

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

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ABSTRACT

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.

Introduction

The uncharacterized protein family 0016 (UPF0016) gathers highly conserved membrane proteins which are widely distributed among kingdoms and defined by the presence of one or two copies of the E-x-G-D-(KR)-(TS) motif (1). It was previously reported that specific mutations in the human UPF0016 member transmembrane protein 165 (TMEM165) cause congenital disorders of glycosylation (CDGs) (2). As the exact function of the UPF0016 members is not deciphered yet, the causal link between a mutated TMEM165 and CDGs 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 E-x-G-D-(KR)-(TS) found in Gdt1p were additionally demonstrated to be essential for proper Ca^{2+} tolerance and Ca^{2+} response to salt stress (5).

Besides 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 due to 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, while 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.

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 intra-vesicular trafficking between the different organelles, indirectly contributes to glycosylation as 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 is dependent 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 (Figure 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 (Figure S1), in order to reflect only Mn^{2+} transport. Addition of $MnCl_2$ at a concentration of 10 μM (Figure 1A) and 25 μM (Figure 2B) in the extracellular medium led to a significantly more pronounced decrease of the fluorescence signal over time for the cells producing Gdt1p compared to the ones transformed with the empty plasmid. While the slight signal 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 μM than of 10 μM of manganese, thereby indicating a dose-dependent response. Similar transport measurements were carried out in the presence of 25 μM 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 (Figure 1C), suggesting that these three ions are not transported by Gdt1p and strengthening that the effect observed for manganese on Figures 1A and 1B 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 μM) and

variable $CaCl_2$ concentrations (0 to 50 μM) with an excitation wavelength at the isosbestic point of the probe (360 nm) to mainly reflect Mn^{2+} transport. As shown in Figure 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 μM) was recorded in the presence of Mn^{2+} (0 to 100 μM) 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 (Figure 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+}

In order 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 μM $CaCl_2$ and from 5 to 500 μM $MnCl_2$). 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. Figure 3A illustrates the time-course measurements at various $CaCl_2$ and $MnCl_2$ concentrations, from which the initial slopes were determined, while Figure 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 ± 9.8 μM , suggesting that the affinity of Gdt1p towards Ca^{2+} is greater than the one towards 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 wild-type 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 Figure 4A, indicate that deletion of *GDT1* led to a 3.5-fold increase of the yeast total Mn^{2+} content compared to the wild-type. The *pmr1Δ* strain showed a higher cellular Mn^{2+} content, with a 20-fold increase compared to the wild-type. 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 TPI1 promoter partially or totally rescued the wild-type phenotype, respectively (Figure 4B). The partial complementation by *GDT1* under the control of its endogenous promoter correlates with an intermediate level of Gdt1p production, between the wild-type and the *gdt1Δ* strain, while the full complementation using the TPI1 promoter results from a higher level of Gdt1p production than in the wild-type. Using the same methodology, we previously reported Gdt1p-dependant effects on calcium accumulation in yeast (4). As a 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, we monitored growth of the four strains in the presence or not of $MnCl_2$ in the extracellular medium. We could not detect any growth difference between the wild-type and the *gdt1Δ* strain when exposed to $MnCl_2$ concentrations of up to 10 mM, higher concentrations being lethal for the wild-type (data not shown). Interestingly, when exposed to 400 μM $MnCl_2$, a sub-lethal concentration for *pmr1Δ*, we identified a more pronounced growth defect for *gdt1Δpmr1Δ* than for *pmr1Δ* (Figure 4C), thereby indicating that Gdt1p is involved in Mn^{2+} tolerance in a *pmr1Δ* background. These growth defects correlate with the fact that the highest Mn^{2+} accumulations were recorded in the *pmr1Δ* and double deletant strain. Most likely, Mn^{2+} 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 Mn^{2+} as a cofactor (21). Luk and Culotta (22) previously reported that Smf2p, a yeast Nramp Mn^{2+} 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 wild-type, *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 $MnCl_2$ (Figures 5A and 5B). The activity of Sod2p could also be restored by addition of $MnCl_2$ (40 mM) to the cell lysates, thereby indicating that the decreased activity of Sod2p does not result from *in vivo* protein degradation (Figure 5C). Luk and Culotta (22) also verified by Western blotting that deleting *SMF2* does not affect the abundance of Sod2p. Interestingly, while 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 wild-type. This result shows that, like *PMR1*, deletion of *GDT1* leads to a better bioavailability of Mn^{2+} 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-fold and 35-fold increase of the cellular manganese content respectively in the *gdt1Δsmf2Δ* and the *pmr1Δsmf2Δ* strain compared to the *smf2Δ* strain (Figure 5D). As 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). Since 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 in order 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 to 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 μM 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 is currently available. We however 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)).

