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## **Chemical biology of glucosylceramide metabolism fundamental studies and applications for Gaucher disease**

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Chemical probing of the catalytic pocket of glucocerebrosidase with photoactivatable glucosylceramide and activity-based probes

# Chemical probing of the catalytic pocket of glucocerebrosidase with photoactivatable glucosylceramide and activity-based probes

Based on

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## Abstract

Glucocerebrosidase (GBA) is the lysosomal retaining  $\beta$ -glucosidase degrading glucosylceramide (GlcCer) and glucosylated cholesterol (GlcChol). Deficiency of GBA causes Gaucher disease. Carriers of this disorder are at increased risk for alpha-synucleinopathies. Information on structure-function relationships in GBA stems from conventional enzymology and crystallography. To extend insight, we investigated human GBA with two new classes of chemical biology tools: pacGlcCer and fluorescent  $\beta$ -glucose configured cyclophellitols binding to the catalytic nucleophile residue E340 in mechanism-based manner (ABP). We here demonstrate that pacGlcCer binds to the catalytic pocket of GBA. Binding requires correctly folded, active enzyme and follows the pH optimum of enzymatic activity. We show that two different kinds of ABP, the irreversible inhibitors cyclophellitol and conduritol B-epoxide and the reversible inhibitor AMP-DNM compete the binding of pacGlcCer. Likewise, pacGlcCer is competed by the substrates glucosylsphingosine, GlcChol and 4-methylumbelliferyl- $\beta$ -glucose. Known lipid acceptors in the transglucosylation reaction of GBA (cholesterol, retinol and sphingosine) also inhibit binding of pacGlcCer but not that of ABP. In conclusion, newly available probes offer novel possibilities to investigate the catalytic pocket of GBA.

## Introduction

Glucocerebrosidase (GBA) is a retaining  $\beta$ -glucosidase encoded by the *Gba* gene at locus q21 of chromosome 1<sup>1,2</sup>. It is synthesized as 497 amino acid polypeptide acquiring 4 N-linked glycans<sup>3-5</sup>. Binding of GBA to LIMP-2 (lysosomes integral membrane protein 2) governs transport to lysosomes independent of the mannose-6-phosphate receptor system<sup>6-10</sup>. In the

acid lysosome, GBA cleaves the glycosphingolipid  $\beta$ -D-glucosyl-ceramide (GlcCer) to ceramide (Cer), an essential step in glycosphingolipid turnover<sup>11</sup>. Mutations in the *Gba* gene cause Gaucher disease (GD)<sup>12</sup>. GBA has a catalytic and presumed folding domain<sup>13,14</sup>. Mutations in the latter domain, such as L444P, may impair folding and result in subsequent proteasome-mediated degradation<sup>15,16</sup>. In contrast, the common N370S substitution in GBA is close to the catalytic pocket and reported to affect kinetic parameters, pH optimum and lysosomal stability<sup>14,17-19</sup>. GBA is activated by saposin C and inherited deficiency of this activator protein also leads to lysosomal GlcCer accumulation as well as GD-like disease manifestations<sup>20-22</sup>. Defects in LIMP-2 cause secondary deficiency of GBA, but patients with the associated disease (action myoclonus renal failure syndrome; AMRF) show no GD-like symptoms<sup>23</sup>. The clinical presentation of GD is heterogeneous, ranging from skin defects incompatible with terrestrial life to an almost asymptomatic course of disease<sup>12</sup>. The correlation between *Gba* genotype and phenotypic manifestations of GD patients is limited. Discordant monozygotic GD twins illustrate most prominently the existence of elusive disease modifiers<sup>24,25</sup>. Carriers of mutant *Gba* alleles do not develop GD but show a significantly increased risk for Parkinsonism and Lewy-body dementia<sup>26</sup>. Prominent in symptomatic GD patients are GlcCer-laden macrophages (Gaucher cells)<sup>27,28</sup>. These alternatively activated lipid-laden macrophages are thought to underlie symptoms like splenomegaly and hepatomegaly<sup>12</sup>. As recently recognized, the catalytic functions of GBA may be broader as hitherto assumed. Tissues contain  $\beta$ -D-glucosyl-cholesterol (GlcChol) which is a substrate for GBA<sup>29</sup>. At normal conditions GBA degrades GlcChol, but during excessive accumulation of cholesterol in lysosomes, as in Niemann Pick type C, GBA can generate GlcChol via transglucosylation<sup>29</sup>. An effective treatment for non-neuronopathic (type 1) GD is enzyme replacement therapy (ERT), based on two-weekly intravenous administration of modified human recombinant GBA to supplement macrophages with enzyme<sup>30</sup>. ERT results in reversal of organomegaly and hematological complications as well as stabilization of skeletal disease<sup>31,32</sup>. An alternative registered treatment is substrate reduction therapy (SRT), based on oral administration of an inhibitor of glycosphingolipid biosynthesis; two drugs (*Miglustat*, *Eliglustat*) have been registered for SRT<sup>33-36</sup>.

