Manganese Redistribution by Calcium-stimulated Vesicle Trafficking Bypasses the Need for P-type ATPase Function\(^*\)

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Background: Yeast is a model system for the study of mechanisms governing eukaryotic Golgi-Mn\(^{2+}\) homeostasis.

Results: We provide evidence that calcium stimulates ER and late endosome/trans-Golgi manganese delivery and bypasses the need for Pmr1.

Conclusion: Vesicle trafficking promotes organelle-specific ion interchange and cytoplasmic metal detoxification.

Significance: Our findings open new perspectives on chemical modifiers of Hailey-Hailey disease.

Regulation of intracellular ion homeostasis is essential for eukaryotic cell physiology. An example is provided by loss of ATP2C1 function, which leads to skin ulceration, improper keratinocyte adhesion, and cancer formation in Hailey-Hailey patients. The yeast ATP2C1 orthologue PMR1 codes for a Mn\(^{2+}/Ca^{2+}\) transporter that is crucial for cis-Golgi manganese supply. Here, we present evidence that calcium overcomes the lack of Pmr1 through vesicle trafficking-stimulated manganese delivery and requires the endoplasmic reticulum Mn\(^{2+}\) transporter Spf1 and the late endosome/trans-Golgi Nramp metal transporter Smf2. Smf2 co-localizes with the putative Mn\(^{2+}\) transporter Atx2, and ATX2 overexpression counteracts the beneficial impact of calcium treatment. Our findings suggest that vesicle trafficking promotes organelle-specific ion interchange and cytoplasmic metal detoxification independent of calcineurin signaling or metal transporter re-localization. Our study identifies an alternative mode for cis-Golgi manganese supply in yeast and provides new perspectives for Hailey-Hailey disease treatment.

The intracellular levels of ions and other micronutrients are closely regulated in eukaryotic cells. This is the case for the trace element manganese (Mn\(^{2+}\)), whose regulation is particularly important. This redox active metal is a key cofactor for a wide range of enzymes located in every cellular compartment (1). However, at high concentrations Mn\(^{2+}\) is toxic and promotes DNA damage coupled to replication defects in yeast (2). In humans, overexposure to Mn\(^{2+}\) results in a neurological syndrome called manganism, whose symptoms resemble those of Parkinson disease (3). In addition, Mn\(^{2+}\) has been shown to favor prion misfolding if it displaces copper as the protein cofactor (4). Hailey-Hailey disease phenotypes have been associated with mutations affecting calcium and/or manganese transport activities of the Golgi Ca\(^{2+}\)/Mn\(^{2+}\) transporter ATP2C1 (5). A representative Hailey-Hailey phenotype caused by alterations in the intracellular Mn\(^{2+}\) flux includes keratinocyte differentiation (6). For these reasons, revealing the intracellular mechanisms that regulate Mn\(^{2+}\) homeostasis pathways is of clinical importance.

Much of our current understanding of eukaryotic manganese homeostatic mechanisms comes from the budding yeast, \textit{Saccharomyces cerevisiae}. Yeast Mn\(^{2+}\) uptake is provided by the plasma membrane transporter Smf1, a member of the natural resistance-associated macrophage protein (Nramp) family (7). Smf2 represents a member of intracellular Nramp Mn\(^{2+}\) transporters essential for the activity of Mn\(^{2+}\)-dependent enzymes, which include the mitochondrial Sod2 protein and Golgi-hosted sugar transferases (8). Smf2 localizes to Golgi-like vesicles, and a drop in whole-cell Mn\(^{2+}\) has been observed upon SMF2 deletion (8). Under physiological conditions, ~90% of newly synthesized Smf1 and Smf2 are directly targeted to the vacuole for degradation, presumably to limit uptake of toxic Mn\(^{2+}\) amounts (9, 10). When Mn\(^{2+}\) becomes limiting, these transporters are delivered to the cell surface (Smf1) and intracellular vesicles (Smf2) to increase Mn\(^{2+}\) uptake (9, 10). In contrast, in conditions of toxic metal concentrations, the vacuolar degradation of the Nramp transporters is enhanced, and Smf1 is virtually eliminated from the plasma membrane (11). Moreover, Mn\(^{2+}\) uptake by manganese-phosphate complexes is facilitated by the high affinity cell surface phosphate transporter Pho84 (12).

