E08-01-0068 Leon

Supplementary material legends.

Table S1. Plasmids used in this study.

Table S2. Yeast strains used in this study.

Figure S1. Multiple sequence alignment of Ear1p/Ssh4p related proteins from yeasts. *S. cerevisiae* Ear1p, Ssh4p (Acc. Nos Q03212 and CAA81964, resp.), their putative homologues from *Kluyveromyces lactis* (XP_453509 and XP_452099), *Ashbya gossypii* (ORF ADL279W / NP_983817 and ORF ABL026W / NP_982921) and the sole Ear1p/Ssh4p-like protein from fission yeast (ORF SPCC285.10c / NP_588338) were aligned using ClustalX. PPxY sequences are indicated in yellow. Predicted transmembrane domains and B30.2/SPRY domains are highlighted in red and blue, respectively, according to InterPro predictions (www.ebi.ac.uk/InterProScan/).

Figure S2. Kinetics of Sit1p-GFP and Gap1p-GFP trafficking in the *ear1*Δ *ssh4*Δ double mutant. Gap1p-GFP (**A**) and Sit1p-GFP (**B**) were induced for the indicated time by addition of galactose to the medium, before being repressed by the further addition of glucose (chase). Cells were grown in conditions that trigger the direct sorting of these transporters from the Golgi to the vacuole *via* the VPS pathway. Subcellular localization of these transporters was studied in the indicated strains at various times during the chase.

Figure S3. *ear1*Δ *ssh4*Δ is not sensitive to cadmium. Serial dilutions of the indicated strains were spotted on SC medium supplemented (right panel) or not (left panel) with 20 µM cadmium, which is transported by Smf1p. Growth was assessed after two days of incubation at 30ºC.

Figure S4. Sna3p ubiquitylation is not affected in the *ear1*Δ *ssh4*Δ double mutant. Crude extracts of wild-type and *ear1*Δ *ssh4*Δ strains expressing Sna3p-GFP were immunoblotted with anti-GFP and anti-PGK antibodies. Higher molecular weight bands of Sna3p-GFP represent ubiquitylated species as demonstrated in Stawiecka-Mirota *et al.* (2007).

Figure S5. Ear1p/Ssh4p are not involved in Fur4p internalization. (**A**) Kinetics of Fur4p (uracil transporter) internalization after triggering (at t=0 min) its endocytosis, as measured by the uptake of radioactive (^{14}C) uracil. Uracil uptake is represented on a logarithmic scale. Error bars represent standard deviation (*n*=3). (**B**) Serial dilutions of the indicated strains were spotted on medium supplemented (right panel) or not (left panel) with 5-fluorouracil, an uracil analogue transported by Fur4p. Growth was assessed after two days at 30ºC. (**C**) Time-course of the endocytosis of the fluorescent marker FM 4-64 in wild-type and *ear1*Δ *ssh4*Δ strains. Cells were incubated for 15 min on ice with 20 µM FM 4-64, were washed twice and incubated at 30ºC for the indicated times.

Figure S6. Ear1p is an endosomal protein. (**A**) Time-course of the endocytosis of the fluorescent marker FM 4-64 in wild-type cells expressing Ear1p-GFP (multicopy plasmid). Cells were treated as described in **Figure S5C**. (**B**) Cells expressing Sec7-mCherry (late Golgi marker), tagged at the endogenous locus, were transformed with the Ear1p-GFP construct (multicopy plasmid).

Figure S7. Ear1p is phosphorylated. Ear1p-3HA was expressed from its endogenous promoter in wild-type cells. A crude extract was treated with calf intestinal phosphatase (+ CIP) or mock-treated (- CIP) and immunoblotted with anti-HA antibodies.

Figure S8. Vacuolar targeting of Ear1p-GFP does not depend on Bsd2p. Wild-type and *bsd2*Δ cells were transformed with the Ear1p-GFP construct (multicopy plasmid).

Figure S9. Overexpression of Ear1p affects Rsp5p localization. Additional pictures related to **Figure 6C**.

Figure S10. Overexpression of Ear1p-mCh does not affect Rsp5p steady-state level. Crude extracts from cells expressing Ear1p-mCh, GFP-Rsp5p or both were immunoblotted with the indicated antibodies. The GFP panels relate to different areas of the gel, showing GFP-Rsp5p (Top) and GFP-containing degradation product (Bottom).

