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Suppression of Coatomer Mutants by a New Protein Family with COPI and COPII Binding Motifs in *Saccharomyces cerevisiae*

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Protein trafficking is achieved by a bidirectional vesicle flow between the various compartments of the eukaryotic cell. COPII coated vesicles mediate anterograde protein transport from the endoplasmic reticulum to the Golgi apparatus, whereas retrograde Golgi-to-endoplasmic reticulum vesicles use the COPI coat. Inactivation of COPI vesicle formation in conditional *sec21* (γ-COP) mutants rapidly blocks transport of certain proteins along the early secretory pathway. We have identified the integral membrane protein Mst27p as a strong suppressor of *sec21-3* and *ret1-1* mutants. A C-terminal KKXX motif of Mst27p that allows direct binding to the COPI complex is crucial for its suppression ability. Mst27p and its homolog Yar033w (Mst28p) are part of the same complex. Both proteins contain cytoplasmic exposed C termini that have the ability to interact directly with COPI and COPII coat complexes. Site-specific mutations of the COPI binding domain abolished suppression of the *sec21* mutants. Our results indicate that overexpression of *MST27* provides an increased number of coat binding sites on membranes of the early secretory pathway and thereby promotes vesicle formation. As a consequence, the amount of cargo that can bind COPI might be important for the regulation of the vesicle flow in the early secretory pathway.

INTRODUCTION

Proteins destined for secretion are first translocated into the endoplasmic reticulum (ER) and subsequently packaged into COPII-coated vesicles that are bound for the Golgi apparatus. At the same time, proteins are retrieved by COPI coated vesicles from the Golgi to the ER to maintain an equilibrium of proteins and membranes between the two organelles. The COPII coat consists of the small GTPase Sar1p and two protein complexes, Sec23/24p and Sec13/31p, whereas the COPI coat contains the small GTPase Arf1p and a heptameric protein complex called coatomer (Orci *et al*., 1986; Serafini *et al*., 1991; Barlowe *et al*., 1994; Bednarek *et al*., 1996). The transport between the early compartments of the secretory pathway is highly intertwined: disturbances in one route lead to a block in the other. Uptake of membrane proteins into COPII vesicles requires either a signal on the proteins to be transported or the interaction with an escort protein. So far, it has been established that a diphenylalanine motif on the cytoplasmic face of membrane proteins favors interaction with the COPII coat. Soluble proteins such as the precursor of the yeast pheromone alpha factor or GPI-anchored proteins such as Gas1p require receptors for efficient uptake into COPII vesicles. The nature of the interaction between the cargo and the receptor remains elusive. Proteins that have escaped the ER or transport factors (such as soluble *N*-ethylmaleimide-sensitive factor attachment protein receptors [SNAREs] or escort proteins) need to be retrieved from the Golgi in COPI vesicles. The uptake into these vesicles also requires a signal. A cytoplasmic exposed terminal KKXX motif allows direct interaction with coatomer (Cosson and Letourneur, 1994, 1997). Other proteins that lack an obvious transport signal such as SNARE proteins as well as the HDEL/KDEL-receptor Erd2p, which is essential for the retrieval of ER-resident soluble proteins, use the help of ARF-GAP for inclusion into COPI vesicles (Aoe *et al*.,

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1997; Rein *et al.*, 2002). Several proteins, which cycle between the ER and the Golgi apparatus, were suggested to function as escorting factors (Herrmann *et al.*, 1999; Muniz and Riezman, 2000). The precursor of the pheromone alpha factor requires a small integral membrane protein, Erv29p (Belden and Barlowe, 2001). The V_0 sector of the vacuolar ATPase uses Vma21p, a protein with two transmembrane domains (Hill and Stevens, 1994). GPI-anchored proteins are sorted and included into COPII vesicles by a multimeric complex of the p24 proteins (Muniz *et al.*, 2000; Muniz and Riezman, 2000). Besides the common KKXX motif, these proteins share very little overall similarity.

In this article, we identified a novel class of membrane proteins that suppresses specific mutants of the COPI coat upon overexpression. These suppressor proteins bind directly COPI coat complexes via a C-terminal KKXX motif and interact with COPII proteins via an unspecified sequence. Other members of this protein family contain a characteristic diphenylalanine motif, which mediates interaction with COPII components. However, these proteins are unable to suppress coatomer mutants. The KKXX motif-containing proteins seem to shuttle between the ER and the Golgi. Overexpression of two family members leads to greatly enlarged vacuoles in large cells. However, the overexpressed proteins remain in the ER. The presence of the KKXX motif is essential for suppression of coatomer mutants, and our results suggest that the increase of COPI binding sites in the ER overcomes the defects in COPI mutants by a stimulation of the vesicular transport. From this, we propose that the vesicle flow between membranes of the early secretory pathway is regulated by the abundance of cargo proteins.

MATERIALS AND METHODS

Strains and Reagents

Escherichia coli BL21(DE3)pLysS (Novagen, Madison, WI) was used for protein expression. The yeast strains used in this study are listed in Table 1. Cultures were either grown in rich medium (1% Bacto-yeast extract and 2% Bacto-peptone [YP]) or minimal medium (0.67% nitrogen base without amino acids) containing either 2% dextrose, or 2% galactose and 1% raffinose as carbon sources at 30°C unless indicated otherwise. To test the utilization of different nitrogen sources 0.17% nitrogen base without amino acids without ammonium sulfate was supplemented with 2% dextrose and 1 mg/ml nitrogen source. Standard genetic techniques were used throughout (Sherman, 1991).

Sec21-3 Suppressor Screen

The strain *sec21-3* was provided from E. Gaynor and S. Emr (University of California, San Diego, CA) and transformed with an YEp24 (*URA3*, 2 μ)-based yeast library (Carlson and Botstein, 1982). Transformants (40,000) were grown at 25°C, replica plated, and screened for growth at 37°C. From growing colonies, plasmids were isolated and retransformed into the *sec21-3* mutant. From transformants that remained temperature-resistant, plasmid DNA was isolated and sequenced. The insert of the suppressing plasmid γ1-25 contained a piece of chromosome VII from base pairs 399250–406876 (according to Stanford Genome Database). For subcloning, the γ1-25 plasmid was digested with *Eco*RV and the resulting fragments cloned into the *Bam*HI site of YEp24. The plasmid containing the 3-kb *Eco*RV insert represented the suppressing sequence. Sequences derived from open reading frames (ORFs) YGL051w, YGL052w, and YGL053w were synthesized by polymerase chain reaction (PCR) by using the primer pairs HH92 (GGGGGATCCCCTCATCTGTTCTCGTACTTTGTTG)/HH93(GGGGGATCCCGGGCCAGTTAGTGCTGATTA), HH10 (GGGGAATTCATGCAGTTGCCCCAAAAACAC)/HH11 (GGGGGATCCCTAGGTTCGTTGAGTGTATCT), or HH102 (GGGGGGATCCGTGTGCTAGTGTCTCCCG)/HH103 (GGGGGGATCCTGAGGATTCCTATATCCT), respectively, cloned into the *Bam*HI site of YEp24, and sequenced. Thereby the ORF YGL051w (*MST27*) was identified as suppressing gene.

Plasmid and Strain Construction

For gene disruption, a *HIS3*-containing cassette was amplified by PCR and chromosomal sequences were replaced by homologous recombination using the strains YPH499 and YPH500. All deletions were verified by PCR with primers inside and outside of the inserted sequences. Δ*mst27*+Δ*mst28* and Δ*prm8*+Δ*prm9* deletions were combined by mating and sporulation of the single mutants.

For the expression of myc-tagged versions of Mst27p and Prm8p, a PCR strategy was used that led to a chromosomal insertion of the *GAL10* promoter followed by three myc-epitopes in front of the ORFs (Lafontaine and Tollervey, 1996). To express GST-Mst27p, GST-Mst27_AAXXp, and GST-Prm8p in *E. coli*, we used sequences encoding the cytoplasmic domains of Mst27p and Prm8p amplified by PCR with the primer pairs HH100 (GGGGAGATCTGGTGATGGTAATCCAAAG)/HH93 (GGGGGATCCCGGGCCAGTTAGTGCTGATTA), HH100/HH115 (GGGGGATCCTATTCCGTCGCCGCAAGAAGCGCATCGAT), and HH101 (GGGGAGATCTAGGTTTGGACCACAGATC)/HH102 (GGGGGGATCCGTGTGCTAGTGTCTCCCG), respectively. The PCR products were digested with *Bgl*II and *Bam*HI and cloned into *Bam*HI cut pETGEXCT (Sharrocks, 1994). For expression of Mst28p, a sequence encoding for the cytoplasmic domain was amplified by PCR from the Δ*mst27*-strain with primers TS045 (CGCGGATCCCTACGCCTTGTTGAGGGAG) and TS021 (CCGGAATTCCGGGCCAGTTAGTGCTGATTA), and after digestion with *Bam*HI and *Eco*RI cloned into vector pGEX-6p (Amersham Biosciences, Freiburg, Germany).

The Emp24p overexpression plasmid was provided by A. Rowley (Glaxo Wellcome Foundation, Stevenage, United Kingdom) and contained the *EMP24* encoding sequence in a pRS426GAL multicopy expression vector (Sikorski and Hieter, 1989). The plasmids for overexpression of Wbp1p and the invertase-Wbp1 fusion were described previously (Gaynor *et al.*, 1994). The 2 μ plasmid containing a N-terminally myc-tagged Sec20 was provided by H. Pelham (MRC Cambridge, Cambridge, United Kingdom)

To generate plasmid pESC-*MST27*, the *MST27* coding sequence was amplified by PCR with primers TS013 (GCGAAGATCTTCATGCAGACCCCTCTAGAA) and TS014 (CGTGCGAGCTCCTATTCCGTCTTTTTAAGAAGC), digested with restriction enzymes *Bgl*II and *Sac*I, and cloned into vector pESC-Trp (Invitrogen, Carlsbad, CA).

Pulse-Chase Analysis

Radiolabeling and lysis of cells and immunoprecipitations were essentially performed as described previously (Hosobuchi *et al.*, 1992), except that for radiolabeling cells were grown in YPD or selective minimal medium overnight to an OD_{600} of 0.2–0.5, harvested, washed, and resuspended to an OD_{600} of 5 in sulfate-free minimum medium containing all amino acids without methionine and cysteine. After a preincubation of 5 min, 20 μCi per OD_{600} unit of tran^{35}S-label (ICN Pharmaceuticals, Costa Mesa, CA) was added. After 3 min, labeling was terminated by addition of methionine and cysteine to 10 mM final concentration. Chase time point aliquots (0.5 OD_{600}) were removed as indicated, cells were lysed, and cell lysates used for immunoprecipitation as described. The antisera used had been described previously (Evan *et al.*, 1985; Kuehn *et al.*, 1998).