Other factors that influence intracellular Mn\(^{2+}\) homeostasis include the putative Mn\(^{2+}\) transporter Atx2. Atx2 localizes to Golgi-like vesicles, but the mechanism by which Atx2 regulates

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Intra-Golgi Manganese Redistribution

Table 1: Yeast strains and plasmids used in this study

| Strain or plasmid | Relevant genotype or description | Source |
|-------------------|---------------------------------|--------|
| BY4741            | MAT a ura3Δ0 leu2Δ0 his3Δ0 met15Δ0 | EUROSCARF |
| BY4742            | MAT a ura3Δ0 leu2Δ0 his3Δ0 lys2Δ0 | EUROSCARF |
| NGY051            | BY4741 pmr1Δ::kan | (2) |
| NGY178            | MAT a ura3Δ0 leu2Δ0 his3Δ0 met15Δ0 lys2Δ0 pmr1Δ::nat | This study |
| YML123C           | BY4741 phe8Δ2::kan | EUROSCARF |
| NGY223            | MAT a ura3Δ0 leu2Δ0 his3Δ0 met15Δ0 pmr1Δ::nat phe8Δ2::kan | This study |
| YHR050W           | BY4741 smf2Δ::kan | EROSCARF |
| NGY183            | MAT a ura3Δ0 leu2Δ0 his3Δ0 met15Δ0 lys2Δ0 pmr1Δ::nat smf2Δ::kan | This study |
| YDL128W           | BY4741 vcx1Δ::kan | EUROSCARF |
| NGY192            | BY4741 pmr1Δ::nat vcx1Δ::kan | This study |
| YGR217W           | BY4741 cch1Δ::kan | EUROSCARF |
| NGY190            | MAT a ura3Δ0 leu2Δ0 his3Δ0 met15Δ0 lys2Δ0 pmr1Δ::nat cch1Δ::kan | This study |
| YLR220W           | BY4741 ccc1Δ::kan | EUROSCARF |
| NGY193            | MAT a ura3Δ0 leu2Δ0 his3Δ0 met15Δ0 pmr1Δ::nat ccc1Δ::kan | This study |
| NGY233            | MAT a leu2Δ0 his3Δ0 met15Δ0 pmr1Δ::nat SMF2-GFP | This study |
| NGY234            | MAT a leu2Δ0 his3Δ0 met15Δ0 pmr1Δ::nat SMF2-GFP | This study |
| NGY241            | MAT a ade2–1 ura3–1 pmr1Δ::HIS3 cnb1Δ::LEU2 | EUROSCARF |
| NGY242            | MAT a ade2–1 ura3–1 pmr1Δ::HIS3 cnb1Δ::LEU2 | EUROSCARF |
| NOL122C           | BY4741 Ilg2Δ::kan | This study |
| NGY208b           | BY4741 pmr1Δ::nat Ilg2Δ::kan | This study |
| RH1737            | MAT a ura3Δ0 leu2Δ0 his4Δ0 bar1–1 sec18–20 | H.Riezman |
| NGY230            | MAT a ura3Δ0 leu2Δ0 pmr1Δ::nat sec18–20 | This study |
| YEL936C           | BY4741 antp1Δ::kan | EUROSCARF |
| Plasmids          |                                   |        |
| p2UGpd            | 2-μm origin, URA3, GPDp | (27) |
| p4TX2             | p2UGpd, GPDp, ATX2 | (2) |
| pTPQ2017          | CEN, LEU2, GPDp−FYVE-dsRed | (70) |
| pTPQ2028          | CEN, LEU2, ADH1p−SEC7−dsRed | (70) |
| Pv2-DsRed-PEP12   | pRS414, TRP1, PHO5p−DsRed-PEP12 | (71) |
| RH3100            | TRP1, mRFP, SED5 | (72) |
| pNG011            | pUG23, MEpTR-SMF2-GFP | This study |
| pNG026            | pUG23, GPDp−ATX2-mCherry | This study |
| pHS15-HT-GFP-cSNC1| CEN, LEU2, HA-GFP-cSNC1 | (53) |

Intracellular Mn²⁺ levels remains unknown (13). Recently, the P-type ATPase Spf1 (hAATP13A1) has been suggested to regulate Mn²⁺ transport into the endoplasmic reticulum (ER) (14), whereas Pmr1, a Golgi-localized P-type Ca²⁺ and Mn²⁺ ATPase, pumps cytosolic Mn²⁺ into the lumen of the Golgi (15–17). Apart from providing sugar transferases with Mn²⁺ as cofactor, Pmr1 has another role in Mn²⁺ detoxification by secretory pathway-mediated excretion (16–18). In addition to Pmr1, Mn²⁺ detoxification can be carried out by the vacuolar iron and manganese transporter Ccc1 (19).

