Supporting Information:

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SI Materials and Methods

Yeast Strains, Plasmids, and Media. The yeast strains used are listed in Table S1. The BY4741 and BY4742 strains were purchased from the Euroscarf systematic deletion library. The double-deletant *gdt1*Δ/*pmr1*Δ was obtained by crossing the single deletants BY4742 *gdt1*Δ and BY4741 *pmr1*Δ.

The plasmids were obtained following standard molecular biology protocols, and all genetic constructs were validated by sequencing. The full-length transmembrane protein 165 (TMEM165) gene was amplified from cDNA, digested with *BamHI*/*XhoI,* and integrated in the pRS416 plasmid containing the TPI1 (triosephosphate isomerase) constitutive promoter. The truncated $^{\Delta 55}$TMEM165 was amplified from the full-length gene, digested with *BamHI*/*XhoI*, and integrated in the same vector. These plasmids were called pRS416–pTPI–TMEM165 and pRS416–pTPI–$^{\Delta 55}$TMEM165, respectively. The GDT1 gene was amplified from yeast genomic DNA and cloned following the same method to obtain the pRS416–pTPI–GDT1 construct. Yeast transformations were performed following the method of Gietz et al. (1).

Nontransformed yeast cells were cultured at 28 °C in YD medium [2% (wt/vol) yeast extract, 2% (wt/vol) glucose]. Cells transformed with plasmids were precultured in synthetic SD medium [0.7% yeast nitrogen base without amino acids (Difco), 2% glucose] supplemented with all amino acids except those used as selection markers for plasmid maintenance. Solid media were produced by addition of 2% agar to the mixture. Calcium chloride was always autoclaved separate from the medium to avoid precipitation, and for the synthetic SD medium containing calcium, yeast nitrogen base was replaced by 0.2% yeast nitrogen base without amino acids and ammonium source (Difco) and 76 mM NH_4Cl. In the presence of 15 mM EGTA, YD was supplemented with 50 mM 2-(*N*morpholino)ethane sulfonic acid (Mes) and 50 mM Tris, and the pH was adjusted to 6.0 with potassium hydroxide (KOH).

Human Cell Culture. HeLa cells and primary skin fibroblasts from healthy controls and Congenital Disorder of Glycosylation (CDG) patients were cultured at 37 °C under 5% CO_2 in DMEM/F12 (Life Technologies) supplemented with 10% FBS (Clone III, HyClones).

Yeast Growth Analysis. Yeast cells were precultured overnight in 5 mL of YD medium or in SD medium without uracil for cells carrying a plasmid. The cultures were then adjusted to an OD_{600} 0.3, and three 10-fold serial dilutions were spotted onto the appropriate solid medium. The plates were incubated at 28 °C for 4–6 d and monitored daily.

Subcellular Fractionation on Sucrose Gradient. Subcellular fractionation was performed according to Powers and Barlowe (2). Briefly, cells were grown in 400 mL of YD medium overnight at 28 °C, harvested by centrifugation for 5 min at 5,000 × *g*, and washed in 50 mL of spheroplasting buffer (0.7 M sorbitol, 50 mM DTT, 10 mM Tris·HCl, pH 7.4). The recovered pellet was resuspended in spheroplasting buffer (2 mL of buffer g^{-1} of wet cells), and Zymolyase 20T was added (2.5 mg·g^{-1} of wet cells), then the suspension was incubated for 45 min at 30 °C. The spheroplasts were then centrifuged at 5,000 × *g* for 5 min at 4 °C, washed twice with 5 mL of spheroplasting buffer, resuspended in precooled lysis buffer [1 mL 0.6 g^{-1} of wet cells; 1 mM EDTA, 12.5% sucrose, 10 mM Hepes pH 7.5 supplemented with 1 mM PMSF, and a protease inhibitor mixture (PIC; Sigma), giving a final concentration of 4 μg/mL of leupeptin, aprotinin, antipain, pepstatin, and chymostatin], and lysed by 20 strokes in a tightly fitting Dounce homogenizer. Cell debris was removed by centrifugations at 1,500 × *g* for 5 min, then at 5,000 × *g* for 5 min, and the supernatant gently layered on top of a discontinuous sucrose gradient (22–54%, in 1 mM $MgCl_2$, 10 mM Hepes pH 7.5, supplemented with 1 mM PMSF and PIC) and centrifuged for 2.5 h at 22,000 × *g* at 4 °C. Eleven fractions of 1 mL were collected from the top of the gradient, and nine were analyzed by SDS/PAGE and immunoblotting.

