**Membrane delivery to the yeast autophagosome from the Golgi-endosomal system.**

Yohei Ohashi and Sean Munro

**Supplemental Material**

**Supplemental Figure S1.** Dependence of autophagy on the ER exit of Atg9 and on the late endosomal SNARE Pep12.

(A) The effect of the ER retention signal KKXX motif on a form of Atg9 with its C-terminal cytosolic tail shortened to 38 residues by truncation at residue 786, a form that retains at least partial activity. Ape1 maturation in cells starved of nitrogen for four hours analyzed by immunoblotting in wild-type cells (WT) or Δatg9 containing a CEN plasmid with no insert (empty) or expressing the indicated forms of Atg9(786). Panels below show fluorescent micrographs of cells expressing GFP-Atg9(786) and GFP-Atg9(786)-KKXX during starvation.

(B) Localization of GFP-Ape1 by fluorescence microscopy in the indicated strains. Cells were grown in (-URA) or starved for nitrogen for four hours (SD(-N)) and labeled with FM4-64. In cells lacking the key vacuolar hydrolase Pep4 undigested autophagosomes accumulate in the vacuole (arrows). With Δpep12 similar structures can be seen in some cells, indicating that vacuole digestion is compromised, however in rich medium the autophagosomes labeled with Ape1 accumulate outside of the vacuole indicating that the Cvt pathway may also be inhibited (asterisks).

**Supplemental Figure S2.** Complementation of Δatg24-containing double mutants by plasmid-borne genes.

(A) An *ATG24*-FLAG plasmid rescues the Ape1 maturation defects of Δcog8Δatg24, Δgos1Δatg24, and Δtlg2Δatg24 to the extent of defects in Δcog8, Δgos1, and Δtlg2, respectively in both vegetative and starvation conditions.

(B) Complementation of the indicated double mutants by plasmids carrying *COG8*, FLAG-*GOS1*, or FLAG-*TLG2*. All of the transgenes were driven by the promoters of corresponding genes in pRS416. Similar complementation was observed of Δcog8Δgos1, Δcog8Δtlg2, Δcog8Δatg20, Δgos1Δatg20, and Δtlg2Δatg20 (data not shown).

**Supplemental Figure S3.** Autophagy defects in the Golgi-endosomal mutants are not due to perturbation of the CPY pathway, vacuolar fusion, or the stability of Atg9.

(A) Pulse-chase immunoprecipitation analysis of CPY maturation in the indicated strains. Cells were pulse-radiolabeled with [$^{35}$S]methionine for 10 min and subjected to a non-radioactive chase for 0, 10 and 20 min for wild-type, and for 20 min for the mutant strains, respectively. CPY was immunoprecipitated and resolved in SDS-PAGE. Defective maturation after 20 minute was only observed with the mutant in the vacuolar SNARE Vam3.

(B) Fluorescent micrographs or DIC images of the indicated strains labeled with the endocytic tracer FM4-64 (30 minutes pulse, 60 minutes chase). For reasons that are unclear the vacuole fragmentation phenotype of Δcog8 is rescued in Δcog8Δatg24.

(C) Growth of dilution series of the indicated strains on YEPD at 30$^{o}$C.

(D) Immunoblotting analysis of Atg9-flag. A plasmid carrying Atg9-flag under the *ATG9* promoter in pRS416 was transformed into the same mutants as (A). Atg9-flag was detected by anti-flag antibody. As a loading control, a ribosomal protein Tcm1 was detected on the same membrane.

