# The Ccz1 protein interacts with Ypt7 GTPase during fusion of multiple transport intermediates with the vacuole in *S. cerevisiae*

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## SUMMARY

**Previously we have shown that the *Saccharomyces cerevisiae CCZ1* (*YBR131w*) gene encodes a protein involved in protein trafficking. Deletion of this gene leads to fragmentation of the vacuole typical of the class B *vps* mutants. Genetic and biochemical data indicated that Ccz1p is required for fusion of various transport intermediates with the vacuole. Here we present data indicating that *CCZ1* is a close partner of the *YPT7* gene, which encodes Rab GTPase and is required for fusion of transport vesicles to vacuole and homotypic vacuole fusion. We isolated extragenic suppressors of *CCZ1* deletion. All these suppressors belong to one complementation group and correspond to mutated alleles of the *YPT7* gene. The mutated residues are located in two Ypt7p domains responsible for guanine binding. These data suggest that Ccz1p and Ypt7p interact physically. Coimmunoprecipitation experiments provide direct evidence that this indeed is the case. A possible mechanism of Ccz1p action is discussed.**

Key words: *Saccharomyces cerevisiae*, vesicular transport, vacuole, Ypt7p

## INTRODUCTION

The yeast vacuole is equivalent to the mammalian lysosome and the vacuole of plant cells. In wild-type cells it takes up as much as 25% of the cellular volume. As the main degradative site in the cell, the vacuole contains a variety of degradative enzymes required for intracellular digestion, including endo- and exoproteases, ribonucleases, polyphosphatases, α-mannosidase, trehalase and alkaline phosphatase. In addition, the vacuole serves as a storage compartment for certain cellular nutrients, such as amino acids, purines, polyamines and polyphosphates, which can be mobilised by the cell. The yeast vacuole, similarly to the plant vacuole, functions as a reservoir for mono- and divalent cations (Jones et al., 1997).

In yeast cells several different transport pathways converge upon the vacuole. Newly synthesised proteins, such as proteases destined for the vacuole, pass through the Golgi apparatus and in late Golgi they are diverted from the secretory pathway. The vacuole is also a recipient of material from the cell surface delivered by endocytosis. These two pathways overlap at the stage of the prevacuolar compartment (PVC), equivalent to the mammalian late endosome. At the late Golgi another pathway diverts, referred to as the 'ALP pathway', which bypasses the PVC when delivering alkaline phosphatase to the vacuole. Yet another vacuolar hydrolase, aminopeptidase I (API) is supplied to the vacuole from the cytoplasm by the cytoplasm-to-vacuole targeting (Cvt) pathway. Genetic data indicate that the Cvt process overlaps with macroautophagy, which nonselectively delivers cytosolic proteins and organelles to the vacuole for degradation and recycling. Ions and small molecules reach the vacuole via fluid-phase endocytosis. About 50% of vacuolar material is transferred from the mother to the daughter cell in the process of vacuolar inheritance (Bryant et al., 1998; Catlett et al., 2000; Jones et al., 1997; Scott and Klionsky, 1998).

The combined methods of classical and molecular yeast genetics, with the tools deriving from complete sequencing of the yeast genome and from genome databases from other organisms, allowed the identification of a substantial number of genes required for vacuole biogenesis, function and protein sorting in yeast. The Vacuolar Protein Sorting (*VPS*) genes constitute the main class of these genes. The *vps* mutants are arranged in six groups depending on the phenotype (classes A-F) (Conibear and Stevens, 1998; Dunn et al., 1994; Klionsky, 1998; Jones et al., 1997; Raymond et al., 1992).

In our previous study we identified a new *VPS* gene named *CCZ1* (*YBR131w*). We showed that Ccz1p is a membranous protein that resides mainly in late endosomes. Deletion of *CCZ1* leads to aberrant vacuole morphology typical for the class B *vps* mutants. Loss of Ccz1p results in a failure to deliver both vacuolar and endocytosed proteins to the vacuole. The fragmented vacuoles account for the increased sensitivity of *ccz1Δ* cells to divalent cations as the vacuolar function and integrity is essential for ion homeostasis.

From the genetic studies the most interesting was the finding that overexpression of Ypt7p suppresses all defects of Ccz1p-depleted cells. Ypt7p, the endosomal/vacuolar Rab GTPase, belongs to the superfamily of *ras*-like GTP-binding proteins that play an essential role in the regulation of vesicular protein transport. It is localised mainly in the vacuolar membrane and

**Table 1. *Saccharomyces cerevisiae* strains**

| Strain | Genotype | Source |
|---|---|---|
| W303 | *Mat a/Mat α ade2-1/ade2-1, can$^R$1-100/can$^R$1-100, his3-11,15/his3-11,15 leu2-3,112/leu2-3,112 trp1-1/trp1-1, ura3-1/ura3-1* | Rothstein collection |
| W303-1B* | *Mat α* | Rothstein collection |
| SIIV09* | *Mat a/Mat α ccz1Δ::kanMX4/ccz1Δ::kanMX4* | Kucharczyk et al., 1999 |
| SIIV07-6C* | *Mat a CCZ1/ccz1Δ::kanMX4* | Kucharczyk et al., 1999 |
| SIIV07-6D* | *Mat α CCZ1/ccz1Δ::kanMX4* | Kucharczyk et al., 1999 |
| RKR1-2B* | *Mat a ccz1Δ::kanMX4 ypt7$^{K127E}$* | This study |
| RKR2-1A* | *Mat α ccz1Δ::kanMX4 ypt7$^{D129G}$* | This study |
| RKR3* | *Mat α ccz1Δ::kanMX4 ypt7$^{D129N}$* | This study |
| RKR4* | *Mat a ccz1Δ::kanMX4 ypt7$^{T157P}$* | This study |
| RKR5* | *Mat α ccz1Δ::kanMX4 ypt7$^{A159P}$* | This study |

*These strains harbor additional mutations: *ade2-1*; *can$^R$1-100*; *αhis3-11,15*; *leu2-3,112*; *trp1-1*; *ura3-1*.

controls transport from the late endosome to the vacuole and homotypic vacuole fusion (Gotte et al., 2000). The *ypt7* mutants were also classified as class B *vps* (Haas et al., 1995; Wichmann et al., 1992). The disturbances in three transport pathways to the vacuole (endocytic, CPY and ALP) caused by Ccz1p depletion were identical to those caused by deletion of *YPT7*, indicating that Ccz1p and Ypt7p mediate a common transport step. We hypothesised that Ccz1p acts as a constituent of an endosome-vacuole-associated complex required for fusion of multiple transport intermediates with the vacuole (Kucharczyk et al., 2000). In this study we present new data concerning the mechanism of Ypt7p and Ccz1p interaction.

## MATERIALS AND METHODS

### Strains

The *Saccharomyces cerevisiae* strains and plasmids used in this study are described in Tables 1 and 2, respectively. *E. coli* DH5α was used for plasmid preparation (Sambrook et al., 1989).

