Cation Diffusion Facilitator Cis4 Is Implicated in Golgi Membrane Trafficking via Regulating Zinc Homeostasis in Fission Yeast

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We screened for mutations that confer sensitivities to the calcineurin inhibitor FK506 and to a high concentration of $MgCl_2$ and isolated the *cis4-1* mutant, an allele of the gene encoding a cation diffusion facilitator (CDF) protein that is structurally related to zinc transporters. Consistently, the addition of extracellular Zn^{2+} suppressed the phenotypes of the *cis4* mutant cells. The *cis4* mutants and the mutant cells of another CDF-encoding gene SPBC16E9.14c (we named *zrg17*⁺) shared common and nonadditive zinc-suppressible phenotypes, and Cis4 and Zrg17 physically interacted. Cis4 localized at the *cis*-Golgi, suggesting that Cis4 is responsible for Zn^{2+} uptake to the *cis*-Golgi. The *cis4* mutant cells showed phenotypes such as weak cell wall and decreased acid phosphatase secretion that are thought to be resulting from impaired membrane trafficking. In addition, the *cis4* deletion cells showed synthetic growth defects with all the four membrane-trafficking mutants tested, namely *ypt3-i5*, *ryh1-i6*, *gdi1-i11*, and *apm1-1*. Interestingly, the addition of extracellular Zn^{2+} significantly suppressed the phenotypes of the *ypt3-i5* and *apm1-1* mutant cells. These results suggest that Cis4 forms a heteromeric functional complex with Zrg17 and that Cis4 is implicated in Golgi membrane trafficking through the regulation of zinc homeostasis in fission yeast.

INTRODUCTION

Zinc is an essential nutrient for all cells. However, a high concentration of Zn^{2+} causes lethal damage to the cell. Therefore, cells require homeostatic mechanisms to regulate the intracellular Zn^{2+} concentration. The Zn^{2+} concentration in the cytoplasm and subcellular organelles is regulated by the activity of transporter proteins that mediate its uptake and efflux (Gaither and Eide, 2001; Kambe *et al.*, 2004; Cousins *et al.*, 2006; Kambe *et al.*, 2006). A number of zinc transporters belonging to different families have been identified. In particular, the cation diffusion facilitator (CDF) family has been implicated in the metal homeostasis of diverse organisms from prokaryotes to eukaryotes (Williams *et al.*, 2000; Gaither and Eide, 2001; Nies, 2003; Kambe *et al.*, 2004; Cousins *et al.*, 2006).

Most CDF family of zinc transporters, which are ubiquitously found, have been characterized to facilitate Zn^{2+} efflux from the cytoplasm or to mobilize the cytoplasmic Zn^{2+} into the intracellular organelles. In budding yeast *Saccharomyces cerevisiae*, two CDF family members, Zrc1p and Cot1p, localize at the vacuolar membrane and they have

been implicated in the storage of excess Zn^{2+} in the vacuole (Kamizono *et al.*, 1989; Conklin *et al.*, 1992, 1994; Li and Kaplan, 1998; MacDiarmid *et al.*, 2000, 2002, 2003). Two other CDF family members, Msc2p and Zrg17p, form a heteromeric zinc transport complex in the endoplasmic reticulum (ER) membrane, and they have been implicated in the homeostatic maintenance of ER function. In mutant yeast deficient in these CDF proteins, the UPR induction by zinc deficiency is exacerbated (Ellis *et al.*, 2004, 2005). In fission yeast *Schizosaccharomyces pombe*, Zhf1, a homolog of *S. cerevisiae* Zrc1p and Cot1p, localizes at the ER, mediates Zn^{2+} storage, and differentially affects tolerance to a range of transition metals (Clemens *et al.*, 2002). In vertebrate cells, three CDF family members, ZnT5, ZnT6, and ZnT7, are located in the Golgi and are implicated in the entry of Zn^{2+} into the Golgi lumen (Kambe *et al.*, 2002; Huang *et al.*, 2002; Kirschke and Huang, 2003). Using DT40 cells, Kambe *et al.* showed that the two oligomeric zinc transport complexes, namely ZnT5/ZnT6 hetero-oligomeric complexes and ZnT7 homo-oligomeric complexes, are required for zinc incorporation into alkaline phosphatases (Suzuki *et al.*, 2005a,b; Ishihara *et al.*, 2006).

In this study, we identified Cis4, a new zinc transporter belonging to the CDF protein family, and we showed that its mutation affects cell wall integrity and secretion in fission yeast. In addition, we also showed that the addition of extracellular Zn^{2+} suppressed the phenotypes of two membrane trafficking mutants, namely *ypt3-i5* and *apm1-1*, alleles of the genes implicated in Golgi membrane trafficking. Taken together, these results highlight the importance of

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Table 1. Strains used in this study