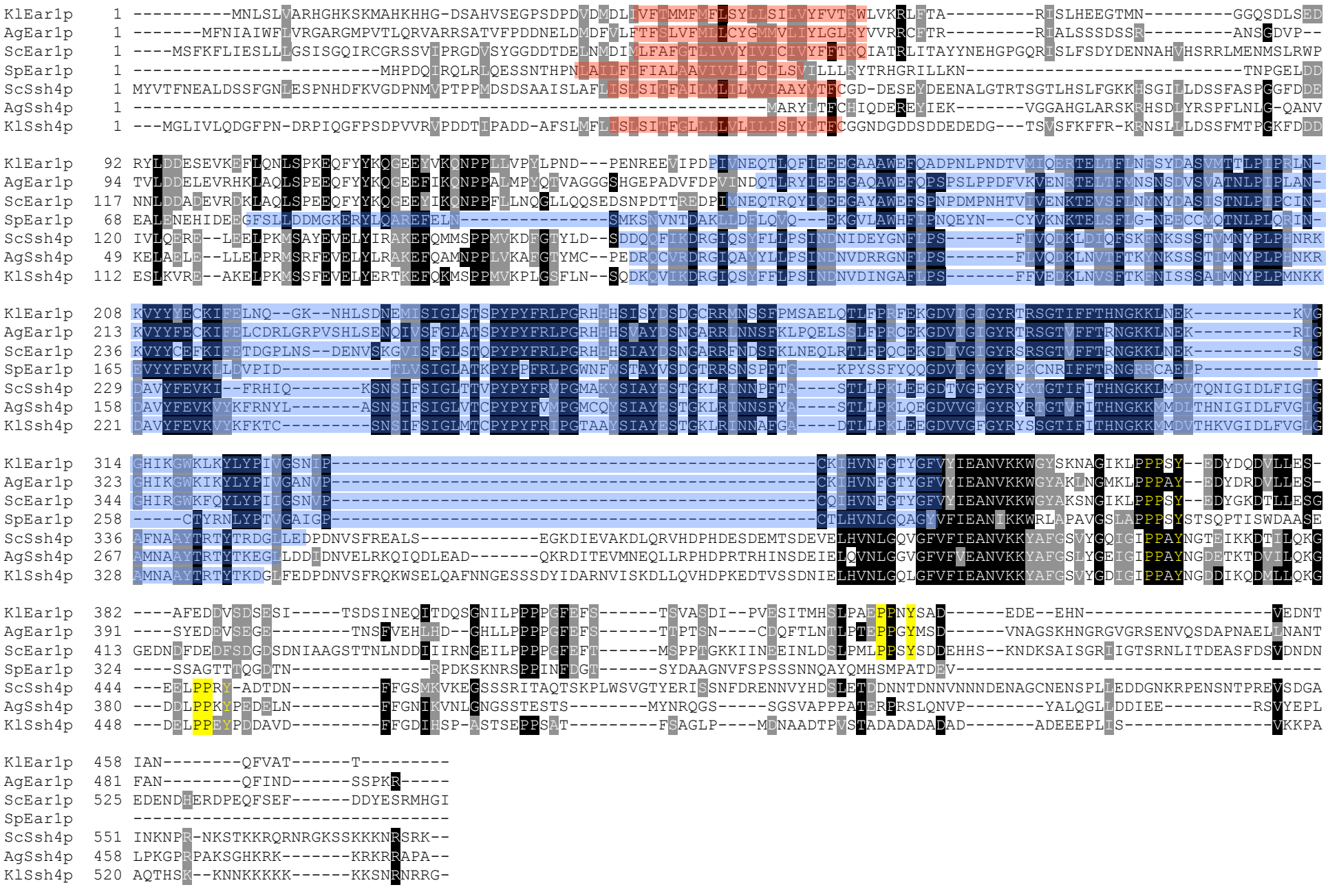
Supplementary Tables

Supplementary table 1.