Membrane fission and fusion are essential processes, allowing the dynamic communication between membrane-bounded organelles in all eukaryotic cells. Lipid vesicles are constantly emerging from one membrane to fuse with another, providing transport shuttles between distinct intracellular compartments. Increasing evidence suggests that calcium (Ca²⁺) plays a role in the regulation of membrane trafficking. For example, Ca²⁺ appears to be involved in ER to Golgi transport (20), intra-Golgi transport (21), and early endosome fusion (22) as well as yeast homotypic vacuole fusion (23).

Although many players involved in the intracellular manganese trafficking network have been characterized in yeast, our understanding of organelle-to-organelle Mn²⁺ flux is far from complete. Here, we report a Pmr1-independent mechanism for cis-Golgi Mn²⁺ supply. This supply depends on the ER Mn²⁺ transporter Spf1 and the Smf2 late endosome/Trans-Golgi Mn²⁺ transport activity and can be counteracted by ATX2 overexpression. In addition, it requires extracellular CaCl₂ in order to stimulate vesicle trafficking and membrane fusion. Based on our observations we propose a model on intracellular manganese homeostasis that provides mechanisms for intragoleral ion flux and manganese detoxification.

Experimental Procedures

Yeast Strains and Plasmids—Yeast strains and plasmids used in this study are listed in Table 1. Gene deletions were constructed by PCR-based methods using pAG25 (EUROSCARF) and pFA6a-::KanMX6 (kindly provided by B. Pardo) as template plasmids. In other cases strains were derived from genetic crosses. The chromosomal SMF2 open reading frame under the control of its own promoter was C-terminal-tagged with enhanced GFP (eGFP) by a PCR-based method using the tagging vector pKT209 (pFA6a-link-YEGFP-CaURA3) (24) as the template plasmid. To generate plasmid pNG011, SMF2 was amplified from genomic DNA, digested with EcoRI/Sall, and inserted into EcoRI/Sall site of pUG23 (25). To generate plasmid pNG026, ATX2 and mCherry were amplified from genomic DNA or pK539 (26), respectively, using overlapping oligonucleotides. The PCR products were mixed and amplified using external oligonucleotides, digested with BamHI/Sacl and inserted into BamHI/Sacl site of pUG23 (27).

Drug Sensitivity Assays—Yeast cells were adjusted in concentration to an initial A₆₀₀ of 0.2, then serially diluted 1:10 and spotted onto plates without or with different drugs at the indicated concentrations (see figure legends). CaCl₂ was added when indicated. Plates were then incubated at 30 °C for 3–4
days, except for temperature-sensitive mutants, which were incubated at the corresponding permissive or semipermissive temperatures.

**Pulse-Chase Analysis of CPY**—Pulse-chase labeling and analysis of immunoprecipitates was done as described previously (28).

**Analysis of Telomere Length**—Genomic DNA was isolated from yeast strains grown in YPAD for 3 days with or without the addition of 10 mM CaCl₂. DNA was digested with XhoI, separated on a 1% agarose-Tris borate EDTA gel, transferred to a Hybond XL (Amersham Biosciences) membrane, and hybridized with a 32P-labeled DNA probe specific for the terminal Y/H₁₁₀₃₂ telomere fragment. The probe was generated by random hexanucleotide-primed DNA synthesis using a short Y/H₁₁₀₃₂ specific DNA template, which was generated by PCR from genomic yeast DNA using the primers Y/H₁₁₀₃₂ up (5’-TGCCGTGCAACAA-ACACTAAAATCA-3’) and Y’ low (5’-CGCTCGAGAAAGTTGAGTTTTTCA-3’). Three independent colonies of each strain were analyzed to ensure reproducibility.

**Fluorescence Microscopy**—Plasmid harboring yeast cells were grown to mid-log-phase in selective Synthetic Complete (SC) medium to maintain the plasmid and fixed in 2.5% formaldehyde and 0.1 M potassium phosphate buffer, pH 6.4, for 10 min. Cells were then washed twice with 0.1 M potassium phosphate buffer, pH 6.6, and finally resuspended in 0.1 M potassium phosphate buffer, pH 7.4. Cells were imaged at 25 °C using a microscope (DM-6000B, Leica) at 100× magnification using L5, N3, and TX2 filters and a digital charge-coupled device camera (DFC350, Leica). Images were taken using LAS AF software (Leica) with the same exposure times for Smf2-GFP (1s) and lower exposure times for different marker proteins in the co-localization analysis. Images were assembled in Photoshop (Adobe) with only linear adjustments. Statistical analysis of co-localization was performed by counting at least 100 cells per marker derived from three independent experiments. Data are shown as the mean ± S.D.

