Impact of disease-causing mutations on TMEM165 subcellular localization, a recently identified protein involved in CDG-II

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TMEM165 has recently been identified as a novel protein involved in CDG-II. TMEM165 has no biological function described so far. Different mutations were recently found in patients with Golgi glycosylation defects and harboring a peculiar skeletal phenotype. In this study, we examined the effect of naturally occurring mutations on the intracellular localization of TMEM165 and their abilities to complement the TMEM165-deficient yeast, gdt1Δ. Wild-type TMEM165 was present within Golgi compartment, plasma membrane and late endosomes/lysosomes, whereas mutated TMEM165 were found differentially localized according to the mutations. We demonstrated that, in the yeast functional assay with TMEM165 ortholog Gdt1, the homozygous point mutation correlating with a mild phenotype restores the yeast functional assay, whereas the truncated mutation, associated with severe disease, failed to restore Gdt1 function. These studies highly suggest that these clinically relevant point mutations do not affect the protein function but critically changes the subcellular protein localization. Moreover, the data point to a critical role of the YNRL motif in TMEM165 subcellular localization.

INTRODUCTION

Congenital disorders of glycosylation (CDG) are severe inherited diseases in which protein N-glycosylation is affected. These result either from a lack of N-glycosylation site occupancy and/or from the presence of abnormal N-glycan structures (1). To date, nearly 40 different causes of CDG have been defined genetically and we recently identified a new subtype of CDG caused by mutations in TMEM165 (2). TMEM165 is a hydrophobic protein of 324 amino acids containing seven predicted transmembrane-spanning domains (Fig. 1). The amino acid sequence of TMEM165 is highly conserved between vertebrates, Caenorhabditis elegans and yeasts. Analysis of the primary sequence did not show obvious targeting signals or motifs indicative of its function or intracellular location. The biochemical role of TMEM165 is currently not known.

According to databases, TMEM165 presents a cytoplasmic loop of 52 amino acids organized in a coiled coil domain, two EXGDK/R motifs and a putative lysosomal-targeting sequence YNRL (belonging to the classical YXXØ lysosomal-targeting signal) (Fig. 1). Lysosome-targeted membranous proteins indeed possess cytosolic tyrosine-based sorting signals, such as NPXY or YXXØ, and di-leucine motifs (DXXLL or [DE]XXXL[LI]) (3). YXXØ and [DE]XXXL[LI] motifs interact with heterotetrameric adaptor protein (AP) complexes AP1, AP2, AP3 or AP4 (3), which recruit clathrin to initiate the formation of coated vesicles (4,5). These motifs function as lysosomal-targeting signals at the Trans Golgi network (TGN) and are usually found in close proximity to a transmembrane segment. Lysosomal membrane proteins can travel from TGN to lysosomes by two major pathways: a direct one to endosomes/lysosomes or an indirect one via...
the plasma membrane (PM) and re-uptake by endocytosis (3,6,7).

Three different CDG-causing mutations have been found in TMEM165 by sequencing this culprit gene at both the genomic DNA and cDNA levels. Based on this approach, a homozygous mutation at the position c.792+182G>A (IVS4+182G>A) was found in three patients, two of them belonging to the same family. The mutation activates a cryptic splice donor site leading to two different transcripts: the wild-type (wt) one and an additional one resulting in the skipping of exon 4 and replacing it by 117 base pairs of intronic sequence. At the protein level, this mutation leads to a truncated protein lacking 94 amino acids in which 27 amino acids changed at the C-terminal part (2). Other mutations in TMEM165 have been described in two different patients: one homozygous patient for the missense mutation c.377G>A (R126H) and another patient compound heterozygous c.376C>T (R126C) and c.911G>A (G304R).

Interestingly, it appears that two of the three identified missense mutations affect the YXXØ-targeting lysosomal signal. We then investigated the impact of TMEM165 mutations on subcellular localization and function. Because of the absence of any known molecular or cellular function of TMEM165, we used yeast to address the latter question. Our results show that mutations mainly affect the subcellular localization of TMEM165. Although the truncated mutation abrogates the function of TMEM165 ortholog in yeast, the missense mutations have no effects.

RESULTS
Subcellular localization of the wt and mutated transiently expressed TMEM165 proteins

Determination of the subcellular localization of wt and mutant TMEM165 was undertaken to further understand the pathophysiology of the CDG linked to this protein. In order to assess the impact of TMEM165 mutations on subcellular protein localization, different clones containing the disease-causing mutations were generated from patients’ cDNA. C-terminal RFP-tagged wt and mutated TMEM165 constructs have been generated, transiently transfected in HeLa cells and visualized by laser scanning confocal microscopy. As shown in Figure 2A, wt and mutated TMEM165 show distinctive staining patterns suggesting differential subcellular localizations.