Table 1. Strains used in this study

Strain	Genotype	Source
BY4741	*MAT* a; *his3Δ1; leu2Δ0; met15Δ0; ura3Δ0*	Euroscarf
BY4742	*MAT* a; *his3Δ1; leu2Δ0; lys2Δ0; ura3Δ0*	Euroscarf
BY4743	*MAT* a/α; *his3Δ1/his3Δ1; leu2Δ0/leu2Δ0; lys2Δ0; met15Δ0/met15Δ0 ura3Δ0/ura3Δ0*	Euroscarf
EGY021.2	*MATα; trp1; leu2; suc2-Δ9; sec21::his3; pRS315-sec21-3*	Erin Gaynor
GPY60	*MATα; ura3-52; leu2,3-112; his4-579; pep4::ura3; prb1; trp4-579*	Randy Schekman
HHY203	*MAT* a; *ade2-101; his3-Δ200; leu2-Δ1; lys2-801; trp1-Δ63; ura3-52; YGL051w::HIS3; YAR033w::HIS3;*	This study
HHY204	*MATα; ade2-101; his3-Δ200; leu2-Δ1; trp1-Δ63; ura3-52; YGL089c::His_{12}-YGL089c(URA3); YGL051w::HIS3; YAR033w::HIS3;*	This study
HHY215	*MATα; trp1; leu2; suc2-Δ9; sec21::his3; pRS315-sec21-3; YEp24-MST27*	This study
HHY216	*MATα; trp1; leu2; suc2-Δ9; sec21::his3; pRS315-sec21-3; YEp24-PRM8*	This study
HHY217	*Matα; ade2-101oc; his3-Δ200; leu2-Δ1; lys2-801; trp1-Δ63; ura3-52; YGL051w::GAL-myc3-YGL051w(HIS3)*	This study
HHY218	*Matα; ade2-101oc; his3-Δ200; leu2-Δ1; lys2-801; trp1-Δ63; ura3-52; YGL053w::GAL-myc3-YGL053w(HIS3)*	This study
HHY251	*MATα; trp1; leu2; suc2-Δ9; sec21::his3; pRS315-sec21-3; YEp24-MST27-AAXX*	This study
Y10422	*Matα; leu2Δ0; lys2Δ0; ura3Δ0; YAR031w::kanMX4*	Euroscarf
Y14419	*Matα; his3Δ1; leu2Δ0; lys2Δ0; ura3Δ0; YGL051w::kanMX4*	Euroscarf
Y14420	*Matα; his3Δ1; leu2Δ0; lys2Δ0; ura3Δ0; YGL053w::kanMX4*	Euroscarf
Y34419	*Mat* a/α; *his3Δ1/his3Δ1; leu2-Δ1/leu2-Δ1; lys2Δ0/LYS2; MET15/met15Δ0; ura3Δ0/ura3Δ0; YGL051w::kanMX4/YGL051w::kanMX4*	Euroscarf
Y34420	*Mat a/α; his3Δ1/his3Δ1; leu2Δ0/leu2Δ0; lys2Δ0/LYS2; MET15/met15Δ0; ura3Δ0/ura3Δ0; YGL053w::kanMX4/YGL053w::kanMX4*	Euroscarf
Y30422	*Mat a/α; HIS3/his3Δ1; leu2Δ0/leu2Δ0; lys2Δ0/LYS2; MET15/met15Δ0; ura3Δ0/ura3Δ0; YAR031w::kanMX4/YAR031w::kanMX4*	Euroscarf
YAS254	*Mat α; ade2-101oc; his3-Δ200; leu2-Δ1; lys2-801; trp1-Δ63; ura3-52; YGL053w::GAL-myc3-YGL053w(HIS3); pPESC-MST27*	This study
YAS276	*Mat a/α; ade2-101/ade2-101; his3-Δ200/his3-Δ200; leu2-Δ1/leu2-Δ1; lys2-801/LYS; trp1-Δ63/trp1-Δ63; ura3-52/ura3Δ2; YGL089c::His_{12}-YGL089c(URA3)/YGL089; YGL051w::HIS3/YGL051w::HIS3; YAR033w::HIS3/YAR033w::HIS3*	This study
YAS277	*Mat a/α; his3Δ1/his3Δ1; leu2Δ0/leu2Δ0; lys2Δ0/LYS2; MET15/met15Δ0; ura3Δ0/ura3Δ0; YGL053w::kanMX4/YGL053w::kanMX4; YAR031w::kanMX4/YAR031w::kanMX4*	This study
YAS286	*MAT α; ade2-101; his3-Δ200; leu2-Δ1, lys2-801; trp1-Δ63; ura3-52; pPESC-MST27*	This study
YAS308	*MAT α; ade2-101; his3-Δ200; leu2-Δ1, lys2-801; trp1-Δ63; ura3-52; YOL044w::GAL-myc3-YOL044w; pPESC-MST27*	This study
YAS314	*Mat α; ade2-101oc; his3-Δ200; leu2-Δ1; lys2-801; trp1-Δ63; ura3-52; YGL051w::GAL-myc3-YGL051w(HIS3); pSTM22 (URA3)*	This study
YAS315	*Matα; ade2-101oc; his3-Δ200; leu2-Δ1; lys2-801; trp1-Δ63; ura3-52; YGL053w::GAL-myc3-YGL053w(HIS3); pSTM22 (URA3)*	This study
YAS316	*MATα; ade2-101; his3-Δ200; leu2-Δ1, lys2-801; trp1-Δ63; ura3-52; pSTM22 (URA3)*	This study
YPH499	*MAT* a; *ade2-101; his3-Δ200; leu2-Δ1, lys2-801; trp1-Δ63; ura3-52*	Phil Hieter
YPH500	*MAT α; ade2-101; his3-Δ200; leu2-Δ1, lys2-801; trp1-Δ63; ura3-52*	Phil Hieter

Protein-binding Assay

Glutathione *S*-transferase (GST)-fusion proteins were purified essentially as described previously (Frangioni and Neel, 1993). Fresh overnight *E. coli* cultures were diluted 100-fold and grown to an OD_{600} of 0.5. Isopropyl β-D-thiogalactoside was added to 0.4 μM final concentration, and cells were incubated at 25°C for 3 h. Cells were harvested, resuspended in lysis buffer to 50 OD_{600}/ml (1 M NaCl, 10 mM EDTA, 5 mM dithiothreitol [DTT], 0.2% laurylsarcosyl, 100 mM Tris-HCl, pH 8.0), and lysed by freeze thawing. The extract was cleared by centrifugation for 5 min at 15,000 × *g*, adjusted to pH 6.8 and 2% Triton X-100, and incubated with glutathione agarose for 1 h at 20°C. The beads were washed five times in 1 M NaCl, 10 mM EDTA, 5 mM DTT, 2% Triton X-100, 100 mM Tris-HCl, pH 6.8, and once in binding buffer [150 mM KOAc, 5 mM $Mg(OAc)_2$, 1 mM EDTA, 1 mM DTT, 0.1% Triton X-100, 2% glycerol, 20 mM HEPES, pH 6.8], followed by an incubation in binding buffer in the presence of either 1.25 or 2.5 mg/ml crude yeast cytosol (Rexach *et al*., 1994) or 25 μg/ml Sec23/Sec24p complex (Barlowe *et al*., 1994) (with or without 20 μg/ml Sar1p) for 1 h at 20°C. The beads were washed five times in binding buffer and bound proteins were resolved by SDS-PAGE.

Gel Filtration

Yeast cells were converted to spheroplasts as described previously (Rexach *et al*., 1994) and lysed in 4% octyl glucoside, 100 mM NaCl, 10% glycerol, 2 mM phenylmethylsulfonyl fluoride (PMSF), 20 mM HEPES, pH 7.5, at a protein concentration of 5 mg/ml. The extract was cleared by centrifugation (100,000 × *g*, 30 min, 4°C), and 200 μl of the resulting supernatant was applied onto a Superose 6 HR 10/30 gel filtration column (Amersham Biosciences). The run was performed in 1% octyl glucoside, 100 mM NaCl, 10% glycerol, 20 mM HEPES, pH 7.5, at a flow rate of 0.25 ml/min, and 500-μl fractions were collected. The fractionation was calibrated by immunoblotting against protein complexes of known size and by a parallel run of molecular weight markers (Amersham Biosciences).

Coimmunoprecipitation

9E10 anti-myc antibody (Roche Diagnostics, Mannheim, Germany) was immobilized on 20% protein A-Sepharose (1.6 μg of antibody + 200 μl of protein A-Sepharose for SDS-PAGE analysis and matrix-assisted laser desorption ionization (MALDI)-identification, 0.8 μg of antibody + 50 μl of protein A-Sepharose for Western blot analysis) at 4°C for 1 h. Yeast cells were converted into spheroblasts and lysed by rotating in 4% octyl glucoside, 100 mM NaCl, 10% glycerol, 2 mM PMSF, 20 mM HEPES, pH 7.5 (24 ml/2 ml) at 4°C for 15°C. The lysate was diluted twofold with 100 mM NaCl, 10% glycerol, 2 mM PMSF, 20 mM HEPES, pH 7.5; cellular debris was removed by centrifugation (30 min, 100,000 × *g*, 4°C); and the supernatant was precleared with 20% protein A-Sepharose at 4°C for 45 min. The fusion proteins were precipitated at 4°C for 3 h, washed five times with 0.5% octyl glucoside, 100 mM NaCl, 10% glycerol, 2 mM PMSF, 20 mM HEPES, pH 7.5, and eluted either by boiling the beads in 30 μl of nonreducing SDS-loading buffer for SDS-PAGE and MALDI-identification or by incubation with 1 mg/ml myc-peptide in 30 μl of wash buffer at 30°C for 30 min for immunoblot analysis.

Protein Identification with Mass Spectrometry

In gel tryptic digestions were performed as described previously (Shevchenko *et al.*, 1996) and modified as outlined below. Briefly, protein bands were excised from gels, fully destained, and digested for 3 h with porcine trypsin (sequencing grade, modified; Promega, Madison, WI) at a concentration of 67 ng/μl in 25 mM ammonium bicarbonate, pH 8.1, at 37°C. Before peptide mass mapping and sequencing of tryptic fragments by tandem mass spectrometry, peptide mixtures were extracted from gels by 1% formic acid followed by two changes of 50% acetonitrile. The combined extracts were vacuum-dried until only 1–2 μl was left, and the peptides were purified by ZipTip according to the manufacturer's instructions (Millipore, Bedford, MA). MALDI-time of flight (TOF) analysis from the matrix α-cyano-4-hydroxycinnamic acid/nitrocellulose prepared on the target by using the fast evaporation method (Arnott *et al.*, 1998) was performed on a Bruker Reflex III (Bruker Daltonik, Bremen, Germany) equipped with a N_2 337-nm laser and gridless pulsed ion extraction.

Sequence verifications of some fragments were performed by nanoelectrospray tandem mass spectrometry on either a Q-Tof I mass spectrometer (Micromass, Manchester, England) or a QStar Pulsar i Qqoa Tof mass spectrometer (Applied Biosystems-MDS Sciex, Weiterstadt, Germany) equipped with a nanoflow electrospray ionization source. Gold-coated glass capillary nanoflow needles were obtained from Protana (Odense, Denmark) (type medium NanoES spray capillaries). Database searches (NCBInr, nonredundant protein database) were done using the MASCOT software (Perkins *et al.*, 1999).

Immunofluorescence

Cells were grown to early log phase in rich medium supplemented either with 2% dextrose or 2% galactose for induction of the *GAL10* promoter. To observe the effects of glucose repression, expression of the respective fusion protein was induced overnight in YP with 2% galactose and afterward repressed by transferring the cells into YP with 2% glucose. Alternatively, protein synthesis was inhibited by addition of rapamycin (Alexis, Grünberg, Germany) to a final concentration of 100 ng/ml. Aliquots were taken at different time points and analyzed by immunofluorescence as described previously (Chuang and Schekman, 1996) by using monoclonal 9E10 anti-myc (Roche Diagnostics) or M2 anti-FLAG-antibodies (Sigma, Taufkirchen, Germany). The secondary antibodies were obtained from Jackson Immunoresearch Laboratories (West Grove, PA).

Electron Microscopy

Yeast cells were cryoimmobilized by high-pressure freezing according to Hohenberg *et al.* (1994). In brief, living specimens were sucked into cellulose microcapillaries of 200 μm diameter, and 2-mm-long capillary tube segments were transferred to aluminum platelets of 200-μm depth containing 1-hexadecene. The platelets were sandwiched with platelets without any cavity and then frozen with a high-pressure freezer (Bal-Tec HPM 010; Balzers, Liechtenstein). The frozen capillary tubes were freed from extraneous hexadecene under liquid nitrogen and transferred to 2-ml microtubes with screw caps containing the substitution medium precooled to −90°C. Samples were kept in 2% osmium tetroxide in anhydrous acetone at −90°C for 32 h, at −60°C and −30°C for 4 h at each step in a freeze-substitution unit (Balzers FSU 010, Bal-Tec; Balzers). After washing with acetone, the samples were transferred into an acetone-Epon mixture at −30°C, infiltrated at room temperature in Epon, and polymerized at 60°C for 48 h. Ultrathin sections stained with uranyl acetate and lead citrate were viewed in a Philips CM10 electron microscope at 60 kV.