Antibodies and Western Blotting. Rabbit polyclonal anti-Gdt1p antibodies were raised against the cytosolic loop of Gdt1p (residues 119–185) expressed in *Escherichia coli* as a fusion product with GST (pGEX, GE Healthcare Life Science), purified using the immunogen coupled to SulfoLink Immobilization resin (Thermo Scientific) following the manufacturer's instructions, and used at a 1:333 dilution. The other primary antibodies used were rat anti-HA (10 ng/mL; Roche Applied Science), mouse anti-Pep12p (0.5 μg/mL; Invitrogen), rabbit anti-carboxypeptidase Y and anti-Emp47p (both 1:2,000; gifts from H. Riezman, Geneva), rabbit anti-Cdc48p (1:2,000; gift from M. Ghislain, Louvain-la-Neuve, Belgium), rabbit anti-Sec22p (1:2,000; gift from C. Barlowe, Hanover, NH) and rabbit anti-Pma1p [1:2,000; produced in our laboratory as described by Szopinska et al. (3)]. Horseradish peroxidase-coupled anti-rabbit IgG (1:10 000) and anti-rat IgG (1:10 000) antibodies were purchased, respectively, from Biognost and Thermo Scientific.

Yeast total protein extracts were prepared from amounts of cells equivalent to 1 mL of a culture with an OD_{600} of 3. The cells were centrifuged at maximum speed for 1 min (Mikro 120, Hettich), then 500 μl of the culture medium was left in the tube and the cells resuspended by vortexing. After addition of 50 μl of 1.85 M NaOH, the cells were lysed for 10 min on ice, then 50 μl of 50% trichloroacetic acid (TCA) was added and the sample mixed and incubated for 10 min on ice. The cell lysate was then centrifuged for 5 min at maximum speed, the supernatant discarded, and the pellet resuspended in 70 μl of a 1:1 mix of 1 M Tris and sample buffer (0.32 M Tris-HCl pH 6.8, 8% SDS, 40% glycerol, 0.02% bromophenol blue). Routinely, 10 μl of the extracts were separated on a 10% SDS/PAGE gel and transferred to a polyvinyl difluoride membrane (Millipore) using a semidry transfer system (Bio-Rad) in 50 mM Tris, 40 mM glycine, 1.3 mM SDS, 20% methanol. The blots were then blocked at room (RT) temperature for 30 min in blocking buffer (20 mM Tris-HCl pH 7.6, 150 mM NaCl, 6% wt/vol low fat dried milk, 0.5% vol/vol Tween 80), then were incubated with the primary antibody for 2 h at RT in TBS-Tween (20 mM Tris-HCl pH 7.6, 150 mM NaCl, 0.1% vol/vol Tween 80) containing 0.5% wt/vol low fat dried milk. After three washes of 5 min with TBS-Tween, the blots were incubated for 45 min at room temperature with the secondary antibody in TBS-Tween. After 3 × 5 min washes in TBS-Tween, bound antibodies were revealed using Lumi-Light Western Blotting Substrate (Roche Applied Science) and the emitted light captured with a Kodak 4000R Image Station, driven by Kodak Molecular Imaging Software v4.0.0.

Immunofluorescence. For yeast immunofluorescence experiments, cells were grown to an OD_{600} of 0.5 in 5 mL of YD medium, then formaldehyde was added to a final concentration of 3.7% directly to the culture and the mixture incubated for 10 min at RT. The cells were then harvested by centrifugation for 5 min at 1500 *g* and incubated in 1 mL of fixation buffer (0.1 M KH_2PO_4, 0.5 M $MgCl_2$, 3.7% formaldehyde, pH 6.5) for 20 min at RT. After centrifugation as above, the cells were resuspended in

1 mL of washing buffer (0.1 M KH_2PO_4, 1.2 M sorbitol, pH 6.5) supplemented with 4 μl of beta-mercaptoethanol and 4 μl of a 5 mg/mL stock solution of Zymolyase 2OT (Seikagaku Biobusiness), and the suspension incubated in a water bath at 37 °C for 20 min. The spheroplasts obtained were then harvested by centrifugation, washed twice with 1 mL of PBS (137 mM NaCl, 2.7 mM KCl, 8.1 mM $Na_2HPO_4 0.2H_2O$, 1.76 mM KH_2PO_4, pH 7.4) and resuspended in 100 μl of PBS supplemented with 0.05% of Tween 20 (PBST), then 4 μl of a 10 mg/mL stock solution of BSA was added and the mixture incubated for 20 min at RT. The primary rabbit anti-Gdt1p, mouse anti-Pep12p, or rat anti-HA antibodies were added at a dilution of 1:50 and the mixture incubated for 2 h at RT. Then the spheroplasts were centrifuged and washed twice with 1 mL of PBST for 5 min and resuspended in 100 μL of PBST. The secondary antibodies (anti-rabbit IgG conjugated to fluorescein, FITC, or TexasRed, anti-mouse IgG conjugated to TexasRed, or anti-rat IgG conjugated to Alexa Fluor 546, all from Invitrogen) were centrifuged at maximum speed for 5 min to pellet insoluble aggregates and added to the cells. From this point, the sample was protected from light. The mixture was incubated for 45 min at RT, then the spheroplasts were washed three times, mounted on a slide, and observed using a Leica Digital-Modul-R epifluorescence microscope with a 100× oil immersion objective, and pictures were taken with an Orca AG Hamamatsu digital charged-coupled device (CCD) camera driven by Wasabi 2.0 software.