### Media and growth conditions

Standard complete YPD, minimal SD and SC-drop-out media were used (Rose et al., 1990). In liquid media, cells were grown at 30°C with vigorous agitation. Growth was followed by measurement of OD at 600 nm.

### Genetic analysis

Standard media and procedures were used for crossing, sporulation and tetrad analysis (Adams et al., 1997). The efficiency of zygote formation and sporulation was assessed by direct microscopic examination.

### Phenotypic characterisation of ion sensitivities

For testing the sensitivity of yeast cells to caffeine, $Ca^{2+}$ and $Zn^{2+}$, YPD solid medium was supplemented with: 7.5 mM caffeine, 500 mM $CaCl_2$ and 5 mM $ZnCl_2$ (Rieger et al., 1997). Sensitivity was determined by the dilution spot assay (Kucharczyk et al., 2000).

### Isolation of spontaneous suppressors of *ccz1Δ* mutant

Homozygous diploid cells *MATa/MATα ccz1Δ/ccz1Δ* (SIIV09) and two haploid *ccz1Δ* strains of opposite mating types SIIV07-6C and SIIV07-6D were grown in liquid YPDA for 48 hours to the late stationary phase. Cells from separate liquid precultures were plated on YPDA plates supplemented with 5 mM $ZnCl_2$ at a concentration of $2\times10^8$ cells/plate. After three days of incubation at 28°C the well growing colonies were isolated, subcloned and tested for growth on plates supplemented with 500 mM $CaCl_2$ or 7.5 mM caffeine. Finally, five independent clones were selected for further analysis.

### DNA manipulations

Routine DNA manipulations: plasmid preparation, subcloning, *E. coli* transformation and agarose gel electrophoresis were carried out as described (Sambrook et al., 1989). Yeast transformations were performed by the improved lithium acetate procedure (Gietz and Woods, 1998). Plasmid DNA from yeast cells was isolated for the transformation of *E. coli* and chromosomal DNA was prepared for PCR, as previously described (Hoffman and Winston, 1987). Oligonucleotide primers were prepared using a Beckman Oligo 1000M DNA Synthesiser according to the manufacturer's instructions. Sequencing reactions were carried out using ABI Prism BigDye terminator cycle sequencing ready reaction kit with unlabelled internal primers. Sequencing reactions were analysed on an ABI310 Genetic Analyser (Perkin-Elmer).

### Tagging of Ccz1p with hemagglutinin epitope

A DNA fragment encoding triple influenza virus hemagglutinin epitope that is recognised by the 16B12 (BabCO) mouse monoclonal antibody was inserted into the *Bgl*II site between nucleotides +42 and +43 of the *CCZ1* gene. The 114 nucleotides encoding the tag were amplified by PCR with primers: 5′CAAGATCTCGCATCTTTTA-CCCATACG3′ and 5′TAGATCTGCAGTGAGCAGCGTAATCTG3′

**Table 2. Plasmids**

| Plasmid | Characterization | Source |
|---|---|---|
| pYCG_YBR131w | Amp$^R$ CEN *URA3 CCZ1* | Kucharczyk et al., 1999 |
| pRK11 | Amp$^R$ 2m *URA3 CCZ1* | Kucharczyk et al., 2000 |
| pRK16 | Amp$^R$ CEN *URA3 YPT7* | This study |
| pRK17 | Amp$^R$ 2μ *URA3 YPT7* | This study |
| pRK18 | Amp$^R$ 2m *URA3* P$_{CCZ1}$-*CCZ1*::*HA* | This study |
| pAS1-*ypt7$^{Q68L}$* | pAS2-1 *ypt7$^{Q68L}$* | T. Lazar, Gottingen |
| pRK40 | Amp$^R$ CEN *LEU2* ypt7$^{Q68L}$ | This study |
| pTL44 | pAS2-1 *ypt7$^{T22N}$* | T. Lazar, Gottingen |
| pRK41 | Amp$^R$ CEN *LEU2 ypt7$^{T22N}$* | This study |
| pTL43 | Amp$^R$ *YPT7* | T. Lazar, Gottingen |
| pRK45 | Amp$^R$ CEN *LEU2 YPT7* | This study |
| pRK16A | Amp$^R$ CEN *URA3 ypt7$^{D129A}$* | This study |
| pRK10S | Amp$^R$ CEN *LEU2 ypt7$^{K127E}$*, isolate from library in pRS315 | This study |
| pRK11S | Amp$^R$ CEN *LEU2 ypt7$^{K127E}$*, isolate from library in pRS315 | This study |
| pRK39S | Amp$^R$ CEN *LEU2 ypt7$^{K127E}$*, isolate from library in pRS315 | This study |
| pRK47S | Amp$^R$ CEN *LEU2 ypt7$^{K127E}$*, isolate from library in pRS315 | This study |

(*Bgl*II restriction sequences underlined), using the pBF30 plasmid as a template (Żołądek et al., 1995), which contains a sequence coding for the HA epitope. The amplified HA sequence was digested with *Bgl*II and cloned into the coding sequence of the *CCZ1* gene (plasmid pRK15; Table 2). The construction was confirmed by DNA sequencing and complementation tests of *ccz1Δ* cells. The CCZ1-HA construct was subcloned into pRS304, a *TRP1* integrative vector (Sikorski and Hieter, 1989), using *Sac*I-*Kpn*I restriction sites, to give pRK20.

### Construction of yeast genomic library

Genomic DNA was isolated from the strain RKR1-2A (Table 1) and partially digested with the endonuclease *Sau3*AI to yield a maximum of fragments in the 6-10 kb range. Gel-purified fragments were cloned into the *Bam*HI site of the shuttle vector pRS315 (Sikorski and Hieter, 1989). The resulting plasmid pools were used to transform *E. coli* by electroporation. After propagation on plates, plasmid DNA was extracted by alkaline lysis. The *S. cerevisiae* SIIV09 strain was transformed with such a library and plasmids were recovered from colonies growing on YPD medium supplemented with 5 mM $ZnCl_2$. Sequencing and restriction analysis of these plasmids revealed that they bear the *YPT7* gene. To identify mutated alleles of the *YPT7* gene that can suppress the effects of *CCZ1* deletion, direct sequencing of PCR products was applied. The *YPT7* gene was amplified with primers oRK25 5′GGAATAACCTCAGAACTCAC3′ and oRK26 5′TTGAAAGGGCCATCACATCC3′ using total DNA from suppressor strains as a template.

