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Multiple functions of the vacuolar sorting protein Ccz1p in *Saccharomyces cerevisiae*

Marta Hoffman-Sommer, Andrzej Migdalski, Joanna Rytka, Róża Kucharczyk*

Institute of Biochemistry and Biophysics PAS, Pawinskiego 5A, 02-106 Warsaw, Poland

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Abstract

The *CCZ1* (*YBR131w*) gene encodes a protein required for fusion of various transport intermediates with the vacuole. Ccz1p, in a complex with Mon1p, is a close partner of Ypt7p in the processes of fusion of endosomes to vacuoles and homotypic vacuole fusion. In this work, we exploited the Ca^{2+}-sensitivity of the *ccz1Δ* mutant to identify genes specifically interacting with *CCZ1*, basing on functional multicopy suppression of calcium toxicity. The presented results indicate that Ccz1p functions in the cell either in association with Mon1p and Ypt7p in fusion at the vacuolar membrane, or—separately—with Arl1p at early steps of vacuolar transport. We also show that suppression of calcium toxicity by the calcium pumps Pmr1p and Pmc1p is restricted only to the subset of mutants defective in vacuole morphology. The mechanisms of Ca^{2+}-pump-mediated suppression also differ from each other, since the action of Pmr1p, but not Pmc1p, appears to require Arl1p function.

Keywords: *Saccharomyces cerevisiae*; *vps* mutants; Vacuole morphology; Calcium sensitivity

In our previous study, we have identified a new *VPS* (vacuolar protein sorting) gene named *CCZ1* (*YBR131w*), encoding a membrane-bound protein that resides mainly in late endosomes. Genetic and biochemical data indicated that Ccz1p is a close partner of Ypt7p, a Rab/Ypt GTPase involved in the fusion of endosomes to vacuoles and in homotypic vacuole fusion [1,2]. Strains lacking the Ccz1 protein exhibit manifold defects in vacuolar protein trafficking and function. Deletion of *CCZ1* leads to aberrant vacuole morphology typical for class B *vps* mutants, severe defects in the endocytic, CPY, and ALP vacuolar transport pathways, and an inability of the homozygous diploid to sporulate [1]. *ccz1Δ* strains also display defects in the *cvt* pathway, autophagy, and pexophagy [3,4]. Progress in the knowledge concerning the mechanisms of membrane fusion pointed to Ccz1p as an important factor in SNARE (soluble NSF attachment protein receptor)-mediated membrane fusion at the vacuole. It has been shown that Ccz1p forms a stable complex with Mon1p. In the process of SNARE pairing Ccz1–Mon1 interacts with the C-Vps/HOPS (homotypic fusion and vacuole protein sorting) proteins [3,5]. It is proposed that the Ccz1–Mon1 complex regulates the Ypt7-dependent tethering/docking stage, which leads to the formation of a *trans*-SNARE complex and subsequent vacuole fusion. On the other hand, the results of Love et al. [6] showed an interaction between *CCZ1* and the gene *ARL1*, which encodes a GTPase associated with the Golgi complex, indicating a possible role of Ccz1p in the regulation of membrane traffic/fusion at the stage of the Golgi compartment.

The C-Vps/HOPS complex is involved in multiple steps of vesicular transport. It is a crucial component required for docking and fusion of vesicles with the vacuole, and for homotypic vacuole fusion [7,8]. It consists of the Vps18, Vps33, Vps11, and Vps16 proteins (class

* Corresponding author. Fax: +48 22 6584636.
E-mail address: roza@ibb.waw.pl (R. Kucharczyk).

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C Vps proteins), and Vps39p–Vps41p, two additional class B Vps proteins. Vps39p binds the GDP-bound and nucleotide-free forms of Ypt7p and stimulates nucleotide exchange on this protein [7]. Besides its role in the endosome-to-vacuole transport, the C-Vps/HOPS complex plays essential roles in docking and fusion of vesicles in Golgi-to-endosome anterograde and retrograde transport, as well as in the endocytic pathway [9–11].

