The *Saccharomyces cerevisiae* protein Ccz1p interacts with components of the endosomal fusion machinery

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Abstract
The yeast protein Ccz1p is necessary for vacuolar protein trafficking and biogenesis. In a complex with Mon1p, it mediates fusion of transport intermediates with the vacuole membrane by activating the small GTPase Ypt7p. Additionally, genetic data suggest a role of Ccz1p in earlier transport steps, in the Golgi. In a search for further proteins interacting with Ccz1p, we identified the endosomal soluble N-ethylmaleimide-sensitive factor attachment protein receptor Pep12p as an interaction partner of Ccz1p. Combining the *ccz1*Δ mutation with deletions of *PEP12* or other genes encoding components of the endosomal fusion machinery, *VPS21*, *VPS9* or *VPS45*, results in synthetic growth phenotypes. The genes *MON1* and *YPT7* also interact genetically with *PEP12*. These results suggest that the Ccz1p–Mon1p–Ypt7p complex is involved in fusion of transport vesicles to multiple target membranes in yeast cells.

Introduction
Intracellular vesicular transport plays a crucial role in the compartmentalization of eukaryotic cells. Selective fusion of transport vesicles with their target organelles is necessary for maintaining compartment identity. Intensive studies on membrane fusion have led to the identification of many proteins involved in this process. The key players include the soluble NSF attachment protein receptor (SNARE) proteins (for a recent review, see Ungermann & Langosch, 2005; Cai *et al*., 2007; Gurkan *et al*., 2007), small GTPases of the Rab/Ypt family (Pfeffer, 2001), members of the Sec1/MUNC18 (SM) family of proteins (Toonen & Verhage, 2003) and the largely unrelated group of multi-subunit-tethering complexes (Whyte & Munro, 2002). The question of which of these components are responsible for ensuring fusion selectivity has been the subject of intense debate over the past years. Reports showing that SNARE interactions are not fully specific, but can be promiscuous (reviewed in Götte & von Mollard, 1998), and also that other components of the fusion machinery may be involved in fusion at multiple sites in the cell (Srivastava *et al*., 2000; Peterson & Emr, 2001; Subramanian *et al*., 2004), are making it increasingly clear that no single factor is sufficient to confer specificity to the fusion reaction. Presumably, specific combinations of all the components mentioned are necessary for correct vesicle targeting.

The yeast protein Ccz1p has previously been shown to function in the fusion of diverse types of compartments, because it has been implicated in the biosynthetic trafficking routes for both carboxypeptidase Y (CPY) and alkaline phosphatase, in the cytoplasm-to-vacuole targeting pathway, autophagy, pexophagy and homotypic vacuole fusion (Kucharczyk *et al*., 2000; Meiling-Wesse *et al*., 2002; Wang *et al*., 2002, 2003). It forms a stable complex with Mon1p (Wang *et al*., 2002, 2003) and interacts physically with the Rab-GTPase Ypt7p (Kucharczyk *et al*., 2001), which regulates the docking of many transport intermediates at the vacuolar membrane (Götte *et al*., 2000). On the other hand, a *ccz1*Δ deletion – but not *mon1*Δ or *ypt7*Δ – is synthetically lethal with an *arl1*Δ mutation, and the cells can be rescued by overexpression of Ypt1p, a Rab-GTPase localized to early Golgi compartments (Love *et al*., 2004; Hoffman-Sommer *et al*., 2005). These interactions suggest an involvement of Ccz1p in transport through the Golgi apparatus, and this
function would be independent of Mon1p and Ypt7p. The interaction of CCZ1 with ARL1 suggests that Ccz1p might play a role in additional trafficking steps, other than vacuolar fusion.

In this study, we show that the endosomal syntaxin Pep12p, functioning as a common heavy chain in many SNARE complexes at the endosome membrane (von Mollard et al., 1997; Lewis & Pelham, 2002; Paumet et al., 2004), physically and genetically interacts with the Ccz1p–Mon1p complex. In addition, genetic interactions of CCZ1 with genes encoding other components of the endosomal fusion machinery are demonstrated. These data suggest that the Ccz1p–Mon1p complex functions at multiple sites in the cell.

