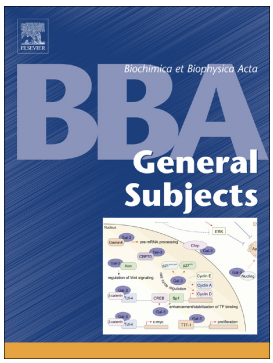

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Investigating the function of Gdt1p in yeast Golgi glycosylation

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Abstract

The Golgi ion homeostasis is tightly regulated to ensure essential cellular processes such as glycosylation, yet our understanding of this regulation remains incomplete. Gdt1p is a member of the conserved Uncharacterized Protein Family (UPF0016). Our previous work suggested that Gdt1p may function in the Golgi by regulating Golgi Ca^{2+}/Mn^{2+} homeostasis. NMR structural analysis of the polymannan chains isolated from yeasts showed that the *gdt1*Δ mutant cultured in presence of high Ca^{2+} concentration, as well as the pmr1Δ and *gdt1*Δ/*pmr1*Δ strains presented strong late Golgi glycosylation defects with a lack of α-1,2 mannoses substitution and α-1,3 mannoses termination. The addition of Mn^{2+} confirmed the rescue of these defects. Interestingly, our structural data confirmed that the glycosylation defect in pmr1Δ could also completely be suppressed by the addition of Ca^{2+}. The use of Pmr1p mutants either defective for Ca^{2+} or Mn^{2+} transport or both revealed that the suppression of the observed glycosylation defect in pmr1Δ strains by the intraluminal Golgi Ca^{2+} requires the activity of Gdt1p. These data support the hypothesis that Gdt1p, in order to sustain the Golgi glycosylation process, imports Mn^{2+} inside the Golgi lumen when Pmr1p exclusively transports Ca^{2+}. Our results also reinforce the functional link between Gdt1p and Pmr1p as we highlighted that Gdt1p was a Mn^{2+} sensitive protein whose abundance was directly dependent on the nature of the ion transported by Pmr1p. Finally, this study demonstrated that the aspartic residues of the two conserved motifs E-x-G-D-[KR], likely constituting the cation binding sites of Gdt1p, play a crucial role in Golgi glycosylation and hence in Mn^{2+}/ Ca^{2+}transport.

Keywords : Gdt1p, Pmr1p, Golgi glycosylation, Mn^{2+} homeostasis

1. Introduction

In 2012, we highlighted TMEM165 as the first member of the Uncharacterized Protein Family 0016 (UPF0016) related to human diseases. Defects in TMEM165 lead to a rare inherited disorder named CDG for Congenital Disorders of Glycosylation in which Golgi glycosylation process is affected. Found in bacteria, archaea, yeast, plants and animals, members of the UPF0016 family share two highly conserved regions as signatures motifs: E-x-G-D-[KR] [1]. Many evidences show that these two motifs form the pore of the protein and thus regulate the functionality of the UPF0016 members. Currently, the precise cellular functions of these proteins remain to be fully characterized and are under debate. In yeasts, it was previously reported that Gdt1p was involved in Ca^{2+} transport then playing an important role in Ca^{2+} signaling and Golgi protein glycosylation thereby supporting the hypothesis that Gdt1p would act as Ca^{2+}/H^{+} antiporter in the Golgi apparatus [2, 3]. The role of TMEM165 as a Golgi Ca^{2+}/H^{+} antiporter can however be questioned. We recently highlighted that the observed glycosylation defect due to TMEM165 deficiencies resulted from a defect in Golgi Mn^{2+} homeostasis [4]. Moreover, we demonstrated that TMEM165 was a novel Golgi protein sensitive to Mn^{2+} as exposition to high Mn^{2+} concentrations lead to lysosomal degradation of TMEM165 [5]. These data reinforced the hypothesis of TMEM165 as being involved in Mn^{2+} transport. This is also currently emphasized by several other studies. In Arabidopsis thaliana, the homologous protein photosynthesis affected mutant 71 PHOTOSYNTHESIS AFFECTED MUTANT 71 (PAM71) has been shown to be required for efficient Mn^{2+}uptake at the thylakoid membrane [6]. Moreover, the Mnx protein of the cyanobacterial model strain *Synechocystis sp*. PCC 6803, also belonging to the UPF0016 family, was recently demonstrated as a Mn exporter [7]. Altogether these data cast doubt about the substrate specificity of the UPF0016 members. From a general point of view, the mechanisms by which yeast cells regulate Golgi Ca^{2+} and Mn^{2+} homeostasis, both critical for many cellular processes and in particular Golgi glycosylation, are not completely deciphered yet.

In this report we have investigated into details the contribution of Gdt1p, Pmr1p and both in Golgi glycosylation processes. We have demonstrated that inactivation of Pmr1 led to strong Golgi glycosylation defects fully reversed by the addition of both Ca^{2+} and Mn^{2+}. Interestingly, in the *gdt1*Δ/*pmr1*Δ double knock-out strain, only the addition of Mn^{2+} was capable to suppress the observed Golgi glycosylation defect thus pointing the critical role of Gdt1p in suppressing the Golgi glycosylation defect in pmr1Δ strains supplemented with Ca^{2+}. We have

also shown that the abundance and function of Gdt1p in Golgi glycosylation was dependent on the function of Pmr1p. By using mutants of Pmr1p specifically defective for transport of either Ca^{2+} ions (Pmr1pD53A), Mn^{2+} ions (Pmr1pQ783A) or both (Pmr1pD778A), our results evidenced that in the case where Pmr1p only transport Ca^{2+} from the cytosol to the Golgi lumen, Gdt1p was necessary to import Mn^{2+} inside the Golgi lumen to suppress the observed Golgi glycosylation defect. Finally, this report demonstrates that the acidic residues of the two conserved motifs E-x-G-D-[KR] of Gdt1p are involved in Golgi glycosylation.

2. Material and methods

Yeast strains and media

Yeast strains used for the experiments are all derivatives of BY4741 and BY4742 and are listed below:

Wild-type (WT) Mata his3Δ1 leu2Δ0 ura3Δ0

pmr1Δ Mata his3Δ1 leu2Δ0 ura3Δ0 pmr1Δ::KanMX4

gdt1Δ Mata his3Δ1 leu2Δ0 ura3Δ0 gdt1Δ::KanMX4

gdt1Δ/pmr1Δ Mata his3Δ1 leu2Δ0 ura3Δ0 gdt1Δ::KanMX4 pmr1Δ::KanMX4

All strains were obtained by backcrossing pmr1Δ (Y04534) and gdt1Δ(Y13327) strains provided by Euroscarf.

Yeast were cultured at 30°C. Cultures in liquid media are done under a light shaking. Rich media, named YEP media, contains yeast extract (10 g.L-1, Difco), Bacto-peptone (20 g.L-1, Difco). YPD media is a YEP media supplemented with 2% D-glucose (Sigma-Aldrich). YPR is YEP supplemented with 2% raffinose (Euromedex). Selection antibiotics were added at 100 µg.mL-1 for nourseothricine, 200 µg.mL-1 for G418 and 300 µg.mL-1 for hygromycin.