Phenotypic analysis has shown that the null mutant *ccz1Δ* exhibits a variety of growth phenotypes. It displays sensitivity to many chemicals, including sensitivity to elevated levels of the divalent cations Ca^{2+} and Zn^{2+} [12]. The vacuole fragmentation phenotype and defective vacuolar transport seem to account for the increased sensitivity of *ccz1Δ* cells to divalent cations, since the vacuole serves as a storage and detoxification site for excess divalent cations. Among divalent cations calcium plays a major role due to its influence on numerous signaling pathways. The Golgi apparatus and the endoplasmic reticulum also serve as exchangeable Ca^{2+} stores. An increase in the cytosolic-free Ca^{2+} concentration may occur as a result of calcium release from intracellular calcium stores or due to high Ca^{2+} concentration in the medium ([12–15], and references therein).

To identify genes specifically interacting with *CCZ1* in functional networks, we searched for multicopy suppressors that alleviated the Ca^{2+}-sensitivity of *ccz1Δ* cells. Previously we have identified three suppressors of the *ccz1Δ* mutation. The genes *PMC1* and *PMR1* recovered the growth of *ccz1Δ* on YPD medium supplemented with 0.5 M calcium but did not suppress other defects of this mutant [1]. These two genes, *PMR1* and *PMC1*, encode Ca^{2+}-ATPases involved in calcium homeostasis. Pmc1p is an integral vacuole membrane protein. It transports Ca^{2+} from the cytosol into the vacuole. Pmr1p is a P-type ion pump of the secretory pathway. Although predominantly localized in the Golgi, it also controls the Ca^{2+} concentration in the ER. Despite their localization in different compartments, the two Ca^{2+}-ATPases have overlapping functions [16–20]. The third suppressor, the *YPT7* gene, encodes a GTPase of the superfamily of *ras*-like GTP-binding proteins. It is localized mainly in the vacuolar membrane and controls transport from the late endosome to the vacuole and homotypic vacuole fusion [21]. The mechanism of Ypt7p-mediated suppression differs from that caused by the *PMR1* and *PMC1* genes, as Ypt7p alleviated all the defects of Ccz1p-depleted cells, not only their calcium sensitivity [1,2].

In this work, we exploited the Ca^{2+}-sensitivity of the *ccz1Δ* strain to analyze genetic interactions of the *CCZ1* gene, basing on multicopy functional suppression of the calcium sensitivity. Our results imply that Ccz1p has multifarious functions in the cell, either in association with Mon1p and Ypt7p, or independently of these two proteins with Arl1p and Ypt1p. We also show that suppression of calcium toxicity by *PMR1* and *PMC1* is restricted only to the subset of *Vps* mutants. The mechanisms of the Ca^{2+}-pump-mediated suppression also differ from each other, since the action of Pmr1p, but not Pmc1p, appears to require Arl1p function.

Materials and methods

Media and growth conditions. The *Saccharomyces cerevisiae* deletion mutants used in this study are derivatives of the BY4741 parental strain from the Euroscarf collection (*MATa*, *his3Δ1*, *leu2Δ0*, *met15Δ0*, and *ura3Δ0*). The strain KT15 (*MATα*, *ade2*, *lys2*, *leu2*, *his3*, *ura3*, and *ccz1*::*KanMX4 arl1*::*HIS3*) was kindly provided by Dr. Anne G. Rosenwald. Plasmids are described in Table 1. *Escherichia coli DH5α* was used for plasmid preparation [22]. Standard complete YEPD, minimal SD, and SC-drop-out media were used [23].

Genetic analysis. Standard media and procedures were used for crossing, sporulation, and tetrad analysis [23]. The efficiency of zygote formation and sporulation was assessed by direct microscopic examination.

Phenotypic characterization of ion sensitivities. For testing the sensitivity to Ca^{2+} and Zn^{2+} ions solid YEPD medium was supplemented with $CaCl_2$ at concentrations from 100 to 700 mM, or $ZnCl_2$ from 3 to 7 mM [24]. The sensitivity was determined by dilution spot assays. For each strain tested, four serial 33-fold dilutions were made from a saturated overnight culture adjusted to a starting concentration of 1×10^8 cells/ml. Five-microliter aliquots of each cell suspension were spotted on the plates. The plates were incubated at 30 °C for 3–5 days. The two divalent ion concentrations that best illustrated the differences between the wild-type and mutant strains were chosen for presentation.

DNA manipulations. Routine DNA manipulations: plasmid preparation, subcloning, transformation, and transfection of *E. coli*, and agarose gel electrophoresis were carried out as described in Sambrook et al. [22]. Yeast transformations were performed by the improved lithium acetate procedure [25].