**Materials and methods**

**Strains, growth conditions and genetic analysis**

The *Saccharomyces cerevisiae* strains used in this study are described in Table 1. Unless indicated otherwise, all strains constructed in this work are isogenic with BY4741. Standard complete YEPD (yeast extract, peptone, dextrose), minimal SD and SC-drop-out media were used for yeast cultivation (Amberg et al., 2005). For testing the sensitivity to Ca^{2+} ions, solid YEPD medium was supplemented with CaCl_2 at the indicated concentration (Rieger et al., 1997). Standard genetic methods for mating, sporulation and tetrad analysis were used (Amberg et al., 2005). For determining the growth phenotypes of double mutants obtained in this study, three separate spores carrying confirmed double deletions were assayed for each strain. Yeast transformations were performed using the improved lithium acetate procedure (Gietz et al., 1995).

**Plasmids**

The plasmids used in this study are described in Table 2. Routine DNA manipulations were performed according to Sambrook et al. (1989).

**Yeast two-hybrid assays**

For the yeast two-hybrid tests, the LexA system (Vojtek & Hollenberg, 1995) and the method of Fields & Song (1989) were used.

**Table 1. Saccharomyces cerevisiae strains used in this study**

| Strains   | Genotypes | Sources |
|-----------|-----------|---------|
| PJ69-4A   | MATα trp1-901 leu2-3,112 ura3-52 his3-A200 gal4Δ gal80Δ GAL2-ADE2 lys2Δ:GAL1-HIS3 met2Δ::GAL7-lacZ | James et al. (1996) |
| L40       | MATα his3-A200 trp1-901 leu2-3,112 ade2 lys2Δ::lexAop4-HIS3 URA3Δ::lexAop8-lacZ gal4Δ gal80Δ | Invitrogen |
| BY4741    | MATα his3Δ1 leu2Δ met15Δ0 ura3Δ0 | Euroscarf |
| Y07164    | MATα his3Δ1 leu2Δ met15Δ0 ura3Δ0 ccz1Δ::kanMX4 | Euroscarf |
| Y14491    | MATα his3Δ1 leu2Δ lys2Δ0 ura3Δ0 mon1Δ::kanMX4 | Euroscarf |
| Y10575    | MATα his3Δ1 leu2Δ lys2Δ0 ura3Δ0 ypt7Δ::kanMX4 | Euroscarf |
| Y08128    | MATα his3Δ1 leu2Δ met15Δ0 ura3Δ0 pep12Δ::kanMX4 | Euroscarf |
| Y00394    | MATα his3Δ1 leu2Δ met15Δ0 ura3Δ0 syn8Δ::kanMX4 | Euroscarf |
| Y16495    | MATα his3Δ1 leu2Δ lys2Δ0 ura3Δ0 vps9Δ::kanMX4 | Euroscarf |
| Y11865    | MATα his3Δ1 leu2Δ lys2Δ0 ura3Δ0 vps21Δ::kanMX4 | Euroscarf |
| Y14462    | MATα his3Δ1 leu2Δ lys2Δ0 ura3Δ0 vps45Δ::kanMX4 | Euroscarf |
| Y12362    | MATα his3Δ1 leu2Δ lys2Δ0 ura3Δ0 vam3Δ::kanMX4 | Euroscarf |
| RKY112-9D  | MATα his3Δ1 leu2Δ lys2Δ0 met15Δ0 ura3Δ0 ccz1Δ::kanMX4 pep12Δ::kanMX4 | This study |
| RKY122-5A  | MATα his1Δ leu2Δ0 lys2Δ0 met15Δ0 ura3Δ0 syn8Δ::kanMX4 | This study |
| RKY111-1D  | MATα his3Δ1 leu2Δ0 lys2Δ0 met15Δ0 ura3Δ0 mon1Δ::kanMX4 pep12Δ::kanMX4 | This study |
| MH24-2A    | MATα his3Δ1 leu2Δ met15Δ0 ura3Δ0 pep12Δ::kanMX4 ypt7Δ::kanMX4 | This study |