**Metal Measurements**—Yeast cells were grown to an A₆₀₀ of 2.5 in YPAD medium or the same medium supplemented with 5 mM CaCl₂. In both cases the growth media was supplemented with 20 μM MnCl₂ to monitor metal accumulation under manganese toxicity conditions. The cultures were harvested and washed with TE (10 mM Tris-HCl and 1 mM EDTA, pH 8), then deionized water, and finally dried. Samples were subjected to acid digestion and applied to an ICP Horiba Jobin Yvon Ultima 2 atomic-emission spectrometer at the Microanalysis Service of University of Seville (Seville, Spain). Manganese and calcium content were measured according to the manufacturer’s specifications.

**Microarray Analysis**—Gene expression profiles were determined by using the “3’-expression microarray” technology by
RESULTS

CaCl₂ Counteracts Mn²⁺ Toxicity—In a previous work we found that an excess of cytosolic Mn²⁺ alters mRNA transcription regulation and challenges genome stability (2). An example is the transcriptional 42-fold down-regulation of the low-affinity plasma membrane Mn²⁺ transporter PHO84 (YML123C). Interestingly, upon CaCl₂ addition, transcriptional down-regulation of PHO84 was reversed, suggesting that extracellular CaCl₂ alters cellular Mn²⁺ levels (see the supplemental data).

To test if this is the case, we first compared the total cellular manganese and calcium levels in wild type and pmr1Δ cells in the presence of extracellular CaCl₂ (Fig. 1A). In accordance with previous studies (16), pmr1Δ cells suffered from a dramatic increase in total manganese and calcium levels. Upon the addition of CaCl₂, the cellular calcium content increased with a concurrent decrease in the manganese content (~8.5-fold). Because Mn²⁺ interferes with telomerase activity leading to telomere shortening (29) we assayed telomere length as an indirect measure for nuclear Mn²⁺ levels (Fig. 1B). We found that telomere shortening in pmr1Δ mutants was alleviated upon CaCl₂ addition, suggesting that the addition of extracellular CaCl₂ either competes with Mn²⁺ uptake or stimulates the removal of toxic Mn²⁺ from the cytoplasm.

Transformation with an SMF1 overexpression vector challenged pmr1Δ viability independently of CaCl₂ supplementation (data not shown), indicating that increased Smf1 levels could lead to uncontrolled and toxic Mn²⁺ uptake. Loss of the Mn²⁺ importer Smf1 should, therefore, impair Mn²⁺ uptake and suppress pmr1Δ phenotypes related to cytosolic Mn²⁺ excess. However, deletion of SMF1 has been shown to be lethal in combination with pmr1Δ (30) (Fig. 1C, left), whereas mutants in PMR1 up-regulate Smf1 protein levels under Mn²⁺ starvation conditions (11). Interestingly, we could recover viable pmr1Δ smf1Δ spores when the tetrads were plated on CaCl₂-containing medium (Fig. 1C, right), suggesting that CaCl₂ is able to facilitate bypass of Mn²⁺ toxicity via an alternative mechanism.

Bypass of pmr1Δ Glycosylation Defects Requires the Putative Mn²⁺ Transporters Spf1 and Smf2—Numerous studies have reported suppression of other pmr1Δ phenotypes by CaCl₂ (31–33). However, the underlying mechanism by which this occurs remains unclear. We asked whether other cation transporters contribute to this phenomenon. First, we set up a targeted, genetic screen for synthetic phenotypes of pmr1Δ with deletion of genes involved in Ca²⁺ or Mn²⁺ homeostasis. As a read-out, we monitored pmr1Δ-dependent loss-of-viability by the cell wall-perturbing agent calcofluor white (CFW) (34, 35) and recovery-of-viability in the presence of CaCl₂. Consistent with glycosylation defects, pmr1Δ shows a weakened cell wall exemplified by hypersensitivity to CFW, Congo Red, and hygromycin B and constitutive activation of the cell integrity pathway (36). Notably, CaCl₂-mediated recovery of viability was not observed in other mutants affected in protein glycosylation such as anp1Δ, lacking a cis-Golgi α-1,6-mannosyltransferase subunit (Fig. 2A). Interestingly, CFW sensitivity of pmr1Δ pho84Δ, pmr1Δ vcx1Δ, and pmr1Δ ccc1Δ double mutants was suppressed by CaCl₂, whereas pmr1Δ spf1Δ and pmr1Δ smf2Δ double mutants failed to grow upon CaCl₂ addition (Fig. 2B).