For human cells, immunofluorescence experiments, cells were grown on glass coverslips for 12–24 h, washed once with PBS, and fixed by incubation for 25 min at RT in 4% formaldehyde in 0.1 M sodium phosphate buffer, pH 7.2. The coverslips were then rinsed twice with 0.1 M glycine in PBS for 15 min at RT, then were incubated for 1 h at RT with primary antibodies diluted in blocking solution [0.1% Triton X-100 (Sigma-Aldrich), 1% BSA (Roche), and 5% normal goat serum (Invitrogen)]. After washing with PBS, Alexa Fluor 488– or Alexa Fluor 568–conjugated anti-rabbit IgG antibodies (Invitrogen) diluted in blocking solution were applied for 1 h at RT. For plasma membrane localization, HeLa cells overexpressing TMEM165–red fluorescent protein (RFP) were labeled at 18 °C with polyclonal antibodies against RFP, washed, and directly fixed, then were labeled with Alexa Fluor 488–conjugated anti-rabbit IgG antibody to visualize TMEM165-RFP at the plasma membrane. Immunostaining was detected using an inverted Leica True Confocal Scanner- 5 SpectroPhotometer confocal microscope. Data were collected and processed in Adobe Photoshop 7.0 (Adobe Systems).

Luminometric Assays. The aequorin-encoding plasmid, pEVP11-Aeq, was a gift from S. Loukin (Madison, WI) (4, 5). Aequorin-based experiments were performed as described by Denis and Cyert (6) with minor modifications. Cells carrying the plasmids were grown overnight, then were used to inoculate a fresh culture to an OD_{600} of 0.005 in slightly modified SD medium (2% glucose, 0.2% yeast nitrogen base without amino acids and ammonium source, 0.35% NH_4Cl, and all amino acids except those required for maintenance of the plasmids) supplemented with 10 μM coelenterazine H (1 mM stock in methanol, Promega) and grown overnight at 28 °C to an OD_{600} of 3.0 to reconstitute aequorin from apoaequorin. For each culture, an aliquot of 200 μL was transferred to a luminometric tube and the baseline luminescence recorded every 0.4 s for 1 min using a Berthold FB12 luminometer. High calcium shocks were performed by injecting 100 μL of saline solution containing three times the desired final concentration (400 mM $CaCl_2$), and the signal induced was monitored over 4 min in the luminometer. To determine the cytosolic calcium concentration ($[Ca^{2+}]_{cyt}$) from the luminescence levels, aequorin was released by lysing the cells by addition of 400 μL of CE solution (2 M $CaCl_2$, 20% ethanol) and $[Ca^{2+}]_{cyt}$ values were calculated using equation derived by Allen et al. (7). All results shown are representative of those seen in at least three replicates.

siRNA. ON-TARGETplus SMARTpool siRNA oligonucleotides directed against TMEM165 mRNA were obtained from Dharmacon (Thermo Fisher Scientific). siRNA duplex (200 picomoles) was transfected into HeLa cells using Lipofectamine 2000, and knockdown efficiency was verified by real-time PCR on RNA harvested from the cells.

Flow Cytometric Analysis. Flow cytometric analysis of surface TMEM165-RFP on HeLa cells was performed by pelleting exponentially growing cells, washing the cell pellet (about 5×10^5 cells) twice with DMEM containing 1% (wt/vol) BSA (DMEM–BSA), and resuspending it in 500 μL of a 1:500 dilution of purified anti-RFP rabbit polyclonal antibodies in DMEM–BSA for 30 min at RT. The control sample was prepared by incubating cells with a 1:500 dilution of nonimmune rabbit antibodies. After three washes with DMEM–BSA, the cells were incubated with a 1:100 dilution of Alexa Fluor 488–conjugated goat anti-rabbit IgG (Molecular Probes) for 20 min at RT, washed twice in DMEM–BSA, and analyzed by flow cytometry.

