Studies on the Roles of Clathrin-Mediated Membrane Trafficking and Zinc Transporter Cis4 in the Transport of GPI-Anchored Proteins in Fission Yeast

Wurentuya Jaiseng2*, Yue Fang1,2a, Yan Ma2, Reiko Sugiura3, Takayoshi Kuno1,2

1 Department of Pharmacology, School of Pharmaceutical Sciences, China Medical University, Shenyang, China, 2 Division of Molecular Pharmacology and Pharmacogenomics, Department of Biochemistry and Molecular Biology, Kobe University Graduate School of Medicine, Kobe, Japan, 3 Laboratory of Molecular Pharmacogenomics, School of Pharmaceutical Sciences, Kinki University, Higashi-Osaka, Japan

Abstract

We previously identified Cis4, a zinc transporter belonging to the cation diffusion facilitator family, and we demonstrated that Cis4 is implicated in Golgi membrane trafficking in fission yeast. Here, we identified three glycosylphosphatidylinositol (GPI)-anchored proteins, namely Ecm33, Aah3, and Gaz2, as multicyclic suppressors of the MgCl2-sensitive phenotype of cis4-1 mutant. The phenotypes of ecm33, aah3 and gaz2 deletion cells were distinct from each other, and Cis4 overexpression suppressed Δecm33 phenotypes but did not suppress Δaah3 defects. Notably, green fluorescent protein-tagged Ecm33, which was observed at the cell surface in wild-type cells, mostly localized as intracellular dots that are presumed to be the Golgi and endosomes in membrane-trafficking mutants, including Δapm1, ypt3-5, and chc1-1 mutants. Interestingly, all these membrane-trafficking mutants showed hypersensitivity to BE49385A, an inhibitor of Its8 that is involved in GPI-anchored protein synthesis. Taken together, these results suggest that GPI-anchored proteins are transported through a clathrin-mediated post-Golgi membrane trafficking pathway and that zinc transporter Cis4 may play roles in membrane trafficking of GPI-anchored proteins in fission yeast.

Citation: Jaiseng W, Fang Y, Ma Y, Sugiura R, Kuno T (2012) Studies on the Roles of Clathrin-Mediated Membrane Trafficking and Zinc Transporter Cis4 in the Transport of GPI-Anchored Proteins in Fission Yeast. PLoS ONE 7(7): e41946. doi:10.1371/journal.pone.0041946

Editor: Christopher Beh, Simon Fraser University, Canada

Received April 16, 2012; Accepted June 29, 2012; Published July 25, 2012

Copyright: © 2012 Jaiseng et al. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

Funding: This work was supported by the National Natural Science Foundation of China (No. 30900795 and No. 31071094) and research grants from Japan Society for the Promotion of Science. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

* E-mail: fangyue@mail.cmu.edu.cn
a These authors contributed equally to this work.

Introduction

Glycosylphosphatidylinositol (GPI) anchoring is a common post-translational lipid modification by which proteins are attached to the cell surface in all eukaryotic cells. GPI-anchored proteins are functionally diverse and are important for signal transduction, cell-cell interaction, cell adhesion, cell surface protection, and cell wall synthesis [1,2,3,4]. In mammalian cells, more than 150 proteins including receptors, adhesion molecules, transcription factors Atf1 and Mbx1 and is involved in the negative feedback regulation of Pmk1 cell integrity signaling [12]. In order to gain further insight into the function of Cis4, we screened for multicopy suppressors of the MgCl2-sensitive phenotype of the cis4-1 mutant cells and identified three genes encoding GPI-anchored proteins, namely Ecm33, Aah3, and an uncharacterized protein, Gaz2.

The ecm33+ gene was previously identified as a target of the two transcription factors Atf1 and Mbx1 and is involved in the negative feedback regulation of Pmk1 cell integrity signaling [12]. The aah3+ gene encodes a α-amylase homolog required for cell wall integrity, morphogenesis and vacuolar protein sorting [13,14]. These three GPI-anchored proteins all suppressed the phenotypes of cis4-1 mutant cells. Furthermore, we showed that GFP-Ecm33 localized at the cell surface in wild-type cells, whereas it mostly localized as intracellular dots which are presumed to be the Golgi and endosomes in membrane-trafficking mutants, including Δapm1, ypt3-5, and chc1-1 mutants. Taken together, these results highlight the importance of the clathrin-mediated post-Golgi membrane trafficking pathway as well as the zinc transporter Cis4 in the intracellular transport of GPI-anchored proteins.
Results

Isolation of the ecm33+, aah3+, and gaz2+ genes as multicopy suppressors of zinc transporter cis4-1 mutant

We have previously demonstrated that Cis4 is a zinc transporter belonging to the CDF protein family, and plays a role in Golgi membrane trafficking in fission yeast [11]. To better understand the function of Cis4, we screened for genes that when overexpressed could suppress the Cl− hypersensitivity of cis4-1 mutant. The cis4-1 mutant cells grew well in rich YPD medium, however, in the presence of 0.15 M MgCl2, the cis4-1 cells failed to grow, whereas wild-type cells grew well (Figure 1A). Notably, overexpression of the ecm33+ gene partially suppressed the MgCl2 sensitivity of cis4-1 mutant, and overexpression of the aah3+ and gaz2+ genes more strongly suppressed the MgCl2 sensitivity of the cis4-1 mutant (Figure 1A). Then we further determined the growth rates of ecm33+ overexpression in liquid media to assess the level of the suppression of the phenotype. Consistently, results showed that cis4-1 mutant cells harboring the multicopy vector grew almost normally but stopped growing 1 h after shift to the YPD media containing 0.15 M MgCl2. However, the cis4-1 mutant cells expressing ecm33+ gene could grow in the presence of 0.15 M MgCl2, although the growth was slower than that of the cis4-1 mutant cells harboring cis4+ gene (Figure 1B). Likewise, these three genes complemented the FK506-sensitive phenotype of the cis4-1 mutant (Figure 1A). Then we examined in Δcis4 deletion mutants the effects of the overexpression of ecm33+, aah3+, and gaz2+ genes, respectively, and results showed that these genes also suppressed the MgCl2-sensitive and FK506-sensitive growth defect of the Δcis4 cells (our unpublished data).

