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Chemical biology studies on retaining α -glucosidases

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Chapter 5

Characterization of *C. elegans* retaining β -glucosidases

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Abstract

The human lysosomal β -glucosidase (hGBA1) degrades the glycosphingolipid (GSL) glucosylceramide (GlcCer). Inherited deficiency in hGBA1 causes Gaucher disease (GD). Like mammals, the nematode *C. elegans* contains glycosphingolipids, but with a C17iso sphingoid base instead of the mammalian C18 one (d17iso-GlcCer). Information on β -glucosidase activities of *C. elegans* that may be involved in d17iso-GlcCer metabolism is limited. Substrates, inhibitors, and activity-based probes (ABPs) that report on hGBA1 and the non-lysosomal hGBA2 were used to identify and characterize β -glucosidase activities in *C. elegans*. Candidate genes for the GBA1-like and GBA2-like proteins in *C. elegans*, as well as characteristics of one candidate orthologue of hGBA1 in *C. elegans* encoded by the *gba3*-gene are described in this Chapter.

Introduction

Humans express three cellular β -glucosidases, all employing a retaining reaction mechanism,¹ termed (h)GBA1 (lysosomal glucosylceramidase), (h)GBA2 (non-lysosomal glucosylceramidase) and (h)GBA3 (a broad-spectrum cytosolic β -glucosidase). Whereas the role of hGBA3 is enigmatic (see also Chapter 4), hGBA1 and hGBA2 have in common that both enzymes process the same substrate, namely, glucosylceramide (GlcCer, **1**, Figure 1) into glucose (**2**) and ceramide (**3**). Inherited defects in the hGBA1 gene are linked to the lysosomal storage disorder Gaucher disease (GD),²⁻⁴ and heteroallelic mutations in the hGBA1 gene are a risk factor for Parkinson's disease (PD).^{5,6} Treatment of non-neuronopathic symptoms of type 1/3 GD patients with recombinant hGBA1 (enzyme replacement therapy – ERT) comprises the most successful of the enzyme replacement therapies (ERTs) to date.⁷⁻⁹ hGBA2 in turn is a cytosol-facing membrane-associated glucosylceramidase and while processing the same substrate as hGBA1, it does so in a different cellular compartment.¹⁰ Therapeutic co-inhibition of hGBA2, jointly with the GlcCer-synthesising enzyme, glucosylceramide synthase (GCS), has recently come to the fore as a potentially improved substrate reduction therapy (SRT) for the treatment of neuropathological (type 3) GD patients that cannot be treated by recombinant hGBA1 and neither by selective inhibition of GCS alone (SRT with Zavesca, as is now practice in the clinic as an alternative for ERT).^{11,12}

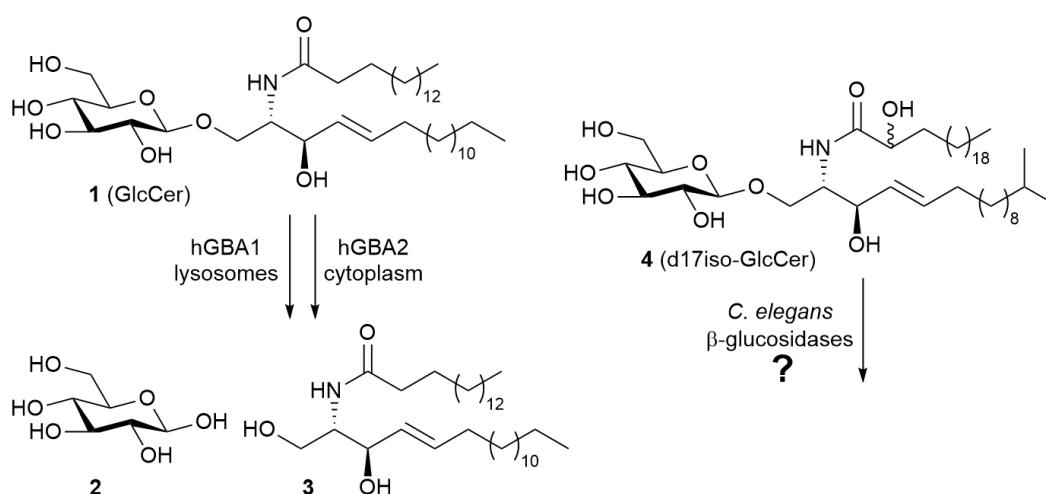


Figure 1. Metabolism of glucosylceramide (GlcCer, **1**) in humans and the closely related *C. elegans* glycosphingolipid, d17iso-GlcCer (**4**, d17iso-GlcCer) a GSL with high abundance in compositions of *C. elegans* GSL. Of note, the side chain fatty acid of d17iso-GlcCer can vary from C12 to C26, with or without the presence of a hydroxyl group¹³. *C. elegans* β -glucosidase activities potentially able to process d17iso-GlcCer are poorly described and subject to the studies presented in this Chapter.

Beyond its key role in these various human pathologies, the importance of GlcCer and its metabolites in nature is reflected by their wide occurrence across the various kingdoms of life. Structural elements in species-specific GlcCer may differ subtly, both in the fatty acid appended to the sphingoid base and the sphingoid base itself (length, branching, saturation, hydroxylation pattern) but the general structure appears conserved. The same appears to be true for the function of GlcCer, which is often utilized as a common starting point in the biosynthesis of a wide array of oligosaccharidic glycosphingolipids (in humans for instance the gangliosides and globosides) that are an integral part of cell membranes where they partake in signalling events.¹⁴ A case in point and subject of the studies described in this Chapter comprises the transparent nematode, *C. elegans* synthesizes d17iso-glucosylceramide (d17iso-GlcCer, **4**, Figure 1),^{13,15,16} which differs from the

mammalian GlcCer by the nature of the sphingoid base: C17iso monomethyl branched-chain sphingosine, as opposed to linear C18-sphingosine in humans. *C. elegans* thus produces a close homologue of the primary storage material in GD (GlcCer). Moreover, it does so in a highly conserved biochemical pathway, starting from serine and a fatty acid, and proceeding through sphinganine which is then acylated, dehydrogenated and glucosylated.^{15,17} The presence of d17iso-GlcCer (**4**) as the central glycosphingolipid in *C. elegans* raises the question whether this species, like humans and other mammals, express d17iso-GlcCer-processing β -glucosidases and if so, whether they possess similar characteristics (cytosolic/acting at neutral pH; lysosomal/acting at acidic pH). Although not studied in detail in biochemical and cell biological experiments, orthologues of human genes encoding lysosomal and cytosolic glucosidases have been identified in *C. elegans* as part of the *C. elegans* Genome Project (see Table 1).¹⁸

Table 1. *C. elegans* genes orthologous to the human genes encoding GBA1 and GBA2. Predicted (BLAST search) homology of proteins and molecular masses. ^aUniprot code, ^bputative description, ^camino acid homology to hGBA1/hGBA2, ^dnumber of amino acids.

Genes			Predicted proteins			
Accession ^a	Description ^b (<i>C. elegans</i>)	Gene	Homology ^c	Size ^d	MW (kDa)	Aligned with
Q9UB00	glucosylceramidase 4	<i>gba-4</i>	42.28%	519	58.24	Human GBA1 (UniProt code: P04062)
G5ECR8	glucosylceramidase 3	<i>gba-3</i>	41.92%	522	59.08	
O16580	glucosylceramidase 1	<i>gba-1</i>	40.00%	523	58.17	
O16581	glucosylceramidase 2	<i>gba-2</i>	38.16%	516	57.78	
Q8WQB2	non-lysosomal glucosylceramidase	<i>hpo-13</i>	40.26%	959	110.85	Human GBA2 (UniProt code: Q9HCG7)
Q6EUT3	non-lysosomal glucosylceramidase	<i>hpo-13</i>	40.26%	922	106.27	
Q6EUT4	non-lysosomal glucosylceramidase	<i>hpo-13</i>	40.26%	930	107.13	
O01893	non-lysosomal glucosylceramidase	<i>R08F11.1</i>	38.77%	819	94.50	

These include the *gba-1*, *2*, *3*, and *4* genes, which have 38-42% sequence homology with hGBA1, and the *hpo-13* and *R08F11.1* genes, with around 40% sequence homology to hGBA2. hGBA1 and hGBA2 possess little sequence homology, if at all, and the presence of *C. elegans* genes with considerable homology to either suggests a similar biochemistry to exist in both species. Information on the catalytic activity and substrate selectivity of these potential hGBA1/hGBA2 analogues however is lacking. With the aim to shed some first light in this, selected fluorogenic and fluorescent substrates, inhibitors, and activity-based probes (ABPs) as also described in the previous experimental chapters that report on, or interfere with, hGBA1/hGBA2 activity were applied to *C. elegans* cell extracts in a variety of biochemical assays to identify and characterize retaining β -glucosidase activities. In this way, as is described below, two candidate-genes were identified with homology for hGBA1 and hGBA2 respectively. The hGBA1-like protein was furthermore cloned and expressed and revealed by competitive activity-based protein profiling (ABPP) to indeed possess characteristics also featured by hGBA1. Altogether the here-presented studies provide circumstantial, but strong evidence of the existence of conserved GlcCer metabolism pathways existing between humans and *C. elegans*, and provide the blueprint and reagents to delve deeper into this matter, possibly also in the context of GD and PD.