Lysotracker and LysoSensor Green DND-189 Studies. In Lysotracker studies on the in vivo pH, cells were seeded into LabTek chamber slide dishes in DMEM/F12 supplemented with 10% FBS; then, the Lysotracker Red DND-99 probe (250 nM) (Invitrogen) was added and the mixture immediately analyzed by fluorescence microscopy, images being recorded every 2 min using a 60× oil objective and a 568 nm laser line for excitation.

The fluorescent probe LysoSensor Green DND-189 (Invitrogen) was used to study lysosomal/endosomal pH homeostasis. Control or TMEM165 or V0-ATPase–deficient patients' fibroblasts, HeLa cells, and siRNA TMEM165-treated cells were seeded in LabTek chamber slide dishes in DMEM/F12 supplemented with 10% FBS. For the experiment, the cells were loaded by 30 min incubation at 37 °C with 1 μM LysoSensor Green in DMEM/F12 Hepes medium without serum and washed twice with the same medium; then pictures of living cells were taken using a Leica AF 6000 LX immunofluorescence microscope. Images were recorded using a 60× oil objective and a 448 nm laser line for excitation and 504 nm for emission.

Electrophysiological Recordings. Patch-clamp recordings were performed in the whole-cell configuration (8) using a RK-300 patch-clamp amplifier (Biologic) as described previously (9). The patch-clamp amplifier was driven by Pulse 8.30 software (HEKA Elektronik). Membrane currents were digitized at 20 kHz using an ITC16 computer interface (Instrutech Corp.), low-pass filtered at 3 kHz, and stored online on the hard drive of the computer. Electrodes were pulled on a pipette vertical puller (PIP5) (HEKA) in two stages from borosilicate glass capillaries (patch clamp glass 52151, World Precision Instruments) to a tip diameter giving a pipette resistance of 5 MΩ. For each cell, the membrane potential was clamped initially at –80 mV, and the passive membrane components (membrane resistance and capacitance) were determined immediately after the establishment of the whole-cell configuration to estimate the cell surface area. Membrane currents were then normalized to the cell surface area.

For measuring membrane currents in the whole-cell configuration, the external buffer was 142 mM NaCl, 1 mM $MgCl_2$, 10 mM Hepes, 5.6 mM glucose, and 2 mM $CaCl_2$ with or without 10 mM TEA-Cl (tetra-ethyl ammonium chloride). The osmolarity and pH of the external buffer were adjusted to 310 mOsm. l^{-1} and 7.4, respectively. The pipette solution was 140 mM K-gluconate or N-Methylglucamine-Gluconate, 0.1 or 10 mM EGTA, 1 mM MgCl2, and 5 mM Hepes. Osmolarity and pH were adjusted to 290 mOsm.l^{-1} and 7.2, respectively.

Calcium Imaging. Cells were plated onto glass coverslips and loaded with 4 μM FURA–2:00 AM (Invitrogen) at RT for 45 min in DMEM containing 10% FBS. Recordings were then performed in HBSS (140 mM NaCl, 5 mM KCl, 2 mM $MgCl_2$, 0.3 mM Na_2HPO_3, 0.4 mM KH_2PO_4, 4 mM $NaHCO_3$, 5 mM glucose, and 10 mM Hepes adjusted to pH 7.4 with NaOH). $CaCl_2$ was adjusted to 2 mM. The coverslips were then placed in a perfusion chamber on the stage of the microscope and fluorescence images recorded with a video image analysis system (Quanticell). FURA–2:00 AM fluorescence at the emission wavelength of 510 nm was recorded by exciting the probe alternately at 340 and 380 nm. When used, thapsigargin was at the final concentration of 1 μM.

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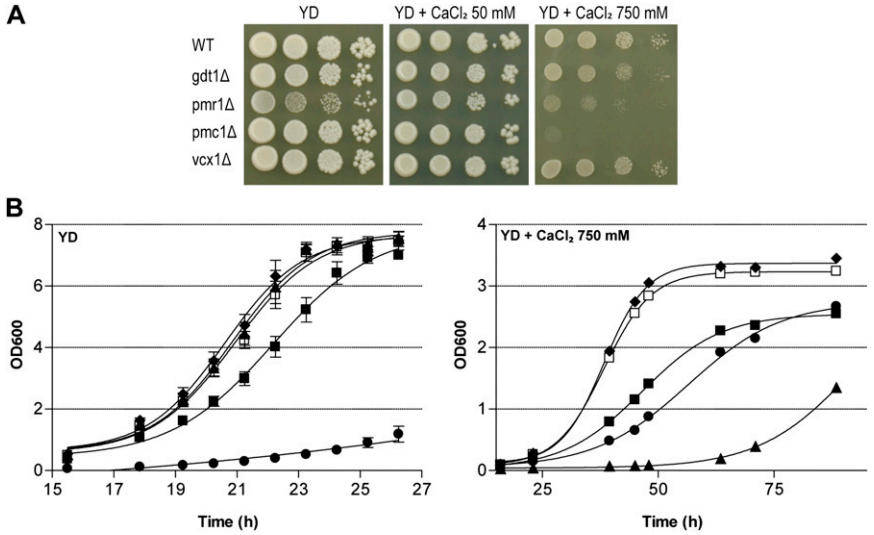