Membrane Flotations

Cells were grown to early to mid-log phase under permissive conditions. Golgi membranes, coatomer, and Arf1p were prepared according to Spang and Schekman (1998), Hosobuchi *et al.* (1992), and Kahn *et al.* (1995), respectively. The Golgi membranes were incubated with 10 μg/ml coatomer, 2 μg/ml Arf1p, and 0.1 mM guanosine 5′-*O*-(3-thio)triphosphate (GTPγS) for 30 min at 30°C in 100 μl of 0.9 M sucrose in B88 [20 mM HEPES, pH 6.8, 150 mM KOAc, 250 mM sorbitol, 5 mM $Mg(OAc)_2$]. The reactions were overlaid with 75 μl of 0.75 M sucrose in B88 and 10 μl of B88. Membranes were floated in a TLA 100 rotor (90 min, 100,000 rpm, 2°C). The top 25 μl was harvested and analyzed by SDS-PAGE and immunoblot.

Budding Assay

Perforated yeast spheroplasts (semi-intact cells) and yeast cytosol were prepared as described by Rexach *et al.* (1994) and Spang and Schekman (1998). Semi-intact cells were incubated with either 25 μg/ml Sar1p, 25 μg/ml Sec23/24p, and 75 μg/ml Sec13/31p (COPII), 25 μg/ml coatomer and 3 μg/ml Arf1p (COPI), or 2 mg/ml cytosol for 30 min at 30°C in the presence of 50 μM GTP and an ATP regeneration system (Baker *et al.*, 1988). The reaction mixture was chilled for 5 min on ice, and subjected to a medium-speed centrifugation (12,000 × *g*, 30 s, 4°C), which retained the vesicles in the supernatant fraction. The vesicles were sedimented by a centrifugation in a TLA 45 rotor (30 min, 45,000 rpm, 2°C). The pellet was resuspended in sample buffer and analyzed by SDS-PAGE followed by immunoblot.

RESULTS

Mst27p Is a Multicopy Suppressor of sec21-3

We performed a multicopy suppressor screen with a *sec21-3* mutant (Gaynor and Emr, 1997; Spang *et al.*, 2001). *SEC21* encodes the γ-subunit of coatomer. The screen should allow the identification of regulators of COPI vesicle budding as well as cargo that binds directly to coatomer. The *sec21-3* allele was chosen because it exhibits an immediate and complete block of transport from the Golgi at the nonpermissive temperature, and a cargo-specific block of anterograde transport (Gaynor and Emr, 1997). The screen led to the identification of Gea2p as an important player in retrograde transport from the Golgi to the ER (Spang *et al.*, 2001). One other plasmid that supported growth of *sec21-3* at the restrictive temperature was named γ1-25. The γ1-25 plasmid did not allow the loss of the *sec21-3* gene containing plasmid in a background where the chromosomal *SEC21* was de-

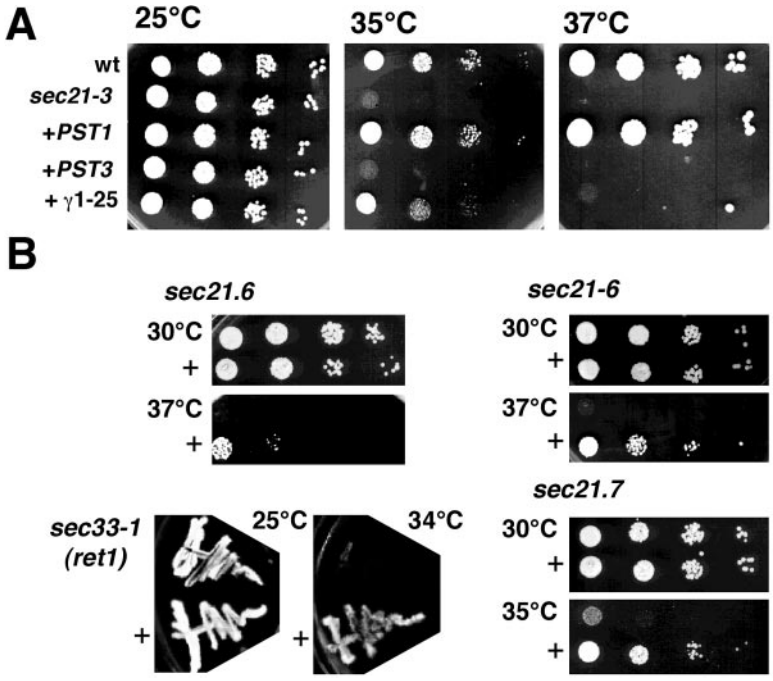

Figure 1. *MST27* is a multicopy suppressor of *sec21-3*. Cells were grown at 25°C to an OD_{600} of ~0.5. Three microliters of these cultures and 10-fold dilutions were spotted and incubated for 2 d at indicated temperatures. (A) From top to bottom, *sec21wt* (wt) and *sec21-3* cells both transformed with an empty YEp24 plasmid, *sec21-3* carrying either *MST27*, or *PRM8* in the YEp24 vector or with the originally isolated γ1-25 plasmid. The γ1-25-harboring suppressor allows growth at 35°C, and overexpression of Mst27p enables the *sec21-3* mutant to grow until 37°C. (B) The presence of γ1-25 (+) allows growth of several other γ-COP (*sec21*) mutants and of the α-COP/ret1 mutant *sec33-1* at otherwise restrictive temperatures.

leted. Thus, this suppressing plasmid did not encode a protein that bypassed the need for Sec21p function.

γ1-25 contained the five ORFs YGL050w to YGL054c. Two of these, YGL051w and YGL053w, are closely related to each other. Subcloning revealed YGL051w as the suppressing gene (Figure 1A). The plasmid containing only the YGL051w gene completely suppressed the *sec21-3* phenotype and allowed nearly wild-type growth at 37°C (Figure 1A). We named YGL051w *MST27* (*m*ulticopy suppressor of *s*ec *t*wenty one of 27 kDa).

To test whether other mutants defective in essential transport proteins in the early secretory pathway were suppressed by *MST27*, we transformed the γ1-25 plasmid into various mutants. Several different temperature-sensitive *sec21* strains were partially or completely suppressed (Figure 1B). In contrast, γ1-25 had no effect on the temperature sensitivity of the *sec21-1* mutant, which might be due to the strong anterograde transport defect observed in this mutant allele. In addition, the α-COP mutant *sec33-1* was suppressed by γ1-25 at a moderate temperature (Figure 1B). The mutants *sec27-1* (β′-COP), *sec12-1*, *sec23-3*, *sec22-2*, and *bet1-1* were not suppressed by γ1-25. Sec12p and Sec23p are the nucleotide exchange factor and the GTPase-activating protein for Sar1p, the small GTPase involved in COPII vesicle formation, respectively. Sec22p and Bet1p are v-SNAREs in the ER-Golgi shuttle. Thus, *MST27* represents a novel gene, which specifically suppresses the growth defects of certain COPI mutants.

Mst27p Belongs to a Large Family of Membrane Proteins

Mst27p belongs to one of the most curious gene families in yeast (Goffeau *et al*., 1996; Feuermann *et al*., 1997): the Ycr7 family comprises 16 members on six chromosomes. Some of them are scattered singly, such as YCR007c on chromosome III, but most are clustered and some even form long arrays (YHL042c through YHL046c or YAR023c through YAR033w). All these genes encode proteins with one or two membrane spanning domains, but nothing is known about their function. As mentioned above, γ1-25 contained the genes of two members of this family: *MST27* (YGL051w) and YGL053w, which is identical to *PRM8* (Heiman and Walter, 2000). *MST27* overlaps with the predicted ORF YGL052w, which is probably not a functional gene (Zhang and Smith, in http://bmerc-www.bu.edu/genome/yeast-analysis.html). Thus, *MST27* and *PRM8* are directly adjacent genes. The ORFs YAR033w (*MST28*) and YAR031w (*PRM9*) are highly homologous to *MST27* and *PRM8*, respectively (Figure 2, A and B). The predicted proteins Mst27p and Mst28p differ in only six amino acid residues. Because the noncoding region is also very highly conserved, the chromosomal region seemed to be subject to gene duplication (Figure 2B; Sonnhammer *et al*., 1998). *MST27*and *MST28* as well as *PRM8* encode proteins of 27 kDa that contain two predicted transmembrane domains separated by approximately six amino acids (Figure 2C). Prm9p contains an N-terminal extension, resulting in apparent molecular mass of ~34 kDa. According to a topology prediction (Hartmann *et al*., 1989), the N- and C-terminal tails face the cytoplasm. Both proteins contain C-terminal domains, which show a high probability to form coiled-coil domains (Lupas *et al*., 1991). Prm8p and Prm9p were identified as membrane proteins that are upregulated in response to mating factor (Heiman and Walter, 2000). Mst27p and Mst28p expression was not significantly altered under these conditions. Neither *MST27* and *MST28* nor *PRM8* and *PRM9* are essential. Even the double deletions of Δ*mst27*Δ*mst28* and Δ*prm8*Δ*prm9* did not show any altered growth phenotype under standard growth condi-

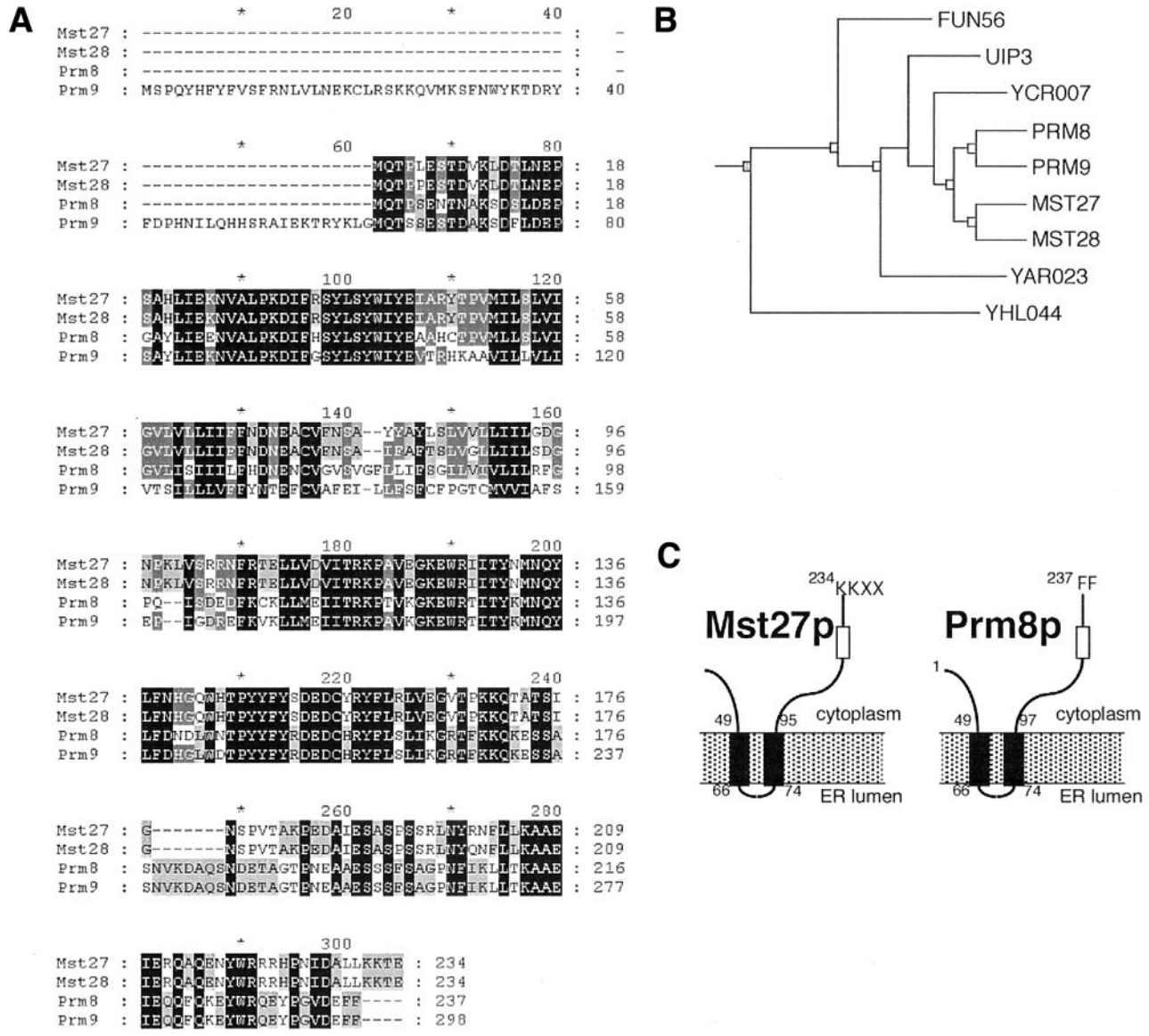

Figure 2. *MST27* and *PRM8* are members of a curious protein family. (A) Sequence alignment of Mst27p with its closest homologs in yeast (Mst28p, Prm8p, and Prm9p). (B) Family tree of a part of the DUP domain containing proteins, modified from PFAM database (Sonnhammer *et al.*, 1998). (C) Predicted topologies of Mst27/28p and Prm8p. The numbers refer to amino acids as counted from the N terminus.

