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ORIGINAL ARTICLE

Loss of glucocerebrosidase 1 activity causes lysosomal dysfunction and α-synuclein aggregation

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Lysosomal dysfunction is a common pathological feature of neurodegenerative diseases. GTP-binding protein type A1 (*GBA1*) encodes β-glucocerebrosidase 1 (GCase 1), a lysosomal hydrolase. Homozygous mutations in *GBA1* cause Gaucher disease, the most common lysosomal storage disease, while heterozygous mutations are strong risk factors for Parkinson's disease. However, whether loss of GCase 1 activity is sufficient for lysosomal dysfunction has not been clearly determined. Here, we generated human neuroblastoma cell lines with nonsense mutations in the *GBA1* gene using zinc-finger nucleases. Depending on the site of mutation, GCase 1 activity was lost or maintained. The cell line with GCase 1 deficiency showed indications of lysosomal dysfunction, such as accumulation of lysosomal substrates, reduced dextran degradation and accumulation of enlarged vacuolar structures. In contrast, the cell line with C-terminal truncation of GCase 1 but with intact GCase 1 activity showed normal lysosomal function. When α-synuclein was overexpressed, accumulation and secretion of insoluble aggregates increased in cells with GCase 1 deficiency but did not change in mutant cells with normal GCase 1 activity. These results demonstrate that loss of GCase 1 activity is sufficient to cause lysosomal dysfunction and accumulation of α-synuclein aggregates.

INTRODUCTION

Parkinson's disease (PD) is one of the most common neurodegenerative diseases, with clinical symptoms of resting tremor, increased muscle tone, bradykinesia and abnormal postural righting reflexes.[1] Pathologically, PD is characterized by loss of dopaminergic neurons in the substantia nigra pars compacta; deposition of α-synuclein, visualized in the form of Lewy bodies (LBs) and Lewy neurites and neuroinflammation, demonstrated by the activation of microglia.[2] In addition to these features, neurons of patients with PD show signs of extensive lysosomal dysfunction, such as accumulation of autophagosomes, a characteristic also seen in other neurodegenerative diseases.[3]

Genetic studies of PD have identified several genes and loci in inherited cases.[4] Recently, genome-wide association studies have identified several risk loci for PD, suggesting that susceptibility to PD (even sporadic PD) is determined by specific allelic combinations.[5–8] Although some of these genes operate independently,[9] there might be interactions among these genes in the pathogenesis of PD. Among the susceptibility loci, the ones most strongly and consistently associated with sporadic PD have been located in *SNCA*, the gene encoding α-synuclein.[6,7]

Gaucher disease (GD), the most common lysosomal storage disease with recessive inheritance is mainly due to loss-of-function of the lysosomal enzyme, glucocerebrosidase (GCase).[10] About 300 different mutations in GTP-binding protein type A1 (*GBA1*) gene, encoding GCase1, are known to cause GD.[11]

Recent studies have suggested the association between mutations in *GBA1* and parkinsonism. Genetic studies have shown that heterozygous carriers of mutations in *GBA1* are at higher risk for PD than the general population.[12] For example, patients with PD are approximately five times more likely to carry *GBA1* mutations than healthy control subjects.[13] Compared with control group, the incidence of parkinsonism is increased 6- to 17-folds in patient group with type1 GD.[14] Brain samples from patients with PD and dementia with LBs with a heterozygous *GBA1* mutation showed a mean of 75% (range, 32–90%) of LBs colocalized with GCase, whereas the

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mean colocalization rate was 4% in patients with PD and dementia with LB without a *GBA1* mutation.[15] In addition, patients with GD and GD carriers with parkinsonism have LB pathology.[16] These results suggest a strong and specific association between *GBA1* mutations and LB diseases (LBDs).

Despite the strong association of *GBA1* mutations with PD and other LBDs, the mechanism underlying the role of these mutations in PD is not clearly understood. Here, we generated human neuroblastoma cell lines harboring nonsense mutations in the *GBA1* gene and analyzed the effects of these mutations on lysosomal function and α-synuclein aggregation.

MATERIALS AND METHODS

Materials

The following antibodies were used in this study: GCase monoclonal antibody 8E4 (from J Barranger, University of Pittsburgh; 1:1000), GCase polyclonal antibody (G4171, Sigma-Aldrich, St Louis, MO, USA; 1:1000), β-actin monoclonal antibody (Sigma-Aldrich; 1:10 000), p62 monoclonal antibody (c2384-0B, BD Transduction Laboratories, Swampscott, MA, USA; 1:1000), ubiquitin polyclonal antibodies (Dako, Glostrup, Denmark and Chemicon, Temecula, CA, USA; 1:1000), α-synuclein monoclonal antibody (610787, BD Biosciences; 1:1500), α-synuclein monoclonal antibody Ab274 (1:1500),[17] α-synuclein monoclonal antibody Ab62 (1:1000),[17] Horse radish peroxidase (HRP)-conjugated goat anti-mouse immunoglobulin G (IgG; H+L) (172–1011, Bio-Rad Laboratories, Hercules, CA, USA; 1:3000) and HRP-conjugated goat anti-rabbit IgG (H+L) (Bio-Rad Laboratories; 1:3000).