Fig. S1. The *gdt1*Δ yeast mutant is sensitive to a high concentration of Ca^{2+}. (*A*) The different mutants were grown in rich liquid medium (YD) to an OD_{600} of 0.3, then serial 10-fold dilutions were dropped onto solid YD medium (*Left*), supplemented with a moderate (50 mM; *Center*) or high (750 mM; *Right*) concentration of $CaCl_2$, and incubated at 28 °C for 4–6 d. (*B*) The different mutants were grown in rich liquid medium (YD) to an OD_{600} of about 3, and then adjusted to the same density in liquid YD medium (*Left*) or liquid YD medium supplemented with high concentration of $CaCl_2$ (750 mM, *Right*). OD_{600} was measured at the indicated times. Error bars represent mean ± SEM (n = 3). WT, ◆; *gdt1*Δ, ■; *pmr1*Δ, ●; *pmc1*Δ, ▲; *vcx1*Δ, □.

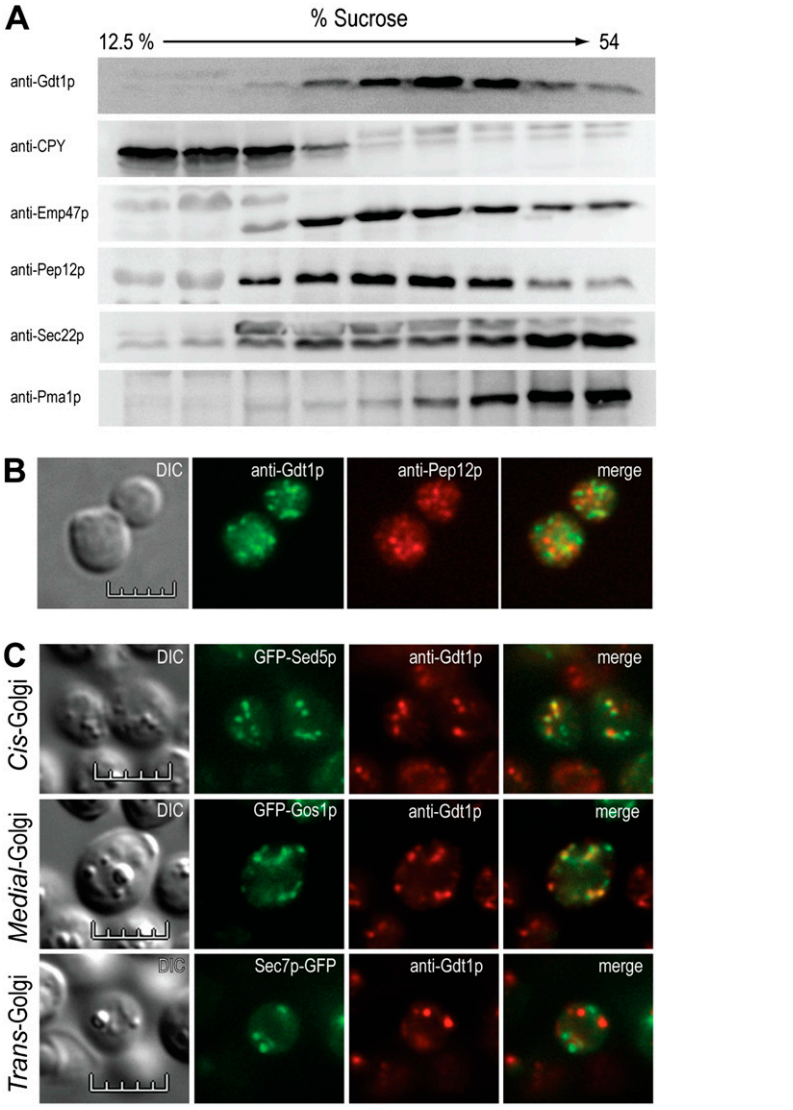

Fig. S2. Gdt1p is localized in the early Golgi apparatus. (*A*) Subcellular fractionation on sucrose gradient. The fractions collected from the top of a discontinuous 10-step sucrose gradient were analyzed by Western blotting using antibodies against Gdt1p or markers of the different subcellular compartments (CPY, vacuole; Emp47p, Golgi apparatus; Pep12p, endosomes; Sec22p, endoplasmic reticulum; Pma1p, plasma membrane). (*B*) Co-immunolocalization of Gdt1p and Pep12p by double staining. Gdt1p and Pep12p were labeled using rabbit or mouse antibodies, respectively, FITC-conjugated anti-rabbit IgG and TexasRed-conjugated anti-mouse IgG antibodies. (*C*) Co-immunolocalization of Gdt1p and GFP-fused markers for different compartments of the Golgi apparatus (Sed5p, *cis*-Golgi; Gos1p, *medial*-Golgi; Sec7p, *trans*-Golgi). Gdt1p was labeled using a mouse primary antibody and a TexasRed-conjugated anti-rabbit IgG antibody, whereas the Golgi markers were visualized by the GFP fluorescence. Differential interference contrast (*DIC*) and merged (*merge*) pictures are also presented. (Scale bar, 5 μm.)

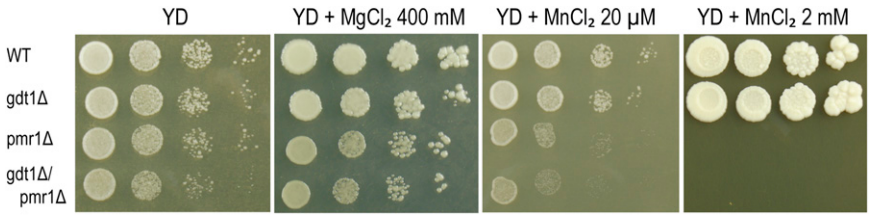

Fig. S3. Growth of the *gdt1*Δ yeast mutant is not affected by Mg^{2+} or Mn^{2+}. The different mutants were grown in rich liquid medium (YD) to an OD_{600} of 0.3, then serial 10-fold dilutions were dropped onto solid YD medium (*Left*), supplemented with 400 mM $MgCl_2$, 20 μM $MnCl_2$, or 2 mM $MnCl_2$, and incubated at 28 °C for 4–6 d.