The ecm33+ gene encodes a 43.3 kDa protein (Ecm33) comprising 421 amino acids and containing a signal peptide for GPI anchor in its N-terminus. The aah3+ gene encodes a cell surface GPI-anchored protein (Aah3) consisting of 564 amino acids (63.2 kDa) and containing an alpha-amylase domain as well as a DUF1966 domain of unknown function. The gaz2+ gene, based on the nucleotide sequence determination, encodes a conserved fungal protein of 317 amino acids. Notably, the N-terminal portion of Gaz2 contains an amino acid signal sequence, and in addition, the gaz2+ gene product (Gaz2) has a serine-rich region. These three proteins are conserved in fungi, and Ecm33 is structurally similar to the budding yeast Pst1p and Ecm33p, while Aah3 and Gaz2 have no apparent S. cerevisiae ortholog. The amino acid sequence similarity among Ecm33, Gaz2, and Aah3 are considerably low, and the domain structure is distinct from each other. Ecm33 is a member of the Ecm33/Sps2 family, Aah3 is an alpha-amylase protein, and Gaz2 seems to be a non-enzymatic serine-rich cell wall protein. The only structural thing they have in common is that they contain signal peptides for ER entry and GPI anchoring. Probably, a common feature of these three proteins is that they are highly glycosylated, and the suppression is related to their glycosylation onto the proteins. As the feature of these three proteins is their high glycosylation, the suppression might be due to an indirect effect of overexpressing the GPI proteins.

Phenotypes of ecm33+, gaz2+, and aah3+ deletion mutants

We constructed a null mutation in the ecm33+ and gaz2+ genes, respectively (see Materials and Methods) and found that the gaz2 deletion mutant was also viable (Figure 2A, upper panel), indicating that Gaz2 is not essential for cell viability. Then we compared the phenotypes of ecm33+, gaz2+, and aah3+ gene deletion mutants. With regard to the cis phenotypes including FK506 sensitivity and MgCl2 sensitivity [11], Δecm33 cells exhibited sensitivity to both FK506 and MgCl2, whereas the Δgaz2 and Δaah3 cells were not sensitive to FK506 or MgCl2. With regard to CaCl2 sensitivity, Δaah3 cells failed to grow on YPD plate containing 0.15 M CaCl2, whereas Δecm33 and Δgaz2 cells grew well on the same plate. With regard to temperature sensitivity, Δaah3 cells were very sensitive to cold temperature while the others were not sensitive, and all were not sensitive to high temperature. With regard to the altered sensitivity to the plasma membrane perturbing agent, sodium dodecyl sulfate (SDS), Δecm33 and Δgaz2 but not Δaah3 cells were significantly more resistant to SDS as compared with that of the wild-type cells (Figure 2A, upper panel).

Because some of the GPI-anchored proteins were found to be involved in cell wall integrity [15], we then examined whether the phenotypes of these three GPI-anchored protein mutants were suppressible by osmotic stabilization of the medium with sorbitol.
Our results showed that in Δecm33 cells, sorbitol suppressed the FK506 sensitivity and MgCl2 sensitivity of the cells. In Δaah3 cells, sorbitol suppressed the cold temperature sensitivity of the cells, whereas sorbitol failed to suppress the CaCl2 sensitivity of the cells (Figure 2A, lower panel). Consistent with these results, Morita et al showed that the morphological defect of Δaah3 cells were not rescued in the presence of 1.2 M sorbitol-YES medium [13].

Analysis of the overlapping functions among the three GPI-anchored proteins

As described above, the domain structures of Ecm33, Aah3, and Gaz2 are distinct, therefore it seems likely that ecm33+, aah3+ and gaz2+ are not functionally redundant. To test this possibility, we examined the effects of the overexpression of gaz2+ and aah3+ on the phenotypes of the ecm33+ deletion mutants, as well as the overexpression of ecm33+ and gaz2+ on the phenotypes of the aah3+ deletion mutants. As shown in Figure 2B, the results showed that overexpression of gaz2+, but not aah3+, suppressed the MgCl2-sensitive growth defect of the ecm33+ gene deletion mutants. On the other hand, overexpression of the ecm33+ or gaz2+ genes failed to suppress the phenotypes of the aah3+ deletion mutants. Thus, these findings suggest that the structures of the three GPI-anchored proteins are distinct from each other, and that these three proteins have only partial overlapping functions. We also examined the effects of the overexpression of cis4+ on the phenotypes of the Δecm33 and Δaah3 mutants. The results showed that overexpression of cis4+ suppressed the MgCl2-sensitive growth defect of the Δecm33 mutants, but failed to suppress the CaCl2-sensitive phenotype of the Δaah3 mutants (Figure 2B).

Deletion analysis of the ecm33+ gene

To determine the functional region of Ecm33, we prepared a series of truncated forms of Ecm33. Structural feature of the deletion mutants employed in this study is illustrated in Figure 3A. Results showed that in Δecm33 mutants, the overexpression of the full-length Ecm33 as well as Ecm33 fragment A, fragment B, fragment C, fragment F, and fragment G suppressed the phenotypes of the mutants (Figure 3B). However, overexpression of Ecm33 fragment D, fragment E, fragment H, fragment I, fragment J, and fragment K failed to suppress the phenotypes of Δecm33 mutants (Figure 3B). Overexpression of these truncated versions of Ecm33 showed similar genetic suppression profile of the cis4-1 mutant as compared with that of the Δecm33 cells (our unpublished data).

