SUPPLEMENTAL MATERIALS (Meiringer et al.)

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Supplemental Methods

IgG pull-down assay from membrane fractions - Cells were grown to log phase (OD_{600}=1.5-2.5) and 1500 OD-units were harvested. The cell pellet was resuspended in 100 mM Tris/HCl, pH 9.4, 10 mM DTT and incubated at 30°C for 10 minutes. Afterwards, cells were retrieved by centrifugation, resuspended in SB-Buffer (0.2x YPD, 600 mM Sorbitol, 50 mM KPi, pH 7.4) and incubated with 2 ml of lyticase at 30°C for 20 minutes. Spheroblasts were recovered by centrifugation and lysed in PEKS-Buffer (10 mM Pipes/KOH pH 6.8, 2 mM EDTA, 50 mM KAc, 200 mM sorbitol, supplemented with Protease Inhibitor Mix FY (Serva), 1 mM PMSF and 1 mM DTT) and 0.4 mg/ml DEAE-Dextran. Unbroken cells and nuclei were removed by centrifugation at 300 g for 10 minutes at 4°C, and the cleared lysate was centrifuged for 15 minutes at 10,000 g. The resulting pellet (P10) was solubilized in L-Buffer (50 mM HEPES/KOH, pH 7.5, 150 mM NaCl, 0.15% Igepal C-630, 1.5 mM $MgCl_2$) supplemented with FY, 1 mM PMSF and 1 mM DTT. After clearing of the lysate at 10,000 g for 15 minutes, it was incubated for 1 hour with IgG-Sepharose (GE Healthcare). IgG-Sepharose beads were then transferred to MoBiCols (MoBiTec GmbH) and washed with 15 ml of L-Buffer without protease inhibitors and only 0.5 mM DTT. Beads were then resuspended in 250 µl of L-Buffer as before and TAP-tagged proteins eluted by incubation with 2.5 µl of TEV-protease either at 4°C over night or at 16°C for 2 hours. The eluate was collected by centrifugation, and TCA-precipitated at 13% TCA final concentration, washed with ice-cold acetone and resuspended in SDS-sample buffer (Invitrogen GmbH). Proteins were separated on 4-12% Tris-Glycine SDS-gels (Invitrogen GmbH), blotted to nitrocellulose membranes and decorated using the described antibodies.

SNARE complex disassembly by Sec18 - Yeast P10-fractions were obtained as described for IgG pull-down assay from membrane fractions. These were resuspended in 0% Ficoll and 1 mg was incubated in a release reaction containing ATP and Sec18 in fusion reaction buffer as described (1). Membranes were re-isolated by centrifugation (13,000g at 4°C) and either processed as described above or subjected after lysis to glycerol gradient analysis as described (2).

Recombinant Proteins - Recombinant Dsl1 complex proteins from pGEX-2TK were prepared from *Escherichia coli* BL21 Rosetta as previously described (3). SNARE proteins without transmembrane domain were expressed from pET32c(-Trx). In brief, cells were grown to OD_{600}=0.4-0.6 and induced with 0.4 mM IPTG over night at 16°C. Cells were harvested, resuspended in Buffer I (50 mM NaH_2PO_4, pH 8.0, 500 mM NaCl, 0.1% IGEPAL C-630, 10% Glycerol) supplemented with 12 mM 2-mercaptoethanol, 1x PIC and 2 mM PMSF and lysed by sonication or Microfluidizer Processor (Microfluidics). Cleared lysate was incubated with Ni^{2+}-NTA (Qiagen GmbH) or GSH-Sepharose (GE Healthcare) and after washing, bound proteins were either eluted with Buffer I containing 250 mM imidazole, or cleaved with thrombin followed by cleanup with benzamidine-agarose (Sigma Aldrich) for pET32c(-Trx) and pGEX-2TK constructs, respectively. Buffer-exchange was performed on PD10 columns (GE Healthcare) to final reaction buffer for GST pull down and gel filtration experiments.

GST pull down - Ten µg each of indicated proteins purified from E. coli in Buffer II (50 mM NaH_2PO_4, pH 8.0, 150 mM NaCl, 0.1% IGEPAL C-630, 5% Glycerol) were incubated in a total reaction volume of 100 µl on 10 µl GSH-Sepharose (GE Healthcare Life Science) over night at 4°C.

GSH-Sepharose with bound proteins were recovered by centrifugation and washed three times each with 800 µl of Buffer II. Proteins were eluted by addition of 15 µl of SDS-sample buffer and boiling for 5 minutes.

