Sphingolipids are essential components of cellular membranes and defects in their synthesis or degradation cause severe human diseases. The efficient degradation of sphingolipids in the lysosome requires lipid-binding saposin proteins and hydrolytic enzymes. The glycosphingolipid galactocerebroside is the primary lipid component of the myelin sheath and is degraded by the hydrolase β-galactocerebrosidase (GALC). This enzyme requires the saposin SapA for lipid processing and defects in either of these proteins causes a severe neurodegenerative disorder, Krabbe disease. Here we present the structure of a glycosphingolipid-processing complex, revealing how SapA and GALC form a heterotetramer with an open channel connecting the enzyme active site to the SapA hydrophobic cavity. This structure defines how a soluble hydrolase can cleave the polar glycosyl headgroups of these essential lipids from their hydrophobic ceramide tails. Furthermore, the molecular details of this interaction provide an illustration for how specificity of saposin binding to hydrolases is encoded.
Sphingolipids are both essential membrane components and bioactive metabolites that regulate critical cell functions. Defects in sphingolipid metabolism underlie a range of diseases, including lysosomal storage diseases, and are implicated in a number of cancers. Sphingolipid degradation occurs in the lysosome and depends upon two families of proteins: glycosyl hydrolases, and lipid-transfer proteins including saposins and the GM2 activator protein. The hydrolases are water soluble while the substrates are embedded in lysosomal intraluminal vesicle membranes. The steric crowding of headgroups and lateral association of sphingolipids into clusters prevents hydrolases from accessing the scissile bonds of their target substrates. Saposins are required to facilitate sphingolipid processing by hydrolases, and extensive work provides evidence for two general models of saposin action (Fig. 1a). The ‘solubiliser’ model proposes the complete extraction of lipids from the membrane, while the ‘lifase’ model envisages saposin proteins binding directly to compatible membranes and improving lipid accessibility by membrane distortion, destabilisation or localised remodelling. The saposin proteins are produced as a precursor prosaposin protein that, upon delivery to the lysosome, is cleaved into the four saposins: A, B, C and D. The functions of these four saposins are distinct, as they cannot compensate for the loss of each other, and saposins appear to function specifically with their associated hydrolases. Lysosomal storage diseases, and more specifically sphingolipidoses, are caused by mutations that inhibit degradation of sphingolipids. For example, Krabbe disease is caused by loss of galactocerebrosidase (GALC) which is responsible for the removal of the terminal galactose from the galactocerebroside (GalCer). Impersonally, mutation of the GALC-associated saposin SapA can also cause Krabbe disease. Similarly, Gaucher disease is caused by loss of glucocerebrosidase activity and also by loss of SapC, highlighting the critical role of saposins in sphingolipid processing.

Several high-resolution structures of saposins have been determined to date, revealing huge conformational variability and a propensity to form oligomeric assemblies. Saposins can broadly be described as existing in two states: a “closed” monomeric form where the helical protein folds back on itself, burying a large hydrophobic core; or a more “open” dimeric form, possessing a hydrophobic cavity into which lipids and detergents can bind. A recent structure of SapA, solved in the presence of the detergent LDAO, revealed that this open conformation can form lipoprotein discs and these discs (also referred to as picodiscs or Salipro nanoparticles) have recently been exploited to aid the determination of challenging membrane protein structures by crystallography and cryo-electron microscopy. However, it has remained unclear what form of saposin–lipid complex mediates binding to their cognate hydrolase. To remedy this deficit we have solved the structure of GALC in complex with SapA, defining how saposins solubilise lipids for processing by soluble hydrolases.

**Results**

**GALC and SapA form a pH-dependent heterotetrameric complex.** Murine GALC and SapA were expressed and purified from mammalian cells and E. coli, respectively. Pulldown assays reveal that SapA binding to GALC is pH-dependent and is optimal at low pH, equivalent to that of the endolysosomal compartments where lipid degradation occurs (Fig. 2a). This interaction depends upon the presence of detergents, suggesting that the “open” (dimeric) lipid-bound form of SapA mediates the interaction (Fig. 2b). The pH-dependency of the interaction, combined with the low pI of SapA (pH 4.5) and the higher pI of GALC (pH 6.1), suggests that the interaction could be mediated by electrostatic interactions. In support of this, the interaction is sensitive to the presence of salt, as increasing concentrations of NaCl reduce binding in these pulldown assays (Fig. 2b). Based on these insights, the GALC–SapA complex was formed at pH 5.0 in

![Fig. 1 Glycosphingolipid processing by saposins and GALC.](Image)
the presence of the detergent LDAO and the X-ray crystal structure of this complex solved and refined to 3.6 Å resolution (Fig. 2c and Table 1). Glycosylation of GALC results in a smeared band on gradient SDS-PAGE gels. b Pulldown assays at pH 5.4 identify that the interaction requires the presence of detergents, such as Tween 20 or LDAO, and is reduced with increasing NaCl concentration (20, 150 and 300 mM NaCl). c The crystal structure of the GALC–SapA complex reveals a pseudo-symmetric 2:2 heterotetramer of SapA (yellow and orange) with GALC (cyan and magenta). d The interacting surfaces of GALC (left) and the SapA dimer (right), rotated by 90° with respect to c to highlight the respective interaction surfaces. On the GALC surface the interacting regions contacted by SapA chain 1 (yellow) and SapA chain 2 (orange) are highlighted, the different domains of GALC are outlined (white lines) and the active site is identified. On the SapA surface the residues that interact with GALC are highlighted for each chain and the different chains of SapA are outlined (white line).