First, no expression could be detected for the TMEM165 (c792+182 G>A) mutant that normally leads to a putative truncated protein. This has been confirmed by western blotting experiments (Supplementary Material, Fig. S1A). The wt-TMEM165 was clearly detected concentrated in unpunctuated region together with vesicular structures localized throughout the cytoplasm (Fig. 2A). In contrast to this, there were clear differences of subcellular localization/expression among TMEM165 (R126H), (R126C), (G304R) and (R126C, G304R) mutants. Although the unpunctuated staining was found decreased, the localization of the missense mutant TMEM165 (R126H) was basically the same as wt (Fig. 2A). In order to study the subcellular localization of patients’ compound heterozygous mutations, TMEM165 (R126C), (G304R) and the double-mutant (R126C, G304R) were observed separately. Localization pattern of TMEM165 (R126C) appeared to be very similar to the R126H mutant. On the other hand, subcellular localization of TMEM165 (G304R) and TMEM165 (R126C, G304R) mutants was found to be highly different from the others with no vesicular staining observed. Only a concentrated staining could be observed for these mutants (Fig. 2A). Interestingly, TMEM165 (R126C, G304R) localization is determined by the G304R mutation.

To further delineate the differential subcellular localizations of the wt and mutated TMEM165, immunofluorescence staining using organelle-specific antibodies followed by confocal microscopy was performed. Colocalization of wt and mutated TMEM165 was then analyzed using the intracellular...
Figure 2. Subcellular localization of transiently expressed wt and mutated TMEM165. (A) Wt-TMEM165 (upper, left panel), TMEM165(c.792+182 G>A) (upper, right panel), TMEM165(R126H) (middle, left panel), TMEM165(R126C) (middle, right panel), TMEM165(G304R) (lower, left panel) and TMEM165(R126C, G304R) (lower, right panel) were coupled to C-terminal RFP tag, transiently expressed in HeLa cells and then visualized by confocal microscopy. (B) Cells transiently expressing wt-TMEM165 (upper, left panel), R126H mutant (upper, right panel), R126C mutant (middle, left panel), G304R mutant (middle, right panel) and R126C, G304R double-mutant (lower, left panel) were double-stained with antibodies against LAMP2 (lysosomal marker) and TGN46 (Golgi marker). Alexa 488- or Alexa 700-conjugated secondary antibodies allowed visualization of LAMP2 (green) and TGN46 (blue), respectively. Cells were then examined using confocal microscopy and colocalizations of fluorescent TMEM165 (in red) with Golgi and endo/lysosomal markers were quantified using the LAS AF Lite software 2.6.0 (Leica Microsystems). Errors bars were determined from 10 transiently transfected cells.
Figure 2. Continued
markers TGN46, LAMP2 and EEA1. TGN46 is a specific marker from the TGN compartment; LAMP2 is known to mark late endosomes and lysosomes, whereas EEA1 resides predominantly within early endosomes. As shown in Figure 2B and Supplementary Material, Figure S2, our data indicate that wt-TMEM165 localizes in the Golgi compartment as well as in late/early endosomes and lysosomes. The level of colocalization was quantified and reveals a partial colocalization of TMEM165 with TGN46 (53%) and with LAMP2 and EEA1 (47%; Fig. 2B). In contrast to the wt form of TMEM165, the Golgi localization was less marked for the mutated TMEM165 (R126H) and (R126C), as proven by the 18 to 28% of colocalization of these forms with TGN46, respectively. As expected, a more pronounced colocalization with LAMP2 and EEA1 is observed for TMEM165 (R126H) and (R126C) (Fig. 2B and Supplementary Material, Fig. S2). Remarkably, mutated TMEM165 (G304R) and (R126C, G304R) were predominantly localized in the Golgi (64 and 78% of colocalization with TGN46, respectively). Only subtle amount of these mutated forms could be found in LAMP2- and EEA1-positive structures (Fig. 2B and Supplementary Material, Fig. S2). All together, these results demonstrate that the identified mutations in TMEM165 affect the protein subcellular localization.

