Chemical biology studies on retaining exo-β-glucosidases

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Chapter 6

Expression and analysis of *Nicotiana tabacum* β-glucosidase B56 in mammalian cells

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Abstract

Gaucher disease (GD) is characterized by deficiency in the lysosomal glucosylceramidase, GBA1, with accumulation of glucosylceramide (GlcCer) as the result. Enzyme replacement therapy (ERT), comprising intravenous administration of GD patients with recombinant human (h)GBA1, is an effective, though costly, therapy for type 1/3 GD patients. In principle, β-glucosidases from other species and that are easier to obtain could work as well, provided they reach Gaucher cell lysosomes, act (all or not assisted by endogenous chaperones, as hGBA1 is) on accumulating GlcCer, and do not elicit an immune response. The *Nicotiana tabacum* β-glucosidase B56 features GBA1-like characteristics: it is a retaining β-glucosidase acting at acidic pH. This Chapter reveals that expression of B56 in hGBA1/hGBA2 double knockout HEK293T cells (a human cancer cell line) reintroduces lysosomal β-glucosidase activity: it is *in vitro* active towards retaining β-glucosidase inhibitors and probes and it proceeds through the secretory pathway (as witnessed by its N-glycosylation) to reach lysosomes. It does not process GlcCer-like substrates in extracts of these transgenic cells, though, and neither possesses transglycosylation activity as is one of the characteristics of hGBA1. The study does show that plant glycosidases can be introduced into human cells while retaining activity, inviting further research towards easy-to-access, lysosomal-active retaining β-glucosidases that do act on GlcCer.

Introduction

Glucosylceramide (GlcCer) is the central glycosphingolipid from which all gangliosides and globosides are derived, and aberrations in glycosphingolipid metabolism is a hallmark of several human diseases.[1,2] Human cells express two retaining β-glucosidases that act on GlcCer in different cellular compartments. Acid glucosylceramidase (GBA1) is the main GlcCer metabolic enzyme, processing GlcCer as the penultimate step in the breakdown of glycosphingolipids within lysosomes.[2] Glucosylceramidase (GBA2) in turn resides within the inner leaflet of (sub)cellular membranes, where it processes GlcCer to give glucose and ceramide in a different topological compartment (cytosol instead of lysosomes) with possibly different cellular consequences.[3] The lysosomal storage disorder, Gaucher disease (GD) is characterized by genetic deficiency in the *GBA1* gene, with different gene mutations leading to different disease states.[4,5] Some mutations (for instance, N370S) lead to relatively mild manifestations (type 1 GD) which can be treated with considerable success by two distinct therapies: enzyme replacement therapy (ERT) and substrate reduction therapy (SRT).[5,6] Other mutations exist that lead to less frequent, but more severe (type 2) GD with neuropathological consequences.[7] Aberrations in GBA1 are moreover also found as a risk factor for Parkinson's disease (PD).[8,9] In all GD types, GlcCer accumulates as the primary storage material and both ERT and SRT are directed to lower GlcCer concentrations in GD patients. In SRT, this is realised by partial inhibition of the GlcCer synthesising enzyme, glucosylceramide synthase (GCS).[5] ERT, which is the clinically more successful of the two therapeutic strategies, pertains the intravenous administration of recombinant human (h)GBA1, which is targeted to Gaucher cells through the mannose receptor (for which purpose rhGBA1 is engineered to display high mannose-type N-glycans).[5,10-12]

Although effective, ERT is a very costly therapy, and costs are in part associated with the production of stable rhGBA1.[13] Originally harvested (as wild-type enzyme) from human placentas, today rhGBA1 is produced in a variety of production platforms, including cells derived from human fibroblasts[14], Chinese hamster ovary cells (CHOs)[5,15] and more recently also carrot cells.[16,17] The non-human production systems are highly engineered to allow the efficient expression of a xenobiotic, human enzyme. On paper, one could also imagine usage of a non-human retaining β-glucosidase in ERT to take over the role of genetically impaired hGBA1 in GD patients. Immune issues aside, such enzymes, which may be harvested from source organisms as wild-type proteins, need to meet several requirements, most predominantly that they are active on GlcCer within lysosomes. The research described in this chapter aimed to establish whether such non-human GBA1 substitutes would be viable *in situ* active retaining β-glucosidases, acting in lysosomes, and attention was turned to the plant world as potentially cheap and easy to manage production vehicles.[18-21] Earlier research identified the existence of a retaining β-glucosidases in the tobacco plant, *Nicotiana tabacum* with some similarities to hGBA1: it has a similar size (about 57 kD) and more importantly has the highest activity at acidic pH.[22] This protein (UniProt code: A0A1S4CL56) classified as a CAZy GH 5 family member, and for the remainder of this chapter termed B56, was putatively classified as a 1,3-β-glucanase.[22] Thus, it is not known whether it is involved in processing of plant GlcCer. Still B56 was considered a good starting point to address the question whether it would be a viable lysosomal β-glucosidase in human cells. The research in this chapter reveals this to be true, in part. It withstands lysosomal conditions and reacts with inhibitors and activity-based probes (ABPs) that also react with hGBA1. It does not hydrolyse fluorescent GlcCer derivatives and is thus not a bona fide hGBA1 substitute. Despite this, the work as presented here does reveal that plant retaining β-glucosidases may become targets for new ERT developments. It moreover provides a platform to study such candidates: by overexpressing, in a human GBA1/GBA2 knockout background, of the candidate enzyme, followed by *in vitro* and *in situ* probing of its stability and activity using the set of substrates, inhibitors and probes that also have shown their value in investigating hGBA1 and hGBA2 in the context of GD and PD.

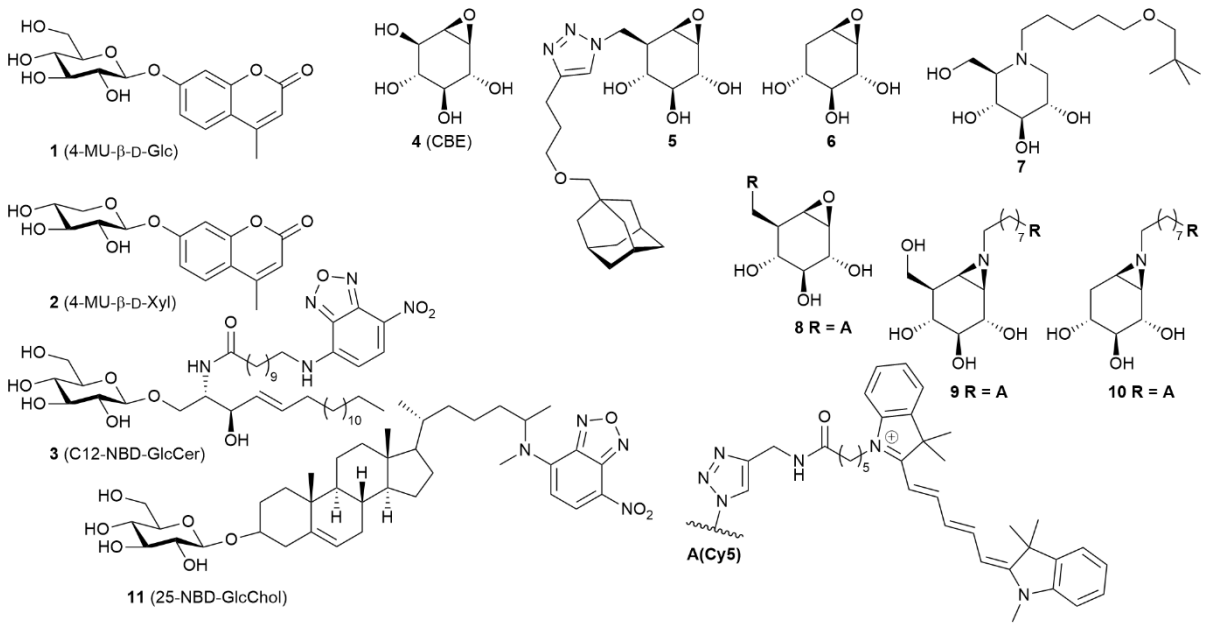


Figure 1. Substrates, inhibitors, and probes used in the here-presented studies.

Results

Chemical proteomics on *Nicotiana tabacum* extracts using a biotin-cyclophellitol aziridine activity-based probe (compound **16** in Chapter 5) in previous studies identified B56 as a retaining β-glucosidase with a pH activity optimum between 4 and 5, thereby indicating this enzyme to be a likely hGBA1 substitute, at least in terms of putative lysosomal activity.[22] Rather than cloning and purifying the enzyme for subsequent exposure to human (Gaucher) cells, it was elected to stably express B56 in GBA1 knockout as well as GBA1/GBA2 double knockout cells as relevant Gaucher models. These cells, and extracts thereof, were interrogated on retaining β-glucosidase activity and on B56 intracellular trafficking and post-translational glycosylation. Selected reagents and probes that also featured in the preceding chapters and that were used to investigate hGBA1, hGBA2 and *C. elegans* GBA1-3 were used in these studies. These comprise (Figure 1) the artificial, fluorogenic substrates, 4-methylumbelliferyl (4-MU-) β-D-glucopyranoside (4-MU-β-D-Glc, **1**), 4-MU-β-D-xylopyranoside (4-MU-β-D-Xyl, **2**), the fluorescent synthetic GlcCer analogue, C12-NBD-GlcCer **3**, and GlcChol analogue, 25-NBD-GlcChol **11**, the hGBA1-selective inhibitors **4** (conduritol B epoxide, CBE),[23] cyclophellitol derivative **5**,[24] and β-D-*xylo*-cyclophellitol **6**,[25] the somewhat hGBA2-selective iminosugar competitive inhibitor **7**[26] as well as the hGBA1-selective (**8**)[24] and broad-spectrum retaining β-glucosidase (**9**[27], **10**[25]) activity-based probes (ABPs).