Strain	Genotype	Reference
HM123	<i>h⁻ leu1-32</i>	Our stock
HM528	<i>h⁺ his2</i>	Our stock
KP456	<i>h⁻ leu1-32 ura4-D18</i>	Our stock
KP928	<i>h⁺ his2 leu1-32 ura4-D18</i>	Our stock
5A/1D	<i>h⁻/h⁺ leu1-32/leu1-32 ura4-D18/ura4-D18 his2/+ ade6-M210/ade6-M216</i>	Our stock
KP457	<i>h⁻ leu1-32 cis4-1</i>	This study
KP537	<i>h⁻ leu1-32 cis4-2</i>	This study
KP680	<i>h⁻ leu1-32 ura4-D18 cis4::ura4⁺</i>	This study
KP2367	<i>h⁺ his2 leu1-32 ura4-D18 arg1-1</i>	Ma et al. (2007)
KP162	<i>h⁻ leu1-32 ypt3-i5</i>	Cheng et al. (2002)
KP1892	<i>h⁻ leu1-32 gdi1-i11</i>	Ma et al. (2006)
KP1447	<i>h⁻ leu1-32 ryh1-i6</i>	He et al. (2006)
KP356	<i>h⁻ leu1-32 cis1-1/apm1-1</i>	Kita et al. (2004)
KP2906	<i>h⁻ leu1-32 ura4-D18 ypt3-i5 cis4::ura4⁺</i>	This study
KP2908	<i>h⁻ leu1-32 ura4-D18 ryh1-i6 cis4::ura4⁺</i>	This study
KP3370	<i>h⁻ leu1-32 ura4-D18 asn1-1 arg1-1 cis4::ura4⁺</i>	This study
KP3412	<i>h⁻ leu1-32 ura4-D18 gdi1-i11 cis4::ura4⁺</i>	This study
KP3439	<i>h⁻ leu1-32 ura4-D18 apm1-1 cis4::ura4⁺</i>	This study
KP3724	<i>h⁻ leu1-32 zrg17::LEU2</i>	This study
KP3725	<i>h⁻ leu1-32 ura4-D18 cis4::ura4⁺ zrg17::LEU2</i>	This study

zinc and a CDF zinc transporter Cis4 in Golgi membrane trafficking in fission yeast.

MATERIALS AND METHODS

Strains, Media, and Genetic and Molecular Biology Methods

S. pombe strains used in this study are listed in Table 1. The complete medium YPD (yeast extract-peptone-dextrose) and the minimal medium EMM (Edinburgh minimal medium) have been described previously (Toda et al., 1996). Standard genetic and recombinant-DNA methods (Moreno et al., 1991) were used except where noted. Tacrolimus (FK506) was provided by Fujisawa Pharmaceutical Co. (Osaka, Japan). Gene disruptions are denoted by lower-case letters representing the disrupted gene followed by two colons and the wild-type gene marker used for disruption (for example, *cis4::ura4⁺*). Gene disruptions are abbreviated by the gene preceded by Δ (for example, $\Delta cis4$). Proteins are denoted by roman letters and only the first letter is capitalized (for example, Cis4).

Isolation of *cis4-1* and *cis4-2* Mutants

The *cis4-1* (KP457) mutant was obtained from a pool of mutants that showed both FK506 sensitivity and $MgCl_2$ sensitivity as described previously (Fujita et al., 2002; Kita et al., 2004). The *cis4-2* (KP537) mutant was obtained from a pool of mutants that showed only $MgCl_2$ sensitivity. The original mutants isolated were backcrossed three times to wild-type strains HM123 and HM528.

Cloning and Knockout of *cis4⁺* and SPBC16E9.14c (*zrg17⁺*) Genes

To clone the *cis4⁺* gene, the *cis4-1* mutant was grown at 30°C and transformed with an *S. pombe* genomic DNA library constructed in the vector pDB248 (Beach et al., 1982). The *Leu⁺* transformants were replica-plated onto YPD plates containing 0.15 M $MgCl_2$, and the plasmid DNA was recovered from the transformants that showed a plasmid-dependent rescue. These plasmids complemented both the immunosuppressant sensitivity and $MgCl_2$ sensitivity of the *cis4-1* mutant. By DNA sequencing, the suppressing plasmids fell into two classes, with one class containing the *cis4⁺* gene (SPAC17D4.03c), and another class containing a gene distinct from the *cis4⁺* gene. Characterization of the gene in the second class will be reported elsewhere. To investigate the relationship between the cloned SPAC17D4.03c gene and the *cis4-1*

mutant, linkage analysis was performed as follows. The entire SPAC17D4.03c gene was subcloned into the pUC-derived plasmid containing the *S. cerevisiae* *LEU2* gene and was integrated by homologous recombination into the genome of the wild-type strain HM123. The integrant was mated with the *cis4-1* mutant. The resulting diploid was sporulated, and tetrads were dissected. A total of 30 tetrads were dissected. In all cases, only parental ditype tetrads were found, indicating the allelism between the SPAC17D4.03c gene and the *cis4-1* mutation (our unpublished data). Similarly, the allelism between the SPAC17D4.03c gene and the *cis4-2* mutation was confirmed (our unpublished data). We therefore named SPAC17D4.03c as the *cis4⁺* gene.

To knockout the *cis4⁺* gene, a one-step gene disruption by homologous recombination was performed (Rothstein, 1983). The *cis4::ura4⁺* disruption was constructed as follows. The 2.1-kb PstI/EcoRV genomic fragment, containing the *cis4⁺* gene excluding the 95 base pairs from the start codon, was subcloned into the PstI/SmaI site of BlueScriptSK(+). Then, a BamHI/PstI fragment containing the *ura4⁺* gene was inserted into the BglII/NsiI site of the previous construct. The construct containing the disrupted *cis4⁺* gene was digested with BamHI and PstI, and the *cis4::ura4⁺* fragment was used to transform the diploids (5A/1D, Table 1). The disruption was verified by Southern hybridization of the *Ura⁺* heterozygous diploids and the *Ura⁺* haploids segregants (our unpublished data).