Gel-Filtration - For reconstitution of Dsl1 complex, 3 µM of recombinantly expressed Dsl1, Dsl3 and Tip20 were incubated in 1 ml reaction volume for 1hr at 4°C, and then centrifuged for 20 min at 20,000 g. Gel-filtration was done on a Sephacryl S-300 HR (GE Healthcare) at 4°C in the same buffer like the incubation before (50 mM Tris/Cl, pH 8.0, 150 mM NaCl, 2 mM DTT) with a flow rate of 0.2 ml/min. One ml fractions were collected and TCA precipitated, and resuspended in SDS-sample buffer. For the reconstitution of the Dsl1 complex together with the SNAREs, fractions of Dsl1 complex reconstitution (45-50 ml) were pooled and concentrated on Vivaspin 2 MWCO 50,000 (GE Healthcare) to 500 µl. Afterwards, the SNAREs were added to the complex, followed by incubation and gel-filtration as before. Samples were analyzed on 12% SDS-PAGE gels.

Subcellular Fractionation - Fractionation of yeast membranes was carried out as described (4). Yeast genetically modified to carry a TAP tag at the C-terminus of either Dsl1, Dsl3 or Tip20 were osmotically lysed and membranes fractionated by differential centrifugation. After a clearing run at 300 g, the supernatant was sequentially spun at 10,000 and 100,000g. The S100 supernatant after the last spin was TCA-precipitated, all pellets were resuspended in SDS-sample buffer, and examined by SDS-PAGE and Western blotting using secondary antibodies coupled with either Alexa 680 or IRDye 800.

SUPPLEMENTAL FIGURES

Fig. S1. Interactions of Ykt6 with the Dsl1 complex. A. Ykt6 is found in association with the Dsl1 complex. Dsl3-TAP was purified either by tandem affinity purification or on IgG-Sepharose alone and subjected to gel filtration on a superose-6 column as described [5]. Aliquots of the resulting fractions were analyzed on SDS-PAGE (Coomassie stained). Fraction numbers are indicated below the gels. Prominent bands of the tandem affinity purification were cut for identification in mass spectrometry. Cbp, calmodulin binding peptide. B. Interaction of the Dsl1 complex with Sec22 and Ykt6. Cells carrying TAP-tagged Dsl3 (D3T) with wild-type or temperature sensitive Sec18 (D3T sec18) were grown at 23°C over night, and heat shocked for 1 hr at 37°C, where indicated. Cells were lysed, and Dsl3 was purified via IgG beads and TEV cleavage from the lysate. Eluates were examined by SDS-PAGE and Western blotting, using antibodies against the indicated proteins. C. Sizing of the Dsl1 complex after Sec18 disassembly. Release reactions were prepared as in Figure 2C with membrane fractions from yeast strain expressing Dsl3-TAP. The re-isolated membranes were lysed and loaded on a 10-30% glycerol gradient and centrifuged for 18 hrs at 250,000 g. One ml fractions were collected, TCA precipitated and analyzed by SDS-PAGE and Western Blot. D. Interaction of full-length Dsl1 complex proteins and SNAREs in yeast two-hybrid assay. E. Depletion of Ypt1. Cells were grown in YPG, washed in PBS and then diluted into YPD and incubated for 9hrs. At indicated time points 1 OD_{600} was spun down, boild in Lämmli buffer and SDS-PAGE was done followed by Western Blotting. Blots were decorated against indicated proteins.

Fig. S2. Localization of Dsl3 and Sec20 under Dsl1 depletion condition. A&B. Depletion of Dsl1 does not disturb the colocalization of Dsl3 (A) or Sec20 (B) with Sec63 at the ER. Colocalization with the Golgi marker protein Mnn9 cannot be observed. Cells were treated like described in methods. Size bar indicates 10 µm. C. Depletion of Dsl1. Cells were grown in YPG, washed in PBS and then diluted into YPD and incubated for 9hrs. At indicated time points 1 OD_{600} was spun down, boiled in Laemmli buffer and SDS-PAGE was done followed by Western Blotting. The blot was decorated against indicated proteins. D. Colocalization of Sec20-GFP in *sec18-1* mutant with Sec63. RFP-tagged Sec63 was co-expressed in the GFP-Sec20 background and analyzed by fluorescence microscopy. Sec20 colocalizes with the ER marker Sec63 in punctuated structures at 23° and more so at 37°C. Size bar indicates 10 µm.