Constructs, vector engineering and mutagenesis

All the constructs allowing *GDT1* (wt (wt-HA) or mutant (mutant-HA)) expression are pRS41H derivatives. First a 1473bp fragment starting 417pb before the Start of *GDT1* was amplified from genomic DNA by PCR and cloned between KpnI and XmaI sites of pRS41H. Mutant and/or HA-tagged versions of *GDT1* (E53G, D56G, E204G, L205W and D207G) were created from this vector using PCR directed mutagenesis by Ezyvec (Lille, France). The HA tag was inserted in the cytoplasmic loop in between the aa 171-172. All the constructs allowing *PMR1* (wt (wt-Myc) or mutant (mutant-Myc)) expression are pRS41N derivatives. First a 4004bp fragment starting 999 pb before the Start of *PMR1* was amplified from genomic DNA by PCR and cloned between EagI and SacI sites of pRS41N. Mutant and/or N-Myc-tagged versions of *PMR1* (D53A, D778A and Q783A) were created from this vector using PCR directed mutagenesis by Ezyvec (Lille, France). All constructs were checked by Sanger sequencing of the full insert.

Extraction and isolation of mannan from yeast

The equivalent of 50 g yeast was suspended in 300 mL of 0.02 M citrate buffer (pH 7), autoclaved at 125°C, 90 min. The solid pellet was then removed by centrifugation and the supernatant collected. An equivalent volume of Fehling solution was added to the supernatant and stirred at room temperature until precipitates form. The precipitates are collected and dissolved with 100 mL of 3N HCl. 300 mL of ethanol are then added to precipitated mannan. The mannan are then dissolved in 50mL water and dialyzed (MWCO 3500) against water overnight at 4°C. The dialyzed mannans are then dried and lyophilized.

Invertase glycosylation analysis

Before any analysis, a preculture in YPD media is done and a volume equivalent to 15 OD600nm units is centrifuged for 3 minutes at 3500g. The supernatant is discarded and the pellet is suspended in YPR media to induce invertase expression. Calcium, manganese and other ions were added at this step at the indicated concentration. After a 20h culture in YPR, yeasts were centrifuged for 5 minutes at 3500g. Supernatant was discarded and the pellet was kept frozen at -20°C. The cells were then resuspensed and lysed by glass-bead agitation in cold TBP buffer (5.52 g of diethylbarbituric acid and 1 g of Tris base per liter of water, pH 7.0; to 100 ml, add 1 ml of stock PMSF (0.174 g of phenylmethanesulfonyl fluoride in 10 ml of absolute ethanol) just before use]. 3µL of the supernatant are loaded on native gel. For the revelation of the invertase activity, the gel is then soaked into a 4°C sucrose solution (0.1 M pure sucrose in 0.1 M sodium acetate, pH 5.1) for 10min and then immediately transferred into a 37°C sucrose solution for 10min to hydrolyze the substrate. The gel is then quickly rinsed twice with water and transferred to a Pyrex dish containing 50 ml of TTC (50 mg of 2,3,5-triphenyltetrazolium chloride (TTC) in 50 ml of 0.5 M NaOH). The dish is boiled until the color appears. To stop the coloration and neutralize the NaOH, the gel is washed with water and stored in 10% acetic acid until imaging.

Western Blotting

Yeasts were centrifuged for 5 minutes at 3500g. Supernatant was discarded and cells were then resuspended in TBP buffer supplemented with a protease cocktail inhibitor (Roche Diagnostics, Penzberg, Germany). Cell lysis was induced by vortexing the cells with beads 1h at 4°C. Cells were centrifuged for 5 min at 3500g. The protein concentration of supernatant was estimated with the micro BCA Protein Assay Kit (Thermo Scientific). 20 µg of total protein lysates were dissolved in NuPAGE LDS sample buffer (Invitrogen) pH 8.4 supplemented with 4% β-mercaptoethanol (Fluka). Samples were heated 10 min at 95°C and then separated on 4%-12% Bis-Tris gels (Invitrogen) and transferred to nitrocellulose membrane Hybond ECL (GE Healthcare, Little Chalfont, UK). The membranes were incubated in blocking buffer (5% milk powder in TBS-T [1X TBS with 0.05% Tween20]) for 1 h at room temperature, then incubated overnight with the anti-HA (Santa Cruz; clone Y-11 used at a dilution of 1:200) or anti-c-myc(Santa Cruz, clone 9E10 used at a dilution of 1:200) or anti-CPY (Abcam; clone 10A5B5 used at a dilution of 1:2000) in blocking buffer, and washed three times for 5 min in TBS-T. The membranes were then incubated with the peroxidase-conjugated secondary goat anti-rabbit (Dako; used at a dilution of 1:10,000) in blocking buffer for 1 h at room temperature and later washed three times for 5 min in TBS-T. Signal was detected with chemiluminescence reagent (ECL 2 Western Blotting Susbtrate, Thermo Scientific) on imaging film (GE Healthcare, Little Chalfont, UK).

Whole cell Mn measurement by ICP-MS

Yeasts were grown in YPD medium and a volume equivalent to 15 OD600nm units was centrifuged for 3 minutes at 3500g. The supernatant was discarded and the pellet was suspended in YPD media containing or not 50 μM MnCl2. After 20h, a volume equivalent to 25 OD600nm units is centrifuged for 3 minutes at 3500g. Yeasts are washed twice with EDTA 1 μM and 3 times with water. Yeasts were suspended in 500 μL of HNO_3 30% and heat at 65°C in a light shaking during 20h. 500 μL of water were added to the mixture. 300 μL were analyzed by ICP-MS (Inductively Coupled Plasma - Mass Spectrometer). Mn analyses were done in the Toxicology Laboratory of the Lille University Hospital. Samples were diluted 50 times with 1.5% (v/v) nitric acid (ultrapure quality 69.5%, Carlo Erba Reagents, Val de Reuil, France) solution in ultrapure water (Purelab Option-Q, Veolia Water, Antony, France) containing 0.1% triton®X-100 (Euromedex, Souffelweyersheim, france), 0.2% butan-1-ol (VWR Chemicals, Fontenay-sous-Bois, France), and 0.5µg/L rhodium (Merk,

Darmstadt, Germany). Assays were performed on an ICP-MS THERMO ICAPTM Q (Thermo Scientific, Courtaboeuf Cedex, France). The limit of quantification was 0.2µg/L).

NMR analyses

All NMR experiments were acquired on Avance II Bruker spectrometer equipped with BBO 5mm probe resonating at 400 MHz for ^{1}H, 100.6 MHz for ^{13}C. Mannans were dissolved in 500 µL $^{2}H_2O$ (99.96% ^{2}H, Eurisotop®), and then transferred into 5 mm Shigemi tubes (Allision Park, USA). NMR experiments were performed at 293K. The ^{1}H chemical shifts were expressed in ppm. related to the methyl signal of acetone (δ ^{1}H 2.225 and δ ^{13}C 31.55 ppm) as internal standards. The COSY90- $^{1}H/^{13}C$-HSQC experiments were performed by using Bruker standard sequences and optimized for each experiment.

