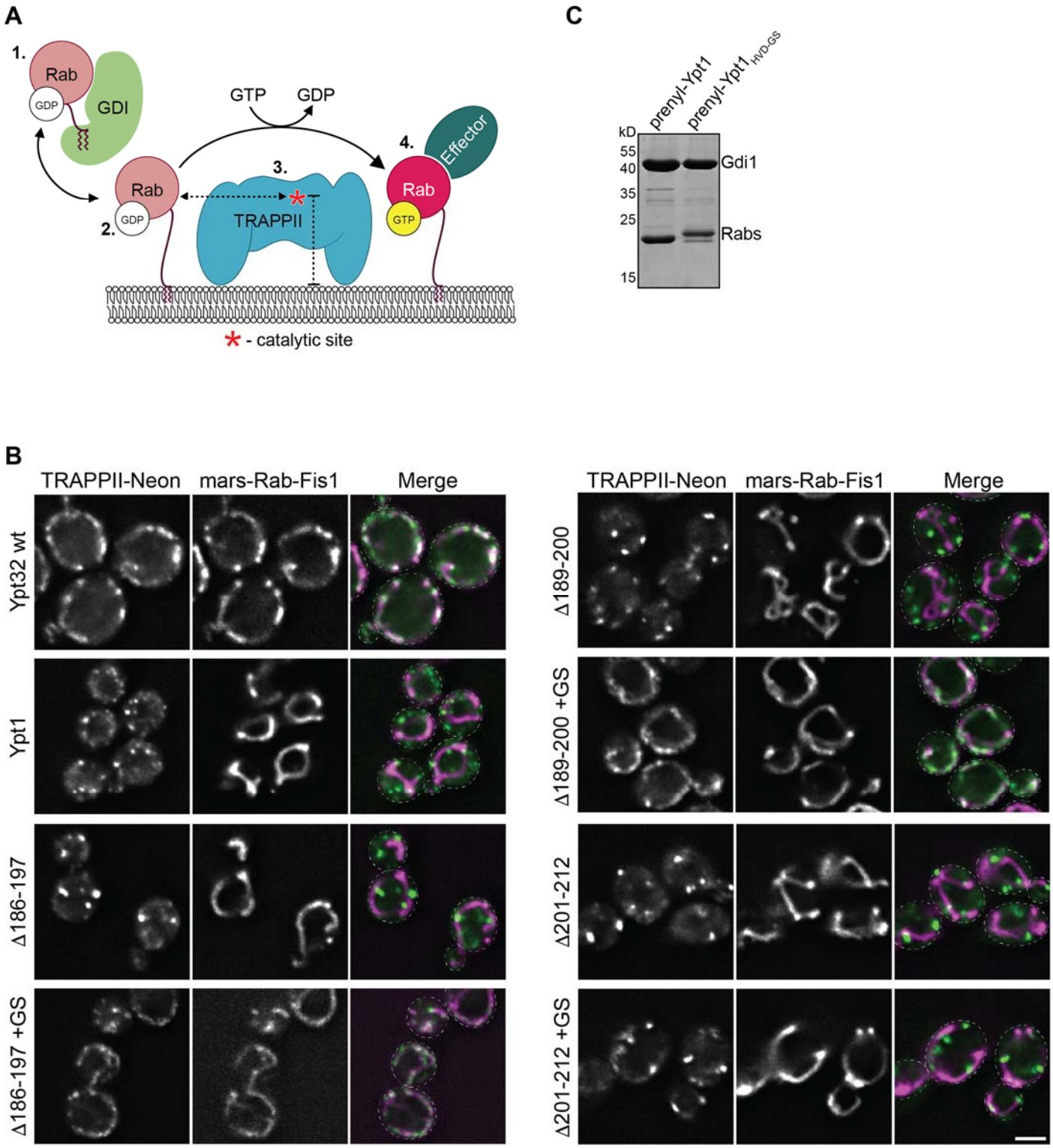
A
1.
Rab
GDP
GDI
GTP
GDP
4.
Effector
3.
Rab
2.
GDP
TRAPPII
Rab
GTP
* - catalytic site
C
prenyl-Ypt1
prenyl-Ypt1 HVD-GS
kD
55
40
35
25
15
Gdi1
Rabs
B
TRAPPII-Neon
mars-Rab-Fis1
Merge
Ypt32 wt
Ypt1
Δ186-197
Δ186-197 +GS
Δ189-200
Δ189-200 +GS
Δ201-212
Δ201-212 +GS