Expression of active B56 enzymes in HEK293T cells

cDNAs encoding B56 (marked as B56 Wild-Type (WT)) or V5/His-tagged B56 (marked as B56 Tag) were transfected into human GBA1/GBA2 KO HEK293T cells. Lysate of HEK293T cells transfected with B56 cDNA showed a considerable increased β-glucosidase activity (40-50 nmol/h/mg) towards 4-MU-β-D-Glc **1** as compared to the non-transfected (mock) cells (< 2 nmol/h/mg) (Figure 2A). Recombinant B56 in cell lysate (both B56 WT and B56 Tag) also hydrolyzed 4-MU-β-D-Xyl **2**, but in an about two-fold lower rate than observed for **1** (Figure 2A). For comparison, hGBA1 activity towards **1** is about 20-fold higher than towards **2**. The transfected hGBA1/GBA2 KO HEK293T cells expressing recombinant B56 (WT or Tag) are for the remainder of this chapter termed as 'B56 (WT or Tag) cells'.

Characterization of B56 expressed in HEK293T cells by activity-based protein profiling (ABPP) and fluorogenic substrate assays

Lysates of B56 (WT or Tag) hGBA1/hGBA2 KO HEK293T cells were incubated with hGBA1-selective ABP **8** or broad-spectrum β-glucosidase ABP **9**. Compared with the absence of ABP-labelling in the non-transfected (mock) cells, ABPs **8** and **9** returned a clear signal in the fluorescence scan of the wet SDS-PAGE gel at around 57 kDa, which corresponds to the molecular weight of B56 (Figure 2B). The signal derived from the B56 Tag transfected cells is slightly higher at around 60 kDa, which would be expected due to the presence of the V5/His tag (Figure 2B). The pH optimum of B56 for ABP labelling and enzyme activity was next examined. To this end, the extracts were treated with 200 nM ABP **2** final concentrations, at a pH range varying from 3 to 8. As shown in Figure 2C, the emitted fluorescence intensity in the resultant SDS-PAGE gels is maximal at pH 5.0, with no signal for the samples incubated at the low and high end of the pH range. The pH-dependent fluorogenic substrate assay using 4-MU-β-D-Glc **1** also shows, both for B56 WT and B56 Tag, a maximal activity at pH 4.5-5. The optimum acidic pH of B56 for β-glucosidase activity thus reflects that of hGBA1, which has an activity maximum at pH 5.2. Next, lysates of B56 cells were incubated with 4-MU-β-D-Glc **1** under variable temperature conditions (20-70 °C) to investigate the optimum temperature in terms of enzyme activity. Intriguingly, B56 (WT or Tag) in HEK293T cell lysate showed maximal 4-MU-β-D-Glc **1** hydrolysis activity at 60 °C (Figure 2D). In sharp contrast, hGBA1 becomes inactive after exposure to 42 °C.

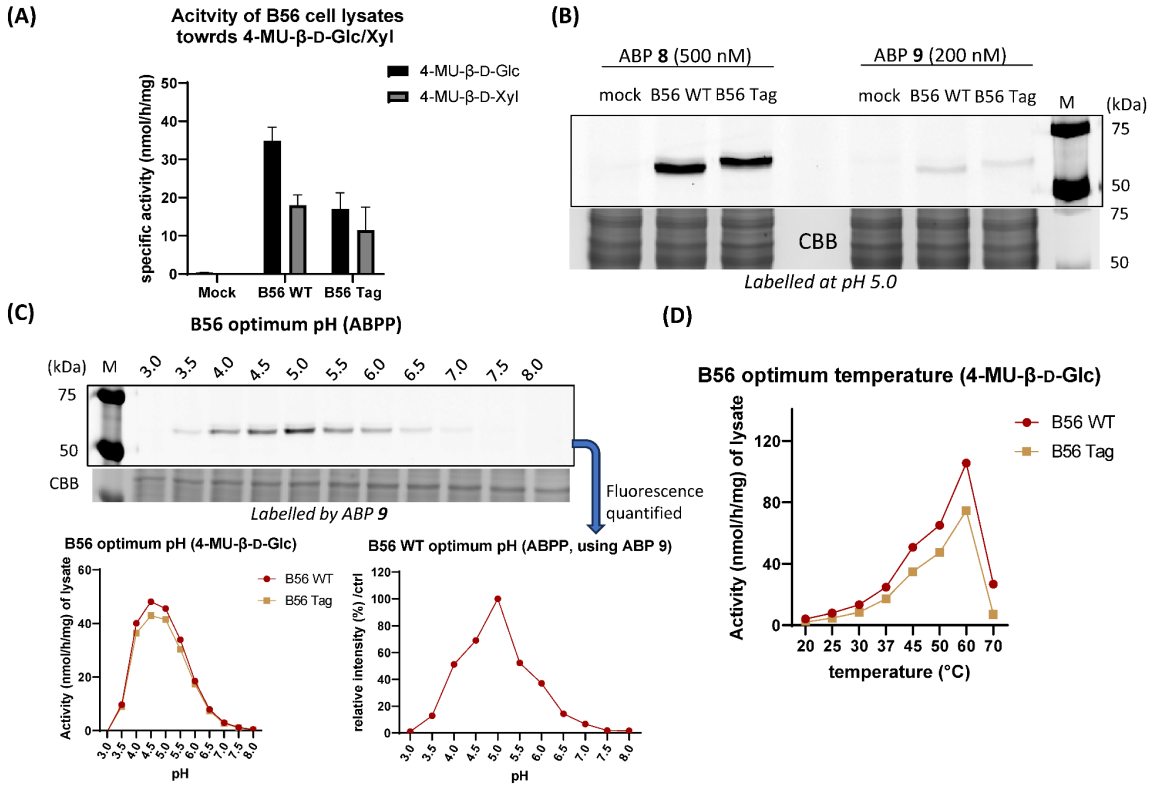
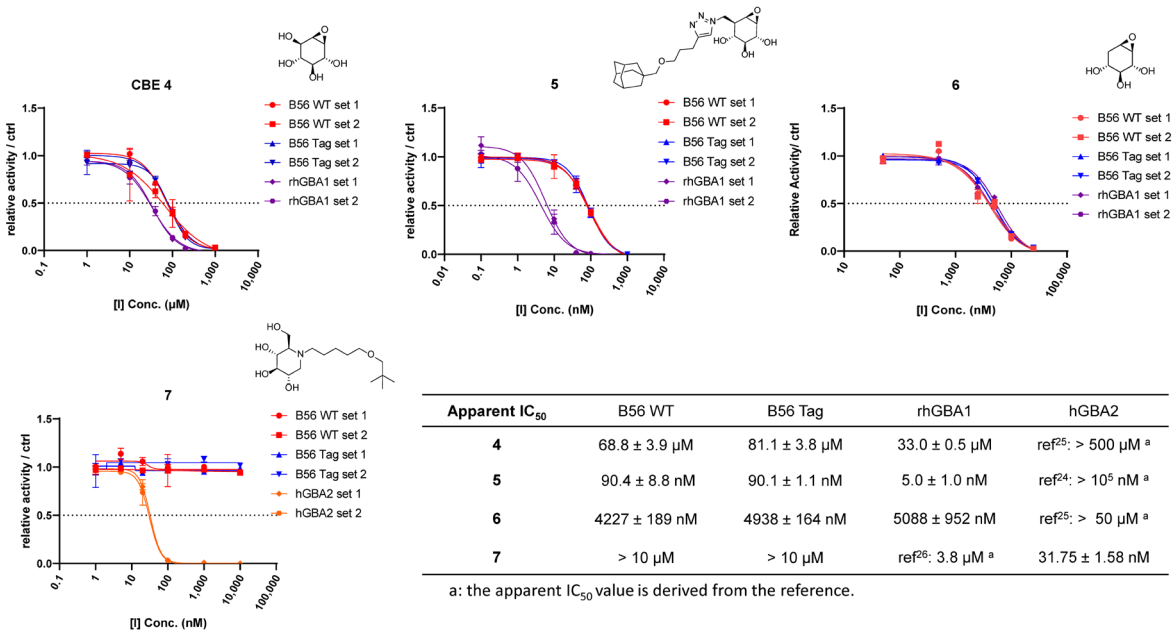


Figure 2. Characterization of B56 (WT and Tag) expressed in HEK293T hGBA1/GBA2 KO cells. Mock = lysate of hGBA1/GBA2 KO HEK293T cells. (A) Activity of B56 in cell lysates towards 4-MU-β-D-Glc **1** and 4-MU-β-D-Xyl **2**. Lysates of B56 cells were incubated with 4-MU substrate **1** or **2** at pH 5.0 for 30 min at 37 °C. (B) Reactivity of ABP **8** and **9** towards B56 (WT and Tag) in cell lysates. Lysates of B56 cells were incubated with 500 nM ABP **8** or 200 nM ABP **9** at pH 5.0 for 30 min at 37 °C and subjected to SDS-PAGE and fluorescence scanning of the wet gel slabs. (C) The optimum pH of B56 for labelling with ABP **9** and 4-MU-β-D-Glc hydrolysis activity, as determined by ABPP and fluorogenic substrate assays, respectively. For ABPP, lysates of B56 cells were incubated with 200 nM ABP **9** for 30 min at 37 °C at various pH values (3-8). For fluorogenic substrate assay, lysates of B56 cells were incubated with 4-MU-β-D-Glc for 30 min at 37 °C at various pH values (3-8). (D) Effect of temperature on enzyme activity of B56 in HEK293T cell lysates. Lysates of B56 cells were incubated with 4-MU-β-D-Glc for 30 min at pH 5.0 at various temperatures (20-70 °C).