tions. The lack of an obvious phenotype for the Δ*mst27* Δ*mst28* deletion might not be very surprising because the expression of *MST27* and *MST28* is down-regulated upon domestication of *Saccharomyces cerevisiae* (Kuthan *et al.*, 2003). The amount and structure of the extracellular matrix seem to change upon domestication. Interestingly, the four proteins contain typical coat binding motifs at their very C-terminus: Mst27p and Mst28p carry a KKXX motif, suggesting an interaction with the COPI coat; Prm8p and Prm9p contain a FF-sequence, a motif that was shown to allow COPII binding (Fiedler and Rothman, 1997; Kappeler *et al.*, 1997).

Mst27p, Mst28p, Prm8p, and Prm9p Form Two Distinct Complexes

Given the COPI binding motifs of Mst27p and Mst28p and the COPII interacting sequences of Prm8p and Prm9, we were wondering whether these proteins could form heteromeric complexes similar to the p24 family of proteins (Marzioch *et al.*, 1999). We solubilized yeast membranes with octylglucoside and performed gel filtration experiments with the detergent extracts. The fractions of the column were analyzed by immunoblot. Because we could not detect any endogenous Mst27p or Prm8p with antibodies raised

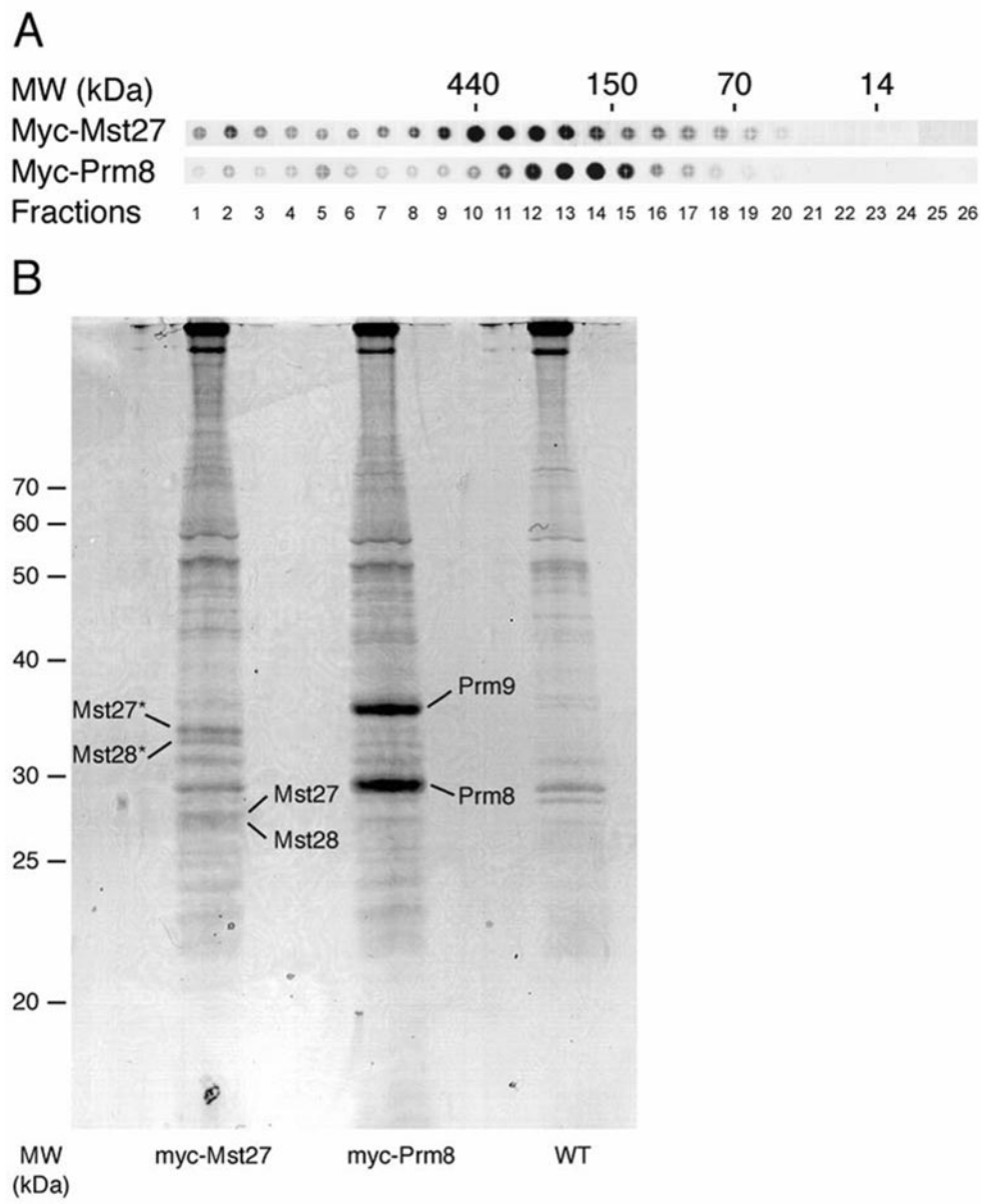

Figure 3. Mst27p and Mst28p as well as Prm8 and Prm9 form complexes. (A) Gel filtration analysis of the Mst27/28p and Prm8/9p complexes. The expression of myc-Mst27p or myc-Prm8p was induced upon the addition of galactose to the medium. An octylglucoside lysate from the different strains was separated in a gel filtration column and the fractions analyzed by dot immunoblot using an anti-myc antibody. (B) Determination of components of the Mst27/28p and the Prm8/9p complexes. Large-scale immunoprecipitation with the overexpressing strains for myc-Mst27p, myc-Prm8p, and a wild type were performed and separated on a large SDS gel and stained with Coomassie Blue. Specific bands were cut out and analyzed by MALDI-TOF.

against specific peptide sequences of Mst27p and Prm8p, we overexpressed Mst27p or Prm8p under the inducible *GAL10* promotor before the fractionation on the gel filtration column. Because we used a chromosomal tagging procedure, the expression of Mst27p or Prm8p was dependent on the addition of galactose. Under these conditions, Mst27p eluted from the column exclusively at ~300 kDa, indicating that Mst27p formed a complex (Figure 3A). We never found any Mst27p signal at around 27 kDa, which would correspond to monomeric Mst27p. Although molecular weight determinations by gel filtration of membrane proteins in detergent solutions are misleading, we took the big discrepancy between the observed and predicted molecular weight as an indication that Mst27p might be part of a complex. To determine the composition of the potential Mst27p complex, we performed large-scale native immunoprecipitations by using a strain carrying a myc-tagged Mst27p under *GAL* promotor control. The precipitate was separated by SDS-

PAGE (Figure 3B). Coomassie Blue-stained bands were excised, digested with trypsin, and subjected to mass spectrometric analysis. Four prominent bands were observed at a mass of ~27 and 34 kDa (Figure 3B), all of which corresponded to either Mst27p or Mst28p. The identification was unambiguously possible because Mst27p contained a myc-tag and thus possessed a slower electrophoretic mobility than Mst28p. Other proteins in the immunoprecipitates were present in much lower amounts than the Mst proteins and might represent contaminations. These results indicate that Mst27p and Mst28p form a complex and that both are at least in part posttranslationally modified. The nature of the modification remains unclear, because we could detect neither ubiquitination nor glycosylation by using different antibodies directed against ubiquitin and a glycosylation detection kit (our unpublished data). The endogenous levels of Mst27p and Mst28p were not detectable. Remarkably, after overexpression of Mst27p, Mst28p was also present in the cell in a higher concentration. Thus, it seems likely that Mst27p and Mst28p form a heteromeric complex and that Mst28p could be stabilized by Mst27p and vice versa. Similar effects have been observed for the p24 family of proteins. The levels of Erp1p and Erv25p are reduced upon deletion of *EMP24* and Erv25p requires Emp24p for its stability (Belden and Barlowe, 1996; Marzioch *et al.*, 1999).

Similar to the Mst27/28p complex Prm8p was part of a complex, though of a different molecular weight. Mass spectrometric analysis of the most prominent bands of this complex revealed Prm8p and Prm9p as major components, indicating a reciprocal stabilization also for these related proteins (Figure 3). We could not detect any Prm8p or Prm9p in the Mst27/28p complex and vice versa. However, upon co-overexpression we observed minor amounts of Mst27p in a co-immunoprecipitation with Prm8p, indicating that these proteins can interact with each other in the cell. In addition, because we overexpressed *MST27* and *PRM8* from the strong GAL promotor, we might have missed other naturally interacting proteins.

The Mst27/28p and the Prm8/9p Complexes Accumulate in the ER upon Overexpression

If the suppression ability of Mst27/28p was due to the KKXX motif, the Mst27/28p complex should at least transiently localize to the Golgi apparatus. Thus, we attempted to determine the localization of the Mst27/28p and Prm8/9p complexes. Because Mst27p and Mst28p form a complex, we assumed that by detecting Mst27p we also could localize Mst28p. Different antibodies that were generated against Mst27p were not able to detect the endogenous protein by immunofluorescence, indicating a very low abundance of this protein. Therefore, we used the myc-tagged Mst27p under *GAL10* promotor control. After induction of the protein, Mst27p was mainly found in the ER (Figure 4A). Because membrane proteins often accumulate in the ER after overexpression, we repressed transcription of *MST27* by addition of either rapamycin or glucose to the medium. Samples were taken after various time points after repression and processed for immunofluorescence. Even after 6 h of repression, a subfraction of the Mst27p persisted in the ER while the remaining Mst27p was chased out of the ER and localized in a punctate pattern, typical for later compartments of the secretory pathway, most likely Golgi or endosomal membranes (Figure 4, compare A with C). Because most of the signal persisted throughout the chase period, we assume that Mst27/28p cycles between the ER and Golgi