Mn²⁺ ions are essential cofactors for the activity of Golgi-hosted mannosyltransferases that progressively and sequentially N-glycosylate proteins in different Golgi compartments (18, 37). Glycosylation events along the secretory route can be followed by analyzing carboxypeptidase Y (CPY) maturation. CPY is subjected to core glycosylation in the ER (p1 form). The core oligosaccharides are extended in the Golgi by the sequen-tial addition of α1,6- and α1,2- linked mannose residues, which results in a mobility shift when analyzed by SDS-PAGE (p2 form). After delivery to the vacuole, the pro region is cleaved to yield mCPY (see Fig. 2C, left) (38). In accordance with a previous report (31), fully glycosylated CPY (p2) was nearly absent in pmr1Δ mutants, but CPY glycosylation recovered upon CaCl₂ addition. We confirmed the previously described CPY glycosylation defect of pmr1Δ spf1Δ double mutants (39), but surprisingly CPY glycosylation was significantly diminished in smf2Δ mutants, and even more interestingly, we observed a CaCl₂ persistent glycosylation defect in smf2Δ, spf1Δ single and pmr1Δ smf2Δ, pmr1Δ spf1Δ double mutants. To further define the protein glycosylation defect of smf2Δ mutants, we compared CPY mannosylation patterns by pulse-chase labeling and sequential immunoprecipitation with antibodies specific to either CPY or α1,6-mannose linkages (Fig. 2D). In contrast to pmr1Δ, smf2Δ isolated CPY can be α1,6-mannosyl-immunoprecipitated, indicating a proficient early (cis-Golgi) α1,6-mannosyl addition. We, therefore, searched for evidence that the N-glycosylation defect of smf2Δ cells might be linked to a late glycosylation event. Consequently, we assessed the subcellular localization of Smf2 by colocalization experiments with protein markers for the trans-
Golgi network (Sec7), late-endosome (Pep12), cis-Golgi (Sed5), or early endosome (FYVE; see Fig. 3). Interestingly, microscopic analysis showed that Smf2 co-localizes with 70 and 90% of late endosome and trans-Golgi markers, respectively. In contrast, Smf2 poorly co-localized with cis-Golgi and early endosomes markers (Sed5 and FYVE, respectively). These findings suggest that Smf2 supplies late endosome and trans-Golgi with Mn\(^{2+}\) and raise the question as to how Smf2 is connected to cis-Golgi Mn\(^{2+}\) homeostasis.

**Smf2 and Atx2 Have Antagonistic Roles in Late Endosome/Trans-Golgi Mn\(^{2+}\) Transport**—Another option would be that the late endosome/trans-Golgi could act as a cellular Mn\(^{2+}\)

![Microscopy images of WT and pmr1Δ cells co-expressing the chromosomal fusion protein Smf2-GFP and different tagged proteins (as indicated). Percentages of co-localization with markers for the trans-Golgi (Sec7), cis-Golgi (Sed5), early endosome (FYVE domain), or late endosome (Pep12) are indicated (bottom). Percentages were quantified with respect to Smf2 or the indicated marker. At least 100 cells per marker were assessed, and errors represent S.D. of three independent experiments. Bar, 5 μm.](image)
storage compartment as previously proposed by Luk and Culotta (8). If so, we reasoned that a Mn\(^{2+}\)/H\(^{10001}\) exporter system might be required to prevent trans/post-Golgi Mn\(^{2+}\)/H\(^{10001}\) overload. A candidate for such activity is Atx2, based on the observations that Atx2 is a Golgi membrane protein whose overproduction provides the cytoplasm with antioxidative Mn\(^{2+}\)/H\(^{10001}\) activities that compensate for the loss of cytoplasmic SOD1, although Atx2 effect seems to require Smf1 function (13). To validate our hypothesis, we determined if ATX2 overexpression counteracts the CaCl\(_2\)-mediated viability (Fig. 4A). In fact, transformation of pmr1\(\Delta\) smf1\(\Delta\) double mutants with an ATX2 overexpressing plasmid conferred lethality in the presence of CaCl\(_2\), indicating a Smf1-independent function of Atx2. In addition, ATX2 overexpression compromised the CaCl\(_2\)-dependent suppression of CFW sensitivity in pmr1\(\Delta\) mutants (Fig. 4B). These observations suggest that Atx2 might expel Mn\(^{2+}\) from the trans-Golgi but also that enough Mn\(^{2+}\) is available for Atx2-mediated Mn\(^{2+}\) transport in CaCl\(_2\)-treated pmr1\(\Delta\) smf1\(\Delta\) cells. We, therefore, determined if Atx2 and Smf2 co-localize to the same compartment (Fig. 4C). This was indeed the case, and based on our experimental evidence we anticipate that Smf2 and Atx2 might have antagonistic roles in trans-Golgi Mn\(^{2+}\) homeostasis such that Smf2 and Atx2 are required for trans-/post-Golgi Mn\(^{2+}\) import and export, respectively.