The clinical interest in GBA has stimulated fundamental investigations on the enzyme, including its catalytic pocket. For catalysis, GBA employs the double-displacement mechanism with E340 as nucleophile E235 and acid/base<sup>37</sup>. As reaction intermediate glucose becomes covalently linked to E340, to be released by attack of a hydroxide. Cyclophellitol irreversibly inhibits GBA by forming a permanent conjugate with E340<sup>38</sup>. Cyclophellitol scaffolds are used in functionalized activity-based probes, allowing *in situ* visualization of GBA and probing of the enzyme's catalytic pocket<sup>39,40</sup>. One class of such ABPs, cyclophellitol-epoxides, requires correctly folded, active enzyme molecules for labeling<sup>40</sup>.

Crystallography has been used to study the catalytic pocket of GBA<sup>13,14</sup>. Although this increased knowledge on structural determinants relevant for the interaction of enzyme and sugar-moiety of substrate, it did not shed light on the aglycon interaction. Only indirect information on this has been acquired by studies on inhibitory effects of lipids<sup>19</sup>. More recently developed photoactivatable lipid analogs can be activated by UV light to form a covalent

linkage to their protein-binding partners<sup>41</sup>. Lipids with small photoactivatable groups, limiting interference with the physicochemical properties of the native molecule, such as diazirine-functionalized and clickable fatty acid (pacFA), sphingosine (pacSph) and GlcCer (pacGlcCer) are available<sup>42,43</sup>. Following UV light-mediated binding to the protein, a fluorescent detection group can be installed by click chemistry<sup>41</sup>.

In theory, pacGlcCer may be very suitable to study features of the catalytic pocket of GBA, including the aglycon binding site. Here we report the probing of human GBA with pacGlcCer. We demonstrate that specific binding of pacGlcCer to GBA occurs via the catalytic pocket. The interaction depends on correctly folded, active enzyme and follows the pH optimum of enzymatic activity. It is competed by the reversible inhibitor AMP-DNM and the irreversible inhibitors conduritol  $\beta$ -epoxide and cyclophellitol<sup>44</sup>. Prior binding of ABPs to GBA also prohibits pacGlcCer labeling. Known acceptors in transglucosylation like sphingosine and retinol but in particular cholesterol<sup>29,45</sup>, also reduce the binding of pacGlcCer to GBA. Thus, the catalytic pocket of GBA can be probed with sugar analogues (ABPs) and with the lipid analogue pacGlcCer, for example to monitor the stabilizing effects of the chaperones N-butyldeoxynojirimycin and Ambroxol.

## Results

### Labeling of GBA with pacGlcCer.

We first examined the feasibility of labeling GBA with pacGlcCer. Pure recombinant enzyme was incubated with pacGlcCer, photo-crosslinked, ligated with fluorophore by click chemistry and subjected to gel electrophoresis and fluorescence scanning (see scheme 1). Increasing the concentration of pacGlcCer led to increased labeling, the maximum reached at 250 pmol per incubation (16,7  $\mu$ M) (figure 1a). Denaturation of GBA prohibited labeling by pacGlcCer (fig 1a). Variation of pH of the incubation revealed optimal labeling at pH 5.2, coinciding with the pH optimum of enzymatic activity of GBA towards 4-methylumbelliferyl- $\beta$ -D-glucose (4MU- $\beta$ -D-Glc)<sup>39</sup> (fig 1b). Next, we examined competition of labeling by substrates of GBA: 4MU- $\beta$ -D-Glc and glucosylsphingosine (GlcSph) (fig 1c). Partial competition of labeling was observed at high substrate concentrations, most prominently with GlcSph. The competitive inhibitor N-adamantanemethyloxypentyl-1-deoxynojirimycin (AMP-DNM, IC<sub>50</sub> 200 nM) led at 500  $\mu$ M to 73 % reduction of pacGlcCer labeling of GBA. Next, we studied whether pacGlcCer serves as substrate for GBA. For this, the lipid was incubated with enzyme and formation of pacCer was detected after click chemistry (fig 1d). We finally compared the labeling of GBA by pacGlcCer, pacCer and pacSpho (fig 1e). The most efficient labeling by far was observed with pacGlcCer.