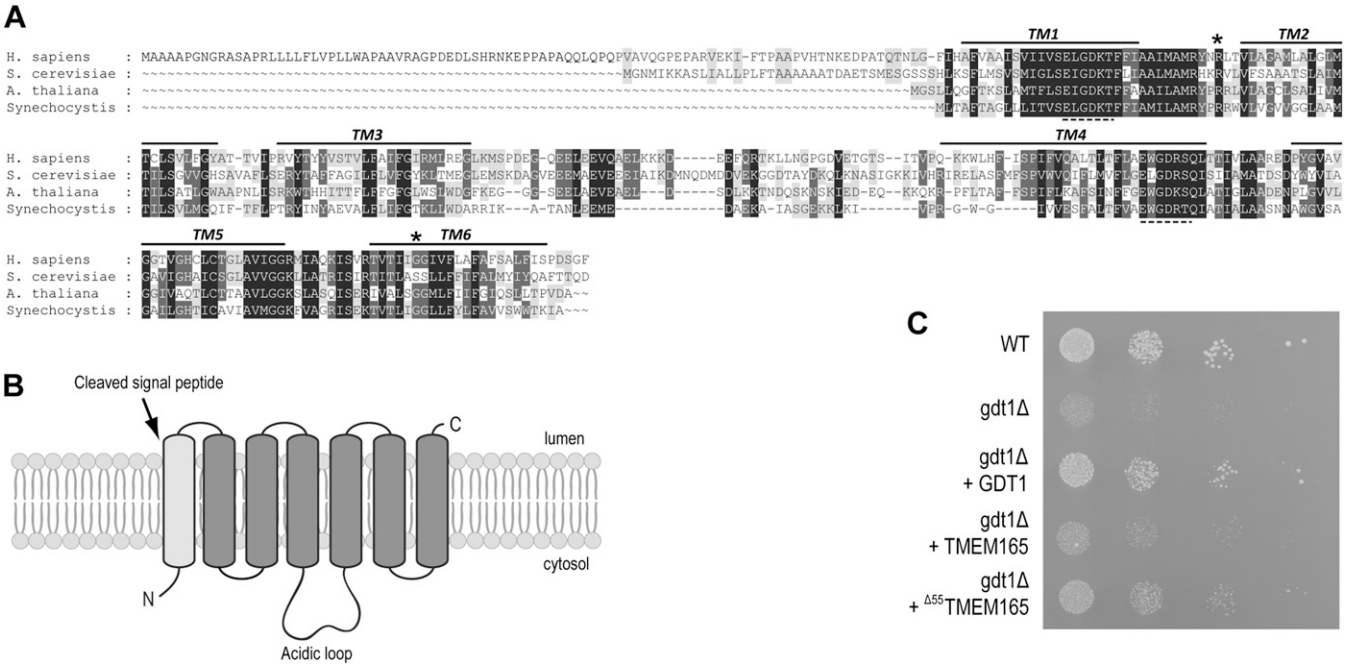

Fig. S4. Members of the Uncharacterized Protein Family0016 family are highly conserved throughout evolution and contain six transmembrane spans. (*A*) Multiple sequence alignment generated using the ClustalW2 program. Residues that are similar in four, three, or two sequences are shaded black, dark gray, or pale gray, respectively. Putative transmembrane spans predicted from the multiple alignment with the program Tmap are indicated by bars on the sequence. The two highly conserved internal E-x-G-D-(KR)-(TS) motifs are indicated by the two dashed lines, and positions of the patients' missense mutations are indicated by stars. National Center for Biotechnology Information accession codes: *Homo sapiens*, NP_060945.2 (transmembrane protein 165); *Saccharomyces cerevisiae*, NP_009746.1 ; *Arabidopsis thaliana*, NP_177032.1; *Synechocystis* sp. *PCC 6803*, NP_442278.1. (*B*) Topology model of the yeast ortholog, Gdt1p. The six transmembrane spans are depicted, the N-terminal signal peptide is predicted to be cleaved (SignalP 