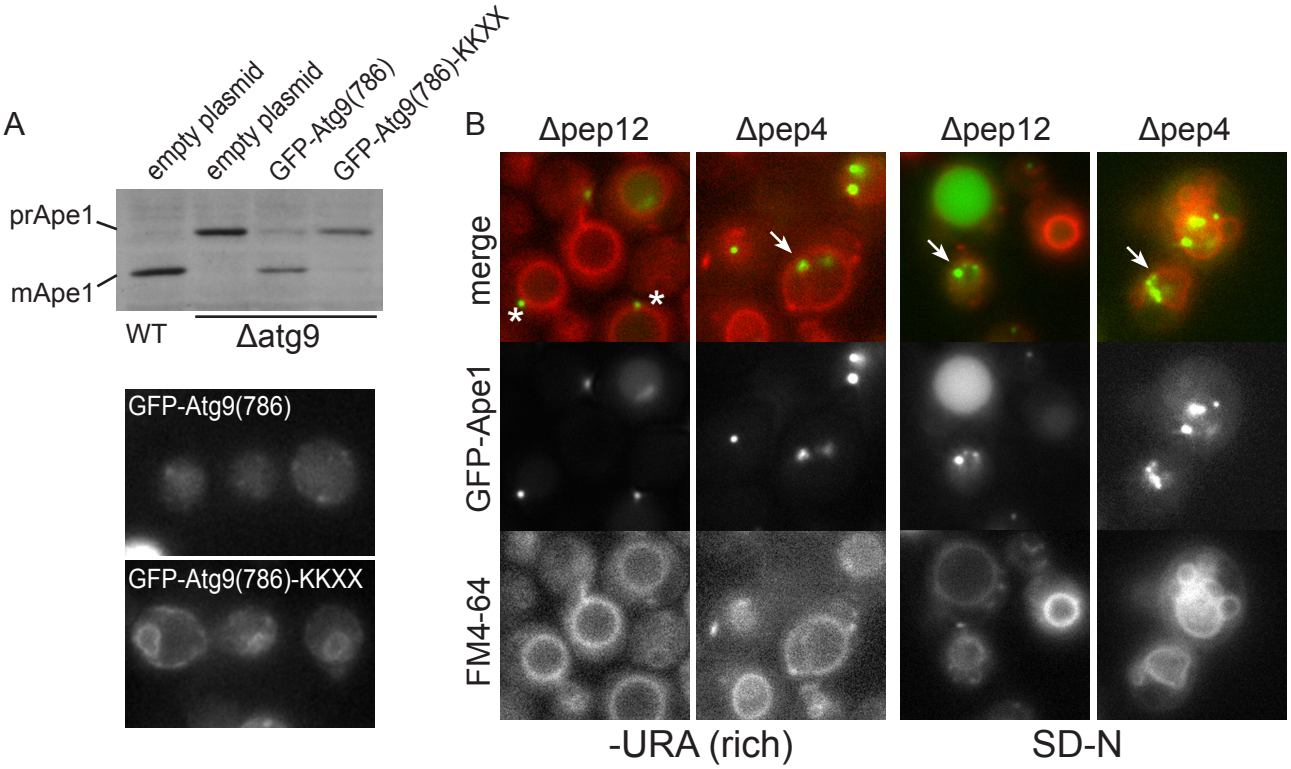
**Supplemental Table S1.** Yeast strains used in this study.

