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<td>Genetics Society of America</td>
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doi:info:doi/10.1534/g3.119.400281
Coordinated Roles of the Putative Ceramide-Conjugation Protein, Cwh43, and a Mn²⁺-Transporting, P-Type ATPase, Pmr1, in Fission Yeast

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ABSTRACT Genetically controlled mechanisms of cell division and quiescence are vital for responding to changes in the nutritional environment and for cell survival. Previously, we have characterized temperature-sensitive (ts) mutants of the cwh43 gene in fission yeast, Schizosaccharomyces pombe, which is required for both cell proliferation and nitrogen starvation-induced G0 quiescence. Cwh43 encodes an evolutionarily conserved transmembrane protein that localizes in endoplasmic reticulum (ER). Defects in this protein fail to divide in low glucose and lose mitotic competence under nitrogen starvation, and also affect lipid metabolism. Here, we identified mutations of the pmr1 gene, which encodes an evolutionarily conserved Ca²⁺/Mn²⁺-transporting P-type ATPase, as potent extragenic suppressors of ts mutants of the cwh43 gene. Intriguingly, these pmr1 mutations specifically suppressed the ts phenotype of cwh43 mutants, among five P-type Ca²⁺- and/or Mn²⁺-ATPases reported in this organism. Cwh43 and Pmr1 co-localized in the ER. In cwh43 mutant cells, addition of excessive manganese to culture media enhanced the severe defect in cell morphology, and caused abnormal accumulation of a cell wall component, 1, 3-β-glucan. In contrast, these abnormal phenotypes were abolished by deletion of the pmr1+ gene, as well as by removal of Mn²⁺ from the culture medium. Furthermore, nutrition-related phenotypes of cwh43 mutant cells were rescued in the absence of Pmr1. Our findings indicate that the cellular processes regulated by Cwh43 are appropriately balanced with Pmr1-mediated Mn²⁺ transport into the ER.

KEYWORDS Cwh43 fission yeast nutrient manganese Pmr1

Cells have the ability to respond and adapt to changes in their nutritional environments. Switching from a proliferative state to quiescence (G0 phase) and vice versa is a principal survival strategy when confronting drastic changes of nutritional availability. The fission yeast, Schizosaccharomyces pombe (S. pombe), is a suitable model organism to study these switching mechanisms, because quiescent cells can easily be induced from proliferative cells by nitrogen (NH₄Cl) deprivation in the culture medium (Su et al. 1996; Yanagida 2009). Conversely, replenishment of the nitrogen source causes quiescent S. pombe G0 cells to restart proliferation. Mechanisms controlling this switching are assumed to be evolutionarily conserved. Taking advantage of this unicellular organism, genetic regulation of mitotic competence (MC) to restart proliferation was investigated in G0 cells (Sajiki et al. 2018), as well as cell-cycle regulation in proliferative cells. Currently, more than 80 “super housekeeping (SHK) genes,” which are essential for both proliferation and quiescence, have been identified (Sajiki et al. 2009). We have been interested in the fission yeast cwh43 gene, an SHK gene that encodes a conserved transmembrane protein, potentially involved in metabolism of a wide range of nutrients (Nakazawa et al. 2018). In addition to the loss of viability under nitrogen-starvation, cwh43 temperature-sensitive mutants fail to divide in low glucose, suggesting that Cwh43 is required for responses to both carbon-
Intriguingly, cwh43 mutant cells significantly altered levels of biomarker metabolites for nutritional stresses, and over-accumulated triacylglycerols (neutral lipids). Cwh43 has been proposed to incorporate the sphingolipid, ceramide, into a lipid moiety of glycosylphosphatidylinositol (GPI)-anchored proteins (GPI-APs) in endoplasmic reticulum (ER) in budding yeast (Martin-Yken et al. 2001; Ghugtyal et al. 2007; Umemura et al. 2007). GPI-anchoring is an evolutionarily conserved post-translational modification, involved in various cellular functions at the plasma membrane, such as signal transduction, cell-cell interaction, cell adhesion, and host defense (Fujita and Jigami 2008). However, the physiological role of Cwh43 protein, the so-called ‘ceramide remodelase’, is still largely unknown.