Fluorescein-conjugated dextran (10 000 molecular weight; D-1821), TO-PRO-3 iodide (T3605) and LysoTracker Red DND-99 (L-7528) were purchased from Invitrogen (Carlsbad, CA, USA).

Generation of *GBA1* knockout cell lines

SH-SY5Y cells (CRL-2266, ATCC, Manassas, VA, USA) were transfected with plasmids encoding zinc-finger nuclease (ZFN) and a magnetic reporter (ToolGen, Seoul, Korea) by using electroporation. After incubation for 48 h, cells were enriched by magnetic separation. After trypsinization, cells were mixed with magnetic bead-conjugated antibody against H-2K^k (MACSelect Kk microbeads, Miltenyi Biotech, Gladbach, Germany), and the mixture was applied to a MACS LS column (Miltenyi Biotech). Single cells were isolated from the eluates and maintained until the clonal colonies were picked from the culture dish. Nonsense mutations in the *GBA1* gene were confirmed by DNA sequencing. Four clones with nonsense mutations in exon 3 and six clones in exon 11 were generated. Among these clones, three clones with mutation in exon 3 and two clones with mutations in exon 11 were further analyzed.

Cell culture

SH-SY5Y human neuroblastoma cell lines were cultured as described previously.[18] Cells were maintained every 2 days at 37 °C in humidified air with 5% CO_2 in Dulbecco's modified eagle's medium (DMEM) (SH30243.01, HyClone, Logan, UT, USA) containing 10% fetal bovine serum (SH30396.03, HyClone), 100 U ml^{-1} penicillin and 100 U ml^{-1} streptomycin (15140-122, Gibco, Grand Island, NY, USA). To differentiate SH-SY5Y cells, cells were maintained in medium with 50 μM all-*trans*-retinoic acid (R2625, Sigma-Aldrich). For overexpression of human α-synuclein, differentiated SH-SY5Y
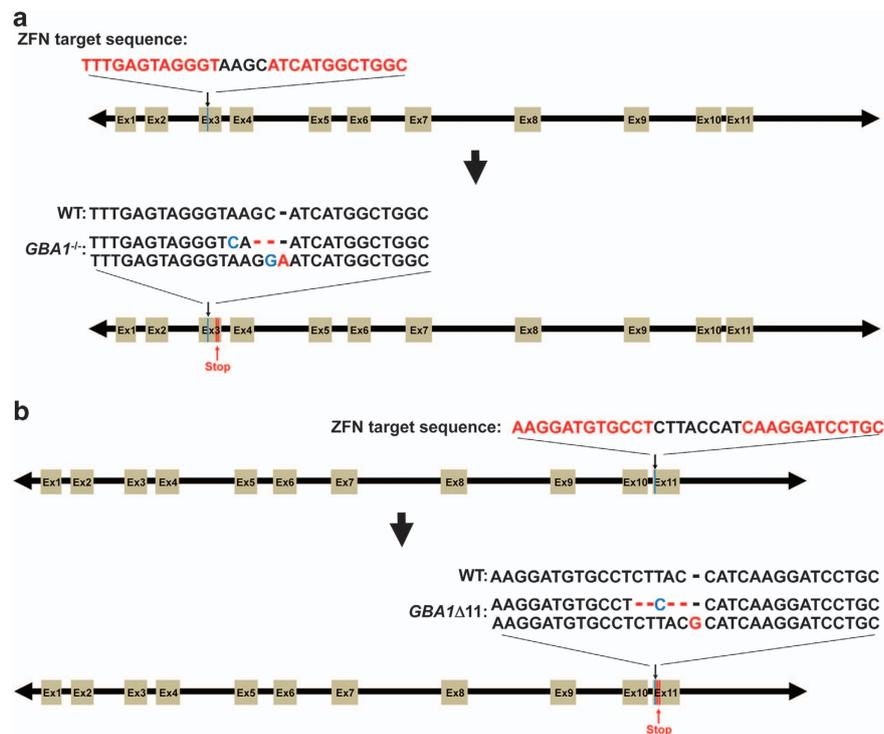

Figure 1 Generation of frame-shift mutations in GTP-binding protein type A1 (*GBA1*) by using zinc-finger nucleases (ZFNs). To generate nonsense mutations in two alleles of the *GBA1* gene, SH-SY5Y cells were transfected with ZFNs targeting either exon 3 (**a**) or exon 11 (**b**). After clonal selection, the nonsense mutations were confirmed by DNA sequencing.

cells were infected with a recombinant adenoviral vector (serotype Ad5, cytomegalovirus promoter) containing human α-synuclein complementary DNA at a multiplicity of infection of 33.3.