Monosaccharide analyses

10 µg of inositol (taken as internal standard) and 10 µg of target mannan were mixed. The monosaccharide composition was established by GC and GC/MS as alditol-acetate derivatives. Briefly, samples were hydrolyzed in 4 M trifluoroacetic acid (TFA) for 4 h at 100°C and then reduced with sodium borohydride (10mg/mL) in 0.05 M NaOH for 4 h. Reduction was stopped by dropwise addition of acetic acid until pH 6 was reached and borate salts were co-distilled by repetitive evaporation in dry methanol. Peracetylation was performed in acetic anhydride at 100°C for 2 h. The derivatized monosaccharides were dissolved in 1 mL of chloroform, and 1 µL of sample was injected into GC-MS (TRACE GC Ultra, Thermo Fisher Scientific). The capillary column is SOLGEL-1MS (Part No. 054795, 30m x 0.25 mm x 0.25 µm). The initial oven temperature was held at 120°C, increased to 230°C at 3°C/min, and then, 270°C for 10 min. The derivatized monosaccharides were separated into individual peaks, and identified by MS. However, the signal of GlcNAc was very low due to low percentage of GlcNAc in mannan. Therefore, selected ion monitor (SIM) was applied to increase sensitivity and quantify Man and GlcNAc. Ions at *m/z* 168, 187, and 144 were used as indicative fragment ions for inositol, Man, and GlcNAc, respectively. The size of isolation window was set 0.2 Da, and the scan time of selected ion was 0.2 sec. The response factor of inositol was set to 1. In this system, response factors of Man and GlcNAc were established to 0.48 and 0.28, respectively. The amount (µg) of mannose in different Mannan samples was

calculated by the formula [(Peak area of selected ion at *m/z* 187 for Man/0.48)/Peak area of selected ion at *m/z* 168 for inositol *10]. Similar calculation was applied to GlcNAc, which is [(Peak area of selected ion at *m/z* 144 for GlcNAc/0.28)/Peak area of selected ion at *m/z* 168 for inositol *10]. We assumed that most of mannan is located on *N*-glycans. Therefore, the number of Man per *N*-glycan was established as [molar of Man/(molar of GlcNAc*2)].

3. Results

3.1. The suppression of the glycosylation defect in pmr1Δ strains supplemented with Ca^{2+} is dependent on the activity of Gdt1p

We have previously reported that the increased mobility of secreted invertase activity by zymography (in native polyacrylamide gel) was a good reporter of Golgi *N*-glycosylation deficiency in yeast [4]. Using this technique, we have demonstrated that the Golgi *N*-glycosylation defect in *gdt1*Δ strains observed on invertase cultured in presence of high Ca^{2+} concentration could efficiently be suppressed by the addition of Mn^{2+}. This was also observed for *pmr1*Δ and *gdt1*Δ/*pmr1*Δ double knock-out strains [4]. Although we demonstrated that high environmental Ca^{2+} concentration in *gdt1*Δ led to strong glycosylation defects, we established here that the addition of Ca^{2+} rescues the glycosylation defect in *pmr1*Δ. Indeed, 10mM Ca^{2+} treatment is sufficient to greatly reduce the invertase mobility to a normal value (Fig. 1). Since Gdt1p and Pmr1p are two Golgi proteins involved in the regulation of the Golgi Ca^{2+}/ Mn^{2+} homeostasis, glycosylation defect in *gdt1*Δ/*pmr1*Δ double knock-out strains was analyzed in the absence and the presence of increasing Ca^{2+} concentrations (from 10mM Ca^{2+} to 300mM) (Fig. 1). Although the invertase mobility is strongly affected in the *gdt1*Δ/*pmr1*Δ double knock-out strains, the Ca^{2+} treatment does not restore it to a normal value (Fig. 1). By contrast and as previously observed, the addition of 50 μM Mn^{2+} is sufficient to fully restore the Golgi *N*-glycosylation in the different yeast strains (Supplementary Fig. 1).

These results points to the crucial requirement of Gdt1p activity in the restoration of the glycosylation in *pmr1*Δ strains supplemented with Ca^{2+}. Altogether these results strongly suggest a functional link between Gdt1p and Pmr1p in maintaining Golgi glycosylation homeostasis.

3.2. General structural analysis of the mannans from wild type and different mutants under different supplement of Ca^{2+}/Mn^{2+}

In order to further delineate the nature of the observed overall Golgi N-glycosylation defects, total mannans were isolated from yeast strains cultured under different Ca^{2+}/ Mn^{2+} conditions, followed by detailed

structural analyses. So called mannans from most yeasts share similar overall architectures. They are made of $Man_8GlcNAc_2$ *N*-linked glycans extended by a α-linked polymannoside containing around 200 mannose residues. In *S. cerevisiae*, the polymannoside is composed of a long stretch of (α-1,6)-linked D-mannopyranose units substituted in C2 positions by short side chains of (α-1,2)-linked mannose units that may be further capped by terminal Man(α-1,3) residues [8]. So called acid-labile mannan domain is further attached to the (α-1,2)-oligomannosides through phospho-di-ester bonds [9] In a first step, we established the structural features of the mannan isolated from WT strain by 1D ^{1}H-NMR experiment (Fig. 2A). Due to its polymeric nature, it is not possible to assign the signals corresponding to all individual monosaccharide residues of mannans. However, five broad signals annotated as I, II, III, IV, V could be detected in the 5.5-4.8 ppm anomeric region , which natures were established by observing their spin systems by ^{1}H-^{1}H COSY, TOCSY and ^{1}H-^{13}C HSQC experiments, based on literature ([10] (Fig. 2B). They were assigned to five major epitopes (**I**), internal -2)Man(α-1,2) residues; (**II**), terminal Man(α-1,3) residues; (**III**), -2,6)Man(α-1,6) branched residues; (**IV**), terminal Man(α-1,2) and -3)Man(α-1,2) residues; (**V**), unbranched -6)Man(α-1,6) residues. Furthermore, terminal Man(α-1,2) (**IV.a**, H2 at 4.06 ppm) and -3)Man(α-1,2) residues (**IV.b**, H2 at 4.22 ppm) could be differentiated by COSY90 spectrum as shown in Fig. 2B. Relative quantification of NMR signals **I** to **V** provides reliable snapshot of the overall mannan structural features. As shown in Fig. 2C, mannan isolated from WT strain is characterized by a high proportion of (α1-2) substitution on the (α 1-6)-mannoside stretch [2,6)Man / 6)Man = 6,4], leaving few un-substituted -6)Man(α-1,6) residues.