Inhibition of B56 in hGBA1/GBA2 KO HEK293T cell extracts by classical hGBA1/hGBA2 inhibitors

In the next set of experiments, the sensitivity of B56 to the established hGBA1 and hGBA2 inhibitors **4**-**7** was assessed. Figure 3 lists the apparent IC_{50} values for inhibitors **4**-**7** as determined in a 4-MU-β-D-Glc **1** fluorogenic substrate assay, in a head-to-head comparison with recombinant hGBA1 (rhGBA1, Imiglucerase) and hGBA2. hGBA1-selective inhibitor **4**-**6** were found to potently inhibit B56 (WT and tagged) and rhGBA1. The hGBA2-selective inhibitor **7** in turn did not inhibit B56 (apparent IC_{50} value > 10 µM). This result underscores that B56 may resemble in selectivity hGBA1, more so at least than it does hGBA2.



Apparent IC_{50}	B56 WT	B56 Tag	rhGBA1	hGBA2
4	68.8 ± 3.9 µM	81.1 ± 3.8 µM	33.0 ± 0.5 µM	ref[25]: > 500 µM [a]
5	90.4 ± 8.8 nM	90.1 ± 1.1 nM	5.0 ± 1.0 nM	ref[24]: > 10^5 nM [a]
6	4227 ± 189 nM	4938 ± 164 nM	5088 ± 952 nM	ref[25]: > 50 µM [a]
7	> 10 µM	> 10 µM	ref[26]: 3.8 µM [a]	31.75 ± 1.58 nM

a: the apparent IC_{50} value is derived from the reference.

Figure 3. Inhibitory potency of β-glucosidase inhibitors **4**-**7** on B56. Lysates of B56 (WT and Tag) cells were incubated with inhibitor **4**-**6** or inhibitor **7** at pH 5.0 for 30 min at 37 °C, followed by incubation with 4-MU-β-D-Glc for 30 min. rhGBA1 = Imiglucerase. hGBA2 = lysate of hGBA1/GBA2 KO HEK293T cells overexpressing hGBA2. Errors range = ± SD, n = 2 replicates.

B56 acquires high-mannose type N-glycans when expressed in hGBA1/hGBA2 KO HEK293T cells

To examine whether B56 undergoes N-glycosylation, extracts of B56 WT cells were treated with Sepharose beads modified with the α-D-mannosyl glycan receptor, Concanavalin A (ConA-beads).[28-30] B56 WT was first incubated with ConA-beads, followed by several washing steps to remove unbound proteins. Following the final washing step, ConA beads were collected and are termed as the 'ConA beads' fraction. Then, the fractions from all washing steps as well as the ConA bead fraction were incubated with ABP **9**, the protein samples resolved by SDS-PAGE, and the resulting wet gel slabs scanned for in-gel fluorescence. As can be seen (Figure 4A) virtually all input signal (left lane) is retained in the ConA-beads fraction with little to no fluorescence in the lanes stemming from the washing steps, indicating that B56 binds ConA-beads and, therefore, contains (high mannose-type) N-linked glycans. To further study the type of N-glycans in B56, N-glycanase digestion experiments with the glycanases, PNGase F and Endo H, were conducted (Figure 4B). PNGase F can remove most N-glycans from glycoproteins whereas Endo H is only able to remove high-mannose-type N-glycans. Treating extracts from B56 WT as well as B56 Tag hGBA1/GBA2 KO HEK293T cells which were pretreated with ABP **9** with PNGase F, followed by SDS-PAGE and in-gel fluorescence scanning revealed several signals corresponding to a lower molecular weight compared to the non-PNGase F treated samples, suggesting partial de-N-glycosylation. Treatment with Endo H in turn returned a major and a minor band for the WT sample and two bands with similar intensity for the Tag one. The

near complete digestion with Endo H suggests that B56 protein predominantly acquired high-mannose type N-glycans.

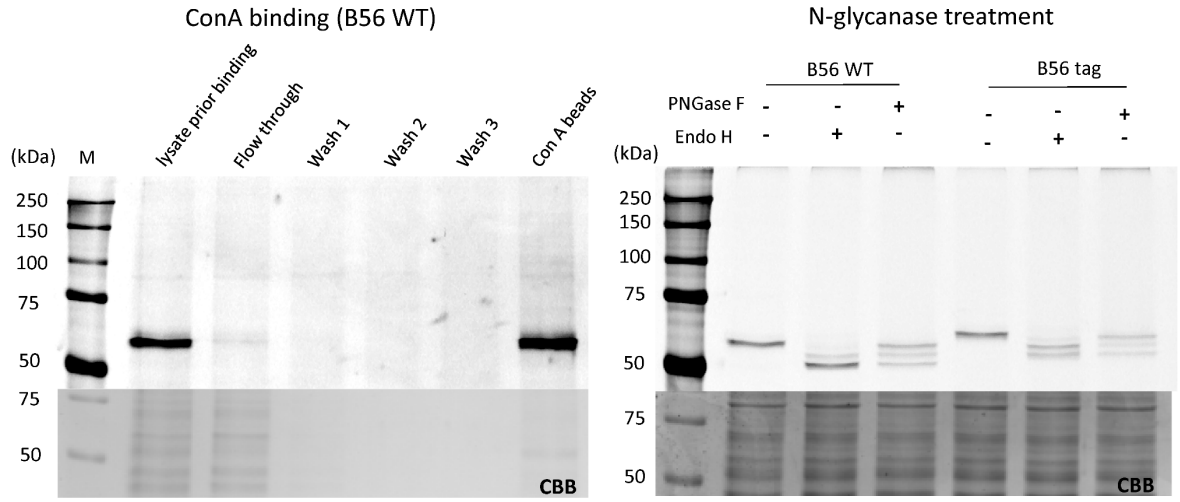


Figure 4. (A) ConA binding to B56 WT in hGBA1/GBA2 KO HEK293T cell extracts. Lysate of B56 WT ('Initial lysate' fraction) were incubated with ConA beads for 2 h at 4°C. After centrifugating, supernatant was collected as 'Flow through' fraction, the ConA beads were subsequently washed for three consecutive times and the washing solutions were collected as 'Wash 1-3' fractions. Following the final washing step, ConA beads were collected as 'ConA beads' fraction. All fractions were incubated with 500 nM ABP **9** for 30 min at pH 5.0 at 37 °C and subjected to SDS-PAGE and fluorescence scanning of the wet gel slabs. (B) PNGase F and Endo H digestion of extracts of B56 expressed hGBA1/GBA2 KO HEK293T cells. Lysates of B56 cells were first incubated with 500 nM ABP **9** for 30 min at 37°C at pH 5.0, followed by incubation with PNGase F or Endo H, and subjected to SDS-PAGE and fluorescence scanning.

Subcellular localization of B56 expressed in hGBA1/GBA2 KO HEK293T cells

To investigate the sub-cellular localization of B56 expressed in HEK293T cells, immunocytochemistry was employed. hGBA1/GBA2 KO HEK293T cells expressing V5-tagged B56 were fixed and stained with fluorescent anti-LAMP1 (lysosomal-associated membrane protein 1) antibody and fluorescent anti-V5 antibody. Subsequently, fluorescence of anti-LAMP1 antibody and anti-V5 antibody were visualized by confocal fluorescence microscopy. As shown in Figure 5, the fluorescence of anti-LAMP1 (indicating sub-cellular localization of lysosomes) and anti-V5 (indicating sub-cellular localization of B56) are overlapping, suggesting that the V5-tagged B56 is at least partly localized in LAMP1-positive lysosomes.