Results

The list of reagents used in the biochemical studies presented in this Chapter are depicted in Figure 1 and comprises a set of fluorescent substrates, fluorogenic substrates, competitive inhibitors, mechanism-based covalent and irreversible inhibitors and ABPs that have previously shown their merit in monitoring and modulating the activity of hGBA1 and/or hGBA2 in *in vitro*, *in situ* and sometimes also in *in vivo* settings (see also the preceding Chapters). These substrates, inhibitors and probes are applied to *C. elegans* extracts in a variety of experiments (see below) with the aim to address the questions whether such extracts contain β -glucosidase activity; whether this activity is (in part) effected by hGBA1/hGBA2-like retaining β -glucosidases; and whether this activity processes GlcCer derivatives. 4-Methylumbelliferyl- β -D-glucopyranoside **5** (4-MU- β -D-Glc) is the standard fluorogenic substrate that is processed by many β -exo-glucosidases including hGBA1/2/3. C12-NBD-GlcCer **6** comprises a fluorogenic GlcCer derivative that acts as an efficient substrate for both hGBA1 and hGBA2. Cyclophellitol **7** and conduritol B epoxide (CBE) **8** are broad-spectrum retaining β -glucosidase inhibitors that block both hGBA1 and hGBA2 in a covalent and irreversible manner.¹⁹ Cyclophellitol derivative **9** with a bulky hydrophobic group appended to C6 (glucopyranose numbering) is selective for hGBA1 over hGBA2,²⁰ whereas the competitive inhibitors **10**²¹ and **11**²² comprise *N*-alkyl deoxynojirimycins with some preference for hGBA2. Cyclophellitol-based ABPs **12**²⁰ and **13**²³, with the reporter moiety (Cy5 in **12**, biotin in **13**) grafted to C6 are selective hGBA1 probes, whereas cyclophellitol aziridines ABPs **14**–**17** (with the reporter moiety attached via the aziridine nitrogen) report on hGBA1 and hGBA2 alike.²⁴ Cy5-modified xylose-configured cyclophellitol aziridine **18** as described in Chapter 2 modifies both hGBA1 and hGBA2, whereas β -D-arabinofuranose-configured (β -D-Araf) cyclophellitol aziridines **19** and **20** report, as described in Chapter 3, with some selectivity on the action of hGBA2.

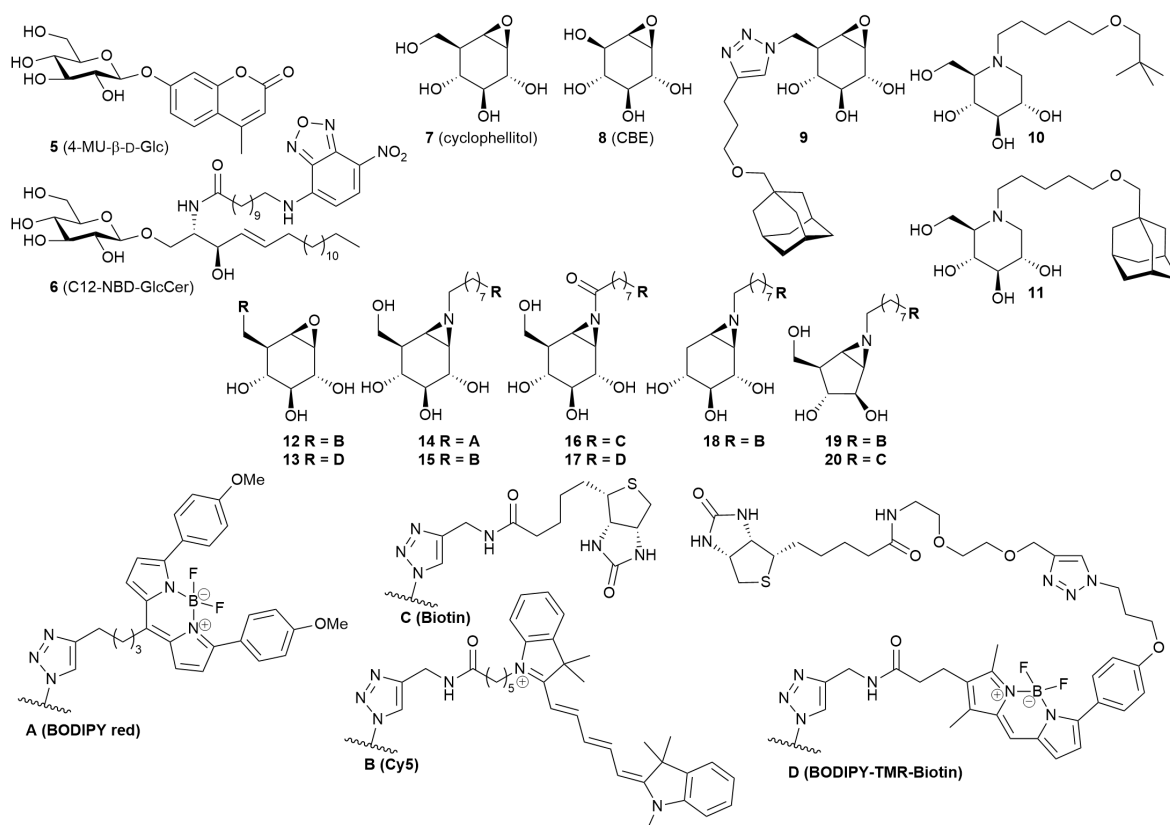


Figure 2. Substrates, inhibitors, and probes used in the here-presented studies.

Fluorogenic and fluorescent β -glucoside processing by *C. elegans* extracts

As the first experiment (Figure 3A), β -glucosidase activity in *C. elegans* homogenate was assessed by incubating *C. elegans* homogenates with 4-MU- β -D-Glc **5** at various pH values (3-7.5). Fluorescence was detected between pH 3 and pH 7.5, with a rather broad area (between pH 4 and pH 6) at which fluorescence is at least 50% of the maximal fluorescence as generated at pH 4.5-5. Next the ability of nematode homogenates to degrade C12-NBD-GlcCer was examined. *C. elegans* homogenate or recombinant hGBA1 (rhGBA1) at pH 5 was incubated for four hours with C12-NBD-GlcCer, after which the lipids were extracted and subjected to high-performance thin layer chromatography (HPTLC). Fluorescence scanning of the TLC plate revealed the formation of NBD-ceramide (NBD-Cer) in both samples.

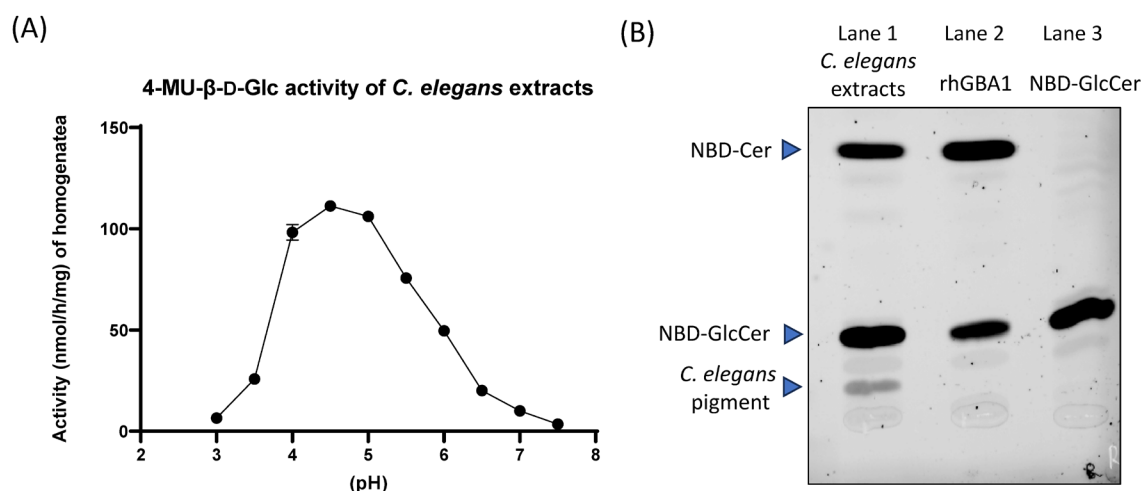


Figure 3. *C. elegans* extracts process 4-MU- β -D-glucopyranoside (A) and C12-NBD-GlcCer (B). (A) *C. elegans* homogenates were incubated with 4-MU- β -D-Glc at various pH values (3-7.5) for 30 min at 37 °C. (B) Lane 1: *C. elegans* homogenates (40-50 μ g) were incubated with 2 μ M C12-NBD-GlcCer in Mcllvaine buffer (150 mM, pH 5.0) for 4 h at 37 °C, followed by lipid extraction and HPTLC separation and fluorescence scanning of the silica gel layers. Lane 2: Around 20 ng isolated recombinant human GBA1 (rhGBA1, Imiglucrase) was used for processing C12-NBD-GlcCer as described above, but now the Mcllvaine buffer (150 mM, pH 5.2) was supplemented with 0.1% (v/v) Trion X-100 and 0.2% (w/v) sodium taurocholate. Lane 3: As the control, 2 μ M C12-NBD-GlcCer in Mcllvaine buffer (150 mM, pH 5.0) was incubated under the conditions described above.

Comparative and competitive ABPP in *C. elegans* extracts

Having established that *C. elegans* extracts contain β -glucosidase activity (Figure 3A) capable of processing the artificial GlcCer derivative **6** (Figure 3B), these extracts were then treated with the panel of retaining glucosidase ABPs **12-20** in a series of comparative and competitive (inclusion of inhibitors **7-11**) ABPP experiments. To this end, *C. elegans* homogenates were first incubated with the ABPs at a pH range of 4 to 7 (Figure 4A) for 30 minutes at 37 °C, after which the homogenates were denatured, the protein content separated by SDS-PAGE and the wet gel slabs scanned for in-gel fluorescence. The two cyclophellitol aziridine probes **14** and **15** as well as the β -xylose-configured one (**18**) return, at pH 5, three distinct fluorescent signals, of which the middle one disappears at elevated pH. These bands indicate modified proteins (or protein mixtures) with molecular weight (MW) of about 52 kDa (Band-1⁺), 95 kDa (Band-2[±]), and 55 kDa (Band-3[•]). Cyclophellitol ABP **12** in contrast predominantly elicits the 52 kDa band together with a faint signal reflecting a higher MW (60 kDa) protein (mixture). β -D-Araf cyclophellitol aziridine **19** also returned one major signal, but now at around 95 kDa. To address the concern that a contamination with *E. coli* may cause some of the

observed labelling is (in part) due to *E. coli* contaminants in the samples (*E. coli* is used to culture *C. elegans*, see experimental procedures), *E. coli* extracts were treated with ABP **14** at pH 4-7. Just one signal at around 70 kDa MW emerged after in-gel fluorescence scanning of the SDS PAGE-separated sample that was treated with probe **14** at pH 5-7 (Figure 4A rightmost lanes). This signal does not overlap with the position of the signals derived from the *C. elegans* homogenates. N-glycanase treatment of probe **14**-treated *C. elegans* extract resolved some, but not all signals, indicating that the different signals do not translate to the same protein present in varying glycoforms (Figure 4B). Finally, inclusion of inhibitors **8-11** in ABP **14**-treated extracts at pH 6 (a pH at which the three predominant signals elicited by **14** are at a maximum signal, see Figure 4A) indicates the presence of at least two distinct retaining β -glucosidases (Figure 4C). It should be noted here that the higher final concentrations of various inhibitors were chosen because these inhibitors did not inhibit the *C. elegans* retaining β -glucosidases at a potency they block human retaining β -glucosidases (the same holds true in selecting the final concentrations of the probes in the comparative ABPP experiments as depicted in Figure 4A). Drawing conclusions should therefore be done with care. Still, the competitive ABPP experiments reveal selective to complete competition of ABP **14**-generated signals, strongly suggesting these signals to relate to distinct retaining β -glucosidases. Iminosugars **10** and **11**, for instance, selectively and almost completely abolish the highest molecular weight signal at 95 kDa. C6-modified cyclophellitol **9** in turn very cleanly block the lower signal at 52 kDa whereas CBE **8** at the applied concentration appears to have little effect. Cyclophellitol **7** finally affects both the high and the low signal while leaving the middle one largely intact at the applied concentration.