**Subcellular localization of the wt and mutated endogenous TMEM165 proteins**

These results, however, seem to be contradictory with the subcellular localization of the endogenous TMEM165 (2). We recently, indeed, found that endogenous TMEM165 is strictly Golgi-localized and no pool could be observed neither in lysosomes nor in early endosomes. We first thought that this discrepancy was due to the RFP tag, but similar results were obtained with green fluorescent protein (GFP) (see further in the paper) or smaller tags (results not shown). This, hence, led us to investigate by biochemical approaches the subcellular localization of the endogenous TMEM165. For this, subcellular organelles from homogenized control and TMEM165-deficient patients’ cells were separated on Percoll density gradients. This allows achieving separation between dense lysosomal endosomes like organelles and low-density organelles such as endoplasmic reticulum and Golgi compartments. After cell fractionation, Golgi and lysosomes/endosomes were resolved by ultracentrifugation in Percoll density gradients, and markers for lysosomes and Golgi were used, respectively. As expected and in all investigated patients’ cells, GPP130, a Golgi marker, was found in the lighter fractions of the gradient and peaked in fractions 1–3 (Fig. 3). The presence of LAMP2, a lysosomal and late endosomal marker, was found in Golgi-positive fractions but also enriched in denser fractions, 4–9 (Fig. 3). In order to check the presence of intact lysosomes in the LAMP2-positive fractions, the activity of β-hexosaminidase in each gradient fraction using p-nitrophenyl-N-acetyl-β-D-glucosaminide as a substrate was determined (Fig. 3C). A beta-hexosaminidase activity was clearly observed in all LAMP2-positive fractions, and ~50% of the beta-hexosaminidase activity was found in fractions 3–9 totally devoid of Golgi as proved by the absence of GPP130 (Fig. 3C). To assess the subcellular localization of TMEM165, the different fractions were probed for TMEM165. Remarkably, for control and patients’ cells, TMEM165 was present in both light and dense gradient fractions. However, a significant fraction of TMEM165 was highly enriched in the dense fractions of the Percoll gradient containing both LAMP2 and the β-hexosaminidase activity (Fig. 3A and C). Importantly, these fractions were not containing any Golgi markers, demonstrating again that endogenously expressed TMEM165, besides its Golgi localization, also localizes in dense lysosomal/endoosomal compartments. Surprisingly it has to be noted that TMEM165 distribution appears to be the same between control and TMEM165-deficient patients’ cells (Fig. 3A). For TMEM165 (R126H), the general distribution could be explained by the lack of resolution of the gradient for the lysosomal fractions, as LAMP2 distribution is widely found from fractions 1–9. Concerning TMEM165 (R126C, G304R), these mutations are compound heterozygotes. TMEM165 (R126C) is known to lead to a more pronounced lysosomal localization, whereas G304R lies in Golgi apparatus. In terms of density gradient, this could explain why the distribution of TMEM165 (R126C, G304R) is found in light as well as dense fractions. Overall, our results demonstrate that endogenous TMEM165 localizes both in Golgi apparatus and in acidic compartments.

**TMEM165 contains two putative lysosomal-targeting signals**

The membrane topology of TMEM165 is characterized by seven transmembrane domains (six if the signal peptide is cleaved off) (2) and three cytosolic loops (Fig. 1). In the predicted cytosolic segments, two motifs could be used to target the protein to lysosomes/endosomes. A potential tyrosine-based sorting motif is present in the first cytosolic loop of TMEM165: Y124NRL127. Moreover, one leucine pair (L209L210) is found in a non-canonical [DE][XXX][L]I signal in the central cytosolic loop. We intended to evaluate the impact of these potential signals on late endosomal/lysosomal targeting of TMEM165 (Fig. 4 and Supplementary Material, Fig. S3). To investigate this, the following mutations have been introduced into TMEM165: Y124S, Y124F, L127G and L209G/L210G. These constructs were fused with C-terminally appended RFP tag, used for transfection in HeLa cells and analyzed by confocal microscopy with Golgi (TGN46) and endo/lysosomal markers (EEA1 and LAMP2, respectively). Interestingly, whereas the wt-TMEM165 shows 53% of Golgi localization, mutant Y124S clearly accumulates in the Golgi compartment with 70% of colocalization with Golgi markers (Fig. 4 and Supplementary Material, Fig. S3). It has to be noted that the tyrosine amino acid of this motif is crucial for the stability of TMEM165, as the Y124F mutant shows undetected signal due to a reduced level expression as confirmed by western blot (Supplementary Material, Fig. S1B). However, mutation L127G in the same tyrosine-based motif does not affect TMEM165 localization. The disruption of the two leucine motif L209G and L210G
**Figure 3.** Fibroblast control and patient cell fractionation on a Percoll density gradient. (A) Organelles of primary skin fibroblast cells from control (upper panel) and patients with mutations R126H (middle panel) and R126C, G304R (lower panel) were separated on a Percoll gradient density gradient (Amersham Biosciences). The indicated fractions were analyzed by SDS–PAGE and immunoblotted using the indicated antibodies. (B) Quantifications of the relative intensities of GPP130 (blue diamond), LAMP2 (red square) and TMEM165 (green triangle) were done with the ImageJ 1.45 software. (C) Lysosomal marker enzyme β-hexosaminidase activity with p-nitrophenyl-N-acetyl-β-D-glucosaminide substrate was measured in each Percoll gradient fraction of control and probed using the indicated antibodies. Relative β-hexosaminidase activity is presented (brown curve). The curve in blue illustrates the relative β-hexosaminidase activity normalized to LAMP2 intensity.
Figure 4. Involvement of putative lysosomal-targeting signals on subcellular localization of TMEM165. Wt-TMEM165 (upper, left panel), Y124S mutant (upper, right panel), L127G mutant (lower, left panel) and L209G, L210G mutant (lower, right panel) were coupled to C-terminal RFP tag and transiently expressed in HeLa cells. These cells were double-labeled with antibodies to LAMP2 (lysosomal marker) and with antibody against TGN46 (Golgi marker). Secondary antibody Alexa 488 was used to highlight LAMP2 in green, whereas Alexa 700 allowed to visualize TGN46 (in blue). Wt and mutated TMEM165 (red) were then examined by confocal microscopy and colocalizations of TMEM165 with Golgi and endo/lysosomal markers were quantified using the LAS AF Lite software 2.6.0 (Leica Microsystem). Errors bars were determined from 10 transiently transfected cells.
does not significantly impair the TMEM165 localization, demonstrating that this motif is not involved in endosomal/lysosomal targeting of TMEM165 (Fig. 4).