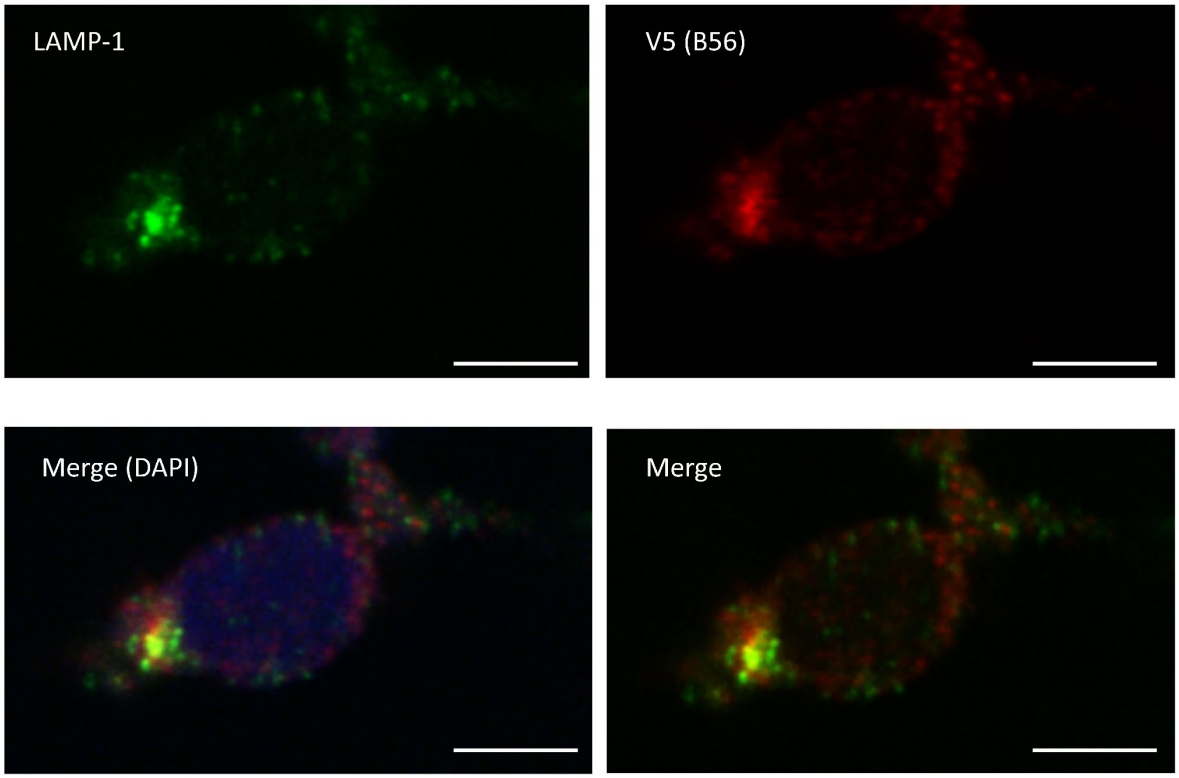


Figure 5. Sub-cellular localization of hGBA1/GBA2 KO HEK293T cells expressing V5-tagged B56. Alexa Fluor™ 488 (green)-tagged anti-LAMP1 antibody was used to visualize sub-cellular localization of lysosomes. Alexa Fluor™ 647 (red)-tagged Anti-V5 antibody was used to visualize sub-cellular localization of V5-tagged B56. Nuclei were stained with 10 µg/ml DAPI (shown as blue). Scale bar = 5 µm.

Processing of C12-NBD-GlcCer by B56-expressing hGBA1/GBA2 KO HEK293T cells and cell extracts

Next, the C12-NBD-GlcCer degradation capacity of B56 expressed in HEK293T cells was examined. Lysates of B56 cells were incubated with C12-NBD-GlcCer for the indicated time, after which lipids were extracted and subjected to high-performance thin layer chromatography (HPTLC) separation and fluorescence scanning of the TLC plates. rhGBA1 (Imiglucerase) was used as positive control to show the position of C12-NBD-Cer. No C12-NBD-Cer formation was observed after 2.5 h incubation *in vitro*, and neither upon prolonged incubation time up to 16 h or using ConA beads enriched B56 (Figure 6A/B). An assay was also conducted to assess whether NBD-GlcCer can be degraded by B56 *in situ*. To this end, 80% confluent hGBA1/GBA2 KO HEK293T cells expressing B56 (WT or Tag) were exposed to C12-NBD-GlcCer for 3 h *in situ*. Afterwards, cells were washed, collected, lysed, and subjected to HPTLC analysis (Figure 6C). Extracts of wild type (WT) HEK293T cells as positive control converted NBD-GlcCer to NBD-Cer *in situ*, while extracts of hGBA1/GBA2 KO HEK293T cells expressing B56 did not. Considering that BY2 cells may contain some components assisting B56 to hydrolyze GlcCer in tobacco, an attempt was conducted to examine whether a mixture of tobacco BY2 cell lysate and B56 expressing HEK293T cell lysate (in hGBA1/hGBA2 KO background) was able to degrade C12-NBD-GlcCer. BY2 cell lysates were treated or not with hGBA1-selective inhibitor **5** (with the aim to inhibit potential β-glucosidases in BY2 lysate), followed by washing with a desalting column to remove unbound inhibitor. Afterwards, the washed BY2 lysate was mixed with lysate of hGBA1/GBA2 KO HEK293T cells expressing B56 WT and incubated with C12-NBD-GlcCer for 3 h *in vitro*. The sample not treated with inhibitor **5** proved to be able to convert NBD-GlcCer, while the **5**-treated samples (including the sample using mixture of BY2 lysate and B56-expressing HEK293T lysate) did not.

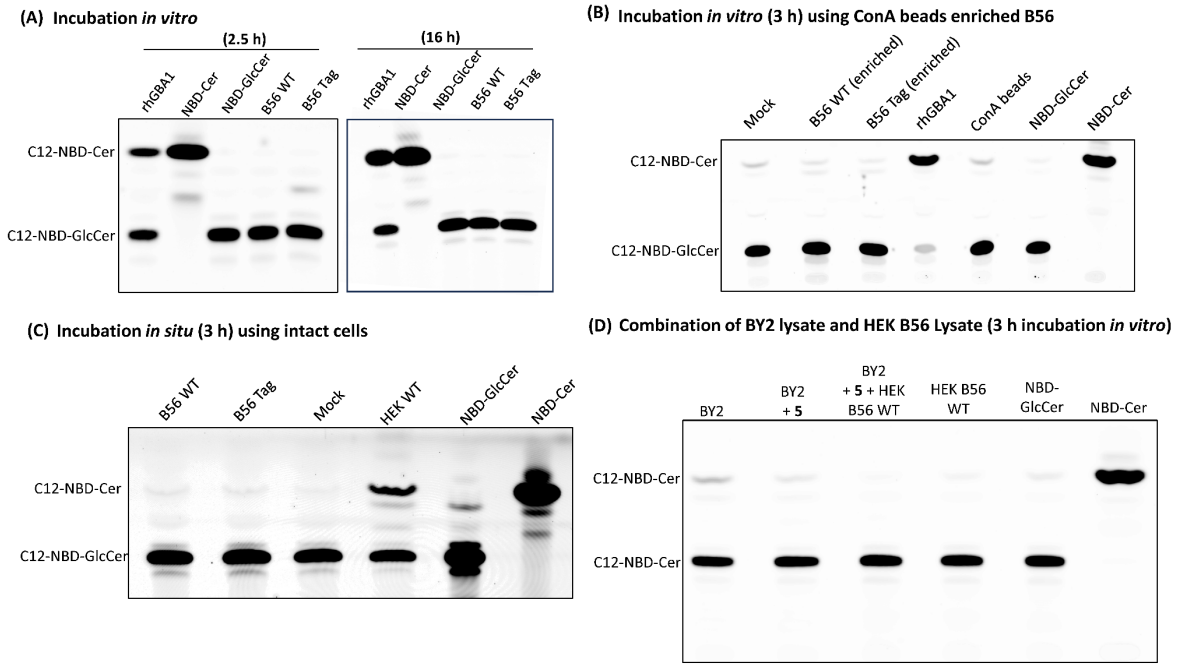


Figure 6. C12-NBD-GlcCer processing by hGBA1/hGBA2 KO HEK293T expressing B56 (WT or Tag) expression. 'rhGBA1' = sample using Imiglucerase. 'B56 WT' or 'B56 Tag' = sample of B56 (WT or Tag) cells or cell lysates. 'Mock' = HEK293T hGBA1/GBA2 KO cells or cell lysates. 'HEK WT' = intact living wild-type HEK293T cells. 'ConA beads' = sample containing ConA beads only. (A) Lysates of B56 cells incubated with 500 nM NBD-GlcCer for 2.5 h or 16 h at pH 5.0 *in vitro*. (B) ConA beads enriching B56 incubated with 500 nM NBD-GlcCer for 3 h at pH 5.0 *in vitro*. (C) Intact WT, hGBA1/GBA2 KO, or B56 expressed HEK293T cells incubated with 1 µM C12-NBD-GlcCer for 3 h *in situ*. Subsequently, cells were washed, collected, lysed, and subjected to HPTLC separation and fluorescence scanning of the TLC plates. (D) BY2 lysate was preincubated with 200 nM inhibitor **5** for 1 h at 37 °C, then sample was washed by a desalting column. Afterwards, BY2 lysate was mixed with lysate of B56 WT cells and incubated with 500 nM NBD-GlcCer for 3 h at 37 °C at pH 4.5. 'BY2' = sample containing BY2 lysate without pre-treatment of **5**. 'BY2 + **5**' = sample of BY2 lysate with pre-treatment of **5** and washing step. 'BY2 + **5** + HEK B56 WT' = lysate of B56 WT cells mixed with BY2 lysate pre-treated with **5**. 'HEK B56 WT' = lysate of B56 WT cells. Lanes: 'NBD-Cer' or 'NBD-GlcCer' = sample containing NBD-Cer or NBD-GlcCer alone.