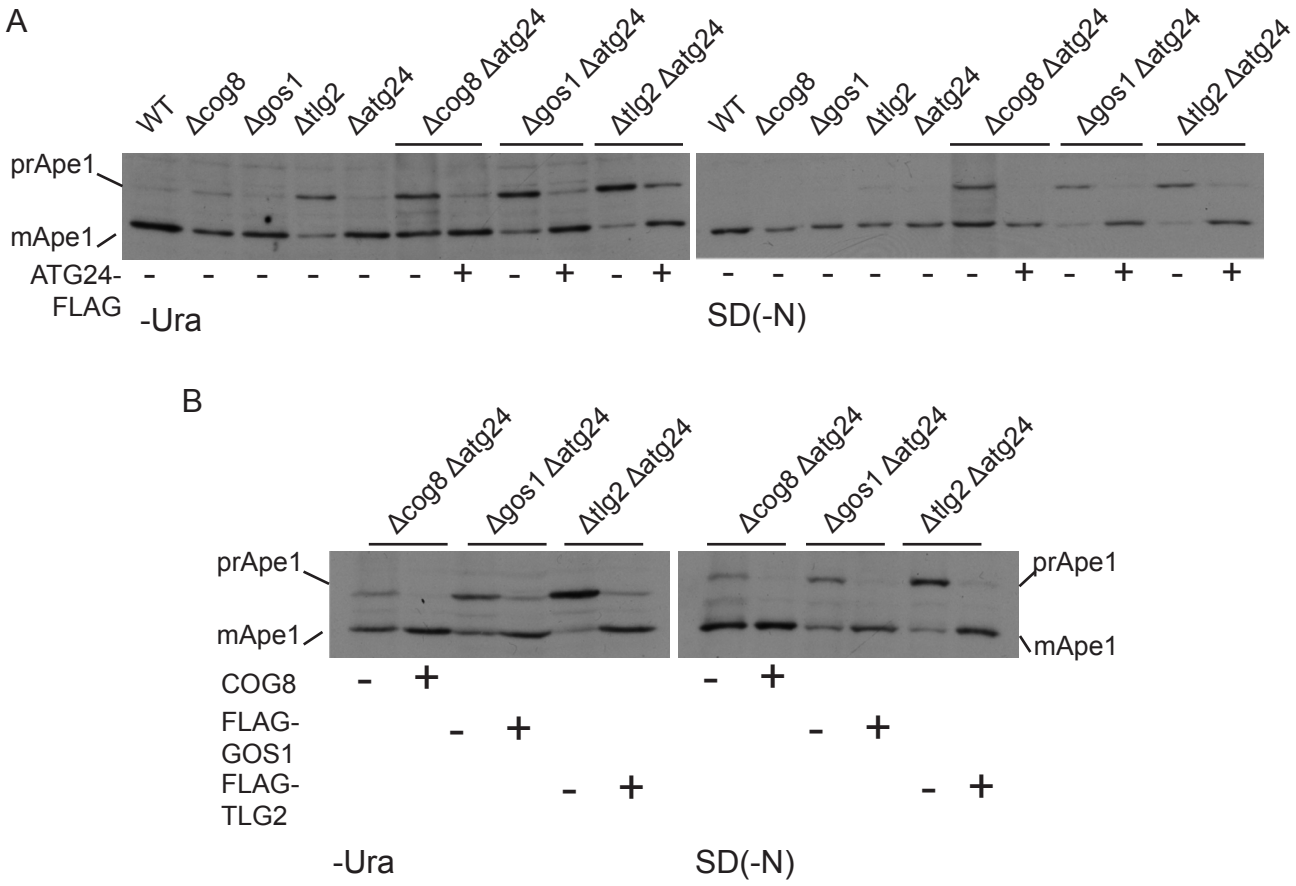
**Supplemental Table S2.** Plasmids used in this study.

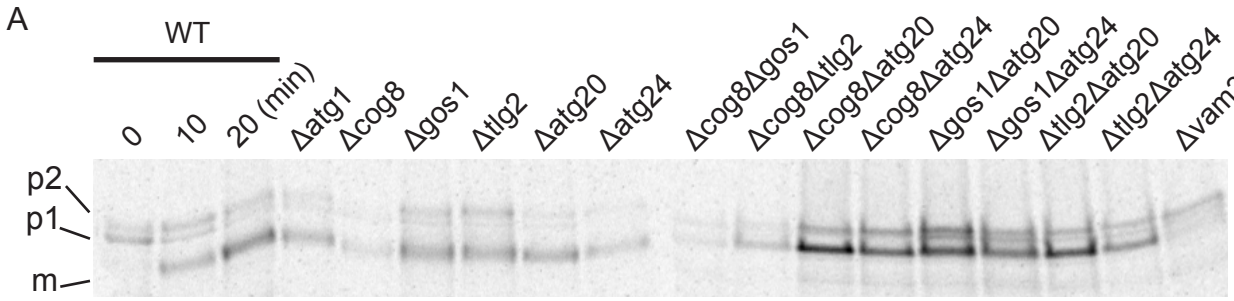
**Supplemental Video 1.** Comparison of Atg9-GFP and mitochondria in living cells.