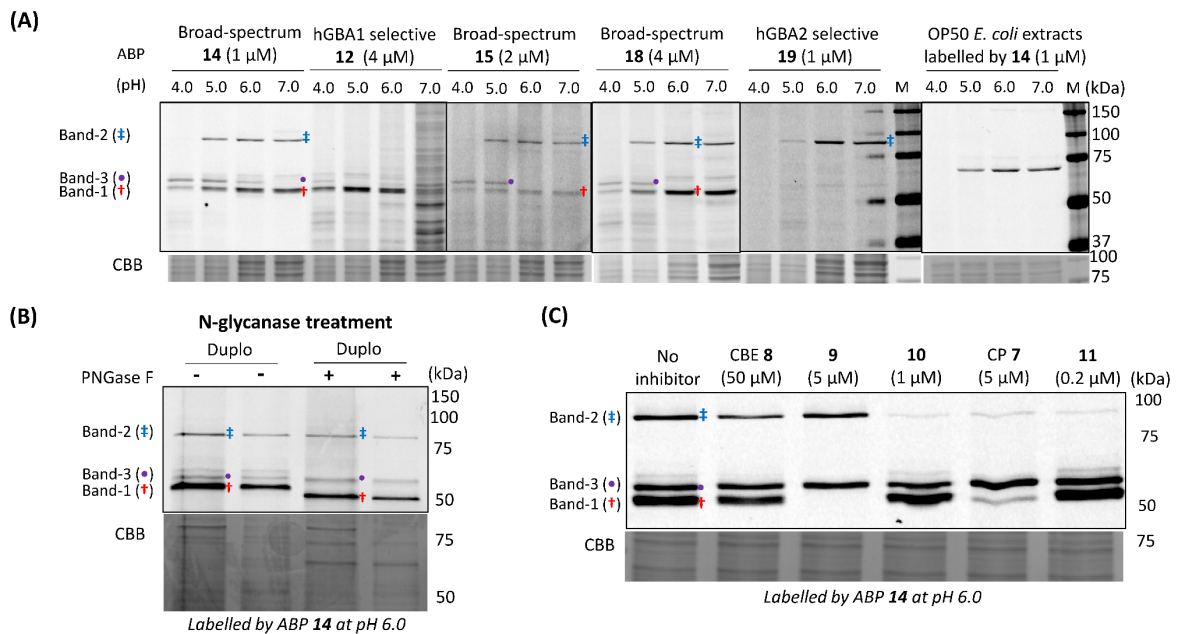
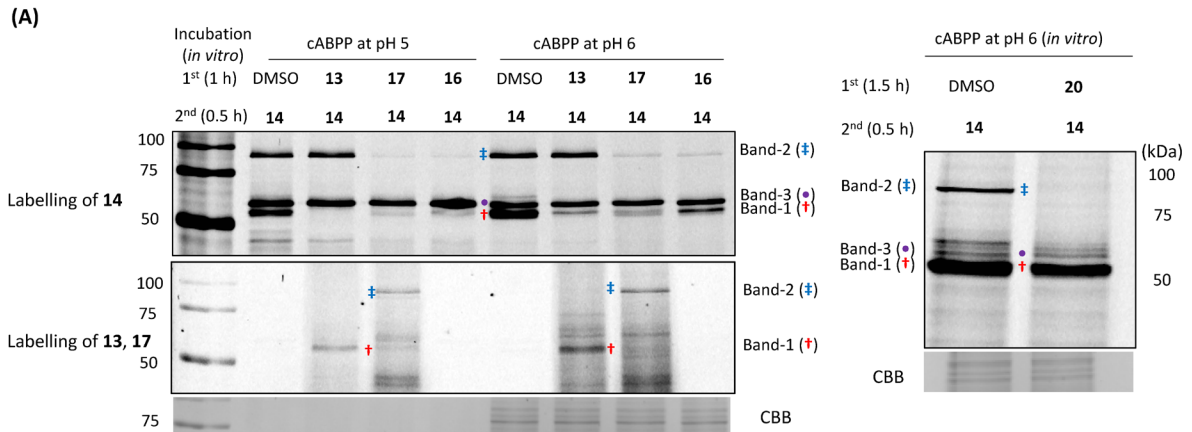


Figure 4. Comparative and competitive ABPP on *C. elegans* extracts. (A) Comparative ABPP. *C. elegans* homogenates were incubated with β -glucosidase ABPs **12**, **14**, **15**, **18**, or **19**, or OP50 *E. coli* homogenates with ABP **14**, for 30 minutes at 37 °C and at various pH values (4-7) *in vitro*, followed by SDS-PAGE, and fluorescence scanning of the wet gel slabs. (B) N-glycanase (PNGase F) treatment. *C. elegans* homogenates were first incubated with ABP **14** at pH 6.0 for 30 min at 37 °C *in vitro*, followed by denaturing of the samples by a 5 min incubation at 98 °C, and subsequent incubation with PNGase F for 2 h, then the samples were subjected to SDS-PAGE and fluorescence scanning. (C) Competitive ABPP. *C. elegans* homogenates were first treated with indicated inhibitor (**7-11**) at pH 6.0 for 30 min *in vitro*, and subsequently incubated with ABP **14** for 30 min, followed by SDS-PAGE and fluorescence scanning.

Chemical proteomics reveals putative hGBA1 and hGBA2 homologues in *C. elegans* extracts

Encouraged by the comparative and competitive ABPP experiments, which indicate the presence of multiple retaining β -glucosidases that moreover show different sensitivities both to pH and selected inhibitors in reacting with the panel of fluorescent probes applied, the biotin ABPs **13**, **16**, **17** and **20** were then investigated on their versatility to isolate and identify these activities (Figure 5 A, B) using a chemical proteomics workflow. In the first instance (Figure 5A) the propensity of the biotin-ABPs to block ABP **14**-mediated generation of the three major fluorescent bands at pH 5 and 6 in *C. elegans* extracts was investigated. As can be seen, neither of the probes were able to block emergence of the middle band. Fluorescent labelling of the upper signal at 95 kDa (by ABPs **16**, **17**, **20**) and the lower signal at 52 kDa (by ABPs **13**, **16**, **17**) in contrast can be competed for, indicating that the protein(s) that are behind these signals may be identified through the biotinylated probes. Probes **13** and **17** moreover besides carrying a biotin are also fluorescent, at a complementary wavelength (both excitation and emission) to that of ABP **14**, allowing to fluorescence-scan the competitive ABPP gel also at the BODIPY-TMR wavelength. As can be seen, the lower signal (now tagged with **13**) and specifically the upper one (with **17**) reemerge at both pH's, indicating that these signals now correspond to proteins that are also biotinylated.

After evaluating the propensity of biotin-ABPs for isolating ABP **14** labelled proteins, *C. elegans* homogenates were then treated with 10 μ M biotin ABPs **13**, **16** or 20 μ M **20** as final concentration for one hour at 37 °C and at pH 5 (for **13**, **16**) or at pH 6 (for **20**). Then the samples were denatured and treated with streptavidin beads. After washing several times to remove unbound proteins, the proteins remaining on the streptavidin beads were digested by trypsin, and the tryptic peptides were analyzed and identified by LC-MS/MS. Usage of ABP **13** yielded the candidate-retaining β -glucosidase gene product encoded by the *gba-3* gene (UniProt code: G5ECR8), and ABP **20** significantly enriched the protein encoded by the *R08F11.1* gene (UniProt code: O01893), while ABP **16** enriched both gene products (see SI Table S1). The *gba-3*-encoded protein has a predicted MW of 59 kDa and the *R08F11.1*-encoded protein a predicted MW of 95 kDa. These predicted protein sizes coincide with the signals as obtained with the comparative ABPP experiments (Figure 4A) with fluorescent ABPs **14** and **15**, and also reflect the MW of hGBA1 and hGBA2.



(B)

Accession	Description	Peptide counts	Sequence coverage	Predicted MW	Score
GSECR8	Putative glucosylceramidase 3 OS=Caenorhabditis elegans OX=6239 GN=gba-3 PE=3 SV=1	22	37.5	59.088	265.07
O01893; A0A168H9S8	Non-lysosomal glucosylceramidase OS=Caenorhabditis elegans OX=6239 GN=CELE_R08F11.1 PE=1 SV=3	28;10	33.5	94.498	216.76

Figure 5. (A) Reactivity of biotin-ABPs (**13**, **16**, **17**, **20**) towards three major fluorescent bands in *C. elegans* extracts as revealed by competitive ABPP. *C. elegans* homogenates were treated with 10 μ M biotin-ABP **13**, **16**, **17**, or 20 μ M **20**, or vehicle alone (DMSO, the final concentration = 0.5%) for 1 h (or 1.5 h for **20**) at 37 °C at the indicated pH, followed by incubation with 1 μ M ABP **14** for 30 min, then samples were subjected to SDS-PAGE and fluorescence scanning of the wet gel slabs. (B) Putative β -glucosidases in *C. elegans* as identified by chemical proteomics.