### Competition of labeling by sugar analogue inhibitors.

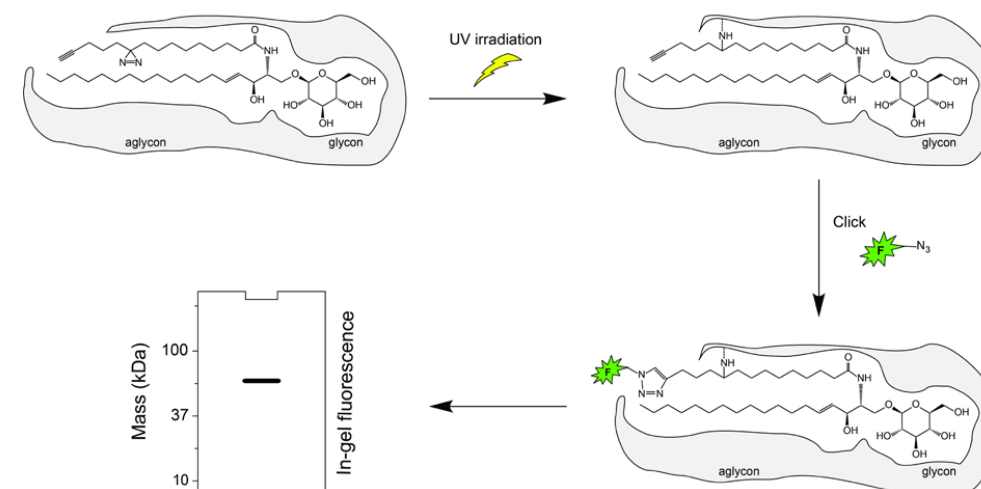
Competition of pacGlcCer labeling of GBA by the irreversible inhibitors cyclophellitol and conduritol  $\beta$ -epoxide (CBE) was prominent following 30 minutes pre-incubation of enzyme with inhibitors (fig 2 a, b). This finding suggests that occupancy of E340 by the relatively small

inhibitors interferes markedly with the interaction of pacGlcCer and GBA. To further substantiate this, we pre-incubated GBA with increasing concentrations of the ABP **1** and ABP **2**. After incubation, click chemistry and gel electrophoresis, fluorescence scanning was performed at respective wavelengths following detection of bound ABP as well as bound pacGlcCer (fig 2c, d).

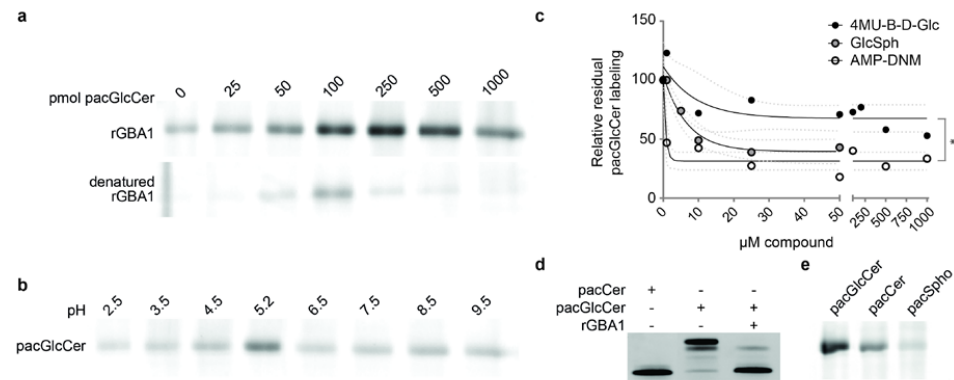
### Efficiency of covalent labeling of GBA with pacGlcCer and ABP.