\[\text{CaCl}_2\]-dependent Suppression Does Not Rely on Calcineurin-mediated Signaling or Smf2 Redistribution from Trans- to Cis-Golgi—Extracellular Ca\(^{2+}\) has been shown to initiate signal transduction events (40). The conserved Ca\(^{2+}\)/calmodulin-dependent protein phosphatase calcineurin plays a critical role in Ca\(^{2+}\)-mediated signaling (41). Therefore, we scored CFW sensitivity of pmr1\(\Delta\) mutants compromised in the calcineurin regulatory subunit Cnb1 or added calcineurin inhibitors (FK506 or cyclosporin A (CsA)) to the growth media (41) (see Fig. 5A). Neither lack of Cnb1 nor the addition of calcineurin inhibitors caused a loss-of-viability in the presence of CFW, suggesting that activation of calcineurin signaling is dispensable for CaCl\(_2\)-mediated suppression of pmr1\(\Delta\) CFW hypersensitivity.

Smf2 could have a dual role in late endosome/trans- and cis-Golgi Mn\(^{2+}\) import if one considers a CaCl\(_2\)-dependent late endosome/trans- to cis-Golgi Smf2 redistribution. We addressed this possibility by determining the Smf2 subcellular localization in the presence of CaCl\(_2\) and found that Smf2 still co-localized with the trans-Golgi marker Sec7 but not with the cis-Golgi marker Sed5 (Fig. 5B). Thus, the CaCl\(_2\)-mediated suppression of cis-Golgi Mn\(^{2+}\) import defect in pmr1\(\Delta\) does not occur through Smf2-mediated Mn\(^{2+}\) redistribution from trans-to the cis-Golgi.
Rescue of pmr1Δ CFW Resistance Relies on a Competent Golgi Retrograde Transport Machinery—In addition to its function in cellular signaling, intracellular Ca$^{2+}$ also plays a regulatory role in membrane trafficking. In particular, Ca$^{2+}$ is thought to participate in different membrane fusion events within secretory and endocytic pathways including intra-Golgi transport (42) (see Fig. 6A). To determine if this is the case, we investigated whether intracellular transport and membrane fusion are essential for CaCl$_2$-dependent suppression of glycosylation defects. First, we benefited from a sec18–20 mutation that has been shown to block many vesicular fusion events (43, 44). Sec18 is an essential ATPase that catalyzes the disassembly and recycling of SNARE complexes for further rounds of vesicle transport (45). Indeed, CaCl$_2$ failed to rescue growth of pmr1Δ sec18–20 double mutants on CFW-containing media, suggesting that vesicle transport is involved in CaCl$_2$-dependent resistance to CFW (Fig. 6A). Next, to broadly assess vesicle trafficking steps, we took advantage of monensin, a Na$^+$/H$^+$ ionophore that interferes with intracellular transport by the neutralization of acidic intracellular compartments (46), blocking intracellular transport in both trans- and post-Golgi compartments (47). Most appealing, CaCl$_2$ failed to rescue the CFW resistance in the presence of monensin (Fig. 6C, left). We then considered that monensin constrains protein glycosylation in pmr1Δ mutants. This was indeed the case, as monensin suppressed the appearance of fully glycosylated CPY (p2CPY) in CaCl$_2$ treated pmr1Δ but not in WT cells (Fig. 6B, right).

Mn$^{2+}$-sensitive mutants were found to be enriched in the functional category of vesicle-mediated transport including late endosome retrograde transport involving Tlg2 (48), a t-SNARE protein needed for the fusion of endosome-derived vesicles with the late Golgi (49, 50). Based on this finding we wondered if the CaCl$_2$-dependent suppression of CFW sensitivity and CPY glycosylation were impaired in pmr1Δ tlg2Δ double mutants. Indeed, although tlg2Δ mutants did not display an obvious CPY glycosylation defect, CaCl$_2$ could not rescue CPY glycosylation defects and viability of CFW-treated pmr1Δ tlg2Δ double mutants (Fig. 6D). To further assess the role of CaCl$_2$ in vesicle transport, we analyzed different mutants defective in the coatomer or COPI coat required for the forma-