To address the role of this enigmatic protein, we employed genetic screens for spontaneous extragenic mutations that recover cell division of the cwh43-G753R mutant at the restrictive temperature (37°C). We identified mutations of an evolutionarily conserved Ca2+/Mn2+-transporting, P-type ATPase, Pmr1, which has been proposed to be involved in regulating cellular Mn2+ levels in S. pombe (Rudolph et al. 1989; Dürr et al. 1998; Maeda et al. 2004; Culotta et al. 2005). We also showed that cwh43 mutant cells are sensitive to excess manganese, but not to calcium. Manganese is a biologically relevant trace metal that is required for growth and survival of most organisms. This trace element acts as a cofactor of many metalloenzymes involved in a wide range of cellular functions, including reactive oxygen species (ROS)-scavenging, protein glycosylation, DNA and RNA biosynthesis, phospholipid biosynthesis, and the urea cycle (Keen et al. 2000; Reddi et al. 2009; Jensen and Jensen 2014). Striking suppression of abnormalities of cwh43 mutants by pmr1 mutations or Mn2+ deprivation highlights the balanced action between Cwh43 and manganese at the ER, and suggests the importance of this metal in proper metabolism of nutrients and lipids.

MATERIALS AND METHODS

Strain constructions

Schizosaccharomyces pombe strains used in this study were derived from haploid wild-type strains 972 (h–) and 975 (h+). Temperature-sensitive (ts) cwh43-G753R and cwh43-G300E strains were constructed by genomic integration of these mutation sites into the wild-type strain (Nakazawa et al. 2018). Deletion of the pmr1Δ, pmc1Δ, cta3Δ, cta4Δ, cta5Δ, and pdt1Δ genes was performed by replacing the entire genomic locus with the hygromycin-resistance gene in a haploid wild-type strain. Strains expressing C-terminal GFP-tagged Cwh43-WT were described previously (Nakazawa et al. 2018). C-terminal mCherry-tagged
Pmr1 strains were made by chromosomal integration under the native promoter with the kanamycin-resistance gene.

**Suppressor screening and identification of mutations**
Suppressor screening, whole-genome sequencing, and mutation identification followed procedures described previously (Xu et al. 2018). Briefly, cwh43-G753R mutant cells were plated on YPD medium and incubated at 37°C for 4 days to obtain revertant colonies (frequency: ~1x 10⁻⁶) that contained suppressor mutations in addition to the original cwh43-G753R ts mutation. Genomic DNA of 30 revertants was extracted, and two genomic DNA mixtures were prepared and each mixture contains equal amounts of genomic DNA from 15 revertants. Then these genomic DNA mixtures were subjected to whole-genome sequencing analysis using Illumina HiSeq 2000 sequencers. Mutation sites and amino acid substitutions in the pmr1 and pga3 genes in the obtained suppressor strains were confirmed by Sanger dideoxy sequencing.

**Growth conditions**
*Schizosaccharomyces pombe* cells were cultivated in YPD (rich medium) or EMM2 (minimal medium) (Moreno et al. 1991) supplemented with extra MnCl₂, MnSO₄, or CaCl₂ as indicated. For Mn²⁺ deprivation, MnSO₄ (original concentration: 2.6 μM) was removed from the recipe for EMM2 medium. To prepare EMM2 medium devoid of Ca²⁺, 0.1 mM CaCl₂ and 2.1 μM calcium pantothenate were deleted from the recipe for EMM2. For the change from normal EMM2 to Mn²⁺-deprived or extra Mn²⁺-containing media, cells cultivated in normal EMM2 at 26°C were harvested by vacuum filtration, washed in the target liquid media twice on the membrane, and transferred to the new media at 26°C for 24 hr. For nitrogen starvation, cells were first cultured in normal EMM2 at 26°C and then transferred to nitrogen-deicient EMM2-N medium at 26°C for 24 hr (Sajiki et al. 2009). Cell viability was calculated as a percentage of the number of colonies formed vs. the number of plated cells. Numbers of liquid-cultured cells were counted using a Multisizer 3 (Beckman Coulter).