To assess the percentage of GBA molecules to which pacGlcCer was covalently bound we determined residual enzyme activity following labeling at optimal conditions as described in Methods. A very high residual enzyme activity was noted at saturating pacGlcCer concentrations (fig 3), suggesting that the efficiency of photo-cross linking of diazirine to protein is not very high. In sharp contrast, the cross-linking of ABP to the catalytic nucleophile E340 of GBA is very efficient and leads to complete loss of enzyme activity at low concentrations (fig 3)<sup>39</sup>.

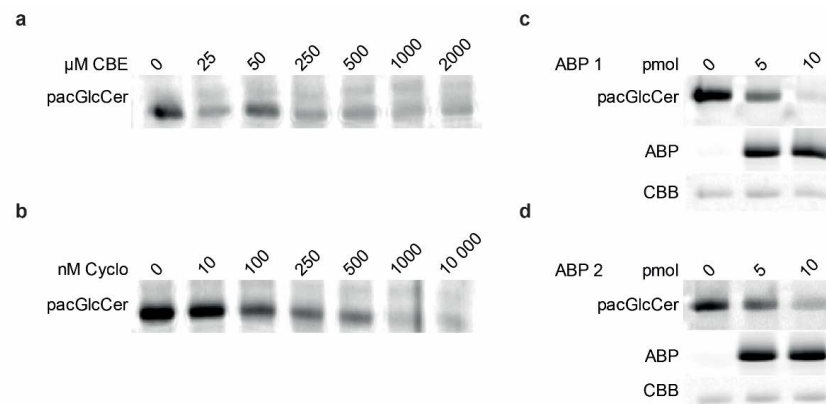
Reaction mechanism pacGlcCer



**Scheme 1. Hypothetical pacGlcCer binding to GBA.** Bifunctional GlcCer is covalently linked to GBA by UV-induced crosslinking. Subsequently, click chemistry is applied to label the alkyne group of the cross-linked GlcCer with azide-conjugated Cy5 (F-N3). PacGlcCer-modified GBA protein is detected by in-gel fluorescence.

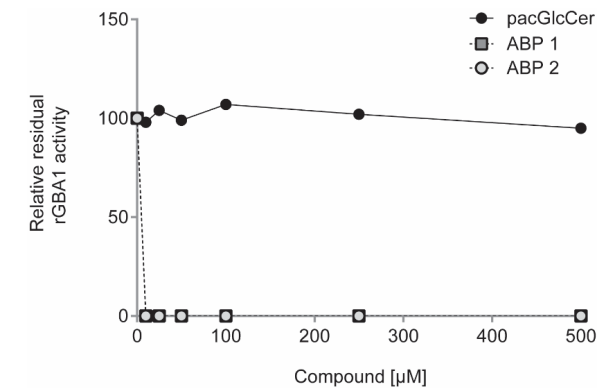


**Figure 1. Binding of pacGlcCer to GBA.** (a) Titration of pacGlcCer. 0.5  $\mu$ g GBA was incubated with 0-1000 pmol pacGlcCer for 15 minutes on ice after which samples were UV irradiated. Lower panel: impact of prior denaturation of GBA on pacGlcCer labeling; (b) pH dependence of pacGlcCer labeling of GBA. For incubations McIlvaine buffer with taurocholate and Triton X-100 was used at indicated pH values. GBA was incubated with 250 pmol pacGlcCer; (c) Competition of pacGlcCer labeling by 4MU- $\beta$ -D-Glc, GlcSph and AMP-DNM. GBA was incubated on ice simultaneously with pacGlcCer and compound. Data, indicated as one phase exponential decay with 90% confidence interval, were analyzed by the Kruskal-Wallis test and subsequent Dunn's multiple comparison test ( $p = 0.0130$  for 4MU- $\beta$ -D-Glc vs. AMP-DNM) and are representative for multiple experiments; (d) GBA degradation of pacGlcCer to pacCer as visualized by HPTLC; (e) Comparative labeling of GBA by pacGlcCer, pacCer and pacSpho.