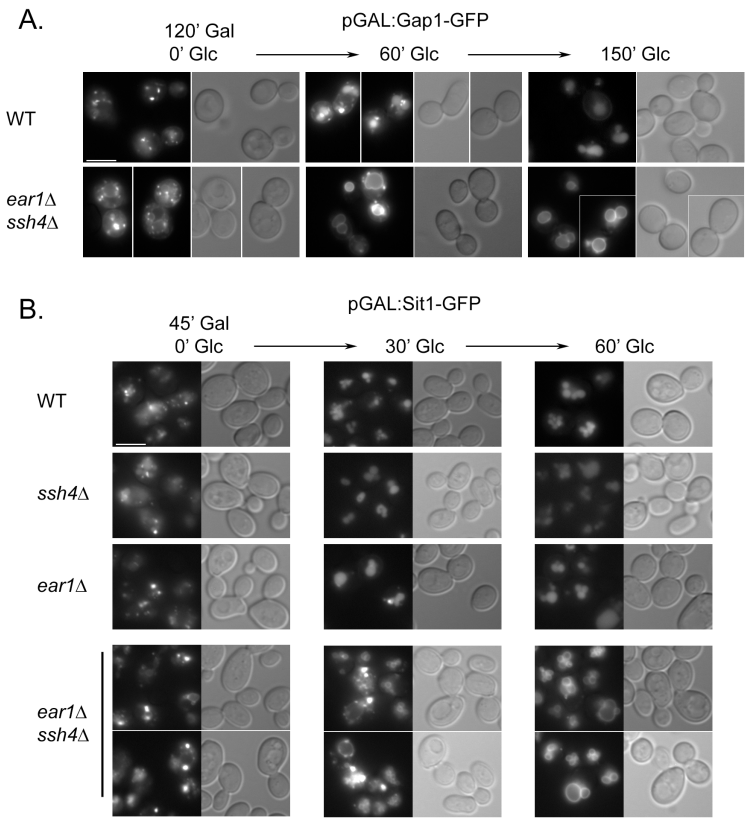
Plasmid name	Feature	Origin (backbone) [Reference]
pRS416-GFP-PHM5	*CEN, URA3*, p_{TPI1}*:GFP-PHM5*	H. Pelham [Reggiori and Pelham, 2001]
pRS416-Ub-GFP-PHM5	*CEN, URA3*, p_{TPI1}*:Ub-GFP-PHM5*	H. Pelham [Reggiori and Pelham, 2001]
pRS416-GFP-CPS1	*CEN, URA3*, p_{TPI1}*:GFP-CPS1*	H. Pelham [Reggiori and Pelham, 2001]
pRS416-GFP-SNA3	*CEN, URA3*, p_{TPI1}*:GFP-SNA3*	H. Pelham [Reggiori and Pelham, 2001]
Sit1-GFP (URA)	*CEN, URA3*, p_{GAL1}*:SIT1-GFP*	This laboratory [Froissard *et al.* 2007]
Sit1-GFP (LEU)	*CEN, LEU2,* p_{GAL1}*:SIT1-GFP*	This laboratory (Erpapazoglou *et al*., unpublished)
YCpJ25	*CEN, URA3*, p_{GAL1}*:GAP1-GFP*	B. André [De Craene *et al.* 2001]
pFL38-gFGFP	*CEN, URA3*, p_{GAL10}*:FUR4-GFP*	This laboratory [Marchal *et al.* 2002]
pSL18	*2µ, LEU2,* p_{EAR1}*:EAR1-3HA*	This study (pRS425 : Christianson *et al.* 1992)
pSL19	*CEN, LEU2,* p_{ADH}*:GFP-RSP5*	This study (p415:p_{ADH}-GFP : Mumberg *et al.* 1994)
pSL21	*CEN, URA3*, p_{GPD}*:(mcs)-mCherry*	This study (p416:p_{GPD} : Mumberg *et al.* 1994)
pSL22	*CEN, URA3*, p_{GPD}*:EAR1-mCherry*	This study (pSL21)
pSL25	*2µ, LEU2,* p_{EAR1}*:EAR1(PY1m)-3HA*	This study (pRS425 : Christianson *et al.* 1992)
pSL26	*2µ, LEU2,* p_{EAR1}*:EAR1(PY2m)-3HA*	This study (pRS425 : Christianson *et al.* 1992)
pSL29	*CEN, LEU2,* p_{CYC1}*:GFP-RSP5*	This study (p415:p_{CYC1} : Mumberg *et al.* 1994)
pSL30	*CEN, LEU2,* p_{ADH}*:GFP-BSD2*	This study (p415-p_{ADH}-GFP : Mumberg *et al.* 1994)
pSL31	*CEN, LEU2,* p_{ADH}*:GFP-SMF1*	This study (p415-p_{ADH}-GFP : Mumberg *et al.* 1994)
pSL34	*CEN, URA3*, p_{GPD}*:EAR1(PY1m)-mCherry*	This study (site directed mutagenesis on pSL22)
pSL35	*CEN, URA3*, p_{GPD}*:EAR1(PY2m)-mCherry*	This study (site directed mutagenesis on pSL22)
pSL37	*CEN, LEU2,* p_{CYC1}*:GFP-RSP5(ΔC2)*	This study (site directed mutagenesis on pSL29)

Supplementary table 2.