| MH24-4B    | MATα his3Δ1 leu2Δ0 ura3Δ0 ccz1Δ::kanMX4 vam3Δ::kanMX4 | This study |
| MH29-10D   | MATα his3Δ1 leu2Δ met15Δ0 ura3Δ0 ccz1Δ::kanMX4 vps21Δ::kanMX4 | This study |
| MH30-4A    | MATα his3Δ1 leu2Δ0 ura3Δ0 ccz1Δ::kanMX4 vps9Δ::kanMX4 | This study |
| MH31-2D    | MATα his3Δ1 leu2Δ met15Δ0 ura3Δ0 ccz1Δ::kanMX4 vps45Δ::kanMX4 | This study |
| MH31-1D    | MATα his3Δ1 leu2Δ met15Δ0 ura3Δ0 pep12Δ::kanMX4 vam3Δ::kanMX4 | This study |
| MH34-12B   | MATα his3Δ1 leu2Δ lys2Δ0 met15Δ0 ura3Δ0 pep12Δ::kanMX4 vps21Δ::kanMX4 | This study |
| MH35-13B   | MATα his3Δ1 leu2Δ lys2Δ0 met15Δ0 ura3Δ0 pep12Δ::kanMX4 vps9Δ::kanMX4 | This study |
| MH36-1C    | MATα his3Δ1 leu2Δ lys2Δ0 met15Δ0 ura3Δ0 pep12Δ::kanMX4 vps45Δ::kanMX4 | This study |
| MHY1269    | MATα his3Δ200 leu2-3,112 lys2-801 ura3-52 trp1-1 lys27Δ::LEU2 | Amerik et al. (2000) |
| RKY127-12C*| MATα his3Δ leu2Δ lys2Δ0 ura3-52 trp1-1 ccz1Δ::kanMX4 vps27Δ::LEU2 | This study |
| RKY127-12D | MATα his3Δ leu2Δ lys2Δ0 ura3-52 trp1-1, syn8::kanMX4 vps27::LEU2 | This study |
| RKY115-1C  | MATα his3Δ, leu2Δ, lys2-801, met15Δ0, ura3Δ, pep12::kanMX4 vps27::LEU2 | This study |
| RKY117-3C  | MATα his3Δ, leu2Δ, lys2Δ, met15Δ0, ura3Δ, ccz1::kanMX4 pep12::kanMX4 vps27::LEU2 | This study |
| RKY126-1B  | MATα his3Δ, leu2Δ, met15Δ0, lys2Δ, ura3Δ, ccz1::kanMX4 syn8::kanMX4 vps27::LEU2 | This study |

*These strains were obtained by crossing MHY1269 with Y07164 and Y01812, respectively.
were used. The plasmids encoding fusions of the LexA DNA-binding domain or Gal4 DNA-binding or activation domains (Table 2) were cotransformed into the L40 or the PJ69-4A strain. Growth was monitored on plates without leucine, tryptophan and histidine, supplemented with 1 mM 3-aminotriazole for tests in the L40 strain.

**Fluorescence microscopy**

Uptake and vacuolar transport of the lipophilic styryl dye FM4-64 (Molecular Probes) was performed as described by Vida & Emr (1995). For vacuole labeling, cells were grown to an OD$_{600}$ nm of 0.8–1.2, harvested and resuspended at an OD$_{600}$ nm of 10–20 $\text{mL}^{-1}$ in fresh YEPD. FM4-64 was added to a final concentration of 40 $\text{mM}$ for a 30-min internalization step on ice. Excess FM4-64 was washed away; the cells were transferred to fresh medium and incubated at 28 °C with vigorous shaking. After 20, 60 and 120 min, samples were drawn from the cultures; the cells were harvested, resuspended in YEPD at an OD$_{600}$ nm of 5–10 $\text{mL}^{-1}$ and incubated at 28 °C with vigorous shaking for 2 h. Cells were centrifuged, resuspended in fresh YEPD at an OD$_{600}$ nm of 10–20 $\text{mL}^{-1}$, placed on slides and viewed with the rhodamine filter under a Nikon Eclipse E800 fluorescence microscope.