**Fluorescence microscopy and live-cell analysis**
Fluorescent staining of 1, 3-β-glucan was performed using aniline blue, as previously described (Okada and Ohya 2016). Lipid droplets were stained with BODIPY 493/503 (Thermo Fisher Scientific, D3922) (Meyers et al. 2016). Procedures for live-cell analysis were carried out using a DeltaVision Elite Microscopy System (GE Healthcare), as described previously (Nakazawa et al. 2016). Silicon objective lenses (UPLSAPO 100XS; NA 1.35; Olympus) were used. All-in-one microscopes, BZ9000 and BZ-X700 (Keyence, Japan), were used to obtain bright field images.

**Data availability**
Illumina sequence data have been deposited in the NCBI Sequence Read Archive under BioProject ID PRJNA533914 with BioSample accessions SANN11471144 and SANN11471145. Strains are available upon request. Supplemental Figure S1 shows rescue of defective phenotypes in the cwh43-G753R mutant cells by pga3-R161C mutation. Supplemental material available at Figshare: https://doi.org/10.25387/g3.8259173.
RESULTS

Identification of extragenic suppressor mutations of the cwh43-G753R mutant

We previously isolated 8 alleles of S. pombe cwh43 temperature-sensitive (ts) mutants (Nakazawa et al. 2018). To identify the genetic interactors of cwh43+ gene, we attempted to isolate extragenic suppressors that rescue the temperature sensitivity of the cwh43-G753R mutant. Among spontaneously isolated revertants of the cwh43 mutant (frequencies; $\sim 10^{-6}$, Materials and Methods) (Xu et al. 2018), 30 strains were able to grow at the restrictive temperature (37°C). These revertant strains were then subjected to whole genome sequencing, and suppressor mutation sites were determined in 13 strains. Three of these suppressor mutations contained missense mutations in the pga3+ gene, encoding GPI-phospholipase A2 activity regulator (Figure 1A). Ten of the remaining 13 suppressors contained 4 missense, 2 nonsense, and 4 nucleotide insertion or deletion mutations in the pmr1+ gene, which encodes a Ca$^{2+}$/Mn$^{2+}$-transporting P-type ATPase (Maeda et al. 2004; Cortés et al. 2004). Identification of pmr1 mutations as cwh43 mutant suppressors was unexpected, because direct involvement of Ca$^{2+}$ or Mn$^{2+}$ in Cwh43-mediated reactions has not been documented in S. pombe. Thus, we performed genetic and cytological analyses to study this suppression.

To confirm suppression of the ts phenotype in the cwh43 mutant by pmr1 mutations, we performed spot test analysis of these revertant strains. Although the single cwh43-G753R mutant failed to form colonies at the restrictive temperature (36°C), all 6 cwh43-G753R pmr1 double mutants strikingly recovered the capacity for colony formation at this temperature (Figure 1B). We further confirmed the recovery of colony formation of the cwh43 mutant by constructing a deletion mutant of the pmr1+ gene (pmr1Δ) and by crossing it with the cwh43 mutant (Figure 1C). Another ts allele of the cwh43 mutant strain, cwh43-G300E, was also suppressed by pmr1Δ, indicating that suppression of cwh43 is not allele-specific (Figure 1D).

Figure 3 Cwh43 co-localizes with Pmr1 in close proximity to the nuclear envelope and the plasma membrane. Intracellular localization of GFP-tagged Cwh43 and mCherry-tagged Pmr1 proteins. Wild-type cwh43+ or pmr1+ genes were tagged with GFP or mCherry, respectively, and integrated into the chromosome under the native promoter with the kanamycin-resistance gene. These cells were cultivated at 26°C in EMM2 media and fluorescent images were captured without fixation. A single focal plane is shown with bright field (BF) images. Inserts correspond to the white dashed boxes. Scale bar, 10 μm.