GCase activity and glycosphingolipid assay

Cellular GCase activity was determined as described previously.[19] Briefly, GCase activity was determined using a synthetic substrate, 10 mM 4-methylumbelliferyl-β-D-glucoside (Sigma, St Louis, MO, USA) in buffer containing 1% bovine serum albumin, at 37 °C for 1 h. To stop the reaction, 0.5 volume of 1 M glycine buffer, pH 12.5, was added. Cleaved 4-methylumbelliferone was measured using a SpectraMax Gemini fluorometer (excitation at 365 nm, emission at 445 nm; Molecular Devices, Sunnyvale, CA, USA). All measurements were done without taurocholate, a detergent that activates GCase. GCase 1 and GCase 2 activity was determined in the presence of either a GCase 1 inhibitor, conduritol-B-epoxide (CBE) or a GCase 2 inhibitor, AMP-deoxynojirimycin. GCase 1 activity was obtained by subtracting the GCase 2 activity from the total GCase activity. Cellular glucocerebroside (GL1) and galactocerebroside (GalCer) levels were measured by mass spectrometry as previously described.[19] Briefly, organic cellular extracts were injected into an Atlantis HILIC silica column (Waters, Milford, MA, USA), and the separated GL1 and GalCer were detected using an AB Sciex API-5000 mass spectrometer (AB Sciex, Framingham, MA, USA).

Preparation of cell extracts

Cells were washed twice with ice-cold phosphate-buffered saline (PBS) and then lysed in extraction buffer (1% Triton X-100 and 1% (v/v) protease inhibitor cocktail (Sigma) in PBS). Cell lysates were incubated on ice for 10 min and centrifuged at 16 000 g for 10 min. The Triton X-100 insoluble fraction was resuspended in 1× Laemmli sample buffer and sonicated briefly.

Western blotting

Western blotting was performed as previously described.[20] Images were obtained and quantified using a Luminescent Image Analyzer (Fuji Film, Tokyo, Japan) (LAS-3000) and MultiGauge version 3.0 software (Fujifilm).

Characterization of lysosomal dysfunction

To analyze the LysoTracker-positive compartments, SH-SY5Y cells were stained with 75 nM LysoTracker solution diluted in growth medium. After incubation for 1 h at 37 °C in a CO_2 incubator, cells were washed with ice-cold PBS and fixed in a 4% paraformaldehyde solution. To determine the degradation ratio of internalized dextran, cells were incubated with 20 µg ml⁻¹ fluorescein isothiocyanate-labeled dextran (Invitrogen) for 2 h. After washing with DMEM, cells were incubated with fresh growth medium for 30 min and fixed with a 4% paraformaldehyde solution. The fluorescence intensity was measured using Olympus FV1000 software (Olympus, Tokyo, Japan). The extent of degradation of internalized dextran–fluorescein isothiocyanate was calculated using the equation $(F_{time0} - F_{time30})/F_{time0}$, where F_{time0} and F_{time30} are the integrated fluorescence intensities at 0 min and 30 min, respectively.

Electron microscopy

Cells were fixed with Karnovsky's fixative solution (2% glutaraldehyde, 2% paraformaldehyde and 0.5% $CaCl_2$). After immersion in 1% osmium tetraoxide for 1.5 h, cells were dehydrated with 50, 60, 70, 80, 90, 95 and 100% absolute ethanol. Cells were infiltrated with propylene oxide and EPON mixture (EPON 812, MNA, DDSA, DMP30) for 10 min before being embedded in EPON mixture. After embedding, cells were sectioned with an EM UC-7 Ultramicrotome (Leica Microsystems, Vienna, Austria) and stained with 6% uranyl acetate and lead citrate. Grids were observed using a transmission electron microscope (JEM-1011, JEOL, Tokyo, Japan) and analyzed using MegaView III software (Soft Imaging System, Münster, Germany). For morphometric analysis, 20 cells were analyzed for each experiment.