Vacuolar staining with FM4-64. The uptake and transport to the vacuole of the styryl dye FM4-64 (Molecular Probes, Eugene, OR) was determined as described by Vida and Emr [26]. Cells grown to an OD_{600} of 0.8–1.2 were harvested and resuspended at 10–20 OD_{600} per milliliter in YEPD. FM4-64 was added to 40 μM from a 4 mM stock in DMSO. After a preliminary labeling step for 30 min at 0 °C, the cells were harvested at 4 °C, resuspended in fresh YEPD at 5–10 OD_{600} per milliliter, and incubated at 28 or 37 °C with vigorous shaking and 100 μl samples were withdrawn after 20, 40, 90, and 120 min. of incubation, centrifuged, resuspended in fresh YEPD at 10–20 OD_{600} per milliliter, placed on standard slides, and immediately viewed with a 546 nm filter under a Nikon Eclipse E800 fluorescence microscope. Images were collected using a Photometrix CH350A camera with QED or Lucia G software and processed using Photoshop 7.0 (Adobe) software.

Table 1
Plasmids used in this study

Plasmid	Characterization	Source
pRK102S	*CCZ1*, 2μ LEU2, pEMBLe31	[1]
pRK104S	*ZRC1*, 2μ LEU2, pEMBLe31	[1]
pRK106S	*YPT7*, 2μ LEU2, pFL46S	[1]
pRK107S	*PMC1*, 2μ LEU2, pEMBLYe31	[1]
pRK109S	*PMR1*, 2μ LEU2, pEMBLYe31	[1]
pMON1	*MON1*, 2μ LEU2, pACT2	D. Klionsky

Results and discussion

Deletion of the MON1 gene has the same phenotypic effects as the ccz1Δ and ypt7Δ mutants

We demonstrated previously that the *ypt7Δ* mutant is a phenocopy of the mutant deleted for the *CCZ1* gene. An increased dosage of the calcium pumps Pmr1p and Pmc1p suppressed the calcium-mediated growth defect of both the *ccz1Δ* and *ypt7Δ* mutants [1]. Since the Ccz1 and Mon1 proteins function as a stable protein complex which interacts with Ypt7p [3,5], we compared the growth phenotypes of the BY strains depleted of Ccz1p, Mon1p, and Ypt7p. We tested zinc and calcium sensitivity, and vacuolar morphology of the *ccz1Δ*, *ypt7Δ*, and *mon1Δ* mutants, and of these deletants transformed with multicopy plasmids bearing the genes *CCZ1*, *YPT7*, *MON1*, *PMR1*, *PMC1*, and *ZRC1*. As shown in Fig. 1, the tested growth phenotypes of the *mon1Δ* mutant did not differ from those of *ccz1Δ* and *ypt7Δ*. An increased level of Pmr1p or Pmc1p also suppressed the calcium sensitivity of Mon1p-depleted cells, and overexpression of *ZRC1* suppressed the zinc sensitivity. In particular, the genetic interactions between the *CCZ1*, *YPT7*, and *MON1* genes appeared interesting. As we have reported previously, overexpression of Ypt7p suppressed both zinc and calcium sensitivity of *ccz1Δ* cells, restored wild-type vacuole morphology, and partially rescued the sporulation ability of the *ccz1Δ*/*ccz1Δ* diploid [1]. As shown in Fig. 1, overproduction of Ypt7p exerted the same effects on Mon1p- as on Ccz1p-depleted cells, including the rescuing of the sporulation ability of *mon1Δ*/*mon1Δ* cells (3% of tetrads in a cell population as compared to 6% for *ccz1Δ*/*ccz1Δ*/*YPT7* and 60% for *CCZ1*/*CCZ1* diploids, not shown). In contrast, the overexpression of neither *CCZ1* nor *MON1* could substitute for the function of Ypt7p, nor could they suppress each other's deletions. To compare the vacuolar morphology and efficiency of endocytosis in these strains, we stained the cells with the dye FM4-64. As shown in Fig. 2, the morphology of the vacuoles in all the mutants analyzed was typical for class B *vps* mutants, but we observed that it was less severe in the BY than in the W303 background (not shown, [1]). The delay in endocytosis of the FM4-64 dye in Ccz1p- and Mon1p-depleted cells was comparable, while depletion of Ypt7p caused a much stronger defect—after 2 h of incubation in fresh YEPD medium the dye was still present in the endosomes. Overexpression of the *YPT7* gene improved the rate of endocytosis in *ccz1Δ* and *mon1Δ* cells, but it did not restore the wild-type morphology of vacuoles in the BY background, unlike what it did in a W303-derived *CCZ1* deletion mutant [1]. Neither of the other plasmids tested influenced endocytosis or vacuolar morphology of the analyzed deletants, and none of the genes functioned as suppressors when expressed from centromeric plasmids (not shown). These data indicate the existence of more general interactions among the *CCZ1*, *YPT7*, and *MON1* genes. The results presented here show that the overproduction of Ypt7p can functionally compensate for the lack of both Ccz1p and Mon1p.