**TMEM165 reaches lysosomes from the PM**

In mammalian cells, two intracellular pathways can be used by newly synthesized proteins to reach lysosomes: a direct pathway in which proteins are directly targeted to late-endosomes/lysosomes from the TGN and an indirect pathway in which newly synthesized proteins are sent to the PM from the TGN before being internalized and delivered to lysosomes (reviewed in 8–10). Given the subcellular localization of TMEM165, we investigated the intracellular pathways by which TMEM165 reaches the endosomes/lysosomes compartments. We first looked for the presence of TMEM165 at the PM. For this, wt-TMEM165 or its mutated forms were fused with a C-terminal GFP tag and transiently expressed in HeLa cells. According to the topology of TMEM165 ([Fig. 1]; (2)), the GFP tag should be exposed to the extracellular space if the protein is targeted to the PM. To check PM localization of TMEM165, cells were first incubated at 18°C with polyclonal antibodies against GFP (anti-GFP). After 10 min, they were fixed, permeabilized and labeled with Alexa 568-conjugated secondary antibody (red) to visualize TMEM165-GFP at the PM by confocal microscopy (Fig. 5A and B, upper panel). The wt-TMEM165, as well as the R126H and R126C mutants, were clearly visible at the PM (Fig. 5A, 0 min). As expected from our previous results, no PM localization of the (R126C, G304R) and (Y124S) TMEM165 mutants was observed (Fig. 5B, 0 min). Interestingly and compared with the wt and with the R126C mutant, the mutated TMEM165(R126H) is found more abundant at the PM.

To confirm these results, wt and mutated TMEM165 were fused with a pH-sensitive GFP tag (pHluorin GFP). This probe is brightly fluorescent when exposed to a neutral pH, as it is the case of the extracellular space, but does not emit fluorescence in acidic compartments (11). HeLa cells were transiently transfected with the different constructs and visualized by live confocal microscopy (Supplementary Material, Fig. S4). The wt-TMEM165 staining, although subtle, was clearly visible at the PM. Interestingly and compared with the wt, the TMEM165(R126H) as well as the TMEM165(R126C) mutants were found much more present at the PM as revealed by the bright staining present at the periphery of the cells. As expected, no PM localization of the TMEM165 (R126C, G304R) and (Y124S) was observed. Altogether, these data strongly support that wt and some of the TMEM165-mutated forms can be targeted to the PM. These results point to a crucial role of the Y124NRL127 of TMEM165 delays the internalization of the protein.

**Complementation analysis in Gdt1-deficient yeast cells**

Our data clearly demonstrate that the missense mutations of TMEM165 found in CDG-II patients affect its subcellular localization. In order to address whether the homozygous mutations could also affect the function of TMEM165, we used the yeast strain lacking GDT1, the yeast ortholog of TMEM165. GDT1 presents 38% identical amino acid sequence with human TMEM165, and gdt1Δ yeasts display a strong growth defect when cultured in the presence of high concentrations of calcium dichloride (CaCl2) (http://www.yeastgenome.org). The wt and mutated TMEM165 human cDNAs were cloned into yeast expression plasmids. The human TMEM165 cDNA was first tested for complementation of the CaCl2-sensitive phenotype conferred by the GDT1 null mutation. Unfortunately, despite the quite high conservation of amino acids (38%), human TMEM165 could not complement the yeast Gdt1 mutant phenotype, which is likely due to the different subcellular localizations of the proteins (data not shown).

