

The yeast protein Gdt1p transports Mn^{2+} ions and thereby regulates manganese homeostasis in the Golgi

Received for publication, February 7, 2018, and in revised form, April 5, 2018. Published, Papers in Press, April 9, 2018, DOI 10.1074/jbc.RA118.002324

Louise Thines¹, Antoine Deschamps, Palanivelu Sengottaiyan, Oksana Savel, Jiri Stribny, and  Pierre Morsomme²

From the Institut des Sciences de la Vie, Université catholique de Louvain, B-1348 Louvain-la-Neuve, Belgium

Edited by Chris Whitfield

The uncharacterized protein family 0016 (UPF0016) is a family of secondary ion transporters implicated in calcium homeostasis and some diseases. More precisely, genetic variants of the human UPF0016 ortholog transmembrane protein 165 (TMEM165) have been linked to congenital disorders of glycosylation (CDG). The *Saccharomyces cerevisiae* ortholog Gdt1p has been shown to be involved in calcium homeostasis and protein glycosylation. Moreover, plant and bacterial UPF0016 members appear to have putative roles in Mn^{2+} homeostasis. Here, we produced the yeast UPF0016 member Gdt1p in the bacterial host *Lactococcus lactis*. Using Mn^{2+} -induced quenching of Fura-2–emitted fluorescence, we observed that Gdt1p mediates Mn^{2+} influx, in addition to its previously reported regulation of Ca^{2+} influx. The estimated K_m values of Gdt1p of $15.6 \pm 2.6 \mu M$ for Ca^{2+} and $83.2 \pm 9.8 \mu M$ for Mn^{2+} indicated that Gdt1p has a higher affinity for Ca^{2+} than for Mn^{2+} . In yeast cells, we found that Gdt1p is involved in the resistance to high Mn^{2+} concentration and controls total Mn^{2+} stores. Lastly, we demonstrated that *GDT1* deletion affects the activity of the yeast Mn^{2+} -dependent Sod2p superoxide dismutase, most likely by modulating cytosolic Mn^{2+} concentrations. Taken together, we obtained first evidence that Gdt1p from yeast directly transports manganese, which strongly reinforces the suggested link between the UPF0016 family and Mn^{2+} homeostasis and provides new insights into the molecular causes of human TMEM165-associated CDGs. Our results also shed light on how yeast cells may regulate Golgi intraluminal concentrations of manganese, a key cofactor of many enzymes involved in protein glycosylation.

The uncharacterized protein family 0016 (UPF0016)³ gathers highly conserved membrane proteins that are widely distributed among kingdoms and defined by the presence of one or two copies of the EXGD(K/R)(T/S) motif (1). It was previously

reported that specific mutations in the human UPF0016 member transmembrane protein 165 (TMEM165) cause congenital disorders of glycosylation (2). Because the exact function of the UPF0016 members is not deciphered yet, the causal link between a mutated TMEM165 and congenital disorders of glycosylation remains unclear. Based on previous studies, the UPF0016 members were hypothesized to act as Ca^{2+}/H^{+} antiporters. This hypothesis arose, among others, from studies carried out on the *Saccharomyces cerevisiae* UPF0016 member Gdt1p. Indeed, we previously reported that Gdt1p localizes at the yeast Golgi membrane and that this protein is required for (i) Ca^{2+} tolerance, (ii) proper Ca^{2+} response after exposure to salt stress, and (iii) proper proteins glycosylation in the presence of a high calcium concentration (3, 4). Transport of Ca^{2+} by Gdt1p was further demonstrated by producing Gdt1p in the bacterial host *Lactococcus lactis* and using the fluorescent Ca^{2+} -sensitive Fura-2 probe (4). The acidic and polar uncharged amino acids of the conserved motif EXGD(K/R)(T/S) found in Gdt1p were additionally demonstrated to be essential for proper Ca^{2+} tolerance and Ca^{2+} response to salt stress (5).

In addition to this role in calcium homeostasis, recent studies on other UPF0016 members suggested an implication of these proteins in manganese homeostasis. Among others, Wang *et al.* (6) and Schneider *et al.* (7) reported an implication of the *Arabidopsis thaliana* CCHA1 in resistance to high concentrations of Ca^{2+} and Mn^{2+} and to pH changes. The UPF0016 members of the bacterium *Vibrio cholerae* and of the cyanobacterial model strain *Synechocystis* sp. PCC 6803 were additionally suggested to function as Mn^{2+} transporters (8–10). Interestingly, members of the photosynthetic *A. thaliana* and *Synechocystis* both localize to the thylakoid membrane and are essential for proper photosynthesis, most likely because of the required presence of Mn^{2+} in the oxygen-evolving complex of photosystem II (6–8, 10). Regarding the yeast Gdt1p, we previously reported that the glycosylation defects observed in a *gdt1Δ* strain grown in the presence of a high calcium concentration were suppressed when manganese was additionally present in the culture medium (4, 11). Manganese cations were also demonstrated to restore glycosylation in TMEM165-deficient cells (11). Interestingly, TMEM165 and a Myc-tagged version of Gdt1p were both reported to be degraded in the presence of high extracellular manganese concentration (12, 13). However, although these studies all established a clear functional link between the UPF0016 members and manganese homeostasis, none of them provided direct evidence for Mn^{2+} transport.

This work was supported by grants from Fonds National de la Recherche Scientifique-FNRS Grant PDR-T.0206.16 and the Communauté Française de Belgique–Actions de Recherches Concertées. The authors declare that they have no conflicts of interest with the contents of this article.

This article contains Fig. S1.

¹ Research fellow at the Fonds pour la Formation à la Recherche dans l'Industrie et dans l'Agriculture (FRIA).

² To whom correspondence should be addressed: Institut des Sciences de la Vie, Université catholique de Louvain, B-1348 Louvain-la-Neuve, Belgium. Tel.: 3210472623; Fax: 3210473872; E-mail: pierre.morsomme@uclouvain.be.

³ The abbreviations used are: UPF0016, uncharacterized protein family 0016; TMEM165, transmembrane protein 165; ICP–AES, inductively coupled plasma atomic emission spectrometry; AM, acetoxymethyl ester.