As the final set of experiments, the *gba-3* protein (cGBA1-3) was brought to expression in both GBA1 KO and GBA1/GBA2 KO HEK293T cell lines. As shown in Figure 6, extracts of *gba-3* cDNA-transfected GBA1/GBA2 KO HEK293T cells give a strong fluorescence signal when treated with 4-MU- β -D-Glc, with maximal fluorescence at pH 4.4 (Figure 6B). This signal is absent in extracts from non-transfected GBA1/GBA2 KO HEK293T cells, indicating that 4MU- β -D-Glc processing in the sample derived from the *gba-3*-transfected cells is caused by the introduced *C. elegans* gene product. The fluorescence signal can be suppressed by inclusion of hGBA1-selective inhibitor **9**, but not by hGBA2-selective inhibitor **10**. Treatment of extracts of the *gba-3*-transfected GBA1 KO and GBA1/GBA2 KO cells with ABP **14** yielded, after SDS-PAGE and fluorescence scanning, two bands at around 54 kDa and 65 kDa MW that are not visible in the samples derived from the non-transfected cell lines (Figure 6C). As in the fluorogenic substrate hydrolysis assay, these signals can be competed for by treatment with hGBA1-selective inhibitor **9** (prior to incubation with ABP **14**), but not with hGBA2-selective inhibitor **10** (Figure 6D). The two labelled bands of recombinant cGBA1-3 can both be reduced molecular weight by N-glycanase (PNGase F) treatment (see SI Figure S4B), but this does not lead to a single signal. Finally, extracts of both transfected cell lines were incubated with C12-NBD-GlcCer, and the lipids extracted and resolved by HPTLC. Fluorescence scanning (Figure 6E) of the TLC plates showed no processing of this artificial GlcCer derivative, this in contrast to rhGBA1-mediated C12-NBD-GlcCer turnover in the control experiment. Altogether these results demonstrate that the *C. elegans gba-3* gene encodes for a retaining β -glucosidase with similarities to hGBA1 in pH optimum and sensitivity to hGBA1-selective inhibitors and ABPs, yet one that is not able to process the artificial substrate, C12-

NBD-GlcCer. Whether it is a *C. elegans* β -glucosidase that processes d17iso-GlcCer remains to be established.

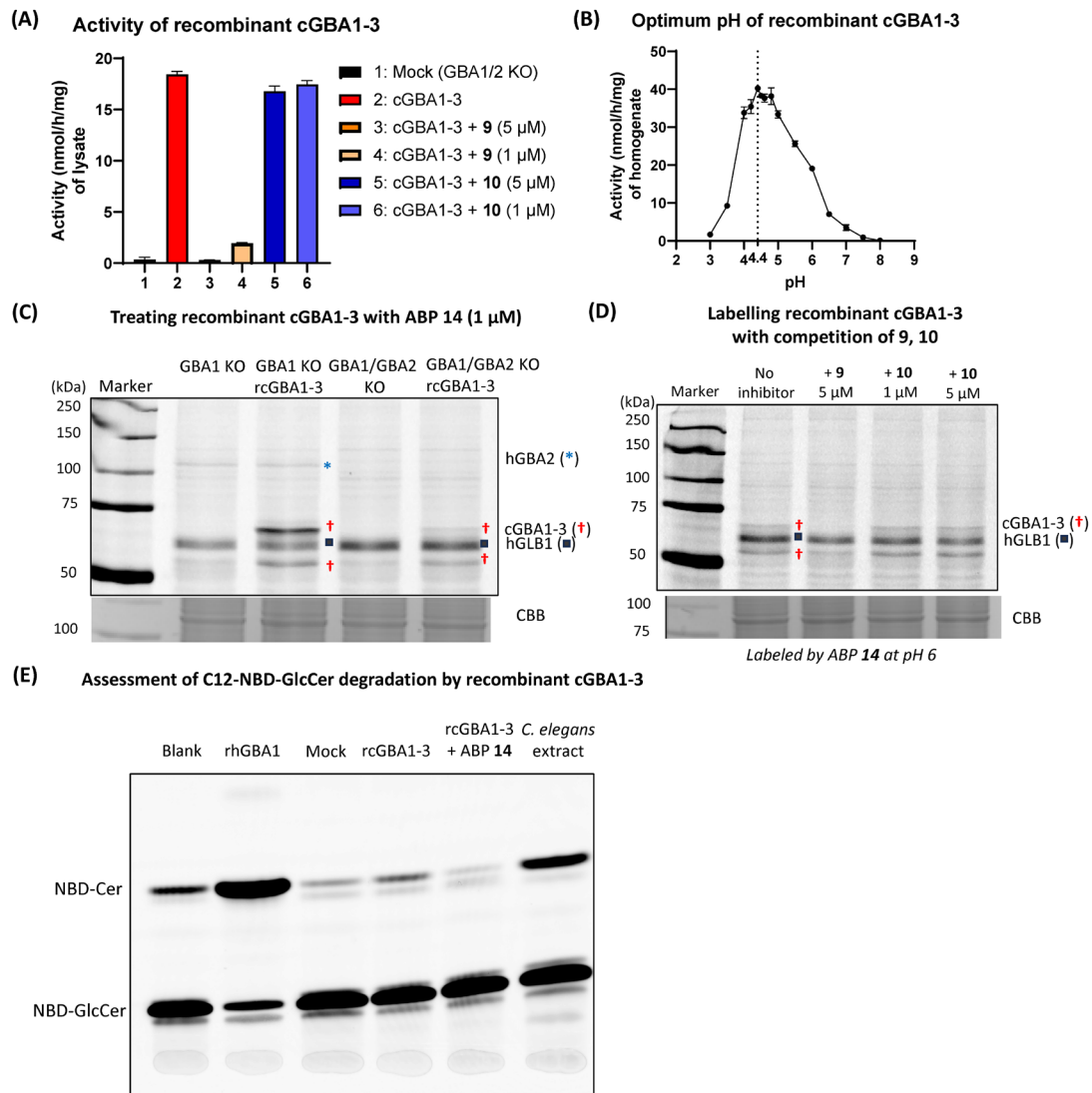


Figure 6. Expression and characterization of cGBA1-3. (A) Enzyme activity of lysates of hGBA1/GBA2 KO HEK293T cells expressing recombinant cGBA1-3 (termed as cGBA1-3 cells), determined by incubation with 4-MU- β -D-Glc for 1 h at pH 5.0 at 37 °C, with or without inhibitor (hGBA1-selective **9** or hGBA2-selective **10**) pre-treatment. Mock = lysate of hGBA1/GBA2 KO HEK293T cells. (B) Optimum pH of rcGBA1-3 for 4-MU- β -D-Glc hydrolysis activity. Lysates of cGBA1-3 cells were incubated with 4-MU- β -D-Glc for 1 h at 37 °C at various pH values (3-8). (C) Lysate of HEK293T cells expressing cGBA-1 (in either hGBA1 KO or hGBA1/GBA2 KO background) were incubated with 1 μ M ABP **14** at pH 6 for 30 min, followed by to SDS-PAGE and fluorescence scanning of the wet gel slabs. hGLB1 = human β -galactosidase (EC 3.2.1.23). (D) lysate of cGBA1-3 cells with or without pre-treatment of inhibitor **9** or **10**, were incubated with 1 μ M ABP **14** at pH 6 for 30 min at 37 °C, followed by SDS-PAGE and fluorescence scanning. (E) Lysates of cGBA1-3 cells was incubated with NBD-GlcCer at pH 4.5 for 4 h at 37 °C. Blank = NBD-GlcCer alone, no enzyme addition. rhGBA1 = 20 ng Imiglucerase (incubated at pH 5.2, supplemented with 0.1% (v/v) Triton X-100 and 0.2% (w/v) sodium taurocholate). Mock = 200 μ g lysates of hGBA1/GBA2 KO HEK293T cells. rcGBA1-3 = 200 μ g lysates of GBA1/GBA2 KO HEK293T cells expressing recombinant cGBA1-3, rcGBA1-3 + ABP **14** = 200 μ g rcGBA1-3 in hGBA1/GBA2 KO HEK293T cell lysates was pre-treated with ABP **14** for 30 min at 37 °C, followed by incubation with NBD-GlcCer. *C. elegans* extracts = 40 μ g *C. elegans* homogenates.

Discussion

Cells of *C. elegans* contain lysosomes with comparable morphology to the ones in vertebrate cells. The here-presented studies comprising measurement of activities in fluorogenic substrate and ABPP assays reveal the presence in *C. elegans* of two putative β -glucosidases with similarities to the human GBA1 and GBA2. Specifically, using biotin-ABPs **13**, **16** and **20** in pull-down chemical proteomics experiments allowed enrichment of a hGBA1-like protein (cGBA1-3) as well as GBA2-like protein (cGBA2). The cGBA1-3 (UniProt code: G5ECR8) encoded by the *gba-3* gene was recombinantly expressed in human HEK293T cells with a hGBA1 as well as a hGBA1/GBA2 KO background. cGBA1-3 shows considerable similarities to human GBA1, in predicted protein structure and polypeptide molecular weight, the presence of N-glycans, and acid pH optimum of enzymatic activity. In addition, the cGBA1-3 resembles hGBA1 in reactivity towards various selective inhibitors and probes. It however does not process (at least in the initial experiments performed) the artificial GlcCer analogue, NBD-GlcCer. This may be an intrinsic property, or the result of the conditions applied, and it is not excluded that cGBA1-3, while inactive towards the artificial substrate (which would set it apart from hGBA1), it does process the *C. elegans* form of GlcCer, d17iso-GlcCer. Alternatively, an endogenous accessory activator protein or lipid activator might be required to allow cGBA1-3, or its orthologs, to be active towards lipid substrates. Future research is needed to address these issues, as indeed glycolipidomics experiments on *C. elegans* extracts need to be done to shed further light into d17iso-GlcCer metabolism and its similarities to human GlcCer metabolism. As well, the enzyme products of the other orthologous genes *gba-1*, *gba-2* and *gba-4* require careful further examination. The identified cGBA2 by biotin-ABP pull down (UniProt code: O01893, encoded by the *R08F11.1* gene) in turn shows similarities to hGBA2 in molecular weight and reactivity with ABPs and inhibitors. More in-depth research, including cloning and expression of this gene, is required to fully comprehend similarities and differences of this *C. elegans* retaining β -glucosidase with hGBA2, as well as its involvement in d17iso-GlcCer metabolism. Such studies may also be geared towards another recent finding regarding human GlcCer metabolism: the ability of hGBA1 and hGBA2 to, besides GlcCer hydrolysis, also perform transglycosylation reactions.²⁵ In this process, GlcCer serves as glucose donor from which glucose is transferred to, instead of water, an acceptor alcohol. In particular, cholesterol was identified as an effective acceptor and β -glucosylcholesterol (GlcChol) has emerged as a natural metabolite in humans that moreover is produced in elevated levels in GD patients.²⁵ In an initial experiment, the presence of GlcChol in *C. elegans* extracts was also established (see SI Figure S5). Further research on d17iso-GlcCer metabolism in *C. elegans*, focusing on the enzymes involved (as initiated by the here-presented studies), but also on the glycosphingolipid pools present under given conditions (inhibitors, genetic manipulations) are needed to establish species-specific events, and whether *C. elegans* would serve as a good model to study cell biological events in the context of GD and PD.