To determine whether the mutations, identified in the patients, effectively affect TMEM165 activity, the corresponding changes were introduced in the Saccharomyces cerevisiae GDT1 gene. Thus, gdt1Δ cells transformed with the pRS416 vector, with pRS416 expressing wt GDT1 or with pRS416 expressing trunc-GDT1 (corresponding to the frame shift induced by c.792+182G>A in the patient), R71H (equivalent to p.R126H) or R71C (equivalent to p.R126C, p.G304R being not conserved in yeast) were grown in the presence of high CaCl2 concentration. The truncated mutant GDT1 failed to complement this growth defect (Supplementary Material, Fig. S5), thus confirming its deleterious effects on the GDT1 protein. Interestingly, R71H and R71C mutations have, however, no effects on the function of the GDT1 protein, since growth of gdt1Δ yeast was restored by the expression...
Figure 5. Localization of TMEM165 to the PM and internalization kinetics of wt and mutated TMEM165. Living cells transiently expressing wt or mutated TMEM165 coupled to C-terminal GFP tag were incubated with antibody raised against GFP that was exposed to the extracellular space. After 0 min to 2 h incubation, cells were immunostained with Alexa 568-conjugated antibody. As a result, TMEM165 from the secretory pathway appears in green, whereas TMEM165 both at the PM and endocyted into the cells is stained in red. (A) Internalization kinetics of wt, TMEM165(R126H) and TMEM165(R126C). (B) Internalization kinetics of wt, TMEM165(Y124S) and TMEM165(G304R, R126C).
Figure 5. Continued
DISCUSSION

The present work relates the impact of TMEM65 patients’ mutations on the subcellular localization of the protein. First, we have shown that TMEM165 localizes both in Golgi apparatus and in acidic compartments, following the indirect pathway to reach endo/lysosomes through the PM. Interestingly, although complementation experiments in yeasts have shown that TMEM165 function is unaffected, its subcellular localization differs according to patients mutations. The use of transiently expressed TMEM165 differs from our previous immunostaining experiments on patients’ cells, showing that endogenous TMEM165 only localizes in Golgi apparatus (2). This discrepancy could either be due to the tag or to the over-expression of wt and mutated TMEM165. However, the same results were obtained with GFP (Fig. 5 and Supplementary Material, Fig. S4) or smaller tags (results not shown) and the pattern of subcellular localization of TMEM165 differs between wt and mutated transiently expressed protein. Moreover, cell fractionation followed by immunoblotting experiments gave evidence that endogenous TMEM165 localizes both in Golgi apparatus and in lysosomes. How can we explain that endogenous TMEM165 can only be observed in Golgi apparatus using immunostaining? One logical explanation could be that the antibody would recognize TMEM165 lying in endo/lysosomes only in denaturing conditions. Interestingly, the modeling software ch.embnet.org, indeed, predicts that the antibody recognition site is structured in a coiled coil, a protein–protein interaction domain. The antibody-targeting sequence (R176 to Q226, corresponding to the second cytoplasmic loop) would be hidden in acidic compartments because of TMEM165 partners, whereas this recognition site would be free in Golgi apparatus. TMEM165 amino acid sequence is well conserved between human and yeast. Putative lysosomal-targeting signal YNRL is, however, not found in the Gdt1 protein sequence (NP_009746.1), the yeast TMEM165 ortholog, as blast analysis revealed the presence of this motif in TMEM165 orthologs only from vertebrates. YXXØ is a tyrosine-based motif involved in the targeting of lysosomal transmembrane proteins that follow either direct or indirect pathway (through the PM) to reach lysosomes (7). Here, we have highlighted that wt and mutated TMEM165(R126H) and (R126C) reach lysosomes by following the indirect pathway. The vesicular pathway that governs sorting of cargo proteins from TGN and endocytosis requires clathrin-coated vesicles. In these vesicles, AP complexes are required for clathrin recruitment. In addition, APs serve as intermediate between coat and cargo proteins, recognizing tyrosine sorting YXXØ signal as well as acidic di-leucine DXXLL and [DE]XXXL[LI] signals (12–15). It has been proposed that these signals would give specificity for each adaptor complex (13,16). Mutations of the putative acidic cluster di-leucine signal of TMEM165 (L209, L210) have no effect on protein localization. DXXLL and [DE]XXXL[LI] signals interact with AP complexes in vitro and this binding is dependent on the acidic residue positions −4 or −5 from the first leucine (15,17–22). Moreover, previous solved crystal structure studies of DXXLL signal peptides with a GGA domain, an ADP-ribosylation factor-dependent clathrin adaptor, show that the critical D must be close to LL residues for enabling interaction (23,24). inside the TMEM165 putative signal, aspartate is located at the −8 position from the first leucine. So the number of residues between D and LL is actually higher than in the conventional DXXLL or [DE]XXXL[LI] signals, likely accounting for lack of mislocalization of TMEM165 after mutations of this motif. It is known that clathrin-mediated internalization mechanisms at the PM exclusively involve AP2 (25). AP1 mediates cargo proteins sorting at the TGN and endosomes (26,27), whereas AP3...
and AP4 are found at TGN and endosomes (28,29). Concerning the YXXØ motif, interaction with AP2 is favored with an arginine for the second X position and a leucine for the O residue (14), suggesting that internalization of wt-TMEM165 could be mediated by AP2 clathrine-coated vesicles. Mutation of R126 to H led to a strong delay in protein internalization from PM, meaning that accumulation of mutated TMEM165 (R126H) to the PM could arise from an impairment between AP2 and TMEM165 (R126H) interaction, R126 being favored for this binding. Interestingly, whereas two hybrid experiments have shown that both H and C were disfavored at Y+2 position (14), we highlighted here a stronger internalization delay of TMEM165 (R126H) compared with TMEM165 (R126C) in human cells. Several APs are likely to drive TMEM165 from the TGN to the PM, but tyrosine signal amino acids composition determines specificity for each adaptor complex (13). A basic residue is indeed located at the second X position of the TMEM165 YXXØ signal, whereas AP3 preferentially interacts with non-polar or acidic residue in this position (30,31). Interaction with AP1 is, however, favored by a leucine residue at Y+3 position (14) as it is found in YNRL of TMEM165. Finally, interaction with AP4 is suggestive for a role in direct lysosomal targeting (29). Consequently, it seems that traveling of TMEM165 between TGN and PM would be mediated by AP1 complex. According to our results, TMEM165 (R126H) and (R126C) were more abundant in acidic compartments, meaning that TMEM165 exit from the TGN would be increased. Mutation of arginine 126 could, therefore, promote exit from TGN by increasing AP1-dependant vesicles budding. On the other hand, it is likely that TMEM165 (R126H) and (R126C) could also interact with another AP, like AP4, favoring TMEM165 leaving TGN to directly reach lysosomes. Interestingly, whereas TMEM165 (R126C) is found in vesicular structures, TMEM165 (R126C, G304R) and (G304R) mainly stay in the Golgi apparatus, meaning that G304R mutation determines the subcellular localization of the mutant. The crucial importance of the YXXØ motif in TGN exit came from the mutation of tyrosine 124 of YNRL that leads to TMEM165 Golgi accumulation. All these results corroborate with previous studies in which the YXXØ motif, similar to Y124NRL-127 from TMEM165, is involved in both lysosomal proteins targeting and endocytosis (32), highlighting both the Golgi exit role of the tyrosine residue and the internalization role of Y+2 residue.