SPBC16E9.14c gene, which we named *zrg17⁺* gene, encodes a homolog of budding yeast *Zrg17p* (Score = 210, Expect = $1.2e^{-15}$). Budding yeast *Zrg17p* is proposed to form a heteromeric zinc transport complex with *Msc2p* in the ER membrane (Ellis et al., 2005). To knockout the *zrg17⁺* gene by homologous recombination, the 2054-base pair HindIII/EcoRI genomic fragment containing the *zrg17⁺* gene was subcloned into the HindIII/EcoRI site of BlueScriptSK(+). The *NcoI* site of *zrg17⁺* gene was changed to BglII site by linker. Then, a BamHI fragment containing the *LEU2* gene was inserted into the BglII site of the previous construct. The construct containing the disrupted *zrg17⁺* gene was digested with HindIII and EcoRI, and the *zrg17::LEU2* fragment was used to transform the diploids (5A/1D, Table 1). Stable integrants were selected on medium lacking leucine. The disruption was verified by genomic Southern hybridization (our unpublished data). The $\Delta cis4\Delta zrg17$ double knockout mutant cells were generated by the genetic cross between *cis4::ura4⁺* and *zrg17::LEU2*.

Gene Expression

Two budding yeast genes, *MSC2* and *ZRG17*, were expressed in fission yeast as follows. The open reading frame (ORF) of *MSC2* was amplified by PCR (forward primer 2149, 5'-CCG CTC GAG ATG GAT AGA GGC AGG TGG TG-3'; reverse primer 2151, 5'-GAA GAT CTT CAA TTT GCT ATA GGC TGT AGC-3') from the genomic DNA of *S. cerevisiae* strain W303-1A and subcloned into the XhoI/BamHI site of pD5473aL expression vector (Forsburg and Sherman, 1997) to give pKB6957. The ORF of *ZRG17* was amplified by PCR (forward primer 2283, 5'-CGC GGA TCC ATG GAG ACG CCG CAA ATG AAC GC-3'; reverse primer 2284, 5'-CGC GGA TCC TTA TAT TCG GTC TAT GTC TAT TGT AGT TTC-3') from the genomic DNA of *S. cerevisiae* and subcloned into BamHI site of BlueScriptSK(+). The fission yeast expression vector pREP1 (Maundrell, 1993) was cut with PstI/SacI, and the fragment containing *nmt1* promoter and stop codon was subcloned into the integration vector containing the *asn1⁺* gene, pKB6864 (Ma et al., 2007), to give pKB7211. Then the above construct containing complete *ZRG17* ORF was digested with BamHI, and the resulting fragment was subcloned into the BamHI site of pKB7211 to give pKB7218. These constructs were used to express the budding yeast genes in fission yeast cells.

To express Cis4 tagged with red fluorescent protein (RFP) from its own promoter, the *cis4⁺* ORF was amplified by PCR (forward primer 2285, 5'-CCG CTC GAG CAC CAT GAA TGT TAA TTC TTC TGC GTT CG-3'; reverse primer 2286, 5'-ATA GTT TAG CGG CCG CCT TTT CCA CAC CAG CAA TTG TTT GGC CG-3') from the genomic DNA and was subcloned into the XhoI/NotI site of BlueScriptSK(+). The cloned ORF of the *cis4⁺* gene was digested with HindIII/NotI and the resulting fragment (1.2 kb), which lacked half of the ORF starting from the 5' end and in addition lacked the stop codon, was inserted at the 5' end of the coding sequence for monomeric RFP gene (GenBank Accession No. AB166761) in the integration vector containing the *ura4⁺* gene to give pKB7269. To obtain the chromosome-borne Cis4-RFP under the control of its own promoter, wild-type cells (KP456, Table 1) were transformed with pKB7269 and were integrated into the chromosome at the *cis4⁺* gene locus of KP456. A successful integration was confirmed by PCR and Southern blot (our unpublished data). All the RFP, GFP-, or GST-tagged versions of Cis4 or *Zrg17* were fully functional as demonstrated by its complementation of *cis4* or *zrg17* mutant phenotypes, respectively (our unpublished data).

GFP-Syb1, Rer1-GFP, and Krp1-GFP were expressed as described previously (Nakamura-Kubo et al., 2003; Kita et al., 2004; He et al., 2006).

Microscopy and Miscellaneous Methods

Acid phosphatase activity in whole cells and cell extracts was determined according to Bergman (1986), and the accumulation of acid phosphatase in cells was estimated by subtracting the cell-extract values from the whole-cell values. Methods in light microscopy, such as fluorescence microscopy which

was used to observe the localization of GFP- or RFP-tagged proteins were performed as described (Kita *et al.*, 2004). Tetrad analysis (Zhang *et al.*, 2000), cell wall digestion by β -glucanase (Zymolyase, Seikagaku Kogyo, Tokyo, Japan, Cheng *et al.*, 2002), GST pulldown assay (Tsutsumi *et al.*, 2007) were performed as previously described. To quantify the GFP-Syb1 localization, at least 50 cells with GFP fluorescence were identified and scored for GFP-Syb1 localization to the cell surface. Most strains were analyzed by independent observers in a double-blind manner.

RESULTS

Isolation of the *cis4-1* Mutant

To isolate new molecules that functionally interact with calcineurin, we further screened for mutations that confer sensitivities to the calcineurin inhibitor FK506 and to a high concentration of MgCl_2 and isolated the *cis4-1* mutant (*cis*: chloride and immunosuppressant sensitive; Kita *et al.*, 2004). As shown in Figure 1, the *cis4-1* mutant cells grew equally well compared with the wild-type cells at 27°C. However, the *cis4-1* mutant cells could not grow on YPD plate containing FK506 or 0.15 M MgCl_2 at the permissive temperature, whereas wild-type cells grew normally (Figure 1). As predicted, no double mutant was obtained at any temperature by the genetic cross between *cis4-1* and calcineurin deletion (Δ *ppb1*; our unpublished data), indicating that *cis4-1* and Δ *ppb1* are synthetically lethal.