SUPPLEMENTAL TABLES

Table S1. Yeast strains used in this study.

Strain	Genotype	Source/Reference
BY4741	MATa *ura3Δ0 leu2Δ0 his3Δ0 met15Δ0*	EuroscarfTM
BY4727	MATalpha *his3Δ200 leu2Δ0 lys2Δ0 met15Δ0 trp1Δ63 ura3Δ0*	EuroscarfTM
PJ69-4A	MATa *trp1-901 leu2-3-112 ura3-52, his3-200 gal4Δ gal80Δ GAL2-ADE2 LYS2::GAL1-HIS3 met2::GAL7-lacZ*	(6)
YSC1178-7502517	MATa *DSL1-TAP::HIS3MX ura3Δ0 leu2Δ0 his3Δ met15Δ0*	(7)
YSC1178-7501954	MATa *DSL3-TAP::HIS3MX ura3Δ0 leu2Δ0 his3Δ met15Δ0*	(7)
YSC1178-7500084	MATa *SEC20-TAP::HIS3MX his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*	(7)
YSC1178-7500501	MATa *TIP20-TAP::HIS3MX his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*	(7)
D3T-s18	MATa *DSL3-TAP::HIS3MX sec18-1 ura3 his3 leu2*	(8)
D3T-d1	MATa *DSL3-TAP::HIS3MX dsl1-22 ura3 his3 leu2*	(8)
D3T-d1N	MATalpha *DSL3-TAP::HIS3MX dsl1Δ ura3 his3 leu2 + pRS315-DSLl1ΔN*	(8)
D3T-s20	MATalpha *DSL3-TAP::HIS3MX sec20-1 ura3 his3 leu2*	(8)
D3T-t20/5	MATa *DSL3-TAP::HIS3MX tip20-5 ura3 his3 leu2*	(8)
D3T-t20/8	MATa *DSL3-TAP::HIS3MX tip20-8 ura3 his3 leu2*	(8)
CUY1416	MATa *his3Δ200 trp1Δ63 ura3Δ0 DSL3::TAP-KanMX6*	this study
CUY1500	MATa *his3Δ200 trp1Δ63 ura3Δ0 DSL1::TAP-KanMX4*	this study
CUY1608	MATa *his3Δ200 trp1Δ63 ura3Δ0 ykt6Δ::KanMX4 pRS424-YKT6pr-GFP-YKT6*	this study
CUY1893	MATa *his3Δ200 trp1Δ63 ura3Δ0 DSL3::TAP-KanMX6 sec22Δ::HIS3*	this study
CUY2615	YSC1178-7501954 *GAL1pr::pRS401-GAL1pr-YKT6*	this study
CUY2710	MATa *his3Δ200 trp1Δ63 ura3Δ0 sec22pr::HIS3-GAL1pr DSL3::TAP-URA3*	this study
CUY2848	MATa/alpha *lys2Δ0/LYS2 HIS3/his3Δ1 LEU2/leu2Δ0 MET15/met15Δ0 URA3/ ura3Δ0 TIP20/TIP20-TAP::HIS3MX*	this study
CUY2847	MATa/alpha *lys2Δ0/LYS2 HIS3/his3Δ200 TRP1/trp1Δ63 URA3/ura3Δ0 DSL1/ DSL1-TAP::KanMX4*	this study
CUY2925	BY4727 *HIS3MX-PHO5pr-GFP::SEC20*	this study
CUY2976	BY4727 *URA3::PHO5pr-GFP-SEC20*	this study
CUY2971	BY4741 *sec22Δ::KanMX4 HIS3MX:: PHO5pr-GFP-SEC20*	this study
CUY2973	BY4741 *KanMX4-GAL1pr::YKT6 pRS416-NOP1pr-YKT6 HIS3MX:: PHO5pr-GFP-SEC20*	this study
CUY3007	RSY271 MATa *sec18-1 his4-619 ura3-52 URA3::PHO5pr-GFP-SEC20*	this study
CUY3010	MATa *his3Δ leu2Δ ura3Δ dsl3-2 URA3::PHO5pr-GFP-SEC20*	this study
CUY3027	BY4741 *TIP20-TAP::HIS3MX DSL1-TAP::KanMX4 DSL3-TAP::URA3*	this study
CUY2393	BY4741 *TIP20:: monoGFP-KanMX4*	this study
CUY2394	BY4741 *DSL3:: monoGFP-KanMX4*	this study
CUY1765	BY4741 *DSL1:: monoGFP-KanMX4*	this study
CUY4810	BY4727 *DSL3::GFP-KanMX4 DSL1::GAL1pr hphNT1::SEC63-MARS*	this study
CUY4811	BY4727 *DSL3::GFP-KanMX4 DSL1::GAL1pr hphNT1::MNN9-MARS*	this study
CUY4812	BY4727 *SEC20::HIS3MX-PHO5pr-GFP DSL1::GAL1pr*	this study