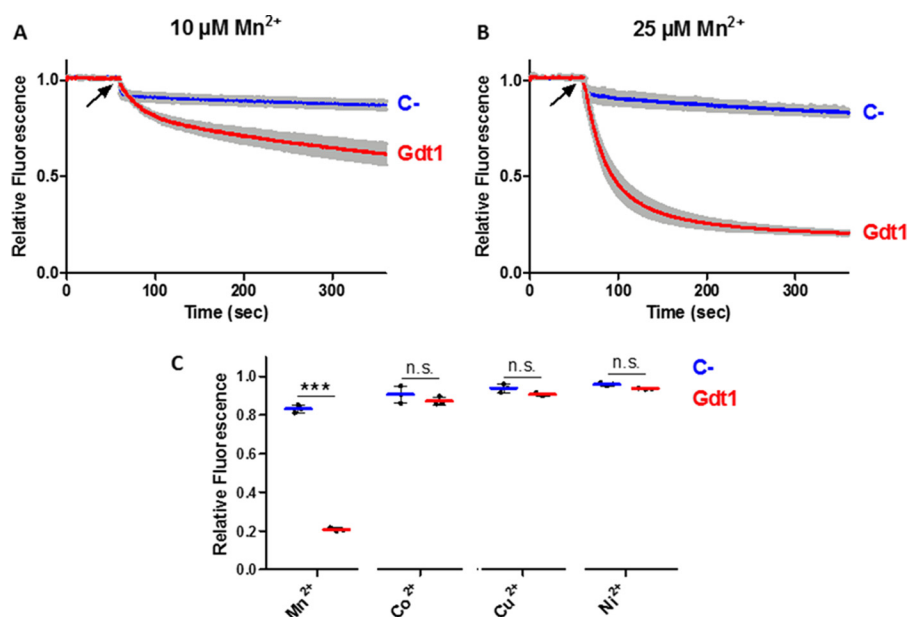


Figure 1. Gdt1p promotes Mn^{2+} influx in *L. lactis*. A, time-course measurement of the quenching by Mn^{2+} of the fluorescence emitted by Fura-2 in *L. lactis* DML1 cells transformed with the empty (C-) or *GDT1*-containing plasmid. The cells were grown to an A_{600} of 0.5, at which expression of *GDT1* was initiated by addition of 2.5 $\mu\text{g/liter}$ nisin. After a 2-h postinduction time, the cells were washed and incubated for 2 h in the presence of Fura-2/AM. The fluorescence measurements were carried out in a calcium-depleted Tris buffer with an excitation and emission wavelength of 360 and 510 nm, respectively. Ten μM MnCl_2 were added in the extracellular medium after 60 s of measurement (indicated by the arrow). The data are represented as the mean fluorescence normalized to the fluorescence at time 0 ($n = 3$, \pm S.D.). B, time-course measurement of the quenching by Mn^{2+} of the fluorescence emitted by Fura-2 in *L. lactis* DML1 cells in the presence of 25 μM MnCl_2 in the extracellular medium. The cells were treated, and the data are represented as described for A. C, normalized fluorescence recorded 5 min after addition of 25 μM MnCl_2 , CoCl_2 , CuCl_2 , or NiCl_2 for the negative control (C-) and the clone producing Gdt1p. The cells were treated as described for A. The results are shown as the mean relative fluorescence \pm S.D. ($n = 3$). ***, $p < 0.001$ (two-way analysis of variance with Bonferroni post hoc test).

In yeast, the mechanisms by which cells regulate their Ca^{2+} and Mn^{2+} homeostasis are not fully characterized yet. This statement also applies to the Golgi where a proper maintenance of the homeostasis of these two ions is known to be essential for numerous cellular processes including glycosylation. Calcium, well described to regulate the intravesicular trafficking between the different organelles, indirectly contributes to glycosylation because the transport of proteins along the secretory pathway is affected by calcium concentration (14). Ca^{2+} is additionally described as essential for the activity and stability of enzymes involved in glycosylation (15, 16). On the other hand, the mannosyltransferases Och1p, Mnn9p, Mnn1p, Mnn2p, and Mnn5p, all implicated in *N*-glycosylation, require Mn^{2+} as cofactor (17–20). A tight regulation of both Ca^{2+} and Mn^{2+} concentrations in the Golgi is therefore essential. By now, the P-type ATPase Pmr1p is the only reported yeast Golgi transporter of Ca^{2+} and Mn^{2+} . By analyzing the contribution of Pmr1p and Gdt1p in the Golgi glycosylation process, Dulary *et al.* (13) suggested that the abundance and function of Gdt1p depends on the function of Pmr1p, thereby creating a functional link between these two Golgi proteins. Our study highly reinforces the hypothesis that Gdt1p and Pmr1p act in concert in the regulation of the yeast Ca^{2+} and Mn^{2+} homeostasis.

In this paper, we combined approaches in bacteria as a system to carry out direct transport assays and in yeast for a better understanding of the physiological role of Gdt1p. First, in bacteria, we (i) directly showed transport of Mn^{2+} by Gdt1p, (ii) highlighted a competition between manganese and calcium for transport by Gdt1p, and (iii) estimated a higher affinity of the transporter for calcium than for manganese. Second, in yeast,

we (i) demonstrated that Gdt1p is involved in resistance to high Mn^{2+} concentration and controls the total Mn^{2+} stores and (ii) showed that the activity of the Mn^{2+} -dependent Sod2p is modulated by a deletion of *GDT1*. Altogether, this study undeniably demonstrates that Gdt1p and most likely other UPF0016 members involved in essential cellular processes like glycosylation or photosynthesis are directly involved in Mn^{2+} transport. Additionally, this study places Gdt1p as a novel yeast Golgi protein which, together with Pmr1p, tightly regulates the Ca^{2+} and Mn^{2+} concentrations at the Golgi level.

Results

Gdt1p mediates Mn^{2+} influx in *L. lactis*

To determine whether Gdt1p is able to transport Mn^{2+} , we took advantage of the fact that the fluorescence emitted by Fura-2 is quenched by manganese (Fig. S1). The transport assays were carried out in the *L. lactis* DML1 strain, which was previously reported to be suitable for heterologous production of Gdt1p (4). More precisely, the fluorescence intensity was monitored over time on Fura-2-loaded *L. lactis* DML1 cells expressing *GDT1* or containing the corresponding empty plasmid. The excitation wavelength was set at 360 nm, the Fura-2 isosbestic point at which its properties are independent on the Ca^{2+} concentration (Fig. S1), to reflect only Mn^{2+} transport. Addition of MnCl_2 at a concentration of 10 μM (Fig. 1A) and 25 μM (Fig. 1B) in the extracellular medium led to a significantly more pronounced decrease of the fluorescence signal over time for the cells producing Gdt1p compared with the ones transformed with the empty plasmid. Although the slight signal

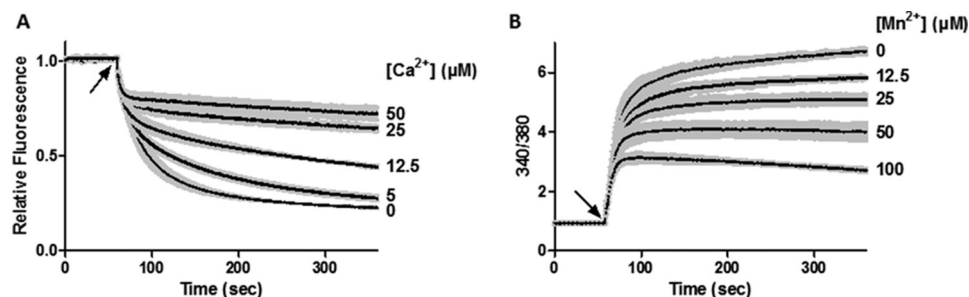


Figure 2. Ca^{2+} and Mn^{2+} influx are both promoted by Gdt1p in *L. lactis*. **A**, time-course measurements of the quenching by Mn^{2+} of the fluorescence emitted by Fura-2 in *L. lactis* DML1 cells producing Gdt1p in the presence of both MnCl_2 (fixed concentration of $25 \mu\text{M}$) and CaCl_2 (variable concentration from 0 to $50 \mu\text{M}$). Measurements were carried out with an excitation wavelength at the isosbestic point of Fura-2 (360 nm). The data are represented as the mean fluorescence normalized to the fluorescence at time 0. **B**, time-course measurements of the ratio of the fluorescence emitted at 510 nm after excitations at 340 and 380 nm (340/380) in the *L. lactis* DML1 clone in the presence of both CaCl_2 (fixed concentration of $25 \mu\text{M}$) and MnCl_2 (variable concentration from 0 to $100 \mu\text{M}$). In the two experiments, the cells were treated as described for Fig. 1A. MnCl_2 and CaCl_2 were added simultaneously after 60 s of measurement (arrow), and the curves are represented as means ($n = 3$) \pm S.D.