While 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. While 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. Due to 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 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+}/Mn^{2+} 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 constitute the next step towards 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 *Saccharomyces cerevisiae* strains used in this study are listed in Table 1. Non-transformed yeast cells were routinely cultured at 28 °C in YD medium (2% yeast extract KAT, 2% glucose) under agitation. 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 *Lactococcus lactis* DML1 strain was kindly provided by B. Poolman (Groningen, Holland). *L. lactis* cells were grown in M17 broth acc. to Terzaghi (Merck Millipore) supplemented with 1% glucose at 28°C. 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/l nisin at the log phase (OD_{600} 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 OD_{600} of 0.5, induction was initiated by adding 2.5 µg/l nisin in the extracellular medium. After a post-induction time of 2 h, cells were harvested (3,000 g - 7 min) and washed twice with the washing buffer (50 mM Tris-HCl, pH 7.4, 100 mM KCl, 1 mM $MgCl_2$). 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. 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 (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. 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, cells were grown in YD or minimal medium to an OD_{600} of 3. 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 H_2O . Cells were collected in heat-resistant beakers in 10 ml 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 pre-cultured overnight in 5 ml minimal dextrose medium without uracil (MD-U). Each culture was then adjusted to an OD_{600} of 0.3. Four μl of

the adjusted culture and of successive ten-fold dilutions were spotted on YD solid medium with or without 400 μM MnCl_2 . Plates were incubated for 2 days at 28°C.

Monitoring of Sod2p activity

To monitor Sod2p activity, cells were grown in YD medium to an OD_{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 $\mu\text{g}/\text{ml}$ PMSF, 4 $\mu\text{g}/\text{ml}$ 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 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.

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Author contributions: L.T., A.D., P.S., and P.M. designed the experiments; L.T., A.D., O.S., and J.S. performed research; L.T., A.D., and P.M. analyzed data; and L.T. and P.M. wrote the paper.

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FOOTNOTES

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The abbreviations used are: TMEM165, transmembrane protein 165; UPF0016, uncharacterized protein family 0016; CDG, congenital disorder of glycosylation; IPC-AES, inductively coupled plasma atomic emission spectrometry.

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::KanMX</i>	Euroscarf
BY4742 <i>sod2Δ</i>	<i>MATa his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 sod2::kanMX4</i>	Euroscarf
BY <i>gdt1Δ pmr1Δ</i>	<i>Mata his3Δ1 leu2Δ0 ura3Δ0 met15Δ0 gdt1::KanMX4 pmr1::KanMX4</i>	(3)
BY <i>smf2Δ pmr1Δ</i>	<i>Mata his3Δ1 leu2Δ0 ura3Δ0 smf2::KanMX pmr1::KanMX</i>	This study
BY <i>smf2Δ gdt1Δ</i>	<i>Mata his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 smf2::KanMX gdt1::KanMX</i>	This study