Suppression of the Ca^{2+}-sensitivity of the ccz1Δ, ypt7Δ, and mon1Δ mutants is specific

As has already been mentioned, the closest known partners of Ccz1p, Mon1p, and Ypt7p are components of the C-Vps/HOPS complex. We therefore investigated if the calcium sensitivity and fragmented vacuolar morphology and their suppression are specific for *ccz1Δ*, *ypt7Δ*, and *mon1Δ* mutants, or if they may also result from defects in other genes structurally or functionally related to them. We tested the above phenotypes in *vps18Δ*, *vps33Δ*, *vps11Δ*, and *vps16Δ* mutants; since the phenotypes of all the class C mutants tested were identi-

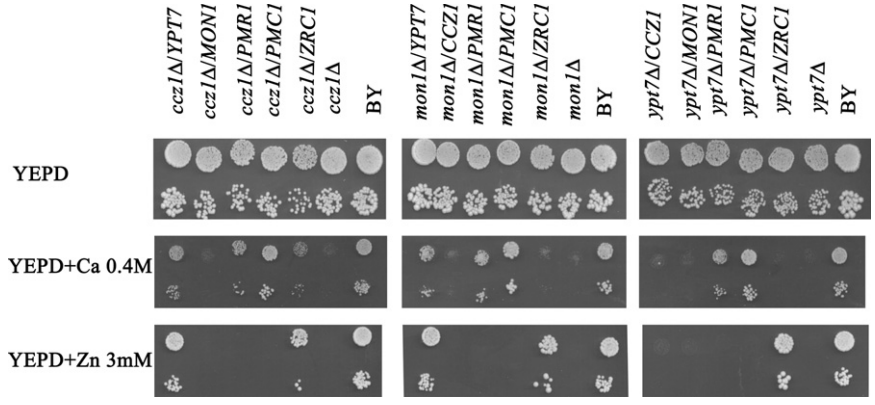

Fig. 1. The *ccz1Δ*, *mon1Δ*, and *ypt7Δ* deletions share the same phenotypes and suppression pattern. Cells were grown for two days at 28 °C in SC-drop-out medium, then the cultures were serially diluted 1:33, starting from 10^8 cells/ml, and 5 μl aliquots of the successive dilutions were spotted on YEPD medium supplemented with 400 mM $CaCl_2$ or 3 mM $ZnCl_2$. Plates were incubated at 28 °C for three days.