decrease observed for the negative control most likely reflects transport of Mn^{2+} by *L. lactis* endogenous transporters, the quenching observed to a greater extent for the clone producing Gdt1p indicates that it mediates Mn^{2+} influx in *L. lactis*. Interestingly, the Gdt1p-dependent quenching of the fluorescence emitted by Fura-2 occurred to a greater extent in the presence of $25 \mu\text{M}$ than in the presence of $10 \mu\text{M}$ manganese, thereby indicating a dose-dependent response. Similar transport measurements were carried out in the presence of $25 \mu\text{M}$ Co^{2+} , Cu^{2+} , and Ni^{2+} , all three ions being also known to quench the fluorescence emitted by Fura-2. In those cases, no significant difference in signal decrease could be observed between the negative control and the cells producing Gdt1p (Fig. 1C), suggesting that these three ions are not transported by Gdt1p and strengthening that the effect observed for manganese on Fig. 1 (A and B) is well specific to Gdt1p.

Ca^{2+} and Mn^{2+} compete for transport by Gdt1p

We previously reported that Gdt1p mediates Ca^{2+} influx when produced in *L. lactis* (4). To highlight a putative competition between calcium and manganese for Gdt1p, we carried out transport assays in the presence of a fixed MnCl_2 concentration ($25 \mu\text{M}$) and variable CaCl_2 concentrations (0– $50 \mu\text{M}$) with an excitation wavelength at the isosbestic point of the probe (360 nm) to mainly reflect Mn^{2+} transport. As shown in Fig. 2A, the higher the CaCl_2 concentration, the lesser the quenching rate, most likely reflecting Mn^{2+} influx to a lesser extent. Inversely, the transport of Ca^{2+} ($25 \mu\text{M}$) was recorded in the presence of Mn^{2+} (0– $100 \mu\text{M}$) in a ratiometric mode (excitation wavelengths of 340 and 380 nm, emission wavelength of 510 nm), this latter being routinely used to monitor Ca^{2+} transport with Fura-2. In that case, the ratio of the fluorescence emitted at 510 nm after excitations at 340 and 380 nm (340/380 ratio), mainly reflecting the intracellular free Ca^{2+} concentration, increased to a lesser extent when the MnCl_2 concentration added in the extracellular medium increases (Fig. 2B). These results reinforce the conclusion that Ca^{2+} and Mn^{2+} are both transported by Gdt1p and suggest that these two cations compete for transport by Gdt1p. Additionally, it indicates that the protein is capable of transporting the two ions in the same direction under the conditions of gradients tested in this study.

Gdt1p displays a higher affinity for Ca^{2+} than for Mn^{2+}

To determine the affinity of Gdt1p for Ca^{2+} and Mn^{2+} , we carried out *in vivo* transport measurements in *L. lactis* in the presence of various concentrations of these ions (from 2.5 to $200 \mu\text{M}$ CaCl_2 and from 5 to $500 \mu\text{M}$ MnCl_2). We then determined the initial slope of the fluorescence signal over time after addition of the cation for each concentration and plotted them according to the free substrate concentration. A Michaelis constant K_m was determined for each cation as the concentration at which half of the maximal initial slope was observed. Fig. 3A illustrates the time-course measurements at various CaCl_2 and MnCl_2 concentrations, from which the initial slopes were determined, whereas Fig. 3B shows the Michaelis–Menten curves that were fitted to the experimental data. The K_m calculated for Ca^{2+} and Mn^{2+} are respectively of 15.6 ± 2.6 and $83.2 \pm 9.8 \mu\text{M}$, suggesting that the affinity of Gdt1p toward Ca^{2+} is greater than the one toward Mn^{2+} in *L. lactis*.

Gdt1p is involved in yeast cellular manganese homeostasis

Our data suggest that Gdt1p could be a new actor involved in Mn^{2+} transport in yeast. To further assess the implication of Gdt1p in regulating yeast Mn^{2+} homeostasis, the total Mn^{2+} levels of the WT strain and of strains deleted for *GDT1*, *PMR1*, or both were evaluated using inductively coupled plasma atomic emission spectrometry (ICP–AES). The results, shown in Fig. 4A, indicate that deletion of *GDT1* led to a 3.5-fold increase of the yeast total Mn^{2+} content compared with the WT. The *pmr1Δ* strain showed a higher cellular Mn^{2+} content, with a 20-fold increase compared with the WT. Interestingly, the *gdt1Δpmr1Δ* strain displayed a higher total Mn^{2+} content than the *pmr1Δ* strain. Additionally, expression of *GDT1* from a plasmid in the *gdt1Δ* strain under the control of the endogenous promoter or of the strong TPII promoter partially or totally rescued the WT phenotype, respectively (Fig. 4B). The partial complementation by *GDT1* under the control of its endogenous promoter correlates with an intermediate level of Gdt1p production, between the WT and the *gdt1Δ* strain, whereas the full complementation using the TPII promoter results from a higher level of Gdt1p production than in the WT. Using the same methodology, we previously reported Gdt1p-dependent effects on calcium accumulation in yeast (4). As a

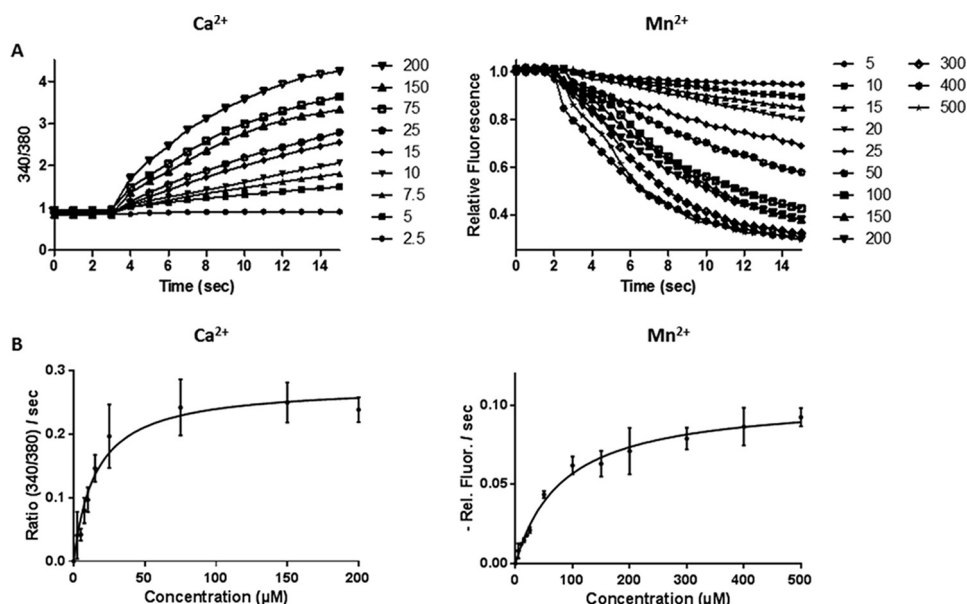


Figure 3. The affinity of Gdt1p for Ca^{2+} is higher than the one for Mn^{2+} in *L. lactis*. A, time-course measurements of the fluorescence emitted at 510 nm after excitations at 340 and 380 nm (340/380) (left panel) or of the fluorescence emitted at 510 nm after excitation at 360 nm normalized to the initial fluorescence (right panel) in *L. lactis* DML1 cells transformed with the *GDT1*-containing plasmid. The cells were treated as described for Fig. 1A. The indicated CaCl_2 (left panel) or MnCl_2 (right panel) concentration (μM) was added in the extracellular medium after 3 s of measurement. The data correspond to a representative set of three repetitions. B, values of the mean initial slopes ($n = 3$, \pm S.D.) as a function of the free Ca^{2+} or Mn^{2+} concentration (left and right panels, respectively). The data were fitted to the Michaelis–Menten equation using GraphPad Prism.