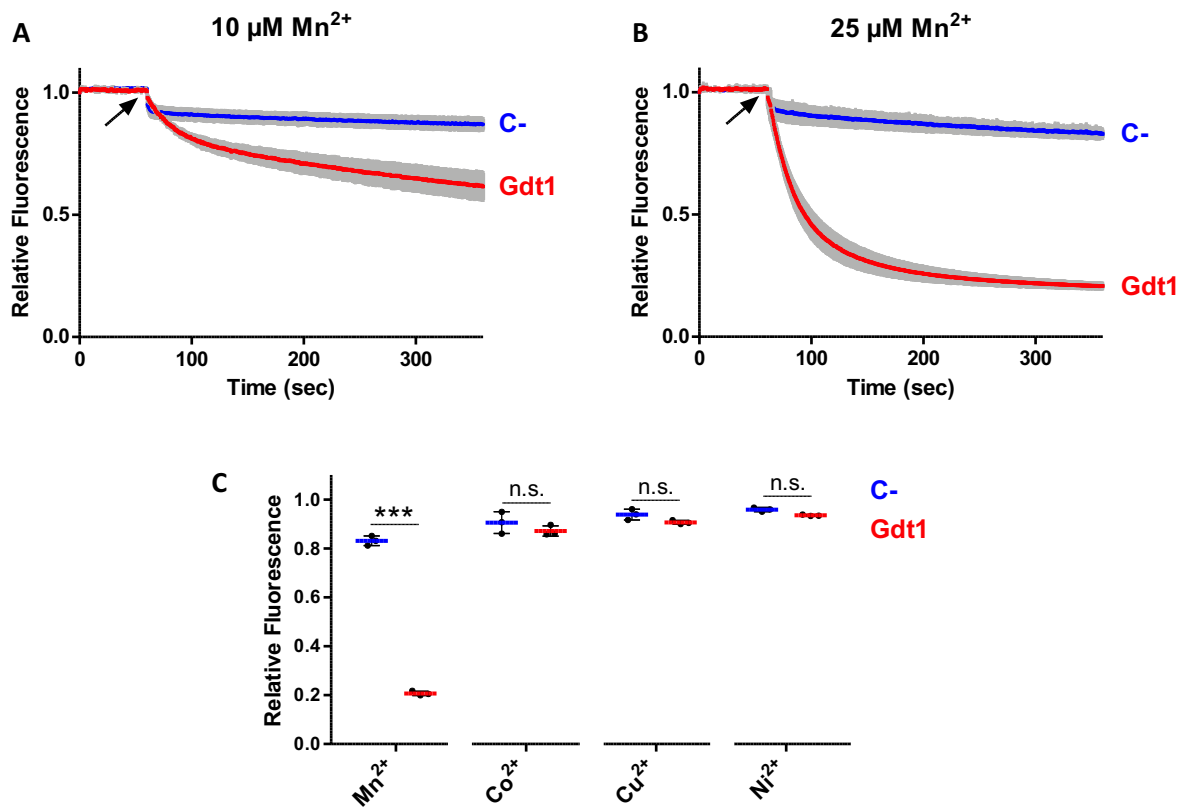


Figure 1. Gdt1p promotes Mn²⁺ influx in *L. lactis*. (A) Time-course measurement of the quenching by Mn²⁺ of the fluorescence emitted by Fura-2 in *L. lactis* DML1 cells transformed with the empty (C-) or *GDT1*-containing plasmid. Cells were grown to an OD₆₀₀ of 0.5 at which expression of *GDT1* was initiated by addition of 2.5 μg/l nisin. After a 2 h post-induction time, 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 MnCl₂ were added in the extracellular medium after 60 s of measurement (indicated by the arrow). Data are represented as the mean fluorescence normalized to the fluorescence at time 0 (n=3, ± SD). (B) Time-course measurement of the quenching by Mn²⁺ of the fluorescence emitted by Fura-2 in *L. lactis* DML1 cells in the presence of 25 μM MnCl₂ in the extracellular medium. Cells were treated and data are represented as described for (A). (C) Normalized fluorescence recorded 5 min after addition of 25 μM CoCl₂, CuCl₂, or NiCl₂ for the negative control (C-) and the clone producing Gdt1p. Cells were treated as described for (A). Results are shown as the mean relative fluorescence ± SD (n=3). ***P<0.001 (two-way ANOVA with Bonferroni post-hoc test).