	hphNT1::SEC63-MARS	
CUY4813	BY4727 *SEC20::HIS3MX-PHO5pr-GFP DSL1::GAL1pr hphNT1::MNN9-MARS*	this study
XII-62	BY4727 *DSL3::GFP-KanMX4 GAL-YPT1::LEU*	this study
XIII-2	BY4727 *SEC20::HIS3MX-PHO5pr-GFP GAL-YPT1::LEU*	this study
CUY5391	BY4727 *SEC20::HIS3MX-PHO5pr-GFP ret2-1 hphNT1::SEC63-MARS*	this study
CUY6270	RSY271 MATa *sec18-1 his4-619 ura3-52 URA3MX-PHO5pr-GFP-myc-tag::SEC20 SEC63::hphNT1-MARS*	this study
CUY6383	RSY271 MATa *sec18-1 his4-619 ura3-52 SEC20::TAP-kanMX*	this study
CUY6384	RSY271 MATa *sec18-1 his4-619 ura3-52 SEC20::TAP-kanMX YKT6::natNT2-GAL1pr*	this study

Table S2. Expression plasmids used for GST and His6-purification from *E. coli*.

Plasmid	Backbone	Insert	Source/Reference
pGEX-2TK DSL1	pGEX-2TK	DSL1 (bp1-2265)	this study
pGEX-2TK DSL3	pGEX-2TK	DSL3 (bp1-2130)	this study
pGEX-2TK TIP20	pGEX-2TK	TIP20 (bp1-2106)	this study
pQLink H USE1-DSL3	pQLink	USE1 (bp1-651) + DSL3 (bp1-2130)	(9)
pET32c(-Trx) UFE1	pET32c(-Trx)	UFE1 (bp1-972)	this study
pET32c(-Trx) SEC20	pET32c(-Trx)	SEC20 (bp1-825)	this study
pET32c(+) SEC20 NT	pET32c(+)	SEC20 (bp1-588)	this study
pET32c(+) SEC20 SD	pET32c(+)	SEC20 (bp571-825)	this study
pET32c(-Trx) SEC22	pET32c(-Trx)	SEC22 (bp1-576)	this study
pETHIS-YKT6	pETHIS	YKT6 (bp1-603)	(10)

Table S3. Plasmids used in yeast two-hybrid analysis

Plasmid	Backbone	Insert	Source/Reference
pACT2 – DSL1	pACT2	DSL1 (bp1-2265)	this study
pACT2 – DSL3	pACT2	DSL3 (bp1-2130)	this study
pACT2 – TIP20	pACT2	TIP20 (bp1-2106)	this study
pACT2 – TIP20 (1-221)	pACT2	TIP20 (bp1-663)	this study
pACT2 – TIP20 (1-391)	pACT2	TIP20 (bp1-1173)	this study
pACT2 – TIP20 (1-612)	pACT2	TIP20 (bp1-1836)	this study
pACT2 – TIP20 (107-701)	pACT2	TIP20 (bp319-2106)	this study
pACT2 – TIP20 (209-701)	pACT2	TIP20 (bp624-2106)	this study
pACT2 – TIP20 (377-701)	pACT2	TIP20 (bp1129-2106)	this study
pACT2 – TIP20 (611-701)	pACT2	TIP20 (bp1831-2106)	this study
pACT2 – TIP20 (Δ80-110)	pACT2	TIP20 (Δbp238-330)	this study
pACT2 – UFE1	pACT2	UFE1 (bp1-972)	this study
pACT2 – UFE1 NT	pACT2	UFE1 (bp1-719)	this study
pACT2 – UFE1 SD	pACT2	UFE1 (bp709-972)	this study
pACT2 – USE1	pACT2	USE1 (bp1-654)	this study
pACT2 – USE1 NT	pACT2	USE1 (bp1-423)	this study
pACT2 – USE1 SD	pACT2	USE1 (bp418-654)	this study
pACT2 – SEC20	pACT2	SEC20 (bp1-588)	this study
pACT2 – SEC20 NT	pACT2	SEC20 (bp1-588)	this study
pACT2 – SEC20 SD	pACT2	SEC20 (bp571-825)	this study
pFBT9 – DSL1	pFBT9	DSL1 (bp1-2265)	this study
pFBT9 – DSL3	pFBT9	DSL3 (bp1-2130)	this study
pFBT9 – TIP20	pFBT9	TIP20 (bp1-2106)	this study
pFBT9 – SEC20	pFBT9	SEC20 (bp1-588)	this study
pFBT9 – USE1	pFBT9	USE1 (bp1-654)	this study
pFBT9 – SEC22	pFBT9	SEC22 (bp1-576)	this study
pFBT9 – YKT6	pFBT9	YKT6 (bp1-603)	this study