For FM4-64 endocytosis, the cells were grown to the early logarithmic phase at 28 °C, washed, and then transferred to fresh medium and incubated at 28 °C with vigorous shaking for 15 min. Half of the cell suspension was incubated at 37 °C for 30 min and then photographed.

**Results and discussion**

**CCZ1 interacts genetically with the PEP12 and SYN8 genes**

We used the yeast two-hybrid system to search for novel interaction partners of the *S. cerevisiae* Ccz1p protein. Among the proteins identified in this screen were the products of the genes *PEP12* and *SYN8*. The Peps12p protein is a t-SNARE of the syntaxin family. It localizes mainly to the membrane of late endosomes and is required for all known trafficking pathways that lead into this compartment in yeast cells (Gerrard et al., 2000). Functions as a common heavy chain in all SNARE complexes that have been described in the endosomal membrane (von Mollard et al., 1997; Lewis & Pelham, 2002; Paumet et al., 2004). The Syn8p protein is a component of two SNARE complexes: with Pep12p, Vti1p and Ykt6p, it forms a complex functioning in the Golgi-to-endosome transport pathway, whereas with Pep12p, Vti1p and Sncl1p it functions in the fusion of plasma membrane-derived endocytic vesicles with the late endosome (Lewis & Pelham, 2002). Tlg1p can substitute for Syn8p in these SNARE complexes – when Tlg1p is present, Syn8p can be removed without loss of Pep12p function or induction of any other trafficking defect.

To confirm the cooperation between Ccz1p, Peps12p and Syn8p, the existence of a genetic interaction between the genes encoding these proteins was tested. Tetrad analysis of the heterozygous diploids *ccz1Δ pep12Δ/CCZ1 pep12Δ* and *ccz1Δ SYN8/CCZ1 syn8Δ* revealed that in both cases all four haploid segregants were viable on standard YEPD media at 28 °C. At 37 °C, the single-mutant pep12Δ grew slower than the wild-type and *ccz1Δ* strains, whereas growth of the *ccz1Δ pep12Δ* cells was completely blocked. Spore clones bearing the *ccz1Δ syn8Δ* mutations grew equally well as the parental strains (Fig. 1a).

Further phenotypic analysis was carried out on YEPD medium supplemented with 0.3 M CaCl$_2$. Under these conditions, growth of the single *ccz1Δ* strain was slowed down (Kucharczyk et al., 2000). As shown in Fig. 1a, the single mutants *pep12Δ* and *ccz1Δ* grew slower than the wild type on YEPD+CaCl$_2$ plates, whereas the double-mutant *ccz1Δ pep12Δ* did not grow at all. Interestingly, introduction of the *syn8Δ* mutation into the *ccz1Δ* strain rescued its calcium sensitivity. This result was corroborated by the discovery that overproduction of Syn8p causes enhancement of the sensitivity phenotype (not shown). It is too early to speculate on the mechanism underlying this phenomenon, but it is interesting in terms of the relation between calcium sensitivity and vacuole morphology. We have previously considered the calcium sensitivity of the *ccz1Δ* strain to be a consequence of its abnormal vacuole morphology.