B56 expressing hGBA1/hGBA2 KO HEK293T cells do not process GlcCer and glucosylsphingosine

Characteristically, GlcCer is elevated in GD patients due to impaired lysosomal degradation, leading to a concomitant marked increase in glucosylsphingosine (GlcSph) through conversion of accumulating GlcCer by acid ceramidase.[2] To investigate whether B56 when expressed in human cells can process these storage glycolipids, LC-MS/MS was employed to detect the changes of glycosphingolipids in hGBA1 KO or hGBA1/GBA2 KO HEK293T cells expressing B56 (WT or Tag). As shown in Figure 7, GlcCer increased 3-fold in both non-B56-transfected hGBA1 KO HEK293T cells and hGBA1/GBA2 KO HEK293T cells, when compared to the wild-type cells. Expression of B56 in these cells did not lead to a decrease of GlcCer levels. GlcSph is present only in very low amounts in wild type HEK293T cells. In contrast, GlcSph levels are drastically increased both in hGBA1 KO HEK293T cells and in hGBA1/GBA2 KO HEK293T cells, resembling the situation of GD patients, and expression of B56 in these cells did not suppress these GlcSph levels. As well, β-glucosylcholesterol (GlcChol), a glycosphingolipid mainly degraded by hGBA1 and generated by hGBA2 via transglycosylation,[31] was not considerably influenced by expression of B56 in hGBA1 KO or hGBA1/GBA2 KO HEK293T cells.

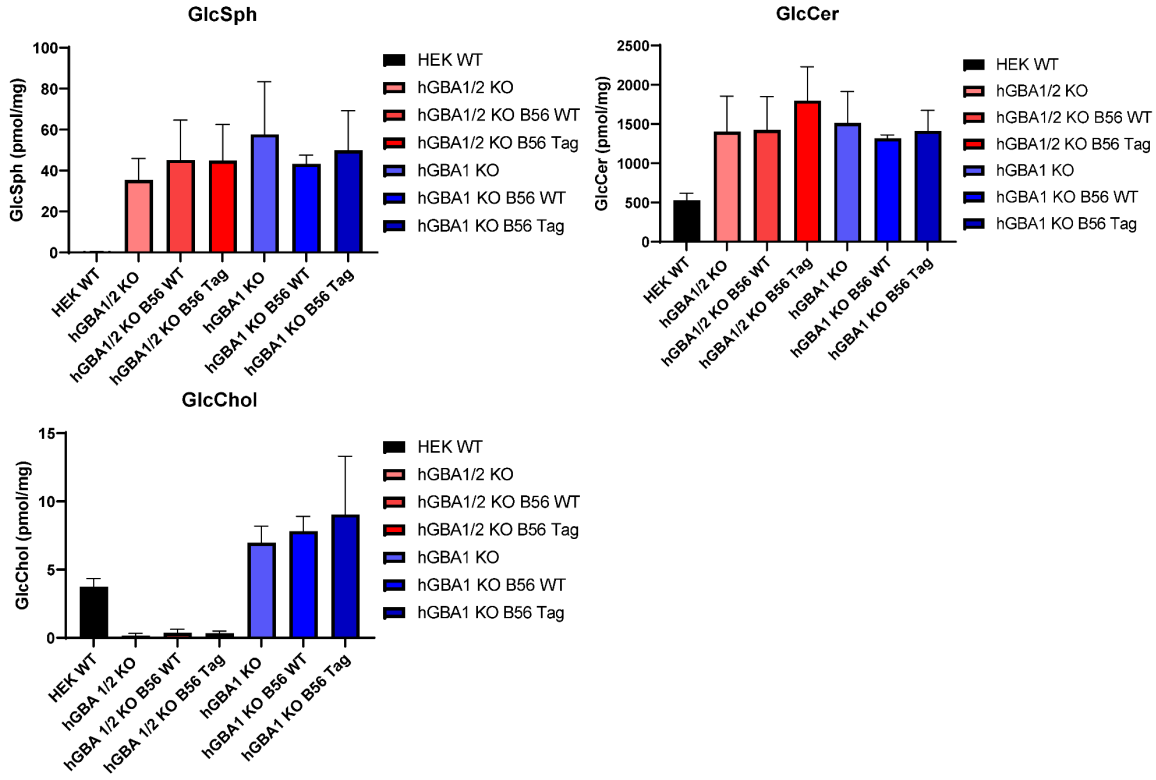


Figure 7. Glycosphingolipid levels in hGBA1 knockout (KO) or hGBA1/GBA2 KO HEK293T cells expressing B56 (WT or tagged), as detected by LC-MS/MS. n = 3 biological replicates. 'HEK WT' = HEK293T wild type cells; 'hGBA1/2 KO' = HEK293T hGBA1/GBA2 KO cells; 'hGBA1 KO' = HEK293T hGBA1 KO cells; 'B56 WT or B56 Tag' = wild type B56 or V5-tagged B56 expressed in HEK293T cells (in either hGBA1 KO or hGBA1/GBA2 KO background).

B56 expressing hGBA1/hGBA2 KO HEK293 cells do not produce GlcChol

Previous studies have revealed that hGBA1 catalyzes transglycosylation *in vitro*, generating β-GlcChol when incubated with cholesterol as an acceptor and 4-MU-β-D-Glc or GlcCer as sugar donors at appropriate conditions.[31] *In vivo* hGBA1 is involved in degrading GlcChol into cholesterol and glucose. hGBA2 as well generates GlcChol via transglucosylation using GlcCer as sugar-donor.[31] Additionally, hGBA1 also efficiently produces β-xylosylated cholesterol (XylChol) and even sequentially Xyl_2Chol *in vitro*. As the final experiment, B56 as produced by hGBA1/hGBA2 KO HEK293 cells studied on its transglycosylation capacity. Enriched B56 (by ConA beads) was incubated with 4-MU-β-D-Glc **1** or 4-MU-β-D-Xyl **2** as sugar donors and 25-NBD-Chol **11** as acceptor for 16 h at 37 °C. As shown in Figure 8, B56 did not generate GlcChol or XylChol. In sharp contrast, recombinant human GBA1 (rhGBA1, Imiglucerase) was able to generate GlcChol, XylChol and Xyl_2Chol as reported earlier.[32]

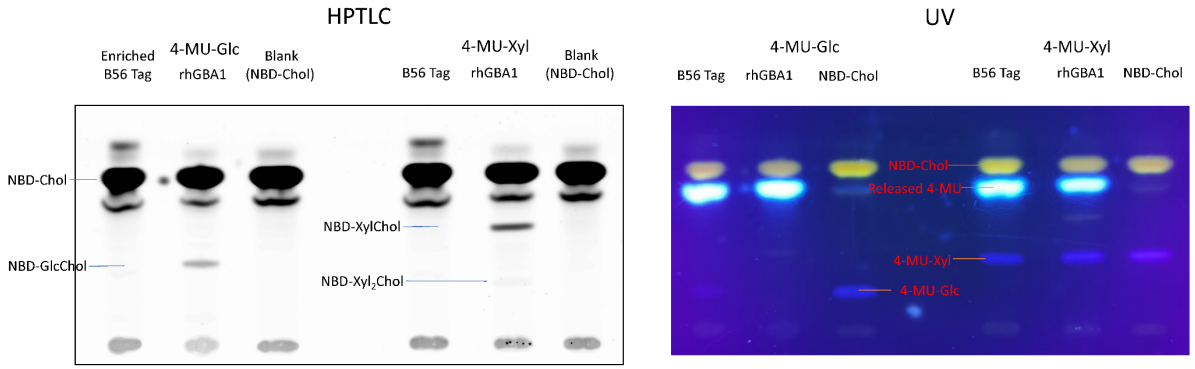


Figure 8. Transglycosylation capacity of HEK293T-produced B56. V5-tagged B56 enriched by ConA beads was incubated with either 4-MU-β-D-Glc or 4-MU-β-D-Xyl as sugar donor and 25-NBD-Chol as acceptor for 16 h at 37 °C at pH 5.0. Lanes: 'B56 Tag' = the enriched B56 (by ConA beads) expressed in hGBA1/GBA2 KO HEK293T cells was incubated with substrates (sugar donor and acceptor). 'rhGBA1' = isolated rhGBA1 (Imiglucerase) incubated with substrates at pH 5.2 (supplemented with 0.1% (v/v) Triton X-100, 0.2% (w/v) sodium taurocholate, and 0.1% (w/v) bovine serum albumin). 'NBD-Chol' = sample containing 25-NBD-Chol alone. Intensity of 'released 4-MU' reflects the amount of hydrolyzed 4-MU-glycoside, as detected under ultraviolet light (UV).