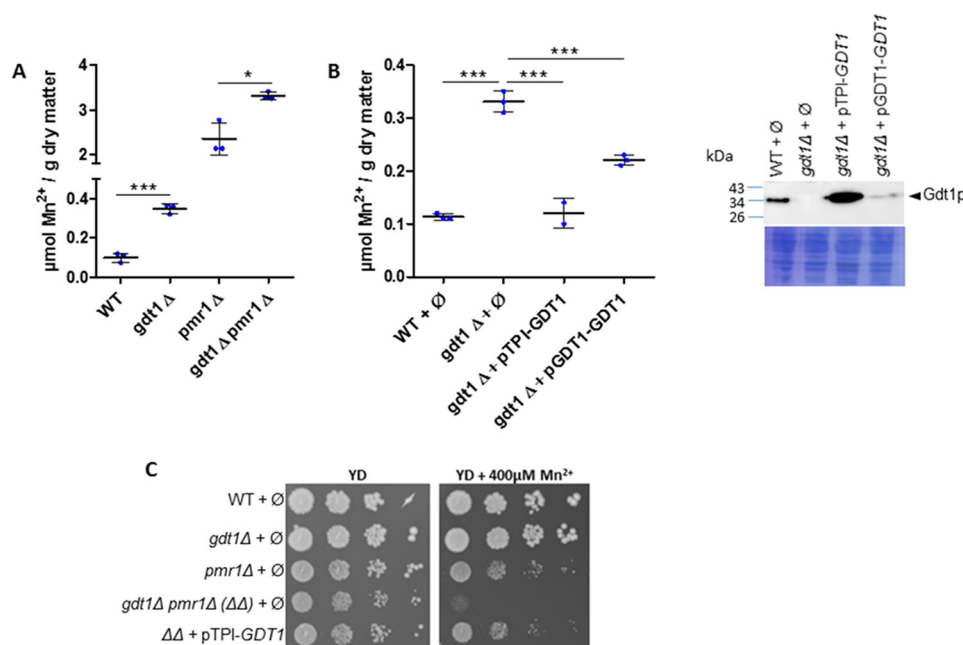


Figure 4. Gdt1p regulates the homeostasis of Mn^{2+} in yeast. A, cellular Mn^{2+} content of the WT, *gdt1Δ*, *pmr1Δ*, and *gdt1Δpmr1Δ* strains. The cells were grown in YD medium to an A_{600} of 3, and the cellular Mn^{2+} content was measured by ICP–AES. The data are shown as means \pm S.D. ($n = 3$). *, $p < 0.05$; ***, $p < 0.001$ (unpaired t tests). B, left panel, cellular Mn^{2+} content of the WT and *gdt1Δ* strains transformed with the empty plasmid (\emptyset) or the plasmid containing *GDT1* under the control of the TPI1 or of the endogenous *GDT1* promoter. The strains were grown in MD–U medium to an A_{600} of 3, and the cellular Mn^{2+} content was measured by ICP–AES. The data are shown as means \pm S.D. ($n = 3$, excepted for *gdt1Δ* + pTPI-GDT1 where $n = 2$). ***, $p < 0.001$ (one-way analysis of variance with Bonferroni post hoc test). Right panel, production of Gdt1p in the corresponding strains. Total protein extracts of cells grown to an A_{600} of 3 were analyzed by Western blotting with antibodies directed against Gdt1p. Coomassie Blue-stained polyvinylidene fluoride membranes were used as loading controls. C, growth of the WT, *gdt1Δ*, *pmr1Δ*, and *gdt1Δpmr1Δ* strains in rich solid medium supplemented or not with 400 μM Mn^{2+} . The strains transformed with the empty (\emptyset) or *GDT1*-containing pRS416 plasmid were precultured in MD–U medium to an A_{600} of 0.3. Serial 10-fold dilutions of the culture were dropped on YD solid medium containing or not 400 μM MnCl_2 . The $\Delta\Delta$ + *GDT1* strain corresponds to the *gdt1Δpmr1Δ* mutant expressing *GDT1* under the control of the constitutive TPI1 promoter. The plates were incubated 2 days at 28 $^{\circ}\text{C}$.

control, the intracellular levels of an ion not transported by Gdt1p, *i.e.* cobalt, were measured. No effect of the deletion of *GDT1* could be observed for this ion (data not shown). These

results support the conclusion that Gdt1p, in addition to Pmr1p, controls the total Ca^{2+} and Mn^{2+} content in yeast. To further evaluate the involvement of Gdt1p in Mn^{2+} tolerance,