4.0), and the central acidic loop is predicted to face the cytosol. (*C*) Expression of a truncated version of TMEM165, the human ortholog of Gdt1p, partially suppresses the Ca^{2+} sensitivity of the *gdt1*Δ yeast mutant. Wild-type (WT) cells and the *gdt1*Δ mutant transformed with empty pRS416 plasmid, pRS416-expressing GDT1, human TMEM165, or its truncated version, $^{\Delta 55}$TMEM165, were precultured in minimal medium without uracil (SD-U) to an OD_{600} of 0.3, then serial 10-fold dilutions were dropped onto solid SD-U medium supplemented with 750 mM $CaCl_2$ and the plates incubated at 28 °C for 4–6 d.

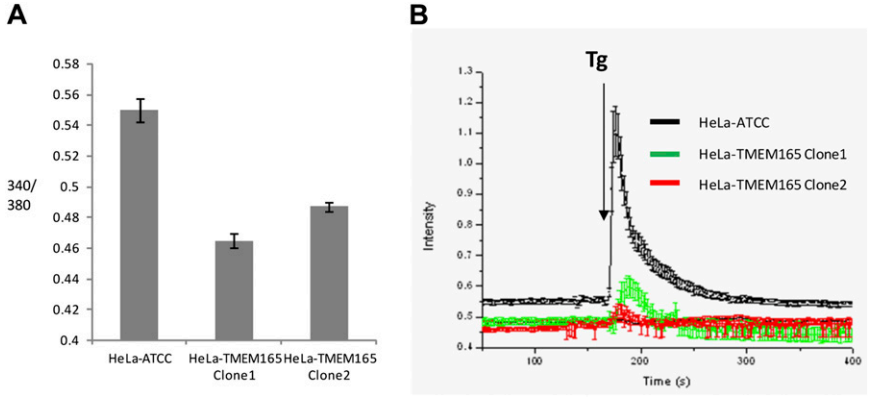

Fig. S5. Cytosolic Ca^{2+} measurements in TMEM165-HeLa cells. (*A*) Basal 340/380 fluorescence intensities in absence of thapsigargin (error bars indicate mean ± SEM; $n = 3$, >70 cells/experiment). (*B*) Intracellular Ca^{2+} recording (340/380 nm) from Fura–2:00 AM loaded HeLa–ATCC cells and two stably overexpressing TMEM165-HeLa cells (clones 1 and 2). ER Ca^{2+} stores were emptied with 1 μM thapsigargin (Tg).