The *cis4*⁺ Gene Encodes a CDF Protein That Is Structurally Related to Zinc Transporters

As described in *Materials and Methods*, the *cis4*⁺ gene (SPAC17D4.03c) was cloned by complementation of the MgCl_2 -sensitive growth defect of the *cis4-1* mutant cells (Figure 1, +*cis4*⁺). The *cis4*⁺ gene also complemented the FK506-sensitive phenotype of the *cis4-1* mutant (Figure 1). Nucleotide sequencing of the cloned DNA fragment revealed that the *cis4*⁺ gene encodes a CDF protein of 732 amino acids that is structurally similar to the budding yeast zinc transporter Msc2p (Score = 361, Expect = 1.3×10^{-35} , BLAST at the Sanger Center) and the human zinc transporters ZnT5, ZnT6 and ZnT7 (Score = 390, Expect = 2.5×10^{-63} , Score = 135, Expect = 5.3×10^{-16} ; Score = 432, Expect = 4.6×10^{-67} , respectively; Figure 2A). To characterize the mutation site in the *cis4-1* mutant, the genomic DNA from the *cis4-1* mutant was isolated, and DNA sequencing of the full-length coding region of the mutant revealed that the G-to-A nucleotide substitution caused a glycine to be altered to a glutamate

Figure 1. Mutation in the *cis4*⁺ gene causes MgCl_2 - and immunosuppressant-sensitive phenotypes. Wild-type (wt), *cis4-1*, or Δ *cis4* cells transformed with the multicopy vector pDB248 or the vector containing the *cis4*⁺ (SPAC17D4.03c) gene were streaked onto the plates as indicated and then incubated for 4 d at 27°C.

codon at the amino acid position 375 (Figure 2A). For the *cis4-2* mutant, similar analysis revealed that the G-to-A nucleotide substitution caused a glycine to be altered to an aspartate codon at the amino acid position 393 in the *cis4-2* mutant (Figure 2A).

Disruption of *cis4*⁺ Gene

We constructed a null mutation in the *cis4*⁺ gene (see *Materials and Methods*) and found that the *cis4* deletion was viable (Figure 1, Δ *cis4* + vector), indicating that Cis4 is not essential for cell viability. The Δ *cis4* cells, compared with the *cis4-1* cells, showed similar sensitivity to MgCl_2 but were less sensitive to FK506 (Figures 1 and 2B). Meanwhile, the *cis4-2* mutants, compared with the Δ *cis4* cells, showed similar MgCl_2 and FK506 sensitivities (Figure 2B).

Figure 2. Cis4 is homologous to zinc transporters and the phenotypes of *cis4* mutants are zinc suppressible. (A) Comparison of the amino acid sequences among fission yeast Cis4, budding yeast Msc2p, and human ZnT7. Amino acid sequences are aligned using the Clustal W program. Filled boxes indicate the mutated amino acids. (B) Phenotypes of the *cis4* mutants are zinc suppressible. A 5- μ l sample of cells was spotted onto the plates as indicated in serial 10-fold dilutions starting with $\text{OD}_{660} = 0.3$ of log-phase cells and then incubated for 4 d at 27°C. (C) Simultaneous expression of budding yeast *MSC2* and *ZRG17* genes suppressed the MgCl_2 -sensitive phenotype of Δ *cis4* cells. A 5- μ l sample of Δ *cis4* cells expressing various genes were spotted onto EMM plates as indicated and incubated for 4 d at 27°C.

Addition of Extracellular Zn^{2+} Suppressed the Phenotypes of *cis4* Mutant Cells

As *Cis4* is structurally related to zinc transporters, we hypothesized that *Cis4* may mobilize the cytoplasmic Zn^{2+} into the intracellular organelles. Mutation or deletion of the *cis4*⁺ gene may cause a shortage of Zn^{2+} in the organelles, and the shortage can be suppressed by the addition of extracellular Zn^{2+} . Then, we examined the effect of the addition of Zn^{2+} on the phenotypes of *cis4* mutants. As shown in Figure 2B, the addition of Zn^{2+} on the YPD medium dramatically suppressed the MgCl_2 sensitivity of all the *cis4* mutants, namely *cis4-1*, *cis4-2*, and *Δcis4* cells. The addition of Zn^{2+} also suppressed the FK506 sensitivity of the *cis4* mutants. The *cis4* mutants showed partial suppression of their phenotypes with the addition of 0.5–1 mM Zn^{2+} and showed full suppression with the addition of 2 mM Zn^{2+} . The removal of zinc from the synthetic medium recipe (a final concentration of Zn^{2+} in EMM was ~0.6 mM) had no effect on the phenotypes (our unpublished data).

When high levels of a metal ion are added to complex media such as YPD, the added metal can titrate other metal ions off of the metal-binding components in the media (e.g., amino acids) and make those other metals more available to the cells. Thus, the suppression of the mutant phenotype observed may not be due to the high amount of zinc per se, but rather it may be due to the increased availability of a different metal ion. Therefore, we tested if high levels of other metal ions can also suppress the mutant phenotype. Neither MnSO_4 (up to 2 mM), nor FeCl_3 (up to 2 mM) or CuSO_4 (up to 0.2 mM), when added to the media, showed suppressive effect on the phenotypes of the *cis4* mutants (our unpublished data). These results strongly suggest that *Cis4* is a zinc transporter that mobilizes the cytoplasmic Zn^{2+} into the intracellular organelles.