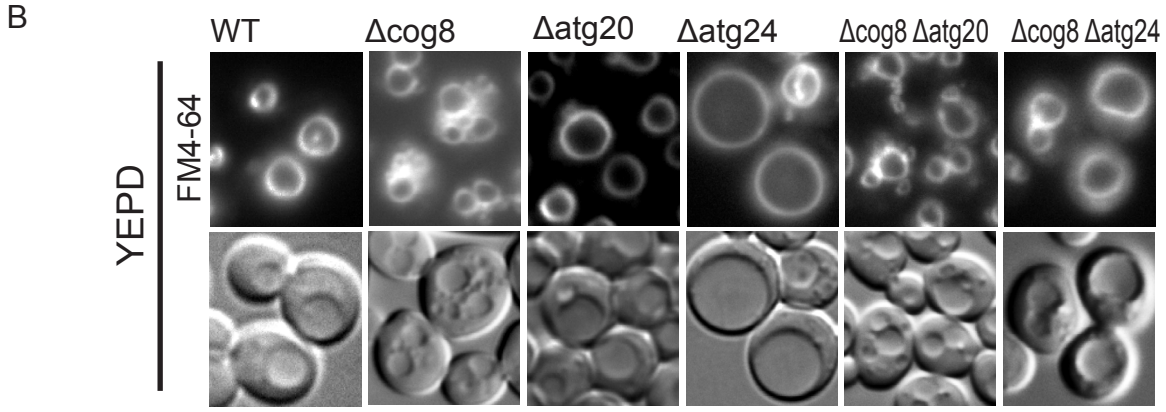
Atg9-GFP and Tom20-RFP were imaged simultaneously using a beam-splitter (Cairn Research) and a CCD camera (Roper CoolSNAP HQ2) attached to a wide-field microscope (Nikon TE2000 with a 60x, 1.4 NA objective). Cells grown to log phase were starved for nitrogen for three hours prior to imaging. 800 ms exposures were obtained continuously (1.25 frames per second) and play back is at 10 frames per second.

Figure S1

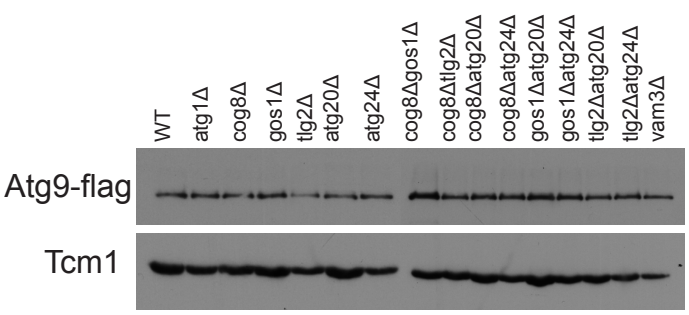

A
empty plasmid
empty plasmid
GFP-Atg9(786)
GFP-Atg9(786)-KKXX
prApe1
mApe1
WT
Δatg9
GFP-Atg9(786)
GFP-Atg9(786)-KKXX
B
Δpep12
Δpep4
Δpep12
Δpep4
merge
GFP-Ape1
FM4-64
-URA (rich)
SD-N


A
WT
Δcog8
Δgos1
Δtlg2
Δatg24
Δcog8 Δatg24
Δgos1 Δatg24
Δtlg2 Δatg24
prApe1
mApe1
ATG24-FLAG
-Ura
SD(-N)
B
COG8
FLAG-GOS1
FLAG-TLG2


A
WT
0
10
20 (min)
Δatg1
Δcog8
Δgos1
Δtlg2
Δatg20
Δatg24
Δcog8Δgos1
Δcog8Δtlg2
Δcog8Δatg20
Δcog8Δatg24
Δgos1Δatg20
Δgos1Δatg24
Δtlg2Δatg20
Δtlg2Δatg24
Δvam3
p2
p1
m


B
WT
Δcog8
Δatg20
Δatg24
Δcog8 Δatg20
Δcog8 Δatg24
YEPD
FM4-64


C
WT
Δgos1
Δtlg2
Δatg24
Δgos1Δatg24
Δtlg2Δatg24


D
WT
atg1Δ
cog8Δ
gos1Δ
tlg2Δ
atg20Δ
atg24Δ
cog8Δgos1Δ
cog8Δtlg2Δ
cog8Δatg20Δ
cog8Δatg24Δ
gos1Δatg20Δ
gos1Δatg24Δ
tlg2Δatg20Δ
tlg2Δatg24Δ
vam3Δ
Atg9-flag
Tcm1