### Change of aspartic acid codon into alanine in position 129 of the YPT7 gene

Plasmid pRK16A, bearing the *ypt7*$^{D129A}$ gene, was constructed by gap repair of PCR-generated fragments. A scheme illustrating the *YPT7* regions targeted for PCR amplification and mutagenesis is shown in Fig. 1. The pRK16 plasmid, bearing wild-type *YPT7* gene cloned into pRS416 (vector), was used as a template. The PCR product from primers oRK26 and mutated oRK28 was used as a primer with oRK25 to generate a fragment of about 1000 bp, encoding the *ypt7*$^{D129A}$ gene. Primers oYPT1 and oRK28 were used to amplify a 6 kb fragment of the pRK16 plasmid, bearing the pRS416 vector flanked with sequences homologous to *YPT7* upstream and downstream regions. The products of these PCR reactions were used to transform the SIIV09 yeast strain. The resulting plasmid pRK16A was recovered and verified by sequencing.

### Light microscopy

A Nikon Microphot-SA microscope equipped with filters for Nomarski optics and for epifluorescence was used. Cells were viewed at ×600 magnification. Photographs were taken with a Nikon FX-35DX camera with Kodak T-Max 400 film.

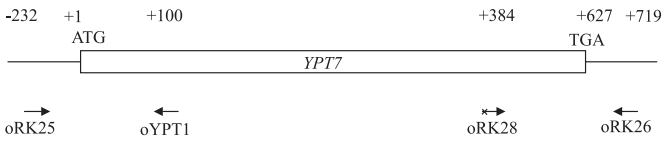



**Fig. 1.** Position of primers used for PCR mutagenesis of *YPT7* gene. x, indicates the *ypt7*$^{D129A}$ mutation. The first base of the *YPT7* gene (A to ATG codon) is designated +1. Nucleotide positions refer to the first 5′ base of oligonucleotide homologous to genomic DNA. For details see Materials and Methods. The sequences of the primers: oRK25: 5′GGAATAACCTCAGAACTCAC3′; oRK26: 5′TTGAAAGGGCCATCACATCC3′; oRK28: 5′TGCTGCCGAAGAATCTAA3′ (the changed base is bold and underlined); oYPT1: 5′AATACTTATCATTGACA3′.

### Visualisation of the yeast vacuole

Endogenous *ade2* fluorophore was used to label vacuoles as described (Weisman et al., 1987). Cells grown on complete SD medium with 12 μg/ml of adenine were collected in the late logarithmic phase of growth and observed under fluorescence microscope by exciting with 450-490 nm light.

### Immunoprecipitation

For immunoprecipitation (IP) experiments, *ypt7Δ* strain and derivatives of the W303-1B strain bearing plasmids: [pRK45], [pRK45,pRK18], [pRK40,pRK18], [pRK41,pRK18] and [pRK39,pRK18] were grown overnight in SC selective medium at 28°C to a density ($OD_{600}$) of 0.4-0.8. Cells were harvested and resuspended in 5 ml of medium. To deplete the cells of ATP and inhibit membrane fusion, the cultures were diluted 10-fold into ice-cold TAF buffer (20 mM Tris-HCl pH 7.5, 20 mM $NaN_3$, 20 mM NaF), pelleted at 4°C and resuspended in 1 ml of ice-cold IP buffer (50 mM HEPES pH 7.4, 150 mM KCl, 1 mM EDTA, 1 mM DTT, 0.5% Triton X-100), supplemented with protease inhibitors (Complete, Protease Inhibitor Cocktail Tablets, Roche Molecular Biochemicals; PMSF 1 mM) (Grote and Novick, 1999). Glass beads (1.1 g) were added to the cells in IP buffer and vortexed 8×30 seconds at 4°C. After centrifugation (13,000 ***g***, 10 minutes, 4°C), the supernatant fraction of lysates was harvested for 5 seconds (13,000 ***g***) to remove unbroken cells and then centrifuged (13,000 ***g***, 15 minutes, 4°C). The supernatant was collected and protein concentration was determined by the Bio-Rad protein assay using BSA as a standard. The samples were adjusted to 10 mg/ml of total protein with ice-cold IP buffer plus protease inhibitors. To minimise the recovery of products that adhere non-specifically to the protein G-Sepharose beads, 0.8-1 ml of cleared lysate (about 10 mg of protein) was mixed gently by shaking (200 rpm on a rotary shaker) at 4°C for 30 minutes with 30 ml of a 50% protein G-Sepharose slurry in IP buffer. The beads, debris and non-specifically bound products were pelleted for 15 minutes at ~13,000 ***g*** in a microcentrifuge at 4°C. The supernatant fractions were transferred to a clean tube, to which 30 ml of the protein G-Sepharose slurry (previously coupled for 2 hours with an anti-HA antibody, clone 16B12) was added. After overnight incubation with shaking at 4°C, the beads and bound proteins were pelleted by centrifugation for 2 minutes at 4°C, each sample was washed three times with 1 ml of IP buffer. Proteins were eluted from the beads by boiling in SDS sample buffer (60 mM Tris, pH 6.8, 100 mg/ml sucrose, 2% SDS, 0.05 mg/ml bromophenol blue and 100 mM DTT) for 5 minutes. Proteins from the eluates were separated by 14% SDS-PAGE, then they were transferred from the gel to nitrocellulose membrane by semi-dry protein transfer for 1 hour at 150 mA per gel. The membrane was probed by western blot analysis with an anti-HA mouse antibody to detect Ccz1-HAp or an anti-Ypt7p serum from rabbit (a gift from Dieter Gallwitz, Gottingen, Germany). In both cases, alkaline phosphatase-conjugated secondary antibodies were used.

### Building the protein model

A homology model of the Ypt7 protein was retrieved from the repository of models generated by the SwissModel software (Guex and Peitsch, 1997; http://www.expasy.ch/). The program used five structures of G-proteins as the templates during modeling. A sequence alignment of Ypt7p and those proteins is shown in Fig. 4. GDP molecule was positioned in the active centre of Ypt7p using the structure of Rap2a (pdb code 1KAO) as a template. Main chain atoms of both molecules were superimposed in the conserved GNKID motif using the InsightII molecular graphics software, then the Rap2a molecule was deleted. The resulting structure of Ypt7p+GDP was subjected to the following refinement. First, 5600 steps of conjugate gradient energy minimisation with CVFF forcefield were performed using the DISCOVER program. To further relax the model molecule, 20 ps molecular dynamics was executed. In the above calculations,

secondary structure elements were fixed in space. Additionally, the two hydrogen bonds between Asp129 and guanine base were constrained. The mutations D129G, D129N, K127E, T157P, A159P and D129A were introduced using the Insight II program. Using the library of side-chain rotamers provided by the software we found and analysed side-chain conformations that do not cause steric overlap with other atoms. Proline mutants were checked for proper proline conformation with the WHAT_CHECK software (Hooft et al., 1996).

