Files in this Data Supplement:

- **Supplemental Figure S1** -

  Figure S1. Erg6 has the biochemical properties of an integral membrane protein. A) Extracts from cells expressing Erg6-GFP were differentially fractionated by centrifugation at the indicated relative centrifugal force (***g***) and equal amounts of proteins were separated and detected with an anti-GFP antibody. Hom, homogenate, Cyt, cytosol. B) Equal amounts of proteins from the 13k microsomal fraction were incubated with buffer (Mock), 1 M NaCl, 0.1 M Na_2CO_3, or 1% Triton X-100 for 30 min, samples were centrifuged at 13k for 15 min and proteins in the pellet (P) and supernatant (S) were analyzed by Western blotting. The integral ER membrane protein and subunit of the oligosaccharyl transferase complex, Wbp1 serves as control.

- **Supplemental Figure S2** - for 15 min and proteins in the pellet (P) and supernatant (S) were analyzed by Western blotting. The integral ER membrane protein and subunit of the oligosaccharyl transferase complex, Wbp1 serves as control.

  Figure S2. The integral membrane proteins Tgl1 and Yeh1 localize to punctate structures upon induction of neutral lipid synthesis. Induction of neutral lipid synthesis results in relocation of Tgl1 and Yeh1. Cells expressing *GAL-LRO1* and either GFP-Tgl1 or GFP-Yeh1 from an *ADH*-promoter were switched from raffinose to galactose containing medium and the localization of GFP-tagged LD membrane proteins was examined at the indicated time points by confocal microscopy. ER localization of marker proteins in the absence of Lro1induction is indicated by arrows, punctate LD localization by arrowheads. Bar, 5 µm.

- **Supplemental Figure S3** -

  Figure S3. TAG synthesis upon Dga1 induction. A) The strain expressing *GAL-GFP-DGA1* was cultured in raffinose medium and switched to either glucose or galactose containing media. Lipids were labeled by the addition of ^{3}Hpalmitate and samples were removed at the time points indicated, lipids were extracted and analyzed by TLC. The position of diacylglycerol and monoacylglycerol (DAG+MAG), free palmitic acid (FPA) and triacylglycerol (TAG) is indicated. B) Quantification of TAG formation upon Dga1 induction. Formation of radiolabeled TAG was quantified by radioscanning of TLC plates as shown in panel A. Means and standard deviation from two independent determinations are shown. C) Expression of Dga1 increases over time. Samples were removed after induction of Dga1 synthesis and protein levels were examined by Western blotting.

- **Supplemental Figure S4** -

  Figure S4. Membrane topology of Dga1. A) Differential fractionation of epitope-tagged versions of Dga1. Homogenates from cells expressing N- or C-terminally tagged versions of Dga1 were differentially fractionated by centrifugation at the relative centrifugal force (***g***) indicated. Equal amounts of proteins were separated and detected with antibodies against GFP or myc. Hom, homogenate, Cyt, cytosol. B) Dga1 is an integral membrane protein. Equal amounts of proteins from the 13k microsomal fraction were incubated with buffer (Mock), 1 M NaCl, 0.1 M Na_2CO_3, or 1% Triton X-100 for 30 min, samples were centrifuged at 13k for 15 min and proteins in the pellet (P) and supernatant (S) were analyzed by Western blotting. The integral ER membrane protein and subunit of the oligosaccharyl transferase complex, Wbp1 serves as control. C) Protease protection assays. Microsomes were incubated with proteinase K (9 µg/ml) in the presence or absence of detergent (0.1% Triton X-100), proteins were separated by SDS-PAGE and probed with antibodies against GFP or myc. Detection of the ER luminal chaperone Kar2 serves as a control for membrane integrity. D) Endo H treatment of Dga1. Cell homogenates were incubated with (+) or without (-) endo H, proteins were TCA precipitated, separated and probed with antibodies against GFP or the glycosylated Wbp1. E) Proposed model for the topology of Dga1. Cytosolic location of the termini and positions of putative transmembrane domains are indicated. A conserved lipid-binding motif within the first transmembrane domain of Dga1 is indicated by an asterisk and a conserved tetrapeptide sequence containing a presumed active site histidine is marked.

- **Supplemental Figure S5** -

  Figure S5. Fluorescence recovery after photobleaching (FRAP) of soluble, membrane associated or chromatin-bound GFP-tagged proteins. A) Cells

expressing soluble GFP (free GFP), a GFP-tagged ER membrane protein and component of the translocon, Sec63-GFP, a raft-associated plasma membrane proton pump (Pma1-GFP), or the chromatin-associated histone H4, Hhf1-GFP, were analyzed by confocal microscopy and the time-dependent FRAP of the indicated proteins was determined (n=9). The mobile fraction and half-life of the fluorescence recovery are indicated. n.d., not determined. B) FRAP of GFP-Dga1 is not due to recovery of the fluorophores. Cells expressing GFP-Dga1 were photobleached and recovery of fluorescence was monitored over time.

- **Supplemental Figure S6** -

  Figure S6. Low temperature and energy depletion arrest uptake of the endocytosed fluorescent lipophilic dye FM4-64. Wild-type cells were incubated with FM4-64 for 30 min on ice, uptake of the dye was allowed to occur for 30 min at 24°C, and cells were examined by fluorescence microscopy. For energy depletion, cells were incubated with FM4-64 on ice, NaN_3 and NaF (20 mM each) were added, and cells were incubated for 30 min at 24°C. For the low temperature treatment, cells were left on ice after incubation with FM4-64. Bar, 5 µm.

- **Supplemental Figure S7** -

  Figure S7. Relocation of Dga1 from the ER to LDs is not affected by mutations that block vesicular transport. A) Conditional mutants in the biogenesis of ER-derived COPII vesicles (*sec12*^{ts}) or in vesicle fusion (*sec18*^{ts}) do not affect the exchange of LD-localized GFP-Dga1. Cells of the indicated genotype expressing GFP-Dga1 were cultured in galactose medium overnight, and fluorescence recovery of LD-localized GFP-Dga1 was determined at 24°C or at the nonpermissive temperature (37°C), after pre-incubating cells at the respective temperature for 15 min. B) Temperature-dependent block of carboxypeptidase Y (CPY) maturation. CPY maturation was monitored by pulse-chase analysis of wild-type, *sec12*^{ts}, and *sec18*^{ts} mutant cells at the temperature indicated. C) Conditional mutants in the biogenesis of COPI vesicles do not affect the exchange of LD-localized GFP-Dga1. Cells of the indicated genotype were treated as described for panel A. D) Temperature-dependent block of CPY maturation in COPI mutants. Maturation of carboxypeptidase Y (CPY) in wild-type, *sec21-1* and *sec27-1*was monitored by pulse-chase analysis.