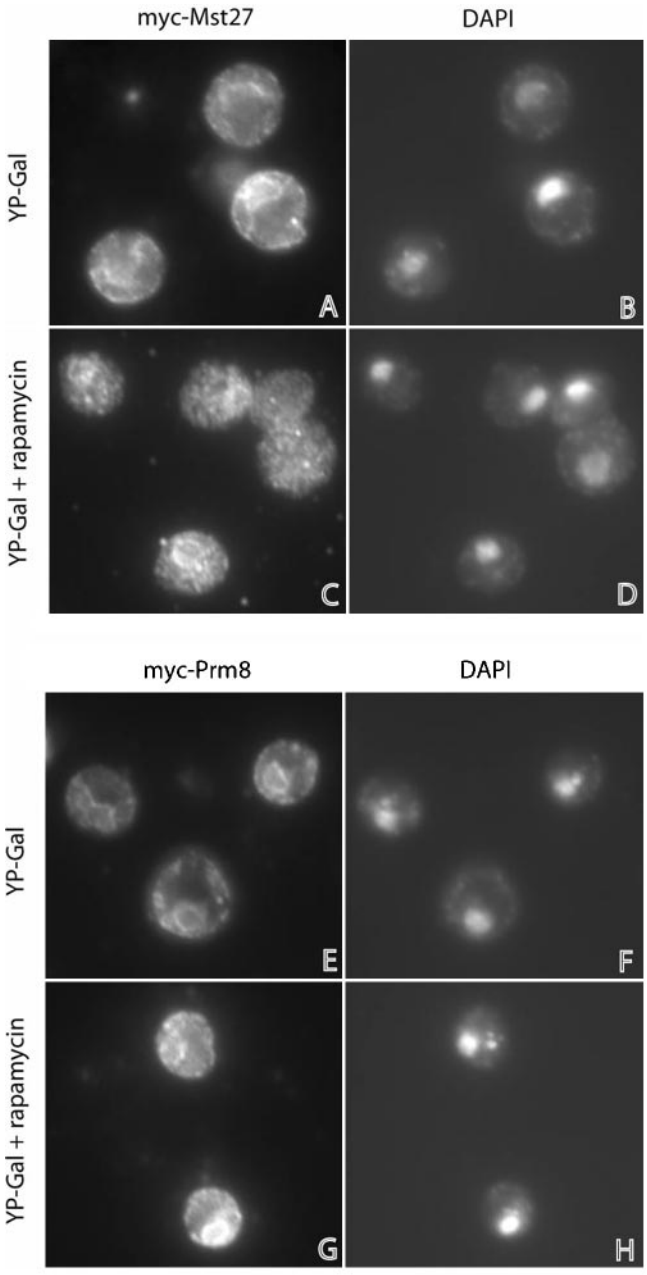

Figure 4. Mst27p cycles between ER and Golgi, whereas Prm8p remains in the ER. Immunofluorescence of strains HHY217 (expressing myc-Mst27p under *GAL10* promotor control) and HHY218 (expressing myc-Prm8p under *GAL10* promotor control). The expression of the myc-tagged proteins was induced overnight. Rapamycin was added to the cultures for up to 6 h. The cells were processed for immunofluorescence with an anti-myc antibody and anti-mouse antibodies coupled to CY3. The DNA was visualized with 4,6-diamidino-2-phenylindole.

apparatus. However, the steady-state localization of Mst27/28p is most likely in the ER.

We extended these experiments by overexpressing Prm8p, which, like Mst27p, accumulated in the ER. In contrast to Mst27p, the entire Prm8p pool remained in the ER during the 6-h chase period (Figure 4, compare C with E and G). This suggests that endogenous Prm8p might localize to the ER at steady state. Our results are consistent with data by Kumar *et al.* (2002), who localized Prm8p in the ER after overexpression in a genome-wide localization approach.

Mst27p Contains a COPI and a COPII Binding Motif

To test whether the C-terminal KKXX motif in Mst27p was able to bind COPI components, we expressed the C-terminal 123 amino acids of Mst27p fused to GST in *E. coli*. As a control, a fusion protein was expressed, in which the lysine residues of the KKXX motif were replaced by alanines (AAXX). Affinity chromatography with immobilized GST fusion proteins revealed a specific interaction of the coatomer complex with Mst27p (Figure 5A, lanes 1 and 2). In contrast, no coatomer binding to GST-Mst27p_AAXX was detected. (Figure 5A, lanes 5 and 6). To rule out that the signal for the AAXX protein was only reduced, we repeated the experiment performing an immunoblot analysis. In addition, we included an Mst28p fusion protein in our assay, which also contains the C-terminal KKXX. Again, only the KKXX containing fusion proteins interacted with coatomer (Figure 5B, compare lanes 1 and 2 to 3 and 4).

Because overexpression of *MST27* resulted in a partial relief from the COPII budding defect in *sec21-3* mutants in vitro, we wondered whether Mst27p was able to bind members of the COPII coat. We performed GST pull-down assays in the presence of the small GTPase Sar1p and the Sec23/24p complex of the COPII coat. No significant amounts of Sec24p bound to GST alone (Figure 5C, lanes 14 and 15). In contrast, all three fusion proteins, GST-Mst27p, GST-Mst28p, and GST-Mst27p_AAXX, recruited the Sec23/Sec24p complex in a Sar1p-independent manner (Figure 5C, lanes 1–9). Sar1p did not bind to the fusion proteins, nor was this reaction guanine nucleotide dependent (our unpublished data). The tails of Mst27p and Mst28p do not contain any obvious COPII binding motif. These results indicate that the Mst proteins expose high-affinity binding sites for both coats in the ER-Golgi shuttle.

Using a similar experimental setup, we determined the coat recruitment abilities of Prm8p. Prm8p contains a C-terminal diphenylalanine (FF) motif, which has been reported to allow interaction with the Sec23/24p complex of the COPII coat. Consistently, Prm8p was able to specifically recruit Sec23/24p complex (Figure 5C, lanes 10–12). This interaction was independent of the small GTPase Sar1p. However, no interaction with coatomer was observed (Figure 5, A and B).

COPI Binding and **sec21-3** *Suppression*

To test whether the COPI binding of Mst27p is crucial for its ability to suppress COPI mutants, we expressed in *sec21-3* cells a version of Mst27p in which the lysines of the KKXX motif were replaced by alanines (AAXX). This mutant Mst27p did not suppress the *sec21-3* strain (Figure 5D). Thus, COPI-binding was required for suppression of the *sec21-3* mutant. However, we wondered whether overexpression of any KKXX-containing membrane protein would be sufficient to suppress the *sec21-3* mutant. Wbp1p is the only subunit of the octameric ER resident oligosaccharyl-transferase complex that contains a KKXX COPI-binding motif and is not transported from the ER to the Golgi apparatus (te Heesen *et al*., 1992; Gaynor *et al*., 1994). Overexpression of Wbp1p failed to increase the temperature resistance of the strain (+*WBP1*; Figure 5D), but even reduced the restrictive temperature of the mutant from 35°C to 32°C. Therefore, it does not seem to be sufficient to provide coatomer binding sites on a membrane per se to rescue the *sec21-3* mutant. Wbp1 is an ER resident protein and does not leave the ER. The increase of COPI binding sites exclusively at the ER might recruit coatomer to the ER and thereby dramatically reduce the COPI vesicle formation at the Golgi. Hence, suppression of coatomer mutants would only be expected if COPI binding sites would be provided by a protein that at least transiently resides in Golgi membranes. In accordance with this hypothesis, overexpression of Emp24p suppressed the *sec21-3* mutant (+*EMP24*). Although, Emp24p does not contain a KKXX sequence, it might interact like its mammalian homolog directly with coatomer (Fiedler and Rothman, 1997). However, overexpression of *ERV25* and *ERP1*, two other members of the p24 family only scarcely suppressed the *sec21-3* phenotype at 35°C. However, Erv25p and Erp1p depend on Emp24p for their stability (Belden and Barlowe, 1996; Marzioch *et al*., 1999). In summary, the up-regulation of only certain COPI-binding proteins can suppress the *sec21-3* mutant. Hence, overexpression of a coatomer binding motif and transient localization of these proteins at the Golgi might not be sufficient to relieve the *sec21-3* defect.

Overexpression of **MST27** *Suppresses Secretion Defects in the* **sec21-3** *Mutant*

After a short incubation at the restrictive temperature, the *sec21-3* mutant shows a cargo-specific anterograde transport defect in vivo. Precursors of α-factor and the vacuolar protease carboxypeptidase Y (CPY) accumulate, whereas other proteins such as invertase are secreted at normal rates. To analyze whether overexpression of *MST27* rescues the secretion defects in *sec21-3*, we grew either wild-type, *sec21-3*, or *sec21-3* (+γ1-25) cells at 25°C, shifted the cultures to 37°C for 5 min, and labeled newly synthesized proteins for 3 min in the presence of [^{35}S]methionine. After the addition of an excess of cold methionine, aliquots were taken after various incubation times. The cells were lysed and the extracts used for immunoprecipitations with α-factor- or CPY-specific antisera (Figure 6A). In wild-type cells, the secretion of α-factor is fast, and after 5 min no glycosylated ER form of the α-factor precursor (gpαF) was detected. In contrast, in the *sec21-3* mutant, gpαF accumulated in the ER, leading to an increased signal that remained stable even after long chase periods. In the presence of the suppressing plasmid, however, gpαF was again efficiently secreted from the ER and completely processed after a 10-min chase.

CPY is synthesized as a proenzyme that is cotranslationally translocated into the ER and glycosylated, leading to the p1 precursor form. On transport to the Golgi apparatus, the glycan chains of CPY are elongated to yield the p2 form, which is finally processed in the vacuole to mature CPY. In