**Figure 2. Prevention of pacGlcCer binding to GBA by irreversible inhibitors.** Inhibition of pacGlcCer labeling by prior labeling with (a) CBE; (b) cyclophellitol (cyclo); (c) ABP 1 (epoxide); (d) ABP 2 (aziridine). Samples were incubated with CBE or cyclophellitol for 30 minutes at 37  $^{\circ}$ C before incubation on ice for 15 minutes with pacGlcCer. Incubation with ABP was

performed for 15 minutes on ice simultaneously with pacGlcCer. ABP and pacGlcCer fluorescence were visualized separately. CBB: Coomassie Brilliant Blue protein stain.



**Figure 3. Inhibition of GBA activity by pacGlcCer, ABP 1 and ABP 2.** GBA was incubated on ice for 15 minutes with pacGlcCer, ABP 1 or 2. Next, all samples were UV irradiated for 10 minutes and incubated with 4MU- $\beta$ -D-Glc for 30 minutes at 37  $^{\circ}$ C after which the reaction was stopped and released 4MU was measured. Data are indicated as residual GBA activity relative to incubation without added compound.

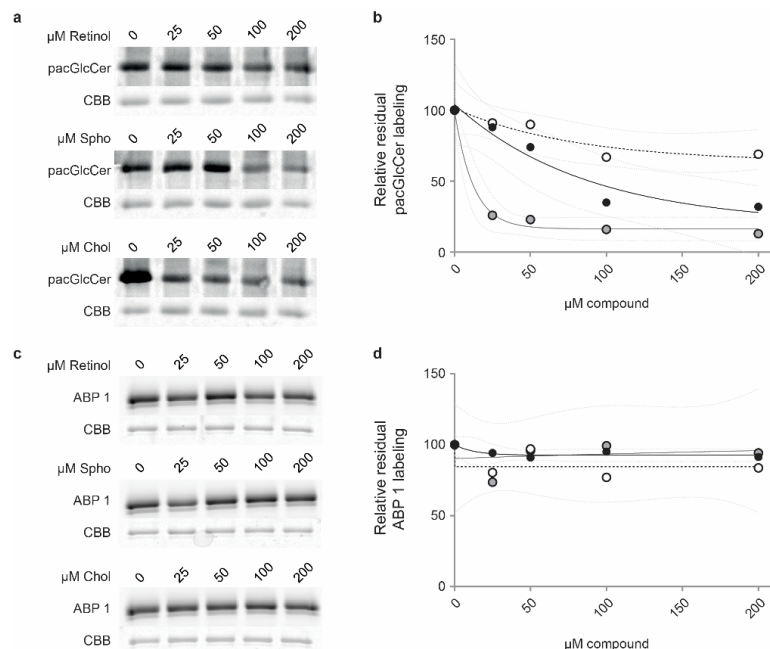
#### Competition of pacGlcCer interaction with GBA by lipid acceptors in transglucosylation.

Since GBA uses acceptors in transglucosylation, we compared the effects of such compounds (cholesterol, retinol and sphingosine) on enzyme labeling with pacGlcCer and ABP 1. For this, enzyme was first shortly exposed to these lipids after which pacGlcCer was added, the mixture incubated and labeled GBA detected (fig 4). Competition of pacGlcCer labeling of GBA was exerted by all lipids tested, being most prominent for cholesterol (fig 4a, b). Of note, as shown in figure 4c, ABP 1 labeling was not competed by the lipid acceptors retinol, sphingosine or cholesterol (fig 4c, d).

#### Discussion

We studied the potential value of a photoactivatable GlcCer analogue, pacGlcCer, as additional tool to probe the catalytic pocket of GBA. Our investigation revealed that the catalytic pocket of GBA is labeled with high affinity by pacGlcCer. Maximal labeling by pacGlcCer was already reached at 250 pmol/15  $\mu$ l (16,7  $\mu$ M). At the same concentration the labeling of enzyme by pacCer and pacSpho was far from maximal. GBA is labeled by pacGlcCer in specific manner via its catalytic pocket as indicated by the following observations. Labeling is most efficient at the pH optimum of activity (pH 5.2) and denaturation of GBA prohibits labeling. GBA substrates glucosylsphingosine and 4-methylumbelliferyl- $\beta$ -D-glucose compete labeling. The hydrophobic iminosugar AMP-DNM, a competitive inhibitor, also competes the