- **Supplemental Figure S8** -

  Figure S8. GFP-Erg6 reaches LDs by an energy-independent process. A) Photobleaching reveals exchange of LD-localized GFP-Erg6. Cells were cultured in galactose medium for 4 h to induce expression of GAL-GFP-Erg6. Individual LDs (circled) were photobleached (T=0 sec), and fluorescence recovery was monitored over time. Recovery of fluorescence after 200 sec is shown, and LDs which were photobleached are indicated by arrowheads. The graph shows time-dependent recovery of fluorescence (n=9 LDs). Bar, 5 µm. B) Temperature and drug-sensitivity of fluorescence recovery was examined as described for GFP-Dga1 in Fig. 6D.

- **Supplemental Figure S9** -

  Figure S9. Transcriptional induction of membrane-anchored LD proteins results in uniform labeling of pre-existing LDs. GFP-Erg6, GFP-Tgl1, or GFP-Yeh1 expression was induced by shifting cells to galactose containing medium for 2 h and the colocalization of the newly induced LD marker protein with pre-existing LDs marked by Erg6-RFP expressed from its native promoter was analyzed by confocal microscopy. The degree of overlap of the two LD markers is indicated in % of LDs that show colocalization between GFP and RFP (n≥100 cells). Bar, 5 µm.

- **Supplemental Figure S10** -

  Figure S10. LDs are associated with the ER membrane. Localization of LD proteins Erg6-GFP, Tgl1-GFP, and Yeh1-GFP and the ER luminal marker Kar2-mRFP-HDEL was analyzed by confocal microscopy. The degree of overlap of LD markers with that of Kar2-mRFP-HDEL (arrowheads) is indicated in % of LDs that show colocalization with the ER marker (n≥100 cells). Bar, 2.5 µm.

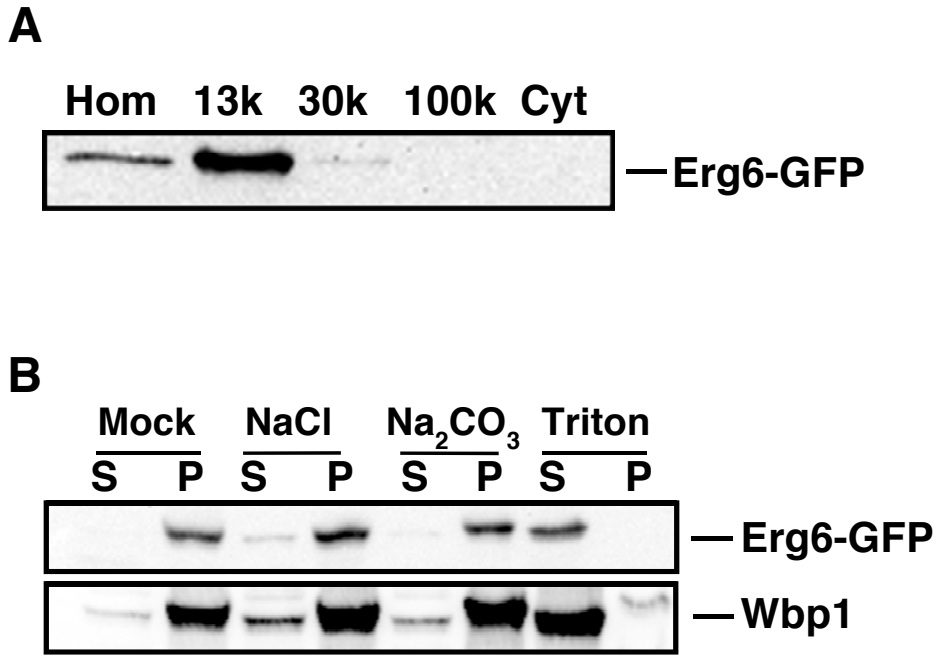
- **Supplemental Figure S11** -

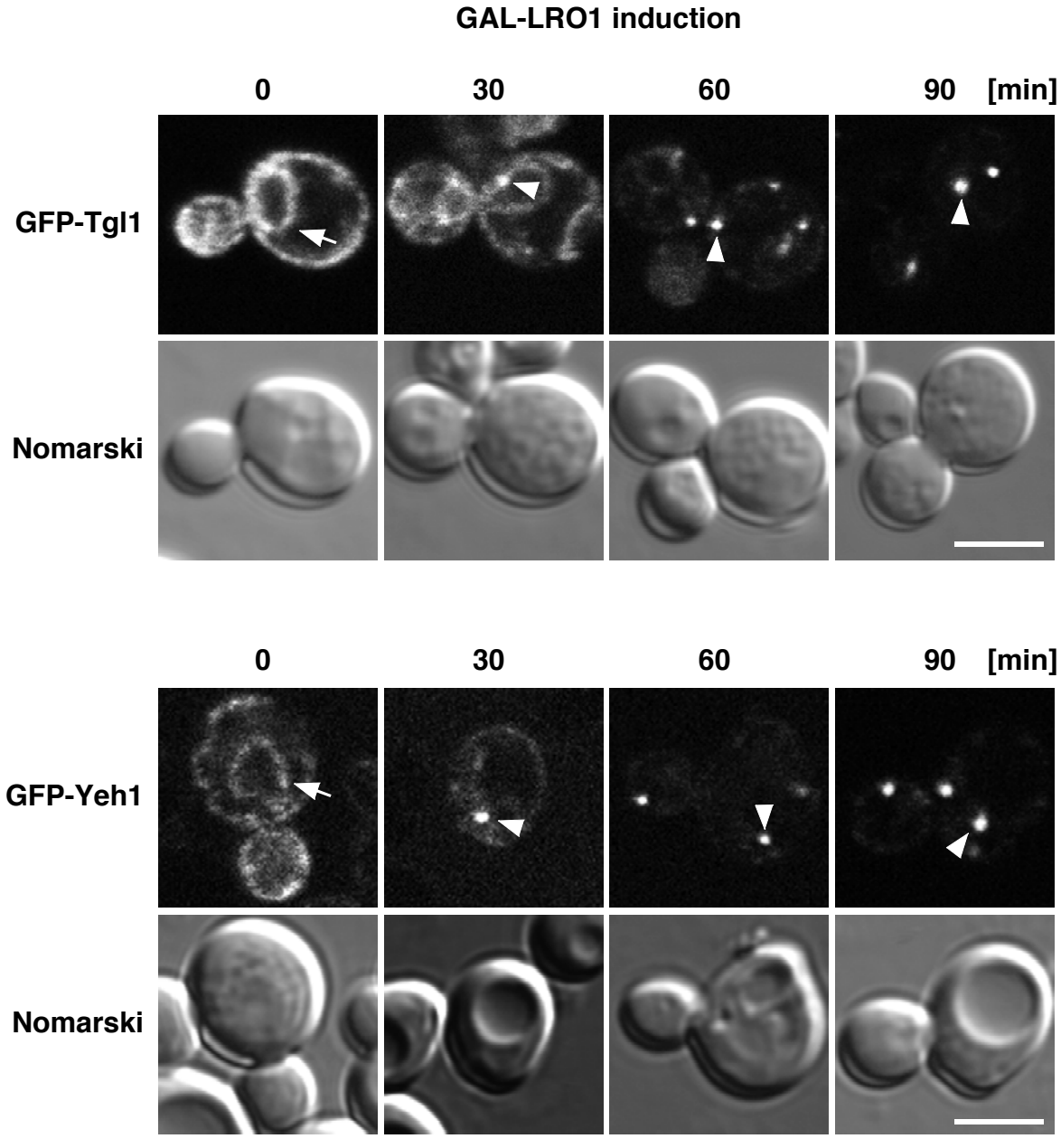
  Figure S11. Seipin is not required for relocation of Dga1 to LDs. Dga1 relocates from the ER to LDs in a seipin deficient mutant. Seipin mutant cells expressing GAL-GFP-Dga1 and Erg6-RFP in a *lro1*Δ *are1*Δ *are2*Δ mutant background were cultured in galactose containing medium for the