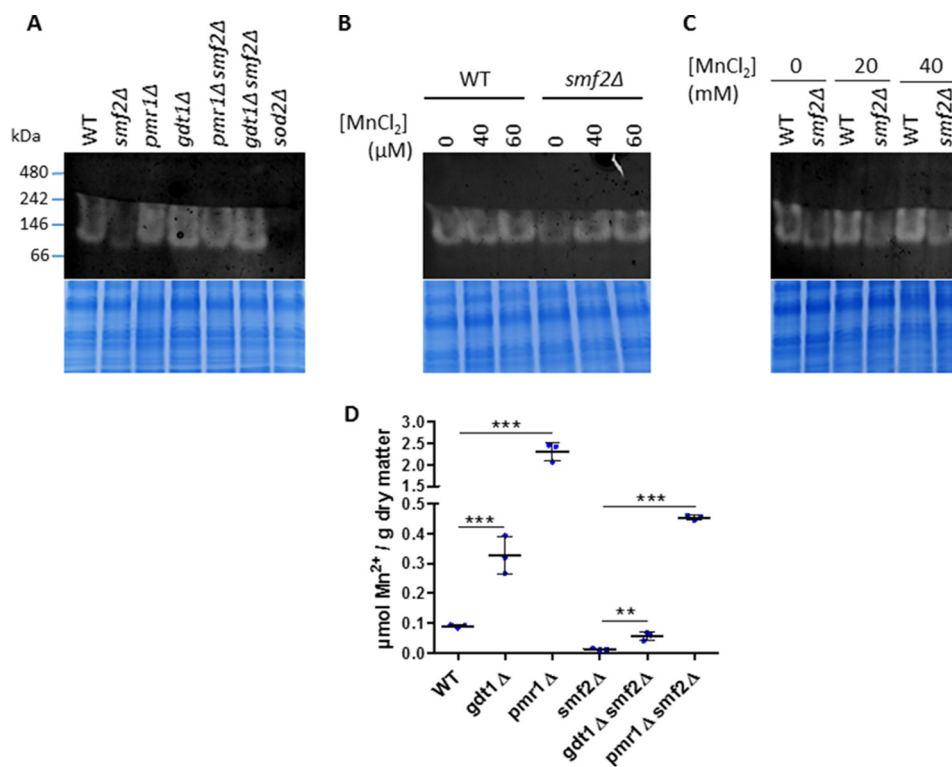


Figure 5. Effect of a *gdt1Δ* mutation on Sod2p activity and manganese accumulation in an *smf2Δ* mutant. A, activity of the Mn²⁺-dependent Sod2p in the indicated yeast strains. The seven yeast strains were grown to an A₆₀₀ of 3. Cell lysates were loaded on a native PAGE gel in which the activity of Sod2p was monitored by nitro blue tetrazolium staining. Colloidal blue-stained gels were used as loading controls. B, activity of Sod2p in the WT and *smf2Δ* strain grown in the presence of 0, 40, or 60 μM MnCl₂. C, activity of Sod2p in cell lysates containing 0, 20, or 40 mM MnCl₂. D, cellular Mn²⁺ content of the indicated strains. The cells were grown in YD medium to an A₆₀₀ of 3, and the cellular Mn²⁺ content was measured by ICP-AES. The data are shown as means ± S.D. (n = 3). **p < 0.01, ***p < 0.001 (unpaired t tests).

we monitored growth of the four strains in the presence or not of MnCl₂ in the extracellular medium. We could not detect any growth difference between the WT and the *gdt1Δ* strain when exposed to MnCl₂ concentrations of up to 10 mM, higher concentrations being lethal for the WT (data not shown). Interestingly, when exposed to 400 μM MnCl₂, a sublethal concentration for *pmr1Δ*, we identified a more pronounced growth defect for *gdt1Δpmr1Δ* than for *pmr1Δ* (Fig. 4C), thereby indicating that Gdt1p is involved in Mn²⁺ tolerance in a *pmr1Δ* background. These growth defects correlate with the fact that the highest Mn²⁺ accumulations were recorded in the *pmr1Δ* and double deletant strain. Most likely, Mn²⁺ accumulation in these strains reaches a threshold from which it becomes toxic for the cell. Similarly to what was observed for the ICP-AES analyses, expression of *GDT1* under the TPI1 promoter could complement the growth defect of the *gdt1Δpmr1Δ* in the presence of manganese. Taken together, these results show that, in complement to Pmr1p, Gdt1p plays a role in yeast cellular manganese homeostasis.

Deletion of *GDT1* restores the Sod2p activity in a *smf2Δ* background

Sod2p encodes a superoxide dismutase that localizes in the mitochondrial matrix and that catalyzes the breakdown of superoxide radicals to dioxygen and hydrogen peroxide. This enzyme requires Mn²⁺ as a cofactor (21). Luk and Culotta (22) previously reported that Smf2p, a yeast Nramp Mn²⁺ transporter found in intracellular Golgi-like vesicles (23), is required

for acquisition by Sod2p of its cofactor. They additionally reported that the Sod2p activity defect was corrected when either growing cells in the presence of manganese or generating an increased intracellular manganese concentration through deletion of *PMR1*. In this study, we evaluated whether deletion of *GDT1* could also restore the activity of Sod2p in the *smf2Δ* strain. To do so, cell lysates of the WT, *smf2Δ*, *gdt1Δ*, and *pmr1Δ* strains, as well as of the double deletants *gdt1Δsmf2Δ* and *pmr1Δsmf2Δ*, were loaded on a native polyacrylamide gel on which the Sod2p activity was monitored by staining with nitro blue tetrazolium (24, 25). As reported by Luk and Culotta (22), we observed a decreased activity of Sod2p in the *smf2Δ* strain that could be restored by deleting *PMR1* and by growing cells with 40 or 60 μM MnCl₂ (Fig. 5, A and B). The activity of Sod2p could also be restored by the addition of MnCl₂ (40 mM) to the cell lysates, thereby indicating that the decreased activity of Sod2p does not result from *in vivo* protein degradation (Fig. 5C). Luk and Culotta (22) also verified by Western blotting that deleting *SMF2* does not affect the abundance of Sod2p. Interestingly, whereas neither Gdt1p nor Pmr1p seem to be critical for delivery of manganese to Sod2p, deletion of *GDT1* in the *smf2Δ* strain led to a Sod2p activity level similar to the one of the WT. This result shows that, like *PMR1*, deletion of *GDT1* leads to a better bioavailability of Mn²⁺ for Sod2p, most likely through an increased cellular manganese content in the *smf2Δ* strain. This latter statement was verified by quantifying by ICP-AES the total manganese content of the strains for which the

Sod2p activity was monitored. By doing so, we observed a 5- and 35-fold increase of the cellular manganese content, respectively, in the *gdt1Δsmf2Δ* and the *pmr1Δsmf2Δ* strain compared with the *smf2Δ* strain (Fig. 5D). Because the effects of deleting *GDT1* or *PMR1* are observed in the same direction both in terms of cellular manganese content and restoration of the Sod2p activity in an *smf2Δ* strain, this suggests that the direction of transport of Mn^{2+} is identical for these two proteins, i.e. from the cytosol to the Golgi.

Discussion

Several recent studies suggested an implication of UPF0016 members in manganese homeostasis. However, no direct evidence of manganese transport by a UPF0016 member has been reported up to now. In this study, using transport assays in the bacterium *L. lactis*, we demonstrated that the yeast Gdt1p mediates Mn^{2+} transport across membranes. The role of Gdt1p in regulating manganese homeostasis was further shown in yeast. As supported by the competition assays carried out in this study, this Mn^{2+} transport activity adds up to the Gdt1p Ca^{2+} transport activity, which was previously demonstrated with a similar *in vivo* assay in *L. lactis* (4). Because Gdt1p was also reported to be involved in pH regulation (4, 26), we hypothesize that this protein acts as a Ca^{2+} - Mn^{2+} /H⁺ antiporter. However, stronger evidence for proton transport by Gdt1p still needs to be obtained.

This study therefore places Gdt1p as a novel protein implicated in both calcium and manganese yeast Golgi and/or cytosol homeostasis. The well characterized Golgi P-type ATPase Pmr1p is known to also regulate the homeostasis of these two ions at the Golgi level. Gdt1p and Pmr1p therefore most likely act in concert to correctly balance the concentration of Ca^{2+} and Mn^{2+} within the Golgi and the cytosol. However, our results suggest that the contribution of Gdt1p in regulating the yeast Mn^{2+} homeostasis is lower than the one of Pmr1p in the conditions tested in this study. Indeed, compared with a *pmr1Δ* strain, the absence of solely *GDT1* does not cause any growth reduction in the presence of 400 μM Mn^{2+} and leads to a less pronounced increase of the total Mn^{2+} content. Additionally, a comparison of the affinity of Gdt1p for calcium and manganese cations estimated via the *in vivo* transport assays (K_m of respectively 15.6 and 83.2 μM) to the ones reported in the literature for Pmr1p (K_m of 0.1 μM for Ca^{2+} (27) and of 0.02 μM for Mn^{2+} (28)) highly suggests a higher affinity of Pmr1p for these two ions. To our knowledge, no data regarding the affinity of other yeast manganese transporters are currently available. However, we noticed that the affinity of Gdt1p for calcium is similar to the one reported for the vacuolar Ca^{2+} /H⁺ antiporter Vcx1p (K_m of 25 μM (29)).