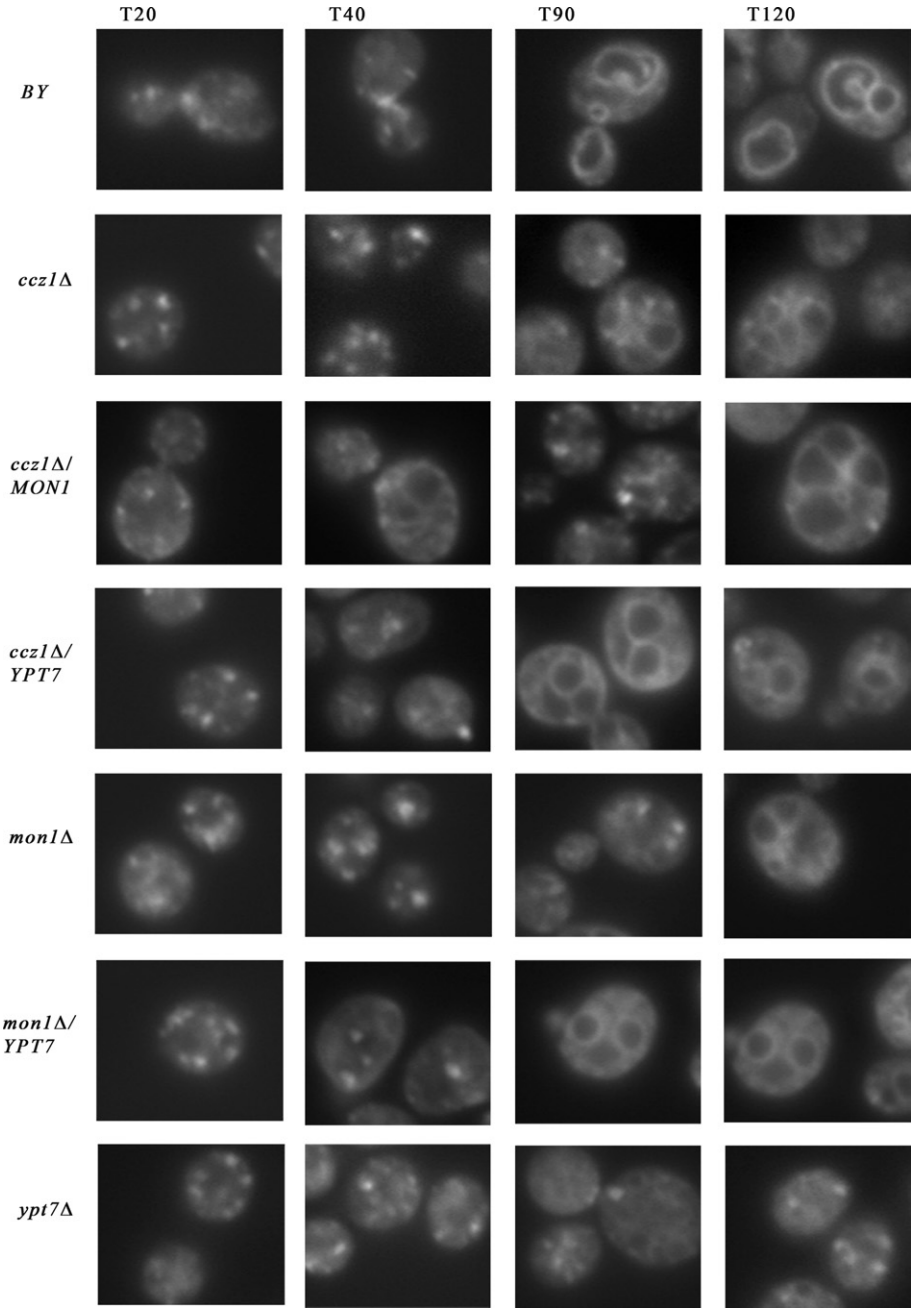

Fig. 2. FM4-64 dye transfer from the plasma membrane to the vacuole is delayed in *ccz1Δ*, *mon1Δ*, and *ypt7Δ* mutants compared to their partially suppressed derivatives and the wild-type. Cells were incubated with 40 μM FM4-64 for 30 min at 0 °C. The dye was removed by centrifugation and cells were incubated in fresh YEPD medium. At the indicated time points portions of the cell suspension were removed, viewed for fluorescence, and photographed.

cal (data not shown), we present here only the results for the *vps18Δ* mutant. Class C *vps* mutants are characterized by a loss of vacuole and accumulation of numerous vesicles (Fig. 3A). As shown in Fig. 3B, depletion of Vps18p caused temperature sensitivity and increased sensitivity to calcium: even 0.1 M $CaCl_2$ inhibited the growth of *vps18Δ* cells. Interestingly, none of the suppressors alleviating the calcium sensitivity of the *ccz1Δ* mutant restored the growth of *vps18Δ* cells on calcium media (not shown). Even overexpression of *YPT7* did not affect the vacuole morphology or calcium sensitivity of the *vps18Δ* strain (Fig. 3B). We also tested the HOPS mutants *vps39Δ* and *vps41Δ*—representatives of class B vacuolar mutants (Fig. 3A). In particular, Vps39p seems to be a close partner of Ccz1p and Ypt7p, as it interacts with Ccz1p in the two-hybrid assay (Kucharczyk, unpublished) and functions as a guanine nucleotide exchange factor (GEF) for Ypt7p [5,6]. The phenotypes