periods of time indicated and the localization of GFP-Dga1 and Erg6-RFP was analyzed by confocal microscopy. Arrows indicate localization of GFP-Dga1 in the ER membrane, arrowheads point to punctate LD localization of GFP-Dga1 or Erg-RFP. Bar, 5 µm.

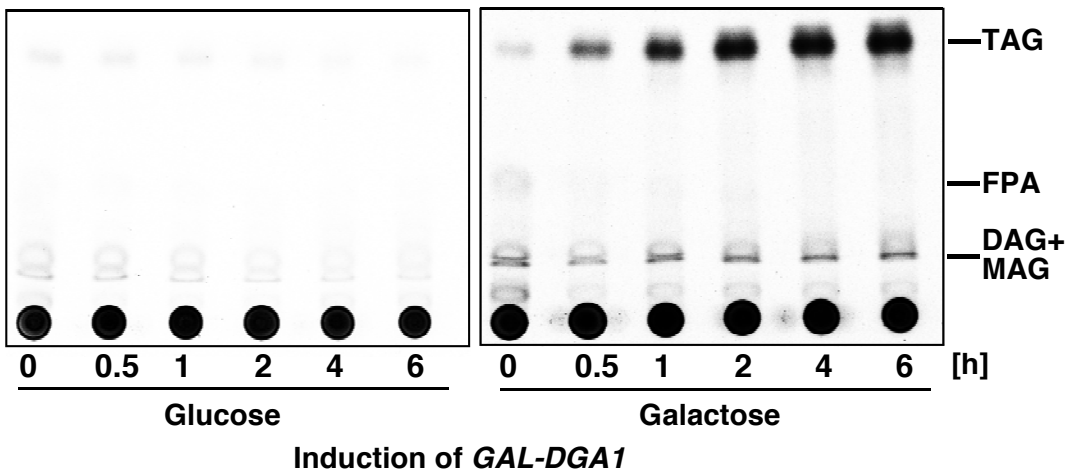
- **Supplemental Table S1** -

  Table S1. *S. cerevisiae* strains used in this study

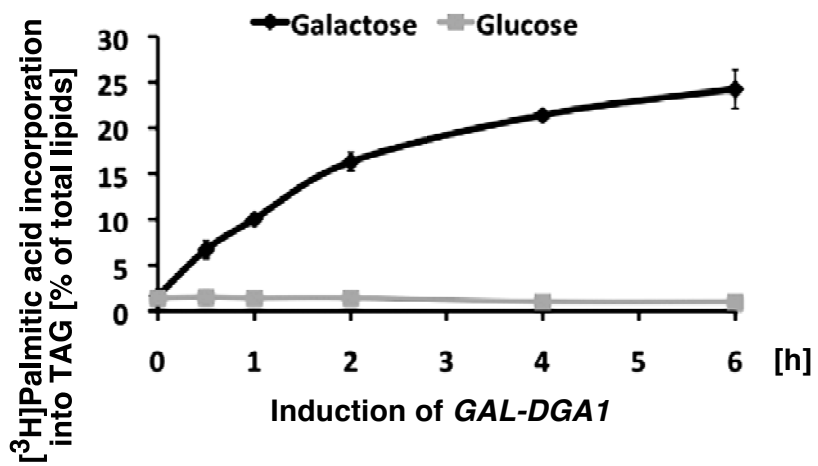

A
Hom
13k
30k
100k
Cyt
Erg6-GFP
B
Mock
NaCl
Na_2CO_3
Triton
S
P
S
P
S
P
S
P
Erg6-GFP
Wbp1


GAL-LRO1 induction
0
30
60
90
[min]
GFP-Tgl1
Nomarski
0
30
60
90
[min]
GFP-Yeh1
Nomarski