cross-linking of pacGlcCer to GBA. Importantly, prior incubation of GBA with two small irreversible inhibitors, conduritol  $\beta$ -epoxide and cyclophellitol, as well as two cyclophellitol-type activity based probes with tagged fluorophores almost completely prevents labeling of enzyme with pacGlcCer. This indicates that occupancy of the catalytic nucleophile E340 and adjacent glycon binding site negatively impacts on the interaction of pacGlcCer with the enzyme. Thus, pacGlcCer seems to preferentially interact with GBA via the catalytic pocket. Indeed, pacGlcCer is found to be an excellent substrate for GBA.



**Figure 4. Competition of pacGlcCer labeling by lipid acceptors in transglucosylation.** Competition of pacGlcCer labeling by retinol (open symbol), sphingosine (Spho) (closed symbol) and cholesterol (Chol) (grey symbol): **(a)** Gel – fluorescence scan and subsequent CBB staining; **(b)** Quantification (Cy5 fluorescence without added lipid is set at 100 %). Shown result is representative for multiple independent experiments and is indicated as one phase exponential decay with 90 % confidence interval. Kruskal-Wallis test and subsequent Dunn’s multiple comparison test did not reveal significant difference between the three competitors; Spho vs. Chol  $p = 0.4674$  and Retinol vs. Chol  $p = 0.1408$ , Spho vs. Retinol  $p > 0.99$ . **(c)** Competition of ABP 1 labeling by retinol, sphingosine and cholesterol. **(d)** Quantification of c.

We comparatively studied the labeling of GBA by fluorescent ABPs<sup>37,39</sup>. An enormous advantage of ABPs to pacGlcCer is found in the efficiency of labeling. All active GBA molecules are labeled by ABPs at very low concentrations whereas the efficiency of cross-linking

pacGlcCer to GBA is low at saturating lipid concentration. Following incubation with excessive amounts of pacGlcCer less than 5 % of enzyme activity was lost. The intensity of protein-bound fluorescence per mg GBA was consistently far less with pacGlcCer (Cy5 tagged) than ABP labeling with BODIPY-FL. The low efficiency of UV-mediated cross-linking of diazirine to protein has indeed been reported earlier<sup>46</sup>. In practice, the poor efficiency of cross-linking pacGlcCer to GBA is a major drawback because of significantly less sensitive detection which is far surpassed by ABP labeling.

Of recent interest are the hydrophobic acceptors in the newly recognized transglucosylation reactions catalyzed by GBA. Labeling of GBA with ABP is not influenced by the presence of known acceptors such as cholesterol, sphingosine or retinol, likely due to fact that bound ABP inhibitor does not overlap with the binding sites of the acceptors. In contrast to this, labeling of GBA by pacGlcCer is competed by lipid acceptors, most prominently by cholesterol and least striking retinol, coinciding with different rates of their respective transglucosylation. Thus, pacGlcCer offers a tool to identify potential acceptors in transglucosylation and could contribute to future identification of yet unknown glucosylated metabolites. Several academic researchers and pharmaceutical companies pursue the development of chemical chaperones, small glycomimetics, which would promote/stabilize folding of GBA through their transient binding within the catalytic pocket<sup>47–49</sup>. Another application for pacGlcCer labeling of GBA might be found in the probing of stabilization of GBA conformation by potential small compound chaperones as for example N-butyldeoxynojirimycin (NB-DNJ) and Ambroxol<sup>50–56</sup>. In conclusion, our investigation provides evidence that  $\beta$ -glucose-configured cyclophellitol-type ABPs and pacGlcCer offer novel possibilities to investigate the catalytic pocket of GBA.