### Table 2. Plasmids used in this study

| Plasmids         | Genetic features                  | Sources               |
|------------------|-----------------------------------|-----------------------|
| pGBT9-2H-CCZ1    | GAL4 BD-CCZ1 2μ TRP1              | This study            |
| pGAD424-2H-CCZ1  | GAL4 AD-CCZ1 2μ LEU2              | This study            |
| pBK165           | LexA BD-SYN8(aa 1–169) 2μ LEU2    | Chidambaram et al. (2007) |
| pBK171           | LexA BD-PEP12(aa 1–200) 2μ LEU2   | Chidambaram et al. (2007) |
| pGAD-MON1        | GAL4 AD-MON1 2μ LEU2              | Wang et al. (2003)    |
| pACT2-YPT7       | GAL4 AD-YPT7 2μ LEU2              | This study            |
| STE2-GFP         | Ste2-GFP Int LEU2                 | Hicke & Riezman (1996) |
intermediary number of round vacuoles, while other cells appeared to resemble either of the parental strains. The fragmented vacuolar morphology of the ccz1Δ mutant was epistatic to that of the syn8Δ mutant in the ccz1Δ syn8Δ mutants (Fig. 1b).

Genetic interactions of MON1 and YPT7 with PEP12

Because Ccz1p is known to regulate the Ypt7p GTPase in a complex with Mon1p (Wang et al., 2003; Hoffman-Sommer et al., 2005), we checked whether MON1 and YPT7 would also display synthetic genetic interactions with PEP12. Tetrads analysis of the heterozygous diploids mon1Δ PEP12/MON1 pep12Δ and ypt7Δ PEP12/YPT7 pep12Δ was carried out. As shown in Fig. 2a, the double mutants mon1Δ pep12Δ and ypt7Δ pep12Δ displayed the same growth defects as ccz1Δ pep12Δ: they were temperature-sensitive and showed additive growth defects on calcium plates. In addition, both mon1Δ and ypt7Δ interacted with Pep12p in the two-hybrid system (Fig. 2b).

The vacuole morphology of mon1Δ pep12Δ was similar to that of ccz1Δ pep12Δ cells. The pep12Δ ypt7Δ double mutant, however, despite displaying the same growth defects as the ccz1Δ pep12Δ and mon1Δ pep12Δ strains (Fig. 2a), differed from them in terms of vacuolar morphology. The pep12Δ ypt7Δ mutant contained almost no discernible vacuoles at all, displaying a vacuolar phenotype even more severe than that of the single ypt7Δ mutant (Fig. 2c). These results indicated that the function of Ccz1p responsible for the synthetic interaction with Pep12p was common to the whole ccz1Δ–Mon1p–Ypt7p complex.

Temperature sensitivity has been reported previously for strains in which fusion both at the endosomal and at the vacuolar membrane is very strongly impaired, such as pep12Δ van3Δ or vps21Δ ypt7Δ (Peterson & Emr, 2001). These strains also exhibit a very severe vacuole morphology phenotype, similar to that of the pep12Δ ypt7Δ mutant, resulting probably from the nearly complete block in vacuolar protein trafficking. A similar mechanism could thus account for the temperature sensitivity of the pep12Δ ypt7Δ strain. In the ccz1Δ pep12Δ and mon1Δ pep12Δ strains, on the other hand, transport of proteins to the vacuole is not completely blocked, because discernible vacuoles are clearly present, and so probably the temperature sensitivity of these strains cannot be attributed solely to their vacuolar-trafficking defects.

The synthetic interaction of CCZ1 with PEP12 is not specific, but also occurs for other components of the vacuolar and endosomal fusion machineries

We assumed that the temperature- and calcium-sensitivity phenotypes of the ccz1Δ pep12Δ, mon1Δ pep12Δ and

(Kucharczyk et al., 2000). Here we show that in the ccz1Δ syn8Δ mutant these two phenotypes are uncoupled: vacuole morphology remains similar as in the ccz1Δ single mutant (Fig. 1b) while calcium sensitivity becomes less severe. This demonstrates that disturbed vacuole morphology and function are only one of the factors contributing to calcium sensitivity in the ccz1Δ strain.

Next, comparison of the vacuolar morphology in the single and double mutants was carried out. For this purpose, the cells were stained with the dye FM4-64 to visualize vacuoles and viewed microscopically. WT, wild type.