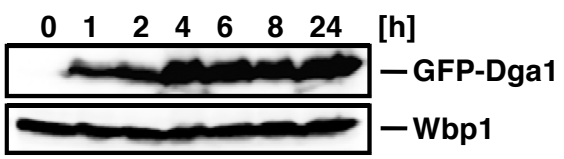
A

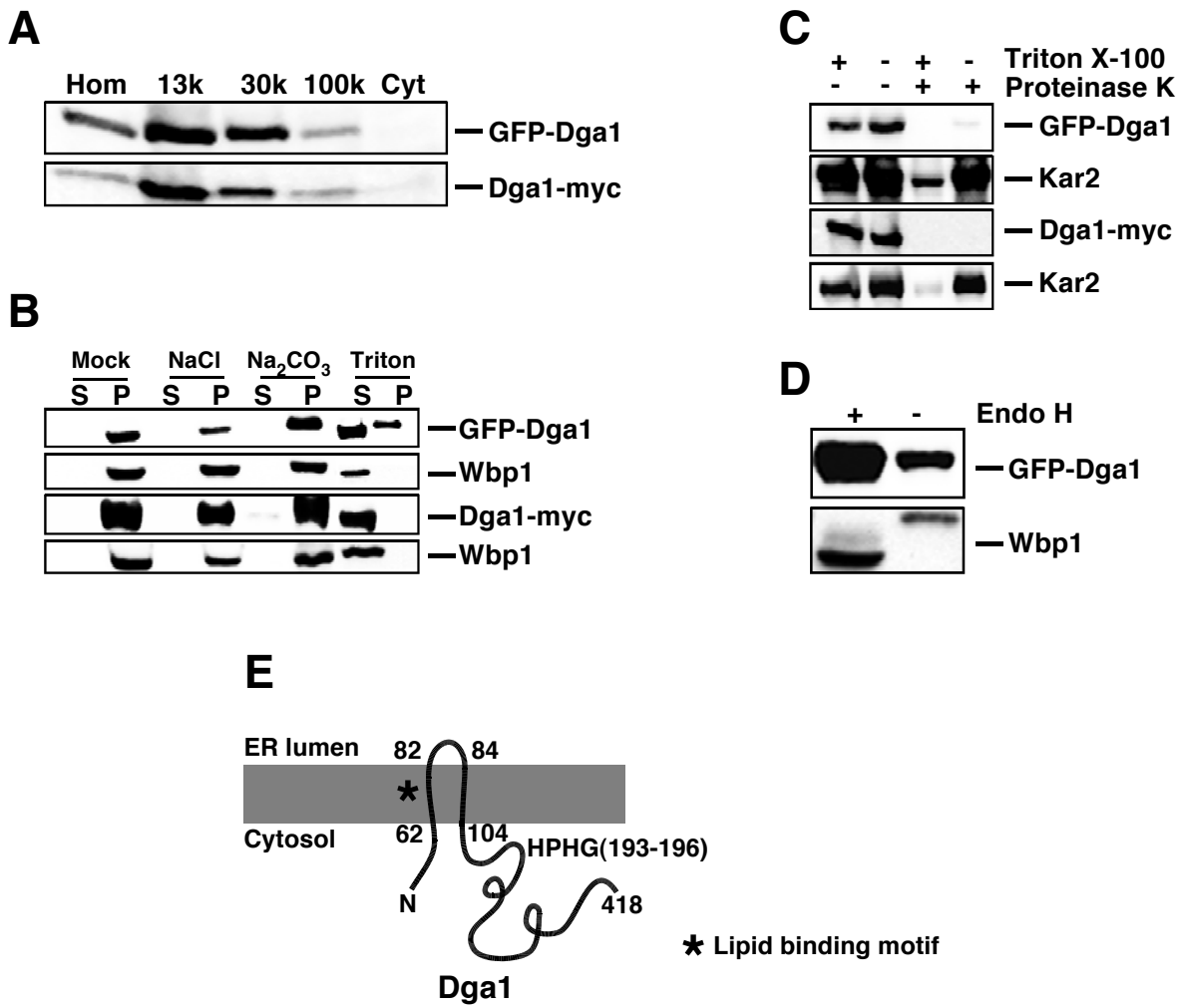

TAG
FPA
DAG+
MAG
0 0.5 1 2 4 6
0 0.5 1 2 4 6 [h]
Glucose
Galactose
Induction of GAL-DGA1