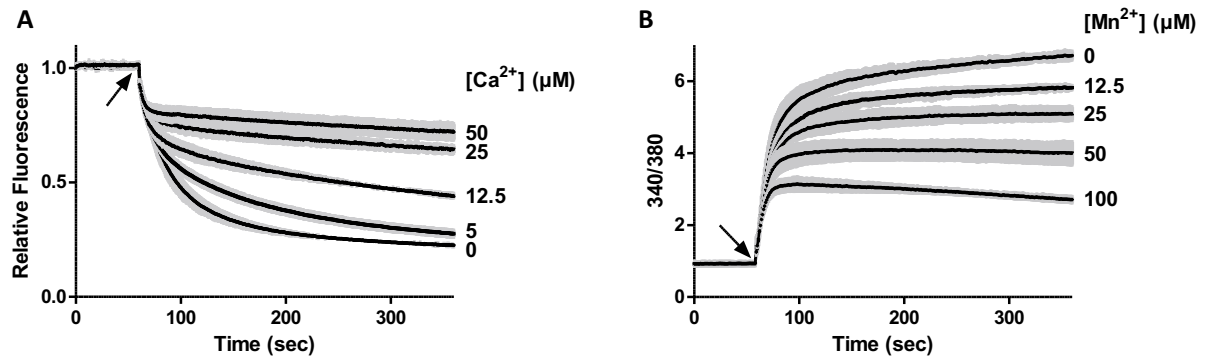


Figure 2. Ca^{2+} and Mn^{2+} influx are both promoted by Gdt1p in *L. lactis*. (A) Time-course measurements of the quenching by Mn^{2+} of the fluorescence emitted by Fura-2 in *L. lactis* DML1 cells producing Gdt1p in the presence of both MnCl_2 (fixed concentration of 25 μM) and CaCl_2 (variable concentration from 0 to 50 μM). Measurements were carried out with an excitation wavelength at the isosbestic point of Fura-2 (360 nm). 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 μM) and MnCl_2 (variable concentration from 0 to 100 μM). In the two experiments, cells were treated as described for Figure 1A. MnCl_2 and CaCl_2 were added simultaneously after 60 sec of measurement (arrow) and curves are represented as means ($n=3$) $\pm$ SD.

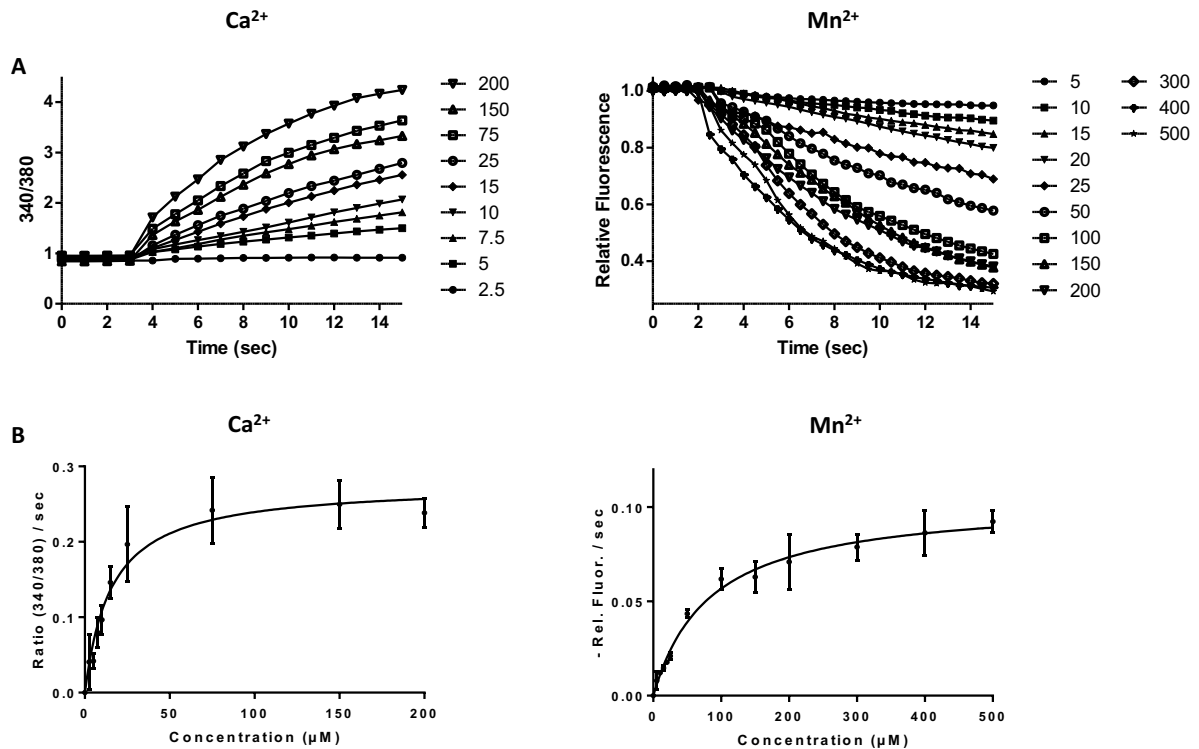


Figure 3. The affinity of Gdt1p for Ca^{2+} is higher than the one for Mn^{2+} in *L. lactis*. (A) Time-course measurements of the fluorescence emitted at 510 nm after excitations at 340 and 380 nm (340/380) (left) or of the fluorescence emitted at 510 nm after excitation at 360 nm normalized to the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

