Supplemental Materials

Molecular Biology of the Cell

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Supplemental Figure Legends

Figure S1. The WD-40 domain of Kog1, a homolog of Raptor binds directly to PI3,5P$_2$. (A) Schematic of Kog1, indicating the peptide (1162-1557 aa) tested for binding to PI3,5P$_2$; and the predicted WD40 domain, 1200-1557aa. Four predicted HEAT repeats (548-586aa, 588-625aa, 777-814aa and 888-925aa) are indicated in red (Loewith et al., 2002). (B) Equilibrium binding assessed with surface plasmon resonance (SPR). A recombinant GST-Kog1 fusion peptide binds PI3,5P$_2$ with a dissociation constant of 19 ± 6 mM (Table 1). Similar to previous studies, recombinant GST-full-length Atg18 fusion protein binds PI3,5P$_2$ with high affinity, with a dissociation constant of 0.046 ± 10 mM (Table S1). There was no significant binding between GST alone and PI3,5P$_2$ (data not shown). Equilibrium state response levels (RU) for each protein concentration were assessed. Data are representative of three independent experiments. For each experiment, the binding between Atg18 (positive control) or GST (negative control) using the same PI3,5P$_2$ surface used for the GST-Kog1 fusion protein was determined (Table 1).

Figure S2. PI3,5P$_2$ does not provide the sole mechanism for Tor1 localization on the vacuole membrane.
(A and B). 3xGFP(D330)-Tor1 as the sole copy of *TOR1* (A), or Kog1-3xGFP as the sole copy of *KOG1* (B) are each functional as measured by resistance to 2 ng/ml rapamycin. Indicated strains were grown to mid-log phase in YEPD medium, then diluted onto SC plates containing DMSO (control) or 2 ng/mL rapamycin. (C and D). Steady-state levels of 3xGFP(D330) or Kog1-3xGFP are similar in wild type, *vac7Δ* or *FAB1^{VLA}* yeast. Loading control: Pgk1. (E) 3xGFP(D330)-Tor1 or Kog1-3xGFP (green) localize on the vacuole membrane (red) in wild-type, *vac7Δ* or *FAB1^{VLA}* yeast. Vacuole membranes were visualized with FM 4-64. (F and G) Independent of the levels of PI3,5P$_2$, 3xGFP(D330)-Tor1 and Kog1-3xGFP are in the pellet [P] fraction, which includes vacuole membranes. Cell lysates were centrifuged at 13,000g for 10 min at 4°C. Supernatant [S] fractions, and whole cell lysates [W] were suspended in the same volume of sample buffer, and pellet [P] fractions were suspended in twice the volume of sample buffer. Loading control for cell fractionation: Vac7 and Vac8, which tightly localize on the vacuole membrane. Red asterisk: Vac7 protein. (H) Kog1 and Tor1 reside in a complex in both wild-type and *vac7Δ* yeast. HA$_3$-Tor1 or mock plasmid were expressed in a Kog1-3xGFP strain, or Kog1-3xGFP, *vac7*Δ strain. HA$_3$-Tor1 affinity isolated using anti-HA antibody. HA$_3$-Tor1 associated with Kog1-3xGFP with or without PI3,5P$_2$.

Figure S3. Phosphorylation of Sch9 is regulated by TORC1 activity.
(A) Wild-type or *gtr1Δ* strains, with a plasmid expressing Sch9-T570A-HA$_5$ were grown on SC-His medium, then either a final concentration of 200 ng/ml rapamycin (+) or DMSO carrier alone (-) were added to the cell culture. Cells were incubated for an additional 2 h. Lysates from 5x10^6 cells from each strain were incubated at 30°C for 1 h with (+) or without (-) 6000 U l-phosphatase (New England Biolabs). The degree of phosphorylation of Sch9 was assessed by migration on 6% SDS-PAGE. Loading controls: Sch9-T570A-HA$_5$ and Pgk1 run on 15% SDS-PAGE. (B) Images from Figure 3D were analyzed by drawing the lines indicated. Lines initiate from the yellow asterisk and cover the same pixel number in both length and width. (C) Line plot indicating fluorescence intensity of green (GFP-Sch9) and red (FM 4-64) pixels of the lines in panel B.