Enzyme-linked immunosorbent assay

Enzyme-linked immunosorbent assay (ELISA) was performed as previously described.[17] Briefly, 96-well ELISA plates (Nalge Nunc International, Rochester, NY, USA) were coated with 1 mg ml⁻¹ capture antibody (Ab62) in 50 mM carbonate buffer (pH 9.6) at 4 °C overnight. After washing with PBS with 0.05% Tween 20 (PBST), SuperBlock T20 (PBS) Blocking Buffer (Thermo Scientific, Rockford, IL, USA) was added for 1 h at room temperature with shaking. Plates were washed five times in PBST and samples and standards were incubated at room temperature for 2.5 h with shaking. After washing
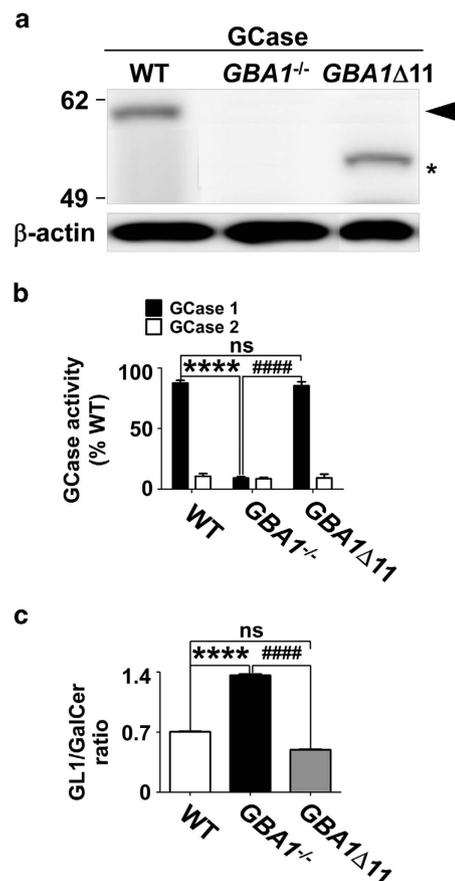

Figure 2 Confirmation of GTP-binding protein type A1 (*GBA1*) deficiency. (**a**) Western blot analysis of glucocerebrosidase 1 (GCase 1) in Triton-soluble fractions. Arrowhead: GCase1, asterisk: C-terminal truncated GCase1. (**b**) Analysis of GCase activity ($n=4$; ****$P<0.0001$, ####$P<0.0001$ by paired, two-tailed Student's *t*-test). (**c**) Accumulation of GCase substrate. Substrate accumulation was assessed by the glucocerebroside (GL1) to galactocerebroside (GalCer) ratio ($n=3$; ****$P<0.0001$, ####$P<0.0001$ by paired, two-tailed Student's *t*-test). NS, not significant.

with PBST, 1 µg ml^{-1} biotinylated Ab62 in blocking buffer was added and incubated at room temperature for 1.5 h. The plates were washed with PBST, and avidin-conjugated peroxidase (ExtrAvidin, Sigma) was added for 1 h at room temperature. After washing with PBST, 100 µl of 3,3′,5,5′-tetramethylbenzidine solution (Sigma) was added to each well and incubated for 15 min with shaking. To stop the reaction, 50 µl of 2N H$_2$SO$_4$ was added to each well. The absorbance was measured at 450 nm.

Statistical analysis

Values shown in the figures are mean ± s.e.m. To analyze the statistical significance, *P*-values were calculated by mean of paired, two-tailed Student's *t*-tests by using InStat version 3.05 software (GraphPad Software, San Diego, CA, USA).