Ellis *et al.* (2005) showed that the budding yeast *zrg17* mutant exhibits the same zinc-suppressible phenotypes as the *msc2* mutant and suggested that *Msc2p* and *Zrg17p* form a heteromeric zinc transport complex. Then, we examined whether the expression of these budding yeast genes could suppress the growth phenotype of *Δcis4* cells. As shown in Figure 2C, the expression of the budding yeast *MSC2* or *ZRG17* alone could not suppress the growth phenotype of *Δcis4* cells. However, when both *MSC2* and *ZRG17* were coexpressed, the growth defect was suppressed.

The *cis4* and *zrg17* Mutant Cells Shared Common and Nonadditive Zinc-suppressible Phenotypes, and *Cis4* and *Zrg17* Physically Interacted

The above findings prompted us to disrupt the *zrg17*⁺ gene and to examine the phenotypes of *Δzrg17* cells and *Δcis4Δzrg17* cells. As shown in Figure 3A, the *Δzrg17* mutant also exhibited MgCl_2 - and FK506-sensitive growth defects, and they were also suppressible by the addition of zinc. Thus, *Δcis4* and *Δzrg17* mutants show very similar zinc-suppressible growth defects. As expected, the *Δcis4Δzrg17* double mutant also showed MgCl_2 - and FK506-sensitive growth defects. The addition of similar concentrations of zinc suppressed the double mutant to the same degree as that of the single mutants, indicating that the effect of zinc on the phenotype of these two mutations are not additive.

Furthermore, the pulldown experiments showed that GFP-*Cis4* associated with GST-*Zrg17* but not with GST, thus indicating the physical interaction between *Cis4* and *Zrg17* (Figure 3B).

Figure 3. The *cis4* and *zrg17* mutant cells share common and nonadditive zinc-suppressible phenotypes (A), and *Cis4* and *Zrg17* physically interact (B). (A) Wild-type, *Δcis4*, *Δzrg17*, or *Δcis4Δzrg17* cells were spotted onto the plates as indicated and then incubated for 4 d at 27°C. (B) In cells expressing GFP-*Cis4* from the chromosomally integrated gene, GST, or GST-*Zrg17* was expressed from the harboring plasmid at 27°C. GST-tagged protein was pulled down by glutathione beads, washed extensively, subjected to SDS-PAGE, and immunoblotted using anti-GFP or anti-GST antibodies.

Intracellular Localization of *Cis4*

To study the intracellular localization of the *Cis4* protein, we tagged the 3' end of the *cis4*⁺ gene with the sequence encoding RFP. This construct is fully functional as cells expressing *Cis4*-RFP complemented the phenotypes associated with the *cis4* mutation, and the phenotypic analysis of the cells expressing *Cis4*-RFP also indicates that tagging did not abolish function (our unpublished data). Then we examined the localization of *Cis4*-RFP expressed from its own promoter in wild-type cells. *Cis4*-RFP localized to the punctate structures that were scattered throughout the cytoplasm (Figure 4). To determine whether these punctate structures indicate the Golgi-associated localization of *Cis4*, the colocalization of *Cis4* with the *cis*-Golgi marker *Rer1* and the *trans*-Golgi marker *Krp1* were examined. *Cis4*-RFP clearly colocalized with *Rer1*-GFP (Figure 4A), but not with *Krp1*-GFP (Figure 4B), indicating that *Cis4* localizes at *cis*-Golgi.

The *cis4* Mutants Showed Defects in Cell Wall Integrity

Because most of the FK506-sensitive fission yeast mutants we have studied showed defects in cell wall integrity (Yada

Figure 4. Cis4 protein localizes at the *cis*-Golgi. Cells expressing Cis4-RFP were transformed with the vector expressing Rer1-GFP (A) or Krp1-GFP (B). Cells were grown to early log phase in EMM containing 4 μ M thiamine. Bar, 10 μ m. DIC, differential interference contrast (microscopy).

et al., 2001; Cheng *et al.*, 2002; Kita *et al.*, 2004), we hypothesized that the *cis4* mutants are also defective in cell wall integrity. Defects in cell wall integrity can sometimes be compensated for by increases in the osmolarity of the growth media (Levin and Bartlett-Heubusch, 1992). Consistent with our hypothesis, all the *cis4* mutant cells were capable of forming colonies on YPD plates containing both 0.15 M MgCl_2 and 1.2 M sorbitol (Figure 5A).

A high septation index (the percentage of cells in a population bearing a septum) also reflect the cell wall alterations (Karagiannis *et al.*, 2002). As shown in Figure 5B, the Δ *cis4* cells exhibited an increased septation index that was suppressed by the addition of Zn^{2+} . Consistently, we found that the *cis4* mutants were highly sensitive to micafungin, a (1,3)- β -D-glucan synthase inhibitor (Figure 5C).

Expression of Constitutively Active Calcineurin Suppressed the Phenotypes of *cis4* Mutant Cells

We then examined the effect of the overexpression of the constitutively active calcineurin on the *cis4-1* mutant. The

cis4-1 mutant cells were transformed with a vector containing the full-length calcineurin gene (CN full), the constitutively active truncated calcineurin gene (CN Δ C), or a control multicopy vector (Figure 5D). The results showed that the overexpression of the constitutively active calcineurin, but not the full-length calcineurin, suppressed the MgCl_2 -sensitive growth defect of the *cis4-1* mutant cells. Overexpression of the constitutively active calcineurin also suppressed the MgCl_2 -sensitive growth defect of the *cis4-2* and Δ *cis4* mutant cells (our unpublished data). These results further suggest that Cis4 is implicated in cell wall integrity.