Figure S4. PI3,5P$_2$ is required for autophagy. (A) Quantification of the number of cells with only GFP puncta. Wild-type, *vac7Δ*, *atg1Δ* or *vac7Δ atg1Δ* strains with plasmids expressing GFP-Atg8 were grown on SC-Ura medium, then a final concentration of 200 ng/ml rapamycin was added. Cells were incubated for an additional 2 h. Three independent trials with more than 100 cells were counted per each trial. Error bars in A represent the SD of triplicate experiments. (B) Microscope images used for quantification in (A) and Figure 6C. Vacuole membranes were visualized with FM 4-64 (red). (C) Examples of microscope images used for quantification in Figure 6E. Vacuole membranes were visualized with FM 4-64 (red). (D) Autophagy was assessed by the degree of release of GFP from GFP-Atg8, monitored by western blot analysis. Loading control: Pgk1.

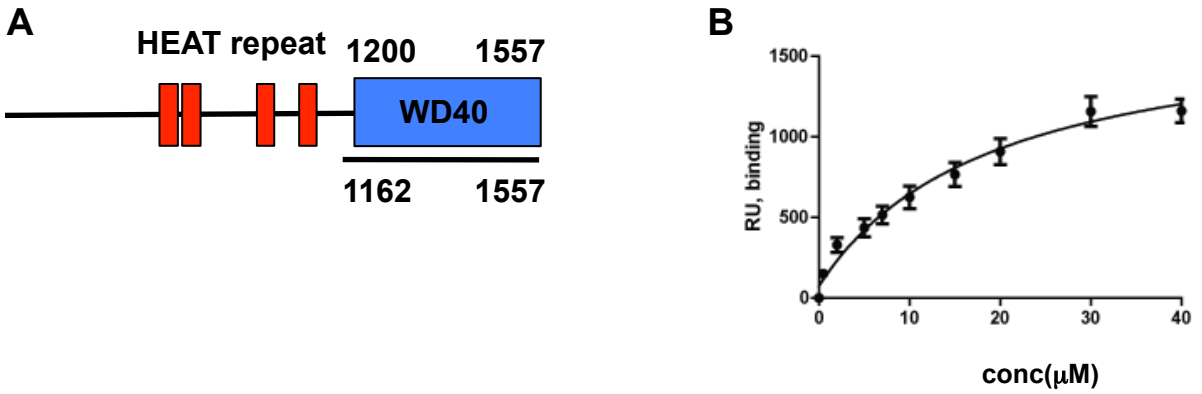
A
HEAT repeat
1200
1557
WD40
1162
1557
B
RU, binding
1500
1000
500
0
0
10
20
30
40
conc(μM)