In a second step, we compared the structures of mannans isolated from all four strains grown in normal conditions by homo- and heteronuclear NMR. 1D ^{1}H-NMR spectra, of mannans islolated from wild type and *gdt1*Δ established that these two strains exhibit very similar mannosylation patterns (Fig. 2A). In contrast, distinctive features were observed in the structures of mannans isolated from *pmr1*Δ and *gdt1*Δ/*pmr1*Δ strains compared to WT, as shown by 1D ^{1}H-NMR spectra (Fig. 2A) and relative quantification of ^{1}H-^{13}C HSQC NMR signals (Fig. 2C). The two most salient features of mannans from *pmr1*Δ and *gdt1*Δ/*pmr1*Δ strains were (i) a sharp decrease in the proportion of internal (α-1,2) oligomannoside side chains and of (α-1,3) capping mannose residues, which correlated with (ii) a large increase in the proportion of unbranched (α-1,6) polymannoside

backbone. Indeed, the proportion of unbranched 6)Man(α-1,6) residues in pmr1Δ and gdt1Δpmr1Δ strains increased by 17 and 28 times compared to WT mannan. Additionally, COSY90 spectra showed an increased proportion of terminal Man(α-1,2) residues on mannans from *pmr1*Δ and *gdt1*Δ/*pmr1*Δ strains, which is correlated with a decreased proportion of terminal Man(α-1,3) (data not shown). Altogether, these experiments show that a lack of pmr1 and both gdt1pmr1 leads to a drastic reduction of the branching pattern of (α-1,6) polymannoside domain of the mannan.

In a third step, we screened by 1D ^{1}H-NMR experiments the structural variability of mannans isolated from all four strains following supplementation with 0.5M Ca^{2+}, 0.05mM Mn^{2+} and 0.5M Ca^{2+}/ 0.05mM Mn^{2+} with a special focus on their branching patterns (Supp Fig. 1) expressed as a % of unbranched -6)Man(α-1,6) residues compared with total residues by quantifying signal **V** (Fig 3A). In accordance with the above results, WT and gdt1Δ grown in normal condition contained less than 2% of -6)Man(α-1,6) residues, whereas mannans from pmr1Δ and gdt1Δpmr1Δ contained 21 and 27% of -6)Man(α-1,6) residues, respectively (Fig. 3A). This branching defect was fully restored in pmr1Δ by the addition of any divalent cation, Ca^{2+} or Mn^{2+}. Contrarily, the glycosylation defect of gdt1Δpmr1Δ strain which is almost entirely restored in presence of Mn^{2+} and both Ca^{2+}+ Mn^{2+} (4% and 7%), is not restored in the sole presence of Ca^{2+}. Then, gdt1Δ cultured in presence of 0.5M Ca^{2+} showed an increased proportion of unbranched -6)Man(α-1,6) backbone, as well as a decreased number of (α-1,3) mannose capping (data not shown). However, these defects were also completely restored by the addition of Mn^{2+}.

NMR analysis established that a lack of Pmr1p leads to strong defects in the mannan synthesis through the decrease of terminal Man(α-1,3) capping, the decrease of side chains (α-1,2) mannosylation and the increase of the proportion of unbranched 6)Man(α-1,6). These characteristics should result in the change of mannan size. In order to determine the average size of mannan domain of *N*-glycans, we quantified Man and GlcNAc residues in all samples by GC/MS analysis, and deduced the average number of mannose per *N*-glycan based on the presence of the chitobiose core. As shown in Fig. 3B, *N*-glycans isolated from WT and *gdt1*Δ strains grown in

normal conditions contained an average of 220-250 Man residues. However, we observed that the size of mannan domain of *gdt1*Δ strain under Ca^{2+}, as well as *pmr1*Δ and *gdt1*Δ/*pmr1*Δ strains in normal conditions and under Ca^{2+} are drastically reduced (Fig. 3A) down to about 40 Man residues. Under Mn^{2+} supplementation, the size of mannans from *pmr1*Δ and *gdt1*Δ/*pmr1*Δ was restored to average sizes. Altogether, structural analysis by NMR and GC/MS showed that the glycosylation defects are due to a reduced synthesis of -2)Man(α-1,2) side chains that results in the synthesis of polymannosylated *N*-glycans of smaller size compared to that of WT strains.

This suggests that a lack of pmr1 and/or gdt1 would affect late Golgi glycosyltransferases such as MMN2/ MNN5/ MNN. 1Altogether, these results demonstrate (i) the crucial requirement of Gdt1p in maintaining Golgi glycosylation when cells are cultured in presence of Ca^{2+} and (ii) that the suppression of the glycosylation defect by the Ca^{2+} in pmr1Δ strains is strictly dependent on the activity of Gdt1p.

3.3. The abundance and function of Gdt1p in glycosylation is dependent on the Pmr1p function

To further investigate the contribution of Ca^{2+} *versus* Mn^{2+} transport activity of Pmr1p to the observed *N*-glycosylation defect, we first transfected *PMR1*-deficient cells with three point mutants of Pmr1p that were defective for transport of either Ca^{2+} ions (Pmr1pD53A), Mn^{2+} ions (Pmr1pQ783A) or both (Pmr1pD778A) [11, 12]. Although both Pmr1pD53A and Q783A can restore the observed initial glycosylation defect, differences can be observed (Fig. 4A). The restoration is total with Pmr1pWT and the Pmr1pD53A and only partial with Pmr1pQ783A (Fig. 4A, left panel). To assess the potential role of Gdt1p in this glycosylation rescue, *gdt1*Δ/*pmr1*Δ strains were transfected with the same Pmr1p mutants. While the expression of the D53A completely restored the glycosylation, the Q783A clearly did not (Fig. 4A, right panel). To confirm these results, the invertase mobility in the pmr1Δ and *gdt1*Δ/*pmr1*Δ double knock-out strains transfected with the Pmr1pQ783A in the presence of increasing Ca^{2+} concentrations was evaluated. In the pmr1Δ yeast strains transfected with the pmr1Q783A, the invertase mobility is strongly reduced both in absence of Ca^{2+} and under increasing Ca^{2+} concentrations (Fig. 4B, left panel). We demonstrated that this effect was due to the activity of Gdt1p, as the Ca^{2+} treatment in the *gdt1*Δ/*pmr1*Δ double knock-out strains transfected with the pmr1Q783A does

not suppress the observed glycosylation defect (Fig 4B, right panel). These results have also been confirmed by glycosylation analysis on carboxypeptidase Y (CPY) and fully support those from the invertase assay experiments (supplementary Fig. 2). We then wondered whether the suppression of the glycosylation defect in pmr1Δ strains supplemented with Ca^{2+}could result from Gdt1p expression changes. The cellular abundance of Gdt1p was then evaluated by Western blotting using specific antibodies directed against Gdt1p in pmr1Δ strains, transfected or not with the different Pmr1p mutants defective for transport of either Ca^{2+} ions (Pmr1pD53A), Mn^{2+} ions (Pmr1pQ783A) or both (Pmr1pD778A). This experiment showed that the abundance of Gdt1p was directly linked to the transport function of Pmr1p. In *pmr1*Δ, the abundance of Gdt1p was greatly reduced (-80% compared to WT) (Fig. 4C). Remarkably, the expression of Pmr1Wt in *pmr1*Δ strains restored the Gdt1p abundance. Interestingly, while the expression of the Pmr1pD53A also completely rescued the Gdt1p level, the Pmr1pD778 mutant had no effects on Gdt1p abundance (Fig. 4C and Supplementary Fig. 3). A slight rescue can be seen with the Pmr1pQ783A mutant. The expression of Pmr1p mutant proteins was confirmed by using myc-tagged versions. As seen in Supplementary Fig. 3, all Pmr1p mutants are expressed. Altogether, these results prove that the abundance of Gdt1p is dependent of the transport function of Pmr1p.