The *cis4* Deletion Cells Showed Defects in Acid Phosphatase Secretion and Membrane Trafficking

In previous studies, we showed that several membrane-trafficking fission mutants exhibited a defect in acid phosphatase secretion (Cheng *et al.*, 2002; Kita *et al.*, 2004; He *et al.*, 2006). Here, to assess whether the *cis4* mutation specifically affects acid phosphatase secretion or whether the mutation decreases the enzyme accumulation, we determined the acid phosphatase activity in whole cells and cell extracts (Bergman, 1986). As shown in Figure 5E, the acid phosphatase secretion and accumulation of Δ *cis4* cells were ~25 and 55% compared with that of the wild-type cells, respectively. These results indicate that the *cis4* deletion affects both the secretion and accumulation of acid phosphatase and that the secretion was more seriously impaired.

Next, we monitored the localization of the exocytic v-SNARE synaptobrevin Syb1 to assess the Golgi-to-endosome or Golgi-to-plasma membrane trafficking pathway (Edamatsu and Toyoshima, 2003; Kita *et al.*, 2004). In wild-type cells, the fluorescence of GFP-Syb1 clearly localized to the cell membrane at the cell ends (Figure 5F, left top panel, arrows). In Δ *cis4* cells, however, the fluorescence of GFP-Syb1 at the cell membrane was markedly reduced (Figure 5F, right top panel). The addition of Zn^{2+} to the medium significantly increased the number of cells that showed the fluorescence of GFP-Syb1 at the cell membrane (Figure 5F, bottom panel). These results suggest that the defective membrane trafficking in the Δ *cis4* cells is due to the low intra-Golgi zinc concentration.

Genetic Interaction between *cis4* and the Membrane-trafficking Mutants

The above findings led us to hypothesize that Cis4 mobilizes the cytoplasmic Zn^{2+} into the Golgi and that the intra-Golgi concentration of Zn^{2+} is important for membrane trafficking associated with the Golgi complex. To examine the genetic interaction between Cis4 and the membrane-trafficking mutants, we constructed several double mutant strains, namely *ypt3-i5* Δ *cis4*, *ryh1-i6f* Δ *cis4*, *gdi1-i11* Δ *cis4*, and *apm1-1* Δ *cis4*. Briefly describing these membrane trafficking components, first, the Rab/Ypt GTPase Ypt3 has been implicated in the membrane trafficking associated with the Golgi complex, and its mutation confers sensitivity to FK506 and defects in cell wall integrity (Cheng *et al.*, 2002). Second, Ryh1, a homolog of mammalian Rab6 and budding yeast Ypt6p, has been implicated in retrograde traffic from endosome to the Golgi (He *et al.*, 2006). Third, Gdi1, a Rab GDP-dissociation inhibitor, plays a role in the recycling of Rabs in the secretory pathway (Ma *et al.*, 2006). Fourth, Apm1, a homolog of the mammalian μ 1A subunit of the clathrin adaptor protein complex 1, is implicated in the Golgi/endosome function (Kita *et al.*, 2004).

Interestingly, results showed that there is a genetic interaction between Cis4 and all of the membrane-trafficking mutants tested. As shown in Figure 6A, the *ypt3-i5* Δ *cis4*

Figure 5. The *cis4* mutants show defects in cell wall integrity, secretion, and membrane trafficking. (A) MgCl_2 -sensitive phenotype of *cis4* mutants were osmoremedial. Wild-type, *cis4-1*, *cis4-2*, or $\Delta cis4$ cells were spotted onto the plates as indicated and then incubated for 4 d at 27°C. (B) The addition of Zn^{2+} suppressed the increased septation index of $\Delta cis4$ cells. Wild-type (wt) or $\Delta cis4$ cells were incubated in EMM with or without 2 mM ZnSO_4 at 27°C. Septation index (■, percentage of cells with a septum) of log phase cells was monitored. At least 50 cells were observed at one time in each experiment. The data represent mean $\pm$ SD of 10 determinations. The reduction in the septation index by the addition of Zn^{2+} was statistically significant ($p < 0.005$). (C) The *cis4* mutants are hypersensitive to micafungin. The *cis4* mutants showed hypersensitivity to micafungin, a (1,3)- β -D-glucan synthase inhibitor. Cells were spotted onto YPD plates and YPD plus 0.9 $\mu\text{g/ml}$ micafungin and incubated at 27°C for 4 d. (D) MgCl_2 -sensitive phenotype of *cis4* mutants were suppressed by the expression of constitutively active calcineurin. Wild-type cells or *cis4-1* mutant cells transformed with a control vector, the vector containing the full-length *ppb1*⁺ gene (CN full), or the vector containing the constitutively active truncated calcineurin gene (CN Δ C) were spotted onto each YPD plate as indicated and incubated for 4 d at 27°C. (E) The *cis4* deletion markedly impaired the secretion of acid phosphatase. Wild-type or $\Delta cis4$ cells were assayed for secreted (left panel) and accumulated (right panel) acid phosphatase activities. The data represent mean $\pm$ SD of five determinations. (F) Defective localization of GFP-Syb1 in $\Delta cis4$ cells. Wild-type (wt) and $\Delta cis4$ cells expressing GFP-Syb1 were cultured in EMM medium containing 4 $\mu\text{g/ml}$ thiamine at 27°C and were examined by fluorescence microscopy. Top panel, arrows indicate GFP fluorescence associated with the plasma membrane. Bar, 10 μm . Bottom panel, cells with clear fluorescence at the cell membrane were counted. At least 50 cells were observed at one time in each experiment. The data represent mean $\pm$ SD of five determinations.

double mutants were viable but showed more marked temperature sensitivity than that of the *ypt3-i5* single mutants. Likewise, other double mutants showed more marked temperature sensitivity compared with that of the single mutants (Figure 6A).