Figure 4 cwh43 mutants are sensitive to excess manganese (A) WT, cwh43-G753R, pmr1Δ, and cwh43-G753R pmr1Δ double-mutant strains were spotted onto EMM2 minimal media, which contains 2.6 μM MnSO$_4$ (normal), or onto EMM2 media containing additional manganese (2, 10, or 40 mM MnCl$_2$) at 26 °C. These strains were also spotted onto manganese-free EMM2 media (Mn$^{2+}$ free). cwh43-G753R is sensitive to excess manganese (arrowhead). (B) cwh43-G753R mutant is sensitive to excess manganese, derived from MnSO$_4$. (C) cwh43-G300E and cwh43-G300E pmr1Δ strains were spotted as in (A). (D) The four strains were spotted onto EMM2 media containing 102 μM CaCl$_2$ (normal), or onto EMM2 media containing additional calcium (2, 10, or 40 mM CaCl$_2$) at 26 °C. These strains were also spotted onto calcium-free EMM2 media (Ca$^{2+}$ free). Cell growth of cwh43 mutants was sensitive to additional MnCl$_2$ or MnSO$_4$, but not CaCl$_2$. 

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We constructed gene deletion mutants of the remaining 4 Ca2+ transporters (Figure 2A). We examined whether suppression of the cwh43-G753R pmr1 Δ mutant, none of resulting 4 double-mutant strains suppressed the failure of colony formation by the cwh43-G753R mutant at 36°C. Thus, suppression of the cwh43-G753R mutant is specifically caused by Pmr1 deletion, but not by deletion of the other Ca2+ transporters. Among the 5 Ca2+/Mn2+ P-type ATPases, thus far, only Pmr1 has been proposed to be involved in regulating cellular Mn2+ levels in S. pombe (Maeda et al. 2004), suggesting that loss of Mn2+ -importing ability is critical for suppression of the cwh43 mutant.

**Nramp-related metal transporter, Pdt1, partly suppresses the ts phenotype of the cwh43 mutant**

In S. pombe, Pmr1 regulates cell morphogenesis and Mn2+ homeostasis by cooperating with the evolutionarily conserved Nramp-related divalent metal transporter, Pdt1, which localizes at the plasma membrane (Tabuchi et al. 1999; Maeda et al. 2004). Pdt1 homologs, Smf1 and Smf2, in budding yeast, S. cerevisiae, take up extracellular Mn2+ into the cytosol with high affinity (Supek et al. 1996; Reddi et al. 2009). To test whether the ts phenotype of the cwh43-G753R mutant is alleviated by the loss of Pdt1, we constructed deletion mutant of pdt1 and crossed them with the cwh43-G753R mutant. The resulting cwh43 pdt1 Δ double mutant partly recovered colony formation capacity at 36°C, compared to that of the cwh43 mutant single mutant (Figure 2D). This result suggests that the defective phenotype of the cwh43 mutant is alleviated by restricting Mn2+ uptake from the extracellular environment into the cytosol.

**Cwh43 co-localizes with Pmr1 at the ER**

Next, we compared the intracellular localization pattern of Cwh43 with that of Pmr1. GFP-tagged Cwh43 localized at the ER, which is continuous with both nuclear and plasma membranes (Nakazawa et al. 2018). GFP-Cwh43 co-localized with mCherry-tagged Pmr1, which has been reported to localize predominantly at the ER in S. pombe (Figure 3) (Cortés et al. 2004), indicating that Cwh43 and Pmr1 coexists at the ER.

**cwh43 mutants are sensitive to excess manganese**

To test the effect of manganese on cwh43 mutant cells, we examined cell growth of this mutant in an excess of manganese. Addition of 2 or 10 mM MnCl2 to normal EMM2 medium, which contains 2.6 μM MnSO4 as source of manganese, scarcely affected colony formation of the wild-type strain (Figure 4A). However, these manganese concentrations severely inhibited growth of cwh43-G753R mutant cells. The cwh43-G753R pmr1 Δ double mutant showed mild sensitivity to the addition of 2 or 10 mM MnCl2, as did the pmr1 Δ single mutant, suggesting that deletion of the pmr1 gene partly rescues the hyper-sensitivity of cwh43 mutant cells to excess manganese. These results were also obtained using MnSO4 instead of MnCl2, as a source of manganese (Figure 4B). The cwh43-G300E mutant strain also showed hyper-sensitivity to excess manganese, which was alleviated in the absence of Pmr1 (Figure 4C). The cwh43-G753R mutant did not show any sensitivity to excess CaCl2 (2-40 mM). Nor did wild type or pmr1 Δ single-mutant cells (Figure 4D). These results indicate that cell growth in the cwh43 mutant is probably inhibited by Pmr1-mediated manganese transport.