We also examined the protein levels of truncated mutants of Ecm33 in the Δecm33 cells by immunoblotting with anti-Ecm33 monoclonal antibody [12] (Figure 3C). The immunoblot analysis detected an appreciable amount of Ecm33 fragments A, C, F, G,  

Figure 2. The Δecm33, Δaah3, and Δgaz2 mutants displayed distinct phenotypes. (A) Phenotypes of the Δecm33, Δaah3, and Δgaz2 mutants. Upper panel, Cells were streaked onto each plate as indicated, and then incubated at 30 °C for 4 days, at 36 °C for 3 days or at 17 °C for 7 days. Lower panel, MgCl2-sensitive and FK506-sensitive phenotypes of Δecm33 and cold temperature-sensitive phenotype of Δaah3 were osmoremedial, whereas CaCl2-sensitive phenotype of Δaah3 was not. Cells were streaked onto each plate as indicated, and then incubated at 30 °C for 4 days or at 17 °C for 7 days. (B) Effects of overexpression of the gaz2+, aah3+, and cis4+ genes on the phenotypes of Δecm33, and effects of overexpression of the ecm33+, gaz2+, and cis4+ genes on the phenotypes of Δaah3. Wild-type cells, Δecm33 or Δaah3 cells transformed with a control vector or the vector containing ecm33+, gaz2+, aah3+, and cis4+ were spotted onto YPD plates or YPD plus 0.15 M MgCl2 and incubated at 30 °C for 4 days. doi:10.1371/journal.pone.0041946.g002
and I, but failed to detect fragment B, D, E, H, J or K. These results are consistent with the above results that overexpression of Ecm33 fragments A, C, F, G except for fragment I suppressed the phenotypes of the Δecm33 cells. In addition, the Ecm33 fragment B was not detected in the cells by immunoblotting, although overexpression of the fragment B suppressed the phenotypes of the Δecm33 cells. It is possible that the Ecm33 fragment B contains the epitope for the monoclonal antibody. The reasons for the inability of the antibody to detect other Ecm33 fragments as well as the functional importance of these fragments are unknown.

Subcellular localization of Ecm33 and Gaz2

In order to investigate the subcellular localization and membrane trafficking of the Ecm33 and Gaz2 protein, plasmids carrying GFP-Ecm33 and GFP-Gaz2 fusions, respectively, were constructed. On Ecm33, a GFP carrying the S65T mutation was inserted into 60 bp from the N-terminus of Ecm33. Fujita et al demonstrated that HA- and mRFP-tagged versions of Gas1, a well-characterized GPI-anchored protein in Saccharomyces cerevisiae, were generated by inserting the tags in Gas1 immediately following the N-terminal signal sequence and both the tagged proteins were functional [16]. Therefore, we constructed GFP-Ecm33 fusion by inserting the GFP tag immediately following the N-terminal signal sequence. Results showed that the construct of GFP-Ecm33 was functional as cells expressing GFP-Ecm33 suppressed the phenotypes of Δecm33 mutants (Figure 4A). Then, we examined the localization of GFP-Ecm33 expressed from its own promoter in wild-type cells, and results showed that GFP-
Ecm33 localized to the cell surface or the medial regions. This observation was similar to the sterol localization of filipin fluorescence that was enriched in the plasma membrane at the growing cell tips and at the site of cytokinesis (Figure 4B). Also, this finding was consistent with the data obtained using anti-Ecm33 antibody by immunofluorescence microscopy [12].

On Gaz2, a GFP was inserted into 600 bp from the N-terminus of Gaz2 because deletion of 500–600 bp of Gaz2 gene did not affect its suppression ability on the phenotype of \( \text{cis4-1} \) mutant. However, the construct was not functional and cells expressing GFP-Gaz2 failed to suppress the phenotypes of the \( \text{cis4-1} \) mutant (our unpublished data). Presumably, this is because the GFP tag is inserted somewhere in the middle of the Gaz2 protein.

Next, we examined the effect of BE49385A, an inhibitor of Its8, on the subcellular localization of GFP-Ecm33 in wild-type cells. We previously identified a mutation in the \( \text{ib}^{+} \) gene that are involved in GPI anchor synthesis, and showed that \( \text{ib}^{+} \) is a molecular target of BE49385A [10]. In wild-type cells, before the addition of BE49385A as shown in Figure 4C, GFP-Ecm33 localized to the cell surface or the medial regions. Then, 1 hour after the addition of 1 \( \mu \text{g/ml} \) BE49385A to the medium, GFP-Ecm33 mostly localized to the nuclear envelope and the peripheral ER rather than the cell surface (Figure 4C, arrows). In \( \text{ib}^{+} \) mutant cells, the subcellular localization of GFP-Ecm33 was also examined. As expected, GFP-Ecm33 primarily localized to the ER and to the cell surface in the \( \text{ib}^{+} \) mutant cells (Figure 4D, arrows), suggesting that the impairment of GPI anchor synthesis caused the defective attachment of GPI-anchor to the Ecm33 protein thereby resulting in the abnormal GFP-Ecm33 localization in the ER. Then we also examined the subcellular localization of GFP-Ecm33 in the \( \Delta \text{cis4} \) cells, and results showed that GFP-Ecm33 was observed at the cell surface or the medial region in the \( \Delta \text{cis4} \) cells (Figure 4D) similar to that observed in the wild-type cells (Figure 4B). We further examined the effect of zinc deficiency on the subcellular localization of GFP-Ecm33 in the \( \Delta \text{cis4} \) cells by removing zinc from the EMM medium. As shown in
Figure 5. Genetic interaction between cis4 and its8 genes. (A) Effect of the addition of extracellular Zn\(^{2+}\) on the phenotypes of its8-1 mutant. Wild-type or its8-1 mutant cells were spotted onto each plate as indicated and then incubated for 4 days at 27 \(^{\circ}\)C or at 35 \(^{\circ}\)C. (B) The its8-1Δcis4 double mutants showed more marked temperature sensitivity than the single mutants, while the double mutants showed similar BE49385A-sensitivity as compared with that of the its8-1 mutant. Wild-type, its8-1, Δcis4, and its8-1Δcis4 cells were spotted onto each plate as indicated and then incubated for 4 days at 27 \(^{\circ}\)C or at 33 \(^{\circ}\)C. doi:10.1371/journal.pone.0041946.g005

Genetic interaction between cis4\(^+\) and its8\(^+\) genes

Then we examined the effect of Zn\(^{2+}\) on the phenotypes of the its8-1 mutant cells. The results showed that the addition of Zn\(^{2+}\) to the medium significantly rescued the high temperature-sensitive and FK506-sensitive phenotypes of the its8-1 mutant (Figure 5A).