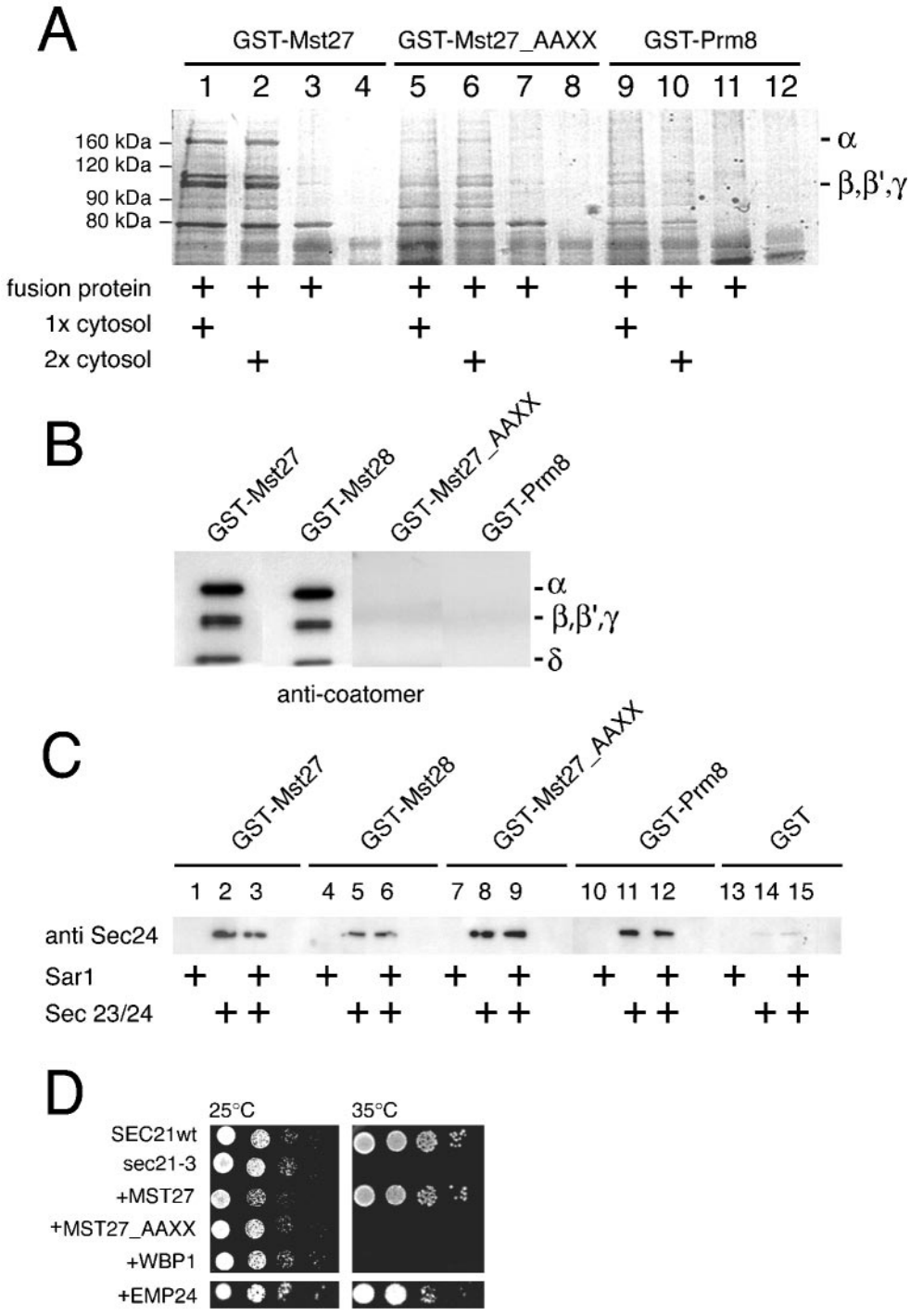

Figure 5. Mst27p and Mst28p bind to COPI and COPII proteins, and the C-terminal KKXX motif is required for the suppression of *sec21-3*. (A) The KKXX motif in Mst27p is responsible for coatomer binding. GST-fusion proteins of Mst27p, Mst27p-AAXX, and Prm8p were expressed in *E. coli*. The proteins were immobilized onto glutathione agarose beads and incubated with cytosol (1× cytosol, 1.25 mg/ml; 2× cytosol, 2.5 mg/ml) where indicated. After washing of the beads, the retained proteins were eluted with Laemmli buffer and analyzed by SDS-PAGE and Coomassie Blue staining. (B) GST-Mst27p-AAXX and GST-Prm8p do not bind to coatomer. Samples were treated as in A but the analysis was performed by immunoblot with antibodies directed against the coatomer complex. (C) Msts and Prm8p recruit Sec23/24p complex in a Sar1p-independent manner. The GST-fusion proteins and GST were immobilized onto glutathione agarose beads and incubated with Sar1p and Sec23/24p complex as indicated. The proteins bound to the beads were analyzed by immunoblot with anti-Sec24p antibodies. (D) Overexpression of Emp24p but not of Mst27-AAXX also suppresses the *sec21-3* mutant. *SEC21*wt or *sec21-3* cells containing an empty YEp24 vector, or *sec21-3* cells overexpressing Mst27p, Mst27p-AAXX, Wbp1p, or Emp24p were grown at 25°C to an OD_{600} of ~0.5. Three microliters of these cultures and 10-fold dilutions were spotted and incubated at indicated temperatures for 2 d.

wild-type cells CPY acquires the Golgi-specific modification after ~5–10 min and after 20 min it is mostly found in its mature form (Figure 6A). However, in the *sec21-3* mutant the p1 form is stable throughout the 40-min chase period and no p2 or mature forms are generated. We also detected a species of higher electrophoretic mobility that may represent a degradation product (Figure 6A, *). Overexpression of *MST27* enabled the *sec21-3* mutant to produce p2 and mature forms. However, the transition from the p1 to the p2 form took about twice as long as in the wild-type, whereas the maturation of the p2 forms occurred without delay. Thus, the presence of increased amounts of Mst27p rescued the α-factor and CPY secretion defects of the *sec21-3* mutant and re-established nearly normal protein transport along the early secretory pathway.

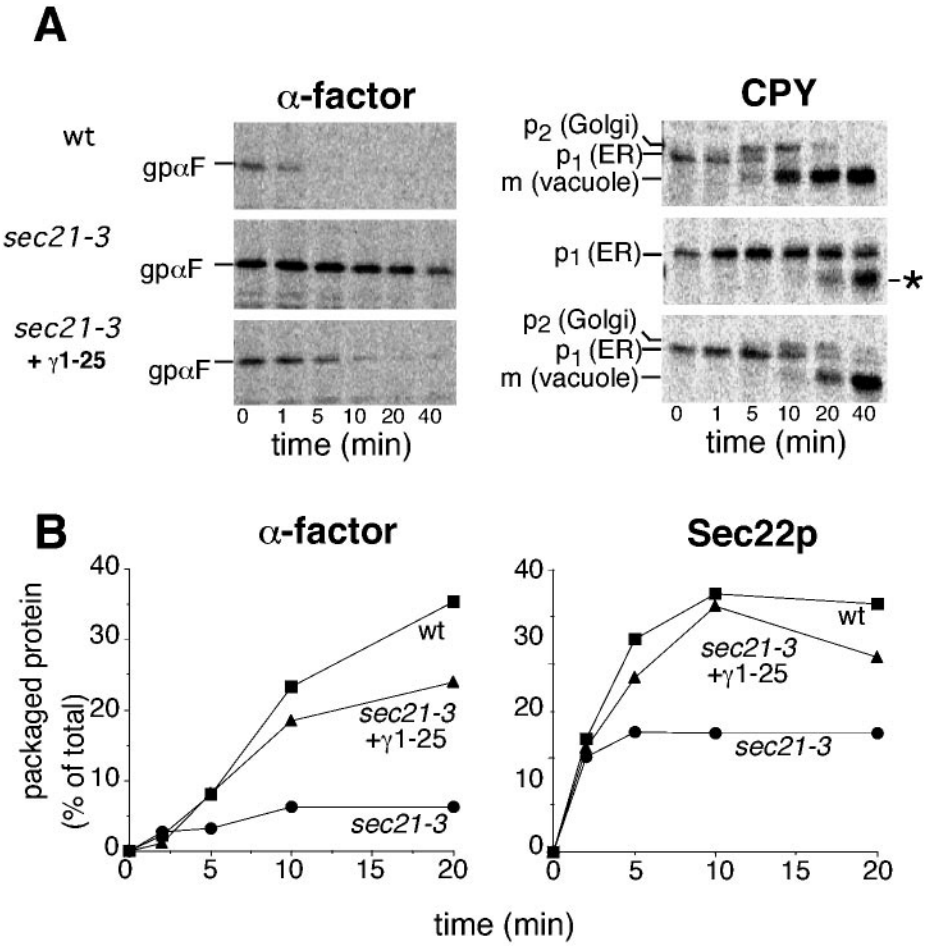

Figure 6. The γ1-25 plasmid suppresses the secretion defects in the *sec21-3* mutant. Wild-type (wt) or *sec21-3* mutant cells without or with the γ1-25 plasmid (+γ1-25) were grown at 25°C and shifted to 37°C for 5 min. The cells were pulse-labeled with tran^{35}S-label for 3 min and chased at 37°C for 0–40 min as indicated. The cells were lysed and the extract used for immunoprecipitations with antiserum specific for α-factor (left) or CPY (right). The different maturation forms of CPY are indicated (p1, ER form; p2, Golgi form; m, mature in the vacuole). (B) Microsomal membranes were isolated from these cells and tested for in vitro packaging of α-factor and Sec22p. After translocation of radiolabeled α-factor, the membranes were incubated at 20°C with 8 μg/ml COPII components for the times indicated. The amounts of ^{35}S-gpαF and Sec22p in the vesicles were quantified.

The observed block in ER to Golgi transport of α-factor precursor and CPY in the *sec21-3* mutant might either be due to a diminished packaging efficiency of these proteins into the vesicles at the ER membrane or to defects in the fusion of ER-derived vesicles with the Golgi. To distinguish between both possibilities, we monitored packaging of gpαF into COPII vesicles generated from microsomes in vitro. Wild-type, *sec21-3* and *sec21-3* (+γ1-25) cells were grown overnight at 25°C. After incubation of cells at 37°C for 20 min, microsomes were isolated and used to incorporate radiolabeled prepro-α-factor in vitro (ppαF). The microsomes were then incubated with COPII components, ATP, and GTP for various times, and the amounts of gpαF and Sec22p were measured by scintillation counting and quantitative immunoblotting. A typical result is shown in Figure 6B. In *sec21-3* mutant microsomes the amounts of gpαF and Sec22p in the vesicle fraction were clearly diminished compared with wild type. We conclude that either vesicle formation or the cargo uptake process into the COPII vesicles is affected in this COPI mutant.

Overexpression of *MST27* almost completely restored the budding efficiency of gpαF and Sec22p from *sec21-3* microsomes (Figure 6B). Thus, overexpression of *MST27* increases either the amount of COPII vesicles generated from the ER or the packaging efficiency of cargo in the *sec21-3* mutant.

MST27 Rescues sec21-3 by Enhancing the Efficiency of COPI and COPII Vesicle Production

We wanted to further investigate the mechanism of the *sec21-3* suppression by *MST27*. Therefore, we used an in vitro budding assay from semi-intact cells. Permeabilized yeast cells from *sec21-3* strains expressing either no gene, *MST27*, *PRM8*, or *MST27-AAXX* from a 2 μ plasmid were incubated with cytosol from *sec21-3* or wild type under conditions that should be restrictive for the *sec21-3* mutant in vitro. The free diffusible vesicles were separated from the membranes by a medium-speed centrifugation. The generated vesicles were enriched by ultracentrifugation and analyzed by immunoblot with antibodies against Sec22p and Emp47p, a KKXX motif-containing protein that cycles between the ER and the Golgi apparatus. As expected, wild-type cytosol resulted in vesicle release from the different semi-intact cells (Figure 7A, lanes 4, 7, 10, and 13). In contrast, in the presence of *sec21-3* cytosol, vesicles were only obtained from membranes containing extra Mst27p (Figure 7A, compare lanes 3, 9, and 12, to lane 6). Although, Prm8p contains a diphenylalanine signal at the C-terminus, which should enhance the recruitment of COPII components, it could not rescue the budding defect. The replacement of KKXX by AAXX in the tail of Mst27p abolished the forma-

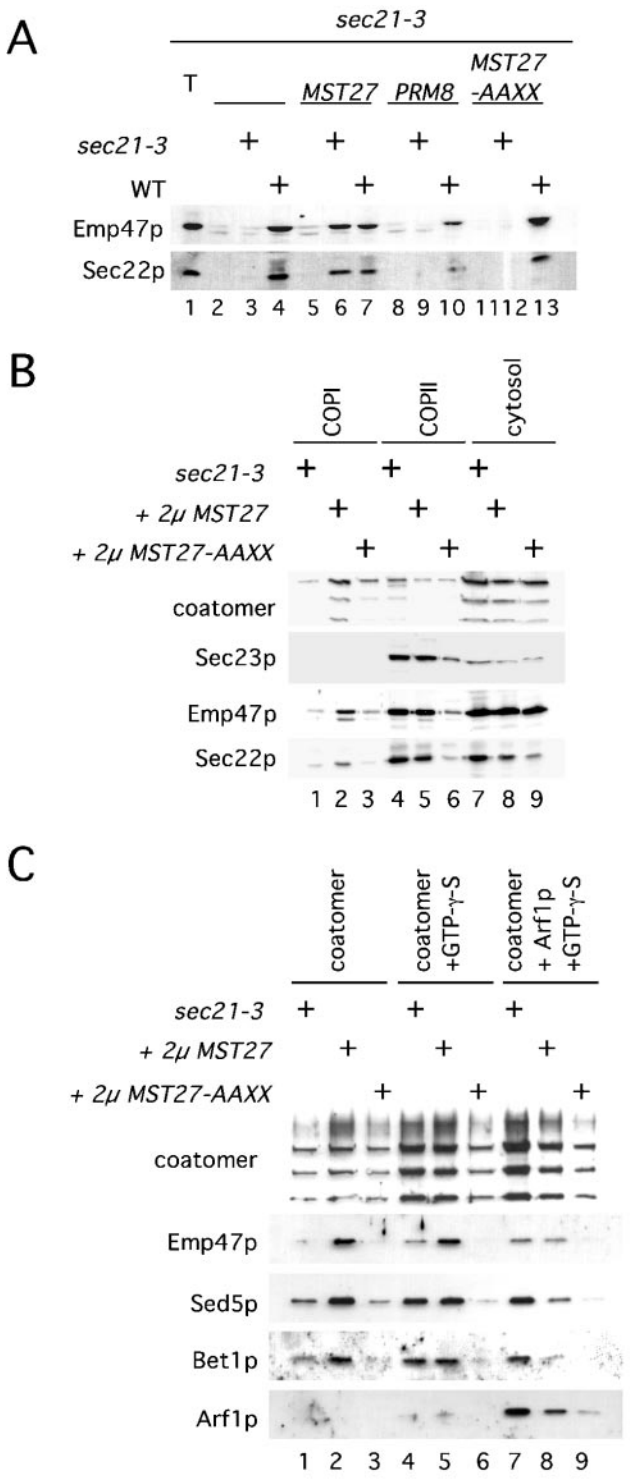

Figure 7. *MST27* facilitates vesicle formation from *sec21-3* membranes. (A) *MST27* rescues budding defects in the presence of *sec21-3* cytosol. Semi-intact cells were prepared from *sec21-3* strains expressing either nothing, *MST27*, *PRM8*, or *MST27-AAXX* from a 2 μ plasmid. These semi-intact cells were incubated with either buffer (lanes 2, 5, 8, and 11), *sec21-3* cytosol (lanes 3, 6, 9, and 12) or wild-type cytosol (lanes 4, 7, 10, and 13) for 30 min at 30°C. Vesicles

tion of vesicles. This result indicates that overexpression of *MST27* leads to the formation of vesicles from *sec21-3* membranes.