When mutated, TMEM165 leads to type II CDG, a glycosylation disorder. It is reasonable to think that the double localization of TMEM165 suggests that this protein would have a function required both in Golgi apparatus and in lysosomes, organelles involved in glycoproteins synthesis and catabolism, respectively. Concerning the function of this protein, the ProtFun software assigns to TMEM165 a role of transporter. Moreover, the enrichment in acidic residues in E108LGDK and E249WGDR motifs, at the end of the first and the fourth transmembrane domains oriented in antiparallel manner, could play a role in putative cation recognition signals. As CDG linked to homeostasis disruption has previously been identified (33), we suppose that the glycosylation defect originates from a homeostasis dysfunction in Golgi apparatus and/or lysosomes. It is tempting to hypothesize that TMEM165 would regulate some cation homeostasis in organelles involved in the metabolism of glycoproteins and in particular Ca²⁺, as gdt1Δ yeasts do not grow in high CaCl₂ concentration. Lysosomes are indeed known to be calcium storage organelles (34), and disruption of calcium signaling has been shown to cause lysosomal storage diseases such as Niemann–Pick type C where calcium uptake and release is defective (35). In our study, we brought evidence that loss of TMEM165 functionality could arise from either a decrease in TMEM165 level, impairment in protein function or a subcellular localization defect. CDG linked to TMEM165 mutations could then be due to a reduced level of the protein. Interestingly, amounts of TMEM165 (c.792+182 G>A), TMEM165(R126H) and compound heterozygous (R126C, G304R) are indeed affected, but differently according to TMEM165 mutations. TMEM165 (c.792+182 G>A) cannot indeed be detected both in patient cells (2) and after transient expression, whereas even levels of endogenous TMEM165(R126H) and (R126C, G304R) are reduced; amounts of these transiently expressed mutants are close to wt (Supplementary Material, Fig. S1). Since we demonstrated that gene expression of TMEM165 (c.792+182 G>A) was not altered, the stability of the protein must be affected, probably due to unstable truncated protein conformation or active degradation. Concerning protein function, complementation assays in yeast have shown that Gdt1(R71H) and (R71C), equivalent to TMEM165(R126H) and (R126C), were able to complement growth defect of gdt1Δ yeast, proving that mutant’s phenotype would not be due to an impairment in TMEM165(R126H) or (R126C) function. However, we have highlighted that protein subcellular localizations are different between wt-TMEM165 and TMEM165 with patients’ mutations. This result fundamentally indicates that the disease could be linked not only to an alteration in protein level, but also to impairments in subcellular localization of mutated TMEM165.