Figure S1

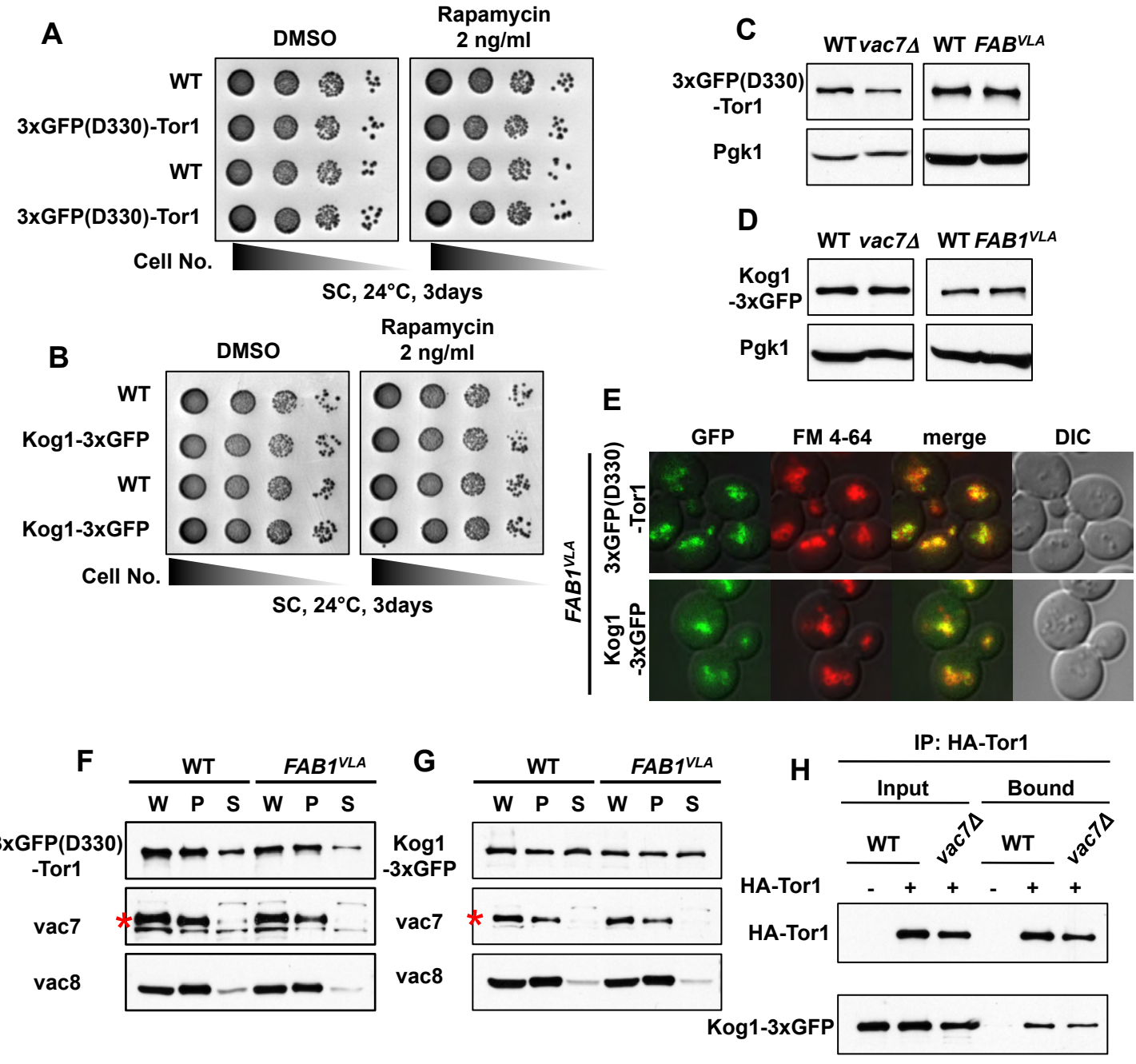
A
DMSO
Rapamycin
2 ng/ml
WT
3xGFP(D330)-Tor1
WT
3xGFP(D330)-Tor1
Cell No.
SC, 24°C, 3days
B
DMSO
Rapamycin
2 ng/ml
WT
Kog1-3xGFP
WT
Kog1-3xGFP
Cell No.
SC, 24°C, 3days
C
WT vac7Δ
WT FAB^VLA
3xGFP(D330)
-Tor1
Pgk1
D
WT vac7Δ
WT FAB1^VLA
Kog1
-3xGFP
Pgk1
E
GFP
FM 4-64
merge
DIC
FAB1^VLA
3xGFP(D330)
-Tor1
Kog1
-3xGFP
F
WT
FAB1^VLA
W P S W P S
3xGFP(D330)
-Tor1
vac7
vac8
G
WT
FAB1^VLA
W P S W P S
Kog1
-3xGFP
vac7
vac8
H
IP: HA-Tor1
Input
Bound
WT
vac7Δ
WT
vac7Δ
HA-Tor1
- + + - + +
HA-Tor1
Kog1-3xGFP