## Materials and Methods.

### Materials.

Recombinant human GBA (Cerezyme<sup>TM</sup>) was a gift from Sanofi-Genzyme (Cambridge, MA, USA). Chemicals were purchased from Sigma-Aldrich if not indicated otherwise. Conduritol  $\beta$ -epoxide (CBE) was from Enzo Life Sciences, ABP MDW933 (epoxide) (**1**) and ABP JJB70 (aziridine) (**2**) (scheme 2)<sup>39,40</sup>, AMP-DNM<sup>57</sup>, N-butyldeoxynojirimycin (NB-DNJ), cyclophellitol and GlcChol were synthesized at the Leiden Institute of Chemistry<sup>29,39</sup>. Retinol and cholesterol were from Sigma-Aldrich. The fluorogenic substrate 4-methylumbelliferyl- $\beta$ -glucose was from Glycosynth (Warrington, UK). Glucosylsphingosine (D-glucosyl- $\beta$ 1-1'-D-erythrosphingosine), sphingosine, pacChol and pacGlcCer were purchased from Avanti Polar Lipids (Alabaster, USA). PacCer and pacSpho were synthesized earlier<sup>42,43</sup>. A SpeedVac Eppendorf concentrator Plus was used to dry lipid compounds in order to dissolve them into reaction buffer. For UV irradiation (350 nm) a Caprotec Caprobox (Berlin, Germany) was used.

## Methods.

### GBA activity measurements.

Enzymatic activity of GBA was measured with 4-methylumbelliferyl- $\beta$ -D-glucose (4MU- $\beta$ -D-Glc) as described previously<sup>58</sup>. Briefly, enzyme was incubated for 30 minutes at 37 °C with 3.7 mM 4MU- $\beta$ -D-Glc in 150 mM Mcllvaine buffer (pH 5.2) with 0.1 % (v/v) Triton X-100 and 0.2 % (w/v) taurocholate. The reaction was stopped with NaOH-glycine (pH 10.3), and fluorescence was measured with a fluorimeter LS55 (Perkin-Elmer, Beaconsfield, UK) at  $\lambda_{ex}$  366 nm and  $\lambda_{em}$  445 nm.

### PacGlcCer labeling experiments.

Binding assays with recombinant GBA were performed at optimal conditions for enzymatic activity: 150 mM Mcllvaine buffer (pH 5.2) with 0.1 % (v/v) Triton X-100 and 0.2 % (w/v) taurocholate was used as reaction buffer. For pacGlcCer binding, in a total volume of 15  $\mu$ L reaction buffer, 0.5  $\mu$ g GBA was incubated on ice for 15 minutes with 0-1000 pmol pacGlcCer, after which samples were UV irradiated for 10 minutes at 4 °C. Subsequently "click-mix" was added as described previously and samples were incubated in the dark at room temperature for 40 minutes<sup>42</sup>. If not indicated differently, 0.5  $\mu$ g GBA labeling was performed with 250 pmol pac GlcCer.

To denature GBA, enzyme was boiled for 5 minutes at 100 °C. For ABP binding, 0.5  $\mu$ g GBA was incubated on ice with probe (**1** or **2**). For competition experiments, 0.5  $\mu$ g GBA was pre-incubated at 37 °C for 30 minutes with CBE or cyclophellitol, or without pre-incubation with ABP **1** or ABP **2**, followed by 15 minute incubation on ice with 250 pmol pac lipid.

For competition with non-covalent inhibitors, substrate and other lipids, 0.5  $\mu$ g GBA was incubated for 15 minutes on ice with 250 pmol pac lipid and 4MU- $\beta$ -D-Glc, AMP-DNM, GlcSph, GlcChol, retinol, sphingosine, or cholesterol in reaction buffer.

### Labeling with other pac-sphingolipids and pac-cholesterol

Labeling of GBA with other pac-sphingolipids and pac-cholesterol (each 250 pmol) was performed similar to that with pacGlcCer as described above.