RESULTS

To address the effects of loss-of-function of GCase1 activity on the lysosomal function and the metabolism of α-synuclein, we have generated SH-SY5Y human neuroblastoma cell lines with nonsense mutations in both copies of the *GBA1* gene using engineered ZFNs. The GBA gene comprises 11 exons and 10 introns. The coding regions of enzymatic active site is located in exon 5–10.[11] The ZFNs were designed to generate mutations at two different sites in the coding region of *GBA1*, one in exon
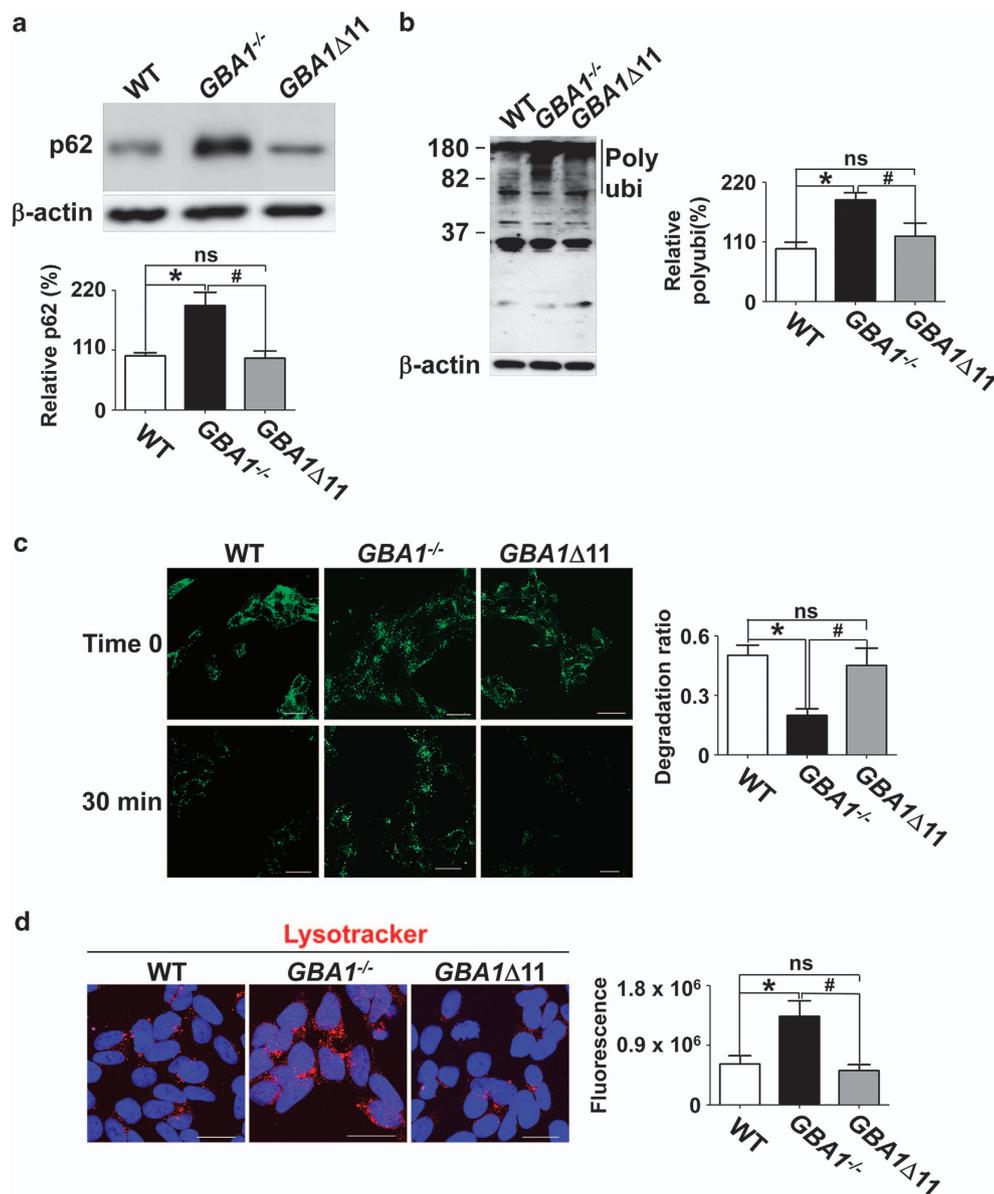

Figure 3 GTP-binding protein type A1 (*GBA1*) deficiency induces lysosomal dysfunction. (**a**, **b**) Levels of p62 (**a**) and polyubiquitinated proteins (**b**) in Triton-insoluble fractions (*n*=3; *P<0.05, #P<0.05 by paired, two-tailed Student's *t*-test). For quantification of polyubiquitinated proteins, the quantified size range is indicated by the line to the right of the blot. (**c**) Degradation of internalized dextran–fluorescein isothiocyanate (*n*=3, 100 cells per experiment; *P<0.05, #P<0.05 by paired, two-tailed Student's *t*-test). Scale bars, 20 µm. (**d**) Quantification of LysoTracker-positive compartments (*n*=3, 100 cells per experiment; *P<0.05, #P<0.05 by paired, two-tailed Student's *t*-test). Scale bars, 20 µm. NS, not significant.

3 and the other in exon 11. Clonal cell lines with nonsense mutations at the intended sites were screened and selected by sequencing (Figure 1). We generated four clones with nonsense mutation in exon 3 and six clones with nonsense mutation in exon 11. To analyze the lysosomal activity, we selected three clones with mutations in exon 3 and two clones with mutations in exon 11. Results from the isogenic clones were identical. Western analysis showed that the translational stop codon in exon 3 caused a loss of GCase 1 expression ($GBA1^{-/-}$), whereas the translational termination signal in exon 11 resulted in a truncated form of GCase 1 ($GBA1\Delta11$) (Figure 2a). $GBA1\Delta11$ contains all the regions necessary for catalytic activity.[21,22] To address the effects of missense mutations in *GBA1* on the GCase activity, we evaluated the catalytic activity