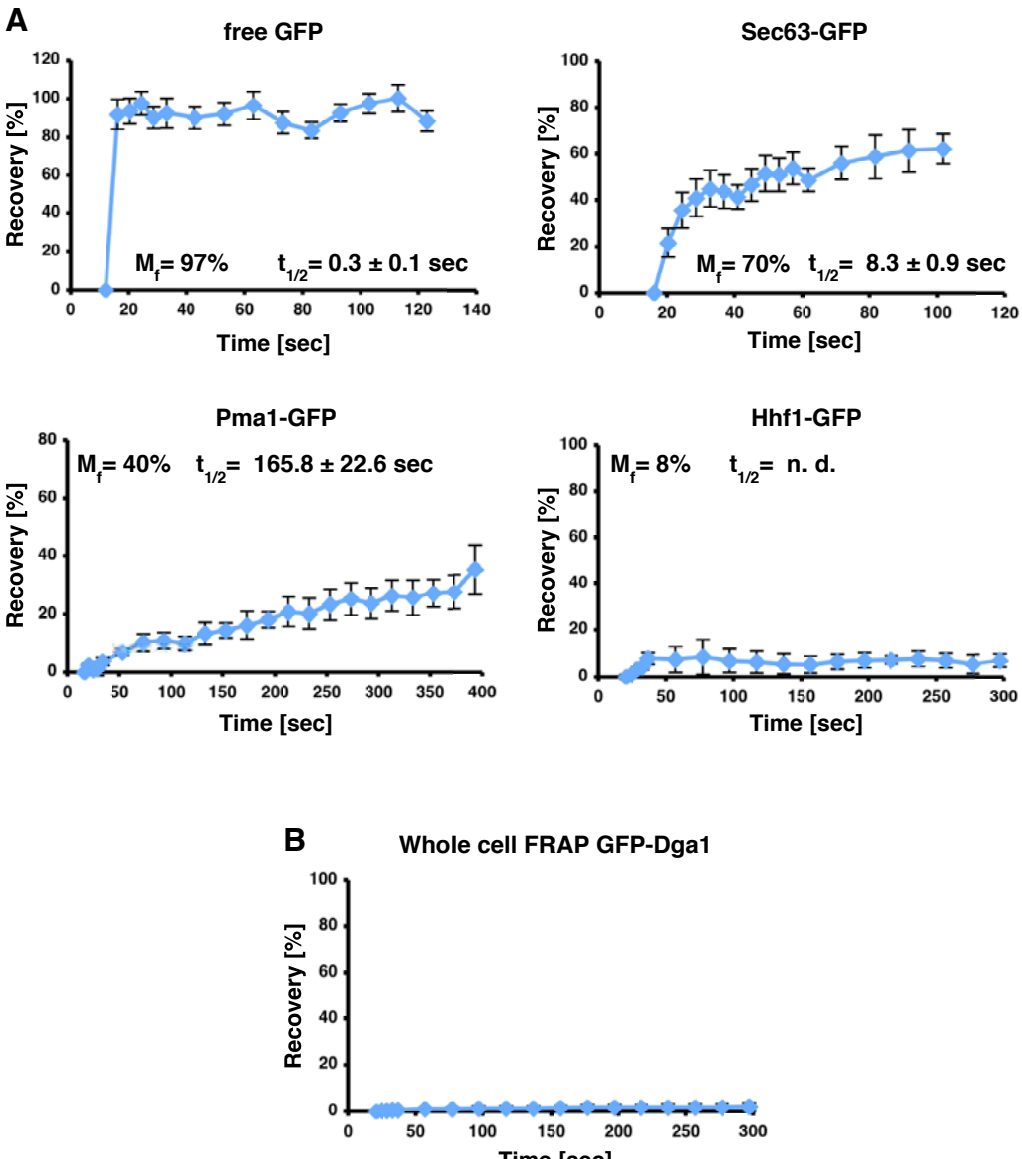
B

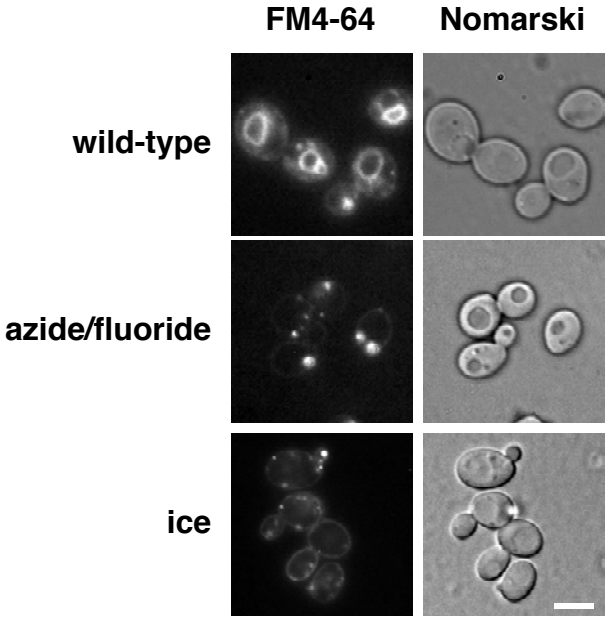

Galactose
Glucose
[3H]Palmitic acid incorporation into TAG [% of total lipids]
30
25
20
15
10
5
0
0 1 2 3 4 5 6 [h]
Induction of GAL-DGA1