Experimental procedures

Materials

Imiglucerase (Cerezyme®), a recombinant human GBA1 (rhGBA1), was kindly provided by Genzyme (Genzyme Nederland, Naarden, The Netherlands). 4-Methylumbelliferyl-glucopyranoside was bought from Glycosynth (Warrington, UK). HEK293T (CRL-3216™) cells were purchased from ATCC (Manassas, VA, USA), and cultured in DMEM medium (Sigma-Aldrich), supplied with 10% (v/v) FCS, 0.1% (w/v) penicillin/streptomycin and 1% (v/v) Glutamax, at 37°C under 5% CO₂. Polytron PT 1300D sonicator (Kinematica, Luzern, Switzerland) and potassium phosphate buffer (25 mM KH₂PO₄-K₂HPO₄, 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 cell and homogenizing *C. elegans* and *E. coli*. Protein concentration was measured using the Pierce BCA assay kit (Thermo Fisher Scientific, Waltham, MA, USA). Harvested cells (cell pellets), cell lysates, and *C. elegans* homogenates not used directly were stored at -80 °C.

Compounds (in Figure 1) were chemically synthesized and obtained in the department of Bio-organic Synthesis at the Leiden Institute of Chemistry, University of Leiden (Leiden, The Netherlands), based on previous reports: **12**²⁶, **15**²⁷, **14**²⁸, **16**²⁴, **18**²⁹, **19** and **20** (Chapter 3), cyclophellitol **7**³⁰, **10**²⁰, **10**²¹, **11**²², and **13**²³. Compound **17** was synthesized as described in the appendix (Scheme S1). Conduritol B epoxide (CBE) **8** was bought from Enzo Life Sciences Inc. (Farmingdale, NY).

C. elegans: cultivation, collection, and homogenizing

C. elegans (wild type, N2 Bristol) and *E. coli* (OP50 and HB101 strains) used in this work were obtained from the Caenorhabditis Genetics Center (CGC). The culture of *C. elegans* and *E. coli* and the preparation of buffers and media for *C. elegans* cultivation were handled as the described in WormBook.³¹ Influences of using either OP50 or HB101 *E. coli* as food for *C. elegans* cultivation have been reported, and no major differences on lipid composition were described.³² Labelling of ABP **14** towards HB101 fed *C. elegans* homogenates showed the same result with OP50 fed *C. elegans* homogenates (data not shown). *C. elegans* was raised on 10 cm plates which contain nematode growth media (NGM) seeded with OP50 or HB101 *E. coli* at 20 °C. For bacteria used as food for seeding worm plates, *E. coli* strains were grown overnight in LB medium at 37 °C with 220 rpm shaking. NGM plates were seeded with 0.1-0.2 ml *E. coli* of overnight bacterial culture and plates were allowed to dry at 37 °C overnight.

To harvest large quantity of *C. elegans*, the nematodes were cultured in liquid medium as described in WormBook.³¹ Briefly, 6-8 plates of *C. elegans* maintained for 4 days after chunking to new plates, were transferred to a 0.5 L or 1 L flask inoculated with S Medium. The flasks were supplemented with OP50 or HB101 *E. coli* and maintained at 20 °C with vigorously shaking for 4-5 days. For harvesting, the flasks were put on ice for 15 min to allow the *C. elegans* to settle at the bottom and subsequently most of the liquid from the flask was aspirated. After removing clumping of *E. coli* bacteria in liquid as much as possible, the remaining liquid was transferred to a 50 mL conical centrifuge tube and spun down to pellet the worms. Subsequently, the remaining liquid was aspirated, and the pellet washed 2 times with M9 buffer and 1 time with ultrapure water by repeated centrifuge and aspiration steps. After aspirating, the pelleted *C. elegans* were stored at -80 °C.

To generate *C. elegans* homogenates, nematodes were taken from -80 °C and thawed on ice, then nematodes were homogenized in potassium phosphate buffer by sonication on ice with a sonicator (40-50 seconds sonication with 3 second pulse on and a 17 second pulse off cycle at a 40-45% amplitude). Afterwards, the homogenized sample was spun down at 15000 rpm for 3 min at 4 °C, and

the supernatant collected. Homogenate of OP50 *E. coli* was prepared with the same method as described above.

Generation of hGBA1 KO and hGBA1/GBA2 KO HEK293T cells

hGBA1 KO and hGBA1/GBA2 KO HEK293T cell line were generated as described in Chapter 2.²⁹ cGBA1-3 (encoded by *gba-3* gene, UniProt code: G5ECR8) was expressed in either hGBA1 KO or hGBA1/GBA2 KO HEK293T by PEI transfection,³³ followed by Zeocin screening to generate HEK293T cell line stably expressing the recombinant cGBA1-3.

cGBA1-3 cDNA construct

cDNA constructs were ordered from GenScript for *C. elegans* cGBA1-3 encoded by the *gba-3* gene. The construct contains the Genbank sequence NM_070718.4 with a C-terminal DYK tag. The signal peptide was predicted by SignalP 5.0.³⁴ The *gba-3* sequence in a zeocin resistance gene containing plasmid was obtained using the Gateway system (Invitrogen). To perform the PCR for the insert with the attB sites, Phusion polymerase (Fisher Scientific) was used. The fragments were isolated from a 1% agarose gel using the Nucleospin gel extraction kit (Machery-Nagel). All plasmids were sent for Sanger sequencing (LGTC) to confirm the sequences of the inserts.

Tabel 2. Primers for Gateway system.

Reaction	Forward	Reverse
Recombination cGBA1-3	5'- GGGGACAAGTTTGTACAAAAAAGCAGGC TCCGCCACCATGTCAAGATGGAAGGTCGT T -3'	5'- GGGGACCACTTTGTACAAGAAAGCTGG GTCTTATTTCTTTTCTTCCAAATCACT -3'

Measurement of enzymatic activities

Enzyme activities were measured by 4-methylumbelliferyl (MU)- β -D-glucopyranoside fluorogenic substrate assays, conducted in 96-well plates as described in Chapter 2 with adaptations as described below. Briefly, *C. elegans* homogenates, *E. coli* homogenates, or lysates of hGBA1/GBA2 KO HEK293T expressing recombinant cGBA1-3 were prepared in 12.5 μ L potassium phosphate buffer, and mixed with 12.5 μ L McIlvaine buffer (150 mM, at the appropriate pH) and incubated with 100 μ L 3.75 mM 4-MU- β -D-Glc dissolved in McIlvaine buffer (150 mM, at the appropriate pH) for 30 min or 60 min at 37 °C. To assess inhibitory effect of inhibitor, above 12.5 μ L hGBA1/GBA2 KO HEK293T cell lysates containing recombinant cGBA1-3 were mixed with 12.5 μ L inhibitor (**9** or **10**) diluted in McIlvaine buffer (150 mM, at the appropriate pH) and incubated for 30 min at 37 °C, then the 25 μ L samples were incubated with 100 μ L 3.75 mM 4-MU- β -D-Glc dissolved in McIlvaine (150 mM, pH 5.0) at 37 °C for 60 min. After stopping the enzyme reaction with 200 μ L 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. Enzyme activities were determined by subtraction of the background signal (measured for incubations without enzyme).

Activity-based protein profiling (ABPP) with SDS-PAGE

ABPP assays were conducted as described in Chapter 2. Briefly, *C. elegans* homogenates were incubated with a fluorescent ABP under the indicated conditions (at the appropriate pH at 37 °C for 30–60 min). The total sample volume was 20–40 µL with a 0.5–1% DMSO (the vehicle) final concentration in Mcllvaine buffer (150 mM, at the appropriate pH). After incubation with the ABP, samples were boiled the using 5× Laemmli buffer and separated by electrophoresis on 10% (w/v) SDS-PAGE gels. Wet gel slabs were scanned on fluorescence using the Typhoon FLA 9500 (GE Healthcare) at λ_{EX} 532 nm and $\lambda_{\text{EM}} \geq 575$ nm for BODIPY-red mediated fluorescence; and at λ_{EX} 635 nm and $\lambda_{\text{EM}} \geq 665$ nm for Cy5 mediated fluorescence. Afterwards, the gels were stained by Coomassie brilliant blue (CBB) G250 or R250 for protein loading control.

Competitive ABPP with SDS-PAGE

Competitive ABPP assays are conducted as described in Chapter 2 with adaptations as described below. Generally, *C. elegans* homogenates or lysates of hGBA1/GBA2 KO HEK293T expressing recombinant cGBA1-3 were first treated with inhibitors (**7–11**) or the vehicle (DMSO) in Mcllvaine buffer (150 mM, pH 6.0) for 30 min incubation at 37 °C. Subsequently, fluorescent ABP **14** was added to samples and incubated for 30 min at 37 °C to reveal residual active enzymes. Afterwards, samples were subjected to SDS-PAGE and fluorescence scanning as described above. Final concentration of DMSO for all samples is 0.5–1%.