of GCase 1 and 2 in the presence of either GCase 1 inhibitor, CBE, or GCase 2 inhibitor, AMP-deoxynojirimycin. AMP-deoxynojirimycin-sensitive GCase 2 activity was much lower than CBE-sensitive GCase 1 activity. About 90% of the total GCase activity was derived from GCase 1 and ~2% from GCase 2, the cytosolic form of GCase encoded by a separate gene *GBA2*.[23] As expected, $GBA1\Delta11$ cells and wild-type cells showed similar levels of GCase activity. In contrast, $GBA1^{-/-}$ cells had a greatly reduced level of GCase activity (Figure 2b). The residual activity detected in $GBA1^{-/-}$ cells was contributed by AMP-deoxynojirimycin-sensitive GCase 2 activity. As a result of reduced GCase activity, $GBA1^{-/-}$ cells showed an increased GL1/GalCer ratio, whereas $GBA1\Delta11$ cells had a GL1/GalCer ratio similar to that of wild-type cells (Figure 2c).
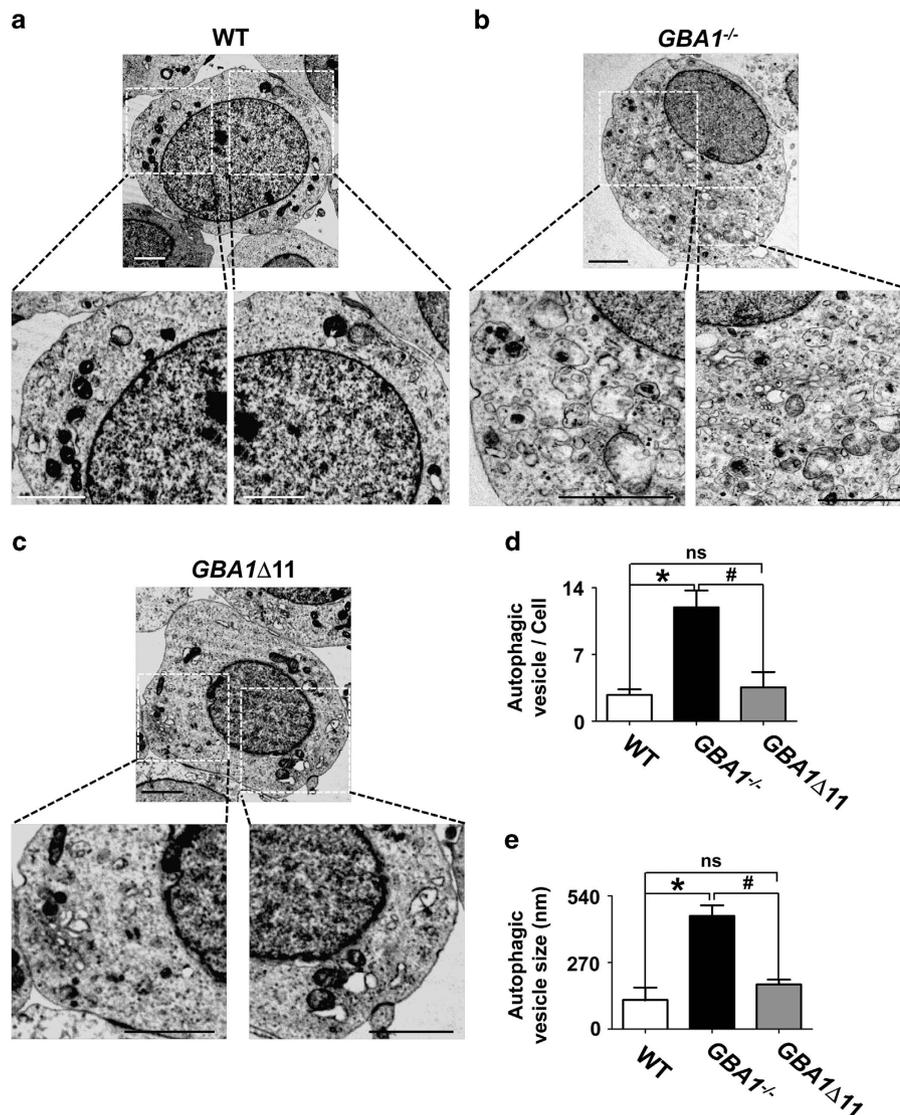