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**FIGURE 5.** CaCl$_2$-mediated suppression of CFW sensitivity is not dependent on calcineurin signaling activation or trans- to cis-Golgi Smf2 redistribution. **A**, top, drop test sensitivity of WT, pmr1Δ, and pmr1Δ cnb1Δ against CFW (10 μg/ml) without (left) or with (right) the addition of 10 mM CaCl$_2$. Bottom, drop test sensitivity of WT and pmr1Δ cells against CFW (15 μg/ml), FK506 (10 μg/ml), CFW + FK506 (15 and 10 μg/ml), cyclosporin A (CsA; 50 μg/ml), and CFW/CsA (15 and 50 μg/ml) without (top) or with (bottom) the addition of 10 mM CaCl$_2$. **B**, microscopic images of WT and pmr1Δ cells co-expressing the chromosomal fusion protein Smf2-GFP and different tagged proteins (as indicated) in the presence of CaCl$_2$ (10 mM). DIC, differential interference contrast.
tion of retrograde transport vesicles from the Golgi to the ER and between Golgi cisternae (intra-Golgi retrograde transport) (51, 52). Again, CaCl₂ failed to rescue growth of pmr1Δ mutants in the absence of Sec28 and in combination with mutations of Cop1 (ret1–1) or Cog3 (sec34–2) (data not shown). Taken together, these results suggest that a functional intra-Golgi ret-

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**DISCUSSION**

Here we dissect a remarkable mechanism by which CaCl$_2$ suppresses pleiotropic phenotypes linked to impaired cis-Golgi manganese transport (Fig. 7; see the figure legend for an explanation). This mechanism relies on functional ER and late endosome/trans-Golgi Mn$^{2+}$ transport, and we provide evidence that calcium stimulates intracellular distribution of manganese and therefore, these results strongly suggest that CaCl$_2$ promotes Golgi-vesicle trafficking overcoming the lack of Pmr1.

**FIGURE 6. Functional vesicle trafficking/fusion is essential for CaCl$_2$-mediated rescue of pmr1Δ glycosylation defects.** A, illustration of the endomembrane system. Organelles (ER, Golgi, endosome, and vacuole) and secretory, endocytic, and CPY pathways are depicted. B, drop test sensitivity of WT, pmr1Δ, sec18Δ–20, and pmr1Δ sec18Δ–20 against CFW (10 μg/ml) without (top) or with (bottom) the addition of 10 mM CaCl$_2$. Cells were grown in permissive (23 °C, left) or semi-permissive (29 °C, right) conditions. C, monensin, a drug that blocks intracellular transport, counteracts CaCl$_2$-dependent suppression of glycosylation defects. Left, WT and pmr1Δ cells were spotted onto YPAD, monensin (25 μg/ml), CFW (10 μg/ml), CFW + MnCl$_2$ (10 μM), and CFW + CaCl$_2$ + monensin. Right, pulse-chase analysis of CPY maturation in WT and pmr1Δ cells without or with the addition of CaCl$_2$ (10 μM) or CaCl$_2$ + monensin (40 μg/ml). E, CaCl$_2$ rescues Sncl protein trafficking. Plasma membrane localization of Sncl-GFP was quantified in WT, pmr1Δ, tlg2Δ and pmr1Δ tlg2Δ cells grown without (white bars) or with the addition of 10 mM CaCl$_2$ (10 μM, black bars). Bar, 5 μm. Error bars represent S.D. Double asterisks (***) indicate p < 0.01. Phase contrast (Ph) and Sncl-GFP images are shown.

**FIGURE 7. A model for Ca$^{2+}$-mediated suppression of pmr1Δ-dependent phenotypes.** Lack of Pmr1 causes cis-Golgi manganese depletion. The addition of CaCl$_2$ stimulates vesicle trafficking and Mn$^{2+}$ retrograde transport from ER to cis-Golgi or late endosome/trans-Golgi. Cis-Golgi Mn$^{2+}$ supply restores sugar transferase and/or Mn$^{2+}$ detoxification activities. This model does not rule out the possibility that CaCl$_2$ could compete with Mn$^{2+}$ uptake or stimulate Mn$^{2+}$ detoxification through the secretory pathway.

retrograde transport is essential for the Ca$^{2+}$-dependent bypass of pmr1Δ glycosylation defects.

Finally, to more directly assess the idea that CaCl$_2$ stimulates intracellular vesicle trafficking, we used the exocytic SNARE Snc1 protein to monitor protein trafficking (53). The chimeric GFP-Snc1 protein is dynamically localized at the plasma membrane by continuous endocytic recycling, via endosomes, to the trans-Golgi, from where it is rapidly trafficked back to the plasma membrane. It has been previously shown that GFP-Snc1 accumulates in internal structures when Golgi function is blocked (54). Consistent with a known defect in Golgi function (31), the absence of Pmr1 leads to the redistribution of GFP-Snc1 to punctuated structures (Fig. 6E). The addition of CaCl$_2$ restored GFP-Snc1 localization to the cell surface, suggesting that CaCl$_2$ indeed rescues Golgi trafficking in pmr1Δ mutant. By contrast, CaCl$_2$ addition could not restore the plasma membrane localization of GFP-Snc1 in a tlg2Δ mutant background, which blocks the transport of GFPSnc1 to the Golgi from endosomes. Therefore, these results strongly suggest that CaCl$_2$ promotes Golgi-vesicle trafficking overcoming the lack of Pmr1.