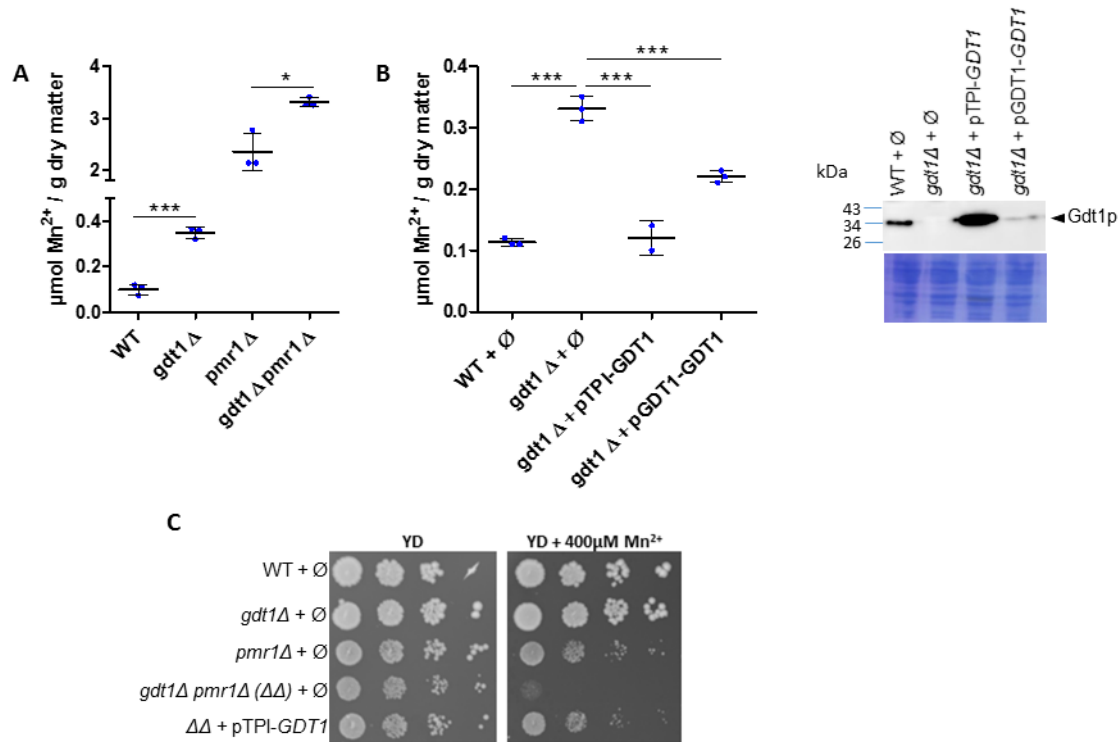


Figure 4. Gdt1p regulates the homeostasis of Mn²⁺ in yeast. (A) Cellular Mn²⁺ content of the wild-type, *gdt1Δ*, *pmr1Δ*, and *gdt1Δpmr1Δ* strains. Cells were grown in YD medium to an OD₆₀₀ of 3 and the cellular Mn²⁺ content was measured by ICP-AES. Data are shown as means ± SD (n=3). *P<0.05, ***P<0.001 (unpaired *t* tests). (B) (left) Cellular Mn²⁺ content of the wild-type and *gdt1Δ* strains transformed with the empty plasmid (∅) 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 OD₆₀₀ of 3 and the cellular Mn²⁺ content was measured by ICP-AES. Data are shown as means ± SD (n=3, excepted for *gdt1Δ* + pTPI-GDT1 where n=2). ***P<0.001 (one-way ANOVA with Bonferroni post-hoc test). (right) Production of Gdt1p in the corresponding strains. Total protein extracts of cells grown to an OD₆₀₀ 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 wild-type, *gdt1Δ*, *pmr1Δ*, and *gdt1Δpmr1Δ* strains in rich solid medium supplemented or not with 400 μM Mn²⁺. The strains transformed with the empty (∅) or *GDT1*-containing pRS416 plasmid were pre-cultured in MD-U medium to an OD₆₀₀ of 0.3. Serial ten-fold dilutions of the culture were dropped on YD solid medium containing or not 400 μM MnCl₂. The ΔΔ+*GDT1* strain corresponds to the *gdt1Δpmr1Δ* mutant expressing *GDT1* under the control of the constitutive TPI1 promoter. Plates were incubated 2 days at 28°C.