To go further, we then evaluated the total cellular Mn^{2+} concentration in the different yeast strains under different conditions by ICP-MS. While under physiological conditions the total cellular Mn^{2+} concentration is similar in the different mutants, a huge increase is observed following Mn^{2+} supplementation in all investigated mutants compared to WT (Fig 4D). After Mn^{2+} supplementation, a 10-fold increase in Mn^{2+} concentration is observed in the *gdt1*Δ/*pmr1*Δ double knock-out mutant, a 5 fold increase in the pmr1Δ mutant and a 2 fold increase in the *gdt1*Δ mutant. These results support the fact that both Pmr1p and Gdt1p are involved in total cellular Mn^{2+} homeostasis maintenance.

Our results demonstrate that (i) the Golgi glycosylation defect observed in pmr1 deficient cells results from a lack of Golgi intraluminal Mn^{2+}, (ii) that the rescue of the glycosylation defect in pmr1Δ strains by the intraluminal Golgi Ca^{2+} requires the activity of Gdt1p.

3.4. Acidic residues of the conserved motifs of Gdt1p are involved in Golgi glycosylation

As previously published [1, 13, 14], members of the UPF0016 family contain two highly conserved consensus motifs E-φ-G-D-[KR]-[TS], predicted to be involved in the transport function of UPF0016 members. Recently these motifs have been shown to be part of the regulatory Ca^{2+} binding domains. In order to evaluate the importance of these two motifs in the maintenance of Golgi glycosylation homeostasis, mutated versions of Gdt1p have been generated (E53G, D56G, E204G, L205W and D207G) and used to complement the observed glycosylation defect in *gdt1*Δ strains cultured in presence of high Ca^{2+} concentrations. The expression level of Gdt1 mutant proteins was first assessed by western blot using the HA-tagged mutated version of Gdt1p (Supplementary Fig. 4). Although the D56G, D207G and L205W were found expressed, the E53G and E204G were surprisingly not. Interestingly none of the mutated Gdt1p, except L205W mutation, complements the observed glycosylation defect on invertase and CPY (data not shown) then demonstrating that the aspartic amino acids are essential for the function of Gdt1p in Golgi glycosylation (Fig. 5 and supplementary Fig. 4). We then wondered whether the activity of Gdt1p was required in the case where Pmr1p would only transport Mn^{2+}. For that, the same mutated versions were expressed in *gdt1*Δ/*pmr1*Δ strains transformed with Pmr1pD53A and the invertase mobility was assessed. As shown in figure 5, the glycosylation was completely restored for all the mutated versions of Gdt1p, demonstrating that, Gdt1p is dispensable when Pmr1p exclusively transports Mn^{2+}. Similar experiment was then performed in *gdt1*Δ/*pmr1*Δ strains transformed with Pmr1pQ783A. Although the expression of Gdt1p wt partially rescues the invertase glycosylation defect, none of the mutated version suppresses the glycosylation defect (Fig. 5). This result clearly demonstrates that the requirement of Gdt1p for Golgi glycosylation depends on the nature of the ion transported by Pmr1p.

4. Discussion

The regulation of Ca^{2+} and Mn^{2+} concentrations in the Golgi apparatus is crucial for many cellular processes particularly the secretion of proteins and the maintenance of Golgi glycosylation. One of the main supplier/ regulator of Ca^{2+}/ Mn^{2+} Golgi homeostasis is the Golgi localized P-type ATPase Pmr1p. Our previous work raised the possibility that Gdt1p may also play a crucial role in Golgi ion homeostasis and Golgi glycosylation. Although the precise cellular function of Gdt1p in the Golgi remains unsolved, results cast doubt about its precise function in the transport of substrates. In this work we show that Gdt1p is a functionally important Golgi protein playing a unique role in Golgi glycosylation. Compared to mammalian cells, yeasts further maturate the *N*-linked glycans with the addition of outer chains that may contain up to ~300 mannose residues [8]. These hypermannosylated structures consist in backbones of α-1,6-linked mannose residues substituted with α-1,2-linked mannose residues themselves branched with terminal α-1,3-linked mannose residues (Fig. 2D). Many Golgi mannosyltransferase complexes are involved in generating these specific structures. In this paper we assessed and compared by using NMR the structural details of polymannan chains of the different yeast strains (*gdt1*Δ, *pmr1*Δ and *gdt1*Δ/*pmr1*Δ strains) under different conditions. The NMR experiments showed strong alteration of the Golgi *N*-linked glycosylation in the different yeast strains under various conditions. While the backbone of α-1,6-linked mannose residues is not altered, strong defects in α-1,3- and α-1,2- branching are mainly observed in *gdt1*Δ strains cultured in presence of high Ca^{2+} concentration, pmr1Δ and *gdt1*Δ/*pmr1*Δ strains. We also confirmed that the addition of Mn^{2+} was sufficient to completely restore the observed branching defects. Interestingly, our data clearly demonstrated that the suppression of the Golgi glycosylation defects by the Ca^{2+} in pmr1Δ yeast strains was dependent on the activity of Gdt1p. Based on the structural analysis of the polymannan chains, we deduced that the defects mainly affected medial and late Golgi glycosylation as only the α-1,2 substitution- and the α-1,3 termination are affected. This points toward an alteration of Mnn2p/ Mnn5p/ Mnn6p and/or Mnn1p activities (Fig. 2D). The α-1,6 initiation/ elongation seems not to be altered in our analysis. Taken together, our structural analysis data showed Gdt1p as well as Pmr1p to be critical participants in medial and late Golgi glycosylation functions.

The identity of Gdt1p as a potential Golgi transporter controlling both Golgi Ca^{2+}/ Mn^{2+} homeostasis arose from our studies and others [3, 4, 14]. In this work we further evaluated the potential role of Gdt1p in importing Mn^{2+}

from the cytosol to the Golgi lumen. As first pointed by us [1] and others [6, 7, 13] members of the UPF0016 family contain two highly conserved consensus motifs E-φ-G-D-[KR]-[TS], predicted to be involved in the transport function of UPF0016 members. Our results show that mutations of the aspartic amino acids of these two conserved motifs (D56A, and D207A) completely abolish the rescue of the glycosylation. Interestingly we did observe that the glutamic amino acids mutated versions of Gdt1p were not expressed then raising the possibility that these two mutated forms are highly sensitive to the availability of Mn2+ in the Golgi lumen or in the cytosol. We did alsodemonstrate that an active form of Gdt1p was exclusively required in case where Pmr1p mainly transports Ca^{2+}. When Pmr1p mainly imports Mn^{2+} inside the Golgi lumen, our results show that Gdt1p is completely dispensable for the Golgi glycosylation. We propose that the aspartic amino acids are part of the cation binding sites of Gdt1p (one for Ca^{2+} and one for Mn^{2+}). We can assume that mutations in any of these amino acids completely abolish the transport function of Gdt1p by impairing cation affinity or conformation changes of the pocket.