Intra-Golgi manganese trafﬁcking when Golgi function is blocked (54). Accordingly, Nramp1, but not Nramp2, can rescue the metal ion stress phenotype of yeast mutants, suggesting that both proteins differ in the direction of transport (60). Notably, when expressed in yeast, Nramp1 localizes to the ER (data not shown) and thereby is unlikely to complement the transport activity of trans-Golgi-localized Smf2. Unfortunately, in contrast to other ions, studies on the abundance and intracellular distribution of manganese are hampered by the lack of chemical or genetically encoded manganese reporters (61).

In this work we specifically localize Smf2 in the late endosome/trans-Golgi, and based on our results, we believe that Smf2 might supply the trans-Golgi with Mn$^{2+}$ needed for the activity of mannosyltransferases such as Mnn1 (37, 62). Neutralization of acidic trans- and post-Golgi compartments by monensin might alter Smf2 flux direction and, therefore, compromise CaCl$_2$-dependent alleviation of CFW sensitivity. In addition, mutations in the vacuolar-type H$^+$-transporting
ATPase (V-ATPase), which alter Golgi acidification, share multiple *pmr1Δ* phenotypes (33). We find that Smf2 co-localizes with Atx2, a poorly characterized, putative trans-Golgi Mn2+ transporter that could function in pumping Mn2+ in the opposite direction to Smf2. Evidence for Atx2 ion transport activity is based on the observation that the protein shares functional characteristics with the SLC39 family of metal ion transporters (63). Consequently, Smf2 and Atx2 might form part of a late endosome/trans-Golgi Mn2+ import/export system required for a stable equilibrium between Mn2+ and other ions in the late endosome/trans-Golgi.

Regulation of Mn2+ homeostasis is highly conserved between yeast and higher eukaryotes, and Mn2+ transport enhancing mutations in the human ortholog of *PMR1*, *ATP2C1* can protect mammalian cells from the cytotoxic effects of Mn2+ (64). The contribution of defective Mn2+ transport on Hailey-Hailey disease progression is still under debate. However, increasing evidence points to the possibility that impaired manganese homeostasis triggers keratinocyte differentiation (6) and causes genetic instability (2).

We first anticipated that CaCl2-dependent suppression of *pmr1Δ* phenotypes could involve signal transduction pathways. However, this seems not to be the case, as CaCl2-mediated rescue of *pmr1Δ* is not coupled to Ca2+/calmodulin-dependent changes in gene expression or protein re-localization. Increasing evidence links Ca2+ to the regulation of membrane trafficking and fusion events (65, 66). The precise mechanism by which calcium regulates membrane trafficking is still poorly understood. It has been proposed that transiently released luminal calcium is required to trigger the last stages of membrane fusion (23). Accordingly, the addition of CaCl2 suppresses the vacuole fragmentation phenotype of *pmr1Δ* mutants (33). In addition, calcium might also regulate the formation of intra-Golgi retrograde transport vesicles as it has been shown to stabilize COPI coat on the Golgi membrane (67). The addition of CaCl2 caused a significant increase in the intracellular calcium levels and might account for a permanent induction of retro- and anterograde pathways. Along this line, we found that CaCl2 decreased intracellular manganese levels and restored Golgi-to-cell surface recycling of the exocytic SNARE Sncl-GFP chimera in *pmr1Δ* but not in *pmr1Δ* tlg2Δ mutants. These results point to the possibility that CaCl2 promotes Golgi to trans-Golgi network, to secretory vesicle, to plasma membrane trafficking of vesicles. Based on our data we suggest that Mn2+-containing vesicles might emerge from the trans- or post-Golgi and fuse with the cis-Golgi, supplying the cis-Golgi with essential Mn2+ for the action of sugar transferases. Consequently, Ca2+ may also stimulate retro- and anterograde trafficking between later secretory pathway organelles and the ER. The results of this study raise the possibility that stimulation of vesicle transport in human cells can bypass *ATP2C1* disease phenotypes or, yet more interestingly, can counteract neurotoxicity upon manganese exposure.

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