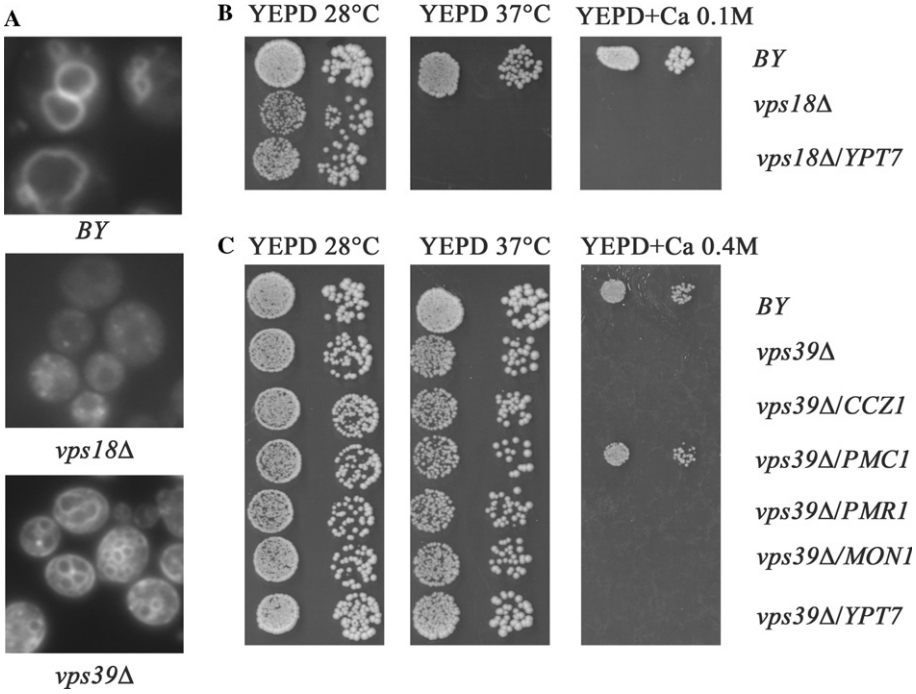

Fig. 3. Phenotypes of representative Vps/HOPS deleted strains. (A) Vacuole morphology, (B) and (C) growth tests. Vacuoles were labeled as described in Materials and methods and viewed after 2 h of incubation in YEPD medium. Experimental procedures are as in Fig. 1.

of the *vps39Δ* and *vps41Δ* mutants were identical, therefore only data for *vps39Δ* are presented. As shown in Fig. 3C, the growth of *vps39Δ* was also inhibited by calcium, however, the inhibitory concentration of $CaCl_2$ was 0.4 M, the same as for *ccz1Δ* (Fig. 1). The inhibition by Ca^{2+} could be alleviated by overexpression of the *PMC1* gene, but not the *PMR1*, *YPT7*, *MON1*, or *CCZ1* genes. Although fragmented and disturbed in their function, the vacuolar compartment is maintained in the class B *vps* mutants, and thus Pmc1p—a calcium pump localized in the vacuolar membrane—could still be functional. The lack of suppression of the calcium sensitivity of the tested class C *vps* mutants by overexpression of Pmc1p probably results from the fact that these mutants have no vacuolar structures. On the other hand, Pmr1p is located in the Golgi apparatus where it plays a crucial role in calcium sequestration [14,16–18]. Deletion of any of the HOPS genes results not only in defects in the processes of endosome-to-vacuole and vacuole-to-vacuole docking and fusion, but it also impairs membrane fusion at the Golgi-to-endosome stage [9]. This defect might be responsible for preventing Pmr1p from exerting its suppressor function in these cells. The lack of *PMR1*- and *PMC1*-mediated suppression of C-Vps/HOPS mutants demonstrates the specificity of Pmr1 and Pmc1, which can only compensate for the defects in a subset of Ca^{2+}-sensitive vacuolar mutants, excluding those in which the Ca^{2+}-sensitivity is linked to a dysfunction of the Golgi compartment as well, as is the case in the HOPS mutants.

The CCZ1 gene, but not YPT7 or MON1, interacts with ARL1

Apart from the interactions of the *CCZ1* gene with *MON1*, *YPT7*, *PMR1*, and *PMC1*, we also investigated the specificity of a synthetic interaction between the *ARL1* and *CCZ1* genes, which has been reported by Love et al. [6]. Arl1p is a member of a family of ADP-ribosylation factors (ARFs), highly conserved guanine nucleotide-binding proteins involved in exocytic and endocytic vesicular transport. Arl1p localizes to the Golgi, where it functions as a component of a cascade of sequentially acting yeast Rab GTPases that regulate Golgi-to-endosome transport and recycling of proteins from endosomal compartments to the Golgi [27].