Fig. 1. Genetic interactions of CCZ1 with PEP12 and SYN8. (a) Growth phenotypes. The double mutants ccz1Δ syn8Δ and ccz1Δ pep12Δ were constructed by crosses and tested for growth under the indicated conditions. The cells were cultured for 2 days at 28 °C in liquid YEPD medium, harvested in the stationary phase of growth, adjusted to a density of 10^6 cells mL^{-1} and serially diluted 1:10. Five-microliter portions of the second and third dilution were spotted onto indicated plates. Photographs were taken after 3 days of incubation at 28 or 37 °C for testing growth at an elevated temperature (upper panel) or after 5 days of incubation at 28 °C for testing growth in the presence of calcium ions (lower panel). (b) Vacuole morphology of the analyzed mutants. Cells were stained with the dye FM4-64 to visualize vacuoles and viewed microscopically. WT, wild type.
pep12Δ ypt7Δ strains are the effect of simultaneous disruption of the vacuolar and endosomal fusion machineries. If this were true, then combining the ccz1Δ mutation with deletions of other genes of the Pep12p-containing endosomal fusion complex should cause similar phenotypes. Therefore, we analyzed the growth of cells that lack Ccz1p in combination with deletions of the genes VPS9, VPS21, and VPS45. These genes code for components of the late-endosomal fusion machinery: Vps21p is the Rab-GTPase responsible for this fusion step (Gerrard et al., 2000), Vps9p is the guanine-nucleotide exchange factor for Vps21p and is necessary for regulation of this protein (Hama et al., 1999) and Vps45p represents the main Sec1/MUNC18-like (SM) protein responsible for the entry of traffic from the biosynthetic route into the late endosome (Bryant et al., 1998; Bryant & James, 2001).

Tetrad analysis of the heterozygous diploids ccz1Δ VPS9/CCZ1 vps9Δ, ccz1Δ VPS21/CCZ1 vps21Δ and ccz1Δ VPS45/CCZ1 vps45Δ revealed that in all cases all four haploid segregants were viable on standard YEPD media at 28°C, whereas at 37°C the growth of spore clones bearing the double mutations ccz1Δ vps9Δ, ccz1Δ vps45Δ and ccz1Δ vps21Δ was blocked (Fig. 3). Further phenotypic analysis was carried out on YEPD medium supplemented with 0.3 M CaCl2. Growth of the single-mutant strains was not affected by calcium, but growth of the double mutants ccz1Δ vps9Δ, ccz1Δ vps45Δ and ccz1Δ vps21Δ was strongly inhibited, as for ccz1Δ pep12Δ.

The synthetic genetic interaction of CCZ1 with PEP12 is therefore not specific for this particular gene, but rather for all components of the late-endosomal fusion machinery. We do not observe such synthetic growth defects when cells are depleted of Ccz1p together with other proteins of the vacuolar fusion complex. The double deletion of CCZ1 with VAM3, which codes for the syntaxin component of the vacuolar fusion machinery, did not lead to synthetic growth defects (Fig. 3), and neither did a double deletion of CCZ1 with YPT7 (Kucharczyk et al., 2000). At the same time, double deletions of PEP12 with the genes coding for other components of the endosomal fusion complex did not cause any additive defects (Fig. 3), while growth of a pep12Δ van3Δ mutant was strongly inhibited (Peterson & Emr, 2001). The observed strong synthetic phenotypes indicate that Ccz1p, besides its role in vacuolar fusion, also influences the same stage of the vacuolar protein sorting (VPS) pathway as the Pep12p, Vps21p and Vps45p proteins.

The genetic data of the interactions of CCZ1 and PEP12 indicate the existence of two functionally distinct complexes.
involved in endosomal fusion: one containing Pep12p, Vps21p, Vps9p and Vps45p, and another containing Ccz1p, Mon1p and Ypt7p. Simultaneous disruption of both of these complexes would cause the synthetic phenotypes. This would explain the genetic interactions of \textit{CCZ1} with \textit{PEP12}, \textit{VPS21}, \textit{VPS9} and \textit{VPS45}, and, similarly, of \textit{MON1} and \textit{YPT7} with \textit{PEP12}.