Our previous study suggested that Cis4 localizes to the cis-Golgi and was involved in Golgi membrane trafficking through regulating the zinc homeostasis [11]. In order to investigate the functional relationship between Cis4 and Its8, we constructed its8-1Δcis4 double mutants, and examined the effect of temperature, BE49385A, and zinc deficiency respectively on these cells. On the effect of temperature, in the its8-1Δcis4 double mutants, these cells exhibited more marked temperature sensitivity than that of the its8-1 single mutants (Figure 5B), suggesting that there is a genetic interaction between Its8 and Cis4. On the effect of BE49385A, in the Δcis4 cells interestingly, the growth of these single deletion cells was significantly inhibited by BE49385A as compared with that of the wild-type cells, although the sensitivity of the Δcis4 cells was not as severe as that of the its8-1 mutant (Figure 5B). In the its8-1Δcis4 double mutants, notably, these cells exhibited the BE49385A-sensitive growth defects similar to that of the its8-1 single mutant. On the effect of zinc deficiency, in the its8-1Δcis4 double mutants interestingly, these cells were observed to have a very small colony size similar to that of the Δcis4 single mutant in the zinc-deficient medium (Figure 5B); these results suggest that the impairment of GPI-anchor synthesis and zinc-ion homeostasis in the double mutant is similar to that of its8-1 and Δcis4 single mutants, respectively.

Furthermore, we examined the effect of overexpression multi-copy suppressors of the cis4-1 mutant on the MgCl\(_2\)-sensitive phenotypes of the apm1-1 mutant, an allele of apm1\(^+\) gene that encodes \(\mu\)A subunit of the clathrin-associated adaptor protein complex 1(AP-1) implicated in the Golgi/endosome function [17]. The multicopy suppressors of cis4-1 mutant identified here were the three genes encoding GPI-anchored protein namely ecm33\(^+\), ahh3\(^+\) and gaz2\(^+\), and in addition, two other multicopy suppressor genes including pmp1\(^+\), and SPCC1322.03 (tp1322\(^+\)) that also suppressed the MgCl\(_2\)-sensitive phenotype of cis4-1 mutant (Materials and Methods). The pmp1\(^+\) gene encodes a dual-specificity MAPK phosphatase that negatively regulates the Pmk1 MAPK signaling [18]. The tp1322\(^+\) gene encodes transient receptor potential (TRP)-like ion channel that mediates the cytoplasmic Ca\(^{2+}\) rise caused by the extracellularly added CaCl\(_2\) [19]. As shown in Table 1, all the multicopy suppressors with the exception of the ahh3\(^+\) gene significantly suppressed the MgCl\(_2\)-sensitive phenotype of the apm1-1 mutant.

Localization of GFP-Ecm33 in various membrane trafficking mutants

As shown above, the overexpression of the GPI-anchored proteins suppressed the MgCl\(_2\)-sensitive phenotype of the mutant allele of the apm1\(^+\) gene. This prompted us to hypothesize that Apm1 may play roles in membrane trafficking of the GPI-anchored proteins. Then, we examined the localization of GFP-Ecm33 in Δapm1 cells. In wild-type cells, GFP-Ecm33 clearly localized at the cell surface and the medial regions as shown in Figure 4B. In Δapm1 cells, in contrast, GFP-Ecm33 primarily localized as dot-like structures that

**Table 1. Complementation of the MgCl\(_2\)-sensitive phenotype of the cis4-1 mutant and apm1-1 mutant.**

| Plasmid | Complementation of the mutants |
|--------|-------------------------------|
| cis4-1 | apm1-1 |
| ecm33\(^+\) | ++ | ++ |
| ahh3\(^+\) | ++ | – |
| gaz2\(^+\) | ++ | ++ |
| pmp1\(^+\) | ++ | + |
| tp1322\(^+\) | ++ | ++ |

Note: Each transformant, carrying various genes on the multicopy plasmids, was streaked onto YPD plates in the presence or absence of MgCl\(_2\) and incubated at 27 \(^{\circ}\)C for 4 days. ++, complemented the 0.15 M MgCl\(_2\)-sensitive phenotype; +, complemented the 0.12 M MgCl\(_2\)-sensitive phenotype; –, did not complement. doi:10.1371/journal.pone.0041946.t001
were observed in the cytoplasm (Figure 6A, arrows) as well as at the cell surface and the division site (Figure 6A, arrowheads). Next, we examined the localization of GFP-Ecm33 in other membrane trafficking mutants namely, ypt3-i5 mutant and chcl-1 mutant. 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 [20]. The chcl-1 gene encodes clathrin heavy chain Chcl involved in intracellular protein transport. Results showed that the chcl-1 mutant exhibited its (to for immunosuppressant- and temperature-sensitive) phenotype [21] (Figure S1A). Sequence analysis of the genomic DNA from the chcl-1 mutant revealed that arginine at 1615 was mutated to a termination codon by a C-to-T transition (CGA to TGA), and resulted in a truncated protein product lacking 51 amino acids downstream of the mutation (Figure S1B).