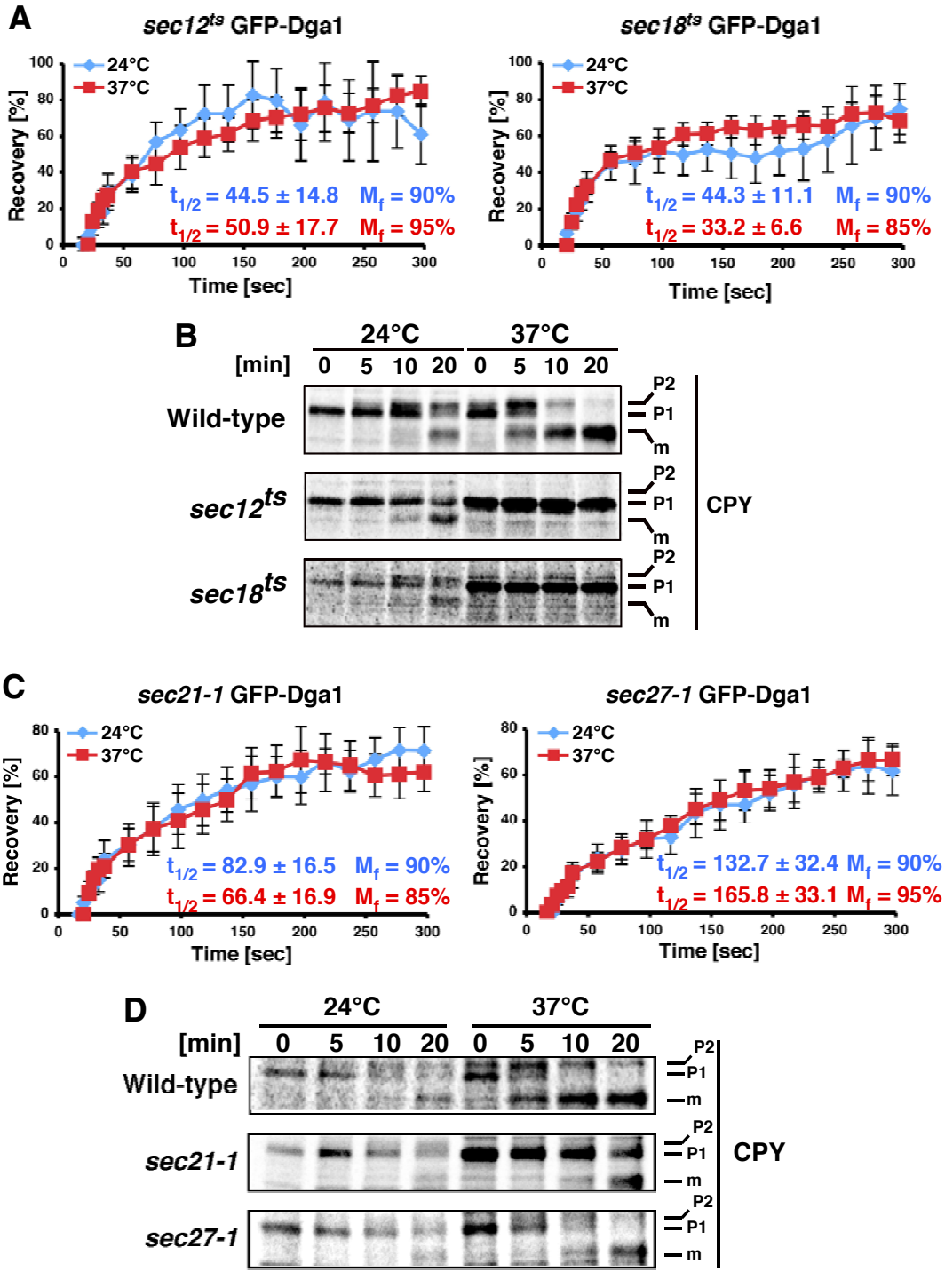
C

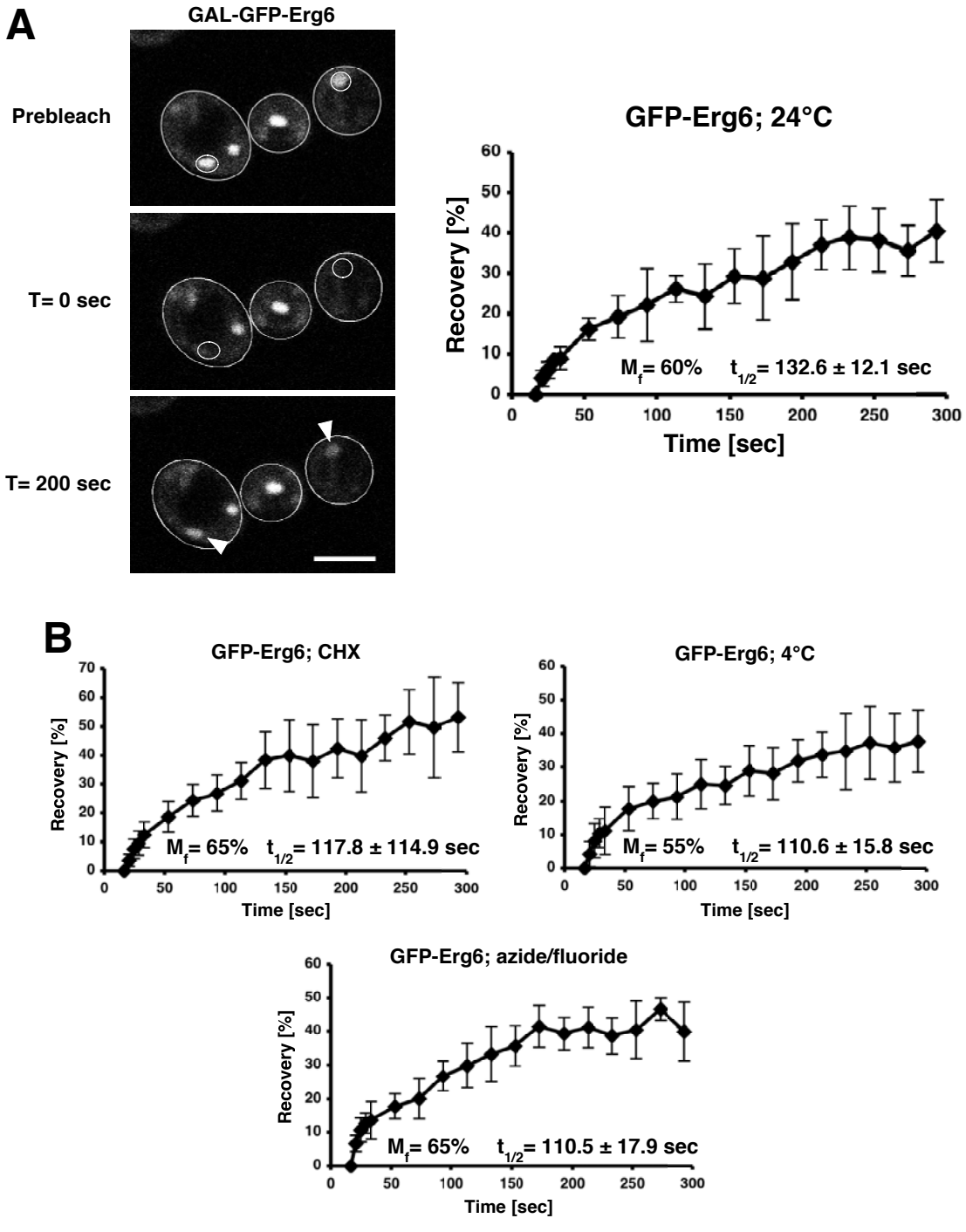

0 1 2 4 6 8 24 [h]
GFP-Dga1
Wbp1

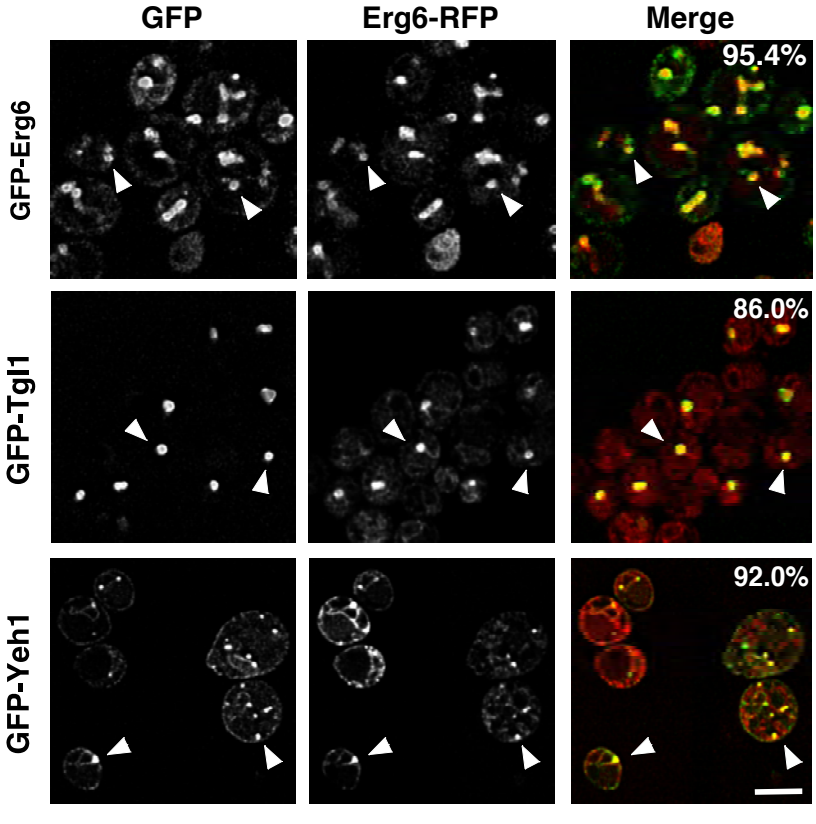

A
Hom
13k
30k
100k
Cyt
GFP-Dga1
Dga1-myc
B
Mock
NaCl
Na2CO3
Triton
S P S P S P S P
GFP-Dga1
Wbp1
Dga1-myc
Wbp1
C
+ - + - Triton X-100
- - + + Proteinase K
GFP-Dga1
Kar2
Dga1-myc
Kar2
D
+ - Endo H
GFP-Dga1
Wbp1
E
ER lumen
82
84
Cytosol
62
104
HPHG(193-196)
N
418
Lipid binding motif
Dga1