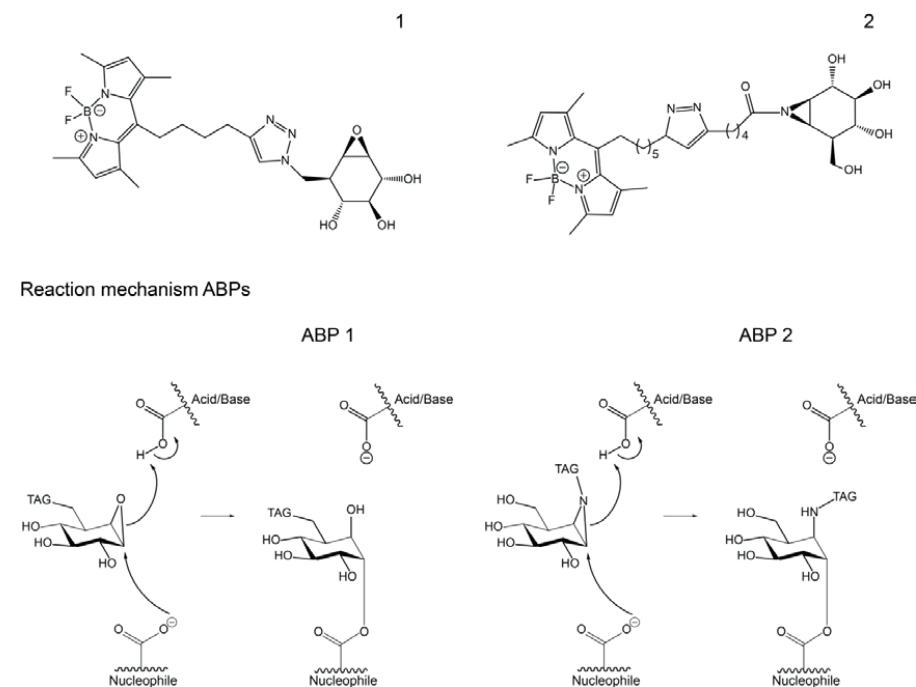
### ABP labeling experiments

To label GBA, 0.5  $\mu$ g enzyme was incubated with 10 pmol ABP for 30 minutes at 37 °C. Labeled GBA was visualized after gel electrophoresis as described below.

### Gel electrophoresis and fluorescence scanning.

Samples were denatured with 5 $\times$  Laemmli buffer (50 % (v/v) 1 M Tris-HCl, pH 6.8, 50 % (v/v) 100 % glycerol, 8 % (w/v) DTT, 10 % (w/v) SDS, 0.01 % (w/v) bromophenol blue), boiled for 5 minutes at 100 °C and separated by electrophoresis on 10 % (w/v) SDS-PAGE gel running at 75 V for 30 minutes and subsequently at 200 V. Wet slab-gels were scanned on fluorescence using the Typhoon Variable Mode Imager (Amersham Biosciences) using  $\lambda_{EX}$  488 nm and  $\lambda_{EM}$  520 nm (band pass filter 40 nm) for green fluorescent ABP **1** and **2** and  $\lambda_{EX}$  635nm and  $\lambda_{EM}$  670nm

(band pass filter 30 nm) for Cy5 labeled pac lipids. After fluorescence scanning, SDS-PAGE gels were stained for total protein for 1 hour using Coomassie brilliant blue (CBB)-R250 and de-stained with 10 % ethanol (96%) in MilliQ water. CBB-stained gels were scanned on a BioRad Universal Hood III.



**Scheme 2. Labeling of GBA with fluorophore tagged  $\beta$ -glucose-configured cyclophellitol-epoxide ABP 1 and cyclophellitol-aziridine ABP 2.** Reaction mechanism involves covalent conjugation of cyclophellitol to nucleophile E340<sup>37</sup>. ABP-labeled protein can be quantitatively detected by in-gel fluorescence.

### Degradation of pacGlcCer by GBA.

PacGlcCer was incubated for 30 minutes at 37 °C with GBA in 150 mM Mcllvaine buffer (pH 5.2) with 0.1% (v/v) Triton X-100 and 0.2 % (w/v) taurocholate. Lipids were extracted, the fluorescent tag was installed by click chemistry, and subjected to HPTLC as described earlier<sup>59</sup>. Fluorescent pacGlcCer and the degradation product pacCer were visualized by fluorescence scanning.

### Data processing and representation.

In-gel fluorescence intensities were quantified using ImageJ software (NIH, Bethesda, USA), acquired data were processed in Microsoft Excel and finally Graphpad Prism 7 (Graph Pad



Software, Inc., San Diego, USA) was used to present quantification data of Cy5 labeled pac lipids and activity measurements. ChemDraw Professional 15.0.0.106 (PerkinElmer Informatics) was used to make the chemical structures presented in scheme 1 and 2.

#### Statistical Analysis.

Data were analyzed by nonparametric Kruskal-Wallis test succeeded by a Dunn's multiple comparison test (fig 1c). *P* values < 0.05 were considered significant; \* *P* < 0.05. Statistics were carried out using GraphPad Prism.

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