Although it is well known that Pmr1p transports Ca^{2+} and Mn^{2+} from the cytosol to the Golgi, the question of the direction of transport of these two ions by Gdt1p remains open. Provided that Gdt1p functions as a Ca^{2+} - Mn^{2+} /H⁺ antiporter, transport of Ca^{2+} and Mn^{2+} from the cytosol to the Golgi or in the opposite direction could both be considered. Gdt1p could also work reversely, depending on the neighboring gradients, or Ca^{2+} and Mn^{2+} could be transported in opposite directions. In this study, the effects of the absence of Gdt1p or Pmr1p on the

cellular manganese content and on Sod2p activity in the *smf2Δ* strain were both observed in the same direction, thereby suggesting manganese transport in the same direction for these two proteins, from the cytosol to the Golgi. The increased cellular manganese content observed in the *gdt1Δ* and *pmr1Δ* strains would hence result from the inability to send Mn^{2+} to the Golgi lumen for further exit from the cell through secretory pathway vesicles. On the other hand, suppression of the Sod2p activity defects by deleting *GDT1* or *PMR1* in an *smf2Δ* background would result from an increased manganese concentration in the cytosol, from which it can be sent to the mitochondrial matrix. More generally, according to this hypothesis of direction of transport, Gdt1p would provide the Golgi with calcium and manganese cations and/or detoxify the cytosol in case of excess of these cations, in complement to Pmr1p. Based on the evaluation of the glycosylation process efficiency as an indicator of the Golgi Mn^{2+} concentration, the same direction of manganese transport was suggested by Dulary *et al.* (13). The entry of Mn^{2+} in the Golgi would then be controlled by an ATPase, Pmr1p, and by a secondary transporter, Gdt1p. Although the ATPases are generally associated with a high affinity but a low capacity of transport, the secondary transporters usually show a low affinity and a high capacity of transport. By comparing the K_m values obtained for Gdt1p to the ones reported in the literature for Pmr1p, we confirmed this trend in terms of affinity. The fact of having two transporters with different transport characteristics most likely enables a tight regulation of Mn^{2+} homeostasis at the Golgi level in a wide range of stresses and concentration gradients, thanks to a complementary action of the two transporters.

In either transport direction, Gdt1p is involved in the crucial regulation of both calcium and manganese, notably in the Golgi where part of the glycosylation process takes place. Because of the implication of the UPF0016 members, including the human TMEM165, in glycosylation, it is of specific interest to examine the link between Gdt1p and glycosylation in the light of its novel manganese transport activity. Indeed, Colinet *et al.* (4) previously reported Gdt1p-dependent glycosylation defects in yeast. More recently, Dulary *et al.* (13) analyzed the structural glycosylation abnormalities observed in a *gdt1Δ* strain cultured in the presence of a high external calcium concentration and reported defects in the α -1,3- and α -1,2-mannose branchings that correspond to deficiencies in late Golgi glycosyltransferases like Mnn2, Mnn5, and Mnn1. Interestingly, these three mannosyltransferases all require Mn^{2+} as a cofactor (18, 20). The Gdt1p-dependent glycosylation defects could be explained by the fact that, in the presence of high external Ca^{2+} concentration, Pmr1p would mainly transport Ca^{2+} , thereby resulting in an excess of Ca^{2+} that could compete with Mn^{2+} for the cofactor binding site of the mannosyltransferases. This illustrates the importance of ensuring tight regulation of this Ca^{2+} /Mn²⁺ ratio for proper protein maturation.

In conclusion, we propose that Gdt1p regulates the calcium and manganese yeast cytosolic and Golgi homeostasis in concert with Pmr1p, through its ability to transport both Ca^{2+} and Mn^{2+} cations. Our results suggest a direction of manganese transport from the cytosol to the Golgi. Confirming the direction of transport of Ca^{2+} and Mn^{2+} by Gdt1p would definitely

Table 1

Yeast strains used in this study

Strain	Description	Source
BY4741	<i>Mata his3Δ1 leu2Δ0 met15Δ0 ura3Δ0</i>	Euroscarf
BY4741 <i>gdt1Δ</i>	<i>Mata his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 gdt1::KanMX4</i>	Euroscarf
BY4741 <i>pmr1Δ</i>	<i>Mata his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 pmr1::KanMX4</i>	Euroscarf
BY4741 <i>smf2Δ</i>	<i>Mata his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 smf2::KanMX4</i>	Euroscarf
BY4742 <i>sod2Δ</i>	<i>MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 sod2::kanMX4</i>	Euroscarf
BY <i>gdt1Δ pmr1Δ</i>	<i>Mata his3Δ1 leu2Δ0 ura3Δ0 met15Δ0 gdt1::KanMX4 pmr1::KanMX4</i>	Ref. 3
BY <i>smf2Δ pmr1Δ</i>	<i>Mata his3Δ1 leu2Δ0 ura3Δ0 smf2::KanMX4 pmr1::KanMX4</i>	This study
BY <i>smf2Δ gdt1Δ</i>	<i>Mata his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 smf2::KanMX4 gdt1::KanMX4</i>	This study

constitute the next step toward a better understanding of its physiological role. Additionally, the results show for the first time direct Mn^{2+} transport by a UPF0016 member, thereby opening new ways to fully unravel the exact function of this protein family involved in essential cellular processes like photosynthesis in plants and cyanobacteria, as well as glycosylation in yeast and human.

Experimental procedures

Strains and culture media

The *S. cerevisiae* strains used in this study are listed in Table 1. Nontransformed yeast cells were routinely cultured at 28 °C in YD medium (2% yeast extract KAT, 2% glucose) under agitation. The cells transformed with plasmids were grown in MD minimal medium (0.7% yeast nitrogen base without amino acids (Difco), 2% glucose, supplemented with all amino acids except those used as a selection marker for plasmid maintenance). Solid media were produced by addition of 2% agar to the mixture. The *L. lactis* DML1 strain was kindly provided by B. Poolman (Groningen, Holland). *L. lactis* cells were grown in M17 broth according to Terzaghi (Merck Millipore) supplemented with 1% glucose at 28 °C. The cells transformed with the pNZ8048 plasmid were grown in the presence of 10 μg/ml chloramphenicol. Expression of genes under the control of the pNisA promoter was induced by 2.5 μg/liter nisin at the log phase ($A_{600} = \sim 0.4-0.5$).