Figure S6

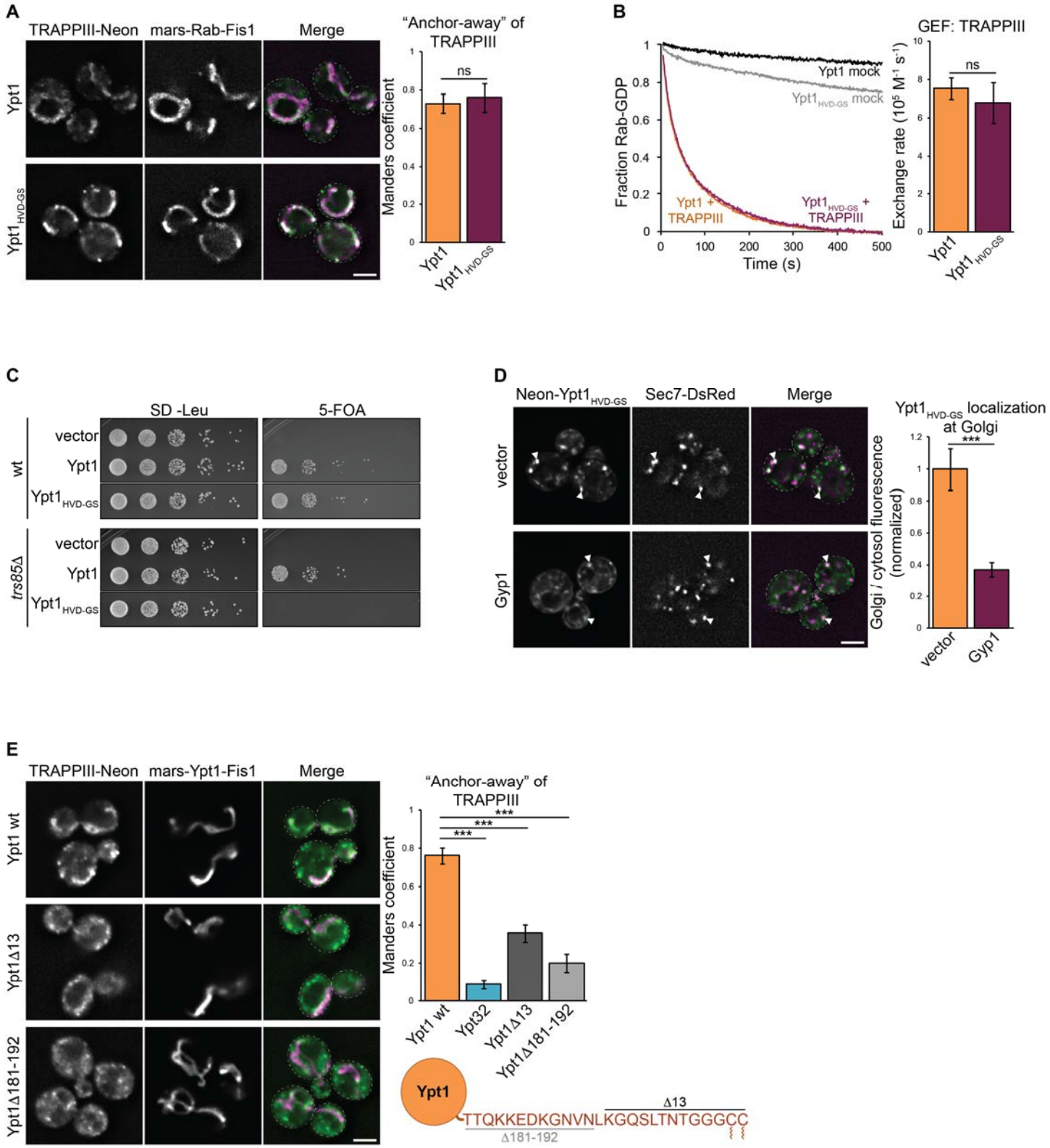
A
TRAPPIII-Neon
mars-Rab-Fis1
Merge
Ypt1
Ypt1HVD-GS
"Anchor-away" of TRAPPIII
Manders coefficient
ns
Ypt1
Ypt1HVD-GS
B
Fraction Rab-GDP
Time (s)
Ypt1 mock
Ypt1HVD-GS mock
Ypt1 + TRAPPIII
Ypt1HVD-GS + TRAPPIII
GEF: TRAPPIII
Exchange rate (10^5 M^-1 s^-1)
ns
C
SD -Leu
5-FOA
wt
trs85Δ
vector
Ypt1
Ypt1HVD-GS
D
Neon-Ypt1HVD-GS
Sec7-DsRed
Merge
vector
Gyp1
Ypt1HVD-GS localization at Golgi
***
Golgi / cytosol fluorescence (normalized)
vector
Gyp1
E
TRAPPIII-Neon
mars-Ypt1-Fis1
Merge
Ypt1 wt
Ypt1Δ13
Ypt1Δ181-192
"Anchor-away" of TRAPPIII
***
***
***
Manders coefficient
Ypt1 wt
Ypt32
Ypt1Δ13
Ypt1Δ181-192
Ypt1
TTQKKEDKGNVNLKGQSLTNTGGGCC
Δ13
Δ181-192