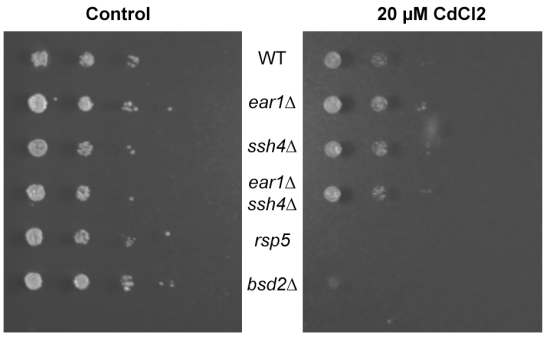
Strain (feature)	Genotype	Reference
BY4741 (WT)	*Mat**a** his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*	Euroscarf
BY4742 (WT)	*Matα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0*	Euroscarf
*vps4*Δ	*Mat**a** his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*	Euroscarf
pep4Δ	*Mat**a** his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 YPL154c::kanMX4*	Euroscarf
bsd2Δ	*Mata his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 YBR290W::kanMX4*	Euroscarf
*ssh4*Δ	*Mata his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 YKL024W::kanMX4*	Euroscarf
rsp5 (*npi1,* BY)	*Mat**a** his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 promYER125W::kanMX4*	Jacob and André, unpublished
*ear1*Δ	*Matα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 YMR171C::His3MX6*	This study
*ear1*Δ*ssh4*Δ	*Matα his3Δ1 leu2Δ0 ura3Δ0 YKL024W::kanMX4 YMR171C::His3MX6*	This study
ySL083 (Ear1-GFP)	*Mat**a** his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 YMR171C::YMR171C-GFP-His3MX6*	Euroscarf
ySL054 (Ear1-3HA)	*Mat**a** his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 YMR171C::YMR171C-3HA-KanMX*	This study
ySL205 (*rsp5,* Ear1-3HA)	*Mat**a** his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 promYER125W::kanMX4 YMR171C::YMR171C-3HA-KanMX*	This study
ySL096 (Vps27-mCh)	*Mat**a** his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 YNR006W:: YNR006W -mCherry-KanMX*	This study
ySL097 (Hse1-mCh)	*Mat**a** his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 YHL002W::YHL002W-mCherry-KanMX*	This study
ySL089 (Sec7-mCh)	*Mat**a** his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 YDR170C::YDR170C-mCherry-KanMX*	This study
ySL057 (Ear1-GFP, Hse1-mCh)	*Mat**a** his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 YMR171C::YMR171C-GFP-His3MX6 YDR170C::YDR170C-mCherry-KanMX*	This study