In vivo transport measurements

The *in vivo* transport measurements were carried out using the fluorescent dye Fura-2/AM according to the method previously described by Colinet *et al.* (4) with slight modifications. Briefly, *L. lactis* DML1 cells transformed with the empty or *GDT1*-containing pNZ8048 plasmid were grown in M17 broth. At an A_{600} of 0.5, induction was initiated by adding 2.5 μg/liter nisin in the extracellular medium. After a postinduction time of 2 h, the cells were harvested ($3,000 \times g$ for 7 min) and washed twice with the washing buffer (50 mM Tris-HCl, pH 7.4, 100 mM KCl, 1 mM $MgCl_2$). The washed cells were resuspended in the same buffer supplemented with 0.2 mM EDTA (pH 8) and incubated for 10 min. The harvested cells were then incubated with the washing buffer supplemented with 10 μM Fura-2/AM and 1.7 mM probenecid for 2 h at 28 °C under agitation. The cells were subsequently washed twice in the presence of 1 mM EGTA, and the final pellet was resuspended in 10 ml of the washing buffer supplemented with 1.7 mM probenecid. Prior to measurements, this latter solution was treated with calcium sponges (1,2-bis(*o*-aminophenoxy)ethane-*N,N,N',N'*-tetraacetic acid (BAPTA) chelator coupled to a polymer matrix;

Invitrogen). The signal was recorded either with a single excitation wavelength of 360 nm (quenching measurements) or with two excitation wavelengths of 340 and 380 nm (ratiometric measurements), the emission wavelength being set at 510 nm. $MnCl_2$ or $CaCl_2$ were added in the extracellular medium at the indicated concentrations. The data were recorded using a JASCO FP8500 fluorometer controlled by the Spectra Manager software.

ICP–AES analyses

For determination of the total Mn^{2+} content of yeast mutant strains, the cells were grown in YD or minimal medium to an A_{600} of 3. The cells were then collected by vacuum filtration using membrane filters (Millipore; 0.45-μm pore size) and washed twice with 2 ml of 1 mM EGTA (pH 8) and twice with 2 ml of H_2O . The cells were collected in heat-resistant beakers in 10 ml of H_2O and dried at 95 °C overnight and then in a dessicator for 24 h. The dry matter was mineralized by heating at 500 °C overnight. The ashed sample was subsequently dissolved in 10 ml 6.5% HNO_3 for analysis on an ICAP 6500 spectrometer (Thermo Scientific).

Western blotting

For Western blotting, 40 μg of proteins were separated on SDS-PAGE gels, and Western blotting was carried out as previously described (3). The primary rabbit antibodies against Gdt1p were previously produced in our lab (3). Horseradish peroxidase-coupled anti-rabbit IgG antibodies were purchased from IMEX.

Yeast drop tests

For drop tests, yeast cells were precultured overnight in 5 ml of minimal dextrose medium without uracil (MD–U). Each culture was then adjusted to an A_{600} of 0.3. Four μl of the adjusted culture and of successive 10-fold dilutions were spotted on YD solid medium with or without 400 μM $MnCl_2$. The plates were incubated for 2 days at 28 °C.

Monitoring of Sod2p activity

To monitor Sod2p activity, the cells were grown in YD medium to an A_{600} of 3 and washed before homogenization by glass-bead agitation in lysis buffer (10 mM sodium phosphate buffer, pH 7.8, 5 mM EDTA, 5 mM EGTA, 50 mM NaCl, 0.1% Triton X-100, 100 μg/ml PMSF, 4 μg/ml leupeptin, aprotinin, antipain, pepstatin, and chymostatin) (22). Proteins (200 μg) were loaded on a native PAGE gel, and migration was carried out at 100 V for 4 h. After migration, the gel was immersed in a 1 mg/ml nitro blue tetrazolium solution for 15 min, followed by an incubation in a solution containing 100 mM potassium phosphate buffer, pH 7.8, 28 mM tetramethylethylenediamine (TEMED), 2.8×10^{-2} mM riboflavin, and 5 mM KCN for

15 min. The gel was finally exposed to light for about 30 min (24, 25). Colloidal blue gels were carried out in parallel as loading controls.

Author contributions—L. T., A. D., P. S., and P. M. conceptualization; L. T., A. D., and O. S. data curation; L. T. and A. D. formal analysis; L. T. validation; L. T. investigation; L. T. and A. D. methodology; L. T. writing-original draft; J. S. visualization; P. M. supervision; P. M. funding acquisition; P. M. writing-review and editing; L. T., A. D., and O. S. data acquisition.

Acknowledgments—We dedicate this publication to the memory of André Goffeau. We thank B. Poolman for providing the DML1 strain, A. Iserentant for technical assistance with the ICP-AES measurements in yeast, and M. Boutry for helpful suggestions and critical reading of the manuscript.