Figure S2

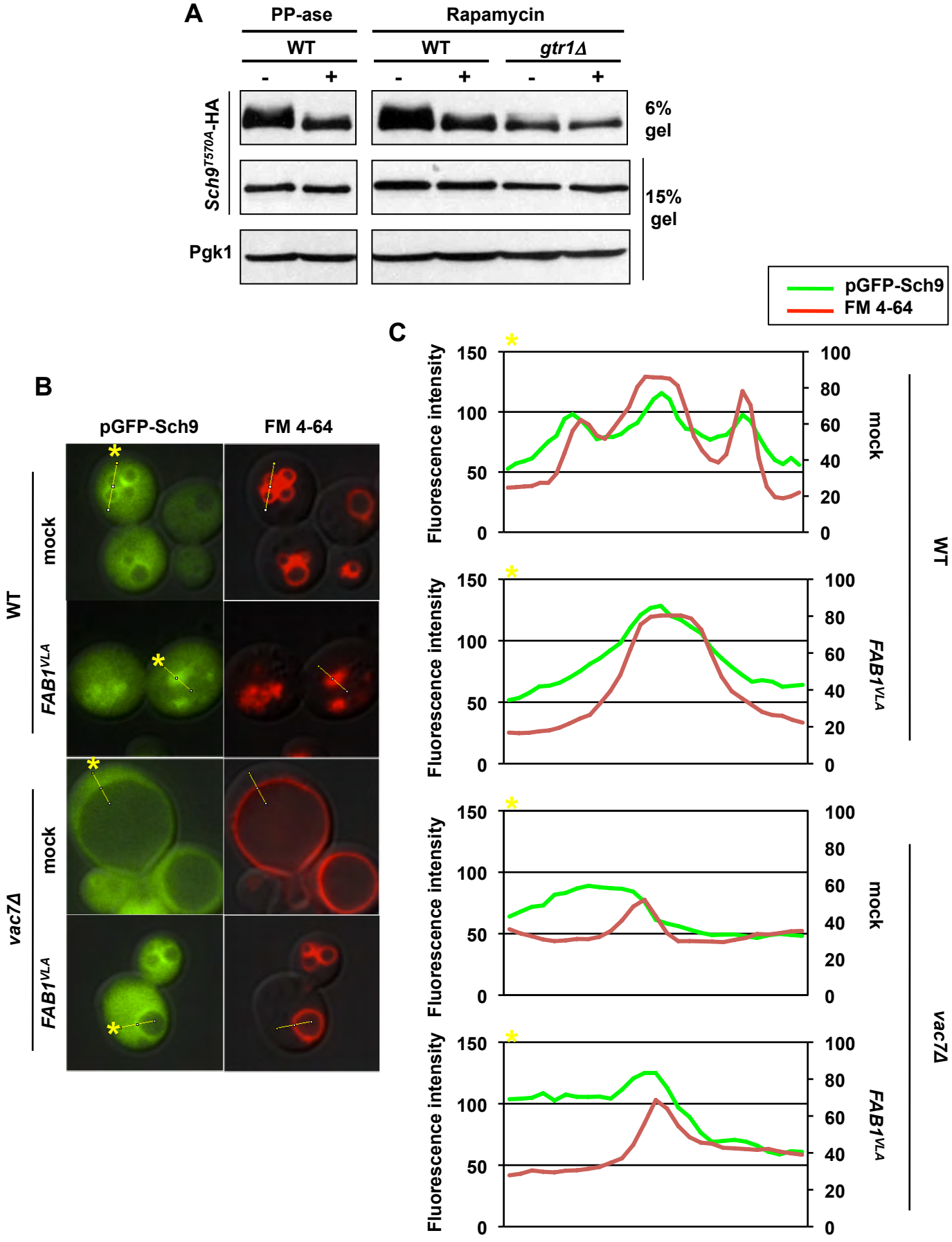
A
PP-ase
Rapamycin
WT
WT
gtr1Δ
-
+
-
+
-
+
Sch9T570A-HA
6% gel
15% gel
Pgk1
B
pGFP-Sch9
FM 4-64
WT
mock
FAB1VLA
vac7Δ
mock
FAB1VLA
C
pGFP-Sch9
FM 4-64
Fluorescence intensity
150
100
50
0
100
80
60
40
20
0
mock
FAB1VLA
WT
vac7Δ