Since Ccz1p interacts closely with Mon1p and Ypt7p in the fusion at the vacuolar membrane, it was interesting to see whether Mon1p and Ypt7p also interact with Arl1p. In order to test this, we crossed a BY derivative strain deleted for *ARL1* with *ccz1Δ*, *ypt7Δ*, and *mon1Δ*. Tetrad analysis of the respective heterozygous diploids revealed that the *ccz1Δ arl1Δ* haploids of the BY background were lethal, whereas the double *ypt7Δ arl1Δ* and *mon1Δ arl1Δ* segregants were viable. Since the loss of *CCZ1* in the *arl1Δ* derivative of BY was lethal, for phenotypic tests we used the KT15 strain (*ccz1Δ arl1Δ* in the PSY316 background), which is characterized by temperature sensitivity [6]. The *ypt7Δ arl1Δ* and *mon1Δ arl1Δ* mutants grew well at 37 °C (Fig. 4A).

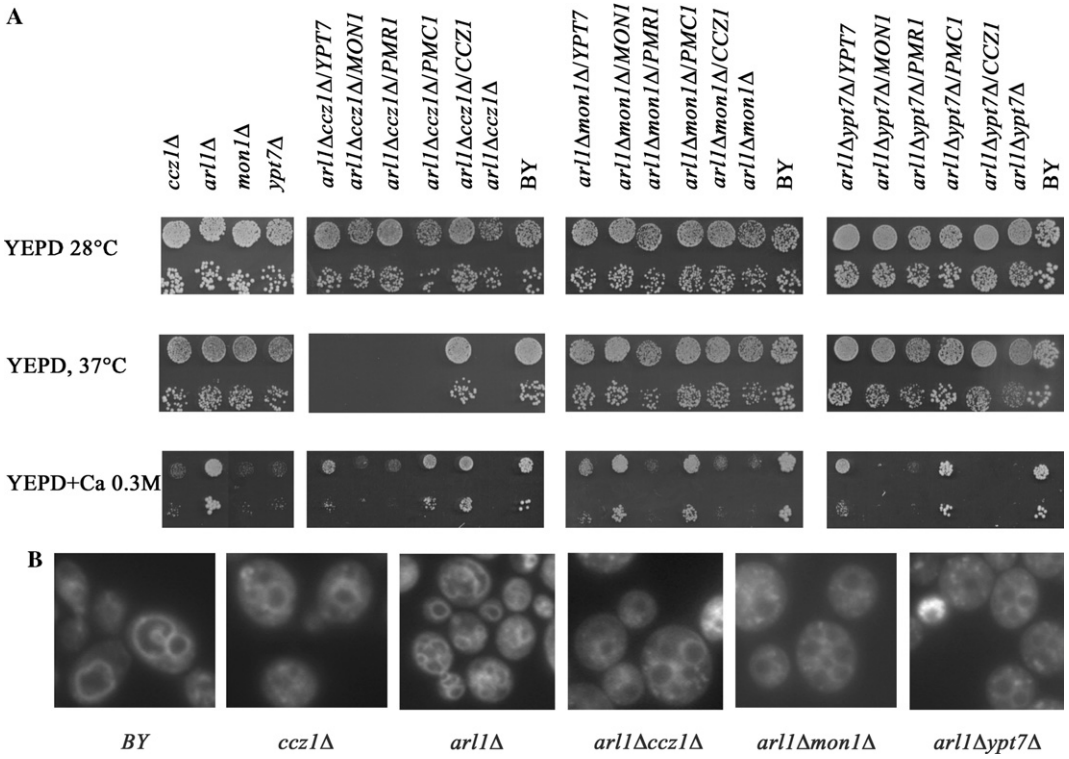

Fig. 4. Phenotypes of double mutants *ccz1Δ arl1Δ*, *mon1Δ arl1Δ*, and *ypt7Δ arl1Δ*, and of the indicated transformants. All analyzed strains are of BY background, except *ccz1Δ arl1Δ* (KT15 strain). (A) Temperature and calcium sensitivity. Experimental procedures are as in Fig. 1. (B) Introduction of the *arl1Δ* mutation into the genetic background of the *ccz1Δ*, *mon1Δ*, and *ypt7Δ* mutants does not exacerbate their aberrant vacuole morphology. Vacuoles were labeled with FM4-64 as described in Materials and methods, and viewed after 2 h of incubation in YEPD medium.