The structure reveals that the complex forms a 2:2 heterotetramer, composed of a central SapA dimer with two molecules of GALC binding its surface. The SapA–GALC heterodimers are related to each other by a pseudo two-fold non-crystallographic symmetry axis. Using a high-resolution GALC structure, and thus high-quality phase information, to solve the complex structure yielded excellent maps: the SapA molecules could be unambiguously positioned due to density for their intramolecular interactions.
Table 1 Data collection and refinement statistics

| Data collection          | Value               |
|-------------------------|---------------------|
| Space group             | P 6_2 2 2          |
| Cell dimensions         | a, b, c (Å)         |
|                         | 187.2, 187.2, 360.2 |
| Resolution (Å)          | 180.12–3.60 (3.70–3.60) |
| Rmerge                  | 0.236 (2.946)      |
| Rwork                   | 0.078 (0.607)      |
| CC1/2                   | 0.945 (0.677)      |
| i/o d                   | 9.3 (1.5)          |
| Wilson B (Å²)α          | 94.5               |
| Completeness (%)        | 100 (100)          |
| Redundancy              | 25.6 (25.2)        |

Refinement

| Resolution (Å)          | 162.11–3.60        |
| No. reflections in working set | 43,825             |
| No. reflections in test set | 2138               |
| Rwork/Rfree             | 0.217/0.231        |
| No. atoms               | Protein 11,522     |
| Other                   | 220                |
| B-factors (Å²)          | Protein 100.9      |
| Other                   | 141.2              |
| Ramachandran            | Favoured (%)       |
|                         | 95.8               |
| Outliers (%)            | 0.5                |
| r.m.s. deviations       | Bond lengths (Å)   |
|                         | 0.008              |
| Bond angles (*)         | 0.94               |

Values in parentheses are for highest-resolution shell
α Calculated using phenix.model_vs_data[10]
β Includes carbohydrate and ions

disulphide bonds, and small GALC conformational changes in the vicinity of the bound SapA molecules were readily visible (Supplementary Fig. 1b–d). By peeling apart the complex structure the details of the surfaces involved in the interaction between GALC and SapA are revealed (Fig. 2d). Importantly, the active site of GALC is buried in the centre of the interaction interface. Not only does the SapA dimer sit over the active site, it makes contact with all three domains of GALC: the TIM barrel, β-sandwich and lectin domains[29]. This extensive interface buries a total surface area of 1366 Å², of which 70% is contributed by one SapA chain and 30% by the other (yellow and orange, respectively, in Fig. 2d). At the optimal pH of the interaction the GALC and SapA surfaces demonstrate extensive charge complementarity, explaining the observed pH dependency of the interaction (Supplementary Fig. 2). The GALC–SapA interaction interface is not in the vicinity of any crystal contacts and thus is not affected by surrounding molecules in the crystal (Supplementary Fig. 1e).

The GALC–SapA complex possesses a hydrophobic channel. Analysis of the SapA dimer in the complex structure suggests the presence of disordered detergent within the core. Hydrophobic residues line the SapA core, consistent with this surface being exposed to non-polar solvent (Fig. 3a, the right panel reveals the internal surface of the dimer). Further, the average electron density in the cavity is lower than the surrounding solvent, consistent with the presence of detergent (Supplementary Fig. 3a). Analysis of the SapA dimer surface reveals a clear hydrophobic patch, within the interaction interface, at the point where the two SapA chains come together to contact the GALC surface (Fig. 3a, circled in the central panel, and Fig. 2d). A cross-section through the structure at this position shows that this hydrophobic patch encircles an opening in the SapA dimer surface that lies directly opposite the GALC active site (Fig. 3b). This reveals a continuous open channel that stretches from the GALC active site through to the hydrophobic cavity buried in the SapA dimer. The hydrophobic acyl chains of glycosphingolipids such as GalCer can thus be shielded from the polar solvent by the SapA dimer, while the hydrophilic glycosyl head groups are presented to the GALC active site. Although GALC–SapA crystals could be obtained in the presence of lipids and substrate-mimics, we were not able to collect data of sufficient quality to yield a structure of the substrate–bound complex. However, our previous work...
order to accommodate lipidated substrate. In particular, residue additional residues in this region may alter their conformation in face. As was observed for substrate-bound GALC, it is likely that structure and is in interaction of W37 and I38 differs from that of the lipoprotein disc complex could function simultaneously. The opening in the SapA dimension of the SapA dimer cavity in our structure is suf