Figure S7

Supplemental Figure Legends

Figure S1 (related to Figure 1). Controls for GRab-IT assays used to measure TRAPP complex substrate recognition. (A) Liposome flotation assay testing TRAPPII association with TGN or phosphatidylcholine (PC) membranes with or without 5% Ni^{2+}-conjugated lipids. (B) Liposome flotation assay testing TRAPPIII association with TGN or PC membranes with or without 5% Ni^{2+}. (C) Liposome flotation assay testing TRAPPII recruitment to TGN (without Ni^{2+}) or Ni^{2+} PC membranes by activated Arf1. GTP* indicates GMP-PNP, a non-hydrolyzable GTP analog. (D) Left: Representative images showing that nucleotide-free Ypt31(D129N) recruits TRAPPII, but not TRAPPIII, to mitochondria. Right: Quantification of TRAPP complex recruitment to mitochondria. Error bars represent 95% CIs for *n > 40* cells. (E) Left: Representative images showing that TRAPPII is recruited to mitochondria by Ypt31(S27N) (GDP-locked), but not wild-type Ypt31 or Ypt31(Q72L) (GTP-locked). Cells were treated with the Sec7 inhibitor MNTC (20 µM, 10 min) to facilitate TRAPPII recruitment by displacing TRAPPII from Golgi compartments. Right: Quantification of TRAPPII recruitment to mitochondria. Error bars represent 95% CIs for *n > 40* cells. Note that TRAPPII remains stably bound to Golgi compartments in control cells. (F) Left: Representative images showing that TRAPPIII is recruited to mitochondria by Ypt1(S22N) (GDP-locked), but not wild-type Ypt1 or Ypt1(Q67L) (GTP-locked). Right: Quantification of TRAPPIII recruitment to mitochondria. Error bars represent 95% CIs for *n > 40* cells. (G) Representative images showing that nucleotide-free Ypt6(D127N) does not recruit TRAPPII or TRAPPIII to mitochondria. See Figure 1 for quantification. (H) Representative images showing that nucleotide-free Sec4(D136N) does not recruit TRAPPII or TRAPPIII to mitochondria. See Figure 1 for quantification. Scale bars, 2 µm.

Figure S2 (related to Figure 1). The GRab-IT assay can be extended to characterize other Rab-GEF pairs. (A) Top: Representative images showing that Sec2 is recruited to mitochondria by Sec4(S34N) (GDP-locked), but not wild-type Sec4. Cells were treated with MNTC (20 µM, 10 min) to facilitate GEF recruitment by displacing Sec2 from secretory vesicles. Bottom: Quantification of Sec2 recruitment to mitochondria. Error bars represent 95% CIs for *n > 40* cells. (B) Top: Representative

images showing that Sec2 is recruited to mitochondria by Sec4(S34N), but not Ypt1(D124N) or Ypt32 (D129N), in MNTC-treated cells. Bottom: Quantification of Sec2 recruitment to mitochondria. Error bars represent 95% CIs for *n > 40* cells. Scale bars, 2 µm.