Figure S3

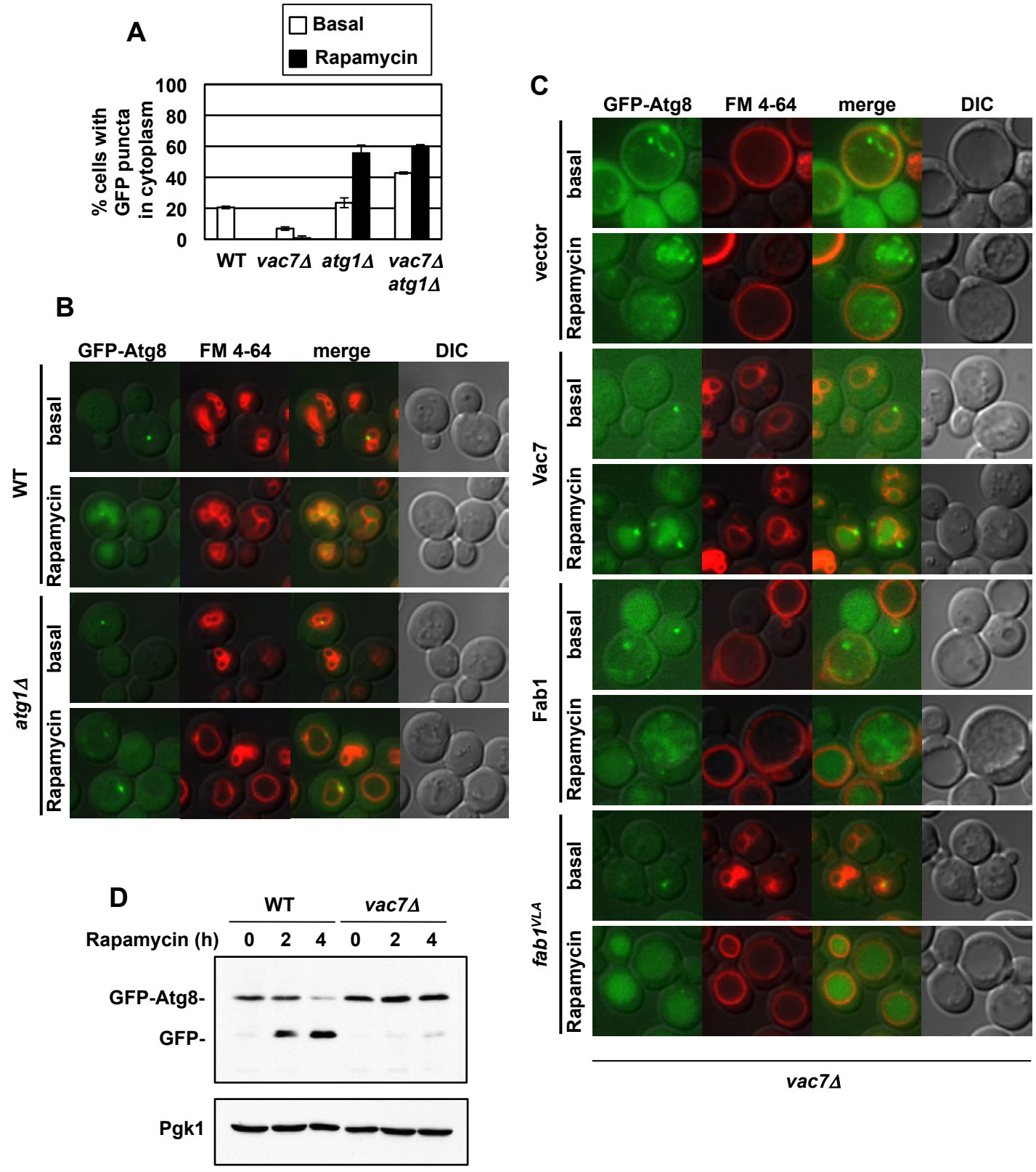
A
Basal
Rapamycin
% cells with GFP puncta in cytoplasm
100
80
60
40
20
0
WT
vac7Δ
atg1Δ
vac7Δ atg1Δ
B
GFP-Atg8
FM 4-64
merge
DIC
WT
atg1Δ
basal
Rapamycin
C
GFP-Atg8
FM 4-64
merge
DIC
vector
Vac7
Fab1
fab1VLA
basal
Rapamycin
vac7Δ
D
WT
vac7Δ
Rapamycin (h)
0
2
4
0
2
4
GFP-Atg8-
GFP-
Pgk1

Figure S4