In the membrane trafficking mutants including chcl-1 mutant and ypt3-i5 mutant, results showed that GFP-Ecm33 also localizes as intracellular dot-like structures (Figure 6A, arrows) in addition to the cell surface and the division site (Figure 6A, arrowheads). So, we examined whether the dot-like fluorescence of GFP-Ecm33 co-localized with the endocytic tracer dye FM4-64 during an early stage of endocytosis in Δapm1, chcl-1 mutant and ypt3-i5 mutants. After 5 min of dye uptake, most of the GFP-Ecm33 dot-like structures co-localized with FM4-64-positive structures in Δapm1, chcl-1 mutant and ypt3-i5 mutants (Figure 6A, Merge). This strongly suggests that the intracellular dot-like fluorescence of GFP-Ecm33 represents Golgi/endosome compartments. Then we further examined the co-localization of GFP-Ecm33 with Krp1 fused to monomeric red fluorescent protein (RFP) at its C-terminus. Krp1 is a furin/Kex2 homolog that resides in the Golgi/endosome [22,23]. As shown in Figure 6B, intracellular GFP-Ecm33 mostly co-localized with Krp1-RFP (Figure 6B). Thus, GFP-Ecm33 localized at Golgi/endosome structures in addition to the cell surface and the division site in these mutants, suggesting that GPI-anchored proteins were not correctly transported and were retained at the Golgi/endosome structures in these membrane trafficking mutants. Similarly, in the wild-type cells, GFP-Gaz2 also clearly localized to the cell surface and medial regions (Figure 6C), while in Δapm1 cells, GFP-Gaz2 localized as intracellular dot-like structures (Figure 6C). Interestingly, all of the membrane trafficking mutants that were tested showed hypersensitivity to BE49385A (Figure 6D).

**Discussion**

Here, we identified three genes encoding GPI-anchored proteins, namely Ecm33, Aah3, and Gaz2 as multicopy suppressors of the MgCl₂-sensitive and FK506-sensitive phenotypes of the cis4-1 mutant. Furthermore, we suggest that GPI-anchored proteins are transported through a clathrin-mediated post-Golgi membrane trafficking pathway in fission yeast. To our knowledge, this is the first report that characterized the roles of clathrin-mediated post-Golgi membrane trafficking pathway as well as the zinc transporter Cis4 in membrane trafficking of GPI-anchored proteins in fission yeast.

**GPI-anchored proteins and clathrin-mediated post-Golgi membrane trafficking**

Important finding of this study is the role of clathrin-mediated post-Golgi membrane trafficking pathway in the transport of GPI-anchored proteins in fission yeast. In budding yeast, GPI-anchored proteins are transported from the ER to the Golgi apparatus in vesicles distinct from those containing non-GPI-anchored proteins such as the general amino acid permease Gap1p and pro-alpha factors [24,25,26], and the transport of GPI-anchored proteins and non-GPI-anchored proteins from the trans-Golgi network (TGN) to the plasma membrane is also regulated by different sorting and trafficking machinery [27]. Consistent with these, Castillon et al observed that GPI-anchored proteins accumulate in ER exit sites (ERES) that are distinct from those in which other secretory proteins accumulate [28]. More recently, Rivier et al reported that in mammalian cells, GPI-anchored and other secretory proteins are not segregated upon exit from the ER, in contrast to the remarkable segregation seen in budding yeast [29].

In this study in fission yeast, we observed the subcellular localization of a GPI-anchored protein Ecm33 using GFP fusion proteins in vivo. Results showed the abnormal localization of GFP-Ecm33 in all of the membrane trafficking mutants tested including ypt3-i5, chcl-1, and Δapm1 mutants. The AP-1 complex that links a clathrin to the membrane plays a role in the post-Golgi membrane trafficking including exit transport from the TGN to endosomes, endosomes to the TGN, and TGN or endosomes to the plasma membrane [23]. In the Δapm1 and chcl-1 mutants, GFP-Ecm33 was primarily seen as dot-like structures presumed to be the Golgi/endosomes, suggesting the delay in the clathrin-mediated post-Golgi membrane trafficking of GPI-anchored protein Ecm33 to the cell surface in these membrane trafficking mutants. Moreover, GFP-Ecm33 also localized at Golgi/endosome structures in addition to the cell surface and the division site in the ypt3-i5 mutant. Ypt3 is involved at multiple steps of the fission yeast membrane trafficking events, namely, at the exit from the trans-Golgi as well as at the later step of the exocytic pathway [20]. Instead, Ypt3p1 and Ypt3p2, the homolog of Ypt3 in S. cerevisiae, have been reported that implicated in the exocytic pathway and mediates intra-Golgi traffic or the budding of post-Golgi vesicles from the trans-Golgi [30,31,32]. Thus, our results suggest that Ypt3 plays roles in transport of GPI-anchored proteins at multiple steps including the exit from the trans-Golgi as well as at the later step of the exocytic pathway. Furthermore, the localization of GFP-Gaz2 in the Δapm1 cells was similar to that of GFP-Ecm33, strongly suggesting that GPI-anchored proteins are transported through a clathrin-mediated post-Golgi membrane trafficking pathway that is required for the efficient transport of other secretory proteins in fission yeast. Of course, our results do not rule out the possibility that some GPI-anchored proteins might still be separately sorted from the secretory proteins in fission yeast. Further studies will be required to clarify the molecular mechanisms of membrane trafficking of GPI-anchored proteins. Given the high similarity between the fission yeast and the mammalian cells, this study may provide a basis for understanding the precise mechanism of membrane trafficking of GPI-anchored proteins in higher eukaryotes.