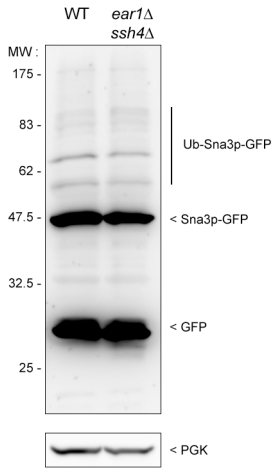
Léon et al. – Figure S1



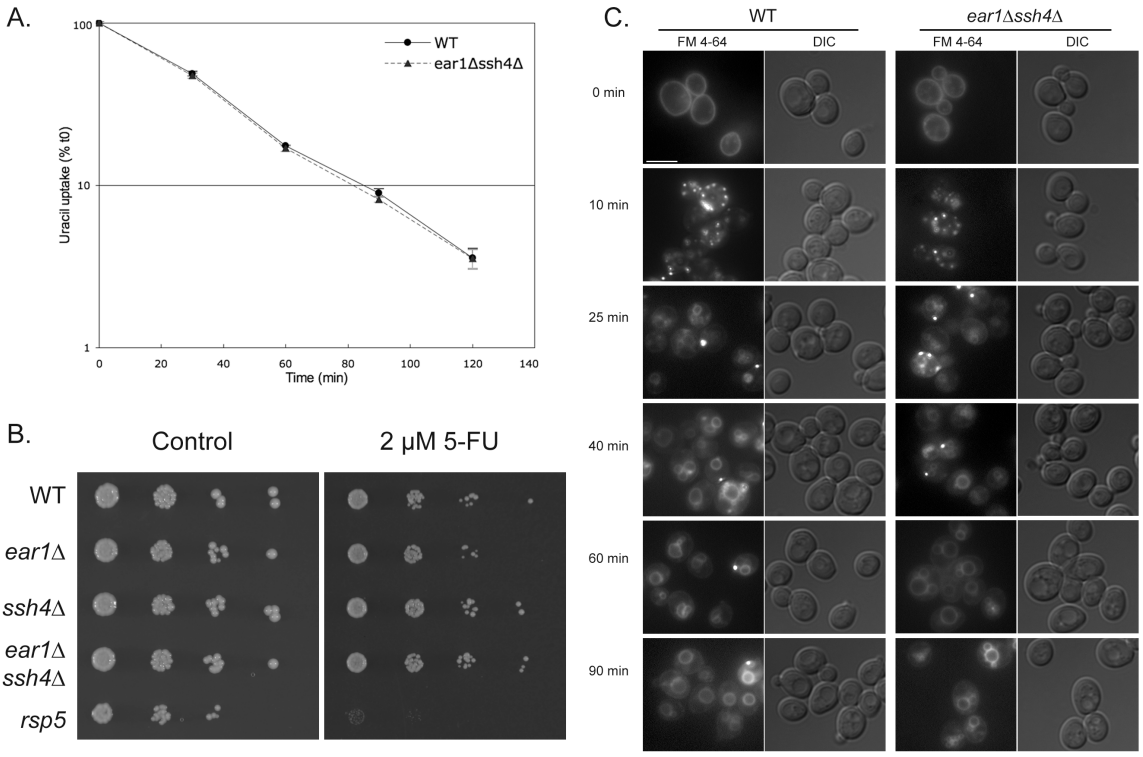
Léon et al., Figure S2



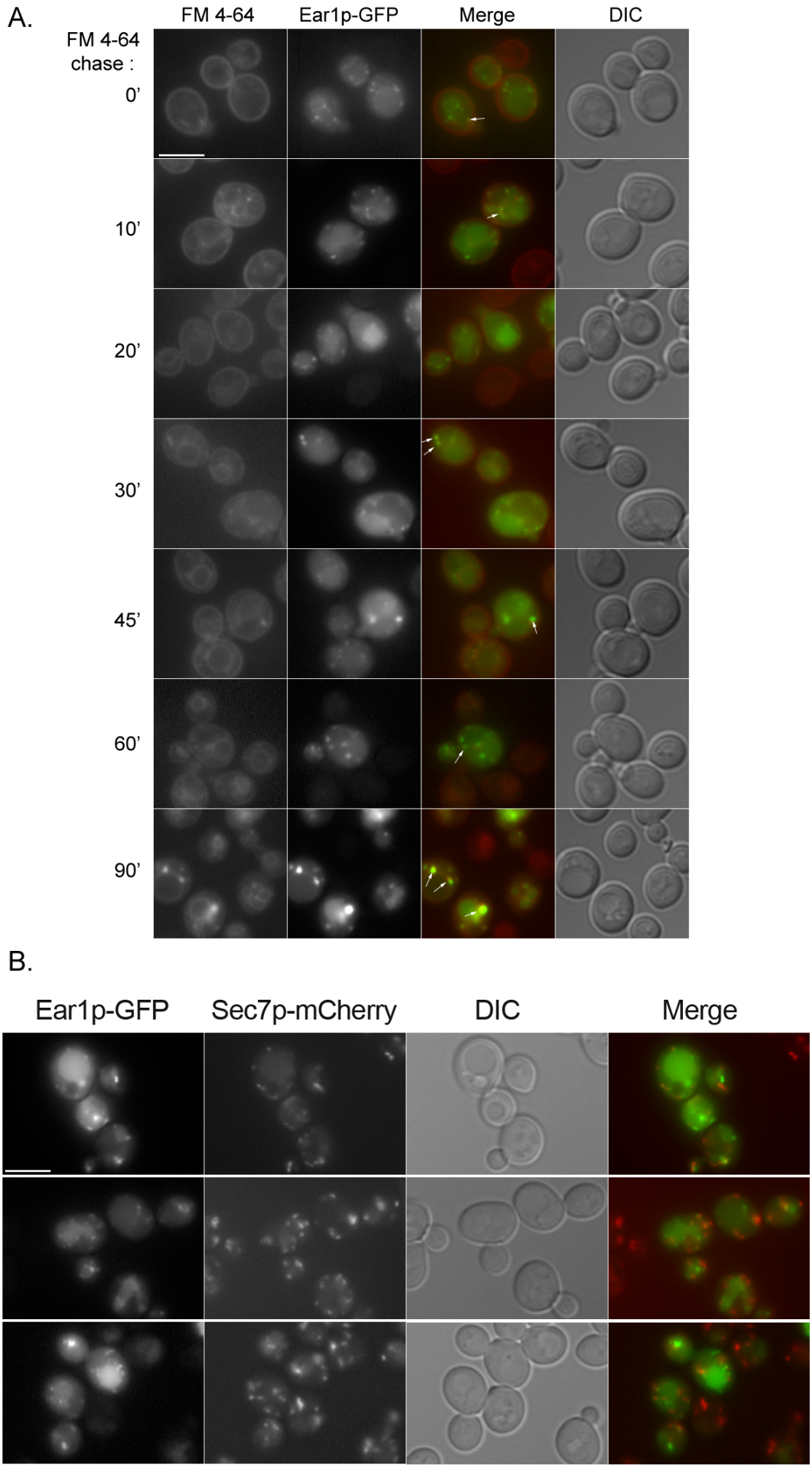
Léon et al., Figure S3



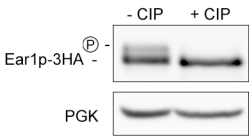
Léon et al. - Figure S4