Figure S3 (related to Figure 4). Controls for microscopy and complementation tests with Ypt31/32 constructs. (A) Western blot showing expression of Ypt32 constructs used in complementation tests and microscopy. G6PDH serves as a loading control. (B) mNG-tagged wild-type Ypt32 complements a *ypt31*Δ*ypt32*Δ mutant in a wild-type background.

Figure S4 (related to Figure 5): The HVD does not mediate substrate specificity for the Ypt1 GAP Gyp1. (A) Western blot showing expression of Ypt1 constructs used in complementation tests and microscopy. G6PDH serves as a loading control. (B) $Ypt1_{HVD32}$ was tested for its ability to complement a *ypt1*Δ mutant in a wild-type background. (C) Top: Localization of mNG-tagged Ypt1 (left) and Ypt32 (right) relative to Sec7-DsRed with or without overexpression of the Ypt1 GAP Gyp1. Bottom left: Localization of mNG-tagged $Ypt1_{HVD32}$ relative to Sec7 with or without Gyp1 overexpression. Bottom right: Quantification of Golgi (active) versus cytosolic/ER-localized (inactive) Rab under each condition. Error bars represent 95% CIs for *n ≥ 25* compartments. Scale bar, 2 µm. White arrowheads denote colocalization of Rabs and Sec7 at late Golgi compartments.

Figure S5 (related to Figure 6). Controls for assays with Ypt32 HVD point mutants. (A) Western blot showing expression of Ypt32 HVD point mutants used in complementation tests and microscopy. G6PDH serves as a loading control. (B) The indicated Ypt32 HVD mutants were tested for their ability to complement a *ypt31*Δ*ypt32*Δ mutant in a wild-type background. (C) The indicated Ypt32 HVD mutants were tested for their ability to complement a *ypt31*Δ*ypt32*Δ mutant in the sensitized *trs130*Δ*33* (TRAPPII mutant) background. Cells were grown at 35ºC. (D) Representative images of mNG-tagged Ypt32 HVD point mutants relative to Sec7-DsRed. See Figure

6C for quantification. (E) Representative images showing recruitment of TRAPPII to mitochondria by the indicated nucleotide-free Ypt32 HVD mutants. See Figure 6D for quantification. (F) Left: Representative liposome pelleting assays with wild-type Ypt32 versus HVD mutant substrates. Prenylated Ypt32/GDI complexes were assayed for membrane binding following EDTA-based exchange with GTP. Efficiently prenylated Rabs bound membranes in a nucleotide-dependent manner (blue boxes), but Ypt32 $_{214}$KKKK>DDDD did not associate with membranes (red box). Failure of Ypt32 $_{214}$KKKK>DDDD to bind membranes could be due to a lack of prenylation, or that the charge reversal prevents the mutant from associating with anionic membranes. Right: Quantification of membrane binding for each construct tested. Values are normalized so that wild-type Ypt32 membrane-binding is equal to 1.0. Error bars represent 95% CIs for $n \geq 4$ pelleting reactions. Scale bars, 2 µm. White arrowheads denote colocalization of Rabs and Sec7 at late Golgi compartments.

Figure S6 (related to Figure 7). Truncating the Ypt32 HVD abolishes substrate recognition by TRAPPII. (A) Schematic depicting the importance of HVD length during Rab activation. Inactive Rabs are bound by GDI (1) and delivered to membranes where they anchor via their prenylated C-terminus (2). The HVD must be long enough to access the GEF active site to permit nucleotide exchange (3). Activated Rabs bind effectors to coordinate membrane trafficking (4). (B) Representative images showing TRAPPII recruitment to mitochondria by nucleotide-free Ypt32 versus the indicated HVD truncation and Gly-Ser rescue constructs. See Figure 7A for quantification. (C) Coomassie-stained SDS-PAGE gel showing prenylated Rab substrates used to test whether Ypt1 HVD length affects TRAPP-mediated nucleotide exchange. Scale bar, 2 µm.