The \textit{ccz1Δ pep12Δ} cells exhibit severe defects in endocytosis

Previously, we analyzed the secretion of CPY in a \textit{ccz1Δ} strain (Kucharczyk \textit{et al}., 2000). This enzyme is present in the endoplasmic reticulum in the so-called p1 form, which is then transported to the Golgi, and there further glycosylated, resulting in an inactive precursor termed p2CPY. This precursor is trafficked from the Golgi to the vacuole via the late endosome. Upon entry into the vacuole, p2CPY is proteolytically cleaved to the active, mature form, mCPY (Bryant & Stevens, 1998). Disruption of the VPS pathway results in the accumulation of p2CPY: strains defective in the Golgi-to-endosome stage of the VPS pathway secrete p2CPY (Burd \textit{et al}., 1997), while strains that are defective in the endosome-to-vacuole step of the pathway retain p2CPY intracellularly, trapped in prevacuolar transport intermediates (Darsow \textit{et al}., 1997). Our results demonstrated that the \textit{ccz1Δ} mutation causes the secretion of small amounts of p2CPY. Secretion of p2CPY in the \textit{ccz1Δ} strain – in addition to measurable intracellular accumulation, which results from a block in vacuolar fusion – also indicated a defect in the entry of p2CPY into the late endosome in this mutant (Kucharczyk \textit{et al}., 2000).

In order to determine whether Ccz1p function is required for transport of vesicles carrying endocytic cargo with the late endosome, the steady state of Ste2-GFP localization was tested in single and double mutants. Ste2-GFP, a chimeric variant of Ste2p, a signaling receptor that is internalized into the endocytic pathway (Hicke & Riezman, 1996), is packaged into endosomal vesicles and delivered to the vacuole lumen through the late endosome/prevacuolar compartment (Odorizzi \textit{et al}., 1998). Thus, it serves as an endocytic marker. We looked at the localization of Ste2-GFP in single and double mutants cultured at 28 °C and after 30 min of incubation at 37 °C. In wild-type and \textit{syn8Δ} mutant cells, Ste2-GFP was transported effectively to the vacuole lumen at both temperatures (Fig. 4, not shown), whereas in the \textit{ccz1Δ} mutant transport of the protein was blocked at the late endosomes and introduction of the \textit{syn8Δ} mutation had no additional effect. The single \textit{pep12Δ} mutant displayed a very severe defect even at the permissive temperature, as the marker protein was present mainly in very small vesicles dispersed in the whole cell, probably early endosomes. In about 15% of the cells, the late-endosome/prevacuolar

Fig. 4. Transport of cargo by receptor-mediated endocytosis. Fluorescence and differential interference contrast images of indicated mutant cells expressing Ste2-GFP grown at 28 °C. Cells were harvested at a cell density of 0.6, washed, resuspended in phosphate-buffered saline buffer and viewed.
compartments were also fluorescent. This is in agreement with the data presented by Gerrard et al. (2000), implicating a block in traffic from early to late endosomes in this mutant. In the double-mutant ccz1Δ pep12Δ, we observed an enhanced defect in Ste2-GFP localization. The diffusible pattern of fluorescence in these cells indicated that both Ccz1p and Pep12p are needed for efficient transit of Ste2-GFP from early to late endosomes.