## RESULTS

### Isolation and genetic analysis of spontaneous suppressors of *ccz1Δ*

In an effort to identify components that may functionally interact with Ccz1p, we searched for extragenic suppressors that suppress the divalent cation sensitivity of *ccz1Δ* cells. We searched for spontaneous mutants that allowed the *ccz1*Δ cells to form colonies on YPD medium supplemented with 5 mM $ZnCl_2$, a concentration that completely inhibited the growth of *ccz1Δ* strain. The colonies grown after 4 days of incubation at 30°C were purified by subcloning. Finally, this screen yielded five independent strains bearing the *CCZ1* null mutation and its suppressor (one *MATa* haploid, two *MATα* haploids and two homozygous diploids). It turned out that all isolates were also resistant to 500 mM $CaCl_2$ and 7 mM caffeine (Fig. 2). The suppressors restored the wild-type vacuole morphology (Fig. 3) and sporulation of homozygous *ccz1Δ /ccz1Δ* diploids, although the sporulation efficiency was rather poor (15% compared with 60% of the *CCZ1/CCZ1* diploid). An analysis of the progeny of RKR1 and RKR2, the $Zn^{2+}$-resistant *ccz1Δ/ccz1Δ* diploids, revealed a 2:2 segregation of the $Zn^{2+}$-resistant:$Zn^{2+}$-sensitive ($Zn^R$:$Zn^S$) phenotype, indicating that the suppression resulted from single dominant mutations.

The haploid strains *ccz1Δ* $Zn^R$ were tested for the dominance or recessiveness of the mutations. Diploids obtained by mating haploid strains RKR3, RKR4 and RKR5 with *ccz1Δ* strain display wild-type phenotype, indicating that all mutations were dominant. To estimate the number of genetic loci represented among the $Zn^R$ revertants, a detailed genetical analysis was performed. Two *ccz1Δ* $Zn^R$ spore clones, RKR1-2A and RKR1-1B, of opposite mating types and derivatives of the RKR1 diploid, were mated with haploid strains RKR2-1A to RKR5 and the meiotic products were analysed for $Zn^{2+}$ and $Ca^{2+}$ resistance. At least 12 tetrads were analysed for each cross and, in all cases, only the parental ditypes were found (four spores resistant to $Zn^{2+}$ and $Ca^{2+}$, data not shown). These results indicate that the five mutants isolated as $Zn^{2+}$-resistant are allelic.

In an attempt to clone the suppressor gene by complementation of the zinc and calcium ions sensitivity of the *ccz1* null mutation, total DNA isolated from the haploid strain RKR1-2A (*ccz1Δ*$Zn^R$) was digested partially with *Sau3*AI and cloned into the *Bam*HI site of the shuttle vector pRS315 (Sikorski and Hieter, 1989). The diploid strain SIIV09 (*ccz1Δ/ccz1Δ*) was transformed with this library. $Leu^+$ colonies were replica-plated onto YPD plates supplemented with 5 mM $ZnCl_2$ and incubated at 30°C for 3 days. Plasmids were recovered from ten colonies and retransformed into SIIV09 cells. Four plasmids: pRK10S, pRK11S, pRK39S and pRK47S (Table 2) were sequenced. From an analysis of overlapping inserts, a mutation in the *YPT7* gene was deduced to be responsible for the suppression. Sequencing of the *ypt7* gene from the four palsmids revealed an A→G transition in the 127th codon resulting in a change of lysine into glutamate (K127E).

To characterise the mutations responsible for the suppression in the RKR2-RKR5 strains, the mutated *ypt7* alleles were sequenced after being amplified using total DNA from the suppressor strains as a template. As shown in Table 3, all mutations are located in two of the five highly conserved G-regions that form part of the guanine nucleotide binding pocket in Rab/Ypt proteins.

Fig. 4 shows the modelled active centre of the *YPT7* protein. The GNKID (G4) and TSAK (G5) motifs interact with the guanine base, the G4 amino acid residues bind the guanine ring, whereas the G5 region is responsible for stabilisation of the G4-interactions (Gotte et al., 2000; Lazar et al., 1997). The mutated residues are located remotely from the part of the active centre responsible for phosphate bond hydrolysis and from the switch I and switch II regions that form the putative sites of interaction with guanine exchange factors (GEFs) and GTP-ase activating proteins (GAPs) (Gotte et al., 2000, Scheffzek et al., 1998). Therefore, the suppressor mutations most probably affect only the interaction of Ypt7p with the base. The most clear is the effect of the $ypt7^{D129G}$ mutation (Fig. 4B). The mutated protein lacks two specific hydrogen bonds formed between the side-chain of aspartic acid and the N1 and N2 nitrogens of the guanine base. No other effects are likely to occur. Consequently, the strength of the interaction between the protein and the nucleotide molecule is decreased without affecting GTP/GDP specificity. It is worth mentioning that two mutants of Ypt7p (i.e. the permanently inactive, GDP-bound ($ypt7^{T22N}$) one and the constitutively active, GTP-bound ($ypt7^{Q68L}$) variant) did not suppress the *ccz1Δ* phenotypes (Fig. 5). The effects of other mutations are more difficult to analyse. In the case of the $ypt7^{D129N}$ mutation (Fig. 4C), only one

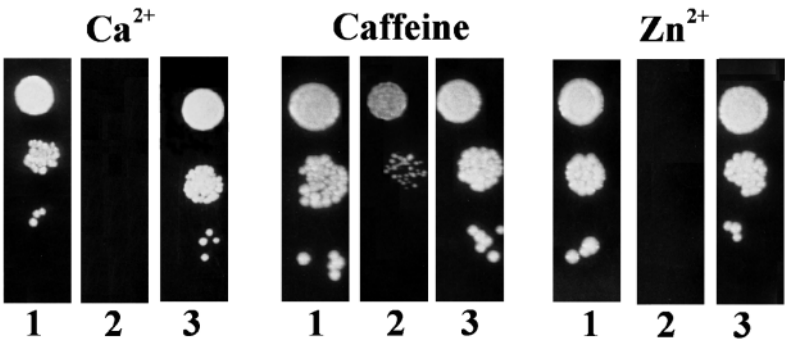



**Fig. 2.** Suppression of *ccz1Δ* phenotypes by representatives of the *ypt7* mutants. (1) *CCZ1/CCZ1*; (2) *ccz1Δ/ccz1Δ*; (3) *ccz1Δ/ccz1Δ, YPT/*$ypt7^{K127E}$. Cells were grown for 2 days at 28°C in YPD medium. For each strain tested, four serial 33-fold dilutions were made starting from $1\times10^8$ cells/ml dilution. 5 μl aliquots of second, third and fourth dilutions were spotted onto YPD plates supplemented with 500 mM $CaCl_2$, 5.14 mM caffeine and 5 mM $ZnCl_2$. Pictures were taken after 4 days of incubation at 28°C.

hydrogen bond is missing; therefore, it is likely that this mutant has a weaker guanine-protein interaction. This is further confirmed by the fact that an equivalent mutation, D138N, in the EF-Tu protein causes lost of guanine specificity (Weijland et al., 1994).