However, because we used semi-intact cells and cytosol and scored for proteins, which cycle between the ER and Golgi, we could not distinguish between COPI or COPII vesicles. Therefore, we aimed to reconstitute vesicle formation with purified proteins. Semi-intact cells were incubated with either components of the COPI or the COPII coat or wild-type cytosol. Only *sec21-3 MST27* membranes were able to efficiently generate COPI-coated vesicles under non-saturating concentrations of coatomer and Arf1p (Figure 7B, compare lanes 1 and 3 to lane 2). Thus, Mst27p enhances the production of COPI vesicles. Next, we added saturating amounts of COPII components to the different semi-intact cells. Under these conditions, *MST27* overexpression did not have a positive effect (Figure 7B, lane 5). However, even from *sec21-3* membranes the COPII vesicles were released quite efficiently (Figure 7B, compare lanes and 5). Surprisingly, the overexpression of *MST27-AAXX* did not promote the formation of COPII vesicles (Figure 7B, lane 6), indicating that Mst27p does not influence the uptake of cargo into COPII vesicles but helps to increase the amount of COPII vesicles. This negative effect of *MST27-AAXX* might be counteracted by one or more cytosolic factors because wild-type cytosol resulted in COPI and COPII vesicle formation from all the membranes (Figure 7, A and B). In addition, Sec21p is part of a cytosolic protein complex, thus adding back wild-type cytosol should rescue the *sec21-3* defect. However, addition of limiting amounts of wild-type COPI (Figure 7B, lanes 1–3) might not be sufficient to alleviate the *sec21-3* phenotype.

We assumed that Mst27/28p cycles between the ER and the Golgi. Therefore, overexpression of *MST27* might result in an increase of coatomer at the Golgi, which could be the explanation for the rescue, we observed. We enriched Golgi membranes from the *sec21-3* strains overexpressing *MST27* or *MST27-AAXX*. We resolved equal amounts of Golgi by SDS-PAGE and compared the relative abundance of different Golgi proteins and coatomer by immunoblot. Although the amount of coatomer and Sed5p, the *cis*-Golgi t-SNARE were comparable in all three Golgi membranes, the amount of Emp47p was increased in Golgi membranes from the *sec21-3 MST27* strain (our unpublished data). Thus, coatomer did not seem to be enriched on the *sec21-3 MST27* Golgi. However, we might have extracted coatomer from the Golgi during the enrichment procedure. Therefore, we added purified coatomer, GTPγS, and Arf1p back to the Golgi membranes and floated the membranes through a

Figure 7 (cont). released into the supernatant of a medium-speed centrifugation were concentrated by ultracentrifugation and analyzed by immunoblot. In lane 1, 10% of the total was loaded. (B) *MST27* increases the release of COPI vesicles. Semi-intact cells were incubated with COPI or COPII components or wild-type cytosol for 30 min at 30°C. The vesicle formation was analyzed as described in A. (C) *MST27* influences cargo uptake at the Golgi membranes. Golgi membranes were prepared from *sec21-3* strains expressing either nothing, *MST27*, or *MST27-AAXX* from a 2 μ plasmid. The Golgi membranes were incubated with coatomer, GTPγS, and Arf1p for 30 min at 30°C as indicated. Membranes were floated through a sucrose cushion, resolved by SDS-PAGE and analyzed by immunoblot.

sucrose cushion. Most of the Golgi would remain on the bottom of the tube together with the unbound cytosolic proteins. If only coatomer was added to the different Golgi membranes, vesicles were released from *sec21-3 MST27* Golgi and floated. They contained Emp47p as well as the SNAREs Sec22p and Sed5p (Figure 7C, lane 2). Although Sed5p is a t-SNARE, it has been shown to recycle through the ER (Wooding and Pelham, 1998). Only a few vesicles were released from the Golgi membranes from the other strains (Figure 7C, compare lanes 1 and 3 to lane 2). On addition of GTPγS, comparable amounts of vesicles were formed from the *sec21-3* and *sec21-3 MST27* Golgi. However, Emp47p seemed to be more concentrated in the vesicles derived from the Golgi that contains more Mst27p (Figure 7C, compare lanes 4 and 5). The addition of Arf1p should accentuate the COPI vesicle production. Under saturating amounts of COPI components and GTPγS, *sec21-3* Golgi could produce even more vesicles than the *sec21-3 MST27* Golgi; however, the cargo packaging as judged by inclusion of Emp47p seemed to be more efficient upon overexpression of *MST27*. The observed effects might have been more pronounced by the use of coatomer derived from a *sec21-3* mutant. As observed for the formation of COPII-coated vesicles from *sec21-3 MST27-AAXX* membranes, COPI-coated vesicle release was abolished from *sec21-3 MST27-AAXX* Golgi membranes. Thus, the change of KKXX to AAXX might even result in a negative effect on COPI and COPII vesicle generation.

Our results indicate that overexpression of *MST27* facilitates the formation of COPI and COPII vesicles in a *sec21-3* mutant. In addition, we provide evidence that at least at the Golgi apparatus inclusion of cargo into COPI vesicles might be more efficient.

Co-overexpression of Mst27p and Prm8p Results in Abnormally Large Vacuoles and Cells

We checked the strains overexpressing *MST27*, *PRM8*, or both under the light microscope. Although single overexpression did not result in any obvious morphological phenotype, the simultaneously increased protein levels of Mst27p and Prm8p resulted in large cells with abnormally big vacuoles (Figure 8, compare F to A to C). This effect was not simply due to overexpression of two transmembrane domain-containing proteins because cells co-overexpressing Sec20p, an ER resident protein that contains an HDEL-ER localization signal, together with Mst27p or Prm8p, were almost indistinguishable from wild-type cells grown under the same conditions (Figure 8, compare D and E to A). We confirmed this phenotype by electron microscopy (Figure 9). The vacuole in the Mst27p- and Prm8p-overexpressing strain seemed to fill almost the entire cell and the nucleus was pushed to the edge of the cell. The vacuoles seemed to be empty, because they contained very little electrodense material in their lumen. One explanation for this phenotype would be that the overexpressed proteins would fill up the vacuole and that their expression rate was so high that they accumulated in the vacuole. Thus, we wondered whether Prm8/9p would piggy-back on Mst27/28p to the vacuole. However, interestingly, both protein complexes remained largely in the ER (Figure 10). Therefore, Mst27/28p and Prm8/9p may act in concert to efficiently export a membrane protein or protein complex that was transported to the vacuole. These membrane proteins might be part of the extracelluar matrix in the wild, but are no longer needed upon domestication and thus transported to the vacuole.

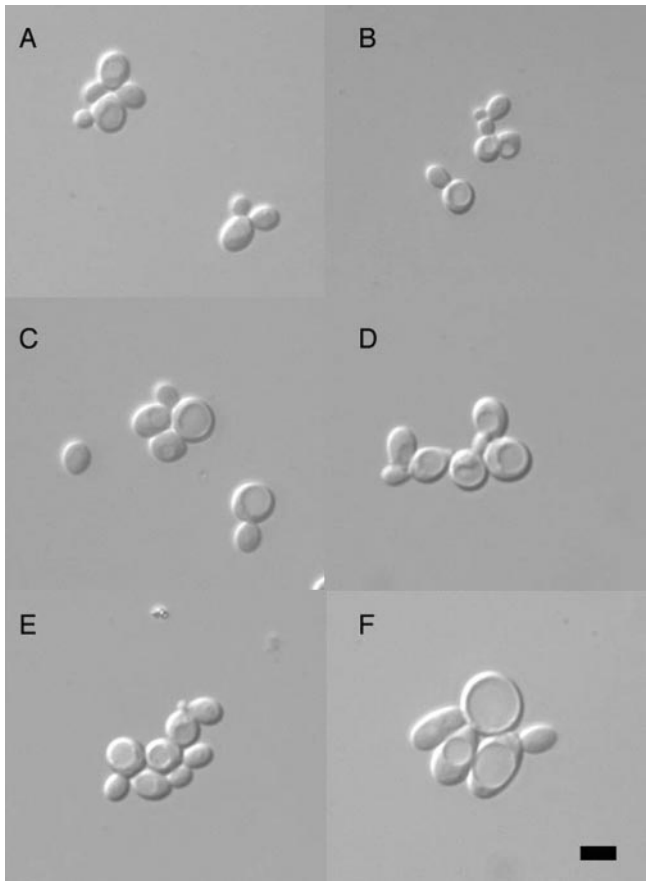

Figure 8. Co-overexpression of FLAG-Mst27p and myc-Prm8p leads to an increase in cell size and large vacuoles. Nomarski pictures of yeast cells. Cells were grown overnight in YP-galactose to induce the expression of FLAG-Mst27p, myc-Prm8p, or myc-Mst27p. The cells were subsequently observed under a light microscope with differential interference contrast. Wild type (A), HHY217 (*GAL10*-myc-Mst27p) (B), HHY218 (*GAL10*-myc-Prm8p) (C), YAS314 (HHY217 + 2 μ myc-Sec20p) (D), YAS315 (HHY218 + 2 μ myc-Sec20p) (E), and YAS254 (HHY218 + *GAL1*-FLAG-Mst27p (F). The bar in F represents 5 μm. The magnification in A–E is the same as in F.