Biotinylated ABP, pull down and proteomics

For pull-down with biotin-ABPs (**13**, **16**, **20**), 500 µg total protein from *C. elegans* homogenates (nematodes were fed with HB101 *E. coli* in liquid NGM) were diluted with Mcllvaine buffer (750 mM, pH 5.0 for ABP **13**, **16**, or pH 6.0 for ABP **20**) to a total volume of 240 µL for each sample and incubated with biotin-tagged ABP (10 µM for **13** and **16**, 20 µM for **20**) at 37 °C for 2 h. For controls, the same volume of DMSO without ABPs was used and all samples have the same final DMSO concentration (0.5–1%). For competition with ABP **14**, the *C. elegans* homogenates were first treated with 5 µM ABP **14** for 1 h incubation at 37 °C, following by incubation with biotin-ABPs (**13**, **16**, **20**) for 2 h at 37 °C. Afterwards, samples were denatured with 10% (w/v) SDS, subjected to chloroform/ methanol precipitation (C/M), reduction/alkylation, C/M precipitation, and pull-down with 75 µL Pierce™ avidin agarose beads (Thermo Scientific) in a volume of 3400 µL pull-down buffer (50 mM Tris-HCl, pH 7.4 with 150 mM NaCl) at 4 °C overnight with a tumbling shaker, as previously described.^{35,36} Afterwards, the samples were subjected to on-bead trypsin digestion and desalted using stage-tips. Desalted peptide samples were reconstituted in 30 µL LC-MS solution (97:3:0.1 H₂O, ACN, FA) containing 10 fmol/µL yeast enolase digest (cat. 186002325, Waters) as injection control. Raw files were analyzed with MaxQuant (Version 2.0.1.0). The following changes were made to the standard settings of MaxQuant: label-free quantification was enabled with an LFQ minimal ratio count of 1. Matches between runs and iBAQ quantification were enabled. Searches were performed against a UniProt database of the *Caenorhabditis elegans* proteome (UP000001940) including yeast enolase (P00924).

N-glycanase treatment

For N-glycans removal, total 20 µg proteins (from *C. elegans* homogenate or lysate of HEK293T hGBA1/GBA2 KO cell expressing recombinant cGBA1-3) in Mcllvaine buffer (150 mM, pH 6.0) were incubated with 1 µM ABP **14** for 30 min at 37 °C. Afterwards, an aliquot of the samples was treated with PNGase F according to the manufacturer's protocol (New England BioLabs). For samples as control

(without incubation with PNGase F), samples with a normalized protein amount were incubated with 1 μ M ABP **14** for 30 min at 37°C in McIlvaine buffer (150 mM, pH 6.0). Subsequently, both the control and PNGase F-treated samples were subjected to SDS PAGE and fluorescence scanning as described above.

Analysis of C12-NBD-Glc degradation using HPTLC

40-50 μ g *C. elegans* homogenates were incubated with 2 μ M C12-NBD-GlcCer in McIlvaine buffer (150 mM) at pH 5.0 for 3-4 h at 37 °C. In the case using lysates of GBA1/GBA2 KO HEK293T expressing rcGBA1-3, a total of 200 μ g lysates were incubated with 2 μ M C12-NBD-GlcCer in McIlvaine buffer (150 mM) at pH 4.5 for 3-4 h at 37 °C. Next, lipids were extracted according to the method of Bligh and Dyer³⁷ by addition of methanol, chloroform, and water (1:1:0.9, v/v/v) and the lower phase was dried under a stream of nitrogen. lipids were separated by HPTLC on silica gel 60 plates (Merck, Darmstadt, Germany) using chloroform/methanol (85:15, v/v) as developing solution followed by detection of NBD-labelled lipids using a Typhoon variable mode imager (GE Healthcare Bio-Science Corp., Piscataway, NJ).³⁸

Analysis of GlcChol by LC-MS/MS

Lipids in *C. elegans* homogenates were extracted and measured according to methods described previously.²⁵ Briefly, 20 μ L of ¹³C-GlcChol (0.1 pmol/ μ L) in MeOH, 480 μ L MeOH, and 250 μ L CHCl₃ were added to the *C. elegans* homogenate samples, stirred, incubated for 30 min at room temperature and sonicated (5 $\times$ 1 min in sonication water bath), followed by centrifugation for 10 min at 15,000 rpm. Supernatants were collected in a clean tube, where 250 μ L CHCl₃ and 450 μ L 100 mM formate buffer (pH 3.2) was added. The samples were stirred and centrifuged, the upper phase was removed. The lower phase was pipetted into a clean tube, to which was added 400 μ L MeOH, 360 μ L formate buffer, and 400 μ L CHCl₃ after which the lower phase was transferred to a clean tube. The lower phase was dried, after which 700 μ L H₂O and BuOH was added and upper phase transferred to a new tube and dried. The residue was dissolved in 100 μ L MeOH. 10 μ L Of these samples were injected into the LC-MS/MS for lipid measurement as previous described.²⁵

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Appendix

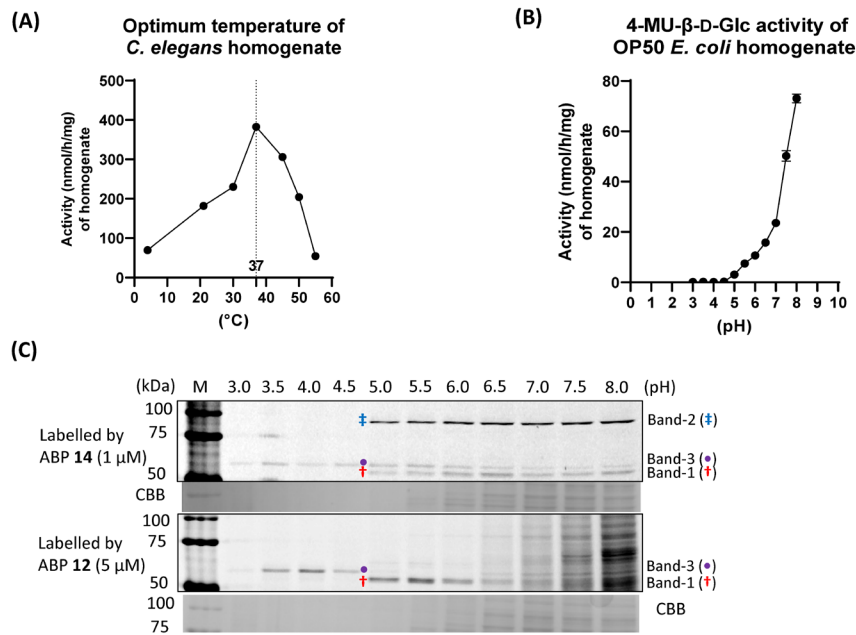


Figure S1. (A) Optimum temperature of *C. elegans* homogenates for hydrolyzing 4-MU- β -D-Glc. *C. elegans* homogenates were incubated with 4-MU- β -D-Glc for 30 min at pH 5.0 under various temperatures (4-55 °C). (B) 4-MU- β -D-Glc hydrolysis activity of OP50 *E. coli* homogenates. OP50 *E. coli* homogenates were incubated with 4-MU- β -D-Glc for 30 min at 37 °C and at various pH values (3-8). (C) *C. elegans* homogenates were incubated with ABP 12 (5 μ M, hGBA1-selective) or 14 (1 μ M, human β -glucosidases broad-spectrum) for 30 min at 37 °C and at various pH values (3-8), subsequently, samples were subjected to SDS-PAGE and fluorescence scanning of the wet gel slabs.

Table S1. Putative β -glucosidases in *C. elegans* homogenates as identified by chemical proteomics, using biotinylated ABP (13, 16, 20). Raw abundance of predicted GBA1-like protein (UniProt code: G5ECR8) and predicted GBA2-like protein (UniProt code: O01893) enriched by ABP 13, 16, 20, indicate by label-free quantification (LFQ) intensity. Higher intensity of LFQ intensity suggests a larger amount of proteins captured and enriched. Samples include the setting groups below. Group 1: Vehicle beads control, pull down (PD) assays only using streptavidin beads, without addition of biotin-ABPs; Group 2-4: PD assays using biotin-ABPs (13, 16, 20); Group 5-6: PD assays with ABP 14 pre-treatment prior to adding biotin-ABPs (16, 20). See detailed conditions in experimental procedures.

			Group 1: Control (without biotin-ABPs)					
Accession	Gene name	Mark	LFQ intensity (3 sets)					
G5ECR8	<i>gba-3</i>	GBA1-like	0	0	0			
O01893; A0A168H9S8	<i>R08F11.1</i>	GBA2-like	0	0	0			
		incubated at	pH 5.0	pH 5.0	pH 6.0			
			Group 2: PD using biotin-ABP 16			Group 5: PD using 16, with ABP 14 pre-treatment		
Accession	Gene name	Mark	LFQ intensity (3 sets)			LFQ intensity (3 sets)		
G5ECR8	<i>gba-3</i>	GBA1-like	2.46E+07	1.68E+07	1.73E+07	6.07E+05	5.18E+05	1.76E+05
O01893; A0A168H9S8	<i>R08F11.1</i>	GBA2-like	9.98E+06	7.89E+06	5.34E+06	1.87E+04	1.07E+04	0
		incubated at	pH 5.0					
			Group 3: PD using biotin-ABP 13					
Accession	Gene name	Mark	LFQ intensity (3 sets)					
G5ECR8	<i>gba-3</i>	GBA1-like	1.99E+07	1.41E+07	1.87E+07			
O01893; A0A168H9S8	<i>R08F11.1</i>	GBA2-like	2.76E+03	5.43E+03	3.66E+03			
		incubated at	pH 5.0					
			Group 4: PD using biotin-ABP 20			Group 6: PD using 20, with ABP 14 pre-treatment		
Accession	Gene name	Mark	LFQ intensity (3 sets)			LFQ intensity (2 sets)		
G5ECR8	<i>gba-3</i>	GBA1-like	6.71E+03	3.25E+03	3.69E+03	2.84E+03	0	
O01893; A0A168H9S8	<i>R08F11.1</i>	GBA2-like	1.64E+07	1.03E+07	8.13E+06	0	4.25E+03	
		incubated at	pH 6.0					