Moreover, the use of different Pmr1p mutants defective for transport of either Ca^{2+} ions (Pmr1pD53A), Mn^{2+} ions (Pmr1pQ783A) or both (Pmr1pD778A) [11 ,12] showed that the observed Golgi glycosylation defect in the *gdt1*Δ/*pmr1*Δ strains only resulted from a lack of intraluminal Golgi Mn^{2+} and not Ca^{2+}. Our data suggest that the activity of Gdt1p in Golgi glycosylation becomes essential only when Pmr1p transports Ca^{2+}. It should also be noted that the suppression of the glycosylation defect is more efficient in pmr1Δ strains complemented with Pmr1pQ783A under Ca^{2+} supplementation. Given the fact that the observed Golgi glycosylation defect was due to a lack of intraluminal Golgi Mn^{2+}, our results strongly suggest that when Pmr1p only transports Ca^{2+} from the cytosol to the Golgi lumen, Gdt1p is necessary to import Mn^{2+} inside the Golgi lumen by exchanging Ca^{2+} (Fig. 6). This model also explains why high environmental Ca^{2+} concentrations in *gdt1*Δ lead to strong *N*-glycosylation deficiencies. When cytosolic Ca^{2+} concentration increases, Pmr1p favors the transport of Ca^{2+} in place of Mn^{2+}. The Golgi luminal pool of Mn^{2+} is then rapidly depleted if Gdt1p is not there to efficiently import Mn^{2+} inside the Golgi lumen. Given the fact that a lack of Pmr1p leads to strong Golgi glycosylation defects, our results suggest that in physiological conditions, Pmr1p preferentially imports Mn^{2+} rather than Ca^{2+} into the Golgi lumen. In such conditions, the role of Gdt1p, at least in Golgi glycosylation, is completely dispensable. Would that suggest that Gdt1p use the Golgi Mn^{2+} gradient generated by Pmr1p to import cytosolic Ca^{2+} inside

the Golgi lumen? The question is completely open. As many antiport transporters can work in reverse if the gradient concentration of the driving ion is reversed, we can reasonably postulate that Gdt1p may also work in both directions. As our results show that the requirement of Gdt1p in Golgi glycosylation depends on the nature of the ion transported by Pmr1p, we propose that Gdt1p would be the leak channel of Pmr1p.

5. Acknowledgements

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6. Conflict of interests

None.

7. References

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8. Figure legends

Figure 1: The suppression of the glycosylation defect in pmr1Δ strains supplemented with Ca^{2+} is dependent of the activity of Gdt1p. Wild-type (WT), *gdt1*Δ, *pmr1*Δ and *gdt1*Δ/*pmr1*Δ yeasts mutants were grown to an OD600 of 0.8 in a YPD medium. Afterwards, yeasts were transferred in YPR medium with an increase of the indicated CaCl2 concentrations to induce Invertase secretion. *N*-glycosylated invertase secreted was analyzed in a native gel as indicated by Ballou et al (15).

Figure 2: Structural analysis of the mannans from *S. cerevisiae* strains depleted or not in Gdt1p and Pmr1p. (A) Comparison of the anomeric regions of ^{1}H-NMR spectra from WT, *gdt1*Δ, *pmr1*Δ and *gdt1*Δ/*pmr1*Δ strains; (B) details of the ^{1}H-^{13}C HSQC and ^{1}H-^{1}H COSY spectra from WT mannan showing the anomeric positions of mannose residues I-V; (C) relative quantifications of mannose residues based on NMR signals intensities. (D) Protein mannosylation pathway in S. cerevisiae. The structures of the N-linked glycans of S. cerevisiae are schematized. The arrow indicates the function of the different mannosyltransferases.

Figure 3: Comparison of mannans isolated from yeasts grown in various conditions: N, non-supplemented; $CaCl_2$, supplemented with 0.5M $CaCl_2$; $MnCl_2$, supplemented with 0.05mM $MnCl_2$; $CaCl_2$+$MnCl_2$, supplemented with $CaCl_2$ 05.N and $MnCl_2$ 0.05mM. (A) The branching pattern of the 1,6-linked mannan backbone is expressed as the proportion of 6)Man(α-1,6) residues in mannans and calculated from the relative integration of signal V from 1H-NMR spectra (see Supp Fig. 1). Its increases is indicative a defect in the synthesis of 2)Man(α-1,2) side chains. (B) The overall size of mannans was established as a number of mannose residues per *N*-glycans. The reduction in the size of mannans correlates with the defect in the synthesis of 2)Man(α-1,2) side chains.

Figure 4: The function and abundance of Gdt1p in glycosylation is dependent on the Pmr1p function (A) The Glycosylation defect in pmr1p mutants depends on its function. Pmr1Δ and *gdt1*Δ/*pmr1*Δ strains were

transformed with pRS41N-pmr1p mutants (pmr1p-WT, pmr1p-D53A, pmr1p-D778A and pmr1p-Q783A). Yeasts were grown in YPR media and *N*-glycosylated invertase profile was performed (B) Ca^{2+} uptake by pmr1p influences the Mn^{2+} uptake by Gdt1p. Yeasts were grown in YPR media supplemented with an increase of the indicated $CaCl_2$ concentrations in the medium and invertase profile was analyzed. (C) Abundance of Gdt1p depends on the Pmr1p function. gdt1Δpmr1Δ strains were transformed with pRS41H-Gdt1p-HA and with pRS41N-Pmr1p mutants (pmr1p-WT, pmr1p-D53A, pmr1p-D778A and pmr1p-Q783A). Yeasts were grown in YPD medium and *GDT1* expression was performed by western Blot using an anti-HA. Quantification of Gdt1p protein after normalization on ponceau red (N = 3). (D) Cytosolic manganese detoxification needs Pmr1p. Wild-type (WT), *gdt1*Δ, *pmr1*Δ and *gdt1*Δ/*pmr1*Δ yeasts mutants were grown to an OD600 of 0.8 in a YPD medium and transferred to a media containing no Mn^{2+} or 50 μM MnCl2. Total Mn^{2+} concentrations were analyzed by ICP-MS. Quantification of the cellular Mn2+ concentration (N = 2).

Figure 5: Acidic residues of the conserved motifs of Gdt1p are involved in Golgi glycosylation. *gdt1*Δ/*pmr1*Δ strains were transformed with pRS41N-pmr1pD53A (middle panel) and pRS41N-pmr1p-Q783A (right panel) and with pRS41H-gdt1p mutants (Gdt1p-E53G, Gdt1p-D56G, Gdt1p-E204G, Gdt1p-L205W and Gdt1p-D207G). Yeasts were grown in YPR medium. *gdt1*Δ strains were transformed with pRS41H-gdt1p mutants (Gdt1p-E53G, Gdt1p-D56G, Gdt1p-E204G, Gdt1p-L205W and Gdt1p-D207G) (right panel) and yeasts were grown in a YPR media supplemented with 200 mM CaCl2.

Figure 6: Proposed model for the function of Gdt1p in regulating Golgi Mn2+ together with the Ca2+/Mn2+ATPase Pmr1p. In this model, Gdt1p would be a Mn^{2+}/Ca^{2+} antiporter whose functions depend on Pmr1p. When Pmr1p would exclusively import Ca^{2+} to the Golgi lumen, Gdt1p is crucial to import Mn^{2+} inside the Golgi lumen by exchanging Ca^{2+}.