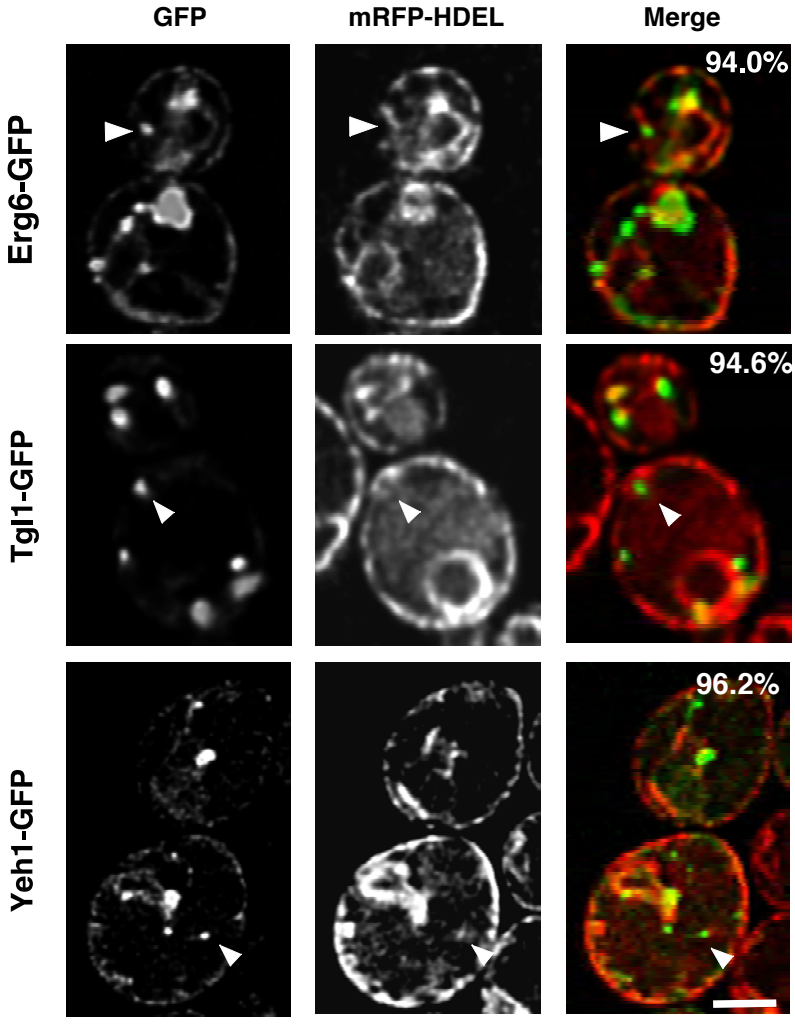

A
free GFP
Recovery [%]
Time [sec]
M_f = 97%
t_1/2 = 0.3 ± 0.1 sec
Sec63-GFP
M_f = 70%
t_1/2 = 8.3 ± 0.9 sec
Pma1-GFP
M_f = 40%
t_1/2 = 165.8 ± 22.6 sec
Hhf1-GFP
M_f = 8%
t_1/2 = n. d.
B
Whole cell FRAP GFP-Dga1


FM4-64
Nomarski
wild-type
azide/fluoride
ice


A
sec12ts GFP-Dga1
sec18ts GFP-Dga1
24°C
37°C
Recovery [%]
Time [sec]
t1/2 = 44.5 ± 14.8 Mf = 90%
t1/2 = 50.9 ± 17.7 Mf = 95%
t1/2 = 44.3 ± 11.1 Mf = 90%
t1/2 = 33.2 ± 6.6 Mf = 85%
B
24°C
37°C
[min]
0 5 10 20 0 5 10 20
Wild-type
sec12ts
sec18ts
P2
P1
m
CPY
C
sec21-1 GFP-Dga1
sec27-1 GFP-Dga1
t1/2 = 82.9 ± 16.5 Mf = 90%
t1/2 = 66.4 ± 16.9 Mf = 85%
t1/2 = 132.7 ± 32.4 Mf = 90%
t1/2 = 165.8 ± 33.1 Mf = 95%
D
sec21-1
sec27-1


A
GAL-GFP-Erg6
Prebleach
T= 0 sec
T= 200 sec
GFP-Erg6; 24°C
Recovery [%]
Time [sec]
M_f= 60%
t_1/2= 132.6 ± 12.1 sec
B
GFP-Erg6; CHX
M_f= 65%
t_1/2= 117.8 ± 114.9 sec
GFP-Erg6; 4°C
M_f= 55%
t_1/2= 110.6 ± 15.8 sec
GFP-Erg6; azide/fluoride
M_f= 65%
t_1/2= 110.5 ± 17.9 sec