**Cis4 is involved in the membrane trafficking of GPI-anchored proteins**

In the present study, we present several lines of evidence that suggests a role of Cis4 in membrane trafficking of GPI-anchored proteins. In our previous study, we established that zinc transporter Cis4 is implicated in Golgi membrane trafficking through the regulation of zinc homeostasis in fission yeast [11]. In this study, we first showed that the overexpression of several GPI-anchored proteins that have distinct structures, namely Ecm33, Aah3, and Gaz2, suppressed the phenotypes of the Δcis4 mutants. Second, there is a genetic interaction between the genes encoding Cis4 and Its8, because the its8ΔΔcis4 double mutant cells were more sensitive to high temperature than that of the single mutants. Third, the Δcis4 mutants were sensitive to BE49385A, an inhibitor of Its8. In particular, the its8ΔΔcis4 double mutants exhibited the
same BE49385A-sensitive growth defects as that of the iso8-1 single mutants, while the double mutant showed defective growth similar to that of the Δcis4 single mutants in the zinc-deficient EMM medium. Fourth, overexpression of a majority of the multicopy suppressors of the cis4-1 mutant complemented the MgCl2-sensitive phenotype of the apm1-1 mutant. The biosynthesis of GPI anchors and its attachment to the target protein are carried out on the ER membrane, and then transported to the plasma membrane by vesicular trafficking [25]. Previous study reported out on the ER membrane, and then transported to the plasma membrane by vesicular trafficking [25].

The transport of GPI-Anchored Proteins

Materials and Methods

Strains, media, genetic and molecular biology techniques

S. pombe strains used in this study are listed in Table S1. The complete medium, YPD, and the minimal medium, EMM, have been described previously [35]. Standard S. pombe genetic and recombinant-DNA methods were performed as described previously except where noted [36]. Gene disruptions are denoted by lowercase letters representing the disrupted gene followed by two colons and the wild-type gene marker used for disruption (for example, gaz2::ura4). Gene disruptions are abbreviated by the gene preceded by a Δ (for example, Δgaz2). Proteins are denoted by roman letters and only the first letter is capitalized (for example, Gaz2). Tacrolimus (FK506) was obtained from Astellas Pharma (Tokyo, Japan). All other chemicals and reagents were purchased from commercial sources.

Multicopy suppressor screen

To identify multicopy suppressors of the high MgCl2 sensitivity of cis4-1 mutant, a genomic library cloned into the vector pDB248 [37] was transformed into the cis4-1 mutant cells. The Leu+ transformants were replica-plated onto YPD plates containing 0.15 M MgCl2 and the plasmid DNA was recovered from transformants that showed a plasmid-dependent rescue. These plasmids complemented MgCl2 sensitivity of the cis4-1 mutant. By DNA sequencing, the suppressing plasmids were found to belong to six classes, with one class containing the cis4+ gene [11], and other classes containing the ecm33+ gene, SPBC1E8.05, aah3+ gene, pmp1+ gene, and typl322+ gene (SPCC1322.03). Here we focus on the ecm33+ gene, SPBC1E8.05, and the aah3+ gene that encodes GPI-anchored proteins, and renamed SPBC1E8.05 gene as gaz2+ (gaz for GPI-anchored protein that suppress the zinc transporter deletion).

Knockout of the ecm33+, gaz2+, and aah3+ genes

To knockout the ecm33+ gene, a PCR-based targeted gene deletion method was prepared by the Crec-loxP-mediated marker removal procedure as described previously [38] using the sense primer 5’-CAT AGC AAG AGC AGC AAC CAA AAG AGA TCC CAA AAC TAA AGC ACC AGC AGT GAA GCC GTT AGA AGC GGC TGA GCC CAA TAG GGC CAA ATC GGC AAA ATC AC-3’, and the antisense primer 5’-GTG GTT GTA ATT CGC TGC CAT TAC TCT TCT TTT CGC CGC AGC TCG TGC ACA AGC TGC TTC CAA CGT CTC CAG CCG CCC GGT CAG GTT TCA CGT AGT GGC CC-3’. The disruption of the gene was checked using genomic Southern hybridization (our unpublished data).

To knock out the gaz2+ gene, one-step gene disruption by homologous recombination was performed [39]. The gaz2::ura4+ disruption was constructed as follows. The open reading frame (ORF) of gaz2+ was amplified by PCR (forward primer2232, 5’-CCG CTC GAG CAC CAT GAA GTT GTC TT TCT TAG TAC TCT CG-3’; reverse primer 2233, 5’-ATA GTT TAG CGG CCG CGA AGA AAC AGG GGA ATG CAG GAA ACA ACA CC-3’) from the genomic DNA and was subcloned into the XhoI/NotI site of BlueScriptSK (+). Then a HindIII fragment containing the ura4+ gene was inserted into the HindIII site of the previous construct. The construct containing the disrupted gaz2+ gene was digested with XhoI/NotI, and the gaz2::ura4+ fragment was used to transform the diploids (5A/1D, Table S1). Stable integrants were subsequently cloned on plates containing the medium lacking uracil, and gene disruption by the gaz2+ derivative containing the ura4+ insertion was verified by genomic Southern blotting (our unpublished data).

The aah3+ gene deletion mutant (h- leu1-32 ura4-D18 ade6-M210 aah3::KanMX4) was purchased from BioNEER (South Korea) [40]. We constructed the aah3+ gene deletion cells that are not auxotrophic for uracil or adenine by the genetic cross between wild-type cells HM123 and the above strain to make KP5075 (h- leu1 aah3::KanMX4) (Table S1).

Plasmids constructions

The ecm33+, gaz2+, and aah3+ genes, respectively, together with their promoter regions were amplified by PCR using the genomic DNA of wild-type cells as a template. The primers used were summarized in Table S3. The amplified products containing the ecm33+ or gaz2+ genes were digested with PstI, while the amplified products containing the aah3+ gene was digested with HindIII. All the resulting fragments were subcloned into BlueScriptSK (+), to give pKB8044, pKB7850, and pKB8147 respectively. A series of truncated Ecm33 mutants were constructed as follows. The ecm33+ gene lacking about 100 base pairs from the predicted region was amplified by PCR using the plasmid pKB8044 as a template, to yield fragment A, fragment B, fragment C, fragment D, fragment E, fragment F, fragment G, fragment H, fragment I, fragment J, and fragment K (Figure 3A). The primers in each mutant were derived from the upstream and the downstream regions to be deleted, as shown in Table S2.