To confirm that the block in endocytic transport occurs at the late endosome, we exploited the phenotype of cells depleted of the VPS27 gene. vps27Δ cells show a defect in traffic out of the late endosome, both in retrograde traffic to the late Golgi as well as in forward transport to the vacuole. As a result, these cells exhibit an enlarged late endosome (class E compartment) in which vacuolar proteins and endocytosed cargo accumulate (Piper et al., 1995; Bryant et al., 1998). By genetic crosses, we constructed the following double and triple mutants: vps27Δ ccz1Δ vps27Δ syn8Δ vps27Δ pep12Δ, vps27Δ ccz1Δ syn8Δ and vps27Δ ccz1Δ pep12Δ. The rate of FM4-64 endocytosis in single, double and triple mutants is presented in Fig. 5. In wild-type cells, after 20 min of chase, the FM4-64 dye was present in endosomes, and in the vacuole membrane after 60 min. The same trend was observed for syn8Δ cells. In ccz1Δ single-mutant cells, the transport to the endosomes proceeded efficiently, because after 20 min of chase the dye was mainly in the late endosomes (2–5 per cell), but traffic from the endosomes was delayed – after 60 min the dye was still in the endosomes and reached the vacuole membrane only after 120 min. In pep12Δ as in wild-type cells, after 20 min of chase the dye was in early and late endosomes; after 60 min of chase, most of the dye was located in the vacuole
membrane and only a few endosomes fluoresced. The double-mutant cells ccz1Δ syn8Δ did not differ from the ccz1Δ mutant, but in the double-mutant ccz1Δ pep12Δ we observed an enhancement in the defect at 20 min of chase – in most cells diffusible fluorescence was observed, indicating that the dye was still blocked in the early endosomes. The vps27Δ mutant transported FM4-64 to the enlarged class E compartment during the first 20 min of the chase and after 60 min the dye localized mainly to the enlarged endosome with some signal in the vacuolar membrane. We observed a different fluorescence profile after introduction of the vps27Δ mutation into the analyzed mutants. In vps27Δ pep12Δ cells, the trafficking of the dye to the endosomes is less efficient, or the morphology of endosomes is altered due to the pep12Δ mutation, because at 60 min the fluorescence signal is still present in small vesicles whereas the enlarged class E compartment is invisible in contrast to faint fluorescence in the vacuolar membrane. In vps27Δ ccz1Δ cells, the defect is stronger – a weak vesicular signal was observed at all time points, and some enlarged endosomes were visible after 60 and 120 min. These results show that in the double mutants, the traffic from early to late endosomes was delayed as compared with the single mutants, indicating that Ccz1p, as Pep12p, is involved in this transport step. Introduction of the vps27Δ mutation into the ccz1Δ pep12Δ and ccz1Δ syn8Δ mutants significantly intensified the defects in FM4-64 endocytosis – we observed a more diffuse labeling pattern. After 20 min, we did not observe any signal in the enlarged endosomes; neither did the staining pattern change during the next 40 min of chase. This indicates that these cells are either not efficient in forming an enlarged endosome as seen in the vps27Δ cells or that trafficking to this endosomal compartment is compromised, or both.

Analysis of FM4-64 trafficking in ccz1Δ vps27Δ cells (Fig. 5) demonstrated that the ccz1Δ mutation slows down the transport of the dye to the enlarged endosomes, although the internalization step proceeds normally (not shown).

A kinetic delay in endocytosis in the ccz1Δ strain has been demonstrated previously (Kucharczyk et al., 2000), but it was considered a result of the vacuolar fusion defect. Now we have shown that a defect occurs also at the endosomal fusion step. Together with CPY secretion in the ccz1Δ mutant (Kucharczyk et al., 2000), these experiments indicate that Ccz1p function is required for efficient entry of both biosynthetic and endocytic cargo into the late endosome. The synthetic phenotypic enhancement, along with the two-hybrid interactions, implies that a functional relationship exists between endosomal/TGN t-SNAREs and the Ccz1p–Mon1p complex.

In this work, we show that the Ccz1p–Mon1p complex is involved in fusion at the endosomal membrane, in addition to its established function in vacuolar fusion. For Ccz1p this is the third potential site of action, because on the basis of genetic interactions between CCZ1, ARL1 and YPT1 it has already been postulated to also function in Golgi trafficking (Love et al., 2004; Hoffman-Sommer et al., 2005). We thus provide a further example of the promiscuity of the membrane fusion machinery, components of which are often present and active in multiple sites in the cell. This report supports the notion that the specificity of membrane fusion depends on a complicated network of protein interactions.

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