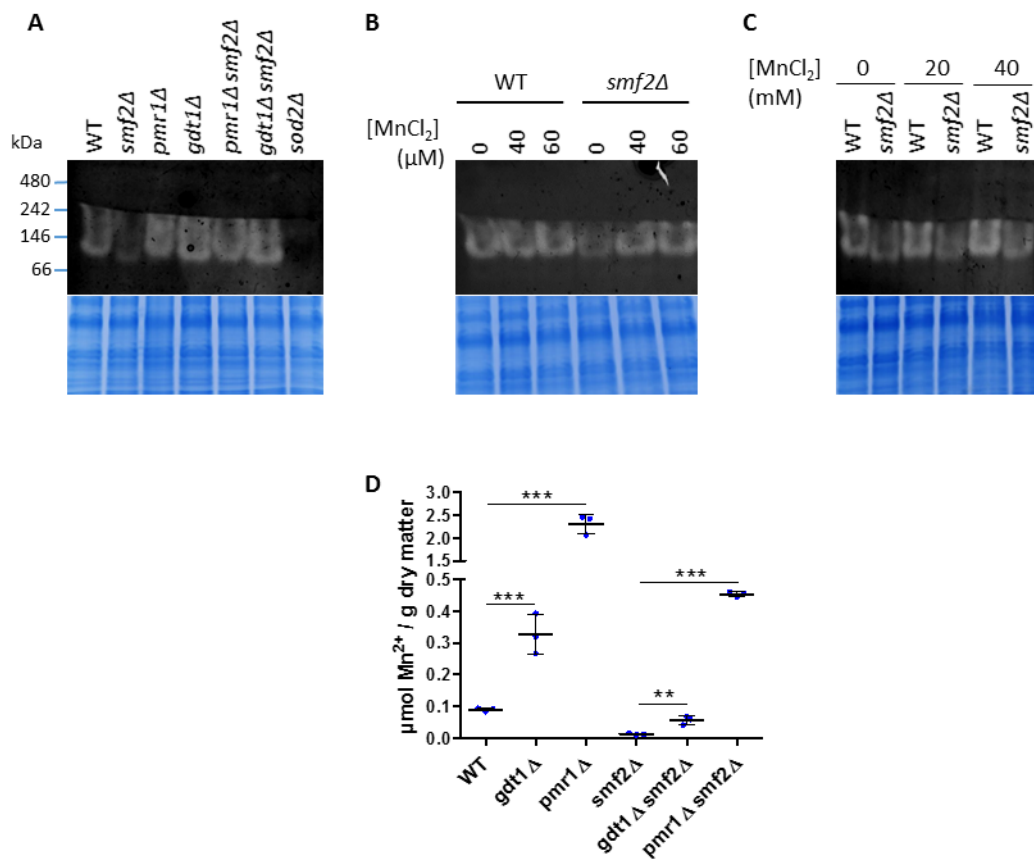


Figure 5. Effect of a *gdt1Δ* mutation on Sod2p activity and manganese accumulation in an *smf2Δ* mutant. (A) Activity of the Mn^{2+} -dependent Sod2p in the indicated yeast strains. The seven yeast strains were grown to an OD_{600} of 3. Cell lysates were loaded on a native PAGE gel in which the activity of Sod2p was monitored by nitro blue tetrazolium staining. Colloidal blue-stained gels were used as loading controls. (B) Activity of Sod2p in the wild-type and *smf2Δ* strain grown in the presence of 0, 40, or 60 μM $MnCl_2$. (C) Activity of Sod2p in cell lysates containing 0, 20, or 40 mM $MnCl_2$. (D) Cellular Mn^{2+} content of the indicated strains. Cells were grown in YD medium to an OD_{600} of 3 and the cellular Mn^{2+} content was measured by ICP-AES. Data are shown as means $\pm$ SD ($n=3$). ** $P<0.01$, *** $P<0.001$ (unpaired t tests).

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

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