Next, we examined whether the *cis4* mutation can alter the resistance of wild-type or the *ypt3-i5* mutant cells to cell wall damage. For this, we used the β -glucanase (Zymolyase) sensitivity as a direct quantitative readout of the cell wall modifications. The *ypt3-i5* cells showed a high sensitivity to Zymolyase as described previously (Cheng *et al.*, 2002). The *ypt3-i5* $\Delta cis4$ double mutants showed more marked sensitivity to Zymolyase than that of the *ypt3-i5* cells, whereas the sensitivity of the $\Delta cis4$ single mutant to Zymolyase was not distinctly different from that of the wild-type cells (Figure 6B). Likewise, the *gdi1-i11* $\Delta cis4$ double mutants showed more marked sensitivity to Zymolyase than that of the *gdi1-i11* cells (our unpublished data).

The Addition of Extracellular Zn^{2+} Suppressed the Phenotypes of *ypt3-i5* and *apm1-1* Mutant Cells

As the phenotypes of the membrane-trafficking mutants were exacerbated by the *cis4* deletion, we next examined the effect of the addition of extracellular Zn^{2+} on various membrane-trafficking mutants. As shown in Figure 7A, the addition of extracellular Zn^{2+} significantly attenuated the temperature sensitivity of the *ypt3-i5* mutant and that of the *apm1-1* mutant, while

only slightly affecting that of the *ryh1-i6* and *gdi1-i11* mutants. The FK506 sensitivity of the *ypt3-i5* and *apm1-1* mutants was also suppressed by the addition of Zn^{2+} (Figure 7B). Consistently, the addition of Zn^{2+} also suppressed the defective septation of the *ypt3-i5* and *apm1-1* mutant cells, while not affecting that of the *ryh1-i6* and *gdi1-i11* mutants (Figure 7C).

We then examined the effect of the overexpression of the constitutively active calcineurin on the membrane-trafficking mutants as described above and as seen in Figure 5D. The mutant cells were transformed with a vector containing the constitutively active truncated calcineurin gene (CN Δ C) or a control multicopy vector (Figure 7D). The results showed that the overexpression of the constitutively active calcineurin significantly suppressed the temperature-sensitive growth defect of the *ryh1-i6* and *gdi1-i11* mutant cells while not affecting that of the *ypt3-i5* and *apm1-1* mutants.

DISCUSSION

In vertebrate cells, the ZnT transporters belonging to the CDF family have been shown to localize at the Golgi to supply Zn^{2+} to zinc-dependent proteins such as alkaline phosphatase (Suzuki *et al.*, 2005a,b; Ishihara *et al.*, 2006); however, evidence on the role of zinc or zinc transporters in membrane-trafficking events remains lacking. Here in fission yeast, we report that Cis4 is implicated in the Golgi membrane trafficking through the regulation of zinc ho-

Figure 6. Genetic interaction between *cis4* and membrane-trafficking mutants. (A) The *ypt3-i5* $\Delta cis4$, *ryh1-i6* $\Delta cis4$, *gdi1-i11* $\Delta cis4$, or *apm1-1* $\Delta cis4$ double mutants showed more marked temperature sensitivity than the single mutants. Wild-type (wt), $\Delta cis4$, *ypt3-i5*, *ypt3-i5* $\Delta cis4$, *ryh1-i6*, *ryh1-i6* $\Delta cis4$, *gdi1-i11*, *gdi1-i11* $\Delta cis4$, *apm1-1*, and *apm1-1* $\Delta cis4$ cells were spotted onto YPD plates and then incubated for 4 d at the temperatures as indicated. (B) The *cis4* deletion markedly enhanced the sensitivity of the *ypt3-i5* cells to Zymolyase. Wild-type, $\Delta cis4$, *ypt3-i5* or *ypt3-i5* $\Delta cis4$ cells exponentially growing in YPD medium were harvested and incubated with β -glucanase (Zymolyase) at 27°C with vigorous shaking. Cell lysis was monitored by the measurement of optical density at 660 nm (the value before adding the enzyme was taken as 100%). The data represent mean $\pm$ SD of five determinations.

meostasis in fission yeast. To our knowledge, this is the first report of the involvement of zinc or a zinc transporter in the regulation of membrane trafficking.

Three lines of evidence support the hypothesis that *Cis4* is a zinc transporter. First, *Cis4* is structurally similar to the budding yeast and human CDF family zinc transporters. Second, the mutations in the *cis4*⁺ gene resulted in several phenotypes that are zinc suppressible. Third, the simultaneous expression of *MSC2* and *ZRG17* that encode the components of the budding yeast zinc transporter complex suppressed the phenotypes of *cis4* mutant cells.