Figure 4 Vacuole accumulation due to GTP-binding protein type A1 (*GBA1*) deficiency. (**a–c**) Electron microscopy of wild-type (**a**), $GBA1^{-/-}$ (**b**) and $GBA1\Delta11$ cells (**c**). The boxed areas in the upper images are magnified in the lower panels. Scale bars, 2 μm. (**d**) Number of autophagic vesicles per cell ($n=3$, 100 cells per experiment; *$P<0.05$, #$P<0.05$ by paired, two-tailed Student's *t*-test). (**e**) Average diameter of autophagic vesicles ($n=3$, 100 cells per experiment; *$P<0.05$, #$P<0.05$ by paired, two-tailed Student's *t*-test). NS, not significant.
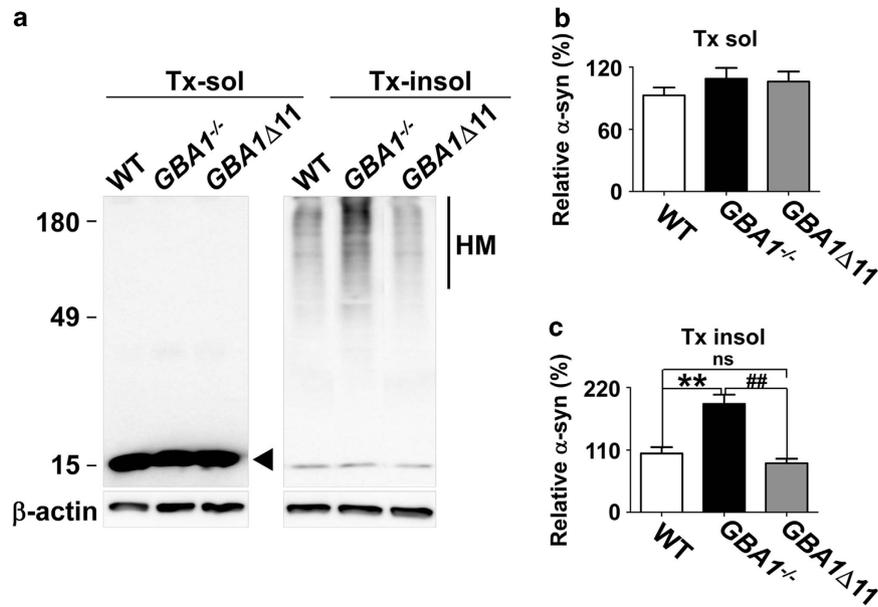
Figure 5 Glucocerebrosidase (GCase) deficiency increases accumulation of α-synuclein aggregates. (**a**) Western blot analysis of α-synuclein in Triton-soluble and Triton-insoluble fractions. HM, detergent-insoluble, high-molecular-weight α-synuclein (the HM regions are quantified in **c**). Arrowhead: detergent-soluble, monomeric α-synuclein. (**b**, **c**) Quantification of α-synuclein in Triton-soluble (**b**) and Triton-insoluble fractions (**c**) ($n = 3$; **$P < 0.01$, ##$P < 0.01$ by paired, two-tailed Student's t-test). NS, not significant.
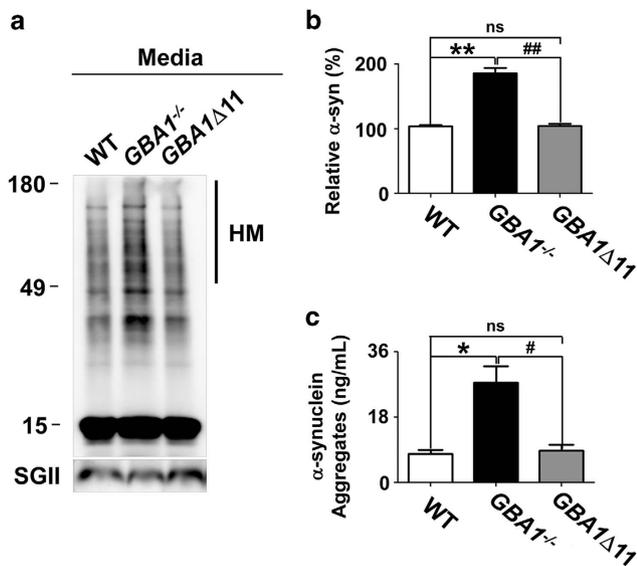

Figure 6 Secretion of α-synuclein aggregates. (**a**) Western blot analysis of α-synuclein in cell culture media. HM, high-molecular-weight α-synuclein (the HM regions are quantified in **b**). SGII, secretogranin II. (**b**) Quantification of high molecular weight α-synuclein aggregates in culture media ($n = 3$, *$P < 0.05$ by paired, two-tailed Student's t-test). (**c**) The levels of secreted α-synuclein aggregates in cell culture media were measured using an enzyme-linked immunosorbent assay specific for aggregated forms of α-synuclein ($n = 4$; *$P < 0.05$, **$P < 0.01$, #$P < 0.05$, ##$P < 0.01$ by paired, two-tailed Student's t test). NS, not significant.

We next examined and compared the lysosomal functions of these cell lines. To assess lysosomal activity, we measured the levels of known lysosomal substrates (p62 and polyubiquitinated proteins) and the degradation rate of exogenously introduced dextran. As shown in Figures 3a and b, both p62 and polyubiquitinated proteins accumulated to significantly higher levels in $GBA1^{-/-}$ cells than in wild-type cells or $GBA1\Delta11$ cells. Dextran degradation was much slower in $GBA1^{-/-}$ cells than in wild-type or $GBA1\Delta11$ cells (Figure 3c). Cells with lysosomal dysfunction often show increased LysoTracker staining, probably because of both reduced breakdown of acidic organelles and increased biosynthesis of lysosomes in an effort to restore lysosomal function.[24] $GBA1^{-/-}$ cells showed a significantly higher Lyso-Tracker signal than wild-type or $GBA1\Delta11$ cells (Figure 3d). Morphological analysis using electron microscopy confirmed lysosomal dysfunction. Compared with wild-type or $GBA1\Delta11$ cells, $GBA1^{-/-}$ cells had abnormal accumulation of enlarged autophagic vesicles and damaged cellular organelles (Figure 4). These morphological changes are akin to previous findings of vacuole accumulation as a hallmark of lysosomal storage diseases.[25]

Lysosomes have crucial roles in the degradation of α-synuclein through macroautophagy and chaperone-mediated autophagy.[26,27] We examined accumulation of α-synuclein in cells with *GBA1* mutations. Neither $GBA1^{-/-}$ nor $GBA1\Delta11$ cells showed changes in the levels of monomeric α-synuclein compared with wild-type cells (Figures 5a and b). However, $GBA1^{-/-}$ cells accumulated SDS-stable α-synuclein oligomers in the Triton-insoluble fractions to a greater extent than did wild-type and $GBA1\Delta11$ cells (Figures 5a and c).