To study the subcellular localization of Ecm33 and Gaz2, plasmids carrying GFP-Ecm33 and GFP-Gaz2 fusions, respectively, were constructed as follows. First, the sequence for GFP lacking the start codon was amplified by PCR (primers shown in Table S3) from the plasmid containing GFP carrying S65T mutation, and was subcloned into BamHI site of BlueScript SK(+). Next, BamHI site was constructed at 60 bp of Ecm33 and at 600 bp of Gaz2, respectively, by PCR technique using the primers shown in Table S3. Then, a BamHI fragment containing GFP S65T mutation was inserted into the BamHI site of the Ecm33 construct and the Gaz2 construct as described above. To obtain the chromosome-borne GFP, the fused gene GFP-Ecm33 was subcloned into the vector containing the ura4+ marker under the control of its own promoter, and was integrated into the chromosome at the ura4+ gene locus of KP1248 as described.
The immunosuppressant and temperature sensitivities of the chl1-
I mutant cells. Cells transformed with the multicopy vector pDB248 or the vector containing the chl1-
I gene were streaked onto each plate containing YPD or YPD plus 0.5 μg/ml FK506,
then incubated for 4 days at 27°C or 3 days at 36°C, respectively.

| Table S1 | Strains used in this study. |
| Table S2 | Primers for construction of truncated Ecm33. |
| Table S3 | Primers for cloning or tagging of the em333+, gaz2b and aah3b genes. |

**Acknowledgments**

We thank Susie O. Sio for critical reading of the manuscript.

**Author Contributions**

Conceived and designed the experiments: YF TK. Performed the experiments: WJ YF YM. Analyzed the data: WJ YF YM RS TK. Contributed reagents/materials/analysis tools: YF RS TK. Wrote the paper: YF TK.

**References**

1. Li, J., Su, X., Jia, J., Shi, Z., Li, Z., Su, J., et al. (2010) The cell extract preparation and immunoblot analysis. Cell extract preparation was performed as described previously [49]. Protein extracts (10–20 μg/5 μl) were subjected to immu-

2. Gyorfi, L., Rodriguez-Boulan, E., Sahiel, A.R. (1990) Emerging functional roles for the glycosylphosphatidylinositol membrane protein anchor. J Membr Biol 117: 1–10.

3. Kapteyn, J.C., Van Den Ende, H., Klis, F.M. (1999) The contribution of cell wall proteins to the organization of the yeast cell wall. Biochim Biophys Acta 1420: 373–383.

4. Kinoshita, T., Ohishi, K., Takeda, J. (1997) GPI-anchor synthesis in mammalian cells: genes, their products, and a deficiency. J Biochem 122: 251–257.

5. Li, F., Fakeek, S.P. (2008) Distinct domains of the Candida albicans adhesin Eaplp mediate cell-cell and cell-substrate interactions. Microbiology 154: 1193–1203.

6. Fujita, M., Kinoshita, T. (2010) Structural remodeling of GPI anchors during biosynthesis and after attachment to proteins. FEBS Lett 584: 1670–1677.

7. Fujita, M., Kinoshita, T. (2012) GPI-anchor remodeling: Potential functions of GPI-anchors in intracellular trafficking and membrane dynamics. Biochim Biophys Acta.

8. Caro, L.H., Tettelin, H., Vossen, J.H., Kam, A.F., van den Ende, H., et al. (1997) In silico identification of glycosyl-phosphatidylinositol-anchored plasma-membrane and cell wall proteins of Saccharomyces cerevisiae. Yeast 13: 1477–1489.

9. Fujita, M., Jigami, Y. (2006) Lipid remodeling of GPI-anchored proteins and its function. Biochim Biophys Acta 1780: 410–420.

10. Fujita, M., Kinoshita, T. (2012) GPI-anchor remodeling: Potential functions of GPI-anchors in intracellular trafficking and membrane dynamics. Biochim Biophys Acta.

11. Caro, L.H., Tettelin, H., Vossen, J.H., Kam, A.F., van den Ende, H., et al. (1997) In silico identification of glycosyl-phosphatidylinositol-anchored plasma-membrane and cell wall proteins of Saccharomyces cerevisiae. Yeast 13: 1477–1489.

12. Fujita, M., Jigami, Y. (2006) Lipid remodeling of GPI-anchored proteins and its function. Biochim Biophys Acta 1780: 410–420.

13. Caro, L.H., Tettelin, H., Vossen, J.H., Kam, A.F., van den Ende, H., et al. (1997) In silico identification of glycosyl-phosphatidylinositol-anchored plasma-membrane and cell wall proteins of Saccharomyces cerevisiae. Yeast 13: 1477–1489.

14. Fujita, M., Jigami, Y. (2006) Lipid remodeling of GPI-anchored proteins and its function. Biochim Biophys Acta 1780: 410–420.

15. Moreno-Ruiz, E., Orito, G., de Grooth, P.W., Cottier, F., Louis, C., et al. (2009) The GPI-modified proteins Pga59 and Pga62 of Candida albicans are required for cell wall integrity. Microbiology 155: 2004–2020.

16. Fujita, M., Yoko, O.T., Jigami, Y. (2006) Inositol decylation by Batlp is required for the quality control of glycosylphosphatidylinositol-anchored proteins. Mol Biol Cell 17: 834–850.

17. Kita, A., Sugira, R., Shoji, H., He, Y., Deng, L., et al. (2004) Loss of Apn1, the micro1 subunit of the clathrin-associated adaptor-protein-1 complex, causes distinct phenotypes and synthetic lethality with calcineurin deletion in fission yeast. Mol Biol Cell 15: 2920–2931.