Furthermore, our results suggest that *Cis4* delivers Zn^{2+} to the lumen of the Golgi to maintain membrane-trafficking function of the cellular compartment. First, *Cis4* localizes to the *cis*-Golgi. Second, the *cis4* mutation affects the membrane-trafficking events related to the Golgi function. Third, the *cis4* mutants show a genetic interaction with several membrane trafficking mutants. These results suggest that there exists in the Golgi as yet unknown zinc-requiring components that are involved in the membrane trafficking function and that these zinc-requiring components and Ypt3, Ryh1, Gdi1, or Apm1 are required in parallel path-

Figure 7. Effect of the addition of extracellular Zn^{2+} on the membrane-trafficking mutants. (A) Effect of Zn^{2+} on temperature sensitivity of the membrane-trafficking mutants. Wild-type, *ypt3-i5*, *ryh1-i6*, *gdi1-i11*, or *apm1-1* mutant cells were spotted onto the plates and then incubated for 3 or 4 d at the temperatures as indicated. (B) Effect of Zn^{2+} on FK506 sensitivity of the membrane-trafficking mutants. Wild-type, *ypt3-i5*, *ryh1-i6*, *gdi1-i11*, or *apm1-1* mutant cells were spotted onto the plates as indicated and then incubated for 4 d at 27°C. (C) Effect of Zn^{2+} on high septation index of the membrane-trafficking mutants. Septation index of the membrane-trafficking mutants cells were monitored as described in Figure 5B. The data represent mean $\pm$ SD of 10 determinations. The reduction in the septation index by the addition of Zn^{2+} was statistically significant ($p < 0.005$) in the case of *ypt3-i5* and *apm1-1* mutant cells. (D) Effect of the expression of constitutively active calcineurin on the temperature-sensitive phenotypes of the membrane-trafficking mutants. Membrane-trafficking mutant cells transformed with a control vector or with the vector containing the constitutively active truncated calcineurin gene (CN ΔC) were spotted onto each YPD plate and incubated as indicated for 3–4 d.

ways with a common essential function in membrane trafficking (Finger and Novick, 2000). In addition, it was observed that the phenotypes of *ypt3-i5* and *apm1-1* mutants were suppressed by the addition of Zn^{2+} , whereas the phenotypes of *ryh1-i6* and *gdi1-i11* mutants were not affected. The results suggest that the *ypt3-i5* or *apm1-1* mutation caused a zinc deficiency that is presumably due to the impairment of the function of unknown zinc transporter(s) or zinc-requiring protein(s) and that zinc is required at multiple pathways in membrane trafficking. These results also suggest a previously undetected interdependency between membrane trafficking and zinc homeostasis in fission yeast.

The overexpression of the constitutively active calcineurin suppressed the temperature-sensitive growth defect of the *ryh1-i6* and *gdi1-i11* mutant cells, whereas it did not affect that of the *ypt3-i5* and *apm1-1* mutants (Figure 7D). Together with above findings, these results suggest that calcineurin also plays a role in membrane trafficking at multiple pathways and that calcineurin-related pathways are distinct from those related to zinc (Figure 8). As shown in Figure 2B, the *cis4-1* allele was more sensitive to the calcineurin inhibitor compared with that of the *cis4-2* or $\Delta cis4$ alleles. The *cis4-1* allele might somehow interfere with other mecha-

Figure 8. Cartoon about relationship between zinc and calcineurin in the regulation of membrane trafficking. Schematic diagram is based on the collective findings in this report combined with what has been reported in the literature. Block arrow with a question mark indicates unknown pathways and the larger arrow indicates stronger effects.

nisms of transporting zinc into the Golgi or that is related to calcineurin.

In fission yeast *S. pombe* genome, there are three genes, namely *cis4*⁺, *zlf1*⁺ (Clemens *et al.*, 2002; Borrelly *et al.*, 2002) and SPBC16E9.14c, that encode CDF proteins, which are structurally related to zinc transporters in other species. On *Zhf1*, the protein localizes to the ER membrane (Clemens *et al.*, 2002) as shown by the immunoelectron microscopic analysis and appears to be required for zinc storage and detoxification, similar to its budding yeast homolog *Zrc1p* and *Cot1p*. Consistently, *Zhf1* had no effect on the phenotypes of the *cis4* mutants (our unpublished data). On SPBC16E9.14c gene that was named *zrg17*⁺ because the gene encodes a homolog of the budding yeast *Zrg17p*, we showed that Δ *cis4* and Δ *zrg17* mutant cells shared common and nonadditive zinc-suppressible phenotypes. We also showed that *Cis4* and *Zrg17* physically interacted. These results suggest that *Cis4* and *Zrg17* form a heteromeric functional complex for zinc transport. The formation of an oligomeric functional complex seems to be an evolutionarily conserved mechanism for zinc transport in the eukaryotic secretory pathway (Ellis *et al.*, 2005; Suzuki *et al.*, 2005a,b; Ishihara *et al.*, 2006).

The *Δcis4* cells showed defects in cell wall integrity (Figure 6). Similar phenotypes were reported by Kumanovics *et al.* (2006). In the same study, it was suggested that the cell wall defect is attributed to the phospholipids biosynthetic pathway because lower concentrations of zinc decreased the phospholipid activity by up to 50%. To test this possibility, we examined the effect of ethanolamine in the growth medium to increase the cellular phosphatidylethanolamine as described by Kumanovics *et al.* However, the phenotypes of the *cis4* mutants were unaffected by the addition of ethanolamine (our unpublished data), suggesting that other enzymatic steps in the phospholipid pathway may also be zinc-dependent.

The *cis4*-mediated $MgCl_2$ sensitivity was suppressed by the expression of the constitutively active calcineurin (Figure 5D). This suggests that calcineurin regulates zinc homeostasis in fission yeast. Alternatively, calcineurin may regulate other cellular events and may indirectly counteract the toxic effects of a high Mg^{2+} concentration. Calcineurin contains a binuclear Fe-Zn center that is required for its

activity (Namgaladze *et al.*, 2002), and a likely reason for the effects observed is that *Cis4* is required to supply Zn directly to calcineurin.

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