Figure S7 (related to Figure 7). Truncating the Ypt1 HVD impairs substrate recognition by TRAPPIII. (A) Left: Representative images showing TRAPPIII recruitment to mitochondria by nucleotide-free Ypt1 versus the longer HVD Ypt1$_{HVD\text{-}GS}$. Right: Quantification of TRAPPIII recruitment to mitochondria. Error bars represent 95% CIs for $n > 40$ cells. (B) Left: Representative traces showing activation of Ypt1 versus

Ypt1$_{HVD-GS}$ by TRAPPIII in the presence of TGN membranes. mock = buffer only control reaction. Right: Rates of TRAPPIII-mediated nucleotide exchange calculated from the traces at left. Error bars represent 95% CIs for $n \geq 3$ reactions. (C) The longer HVD Ypt1$_{HVD-GS}$ was tested for its ability to complement a *ypt1*Δ mutant in either a wild-type or *trs85*Δ (TRAPPIII mutant) background. (D) Left: Localization of mNG-tagged Ypt1$_{HVD-GS}$ relative to Sec7-DsRed with or without overexpression of the Ypt1 GAP Gyp1. Right: Quantification of Golgi (active) versus cytosolic/ER-localized (inactive) Ypt1$_{HVD-GS}$ with or without Gyp1 overexpression. Error bars represent 95% CIs for $n \geq 25$ compartments. (E) Left: Representative images showing TRAPPIII recruitment to mitochondria by nucleotide-free Ypt1(D124N) with a full-length HVD versus the indicated truncation mutants. Right: Quantification of TRAPPIII recruitment to mitochondria. Error bars represent 95% CIs for $n > 40$ cells. Scale bars, 2 μm. White arrowheads denote colocalization of Rabs and Sec7 at late Golgi compartments.

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Supplemental Tables

Table S1 (related to STAR Methods, Liposome Preparation). Composition of liposomes used in this study.

Lipid	TGN	Ni^{2+} TGN	PC	Ni^{2+} PC
DOPC	24%	24%	99%	94%
POPC	6%	6%		
DOPE	7%	7%		
POPE	3%	3%		
DOPS	1%	1%		
POPS	2%	2%		
DOPA	1%	1%		
POPA	2%	2%		
PI	29%	24%		
PI(4)P	1%	1%		
CDP-DAG	2%	2%		
DO-DAG	2%	2%		
PO-DAG	4%	4%		
Ceramide (C18)	5%	5%		
Cholesterol	10%	10%		
Ni^{2+}-DOGS		5%		5%
DiR dye	1%	1%	1%	1%

Table S2 (related to Figure 2). Sequences of Rab chimeras used in this study.

Rab chimera	NBD residues	HVD residues (not including C-terminal cysteines)
Ypt1$_{HVD31}$	Ypt1(1-180)	Ypt31(186-221)
Ypt1$_{HVD32}$	Ypt1(1-180)	Ypt32(186-220)
Ypt1 +30	Ypt1(1-180)	Ypt1(181-204) + SGGGSGGSGSGGSGG SGGGSGGSGSGGSGG
Ypt1 +15	Ypt1(1-180)	Ypt1(181-204) + SGGGSGGSGSGGSGG
Ypt1$_{HVD-GS}$ (+12)	Ypt1(1-180)	Ypt1(181-204) + SGGGSGGSGSGG
Ypt1 +9	Ypt1(1-180)	Ypt1(181-204) + SGGGSGGSG
Ypt1 +6	Ypt1(1-180)	Ypt1(181-204) + SGGGSG
Ypt1 +3	Ypt1(1-180)	Ypt1(181-204) + SGG
Ypt31$_{HVD1}$	Ypt31(1-185)	Ypt1(181-204)
Ypt32$_{HVD1}$	Ypt32(1-185)	Ypt1(181-204)
Ypt6$_{HVD31}$	Ypt6(1-185)	Ypt31(186-221)
Ypt6$_{HVD1}$	Ypt6(1-185)	Ypt1(181-204)

Table S3 (related to Figure 1). Summary of results from GRab-IT experiments, GEF reconstitution assays, complementation tests, and microscopy of mNG-Rabs. We note that Ypt31 and Ypt32 are genetically redundant paralogs and very similar results were obtained whenever direct comparisons were made between these paralogs.