To verify this hypothesis we mutated the invariant aspartic acid at position 129 into alanine. The phenotype of the engineered *ypt7*$^{D129A}$ mutant (Fig. 4G) appeared to be the same as the phenotypes of the spontaneous *ypt7*$^{D129N}$ and *ypt7*$^{D129G}$ mutants. The *ccz1Δ/ccz1Δ* diploid transformed with a plasmid bearing the *ypt7*$^{D129A}$ mutated allele sporulated, formed colonies on YPD medium supplemented with 5 mM $ZnCl_2$ (Fig. 5), was resistant to 500 mM $CaCl_2$ and 7 mM caffeine

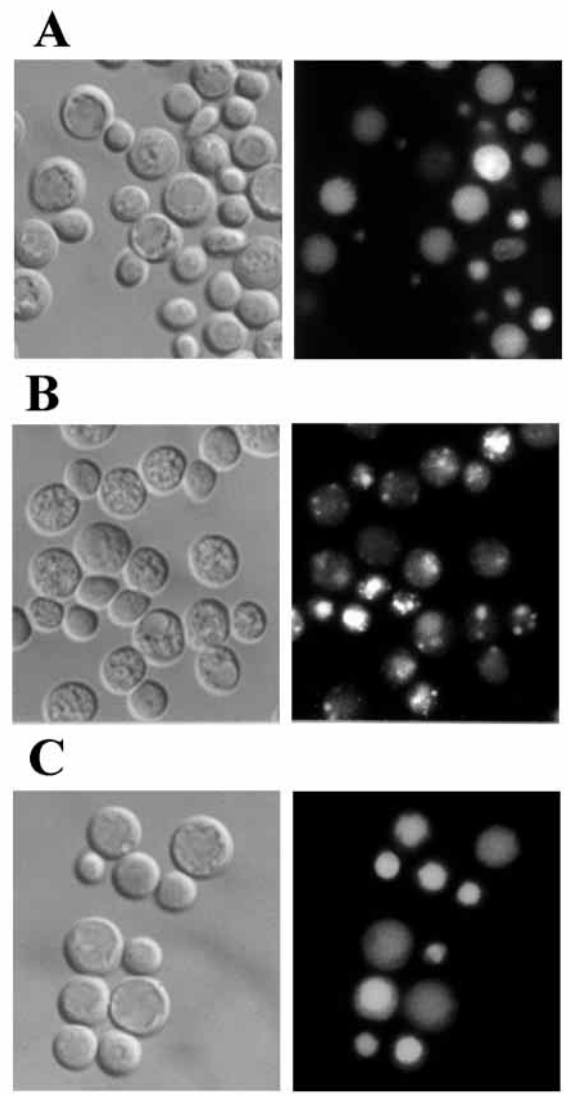



**Fig. 3.** The *ypt7* suppressors restore wild-type morphology of vacuoles. Abnormal vacuolar morphology of *ccz1Δ/ccz1Δ* cells (B), compared with wild-type *CCZ1/CCZ1* (A) and suppressed cells *ccz1Δ/ccz1Δ YPT7/ypt7*$^{K127E}$ (C). Vacuoles were labelled with *ade2* endogenous fluorophore. Cells were viewed by Nomarski optics (left) and the same fields were viewed for fluorescence (right). Wild-type vacuoles appear as large fluorescent spots corresponding to circular indentation in Nomarski. Vacuoles of mutant cells appear as numerous, fluorescent spots corresponding to irregular structures in Nomarski.

and had the wild-type vacuole morphology (data not shown). It is very unlikely that substitution of aspartic acid by alanine has any other effect on the protein-guanine interaction than removing the two hydrogen bonds.

The aliphatic part of the side-chain of lysine 127 forms a hydrophobic contact with the plane of the guanine ring. Such a contact is impossible between the side-chain of glutamic acid placed in the same position. Therefore the mutation *ypt7*$^{K127E}$ (Fig. 4D) again weakens the interaction of the protein and the base.

Mutations that introduce proline in the TSAK motif do not show any clear influence on the protein-ligand interactions. According to WHAT_CHECK analysis, both proline residues have acceptable conformations without changing the local conformation of the side-chain. Therefore, the hydrogen bond between Ser158 and Glu129 should be maintained. This interaction probably stabilises the Glu129 sidechain in the position in which it forms favourable hydrogen bonds with the guanine ring. The only effect of the proline substitutions could be to constrain the flexibility of the loop in this region. Consideration of the subtle, dynamic effects that occur in this situation is beyond the scope of this work.

The genetic data supported by the presented model suggest a physical interaction between the Ccz1 and Ypt7 proteins. Therefore we performed immunoprecipitation experiments in an attempt to detect complex formation between these two proteins.

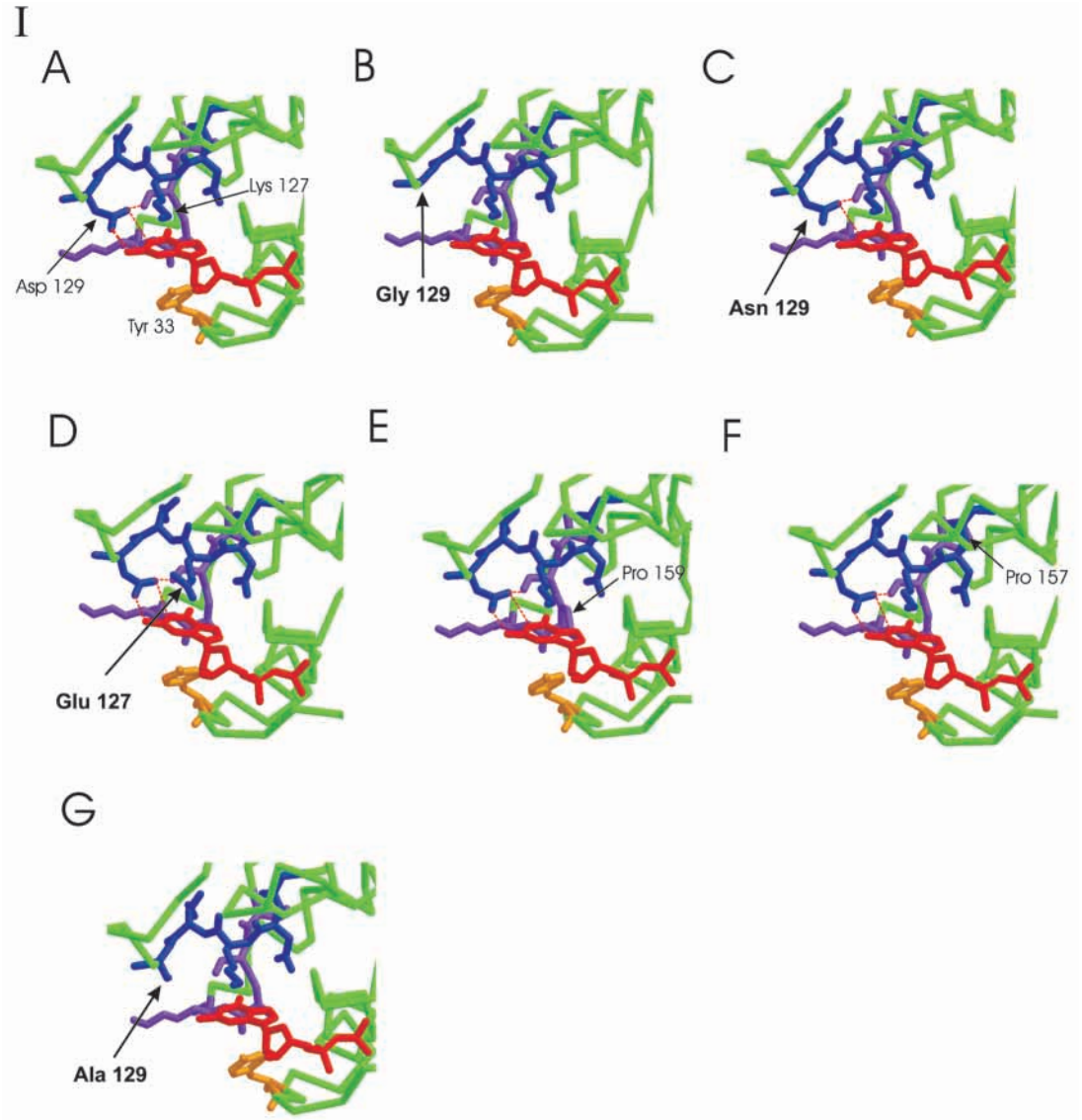
## Ccz1p physically interacts with YPT7p