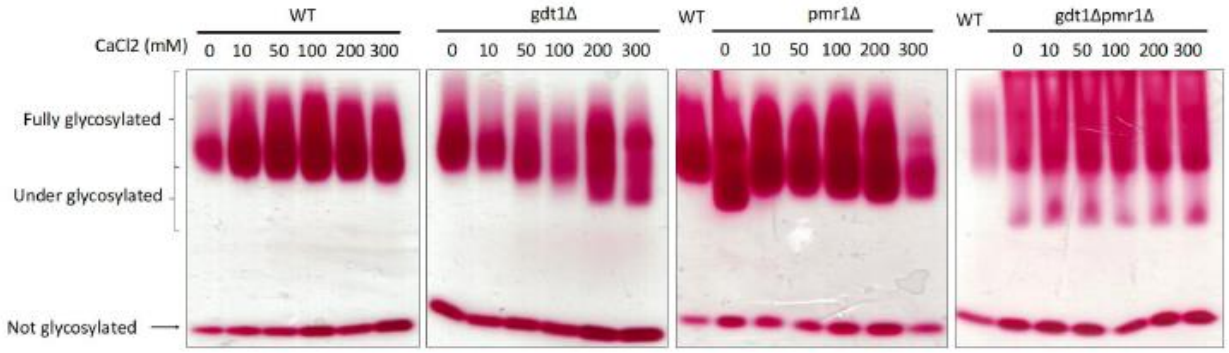

FIGURE 1

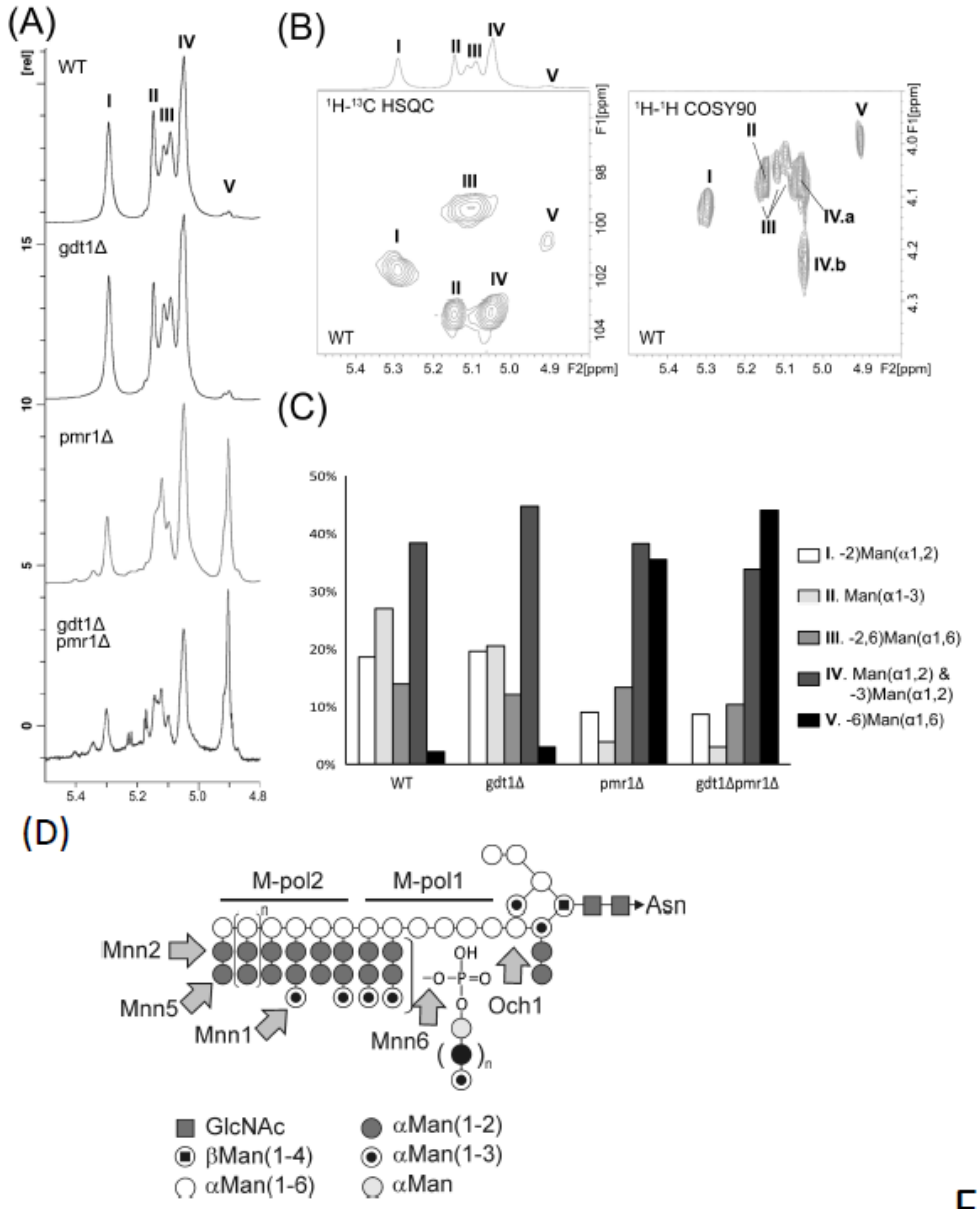

FIGURE 2

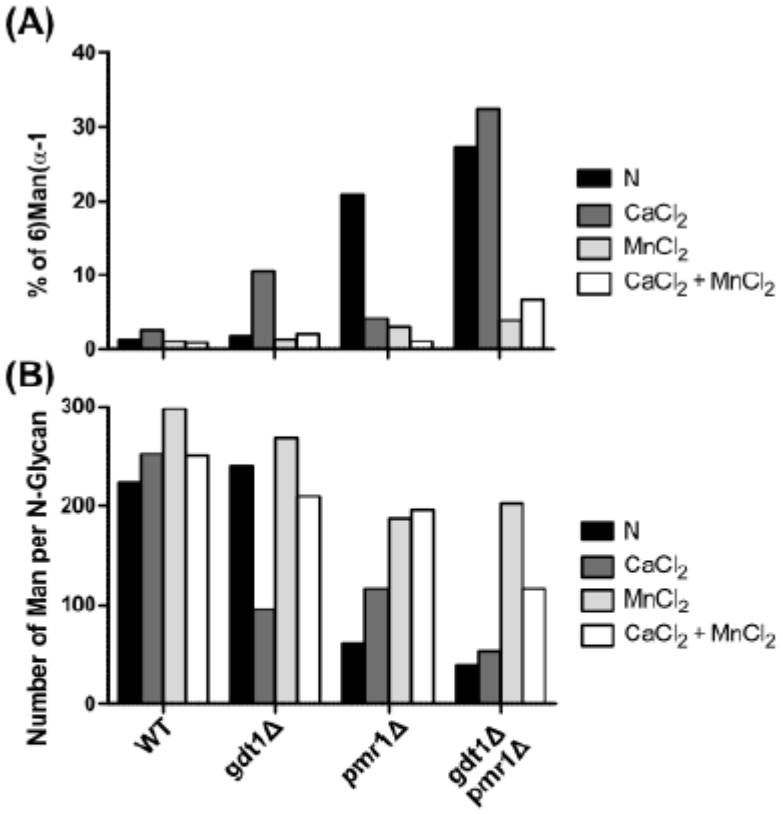

FIGURE 3

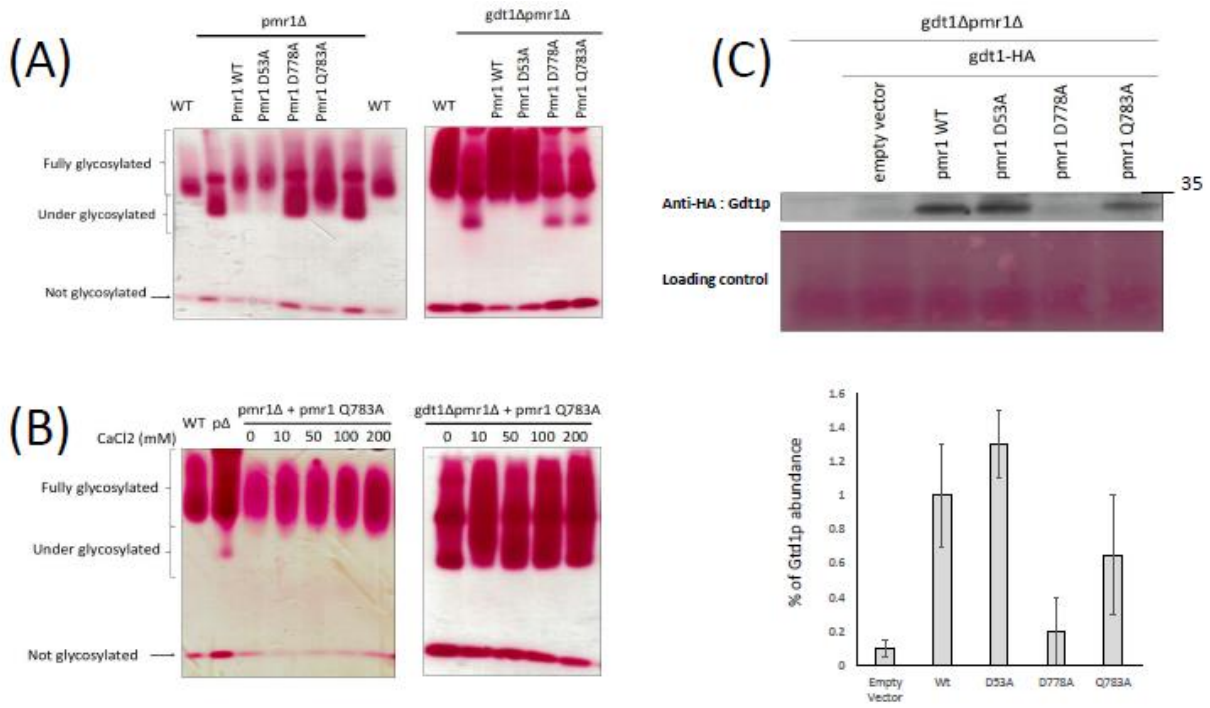

FIGURE 4

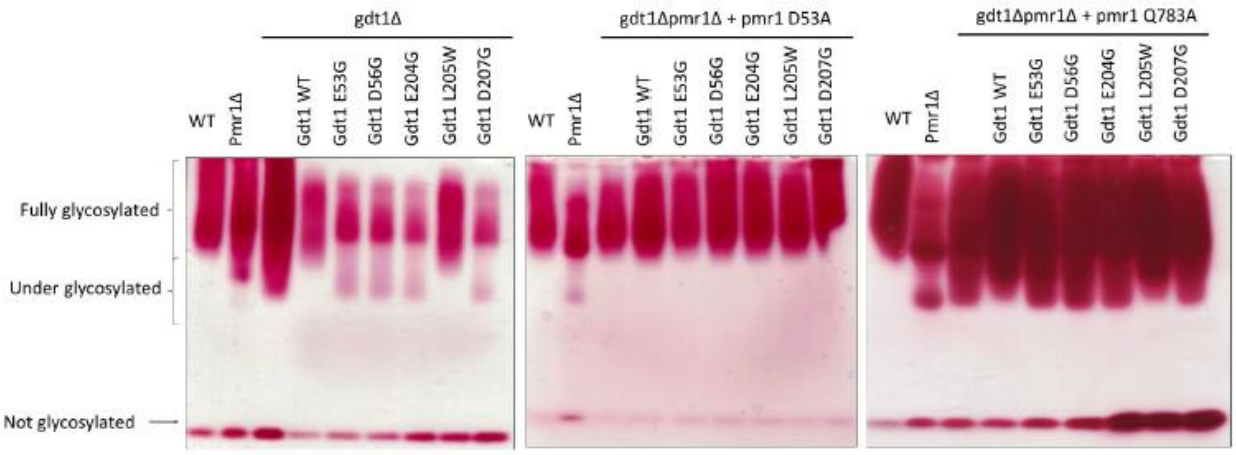

FIGURE 5