The double mutants were transformed with high-copy vectors bearing the genes which alleviated the Ca^{2+}-sensitivity of *ccz1Δ* cells. Growth of the mutants and their transformants was tested on YEPD medium at 37 °C and on YEPD medium supplemented with 0.3 M $CaCl_2$ (Fig. 4A). By means of FM4-64 staining, the vacuole morphology of the strains was inspected (Fig. 4B).

As expected, *PMR1* and *PMC1* did not revert the *ts* phenotype of the double *ccz1Δ arl1Δ* mutant. Interestingly, the same was true for overexpression of *MON1* or *YPT7*. The Ca^{2+}-sensitivity and vacuole morphology of the double mutants *ccz1Δ arl1Δ*, *mon1Δ arl1Δ*, and *ypt7Δ arl1Δ* was similar to those of single mutants (Figs. 4A and B), however, the introduction of an *ARL1* deletion alleviated the suppression effect of *PMR1*. The results presented in Fig. 4A demonstrate that the genetic interaction between *CCZ1* and *ARL1* is specific and can be separated from that occurring between *CCZ1* and *MON1*, and/or *YPT7*. At present, a search for suppressors of the *ts* phenotype of the *ccz1Δ arl1Δ* double mutant is being performed. Preliminary data indicate that overexpression of *YPT1* specifically alleviates the temperature sensitivity of the mutant but does not revert the calcium or zinc sensitivity (not shown). Ypt1p is a *ras*-like GTPase involved in the ER-to-Golgi and *cis*-to-*medial* Golgi vesicular transport [21]. This raises the possibility that Ccz1p, similar to Arf-family proteins, plays additional roles in trafficking steps other than the endosome-to-vacuole one. Moreover, the finding that *PMR1* did not suppress the Ca^{2+}-sensitive phenotype of any of the double mutants demonstrated a requirement for Arl1p in *PMR1*-mediated suppression. This unforeseen interaction between the *PMR1* and *ARL1* genes confirms that the Pmr1p-mediated suppression is based on a functional Golgi compartment.

In our work, we found that overexpression of *PMR1* or *PMC1* can specifically alleviate calcium sensitivity of *ccz1Δ*, *ypt7Δ*, and *mon1Δ* cells, all of which represent class B vacuolar morphology mutants. However, in two other class B mutants, *vps41Δ* and *vps39Δ*, overexpression of *PMR1* did not suppress the calcium sensitivity, whereas overexpression of *PMC1* did. The results of Cunningham and Fink [20] indicate that the functions of Pmr1p and Pmc1p in Ca^{2+} homeostasis overlap to some extent. On the other hand, Pmr1p is also involved in the process of Ca^{2+} and Mn^{2+} transport into the Golgi lumen, where Mn^{2+} is required for protein glycosylation. Pmr1p regulates the lumenal calcium pool of the ER and is involved in Ca^{2+}-dependent protein processing and degradation [15,20,28]. When viewed in the light of these data, our results suggest mechanisms of

Ca^{2+}-sensitivity suppression exerted by the *PMR1* and *PMC1* genes that are similar to each other in some aspects and differ in others. Suppression by either pump requires functional storage compartments, pointing to the transport of surplus cytosolic Ca^{2+} into intracellular stores as the main rescue factor. In the case of *PMR1*, this is probably the Golgi/ER compartment, as indicated by the loss of suppression in the *arl1Δ* and class B Vps/HOPS deleted strains. This is consistent with the fact that Pmr1p is absent from the vacuolar membrane. Pmc1p could potentially be present both in the vacuolar membrane and—especially under the conditions of overproduction—in the ER and Golgi, which it traverses on its way to the vacuole. It could thus exert its function in all these compartments. However, the fact that *PMC1*-mediated suppression still occurs in the *arl1Δ*, *vps39Δ*, and *vps41Δ* backgrounds suggests that Pmc1p functions mainly at the physiological, vacuolar site. The inability of the Pmc1p pump to suppress the Ca^{2+}-sensitivity of class C *vps* mutants would then be explained by the severity of the vacuolar defects in these mutants, whereas the lack of suppression by Pmr1p would be due to malfunctions of Golgi trafficking, which occur in *arl1Δ* cells and in all HOPS mutants.

Acknowledgments

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