**The Pmr1 deletion mutant is epistatic to the cwh43-G753R mutant in cell morphology under surplus or inadequate manganese**

Next, we examined the cell morphology of wild-type, cwh43-G753R, pmr1 Δ, and cwh43-G753R pmr1 Δ mutants under different concentrations of manganese in the culture media. We cultivated these 4 strains...
in normal EMM2 liquid media that contained 2.6 μM MnSO₄ and then shifted to 2 mM MnCl₂-containing EMM2, Mn²⁺-free EMM2, or normal EMM2, after washing the cells with the corresponding media (Figure 5A). After cultivation for 24 hr at 26⁰C, cell shape was not significantly affected in wild type cells in Mn²⁺-free or 2 mM MnCl₂-EMM2 media (Figure 5B, 1st row). However, in the cwh43-G753R mutant, abnormal cell morphology was enhanced with swollen and disorganized cell shapes in the presence of 2 mM MnCl₂ (Figure 5B, 2nd row). This aberrant morphology disappeared in the cwh43-G753R pmr1Δ double mutant in 2 mM MnCl₂-containing EMM2, as well as in pmr1Δ single-mutant cells (Figure 5B, 3rd and 4th rows). Instead, the pmr1Δ and cwh43-G753R pmr1Δ mutants had round cells in Mn²⁺-free EMM2, consistent with previous observations (Maeda et al. 2004). Taken together, the cwh43 pmr1Δ double mutant resembled the pmr1Δ single mutant in cell morphology, regardless of the presence of manganese, suggesting that deletion of pmr1 is epistatic to the cwh43 mutant, in terms of cell morphology.

Manganese enhances 1, 3-β-glucan accumulation in cwh43 mutant cells

An obvious defective phenotype of cwh43 mutant cells is the over-accumulation of a cell wall component, 1, 3-β-glucan, which enriches at cell septa (Nakazawa et al. 2018). Hence, we examined whether manganese concentration in culture media affects accumulation of this glucan in cwh43 mutant cells. Wild-type and cwh43-G753R mutant strains were pre-cultivated in normal EMM2 media, and then shifted to Mn²⁺-free, 2 mM MnCl₂-containing, or normal EMM2 media. After 24 hr at 26⁰C, intracellular localization of 1, 3-β-glucan was stained with specific fluorescent dye, aniline blue. In wild-type cells, localization of 1, 3-β-glucan at cell septa was not obviously altered at the three Mn²⁺ concentrations (Figure 6A, left). On the other hand, in the cwh43 mutant, the glucan signal at the cell surface was weaker in Mn²⁺-free medium than in normal or MnCl₂-enhanced media (Figure 6A, right). Spotted cell pellets of aniline blue-stained cwh43 mutant showed a paler blue color in Mn²⁺-free medium, compared to the two Mn²⁺-containing media (Figure 6C). These results suggest that abnormal accumulation of this glucan in Cwh43-defective cells is alleviated by manganese deprivation in the culture media.

As deletion of the pmr1 gene partly suppressed Mn²⁺ sensitivity of cwh43 mutants, we stained 1, 3-β-glucan in cwh43-G753R pmr1Δ double and pmr1Δ single mutants. Aniline blue staining clearly showed that over-accumulation of the glucan in cwh43 mutant cells did not occur in the pmr1Δ mutant background (Figure 6B and C), indicating that glucan accumulation in defective Cwh43 is caused by Mn²⁺, which is presumably transported by Pmr1.

Low-glucose sensitivity, loss of viability under nitrogen-starvation, and lipid accumulation in cwh43 mutant cells were abolished in the absence of Pmr1