Ccz1p was tagged with the triple HA epitope. The tagged protein contained the 38-amino-acid HA segment between the 14th and 15th amino acid of Ccz1p. Expression of the tagged construct was directed from the CCZ1 promoter. This construct fully complemented the CCZ1 deficiency in the growth tests; it also complemented the sporulation defect of the ccz1Δ/ccz1Δ homozygous diploid and restored normal morphology of vacuoles (Kucharczyk et al., 2000). For the IP experiments we expressed CCZ1HA from a multicopy plasmid because of the low level of Ccz1p. The YPT7 gene was cloned in a centromeric plasmid under its own promoter. The complex formation was tested between the Ccz1-HAp and wild-type Ypt7p, one of the suppressors Ypt7$^{K127E}$ (Fig. 6B, lanes 3,4) and two mutated forms Ypt7$^{Q68L}$ (GTP-bound) (Fig. 6B, lane 5) and Ypt7$^{T22N}$ (GDP-bound) (Fig. 6B, lane 6).

Protein extracts from the *ypt7Δ* and *ccz1*Δ strains bearing appropriate constructs were incubated with protein G-Sepharose coated with anti-HA monoclonal antibody 16B12, and the precipitates were collected. The monoclonal HA

**Table 3. Mutations identified in *ypt7* suppressors of *ccz1Δ***

| Strain | YPT7 allele | Codon # | Mutation | Amino acid change G4 | Amino acid change G5 |
|---|---|---|---|---|---|
| SIIV07-6C | *YPT7* | | | GNKID | FLTSAK |
| RKR1-2A | *ypt7*$^{K127E}$ | 127 | AAA→GAA | GN***E***ID | FLTSAK |
| RKR2-1A | *ypt7*$^{D129G}$ | 129 | GAT→GGT | GNKI***G*** | FLTSAK |
| RKR3 | *ypt7*$^{D129N}$ | 129 | GAT→AAT | GNKI***N*** | FLTSAK |
| RKR4 | *ypt7*$^{T157P}$ | 157 | ACA→CCA | GNKID | FL***P***SAK |
| RKR5 | *ypt7*$^{A159P}$ | 159 | GCC→CCC | GNKID | FLTS***P***K |
| SIIV09/ pRK16A | *ypt7*$^{D129A}$ | 129 | GAT→GCT | GNKI***A*** | FLTSAK |




II

| | | G1 | | | | G2 | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| YPT7 | 1 | NILKVIILG | DSGVGK | TSLM | HRYVNDKYSQ | Q-YKATIGAD | FLTKEVTVDG | DKVATMQV | WD | | |
| H-Rap2a/GTP | 1 | MREYKVVVLG | SGGVGK | SALT | VQFVTGTFIE | K-YDPTIE-D | FYRKEIEVDS | SP-SVLEI | LD | | |
| H-Ras p21/GTP | 1 | MTEYKLVVVG | AGGVGK | SALT | IQLIQNHFVD | EYDPT-IE-D | SYRKQVVIDG | ET-CLLDI | LD | | |
| C-H-Ras(1-171)/GTP | 1 | MTEYKLVVVG | AGGVGK | SALT | IQLIQNHFVD | E-YDPTIE-D | SYRKQVVIDG | ET-CLLDI | LD | | |
| C-H-Ras p21G12D/GTP | 1 | MTEYKLVVVG | ADGVGK | SALT | IQLIQNHFVD | EYDP-TIE-D | SYRKQVVIDG | ET-CLLDI | LD | | |
| C-H-Ras p21/GDP | 1 | MTEYKLVVVG | AGGVGK | SALT | IQLIQNHFVD | E-YDPTIE-D | SYRKQVVIDG | ET-CLLDI | LD | | |
| | | G3 | | | | | | | | | |
| YPT7 | 69 | TAGQE | RFQSL | G--VAFYRGA | DCCVLVYDVT | NASSFENIKS | WRDEFLVHAN | VNSPETFPFV | | | |
| H-Rap2a/GTP | 68 | TAGTE | QFASM | R--DLYIKNG | QGFILVYSLV | NQQSFQDIKP | MRDQII---R | VKRYEKVPVI | | | |
| H-Ras p21/GTP | 68 | TAGQE | EYSAM | R--DQYMRTG | EGFLCVFAIN | NTKSFEDIHQ | YREQIK---R | VKDSDDVPMV | | | |
| C-H-Ras(1-171)/GTP | 68 | TAGQE | EYSA- | -MRDQYMRTG | EGFLCVFAIN | NTKSFEDIHQ | YREQIK---R | VKDSDDVPMV | | | |
| C-H-Ras p21G12D/GTP | 68 | TAGQE | EYSAM | R--DQYMRTG | EGFLCVFAIN | NTKSFEDIHQ | YREQIK---R | VKDSDDVPMV | | | |
| C-H-Ras p21/GDP | 65 | TA--G | QEEYS | AMRDQYMRTG | EGFLCVFAIN | NTKSFEDIHQ | YREQIK---R | VKDSDDVPMV | | | |
| | | G4 | | | | G5 | | | | | |
| YPT7 | 127 | IL | GNKID | AEE | SKKIVSEKSA | QELAKSLGDI | PLFL | TSAK | NA | INVDTAFEEI | ARSALQQNQA DTEA |
| H-Rap2a/GTP | 122 | LV | GNKVD | LES | ERE-VSSSEG | RALAEEWG-C | PFME | TSAK | SK | TMVDELFAEI | VRQMNYA--- - |
| H-Ras p21/GTP | 121 | LV | GNKCD | LA- | ART-VESRQA | QDLARSYG-I | PYIE | TSAK | TR | QGVEDAFYTL | VREIRQH--- -- |
| C-H-Ras(1-171)/GTP | 122 | LV | GNKCD | LA- | ART-VESRQA | QDLARSYG-I | PYIE | TSAK | TR | QGVEDAFYTL | VREIRQHKLR KL |
| C-H-Ras p21G12D/GTP | 121 | LV | GNKCD | LAA | -RT-VESRQA | QDLARSYG-I | PYIE | TSAK | TR | QGVEDAFYTL | VREIRQH--- -- |
| C-H-Ras p21/GDP | 120 | LV | GNKCD | LA- | ART-VESRQA | QDLARSYG-I | PYIE | TSAK | TR | QGVEDAFYTL | VREIRQHKLR -- |