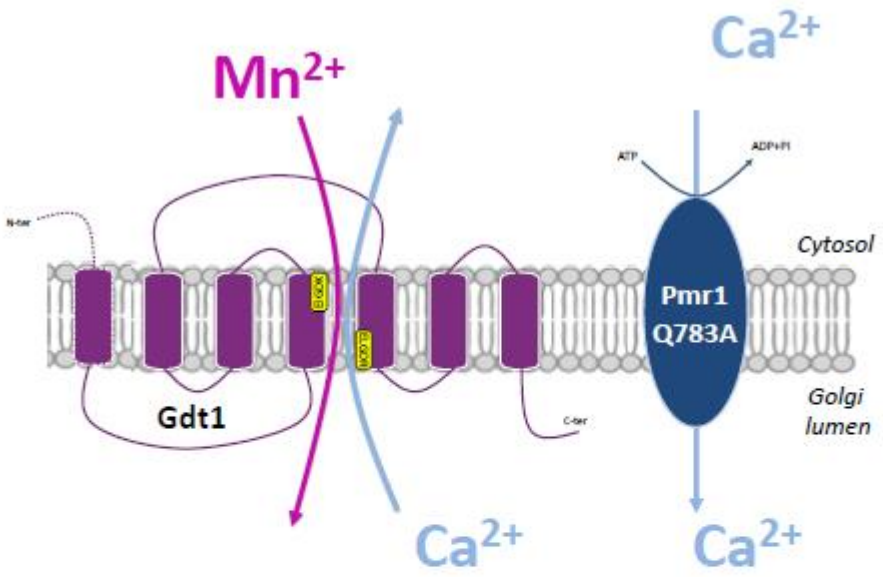

FIGURE 6

Highlights

1_ Functional link between Gdt1p and Pmr1p

2_ Gdt1p imports Mn2+ inside the Golgi lumen when Prm1p exclusively transports Ca2+

Keywords

N-glycosylation, Gdt1p, Pmr1p, Golgi, Mn2+ homeostasis