Discussion

The *Nicotiana tabacum* β-glucosidase B56 was previously identified as a potential analogue of the human lysosomal enzyme, deficient in GD, hGBA1. It has about the same molecular weight; it employs a retaining mechanism; and is active at lysosomal pH.[22] At the onset of the studies described here, also some notable differences were apparent. It is a member of the GH5 CAZy family and therefore has a different fold compared to the GH1 family to which hGBA1 belongs, and indeed there is little to no sequence homology between the two enzymes (see SI Figure S1). As well, no GlcCer metabolizing activity has been ascribed to B56 for which no substrate is known, though B56 is listed as probable glucan 1,3-β-glucosidase A isoform X2 in UniProt. With this prior information available, a research campaign was started to investigate the possibility to express B56 in human cells, and to investigate its fate and catalytic activity in this foreign environment. The results, as described here, reveal that B56 is readily expressed in hGBA1 as well as hGBA1/hGBA2 KO HEK293 cells. It moreover acquires N-glycans and ends up in lysosomes, suggesting it is expressed and processed like hGBA1 (expression in the ER, where it is N-glycosylated and then rooted to lysosomes). Once in lysosomes, it is active both towards fluorogenic substrates and retaining β-glucosidase ABPs, as well as susceptible to hGBA1-selective β-glucosidase inhibitors. It is, however, not able to process either natural or synthetic GlcCer derivatives and neither possesses transglycosylation activities. These last two features distinguishes B56, in this setting, from hGBA1 and also hGBA2. Functionally, B56 can therefore not be seen as a viable biological for new ERT strategies for treating GD patients. This important caveat aside, the work presented here does imply that true plant-origin hGBA1 homologues may be considered for this purpose. Obviously, such species need to be engineered in such way that the host immune system is not activated (this issue is not addressed in this chapter). Should such entities emerge, the workflow presented here including the *in vitro* and *in situ* assays reporting on enzyme activities as well as substrate turnover rates should be of help in evaluating their ability to repair, in Gaucher cells, impaired GlcCer turnover.

Experimental procedures

Materials

Recombinant human GBA1 (rhGBA1, Imiglucerase) was kindly provided by Genzyme (Genzyme Nederland, Naarden, The Netherlands). 4-MU-β-D-glucopyranoside (4-MU-β-D-Glc) and 4-MU-β-D-xylopyranoside (4-MU-β-D-Xyl) were purchased from Glycosynth (Warrington, UK). Wet gel slabs in the ABPP experiments were imaged using a Typhoon FLA 9500 scanner (GE Healthcare) at λ_{EX} 635 nm and $\lambda_{EM} \geqslant$ 665 nm for Cy5 fluorescence. SDS-PAGE gels were stained for loading control of proteins with Coomassie G250 and scanned on a ChemiDoc MP imager (Bio-Rad, Hercules, CA, USA). Fluorescent NBD-lipids (C12-NBD-GlcCer, 25-NBD-Chol) were purchased from Avanti (Alabama, USA). Antibodies purchased from Abcam (Cambridge, MA, USA). PNGase F and Endo H were purchased from New England Biolabs (Ipswich, USA). Polytron PT 1300D sonicator (Kinematica, Luzern, Switzerland) and potassium phosphate buffer (25 mM KH_2PO_4-K_2HPO_4, pH 6.5, supplemented with protease inhibitor cocktail (EDTA-free, Roche, Basel, Switzerland) and 0.1% (v/v) Triton X-100) were used for lysing cells. Harvested cells (cell pellets) and cell lysates not used directly were stored at -80 °C. The protein concentration of the lysates was determined using Pierce BCA Protein Assay Kit (Thermo Fisher Scientific; Waltham, USA). HEK293T cells were cultured and lysed as described in Chapter 2. CBE (conduritol B epoxide) was purchased from Enzo Life Sciences (Farmingdale, NY, USA). All other compounds were synthesized at the department of Bio-organic Synthesis of the Leiden Institute of Chemistry (LIC), Leiden University

Expression of B56 with or without V5-tag in HEK293T cells

The cDNA of B56 was purchased from GenScript. The B56 wild type gene and the wild type gene with C-terminal V5 epitope and a His tag (tagged B56) were amplified from pcDNA3.1+/C-(K)-DYK vectors in DH5α *E. coli*. After amplification, a BP recombination reaction was conducted according to the Gateway® Technology with Clonase II (Invitrogen™). The produced donor vector pDONR221 -wild type and -tagged were transformed into DH5α *E. coli* via heat shock. The plates with kanamycin were used to select the positive transformants and the plasmids were sequenced. Then a LR reaction was performed to clone both genes into the destination vector pDEST40. Then the pDEST40 wild type or V5-tagged plasmids were then transfected into the HEK293T cells (in either hGBA1 KO or hGBA1/GBA2 KO background) using polyethylenimine (PEI)[33] for B56 WT and B56 V5-tagged expression. Primers are shown in Table 1 below. hGBA1 KO and hGBA1/GBA2 KO HEK293T cell lines were generated as described in Chapter 2.

Table 1: Primers for the amplification and recombination of WT and tagged version.

Reaction	Forward	Reverse
Amplification	5'-GCACCAAAATCAA CGGGACT-3'	5'-TTGTCTTCCCAAT CCTCCCC-3'
Recombination for WT	5'-GGGGACAAGTTTG TACAAAAAAGCAGGCT ACCACCATGGCAAGCT ACTCATGG-3'	5'-GGGGACCACTTTGT ACAAGAAAGCTGGGTC TTAAAGCTTGATATAGC CATTC-3'
Recombination for tagged	5'-GGGGACAAGTTTG TACAAAAAAGCAGGCT ACCACCATGGCAAGCT ACTCATGG-3'	5'-GGGGACCACTTTGT ACAAGAAAGCTGGGTC AAGCTTGATATAGCCAT TCTTG-3'

4-MU fluorogenic substrate assays

Enzyme activity and apparent IC_{50} of B56 in cell lysates were determined by 4-methylumbelliferyl-glycoside fluorogenic substrate assays as described in Chapter 2. To determine enzyme activity, lysate of B56 cells were prepared in 12.5 µL potassium phosphate buffer, and mixed with 12.5 µL McIlvaine buffer (150 mM, pH 5.0) and incubated with 100 µL 3.75 mM 4-MU-β-D-Glc or 3 mM 4-MU-β-D-Xyl dissolved in McIlvaine buffer (150 mM, pH 5.0) for 30 min at 37 °C. To determine apparent IC_{50}, 12.5 µL lysate of B56 cells were mixed with 12.5 µL inhibitor (**4**-**7**) diluted in McIlvaine buffer (150 mM, pH 5.0) and incubated for 30 min at 37 °C, then the 25 µL samples were incubated with 100 µL 3.75 mM 4-MU-β-D-Glc dissolved in McIlvaine (150 mM, pH 5.0) at 37 °C for 30 min. After stopping the enzyme reaction with 200 µL 1 M NaOH-glycine (pH 10.3), 4-Methylumbelliferone fluorescence was measured with a fluorimeter LS55 (Perkin Elmer, Waltham, MA, USA) with λ_{EX} 366 nm and λ_{EM} 445 nm. Where indicated, the pH of McIlvaine buffer, incubation temperatures, or the percentage of additives (Triton X-100, sodium taurocholate) in McIlvaine buffer was varied.

***In vitro* ABPP with SDS-PAGE**

ABPP were conducted as described in Chapter 2. Lysates of B56 cells were incubated with a ABP (**8**-**10**) in McIlvaine buffer (150 mM, pH 5.0 or at indicated pH) at 37 °C for 30 minutes, and subjected to proteins denature, SDS-PAGE, and fluorescence scanning of the wet gel slabs.

Immunocytochemistry

HEK293T cells expressing B56 containing a V5-tag were used for immunochemistry using confocal fluorescence microscopy. Sterile coverslips were put into the wells of 6-well plates and 0.01% of poly-L-lysine (PLL) milliQ solution was applied on the coverslips for coating the coverslips aseptically. The cell homogenate was added on the coverslips and DMEM medium (Sigma-Aldrich) was added into wells for cell growing. When the confluency reached 80%-90%, cells were fixed on the coverslips with 4% (w/v) paraformaldehyde (PFA). After rinsing with phosphate-buffered saline (PBS), permeabilization buffer (0.1% Triton X-100 in Tris-buffered saline (TBS)) was applied on cells and cells were incubated in dark for 7 minutes. Then the blocking buffer (3% (w/v) BSA in PBS) was used and plate was kept in dark for 30 minutes or longer. Primary antibodies, mouse anti-V5 (Fisher) and rabbit anti-LAMP1 (SouthernBiotech) in blocking buffer (1% and 0.5%, respectively), were then applied on the coverslips. Incubation was performed in dark at room temperature for 2 hours. Secondary antibodies, donkey-anti-mouse-Alexa 647 antibody (Invitrogen) and donkey-anti-rabbit-Alexa 488 antibody (Invitrogen) in blocking buffer (0.1% and 0.1%, respectively), were applied on coverslips to bind with the primary antibodies and cells were incubated in dark at room temperature for 1 hour. Prolong gold with DAPI was applied then. Nail polish was used to seal the dried coverslips and the fluorescence of the coverslips was detected by Leica confocal SP8 microscope.