**Supplemental Table S1.** Yeast strains used in this study.

| strain name | genotype |
|---|---|
| BY4741 | MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 |
| YOY010 | BY4741 Δcog8::HIS Δgos1::KanMX |
| YOY011 | BY4741 Δcog8::HIS Δtlg2::KanMX |
| YOY012 | BY4741 Δcog8::HIS Δatg20::KanMX |
| YOY013 | BY4741 Δcog8::HIS Δatg24::KanMX |
| YOY014 | BY4741 Δgos1::HIS Δatg20::KanMX |
| YOY015 | BY4741 Δgos1::KanMX Δatg24::HIS |
| YOY016 | BY4741 Δtlg2::HIS Δatg20::KanMX |
| YOY017 | BY4741 Δtlg2::HIS Δatg24::KanMX |
| YOY020 | BY4741 SEC7-RFP::KanMX ATG9-EGFP::NatMX |
| YOY022 | BY4741 TOM20-RFP::LEU2 ATG9-EGFP::NatMX |
| YOY023 | BY4741 mRFP-APE1::LEU ATG9-EGFP::NatMX |
| YOY024 | BY4741 Δatg1::KanMX mRFP-APE1::LEU ATG9-EGFP::NatMX |
| YOY025 | BY4741 Δcog8::KanMX mRFP-APE1::LEU ATG9-EGFP::NatMX |
| YOY026 | BY4741 Δgos1::KanMX mRFP-APE1::LEU ATG9-EGFP::NatMX |
| YOY027 | BY4741 Δtlg2::KanMX mRFP-APE1::LEU ATG9-EGFP::NatMX |
| YOY028 | BY4741 Δatg20::KanMX mRFP-APE1::LEU ATG9-EGFP::NatMX |
| YOY029 | BY4741 Δatg24::KanMX mRFP-APE1::LEU ATG9-EGFP::NatMX |
| YOY030 | BY4741 Δvam3::KanMX mRFP-APE1::LEU ATG9-EGFP::NatMX |
| YOY031 | BY4741 Δcog8::HIS Δgos1::KanMX mRFP-APE1::LEU ATG9-EGFP::NatMX |
| YOY032 | BY4741 Δcog8::HIS Δtlg2::KanMX mRFP-APE1::LEU ATG9-EGFP::NatMX |
| YOY033 | BY4741 Δcog8::HIS Δatg20::KanMX mRFP-APE1::LEU ATG9-EGFP::NatMX |
| YOY034 | BY4741 Δcog8::HIS Δatg24::KanMX mRFP-APE1::LEU ATG9-EGFP::NatMX |
| YOY035 | BY4741 Δgos1::HIS Δatg20::KanMX mRFP-APE1::LEU ATG9-EGFP::NatMX |
| YOY036 | BY4741 Δgos1::KanMX Δatg24::HIS mRFP-APE1::LEU ATG9-EGFP::NatMX |
| YOY037 | BY4741 Δtlg2::HIS Δatg20::KanMX mRFP-APE1::LEU ATG9-EGFP::NatMX |
| YOY038 | BY4741 Δtlg2::HIS Δatg24::KanMX mRFP-APE1::LEU ATG9-EGFP::NatMX |
| YOY039 | BY4741 Δatg20::HIS Δvps51::kanMX |
| YOY040 | BY4741 Δatg24::HIS Δvps51::kanMX |
| YOY041 | BY4741 Δatg20::HIS Δypt6::kanMX |
| YOY042 | BY4741 Δatg20::HIS Δypt6::kanMX |
| YOY043 | BY4741 Δatg20::HIS Δatg21::kanMX |
| YOY044 | BY4741 Δatg24::HIS Δatg21::kanMX |
| YOY045 | BY4741 Chry-TLG1::LEU2 ATG9-EGFP::NatMX |
| YOY046 | BY4741 Chry-TLG1::LEU2 ATG9-EGFP::NatMX Δvps4::KanMX |
| YOY047 | BY4741 TOM20-RFP::LEU2 ATG9-EGFP::NatMX Δugo1::KanMX |
| YOY048 | BY4741 RFP-SCS2::LEU |
| YOY049 | BY4741 mRFP-APE1::LEU |
| YOY050 | BY4741 Δatg9::KanMX::TPI-EGFP-ATG9-MYC-NatMX |
| YOY051 | BY4741 Δatg9::KanMX::TPI-EGFP-ATG9-KKXX-NatMX |
| YOY052 | BY4741 Δatg9::KanMX::TPI-EGFP-ATG9-RxR-NatMX |
| YOY053 | BY4741 Δatg9::KanMX::TPI-EGFP-ATG9-MYC-NatMX Δpho13::MET15 Δpho8::pho8 60-LEU |
| YOY054 | BY4741 Δatg9::KanMX::TPI-EGFP-ATG9-KKXX-NatMX Δpho13::MET15 Δpho8::pho8 60-LEU |
| YOY055 | BY4741 Δatg9::KanMX::TPI-EGFP-ATG9-RxR-NatMX Δpho13::MET15 Δpho8::pho8 60-LEU |
| YOY056 | BY4741 Δpho13::MET15 Δpho8::pho8 60-LEU |