References

- Demaegd, D., Colinet, A. S., Deschamps, A., and Morsomme, P. (2014) Molecular evolution of a novel family of putative calcium transporters. *PLoS One* **9**, e100851 [CrossRef Medline](#)
- Foulquier, F., Amyere, M., Jaeken, J., Zeevaert, R., Schollen, E., Race, V., Bammens, R., Morelle, W., Rosnoble, C., Legrand, D., Demaeagd, D., Buist, N., Cheillan, D., Guffon, N., Morsomme, P., et al. (2012) TMEM165 deficiency causes a congenital disorder of glycosylation. *Am. J. Hum. Genet.* **91**, 15–26 [CrossRef Medline](#)
- Demaegd, D., Foulquier, F., Colinet, A. S., Gremillon, L., Legrand, D., Mariot, P., Peiter, E., Van Schaftingen, E., Matthijs, G., and Morsomme, P. (2013) Newly characterized Golgi-localized family of proteins is involved in calcium and pH homeostasis in yeast and human cells. *Proc. Natl. Acad. Sci. U.S.A.* **110**, 6859–6864 [CrossRef Medline](#)
- Colinet, A. S., Sengottaiyan, P., Deschamps, A., Colsole, M. L., Thines, L., Demaeagd, D., Duchêne, M. C., Foulquier, F., Hols, P., and Morsomme, P. (2016) Yeast Gdt1 is a Golgi-localized calcium transporter required for stress-induced calcium signaling and protein glycosylation. *Sci. Reports* **6**, 24282 [CrossRef Medline](#)
- Colinet, A. S., Thines, L., Deschamps, A., Flémal, G., Demaeagd, D., and Morsomme, P. (2017) Acidic and uncharged polar residues in the consensus motifs of the yeast Ca^{2+} transporter Gdt1p are required for calcium transport. *Cell. Microbiol.* **19**, 12729 [CrossRef Medline](#)
- Wang, C., Xu, W., Jin, H., Zhang, T., Lai, J., Zhou, X., Zhang, S., Liu, S., Duan, X., Wang, H., Peng, C., and Yang, C. (2016) A putative chloroplast-localized $\text{Ca}^{2+}/\text{H}^{+}$ antiporter CCHA1 is involved in calcium and pH homeostasis and required for PSII function in *Arabidopsis*. *Mol. Plant* **9**, 1183–1196 [CrossRef Medline](#)
- Schneider, A., Steinberger, I., Herdean, A., Gandini, C., Eisenhut, M., Kurz, S., Morper, A., Hoecker, N., Rühle, T., Labs, M., Flüge, U. I., Geimer, S., Schmidt, S. B., Husted, S., Weber, A. P., et al. (2016) The evolutionarily conserved protein PHOTOSYNTHESIS AFFECTED MUTANT71 is required for efficient manganese uptake at the thylakoid membrane in *Arabidopsis*. *Plant Cell* **28**, 892–910 [Medline](#)
- Brandenburg, F., Schoffman, H., Kurz, S., Krämer, U., Keren, N., Weber, A. P., and Eisenhut, M. (2017) The *Synechocystis* manganese exporter mnx is essential for manganese homeostasis in cyanobacteria. *Plant Physiol.* **173**, 1798–1810 [CrossRef Medline](#)
- Fisher, C. R., Wyckoff, E. E., Peng, E. D., and Payne, S. M. (2016) Identification and characterization of a putative manganese export protein in *Vibrio cholerae*. *J. Bacteriol.* **198**, 2810–2817 [CrossRef Medline](#)
- Gandini, C., Schmidt, S. B., Husted, S., Schneider, A., and Leister, D. (2017) The transporter SynPAM71 is located in the plasma membrane and thylakoids, and mediates manganese tolerance in *Synechocystis* PCC6803. *New Phytol.* **215**, 256–268 [CrossRef Medline](#)
- Potelle, S., Morelle, W., Dulary, E., Duvet, S., Vicogne, D., Spriet, C., Krzewinski-Recchi, M. A., Morsomme, P., Jaeken, J., Matthijs, G., De Bettignies, G., and Foulquier, F. (2016) Glycosylation abnormalities in Gdt1p/TMEM165 deficient cells result from a defect in Golgi manganese homeostasis. *Hum. Mol. Genet.* **25**, 1489–1500 [CrossRef Medline](#)
- Potelle, S., Dulary, E., Climer, L., Duvet, S., Morelle, W., Vicogne, D., Lebretonchel, E., Houdou, M., Spriet, C., Krzewinski-Recchi, M. A., Peanne, R., Klein, A., de Bettignies, G., Morsomme, P., Matthijs, G., et al. (2017) Manganese-induced turnover of TMEM165. *Biochem. J.* **474**, 1481–1493 [CrossRef Medline](#)
- Dulary, E., Yu, S.-Y., Houdou, M., de Bettignies, G., Decool, V., Potelle, S., Duvet, S., Krzewinski-Recchi, M.-A., Garat, A., Matthijs, G., Guerardel, Y., and Foulquier, F. (2018) Investigating the function of Gdt1p in yeast Golgi glycosylation. *Biochim. Biophys. Acta* **1862**, 394–402 [Medline](#)
- Chen, J. L., Ahluwalia, J. P., and Stamnes, M. (2002) Selective effects of calcium chelators on anterograde and retrograde protein transport in the cell. *J. Biol. Chem.* **277**, 35682–35687 [CrossRef Medline](#)
- Antebi, A., and Fink, G. R. (1992) The yeast Ca^{2+} -ATPase homologue, PMR1, is required for normal Golgi function and localizes in a novel Golgi-like distribution. *Mol. Biol. Cell* **3**, 633–654 [CrossRef Medline](#)
- Vallée, F., Lipari, F., Yip, P., Sleno, B., Herscovics, A., and Howell, P. L. (2000) Crystal structure of a class I α 1,2-mannosidase involved in N-glycan processing and endoplasmic reticulum quality control. *EMBO J.* **19**, 581–588 [CrossRef Medline](#)
- Rayner, J. C., and Munro, S. (1998) Identification of the MNN2 and MNN5 mannosyltransferases required for forming and extending the mannose branches of the outer chain mannans of *Saccharomyces cerevisiae*. *J. Biol. Chem.* **273**, 26836–26843 [CrossRef Medline](#)
- Romero, P. A., and Herscovics, A. (1989) Glycoprotein biosynthesis in *Saccharomyces cerevisiae*: characterization of α -1,6-mannosyltransferase which initiates outer chain formation. *J. Biol. Chem.* **264**, 1946–1950 [Medline](#)
- Striebeck, A., Robinson, D. A., Schüttelkopf, A. W., and van Aalten, D. M. (2013) Yeast Mnn9 is both a priming glycosyltransferase and an allosteric activator of mannan biosynthesis. *Open Biol.* **3**, 130022, [CrossRef Medline](#)
- Wiggins, C. A., and Munro, S. (1998) Activity of the yeast MNN1 α -1,3-mannosyltransferase requires a motif conserved in many other families of glycosyltransferases. *Proc. Natl. Acad. Sci. U.S.A.* **95**, 7945–7950 [CrossRef Medline](#)
- van Loon, A. P., Pesold-Hurt, B., and Schatz, G. (1986) A yeast mutant lacking mitochondrial manganese-superoxide dismutase is hypersensitive to oxygen. *Proc. Natl. Acad. Sci. U.S.A.* **83**, 3820–3824 [CrossRef Medline](#)
- Luk, E. E., and Culotta, V. C. (2001) Manganese superoxide dismutase in *Saccharomyces cerevisiae* acquires its metal co-factor through a pathway involving the Nramp metal transporter, Smf2p. *J. Biol. Chem.* **276**, 47556–47562 [CrossRef Medline](#)
- Portnoy, M. E., Liu, X. F., and Culotta, V. C. (2000) *Saccharomyces cerevisiae* expresses three functionally distinct homologues of the nramp family of metal transporters. *Mol. Cell. Biol.* **20**, 7893–7902 [CrossRef Medline](#)
- Page, M. M., Salway, K. D., Ip, Y. K., Chew, S. F., Warren, S. A., Ballantyne, J. S., and Stuart, J. A. (2010) Upregulation of intracellular antioxidant enzymes in brain and heart during estivation in the African lungfish *Protopterus dolloi*. *J. Comp. Physiol. B* **180**, 361–369 [CrossRef Medline](#)
- Flohé, L., and Otting, F. (1984) Superoxide dismutase assays. *Methods Enzymol.* **105**, 93–104 [CrossRef Medline](#)
- Snyder, N. A., Stefan, C. P., Soroudi, C. T., Kim, A., Evangelista, C., and Cunningham, K. W. (2017) H^{+} and Pi byproducts of glycosylation affect Ca^{2+} homeostasis and are retrieved from the Golgi complex by homologs of TMEM165 and XPR1. *G3 (Bethesda)* **7**, 3913–3924 [CrossRef Medline](#)
- Sorin, A., Rosas, G., and Rao, R. (1997) PMR1, a Ca^{2+} -ATPase in yeast Golgi, has properties distinct from sarco/endoplasmic reticulum and plasma membrane calcium pumps. *J. Biol. Chem.* **272**, 9895–9901 [CrossRef Medline](#)
- Mandal, D., Woolf, T. B., and Rao, R. (2000) Manganese selectivity of pmr1, the yeast secretory pathway ion pump, is defined by residue gln783 in transmembrane segment 6: Residue Asp⁷⁷⁸ is essential for cation transport. *J. Biol. Chem.* **275**, 23933–23938 [CrossRef Medline](#)
- Ohsumi, Y., and Anraku, Y. (1983) Calcium transport driven by a proton motive force in vacuolar membrane vesicles of *Saccharomyces cerevisiae*. *J. Biol. Chem.* **258**, 5614–5617 [Medline](#)

The yeast protein Gdt1p transports Mn²⁺ ions and thereby regulates manganese homeostasis in the Golgi

Louise Thines, Antoine Deschamps, Palanivelu Sengottaiyan, Oksana Savel, Jiri Stribny and Pierre Morsomme

J. Biol. Chem. 2018, 293:8048-8055.

doi: 10.1074/jbc.RA118.002324 originally published online April 9, 2018

Access the most updated version of this article at doi: [10.1074/jbc.RA118.002324](https://doi.org/10.1074/jbc.RA118.002324)

Alerts:

- [When this article is cited](#)
- [When a correction for this article is posted](#)

[Click here](#) to choose from all of JBC's e-mail alerts

This article cites 29 references, 19 of which can be accessed free at <http://www.jbc.org/content/293/21/8048.full.html#ref-list-1>