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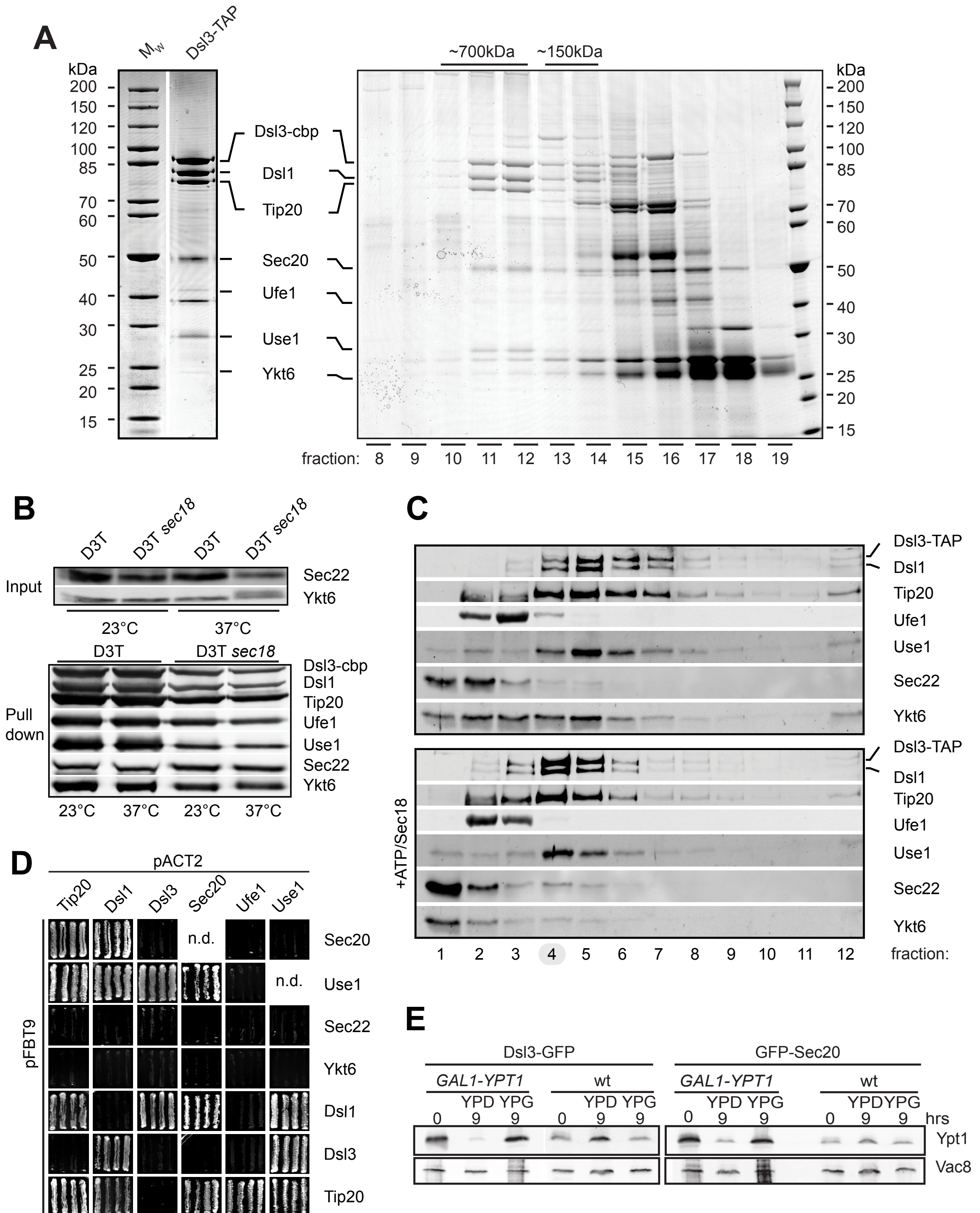
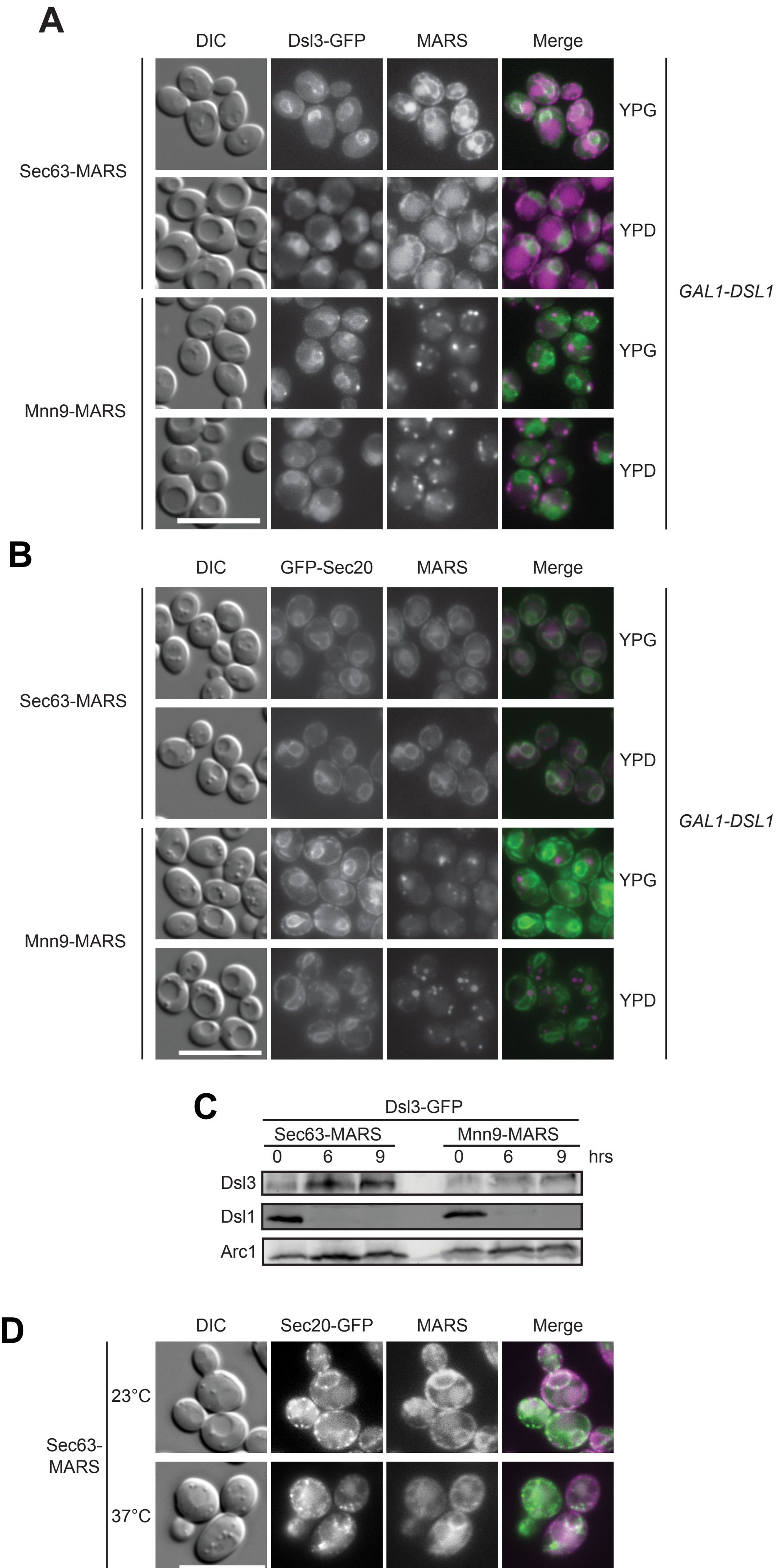
Figure S1, Meiringer et al.

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MARS
Merge
Sec63-MARS
Mnn9-MARS
YPG
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YPG
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GAL1-DSL1
B
DIC
GFP-Sec20
MARS
Merge
Sec63-MARS
Mnn9-MARS
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YPD
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C
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