**MATERIALS AND METHODS**

**Constructs, vector engineering and mutagenesis**

Gene coding wt, R126H- and G304R-mutated TMEM165 were isolated from cDNA of primary skin fibroblasts from healthy and patients. Expression vectors were generated using Qiagen PCR Cloning Kit according to the manufacturer’s instructions. Briefly, TMEM165 was inserted into pcDNA3.1 (Invitrogen) upstream a sequence coding for monomeric red fluorescent protein 1 (mRFP1), GFP or GFPpHluorin. Wt construct is named TMEM165, whereas TMEM165(G304R). The R126C mutation and TMEM165(R126H) and compound heterozygous (R126C, G304R) mainstay in Golgi apparatus, meaning that G304R mutation determines the subcellular localization of the mutant. TMEM165 functionality could arise from either a decrease in TMEM165 level, impairment in protein function or a subcellular localization defect. CDG linked to TMEM165 mutations could then be due to a reduced level of the protein. Interestingly, amounts of TMEM165 (c.792+182 G>A), TMEM165(R126H) and compound heterozygous (R126C, G304R) are indeed affected, but differently according to TMEM165 mutations. TMEM165 (c.792+182 G>A) cannot indeed be detected both in patient cells (2) and after transient expression, whereas even levels of endogenous TMEM165(R126H) and (R126C, G304R) are reduced; amounts of these transiently expressed mutants are close to wt (Supplementary Material, Fig. S1). Since we demonstrated that gene expression of TMEM165 (c.792+182 G>A) was not altered, the stability of the protein must be affected, probably due to unstable truncated protein conformation or active degradation. Concerning protein function, complementation assays in yeast have shown that Gdt1(R71H) and (R71C), equivalent to TMEM165(R126H) and (R126C), were able to complement growth defect of gdt1Δ yeast, proving that mutant’s phenotype would not be due to an impairment in TMEM165(R126H) or (R126C) function. However, we have highlighted that protein subcellular localizations are different between wt-TMEM165 and TMEM165 with patients’ mutations. This result fundamentally indicates that the disease could be linked not only to an alteration in protein level, but also to impairments in subcellular localization of mutated TMEM165.
TTACGCAGTGTTT GTACAAGACCACAAGTCG-5' and [3'-GTACCGTACGC \\
GAAAATTGGCGAGTGGC-3'], [5'-TGCGCTATAACCGC \\
GGGACCGGTGTTCTCAGC-3'] and [3'-ACGGATATTGGC \\
GTTTTGCAGCTGAGAGCCAG-5'], [5'-CAACCGAAGAAA \\
GGGT GAAATGGACCGGGA-3'] and [3'-GTTGCAGT \\
GTTTCCACCT TTACCTGGCACC-5'] were used to yield \\
mutated proteins TMEM165(Y124S), TMEM165(Y124F), \\
TMEM165(Y124C) and TMEM165(Y124I).

Antibodies

Anti-LAMP2 and anti-Actin antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-TMEM165 and anti-GFP antibodies were from Sigma–Aldrich (St Louis, MO, USA). Anti-TGN46 and anti-EEA1 antibodies were from BD Biosciences (Franklin lakes, NJ, USA). Anti-GFP antibody was purchased from Covance (Princeton, NJ, USA) and living color DsRed antibody from Clontech Laboratories (Mountain View, CA, USA).

Cell culture and transfections

HeLa cells (human cervix epithelial carcinoma) and primary skin fibroblasts were maintained in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum (Lonza), at 37°C in humidity-saturated 5% CO2 atmosphere. For protein transient expression in HeLa cells, transfections were performed using lipofectamine (Invitrogen) following the instructions of the manufacturer.

Endocytosis kinetics

Transiently TMEM165-GFP-expressing cells were incubated for 10 min at 18°C, with anti-GFP antibody diluted at 1:100 in DMEM, then washed and incubated at 37°C for 5 to 120 min. They were then washed five times with phosphate buffered saline (PBS) before paraformaldehyde fixation. Immunostaining was done directly with secondary Alexa-568 antibody raised against anti-GFP antibody.