GFP
Erg6-RFP
Merge
GFP-Erg6
GFP-Tgl1
GFP-Yeh1
95.4%
86.0%
92.0%


GFP
mRFP-HDEL
Merge
Erg6-GFP
Tgl1-GFP
Yeh1-GFP
94.0%
94.6%
96.2%

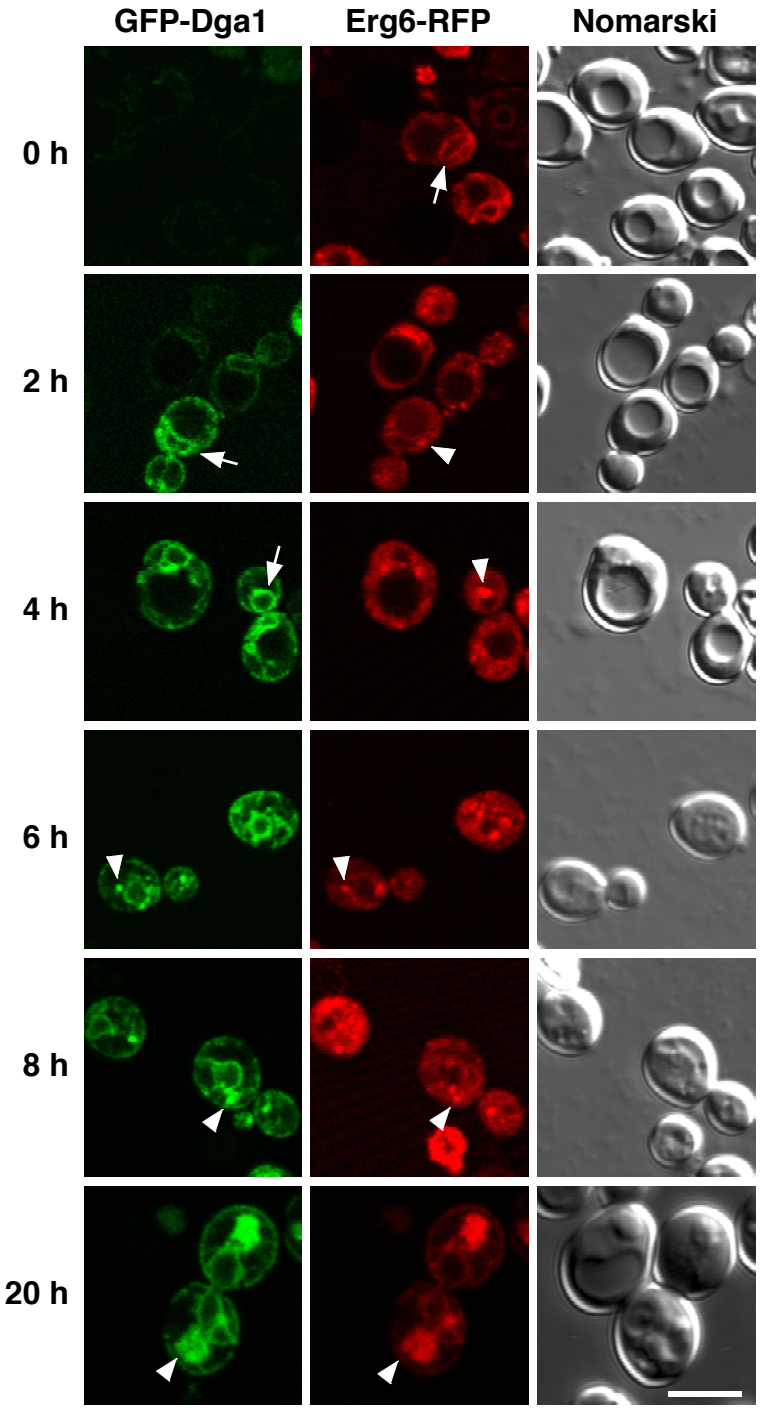

GFP-Dga1
Erg6-RFP
Nomarski
0 h
2 h
4 h
6 h
8 h
20 h

Table S1. *S. cerevisiae* strains used in this study

Strain	Relevant Genotype	Source
RSY1533	*MATα leu2Δ0 ura3Δ0 his3Δ1 lys2Δ0 MET15*	Euroscarf
RSY2307	*MATα leu2Δ0 ura3Δ0 his3Δ1 lys2Δ0 pGAL-PMA1-GFP-URA3*	This Study
RSY3000	*MATα dga1::URA3 lro1::TRP1 are1::HIS3 are2::LEU2 ADE2 HDEL-RFP-URA3 ERG6-GFP::KanMX*	This Study
RSY3021	*MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 are1::KanMX are2::KanMX trp1::URA3 GAL-LRO1::TRP1 dga1::loxP*	This Study
RSY3097	*MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 are1::KanMX are2::KanMX ERG6-GFP::HIS3*	This Study
RSY3272	*MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 ERG6-GFP::HIS3*	This Study
RSY3292	*MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 are1::KanMX are2::KanMX trp1::URA3 lro1::loxP GAL-GFP-DGA1::HIS3*	This Study
RSY3337	*MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 are1::KanMX are2::KanMX trp1Δ lro1::loxP GAL-GFP-DGA1::HIS3 pURA3-GAL-ERG6-RFP*	This Study
RSY3402	*MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 pURA3-GAL-GFP-ERG6*	This Study
RSY3482	*MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 are1::KanMX are2::KanMX lro1::loxP DGA1-myc::HIS3*	This Study
RSY3604	*MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 are1::KanMX are2::KanMX trp1::URA3 lro1Δ::TRP1 dga1::loxP pLEU2-GAL-GFP-TGL1 pURA3-HDEL-RFP*	This Study
RSY3605	*MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 are1::KanMX are2::KanMX trp1::URA3 lro1Δ::TRP1 dga1::loxP pLEU2-GAL-GFP-YEH1 pURA3-HDEL-RFP*	This Study
RSY3755	*MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 are1::KanMX are2::KanMX lro1::loxP HIS3-GAL-DGA1*	This Study
RSY3758	*MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 dga1::KanMX lro1::KanMX ERG6-GFP::HIS3*	This Study
RSY3770	*MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 trp1Δ are1::KanMX are2::KanMX GAL-LRO1::TRP1 dga1::loxP pERG6-RFP-URA3*	This Study
RSY4359	*MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 pGAL-GFP-DGA1-URA3*	This Study
RSY4399	*MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 pGAL-GFP-URA3*	This Study
RSY4452	*MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 pSEC63-GFP-URA3*	This Study
RSY4461	*MATa sec12^{ts} ade2 his3 ura3 can1 pGAL-GFP-DGA1-URA3*	This Study
RSY4465	*MATα sec18^{ts} his3Δ1 leu2Δ0 LYS2 ura3Δ0 pGAL-GFP-DGA1-URA3*	This Study
RSY4509	*MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 pGAL-GFP-DGA1-*	This Study

	URA3 pERG6-RFP-LEU2	
RSY4534	*MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 trp1Δ are1::KanMX are2::KanMX GAL-LRO1::TRP1 dga1::loxP pADH-GFP-TGL1-HIS3*	This Study
RSY4535	*MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 trp1Δ are1::KanMX are2::KanMX GAL-LRO1::TRP1 dga1::loxP pADH-GFP-YEH1-HIS3*	This Study
RSY4612	*MATα leu2Δ0 ura3Δ0 his3Δ1 lys2Δ0 fld1::KanMX pGAL-GFP-DGA1-URA3*	This Study
RSY4695	*MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 are1::KanMX are2::KanMX trp1Δ lro1::loxP fld1::loxP GAL-GFP-DGA1::HIS3 pURA3-GAL-ERG6-RFP*	This Study
RSY4757	*MAT? sec21-1 his3Δ1 leu2Δ0 ura3Δ0 pGAL-GFP-DGA1-URA3*	This Study
RSY4758	*MAT? sec27-1 his3Δ1 leu2Δ0 ura3Δ0 pGAL-GFP-DGA1-URA3*	This Study
RSY4842	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 HHF1-GFP-HIS3*	This Study