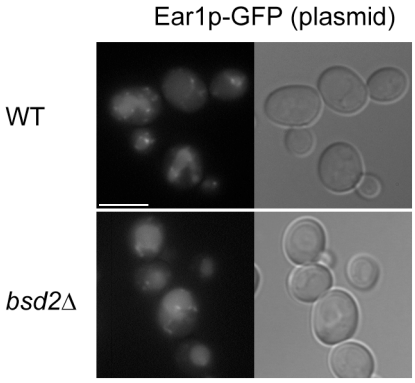
Léon et al. - Figure S5



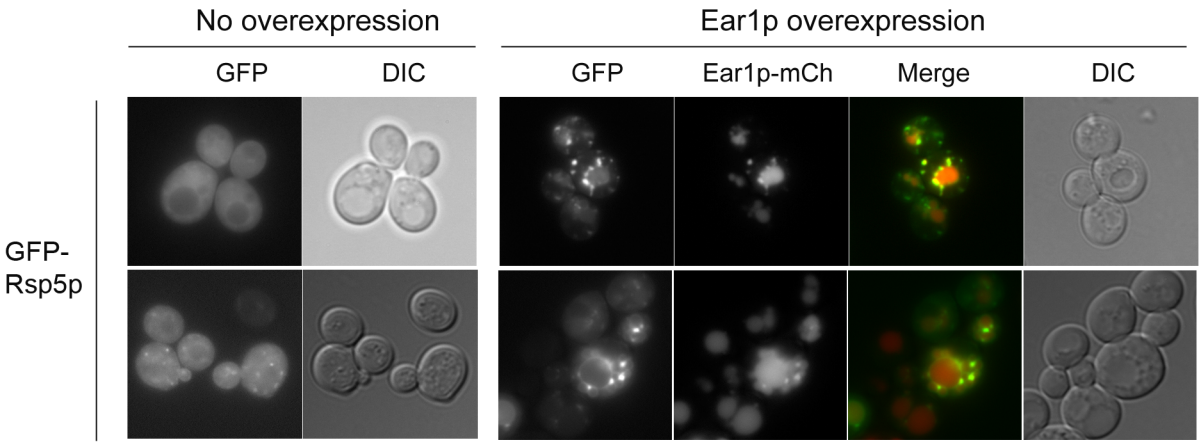
Léon et al. - Figure S6



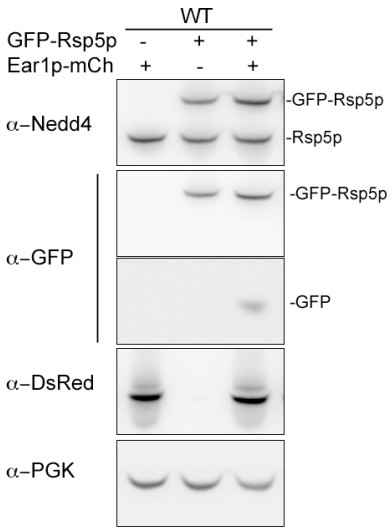
Léon et al. - Figure S7



Léon et al. - Figure S8



Léon et al. - Figure S9



Léon et al. - Figure S10