**Fig. 4.** Guanine binding site of the YPT7 protein (I) and sequence alignment used during the homology modeling (II). (I) A, the wild-type protein. B,C,D,E,F,G the D129G, D129N, K127E, A159P, T157P and D129A mutated proteins, respectively. Residues belonging to GNKID and TSAK motif are marked blue. GDP is shown in purple and Tyr 33 in orange. The hydrogen bonds between Asp129 and the guanine base and Asp129 and Ser158 are marked. (II) G1 to G5 conserved residues are bold.

antibody almost totally precipitated Ccz1-HAp since the protein was not detected in supernatants after IP. Upon analysis of the immunoprecipitate by western blotting, one major band of about 85 kDa and two weak bands with higher mobility, which are likely to be proteolytic fragments of Ccz1-HAp, were observed (Fig. 6B). None of the bands was observed in

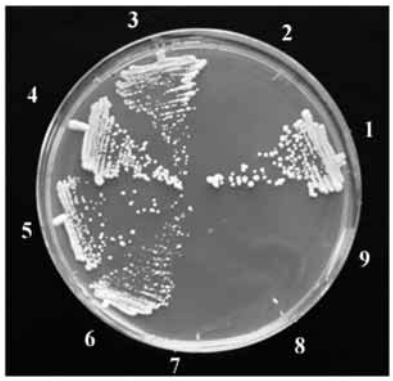



**Fig. 5.** The zinc sensitivity of *ccz1Δ* cells bearing mutated alleles of *YPT7* gene. (1) *CCZ1/CCZ1*; (2) *ccz1Δ/ccz1Δ*; (3) *ccz1Δ/ccz1Δ, YPT/ypt7*$^{K127E}$; (4) *ccz1Δ/ccz1Δ* [*ypt7*$^{K127E}$ CEN]; (5) *ccz1Δ/ccz1Δ* [*ypt7*$^{D129A}$ CEN]; (6) *ccz1Δ/ccz1Δ* [*YPT7* 2μ]; (7) *ccz1Δ/ccz1Δ* [*YPT7* CEN]; (8) *ccz1Δ/ccz1Δ* [*ypt7*$^{Q68L}$ CEN]; (9) *ccz1Δ/ccz1Δ* [*ypt7*$^{T22N}$ CEN].

immunoprecipitates from the control isogenic strain, which lacks Ccz1-HAp (Fig. 6B, lane 2). Using an anti-Ypt7p serum, we detected a band corresponding to Ypt7p and Ypt7$^{K127E}$p in the anti-HA precipitates from the strain bearing the *CCZ1-HA* construct (Fig. 6B, lanes 3,4), but no Ypt7p band was detected in the control strain (Fig. 6B, lane 2), confirming a specific interaction between these proteins in cell lysates. The GTP- and GDP-bound Ypt7p forms did not form complexes with Ccz1-HAp.

## DISCUSSION

Extragenic suppression of a mutant phenotype is a classical way of identifying genetic interactions. The clear cut phenotypes of the *ccz1*Δ mutant made it possible to use a genetic screen to select extragenic suppressors that bypassed the defects caused by the deletion and enabled the mutant to grow on high-calcium and/or high-zinc media. This led to the identification of *ypt7* mutants that had alterations in the conserved sequence motifs GNKID and FLTSAK (Table 3), which are identical in the great majority of guanine nucleotide binding proteins.

The product of the *YPT7* gene is a small monomeric GTPase of the Rab family called Ypt in yeast. The Rab/Ypt proteins act as regulators at specific steps of vesicular traffic assuring proper delivery of the cargo. There is a total of 11 proteins in the yeast Rab/Ypt family. Ypt7p is a vacuolar membrane protein essential for the endosome-vacuole and vacuole-vacuole fusion. Similar to other Rab/Ypt GTPases, Ypt7p cycles between a GTP-bound (active) and GDP-bound (inactive) form and its functional cycle involves a number of proteins that enhance GTP hydrolysis and promote GDP/GTP exchange. These include GAPs, the guanine nucleotide dissociation inhibitors (GDI) maintaining the GDP-bound inactive conformation, one or more factors that recognise and disrupt the Ypt-GDI complex (GDI-displacement factor; GDF), and the guanine nucleotide exchange factors (GEFs), which accelerate the dissociation of GDP and its replacement by GTP (Pryer et al., 1992; Shirtaki et al., 1993; Horiuchi et al., 1997; Lazar et al., 1997; Walch-Solimena et al., 1997; Albert et al., 1999). The analysis of the model structure of mutated Ypt7 proteins that corrected the *ccz1Δ* phenotype raised the possibility that Ccz1p functions as one of the factors regulating GDP/GTP exchange. The loss of the hydrogen bonds formed between the side-chain of aspartic acid and the N1 and N2 nitrogens of the guanine base in Ypt7p of suppressor mutants (Fig. 4), is expected to result in a protein with a decreased strength of the protein-base interaction. Consequently, the release of the nucleotide from the active centre of the protein is faster, which may increase turnover between the GDP- and GTP-bound states with respect to the wild-type protein. Thus the mutations in the *YPT7* gene that confer the wild-type phenotype of *ccz1Δ* cells exert an effect similar to that of guanine nucleotide exchange factors, since the primary function of GEFs is the release of bound guanine nucleotide followed by the formation of the GTP-bound active form. Overexpression of wild-type *YPT7* gene can also lead to an increased amount of the active form of Ypt7p, resulting in a partial suppression of the *ccz1Δ* mutant phenotypes (Fig. 5).