Neuronal cells secrete monomeric and oligomeric α-synuclein,[28] and the secretion of these proteins increases when cellular quality-control systems, such as the ubiquitin–proteasome system and autophagy, are compromised.[29,30] Consistent with these findings, secretion of α-synuclein

aggregates was significantly increased in $GBA1^{-/-}$ cells compared with that in wild-type and $GBA1\Delta11$ cells (Figure 6).

DISCUSSION

In the current study, we demonstrated that *GBA1* deficiency caused by the targeted introduction of nonsense mutations leads to lysosomal substrate accumulation, global lysosomal dysfunction and α-synuclein aggregation. These cellular changes are associated with the loss of GCase activity; a nonsense mutation leading to the production of a C-terminal truncated form of GCase with intact enzyme activity resulted in none of the changes observed with the GCase-deficient mutant. In a previous study, lysosomal dysfunction and accumulation of α-synuclein and glucosylceramide, a substrate of GCase, were observed in GCase-deficient primary neurons and in induced pluripotent stem cells derived from fibroblasts of a patient with GD.[31] More recently, neurons derived from induced pluripotent stem cells from patients with *GBA1* mutations were shown to be defective in autophagy.[32] Taken together, these results indicate that defects in lysosomal and lysosome-related functions may be an important underlying mechanism for human diseases associated with *GBA1* mutations, such as PD and other LBDs.

Our study demonstrated that *GBA1* deficiency can cause lysosomal dysfunction, while cells with a mutation in *GBA1* that maintains GCase activity have normal lysosomal function. These results suggest that GCase activity is critical for maintaining normal lysosomal function. Consistent with our results, decreased GCase activity is associated with PD. The amount and activity of GCase were significantly reduced in several brain regions of PD patients carrying heterozygous *GBA1* mutations and, more importantly, in brain regions of sporadic PD patients.[33] Furthermore, reduction of GCase was associated with accumulation of α-synuclein in sporadic PD.[34] These studies in patients and in our cell lines provide a rationale for therapeutic approaches involving enzyme replacement and chaperoning for GCase.

The mechanism by which *GBA1* mutations cause lysosomal dysfunction and neuropathy is not clearly understood. A loss-of-function mechanism would predict substrate accumulation. In patients with GD, levels of glucosylsphingosine, one of the substrates of GCase, were drastically increased compared with levels of glucosylsphingosine in control subjects.[35] In addition to affecting GCase substrates, *GBA1* mutations may also cause global changes in cellular lipid composition.[36] Our current study also showed substrate accumulation and lysosomal dysfunction in cells with GCase deficiency, supporting a loss-of-function mechanism. However, when it comes to neurodegeneration, a gain-of-function mechanism may also have a role, perhaps through accumulation of misfolded GCases in the endoplasmic reticulum.[37,38] Both loss-of-function and gain-of-function mechanisms may contribute to the role of *GBA1* mutations in increased risk of PD.[19]

Neuropathological examinations of patients with dementia showed that those with LB-type pathology were more likely to carry *GBA1* mutations than those with Alzheimer-type pathology, which showed no difference from control subjects.[39] Furthermore, a decrease in GCase activity in the cerebrospinal fluid was observed in patients with PD[40] and dementia with LB[41] but not in patients with Alzheimer's disease or frontotemporal dementia.[41] These results may provide insights into the origin of disease specificity. For instance, different types of lysosomal dysfunction might lead to defects in metabolism of specific disease-linked proteins. Our study showed that *GBA1* deficiency increased α-synuclein aggregation. Others have shown that mutations in *GBA1* resulted in reduced α-synuclein degradation.[31] One could postulate that the effects of *GBA1* mutations on protein degradation are limited to a certain group of proteins that includes α-synuclein.[42] This idea needs to be validated.

In conclusion, *GBA1* deficiency is sufficient to cause lysosomal dysfunction and promote α-synuclein aggregation. These results suggest that *GBA1* mutations exert pathogenic actions through a loss-of-function mechanism. Several potential mechanisms leading to α-synuclein aggregation have been proposed.[12] Elucidation of the mechanism underlying the pathogenic actions of *GBA1* mutations will help us identify rational therapeutic strategies for LBDs and GD.

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