(A)	AA number
hGBA1 : MEFFSSPSREECPKPLSRVSIAGSLTGLLLQAVSWASGARPCLPKSF--GYSSVVCVCNATYCDSEFDPPTFPALCTFSRYESTRSCRRM : 89	
cGBA1-3 : -----MSRWKVVLCLLSFMFEIGHASFOCNPKTYDGAFLNIVCVCNATFCDEIEPIGEIAGEKAIVYRSLDGDRL : 73	
hGBA1 : ---ELSMGPQANHTGTGLLLTLOPEQKFKVKFGGAWTDAALNIALSPPAONLLLSYFSEEIGYNIIRVPMASCDFSIRTWTYA : 176	
cGBA1-3 : KRMSMKWKEKLKRNESVNVITIDASERFONIFGFGGAFDTSAGDQFVSLSETLQNYIVDSYFGKNCLEYNICRVPTASCDFSTHEYSVD : 163	
hGBA1 : DTPDDFOLHNFSLEEDTKLKIPLIHRAQLAORPVSLASPTTSTFWLKTNCVAVNCKGSLKGQPGDIYHOTWARYEVKELDAYAEHLQ : 266	
cGBA1-3 : DVHDDFELKHFALEDEDLKLIKIPKKAIEKTEGNIQLFASPWASAPGWMKVTGRMRGGAMRNDK--RVYQAYADYEFKEFEAYSSHAIT : 251	
hGBA1 : FWAVTAENFPSACLLSGYPFOCLGFPEHQRDEIARDLGETIANST-HHNVRLLMLDDORLLLPWAKVVLTDPEAAKYVHGTAVHWYLD : 355	
cGBA1-3 : FWGLTIQNEPSTCADMAWRWQTMNYTAETMRDELKYLGPKLKENKLTETLKVMVLDDCRGLLEGWADTIFNDPEATKYADGVAVHWYGN : 341	
hGBA1 : FLAPAKATIGETHRLPENTMLFASACVCSKFEQSVRLCSWDRGMOYSHSITNLLYHVVGWTDWNLAENPEGGNWVRNFDSPITVD : 445	
cGBA1-3 : LYSFAV-LLDITCRHHETKFIFGTACAGY-FGHHGPIMDWFRAESYADDIITDINHHVTGWTDWNLCDEDEGGPNWALNVDSPITVN : 429	
hGBA1 : ITKDTFYKQPMFYHLGHFSKFIPEGSORVGLVASQKNDLDAVALMHPDGSVVVVLNRSKDVPLTIKDPAVGLET-ISPQYSIHTYLW : 534	
cGBA1-3 : RTAQEFYKQPMFYALGHFSKFLPRGSTRVFTKIEGNLAVSATSVVIEGCRRAVILSKASNSLLTRIVDSSTGHSIVLNLPPIRSHIVIN : 519	
hGBA1 : RRQ-	: 537
cGBA1-3 : KKRK	: 523

(B)

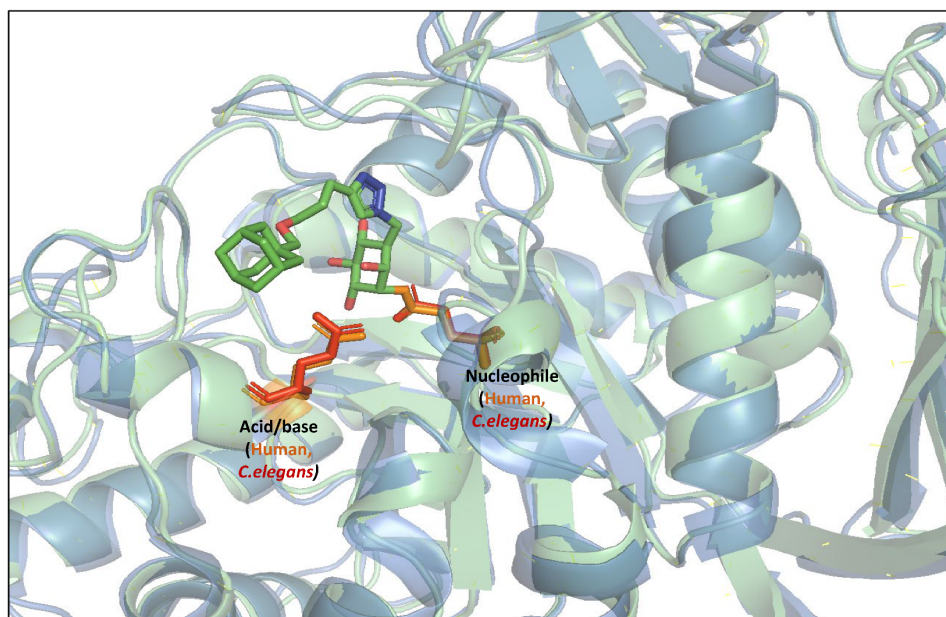


Figure S2. (A) Amino acid (AA) alignment of hGBA1 (UniProt code: P04062) and cGBA1-3 (UniProt code: G5ECR8). Signal peptides predicted by SignalP 5.0 are marked as blue, identical AA are marked with a black box, catalytic active residues are marked as red. (B) Overlap of the AlphaFold¹ predicted protein structure of cGBA1-3 (aqua green) and the complex crystallization of hGBA1 (nattier blue, PDB 6Q6L) with compound **13** (sticks, C = bottle green, O = red, N = blue), processed by PyMOL 2.0. Catalytic active site of (AA numbers without counting signal peptides): hGBA1 (orange sticks), nucleophile = E340, acid/base = E235; cGBA1-3 (red sticks): nucleophile = E343, acid/base = E238.

	AA number
hGBA2 : MGTQDPGNMGTGVPASEQISCAGEDPQVYCPEETGGTKDVQVTDCKSPEDSRPPKETDCCNPEDSGQLMVS Y GKAMGYQVPPFG R ICL :	90
cGBA2 : -----MS F EH R D--RMLE G IG W K A R G :	19
hGBA2 : --A H E F TE R K R K P FO A NN V LS N MIKH I GM G -L R Y L Q W Y R K T H V E K K T P F ID M IN S V L R Q I Y G C PLGGIGGG T IT R G W R G O F CR W Q L N F :	177
cGBA2 : DR I P E EP N EP R PF P RL K HS I K L I P L F VR V AL H T F VE W NG-----RE A F I D I F N VER H F T Y T G V PLGGIG C SI G T D ER G CG N R F SI P :	103
hGBA2 : G M Y Q H R T V -I A D O F T V C L--R R E G Q T V Y Q O V L S L E--R P S V I R S W N W G L C G Y F A F Y H A L Y P R A W T V Y Q L P G Q N V L T C R Q I T S I L E H D W Q :	262
cGBA2 : G I K E Q T E T Q K C N O I V T V H S K K T F E L I Y Q S I L S C A E F P A T V L P K W DT T I P A E D V R I R G L F P R A W Q E F R L G S S C V T V V E H L S V I P G D Y S :	193
hGBA2 : D S S L E V G V F V W D V E N E G D A L D V S I M F S M R N C L G G D D A P G L W N E P F L E R ----S G E T V R G L L H H P T L E N P V T M A V A R V T A A T T V T :	348
cGBA2 : D S S L E L A N E F H V F N D S Y E E V S I T M S F R N C T E N R K W N D E ----N L C S Q S K I Q K D T M V V R T L A H T V K G M P V T V A I G T E E K --N - G S K V :	275
hGBA2 : H I T A F D E D S T G Q Q V W D L L Q D C O L D S P T G S T P T Q L G V G I A C A V C S S K L R E R G C R L E F S L A W D M E R I M F G A K G V H Y R R Y T R F F G Q D G :	438
cGBA2 : T T C L F D P N G T G R L N S D L E A Y C H L S S Y D H L P ---S R P K E L G T A V C S S F F V P D C A H N T O F S L T W Y M P Q V H F G T A E R F Y N R R Y C R F E N G P D :	362
hGBA2 : D--A A P A L S H Y A L C R Y A E E E R I S A W S P V L D R S L E P A N Y K S A L F N E L Y F L A D C G T V W L E V L E D S L P E L G R N M C H L R P T L R D Y G R F G Y L :	526
cGBA2 : A D E V T A A T C R H L O N F S T W O K K I E D W O A P V L N D Q K L E W Y K S A I F N E L Y I V D G S T V W F E Y D P D W K T E S -L M S E Q T E K Q F K Y G R F G Y M :	451
hGBA2 : R G O E Y R M Y N T Y D V H F Y A S F A L I M L W E K L E L S L O Y D M A L T L R E D L T R R Y L M S G V M A P V R R R N V I P H D I C D P D E P W L R V N A L I H D T A D :	616
cGBA2 : R S W E Y F M I N T Y D V H F Y S S A I L K N W Q I E M S M O L D E A D Q M D R V D N N I A T S I A D G E Q M T I R S V D R I P H D M C H E M A D P W I H T N A L I L H D T G R :	541
hGBA2 : W K D L N L K F V L Q V R D Y Y L T G D Q N---F I K D M W P V C L A V M E S E -M K F D K D H D G L I E N G C A D Q T Y L G W V T G S A Y C G L W L A A V A V M V Q :	701
cGBA2 : W K D L N L K F V I S C Y R R K I E L G S E K G Q V E F F L G K T K I V D G A L E C W D K D N D G M I E N D C A D Q T Y L V W K M T G S A Y C G S L W I A L S S Y I E :	631
hGBA2 : M A A L C Q A D I Q D K F S S I L S R G Q E A Y E R L L W N C R Y Y N D S S R P S R S V M S D C A G Q W F L K A C G L G E G D T E V F T Q H V V R A L O I T F E L N V Q :	791
cGBA2 : M L K Q S L P T--K H Y E E K L E M A Y D A I G K L W N C T F F K F D E L P E -N S K I V A D Q L C C F W A M T A M D E ---P V Q I S K D K M K S A L D I T E K Y N V Q :	714
hGBA2 : A F A G C A M G A V N C M Q P H G V P D K S S V Q S D E V V W G V V Y G L A T M I Q E L T W E G F O T A E C C Y R T V W E R L C L A F O T P E A Y C Q Q R V F S L A Y M R P L :	881
cGBA2 : M Y N N R C R C A V N G Y L T S E R V D G S S I Q S E V W A C I T A L S A M M I E K C M D E Q A E K T S E L F E S I N H R F L Q Y O T P E A I T S D G M Y R A L G Y M R P L :	804
hGBA2 : S I W A L Q L A L Q Q Q H K A S W P K V K Q G T G L R T G P M F G P K E A M A N L S P E :	927
cGBA2 : S I W A L Q H A L D R R Y R E ----- :	819

Figure S3. Amino acids (AA) alignment of hGBA2 (UniProt code: Q9HCG7) and cGBA2 (UniProt code: O01893). Identical AA were marked with black box. Catalytic active site of hGBA2 and cGBA2 (predicted) are marked as red: hGBA2, nucleophile = E527, acid/base = D677; cGBA2, nucleophile = E452, acid/base = D607.