Characteristics of the cwh43 mutant phenotype include sensitivity to both nitrogen starvation and glucose limitation (Nakazawa et al. 2018). To examine whether these cwh43 phenotypes under nutrient deficiency are affected by deletion of Pmr1, we spotted the cwh43-G753R pmr1Δ double mutant on solid EMM2 media containing 0.04–2% glucose (2.2–111 mM glucose). Although the cwh43 single mutant failed to form colonies on 0.04 and 0.06% low-glucose media, cwh43 pmr1Δ double mutant divided under these conditions to the same extent as wild type (Figure 7A). Under nitrogen-starvation, the cwh43 mutant was
incapable of producing spherical G0 quiescent cells like those of wild type (Su et al. 1996). Contrarily, the cwh43 pmr1Δ double mutant, as well as the pmr1Δ single mutant, presented spherical cells in the absence of nitrogen (Figure 7B). Mitotic competence (MC), which represents the regeneration capacity of G0 quiescent cells after nitrogen replenishment (Sajiki et al. 2018), diminished to 31% in the cwh43 mutant, but was alleviated by deleting the pmr1Δ gene (60%). Therefore, low-glucose sensitivity and the loss of MC in cwh43 mutant cells require the presence of Pmr1.

We previously found that cwh43 mutant cells overproduce triacylglycerols accompanied by lipid droplet (LD) accumulation (Nakazawa et al. 2018). Finally, we have verified the effect of Pmr1 deletion on lipid accumulation within cwh43 mutant cells. Increased numbers of BODIPY 493/503-stained LDs in the cwh43 mutant was suppressed in cwh43 pmr1Δ double-mutant cells (Figure 7C). Altogether, these data suggest that Pmr1 evokes abnormal responses to nutrient deficiencies and altered lipid metabolism in Cwh43-deficient cells.

**DISCUSSION**

Among the five reported Ca^{2+}- and/or Mn^{2+}-transporting P-type ATPases in *S. pombe*, we found that Pmr1 specifically recovered the defective cell growth of cwh43 mutant cells. Our results support the idea that Pmr1 is the most relevant Mn^{2+}-transporting P-type ATPase in *S. pombe*, as reported previously (Maeda et al. 2004; Cortés et al. 2004). In human cells, several P-type ATPases have been reported to facilitate Mn^{2+} uptake; however, two animal homologs of Pmr1, ATP2C1/SPCA1 and ATP2C2/SPCA2, are the only known P-type ATPases that transport Mn^{2+} into ER and Golgi with high affinity (Van Baalen et al. 2001; Ton et al. 2002; Vangheluwe et al. 2009; van Veen et al. 2014). Cortés et al. (2004) and this study indicate that *S. pombe* Pmr1 localizes at the ER, closely situated to peripheral regions of the nuclear envelope and plasma membrane. Taken together, restricted incorporation of Mn^{2+} from the cytosol into the ER is likely to substantially suppress the defective phenotype of cwh43 mutant cells (Figure 8).

In other words, Cwh43 function may be indispensable for cell proliferation when Mn^{2+} is abundant at the ER.

Manganese is involved in metabolism of carbohydrates and lipids (Keen et al. 2000; Aschner and Aschner 2005). Recently, it was proposed that manganese stress induces cellular toxicity by affecting wide range of metabolic reactions in bacterial cells (Kaur et al. 2017). Considering that sensitivities to nutrient deficiencies and lipid accumulation in cwh43 mutant are clearly rescued by Pmr1 deletion, these metabolic disorders may be caused by Pmr1-mediated Mn^{2+} transport into the ER. cwh43 mutants present a 1000-fold reduction in the ratio of acetyl-CoA to free CoA, relative to wild-type (Nakazawa et al. 2018). The acetyl-CoA/CoA ratio is believed to reflect the energy status of the cell, as does the ATP/AMP ratio (Gumaa et al. 1973), implying that excess Mn^{2+} in the ER perturbs nutrient metabolism for generation of high-energy compounds.

Our results raise the possibility that Cwh43 function is closely correlated with the intracellular Mn^{2+} level, particularly in the ER. Since excessive intracellular Mn^{2+} is assumed to cause cytotoxicity, regulation of this divalent cation is probably critical to manganese homeostasis. Excess intracellular manganese causes severe neurological damage, such as a Parkinson’s disease-like condition (Olanow 2004; Peres et al. 2016). We speculate that a potential role of Cwh43 is consumption of intracellular manganese, which is incorporated into cytoplasm and ER by Pdt1 and Pmr1, respectively. Before Cwh43-mediated ceramide conjugation, biosynthesis of GPI-anchor proteins (GPI-APs) comprises...
Costanzo et al. 2016). Although it is unclear whether the suppression of \( S. \) pombe \( cwh43 \) mutant by \( pga3 \) mutations occurs in a similar mechanism by the loss of \( Pmr1 \), \( Pga3 \) might thus affect the \( Mn^{2+} \) levels in the upstream process of \( Cwh43 \)-mediated reaction.