DISCUSSION

We performed a multicopy suppressor screen with a temperature-sensitive coatomer mutant, *sec21-3* (Spang *et al.*, 2001). The objective of the screen was to identify regulators of COPI vesicle formation as well as novel coat-interacting proteins. The screen led to the identification of two suppressors: Gea2p, a guanine nucleotide exchange factor for Arf1p; and Mst27p, an uncharacterized protein. Gea2p can be considered as a positive regulator of COPI vesicle formation because it activates Arf1p and helps inserting the small

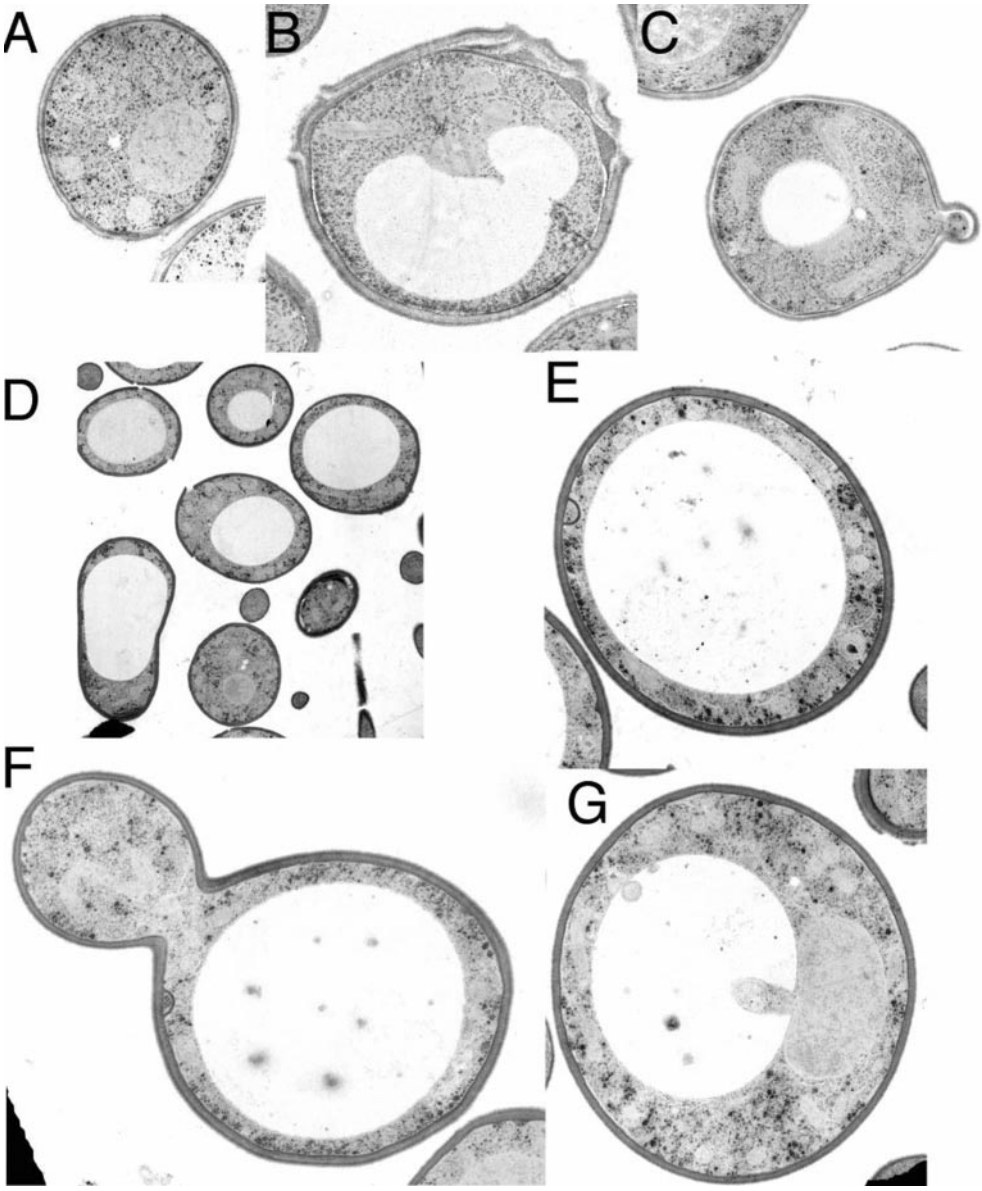

Figure 9. Electron microscopy of the strains YPH500, HHY218, and YAS254. Cells were grown under the same conditions than in Figure 8 and prepared for electron microscopy. Wild type (A), HHY218 (B and C), and YAS254 (D–F). The magnification in A–C, E, and F is the same as in G. The bars in D and G represent 1.87 μm and 685 nm, respectively.

GTPase into the membrane. Herein, we characterized Mst27p and could show that the C-terminal cytoplasmic exposed KKXX motif of the membrane protein is necessary to rescue a *sec21-3* mutant. Mst27p exists in a complex with Mst28p, with which it shares 97% identity. Both proteins contain two transmembrane domains, and our genetic data as well as the localization data suggest that Mst27/28p cycles between the ER and the Golgi apparatus. The suppression ability of Mst27p is probably due to stabilization of coatomer at the Golgi membrane and thus allowing the efficient formation of COPI coated vesicles. In addition, the formation of COPII vesicles was also increased upon *MST27* overexpression. Therefore, Mst27p might only be able to suppress mutants where the affinity of coatomer toward a KKXX binding motif is reduced. This is supported by overexpression data of Emp24p, which also rescues the *sec21-3* phenotype. However providing more coatomer binding sites on a membrane per se was not sufficient for the rescue of the mutant cells. The ER resident subunit of the oligosaccharyl transferase, Wbp1p, which possesses a C-terminal KKXX, was unable to relief the *sec21-3* phenotype. Wbp1p differs from Mst27/28p and Emp24p by at least two features: 1) Wbp1p does not contain a COPII binding motif and thus is unable to leave the ER. In contrast, suppressing proteins expose COPII binding sites. They apparently cycle between the ER and the Golgi. 2) Wbp1p does not form a complex with related proteins, and none of the other members of the oligosaccharyltransferase complex contains a coat binding signal. The Mst proteins and the p24 family members form oligomeric complexes with close relatives and expose coat-binding signals (Dominguez *et*

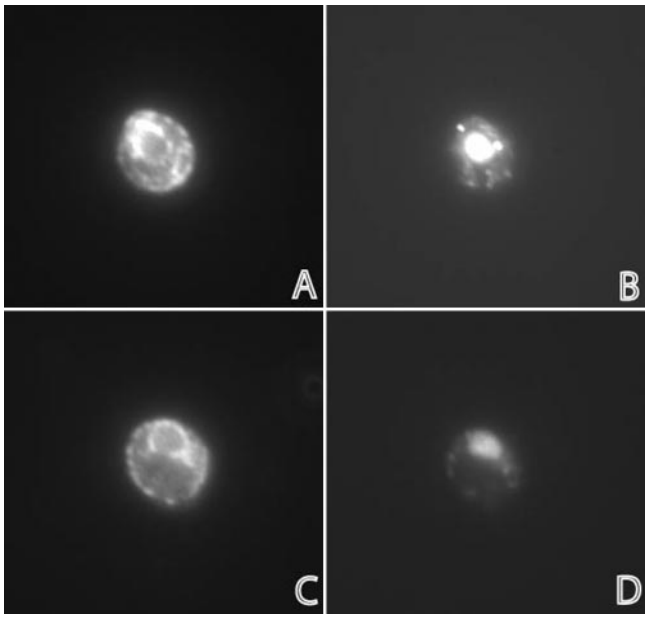

Figure 10. Co-overexpression of Mst27p and Prm8p does not result in the accumulation of the Mst27p or Prm8p complexes in the vacuole. Expression of Mst27p and Prm8p was induced overnight in strain YAS254. Cells were harvested and prepared for immunofluorescence. (A) Mst27p was visualized with an anti-FLAG antibody and CY3 coupled secondary antibodies. (C) Prm8p was stained with anti-myc and CY3 coupled secondary antibodies. (B and D) The DNA was stained with 4,6-diamidino-2-phenylindole.

al., 1998). Reinhard *et al.* (1999) have shown that the γ-COP (Sec21p) only interacts with the dimeric form of p23.

We propose that the Mst27/28p and p24 complexes can provide nucleation sites, which recruit and locally concentrate cytosolic coat complexes onto the membranes of the early secretory pathway. This stimulates the formation of vesicles and thereby mitigates the decreased vesicle flow in *sec* mutants. This hypothesis is supported by the strict dependence of the COPI-binding ability. Although the minimal machinery necessary for COPI vesicle formation in vitro are Arf1p, coatomer and guanine nucleotides (Spang *et al*., 1998), in vivo, the amount of vesicles formed might also be dependent on the amount of cargo to be transported. Currently, it remains unclear whether there are vesicles running between different compartments on a specific schedule or whether the vesicles are formed upon demand when cargo is present to be transported. These possibilities are difficult to distinguish because in vivo vesicle emergence and consumption is a fast and highly dynamic process. Yeung *et al.* (1995) have shown that ER membranes devoid of cargo are still competent for COPII vesicle formation in vitro. However, the amount of COPII vesicles might have been reduced under these conditions. For different vesicle populations regulatory proteins have been identified, which help the recruitment of cargo (for reviews, see Aridor and Traub, 2002; Spang, 2002). These might have a positive effect in the budding process. Our data provide evidence that cargo itself can act as driving force for vesicle formation.

The Mst27/28p complex is part of a large family of proteins, the DUP family, indicating that their members are scattered throughout the genome by gene duplication. So far, no close homologs have been identified in any other organism. Why is this protein family so large and well maintained in *S. cerevisiae*, but is not conserved in any other species? The easy answer could be that the role of the DUP family proteins is a highly specialized task. This would also explain why the phenotypes of the deletion mutants were so difficult to characterize. The Δ*mst27*Δ*mst28* homozygous diploid mutant was sensitive toward PMSF, $ZnCl_2$, EDTA, and H_2O_2 (Sandmann and Spang, unpublished data). The haploid cells did not show any growth defects when compared with the isogenic wild-type. Thus, the function of Mst27/28p complex might be more important in diploids than in haploid cells. This seems indeed to be the case. Recently, Kuthan *et al.* (2003) reported that wild *S. cerevisiae* possessed a fluffy colony morphology that was lost upon domestication of the yeast in the laboratory. The domesticated strains showed a smooth colony morphology. The fluffy colony appearance was due to extracellular matrix unrelated to the flocculins. They analyzed the expression pattern from wild and domesticated *S. cerevisiae* and showed that *MST27* and *MST28* were down-regulated upon domestication. This explains why we could not detect Mst27/28p without overexpression and why we could not find strong phenotypes. We observed only defects in diploid yeast. This might be because in the wild, *S. cerevisiae* exists mostly as diploid and only switches to the haploid cycle upon starvation and spore formation.

The next close homologs of Mst27/28p in yeast are Prm8p and Prm9p. They are induced upon treatment of cells with pheromone (Heiman and Walter, 2000). Similar to Mst27/28p, Prm8p and Prm9p form a complex with each other. Because overexpression of either *MST27* or *PRM8* resulted in higher levels of Mst28p and Prm9p in the cell, respectively, we assume that Mst27p and Prm8p stabilize their counterpart in the complex. Mst27p and Mst28p are both in part posttranslational modified. However, we were unable to determine the nature of the modification. Although our results exclude polyubiquitination of Mst27p and Mst28p, the addition of a single ubiquitin residue cannot be formally ruled out, because the antibodies that are commercially available are not very sensitive toward monoubiquitination. Monoubiquitination was shown to function as a signal for degradation of Ste2p in the vacuole (Hicke and Riezman, 1996). Degradation of Mst27p in the vacuole is supported by the observation that overexpression of Mst27p in a Δ*pep4* background, where the major vacuolar protease is deleted is lethal (Sandmann and Spang, unpublished data). Alternatively the mobility shift, we observed for a fraction of the Mst27/28p complex, might be of a different nature.

What are the functions of the Mst27/28p and Prm8/9p complexes? Both are located in the ER; one complex seems to cycle between ER and Golgi apparatus (Mst27/28p), whereas the other (Prm8/9p) resides stably in the ER. However, because we were unable to detect endogenous Mst27/Mst28p, we cannot exclude that this complex is ER resident and not cycling. The enlarged vacuole phenotype after overexpression of both complexes points toward a function in ER exit of other proteins. This would be in analogy to the exit of the V_0 sector of the vacuolar H-ATPase. There, Vma22p is

required for the assembly for the V_0 sector. Then Vma21p is needed for the export out of the ER of the assembled protein complex. Vma21p is a membrane protein containing a KKXX motif (Hill and Stevens, 1994). On arrival of the V_0-Vma21p complex at the *cis*-Golgi, Vam21p dissociates from the V_0 complex. The V_0 sector continues its journey to the vacuole, whereas Vam21p returns to the ER for another round of transport out of the ER. Similarly, the members of the p24 family are, at least in yeast, required for the efficient export of GPI-anchored proteins (Muniz *et al*., 2000; Muniz and Riezman, 2000). The role of the Mst27/28p complex might be to escort extracellular matrix proteins out of the ER. Alternatively, the passengers might be special Golgi enzymes that allow for a different branching of sugars of glycosylated proteins in the Golgi. The Prms might be required to assemble protein complexes for the export out of the ER. However, the precise role of the Mst and Prm proteins still awaits discovery.

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