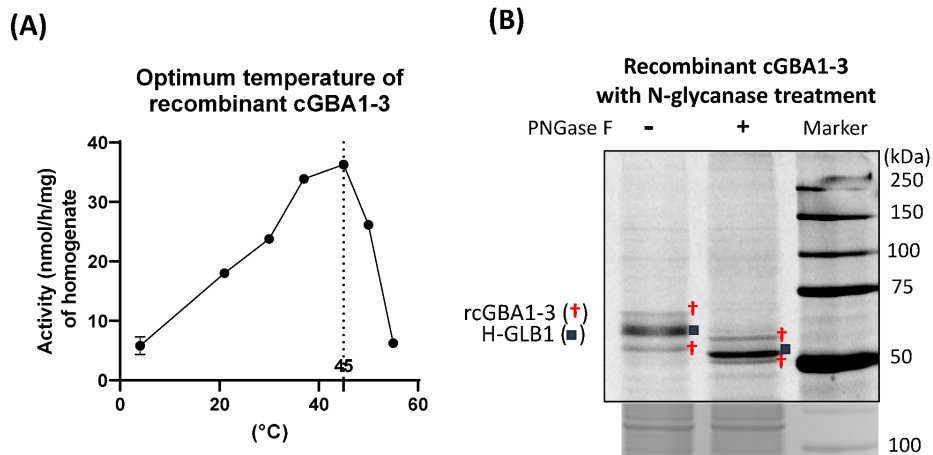


Figure S4. (A) Optimum temperature of recombinant cGBA1-3 for hydrolyzing 4-MU- β -D-Glc. Lysates of hGBA1/GBA2 KO HEK293T cell expressing rcGBA1-3 were incubated with 4-MU- β -D-Glc for 45 min at pH 5.0 under various temperatures (4 °C-55 °C). (B) Treating rc-GBA1-3 with PNGase F. Lysates of hGBA1/GBA2 KO HEK293T cell expressing rcGBA1-3 were incubated with ABP **14** for 30 min at 37 °C at pH 5.0, followed by incubation with PNGase F for 2 h, then samples were subjected to SDS-PAGE and fluorescence scanning of the wet gel slabs.

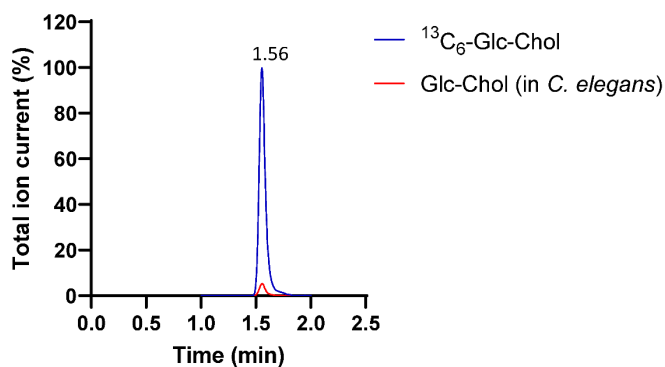
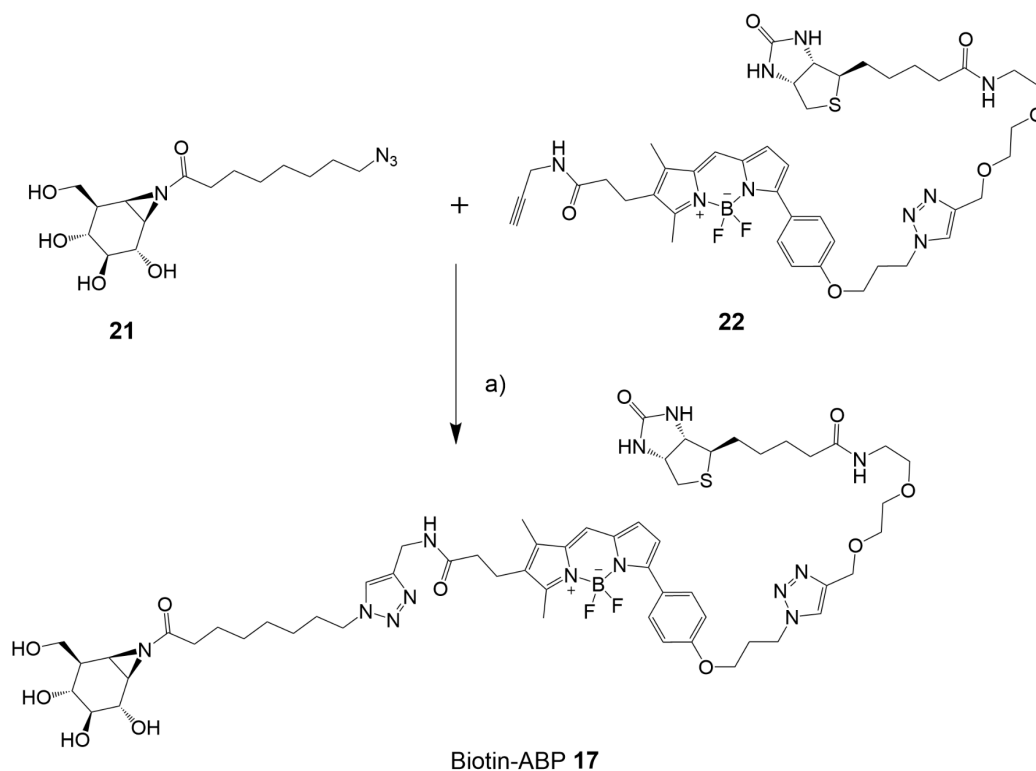
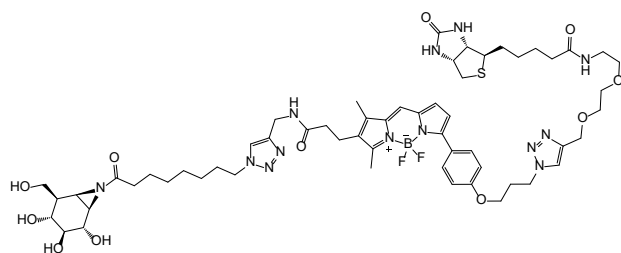
GlcChol of *C. elegans* detected by LC-MS/MS

Figure S5. Presence of GlcChol in *C. elegans* homogenates as detected by LC-MS/MS, using total 2 pmol $^{13}\text{C}_6$ - β -GlcChol as internal standard, $n = 2$ replicates. Quantification reveals the level of GlcChol in *C. elegans* extracts is around 0.1 pmol/ μg .

Synthesis of ABP 17



Scheme S1. Synthesis of ABP 17. (a) CuSO_4 , sodium ascorbate, rt, 15%.



A solution of $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ and sodium ascorbate (0.1 M in MilliQ water) was freshly prepared. Cyclophellitol-aziridine azide **21**² (4.71 mg, 14 μmol , 1 eq.) was dissolved in DMF (2 mL), $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ (0.1 M, 62 μL , 6.2 μmol , 0.45 eq.) and sodium ascorbate (0.1 M, 62 μL , 6.2 μmol , 0.45 eq.) were added to the solution

under argon atmosphere. Then, a solution of **22**² alkyne biotin (12 mg, 14 μmol , 1 eq.) in 1 mL of DMF was added and the reaction mixture was stirred at rt overnight. The resulting mixture was checked with LC/MS within the elution system of 10% NH_4OAc . Starting material was consumed and the reaction mixture was then concentrated under reduced pressure and purified by semi-preparative reversed HPLC (linear gradient: 32% $\rightarrow$ 35% B in A, 12 min, solutions used A: 50 mM NH_4HCO_3 in H_2O , B: acetonitrile), the fractions were concentrated and lyophilized to the final product (2.52 mg, 14 μmol , 15%) which was freeze-dried and aliquoted in 100 nmol tubes. **¹H NMR** (850 MHz, MeOD) δ 8.02 (s, 1H), 7.93 – 7.89 (m, 2H), 7.45 (d, J = 8.2 Hz, 1H), 7.13 – 7.10 (m, 1H), 6.98 (d, J = 8.4, 1.2 Hz, 2H), 6.68 (dd, J = 6.1, 4.1 Hz, 1H), 4.67 (td, J = 6.8, 1.7 Hz, 2H), 4.65 (d, J = 2.1 Hz, 2H), 4.46 – 4.43 (m, 1H), 4.39 (d, J = 5.5 Hz, 2H), 4.25 (ddd, J = 7.8, 4.3, 1.1 Hz, 1H), 4.11 (t, J = 7.2 Hz, 1H), 4.07 (t, J = 5.6 Hz, 2H), 3.66 – 3.64 (m, 2H), 3.62 – 3.60 (m, 2H), 3.52 – 3.49 (m, 2H), 3.33 (td, J = 5.3, 1.2 Hz, 2H), 3.16 – 3.14 (m, 1H), 2.88 (ddd, J = 12.7, 5.0, 1.0 Hz, 1H), 2.81 (td, J = 7.1, 1.6 Hz, 2H), 2.67 (d, J = 12.7 Hz, 1H), 2.53 – 2.51 (m, 3H), 2.45 – 2.41 (m, 3H), 2.25 – 2.22 (m, 3H), 2.18 (td, J = 7.4, 3.5 Hz, 2H), 2.11 – 2.08 (m, 1H). **¹³C NMR** (214 MHz, MeOD) δ 188.5, 176.1, 174.6, 166.1, 160.9, 156.6, 146.4, 145.8, 142.2, 136.7, 135.6, 131.9, 131.6, 129.6, 127.0, 125.6, 124.8, 123.4, 119.3, 115.3, 115.3, 79.2, 76.0, 74.1, 73.4, 72.2, 71.2, 70.7, 70.6, 69.3, 65.7, 64.9, 63.7, 63.6, 63.3, 61.6, 57.0, 51.3, 51.2, 45.4, 45.3, 42.4, 41.1, 40.3, 35.6, 29.7, 29.5, 27.6, 27.3, 26.9, 25.9, 21.1, 13.3, 9.6.

Supplementary references

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