Identification of \( Pmr1 \) as an extragenic suppressor of \( cwh43 \) mutants provides an initial clue to the unexpected link between manganese homeostasis and ceramide metabolism. Ceramide has medical and dermatological importance because it blocks invasion of pathogens, allergens, and toxic compounds, and also renders the stratum corneum less susceptible to water loss (Meckfessel and Brandt 2014). Reduced ceramide abundance is correlated with atopic dermatitis (Hu et al. 2000; Ton et al. 2002; Micaroni et al. 2016). Therefore, this potential role of ceramide metabolism in controlling manganese concentration may shed light on its cosmetic applications. Further study is required to understand the molecular mechanism underlying coordination between \( Cwh43 \) and \( Pmr1 \) in regard to manganese homeostasis.

**Figure 8** Balanced action between \( Cwh43 \) and \( Pmr1 \)-mediated \( Mn^{2+} \) transport. Extracellular manganese is incorporated into cytoplasm and ER by \( Pdt1 \) and \( Pmr1 \) transporters, respectively. The putative ceramide-conjugation protein, \( Cwh43 \), is required for nutrient utilization, lipid metabolism, and cell morphology, presumably by regulating nutrient uptake into cells (Nakazawa et al. 2018). Defective phenotypes in \( cwh43 \) mutants are strikingly rescued by \( Pmr1 \) deletion, indicating that the balanced action between \( Cwh43 \) and \( Pmr1 \)-mediated \( Mn^{2+} \) transport at the ER controls proper nutrient and lipid turnover, as well as cell morphology, PM: plasma membrane.

more than 20 reactions at the ER (Pittet and Conzemmlmann 2007; Kinosita and Fujita 2016). GPI-AP biosynthesis includes several reactions mediated by \( Mn^{2+} \)-requiring enzymes, such as glycosyltransferase (Wiggins and Munro 1998; Roseman 2001). Moreover, \( Pmr1 \) is required for protein glycosylation (Maeda et al. 2004; Cortés et al. 2004). Thus, we assume that \( Cwh43 \) is essential under conditions in which protein glycosylation is accelerated by abundant \( Mn^{2+} \) at the ER. \( Cwh43 \)-mediated formation of ceramide-type GPI-APs may be linked to efficient processing of glycosylated proteins.

The \( pga3 \) mutations were identified as \( cwh43 \) suppressors along with \( pmr1 \) in this study. A suppressor mutation, \( pga3-R161C \), rescued colony formation at high temperature, hyper-sensitivity to excess manganese, and abnormal accumulation of 1, 3- \( \beta \)-glucan in the \( cwh43G753R \) mutant, to a similar extent as \( Pmr1 \) deletion (Supplemental Fig. S1). Budding yeast \( PER1 \), the ortholog of \( S. \) pombe \( Pga3 \), is required for a precursor step of \( Cwh43 \)-mediated reaction (Fujita et al. 2006). \( PER1 \) appeared to be involved in \( Mn^{2+} \) homeostasis through \( Cdc1 \) protein, which acts in a GPI-AP maturation step at upstream of \( PER1 \) (Paidhungat and Garrett 1998). In addition, budding yeast \( CWH43 \) and \( PER1 \) show genetic interaction with a cation-transporting P-type ATPase, \( SPF1 \) (Schuldiner et al. 2005; Surma et al. 2013; 2016). Although it is unclear whether the suppression of \( S. \) pombe \( cwh43 \) mutant by \( pga3 \) mutations occurs in a similar mechanism by the loss of \( Pmr1 \), \( Pga3 \) might thus affect the \( Mn^{2+} \) levels in the upstream process of \( Cwh43 \)-mediated reaction.

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**ACKNOWLEDGMENTS**

We are indebted to Dr. Susumu Morigasaki for helpful comments on this work. We thank Dr. Steven D. Aird for editing the manuscript. We are also grateful for the generous support of OIST Graduate University.

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