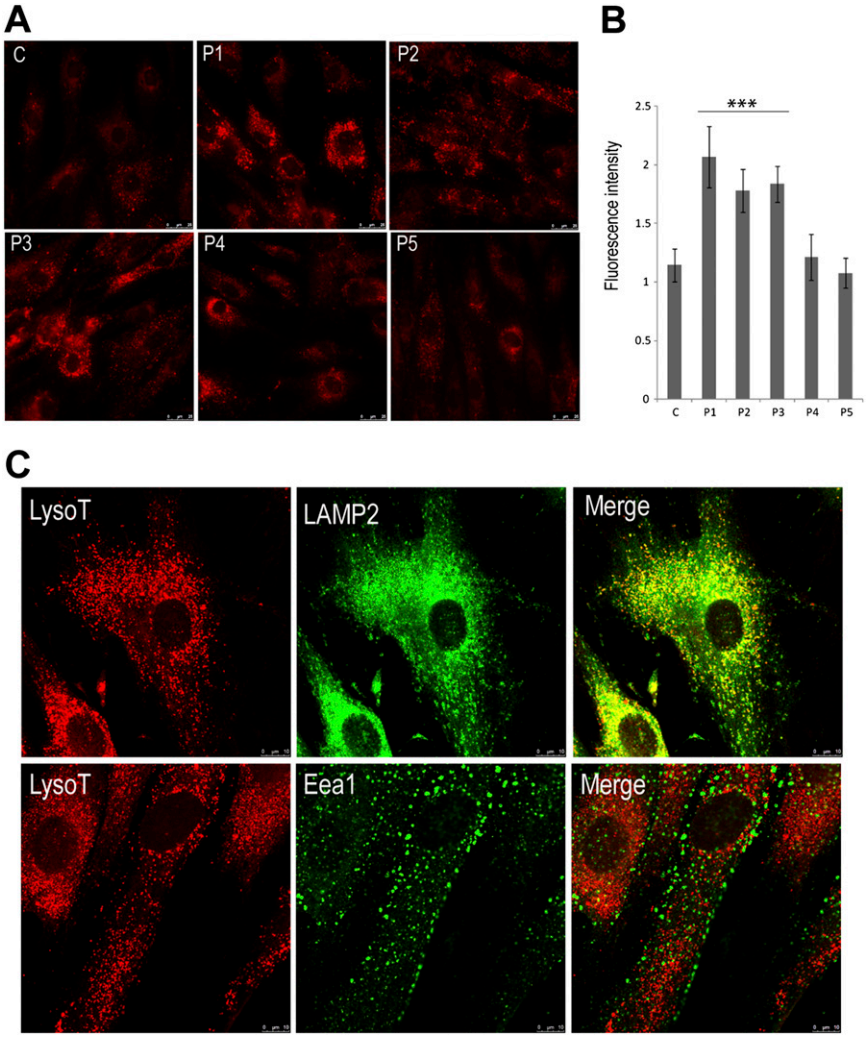

Fig. S6. Increased lysosomal lysotracker red staining in TMEM165-deficient patients' cells. (*A*) Live cell fluorescent images of lysosomes using Lysotracker Red DND-99 in fibroblasts from a control and five CDG patients (P1, P2, and P3 with the c.792+182G > A mutation, P4 with the homozygous c.377G > A; p.R126H mutation, and P5 a compound heterozygous for the c.376G > A; p.R126C and the c.910G > A; p.G304R missense mutations). The results shown are representative of those obtained with multiple cells assessed in independent experiments. (*B*) Quantification of the red fluorescence of the cells in *A*. Error bars indicate mean ± 95% confidence interval (n = 3, 10 cells per experiments). Data were analyzed by one-way ANOVA followed by Student post hoc test to identify mean differences between patients' cells and positive control. *P* values less than 0.001 were considered as significant and indicated by ***. Statistical analyses were performed using the software JMP 10 (SAS Institute). (*C*) Lysotracker and either *lysosomal-associated membrane protein 2* or Eea1 immunostaining of fibroblasts from a c.792+182 G > A mutation patient (P1).

Table S1. Yeast strains and mutants used in this study

Strain	Genotype	Source
BY4741	*Mata his3Δ1 leu2Δ0 ura3Δ0 met15Δ0*	Euroscarf
BY4742	*Matα his3Δ1 leu2Δ0 ura3Δ0 lys2Δ0*	Euroscarf
BY4741 *gdt1Δ*	*Mata his3Δ1 leu2Δ0 ura3Δ0 met15Δ0 gdt1::KanMX4*	Euroscarf
BY4742 *gdt1Δ*	*Matα his3Δ1 leu2Δ0 ura3Δ0 lys2Δ0 gdt1::KanMX4*	Euroscarf
BY4741 *pmr1Δ*	*Mata his3Δ1 leu2Δ0 ura3Δ0 met15Δ0 pmr1::KanMX4*	Euroscarf
gdt1Δ/pmr1Δ	*Mata his3Δ1 leu2Δ0 ura3Δ0 lys2Δ0 met15Δ0 gdt1::KanMX4 pmr1::KanMX4*	This study
BY4742 *pmc1Δ*	*Matα his3Δ1 leu2Δ0 ura3Δ0 lys2Δ0 pmc1::KanMX4*	Euroscarf
BY4741 *vcx1Δ*	*Mata his3Δ1 leu2Δ0 ura3Δ0 met15Δ0 vcx1::KanMX4*	Euroscarf