Binding of B56 to Concanavalin A-Sepharose 4B beads

A slurry of Concanavalin A-Sepharose 4B beads (GE healthcare Bio-Sciences) was prior washed with washing buffer (0.1 M sodium acetate, 0.1 M NaCl, 1 mM $MgCl_2$,1 mM $CaCl_2$,1 mM $MnCl_2$, (pH 6.0)) and then mixed with cell lysates. The mixture was incubated at 4 °C for 2 hours on a tumbling shaker. After centrifugation, supernatant was collected as flow-through and beads were washed for three time by washing buffer (collected as wash fractions). Eventually, beads were separated and collected as beads fraction.[34] Fractions were subjected to 500 nM ABP **9** labelling for 30 min at pH 5.0 at 37 °C, SDS-PAGE and fluorescence imaging of wet gel slabs. For experiments in Figure 6B using B56 enriched by ConA beads, beads were preincubated with B56 cell lysate for 2 h at 4 °C.

N-glycanases treatment

In total 20 µg proteins of B56 cell lysates in McIlvaine buffer (150 mM, pH 5.0) were incubated with 500 nM ABP **9** for 30 min at 37 °C. Afterwards, an aliquot of the samples were treated with PNGase F or Endo H according to the manufacturer's protocol (New England BioLabs). For samples as control (without N-glycanases treatment), samples with a normalized protein amount were incubated with 500 nM ABP **9** for 30 min at 37 °C in McIlvaine buffer (150 mM, pH 6.0). Subsequently, both the control and N-glycanase treated samples were subjected to ABPP as described above.

Assessment of NBD-lipid metabolism

C12-NBD-GlcCer degradation: lysate of hGBA1/GBA2 KO cells expressing B56 (100 µg) were incubated with NBD-GlcCer (500 nM) in McIlvaine buffer (150 mM, pH 5.0) for 2.5 h or 16 h at 37 °C. For rhGBA1, sample containing 10-20 ng rhGBA1 was incubated with NBD-GlcCer in McIlvaine buffer (150 mM, pH 5.2, supplemented with 0.2% (w/v) sodium taurocholate, 0.1% (v/v) Triton X-100, and 0.1% (w/v) bovine serum albumin). Afterwards, lipids were extracted by Blight and Dyer method[35] and subjected to HPTLC, using developing solvent ($CHCl_3$: methanol, 85:15 (v/v)), followed by fluorescence scanning at λ_{ex} = 473 nm and $\lambda_{em} \geq$ 510 nm for detection of NBD fluorescence. For *in situ* assay, 80% confluent hGBA1/GBA2 KO HEK293T cells expressing B56 (WT or Tag) were exposed to 1 µM NBD-GlcCer for 3 h *in situ*, and subjected to lipid extraction and HPTLC. For assessment of NBD-GlcCer degradation by mixture of BY2 cell lysate and B56 WT cell lysate, BY2 cell lysate prepared in potassium phosphate buffer was preincubated with 200 nM inhibitor **5** or vehicle (DMSO, final concentration is 0.5%) for 1 h at 37 °C, then the sample was washed by passing through a Zeba™ spin desalting column with 7K molecular weight cutoff (MWCO). Afterwards, 35 µL BY2 lysate (around 50 µg) pre-inhibited by **5** was mixed with 50 µL lysate of B56 WT cells (70 µg) and incubated with 500 nM NBD-GlcCer in 150 µL McIlvaine buffer (150 mM, pH 4.5) for 3 h at 37 °C.

For the identification of newly formed fluorescent NBD-lipid, using 25-NBD-cholesterol as acceptor, ConA beads were pre-incubated with lysate of B56 (WT or Tag) cells for 2 h at 4 °C, followed by incubation with 400 µM 4-MU-β-D-Glc and 20 µM 25-NBD-cholesterol for 16 h at 37 °C at pH 5.0. For isolated rhGBA1 (Imiglucerase), samples were incubated in McIlvaine buffer (150 mM, pH 5.2, supplemented with of 0.2% (w/v) sodium taurocholate, 0.1% (v/v) Triton X-100, and 0.1% (w/v) bovine serum albumin) for 16 h at 37 °C. Afterwards, samples were subjected to lipid extraction and HPTLC as described above. Hydrolysis of 4-MU-β-D-Glc or 4-MU-β-D-Xyl was indicated by the released 4-methylumbelliferone (released 4-MU) detected under ultraviolet light (UV).

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Appendix

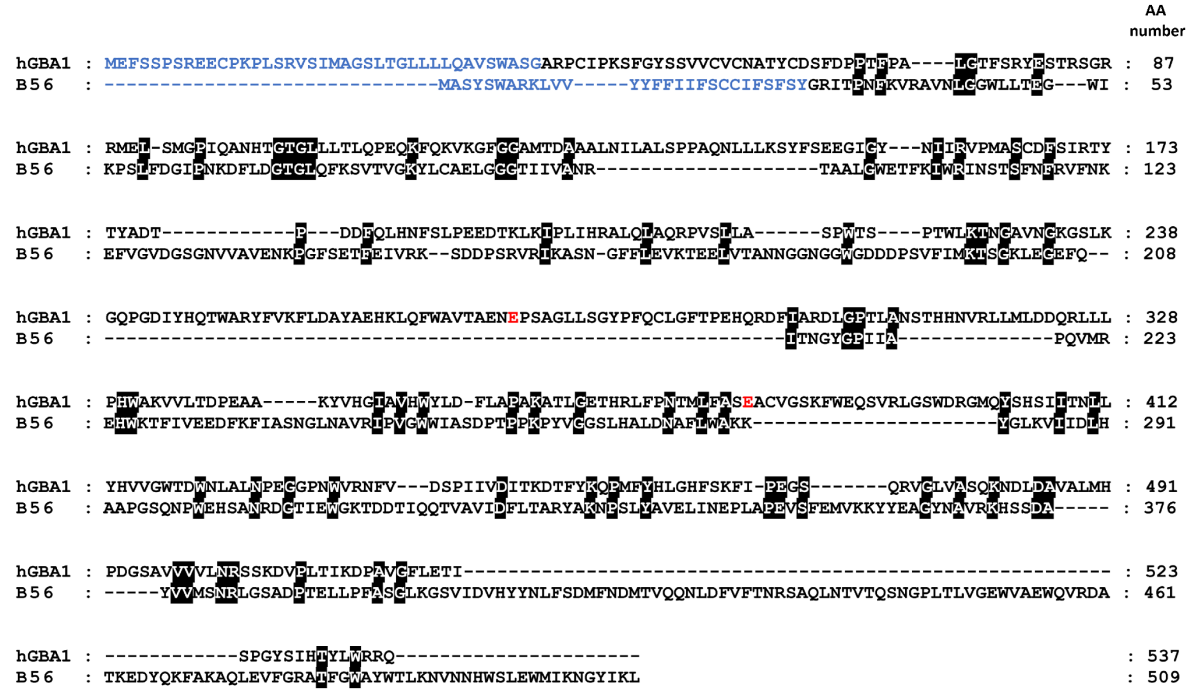

Figure S1. Amino acid (AA) allignment of huaman GBA1 (hGBA1, UniProt code: P04062) and B56 (UniProt code: A0A1S4CL56) by BLAST, no significant similarity was found. Signal peptide (predicted by SignalP 5.0) is marked as blue. hGBA1 catalytic residues (without counting signal peptides), nucleophile E235 and acid/base E340, were marked as red.

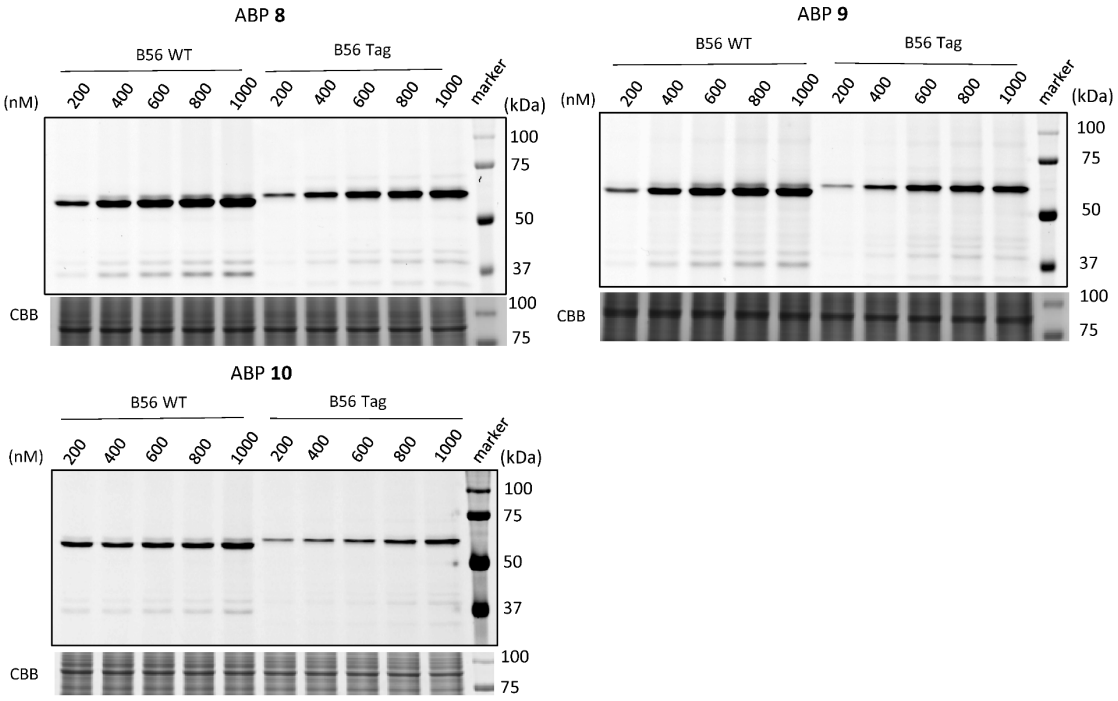

Figure S2. B56 (WT and Tag) reacted with varying concentrations of ABPs **8**-**10**. Lysates of B56 cells were incubated with indicated concentration of ABP **8**-**10** for 30 min at pH 5.0 at 37 °C and subjected to SDS-PAGE and fluorescence scanning of the wet gel slabs.