| | |
|---|---|
| YOY057 | BY4741 Δatg1::KanMXΔpho13::MET15 Δpho8::pho8 60-LEU |
| YOY058 | BY4741 Δgos1::KanMXΔpho13::MET15 Δpho8::pho8 60-LEU |
| YOY059 | BY4741 Δatg24::KanMXΔpho13::MET15 Δpho8::pho8 60-LEU |
| YOY060 | BY4741 Δgos1::KanMX Δatg24::HIS Δpho13::MET15 Δpho8::pho8 60-LEU |
| YOY061 | BY4741 Δatg11::KanMX ATG9-EGFP::NatMX Chry-TLG1::LEU2 |
| YOY062 | BY4741 Δatg11::KanMX ATG9-EGFP::NatMX |
| YOY063 | BY4741 Δatg11::KanMX mRFP-APE1::LEU ATG9-EGFP::NatMX |
| YOY064 | BY4741 Δatg11::KanMX mRFP-APE1::LEU ATG9-EGFP::NatMX Δatg1::MET15 |
| YOY065 | BY4741 Δgos1::KanMX Δatg24::HIS mRFP-APE1::LEU ATG9-EGFP::NatMX Δatg1::MET15 |
| YOY066 | BY4741 Δtlg2::HIS Δatg24::KanMX mRFP-APE1::LEU ATG9-EGFP::NatMX Δatg1::MET15 |
| YOY067 | BY4741 Δgsg1::KanMX Δatg24::HIS |

**Supplemental Table S2.** Plasmids used in this study.

| Plasmid name | Construct | Backbone | Promoter | Application |
|---|---|---|---|---|
| pRS416 | empty | | none | CEN, *URA3* |
| pYOintF8 | ATG9-EGFP-NatMX | pBluescriptII | *TPI1* | Integrate in *ATG9* |
| pYOint2D1 | mCherry-TLG1-LEU2 | pBluescriptII | *TPI1* | Integrate in *TLG1* |
| pYOintC9 | TOM20-tdTom-LEU2 | pBluescriptII | *TOM20* | Integrate in *TOM20* |
| pYOFLD4 | GFP-APE1 | pRS416 | *APE1* | CEN, *URA3* |
| pYOotA8 | COG8 | pRS416 | *COG8* | CEN, *URA3* |
| pYOtagE2 | ATG24-FLAG | pRS416 | *ATG24* | CEN, *URA3* |
| pYOtagC2 | FLAG-GOS1 | pRS416 | *GOS1* | CEN, *URA3* |
| pYOtagC6 | FLAG-TLG2 | pRS416 | *TLG2* | CEN, *URA3* |
| pYOFLI6 | EGFP-ATG9-MYC | pRS416 | *TPI1* | CEN, *URA3* |
| pYOFLI7 | EGFP-ATG9-KKXX | pRS416 | *TPI1* | CEN, *URA3* |
| pYOFL2C6 | EGFP-ATG9-RxR | pRS416 | *TPI1* | CEN, *URA3* |
| pYOFLG8 | EGFP-ATG8 | pRS416 | *ATG8* | CEN, *URA3* |
| pYOint2B7 | EGFP-ATG9-MYC-NatMX | pBluescriptII | *TPI1* | Integrate in Δatg9 |
| pYOint2F8 | EGFP-ATG9-KKXX-NatMX | pBluescriptII | *TPI1* | Integrate in Δatg9 |
| pYOint2F9 | EGFP-ATG9-RxR-NatMX | pBluescriptII | *TPI1* | Integrate in Δatg9 |
| pYOFLI3 | EGFP-ATG9(786)-MYC | pRS416 | *TPI1* | CEN, *URA3* |
| pYOFLI4 | EGFP-ATG9(786)-KKXX | pRS416 | *TPI1* | CEN, *URA3* |
| pYOintD2 | tdTom-SCS2-LEU | pBluescriptII | *PHO5* | Integrate in *SCS2* |
| pYOintG8 | TagRFP-APE1-LEU | pBluescriptII | *APE1* | Integrate in *APE1* |
| pYOFL2E6 | NHX1-td-Tom | pRS316 | *NHX1* | CEN, *URA3* |