Rab construct	TRAPPII recruitment (GRab-IT; Average MOC)	TRAPPIII recruitment (GRab-IT; Average MOC)	TRAPPII-mediated activation (GEF reconstitution; Exchange rate)	TRAPPIII-mediated activation (GEF reconstitution; Exchange rate)	Complementation tests (plasmid shuffling; cell growth)	Activated *in vivo* (live cell imaging; Average Golgi/cytosol fluorescence)	Overlap with Sec7 (live cell imaging; Average MOC)
Ypt1	0.09 ± 0.02	0.73 ± 0.05	n.d.	$5.39x10^5 \pm 0.43x10^5$	+++*		0.39 ± 0.03
Ypt1$_{HVD31/32}$	0.60 ± 0.03	0.75 ± 0.05	$4.42x10^5 \pm 0.20x10^5$	$2.76x10^5 \pm 0.38x10^5$	+/-*		0.49 ± 0.05
Ypt1$_{HVD-GS}$	0.50 ± 0.04	0.76 ± 0.07	$7.59x10^5 \pm 0.74x10^5$	$6.82x10^5 \pm 1.07x10^5$	-*		0.53 ± 0.05
Ypt31/32	0.71 ± 0.05	0.06 ± 0.02	$11.82x10^5 \pm 0.51x10^5$	n.d.	++++	7.94 ± 1.23	
Ypt31/32$_{HVD1}$	0.07 ± 0.03	0.54 ± 0.06	n.d.	n.d.	-	2.42 ± 0.18	
Ypt6	0.06 ± 0.01	0.05 ± 0.02					
Ypt6$_{HVD1}$		0.07 ± 0.02					
Ypt6$_{HVD31}$	0.05 ± 0.04						
Sec4	0.11 ± 0.04	0.06 ± 0.04					
Ypt1 wt	0.09 ± 0.02						
Ypt1 +3	0.10 ± 0.02						
Ypt1 +6	0.16 ± 0.05						
Ypt1 +9	0.41 ± 0.11						
Ypt1$_{HVD-GS}$ (+12)	0.55 ± 0.10						
Ypt1 +15	0.67 ± 0.08						
Ypt1 +30	0.65 ± 0.08						
Ypt1 wt		0.76 ± 0.04					
Ypt1Δ13		0.36 ± 0.05					
Ypt1Δ181-192		0.20 ± 0.05					
Ypt32 wt	0.66 ± 0.05		$12.59x10^5 \pm 2.63x10^5$		++++**	11.63 ± 1.61	
Ypt32 $_{194}$GSN>AAA	0.68 ± 0.04		$13.67x10^5 \pm 2.32x10^5$		++++**	11.33 ± 1.49	
Ypt32 $_{202}$GPT>AAA	0.54 ± 0.04		$5.92x10^5 \pm 0.16x10^5$		+**	4.73 ± 1.07	
Ypt32 $_{205}$ISL>AAA	0.18 ± 0.04				-**	n/a	
Ypt32 $_{208}$TPAP>AAAA	0.64 ± 0.03				++++**	11.88 ± 1.51	
Ypt32 $_{214}$KKKK>DDDD	0.37 ± 0.04				++**	6.62 ± 0.96	
Ypt32 wt	0.75 ± 0.03						
Ypt32Δ186-189	0.08 ± 0.02						
Ypt32Δ186-189 +GS	0.73 ± 0.05						
Ypt32Δ189-200	0.08 ± 0.02						
Ypt32Δ189-200 +GS	0.07 ± 0.05						
Ypt32Δ201-212	0.06 ± 0.02						
Ypt32Δ201-212 +GS	0.06 ± 0.02						

**trs85*Δ TRAPPIII mutant shuffling background
***trs130Δ33* TRAPPII mutant shuffling background#