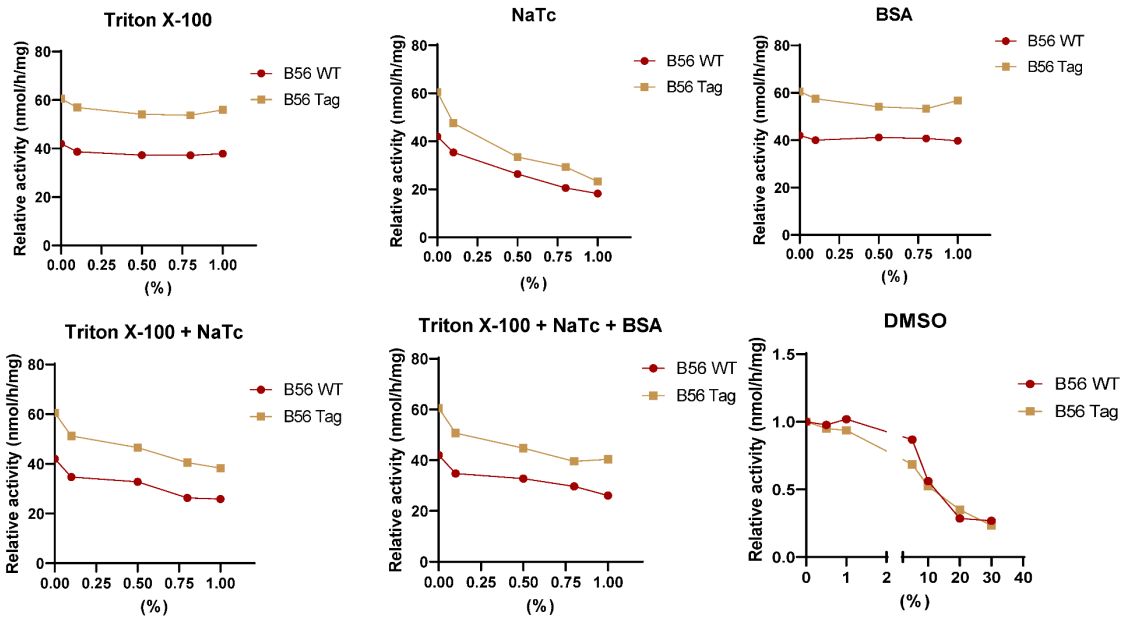

Figure S3. Influence of varying concentrations of various additives on B56 proteins activity, revealed by 4-MU fluorogenic substrate assay. B56 (WT and Tag) in hGBA1/GBA2 KO HEK293T cell lysate was incubated with 4-MU-β-D-Glc for 30 min at 37 °C at pH 5.0 in the presence of Triton X-100, sodium taurocholate (NaTc), bovine serum albumin (BSA), dimethyl sulfoxide (DMSO) alone, or a combination of these additives as indicated.

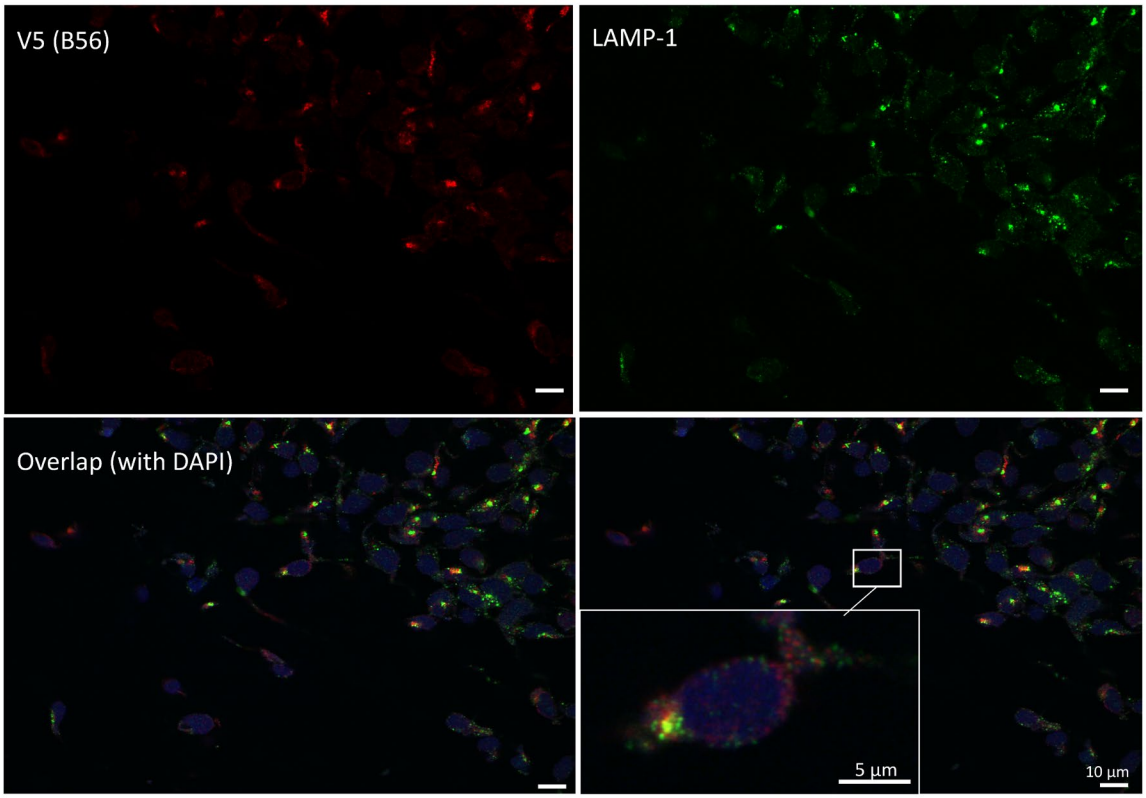

Figure S4. Zoom-out confocal microscopy of Figure 5, showing subcellular localization of V5-tagged B56 (shown as red) expressed in hGBA1/hGBA2 KO HEK293T cells, comparable to subcellular localization of LAMP1 (shown as green).

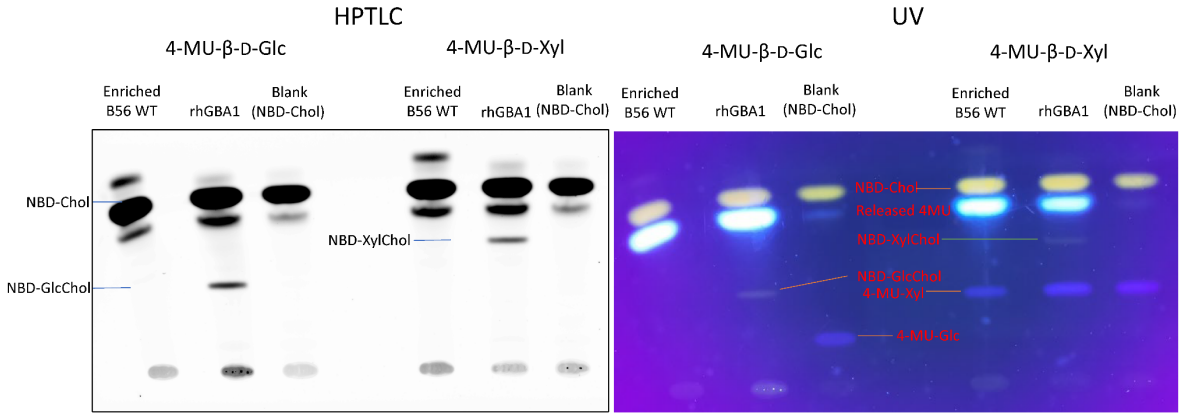

Figure S5. Transglycosylation assessment of B56 WT (enriched by ConA beads). B56 WT enriched by ConA beads was incubated with either 400 µM 4-MU-β-D-Glc or 4-MU-β-D-Xyl as sugar donor and 20 µM 25-NBD-Chol as acceptor for 16 h at 37 °C at pH 5.0. Assay was conducted as described in Figure 8.