18. Sugira, R., Toda, T., Shunth, H., Yanagida, M., Kuno, T. (1998) popp1+, a suppressor of calcineurin deficiency, encodes a novel MAP kinase phosphatase in fission yeast. EMBO J 17: 140–148.

19. Ma, Y., Sugira, R., Koike, A., Ebina, H., Sio, S.O., et al. (2011) Transient receptor potential (TRP) and Cch1-Yam11 channels play key roles in the regulation of cytoplasmic Ca2+ in fission yeast. PLoS One 6: e22421.

20. Cheng, H., Sugira, R., Wu, W., Fujita, M., Lu, Y., et al. (2002) Role of the Rab GTP-binding protein Ypr3 in the fusion yeast exocytic pathway and its connection to calcineurin function. Mol Biol Cell 13: 2963–2976.

21. Zhang, Y., Sugira, R., Lu, Y., Asami, M., Maeda, T., et al. (2000) Phosphatidylinositol 4-phosphate 5-kinase Ic and calcineurin Ppb1 coordinately regulate cytokinesis in fission yeast. J Biol Chem 275: 35600–35606.

22. Powner, D., Dawsey, J. (1998) Activation of the lexin from Schizosaccharomyces pombe requires internal cleavage of its initially cleaved precursor. Mol Cell Biol 18: 400–406.

23. Ma, Y., Takeuchi, M., Sugira, R., Sio, S.O., Kuno, T. (2009) Deletion mutants of AP-1 adaptin subunits display distinct phenotypes in fission yeast. Genes Cells 14: 1015–1028.

24. Muniz, M., Morosonne, P., Riezman, H. (2001) Protein sorting upon exit from the endoplasmic reticulum. Cell 104: 313–328.

25. Mayor, S., Riezman, H. (2004) Sorting GPI-anchored proteins. Nat Rev Mol Cell Biol 5: 110–120.

26. Watanabe, R., Riezman, H. (2004) Differential ER exit in yeast and mammalian cells. Curr Opin Cell Biol 16: 350–355.

27. Simon, K., van Meer, G. (1988) Lipid sorting in epithelial cells. Biochemistry 27: 6197–6202.

28. Castillo, G.A., Watanabe, R., Taylor, M., Schwabe, T.M., Riezman, H. (2009) Concentration of GPI-anchored proteins upon ER exit in yeast. Traffic 10: 198–206.

29. Rivier, A., Castillo, G.A., Michon, L., Fukasawa, M., Romanova-Michaelides, et al. (2011) Exit of GPI-anchored proteins from the ER differs in yeast and mammalian cells. Traffic 11: 1017–1033.
30. Wang W, Ferro-Novick S (2002) A Ypt32p exchange factor is a putative effector of Ypt1p. Mol Biol Cell 13: 3336–3343.
31. Jedd G, Mullholland J, Segev N (1997) Two new Ypt GTPases are required for exit from the yeast trans-Golgi compartment. J Cell Biol 137: 565–580.
32. Brul M, Doring F, Robinson DG, Yang X, Gallwitz D (1996) Two GTase isoforms, Ypt31p and Ypt32p, are essential for Golgi function in yeast. EMBO J 15: 6460–6465.
33. Sedever D, Mann KJ, Medof ME (2001) Differential effect of 1,10-phenanthroline on mammalian, yeast, and parasite glycosylphosphatidylinositol anchor synthesis. Biochem Biophys Res Commun 286: 1112–1118.
34. Mann KJ, Sedever D (2001) 1,10-Phenanthroline inhibits glycosylphosphatidylinositol anchoring by preventing phosphoethanolamine addition to glycosylphosphatidylinositol anchor precursors. Biochemistry 40: 1205–1213.
35. Toda T, Dhut S, Superti-Furga G, Gotoh Y, Nishida E, et al. (1996) The fission yeast pmk1+ gene encodes a novel mitogen-activated protein kinase homolog which regulates cell integrity and functions coordinately with the protein kinase C pathway. Mol Cell Biol 16: 6752–6764.
36. Moreno S, Klar A, Nurse P (1993) Molecular genetic analysis of fission yeast Schizosaccharomyces pombe. Methods Enzymol 194: 795–823.
37. Beach D, Piper M, Nurse P (1982) Construction of a Schizosaccharomyces pombe gene bank in a yeast bacterial shuttle vector and its use to isolate genes by complementation. Mol Gen Genet 187: 326–329.
38. Ma Y, Sugiuara R, Saito M, Koike A, Sio SO, et al. (2007) Six new amino acid auxotrophic markers for targeted gene integration and disruption in fission yeast. Curr Genet 52: 97–105.
39. Rothstein RJ (1983) One-step gene disruption in yeast. Methods Enzymol 101: 202–211.
40. Kim DU, Hayles J, Kim D, Wood V, Park HO, et al. (2010) Analysis of a genome-wide set of gene deletions in the fission yeast Schizosaccharomyces pombe. Nat Biotechnol 28: 617–623.
41. He Y, Sugiuara R, Ma Y, Kita A, Deng L, et al. (2006) Genetic and functional interaction between Ryh1 and Ypt3: two Rab GTPases that function in S. pombe secretory pathway. Genes Cells 11: 207–221.
42. Fang Y, Imagawa K, Zhou X, Kita A, Sugiuara R, et al. (2009) Pleiotropic phenotypes caused by an opal nonsense mutation in an essential gene encoding HMG-CoA reductase in fission yeast. Genes Cells 14: 759–771.
43. Sio SO, Sakehiro T, Sugiuara R, Takeschi M, Mukai H, et al. (2005) The role of the regulatory subunit of fission yeast calcineurin for in vivo activity and its relevance to FK506 sensitivity. J Biol Chem 280: 12231–12238.