Immunofluorescence imaging

Twenty-four hours after transfection, cells were seeded on coverslips, fixed with 4% paraformaldehyde in 0.1 M sodium phosphate buffer (PBS, pH 7.2) for 30 min at room temperature and then washed twice with PBS. After permeabilization in 0.5% Triton X-100 and 2 h saturation in blocking buffer [0.2% gelatin, 1% BSA and 2% normal goat serum (Invitrogen) in PBS], fixed cells were incubated overnight with primary antibody diluted at 1:100 in blocking buffer. After washing with PBS, cells were incubated for 1 h with Alexa 488-, Alexa 568- or Alexa 700-conjugated secondary antibodies (Molecular Probes) diluted at 1:600 in blocking buffer. Visualization of GFP pHluorin-coupled proteins was done after excitation at 488 nm, signal being collected at 510 nm. Immunostaining and fluorescent proteins were detected through an inverted Leica TCS-SP5 confocal microscope. Data collection and quantifications were done using the LAS AF Lite software 2.6.0 (Leica Microsystems, Wetzlar, Germany).

Cell fractionation

Sedimentation on the silica colloid Percoll gradient (Amer sham Biosciences) was used to isolate dense lysosomes from lower density Golgi apparatus, according to the isopycnic position of particles. Primary skin fibroblasts were lysed in homogenization buffer [HB: 250 mM sucrose, 1 mM EDTA and complete protease inhibitors (Roche)] in a ball-bearing homogenizer with a clearance of 12 μm, followed by a centrifugation at 12 000g for 15 min at 4°C. Supernatants were layered on the top of a Percoll gradient of 17.5% and centrifuged for 8 h at 30 000g and 4°C without break. Fractions of 1 ml were carefully collected from the top (low density) to the bottom (high density) of the gradient. Membranes were then solubilized with 1% Triton X-100 in HB, proteins were concentrated using methanol/chloroform precipitation (36) and loaded on SDS–PAGE (precast 4–12% polyacrylamide gradient gels, Invitrogen). LAMP2, GPP130 and TMEM165 were revealed using western blotting. Signal intensity quantifications were done using the ImageJ 1.45 software. Lysosomal marker enzyme β-hexosaminidase activity was measured in each Percoll gradient fraction according to the following protocol. Eighty microliters of each fraction was incubated with 2 mM p-nitrophenyl-N-acetyl-β-D-glucosaminide, 0.1% (v/v) Triton X-100, in 0.5 mM sodium citrate buffer, pH 4. After 30 min at 37°C, the β-hexosaminidase catalyzed reaction was stopped by 150 mM Na2CO3 and then optical density was read at a wavelength of 400 nm. Relative β-hexosaminidase activity was represented in conjunction with relative activity normalized to LAMP2 intensity.

Western blotting

Cells lysis with transiently expressed wt or mutated TMEM165 coupled to a C-terminal RFP tag was done in lysis buffer (25 mM Tris–HCl, 150 mM NaCl, pH 7.6, supplemented with 1% Triton X-100, 1% sodium deoxycholate and 0.1% SDS) (Supplementary Material, Fig. S1). Protein content was measured using the Micro BCA Protein Assay Kit (Thermo Fisher Scientific, Brebieres, France) and then samples were separated by electrophoresis on 12% SDS–PAGE mini-gel and transferred to nitrocellulose membranes (GE Healthcare). Membranes were blocked in blocking buffer: 5% milk powder in TBS-T (1× TBS with 0.05% Tween20) for 1 h at room temperature, incubated for the same duration with the primary rabbit anti-DsRed or actin antibody used at a dilution of 1:1000 in blocking buffer, and washed two times for 5 min in TBS-T. The membranes were then incubated with the peroxidase-conjugated secondary antibody (PO448, Dako; used at a dilution of 1:10 000) in blocking buffer for 1 h at room temperature and afterward washed two times for 5 min in TBS-T. The signal was detected using an electrochemiluminescence reagent (ECL, PerkinElmer) on
imaging film (GE Healthcare). Quantitative analysis of wt and mutants TMEM165 transiently expressed in HeLa cells was performed using the Quantity One software on a GS800 Calibrated Imaging Densitometer (Bio-Rad Laboratories, UK) and normalized to the actin intensity.

**Complementation of Gdt1-deficient yeast cells**

The wt and /gdt1/:: KanMX/ /yeast strains (BY4742 background; Euroscarf) were transformed using the standard lithium acetate method with a yeast centromeric expression plasmid (pRS416) containing the wt-GDT1 or mutated GDT1 with the corresponding mutations found in humans. For the complementation assays, liquid cultures were grown to saturation in minimal medium lacking uracil. Cells were diluted to an OD600 of 0.3, and serial dilutions of 1:10 were spotted on 900 mM CaCl2-containing minimal medium [0.2% yeast nitrogen base without amino acids and ammonium sulfate (Difco), 2% glucose, 2% agar and 0.4% ammonium chloride, supplemented with all amino acids except uracil].

**SUPPLEMENTARY MATERIAL**

Supplementary Material is available at HMG online.

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**Conflict of Interest statement**

None declared.

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