Depletion of Ccz1p results in highly fragmented vacuoles paralleled by disturbances in three different vesicular transport pathways: the endocytic, CPY and ALP pathway (Kucharczyk et al., 2000); these phenotypes are identical to those of the *ypt7Δ* mutant strain (Wichmann et al., 1992). Interestingly, the same defect in vacuolar transport was demonstrated for the mutant *ypt7*$^{T22N}$ encoding the inactive, GDP-bound form of Ypt7p (Wada et al., 1996). As shown in Fig. 5, the three mutants *ccz1Δ*, *ypt7Δ* and *ypt7*$^{T22N}$ also share the growth

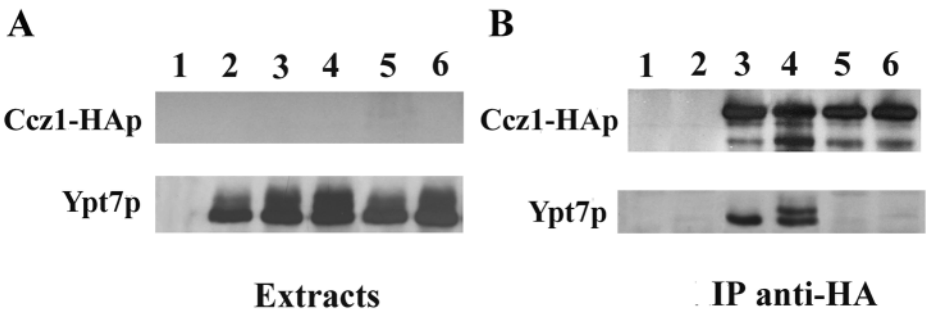



**Fig. 6.** Physical association of Ccz1p with Ypt7p. Total extracts from yeast cells were immunoprecipitated with anti-HA antibody as described in Materials and Methods. Total extracts, 3 μl (A), and immunoprecipitates from 1 ml of extracts (B) were analyzed by SDS-PAGE and immunoblotting with anti-HA antibody (upper panel) to detect Ccz1-HAp and with antiserum to Ypt7p (lower panel). The band corresponding for Ccz1-Hap is not detectable even for 20 μl of extracts loaded on gel. Lanes represent preparations from strains: (1) *ypt7Δ;* (2) *ccz1Δ* [*YPT7*]; (3) *ccz1Δ* [*YPT7/CCZ1-HA*]; (4) *ccz1Δ* [*ypt7*$^{K127E}$ */CCZ1-HA*]; (5) *ccz1Δ* [*ypt7*$^{Q68L}$*/CCZ1-HA*]; (6) *ccz1Δ* [*ypt7*$^{T22N}$ */CCZ1-HA*]. The double bands visible for Ypt7$^{K127E}$p probably represent geranylgeranylated and unmodified forms.

phenotypes. By contrast, mutant *ypt7*$^{Q68L}$ encoding a protein remaining predominantly in the GTP-bound state did not display a changed phenotype, moreover, the Ypt7$^{Q68L}$ protein complemented the defect of the *ypt7Δ* mutation (Wada et al., 1996). The genetic data indicate that Ccz1p is required in the process of activation of the Ypt7 protein by stimulating GDP release, which favours the formation of the GTP-bound form of the protein. Therefore, in the cell, the two proteins Ccz1p and Ypt7p, residing in opposite membranes of the donor (biosynthetic transport vesicles and late endosomal compartment) and acceptor (vacuole) compartment, respectively, should physically interact at membrane fusion. The co-immunoprecipitation experiments strongly support this hypothesis. Our results indicate that Ccz1p forms a complex with wild-type Ypt7 and suppressor Ypt7$^{K127E}$ proteins, although it has very low, if any, affinity for GDP- and GTP-bound Ypt7p (Fig. 6B), which correlates with the genetic data since, in growth experiments, neither *ypt7*$^{T22N}$ nor *ypt7*$^{Q68L}$ mutations suppressed the *ccz1Δ* phenotype.

In vitro experiments indicate that some GTPases interact with their GEFs irrespective of the bound guanine nucleotide (Burton et al., 1994; Collins et al., 1997; Lai et al., 1993). However, GDP release also requires the action of a GDF (Dirac-Svejstrup et al., 1997; Soldati et al., 1994; Ullrich et al., 1994), whereas GTP release also requires the activity of GAPs. In wild-type yeast cells, the stability of the active, GTP-bound Ypt7 protein form appears to be controlled by Gyp7p, classified as a GAP-factor (Vollmer and Gallwitz, 1995). According to the model presented, the domains responsible for GTPase activity are unaffected in the *ypt7* suppressor mutants.

The preference of Ccz1p for wild-type Ypt7p and the mutated form characterised by a decreased affinity to guanine, may reflect the state in which GTPase, devoid of GDP, forms a transient complex with its nucleotide exchange factor followed by GTP binding (Bischoff and Ponstingl, 1991; Burton et al., 1994; Lai et al., 1993; Romero et al., 1985; Sprang and Colman, 1998; Wurmser et al., 2000). Such an association has been demonstrated for Cdc25p, which binds tightly to the nucleotide-free form of the Ras2 protein. The authors suggest that Cdc25p functions as a GEF by stabilisation of transitory nucleotide-free state (Lai et al., 1993).

In *S. cerevisiae*, GEFs have been identified for three Ypt-family GTPases: Sec2p is the GEF for Sec4p, Vps9p for Vps21p (Hama et al., 1999; Walch-Solimena et al., 1997), and a large protein complex, TRAPP, acts as a GEF for the Ypt1 and Ypt31/32 proteins (Jones et al., 2000). In contrast to the Ypt/Rab proteins, GEFs do not share homology with one another (Hama et al., 1999; Horiuchi et al., 1997; Wada et al., 1997; Walch-Solimena et al., 1997).

The GEF for Ypt7p has been identified recently (Wurmser et al., 2000). The authors demonstrated that a class C-Vps complex, containing the Vps11, Vps16, Vps18, Vps33, Vps39 and Vps41 proteins, not only activates Ypt7p through Vps39p, but also acts as an Ypt7p effector through as yet unidentified protein partner(s) (Price et al., 2000). Purified Vps39p binds Ypt7p in its GDP-bound and nucleotide-free forms and stimulates nucleotide exchange on Ypt7p in vitro (Wurmser et al., 2000). Independently, it was shown that class C-Vps complex associates with GTP-bound form of Ypt7p, and thus acts as a downstream effector of Ypt7p (Price et al., 2000).

Since, in general, the known Ypt/Rab GEFs are part of large protein complexes (Burstein and Macara, 1992; Horiuchi et al., 1997; Nair et al., 1990), Ccz1p may be one of the components of a complex activating the GDP/GTP exchange on Ypt7p. A class C-Vps complex functions as a GEF for Ypt7p in the process of homotypic vacuole fusion (Wurmser et al., 2000) but it is also required for the fusion of the CPY, ALP and API transport intermediates with the vacuole (Price et al., 2000; Rieder and Emr, 1997); therefore, it plays a role in the heterotypic membranes fusion as well. The localisation of Ccz1p in the late endosome and transport vesicles suggests that of the two processes regulated by Ypt7p (homotypic and heterotypic membrane fusion) Ccz1p plays a role in the latter one. We are currently using the two-hybrid system to detect other proteins interacting with Ccz1p.

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