Although this plasticity near the active site and the conformational flexibility of the GalCer acyl chains hindered accurate modelling of the conformation of an entire GalCer molecule, the dimensions of the channel and hydrophobic cavity could accommodate the lipidated substrate (Fig.3c). Based on the SapA lipoprotein disc structure containing ordered LDAO molecules, the volume of the SapA dimer cavity in our structure is sufficient to contain between 10 and 14 molecules of LDAO (Supplementary Fig.3b) and therefore would be capable of binding at least 2 molecules of GalCer, meaning both GALC molecules in the complex could function simultaneously. The presence of this continuous channel from the active site to the hydrophobic cavity, with sufficient space to accommodate glycosylated sphingolipid substrates, supports that this arrangement of GALC and SapA represents the functional complex that facilitates glycosphingolipid catabolism in vivo.

**Specific mutations of SapA inhibit binding to GALC.** Analysis of the molecular details at the interface reveals that several sidechains of residues along the length of one SapA chain form critical hydrogen bonds, electrostatic and hydrophobic interactions with residues in GALC (Fig. 4a and Supplementary Fig. 4). These interactions span two stretches of this SapA chain: residues 19–32 and 49–63. The interactions contributed by the second SapA chain encompass residues 36–40 and involve several backbone hydrogen bonds, as well as sidechain interactions with W37. To confirm that the interface observed in our crystal structure mediates the interaction in solution, a series of point mutations were made in SapA and tested for their ability to bind GALC (Fig. 4b, c). Several of the SapA residues that lie at the interface with GALC are acidic: E25, E32, E49, D52 or D60. Single-charge inversions at these sites each abolish or significantly reduce binding to GALC, highlighting the electrostatic nature of the interface. To ensure that alteration of surface charge alone is not sufficient to abolish binding we mutated residue E65, which does not lie in the interface, to lysine and confirmed it does not

**Fig. 4** Critical residues at the GALC-SapA interface. **a** Schematic diagram illustrating the backbone (bb) hydrogen bonds, sidechain (sc) hydrogen bonds and salt bridges observed between GALC (cyan) and specific residues of SapA chains 1 (yellow) or 2 (orange). Note that SapA residues Q24 and W37 interact with multiple GALC residues. **b** Coomassie-stained SDS-PAGE of pulldown assays with immobilised GALC performed in the presence of Tween-20 identifies critical SapA mutations at the interface that abolish GALC binding in solution. **c** Quantification of SapA binding in pulldown assays (n = 3, error bars represent SEM)
Saposin specificity. a Sequence alignment of human and murine saposins A–D coloured by conservation. Important residues at the GALC–SapA interface are highlighted (*). Coloured bars below the sequence identify the regions that interact with GALC: two regions from SapA chain 1 (yellow) and the one from SapA chain 2 (orange) as detailed in Fig. 4a. The position of α-helices in SapA are highlighted (green cylinders). b Critical residues that may determine binding specificity are shown (sticks) on SapA (yellow) at the interface with GALC (cyan surface, key residues depicted as transparent sticks). Hydrogen bonds between GALC and SapA residues are highlighted (orange dotted lines). c Coomassie-stained SDS-PAGE pulldowns with immobilised murine GALC (mGALC) illustrating specific binding of murine SapA (mSapA), but not mSapB or mSapD.
the absence of detergents remain closed\textsuperscript{23,31} whereas in the presence of detergents SapA adopts an open conformation (this study and Popovic et al.\textsuperscript{22}, suggesting that detergent mediates the formation of open SapA dimers essential for the interaction. The crystal structure of GALC–SapA determined here reveals the complex to be a heterotetramer with an extensive interaction interface that involves residues from all domains of GALC and both chains of the SapA dimer. There is an open channel running from the GALC active site through to the hydrophobic cavity of the SapA dimer, suggesting a clear mechanism for glycosphingolipid binding and processing. The combination of structure analysis, pulldown assays and sequence comparison of the saposin family members identifies residues 19, 24, 25, 28, 32, 49, 52 and 60 of SapA as essential for binding to GALC. Despite the structural homology of the saposin proteins it is clear that the distinct charge and shape complementarity of the SapA surface is critical for specific binding to GALC.

The specific importance of SapA for mediating GALC activity in vivo is highlighted by two key observations: clinical data has identified that a mutation in SapA causes Krabbe disease despite normal GALC activity\textsuperscript{17}, and a transgenic model with defective SapA develops a phenotype resembling late-onset Krabbe disease\textsuperscript{18}. In support of this specificity, mutations that result in defective SapC cause Gaucher disease, not Krabbe disease\textsuperscript{19–21}. However, in vitro studies of saposin-mediated degradation of gangliosides have shown that both SapA and SapC can stimulate GALC activity\textsuperscript{2,23}. Our pulldowns studies using recombinant GALC and saposins (Fig. 5c and Supplementary Fig. 5) suggest that, unlike SapA, SapC does not directly bind GALC. Taken together, this suggests that although SapC is not necessary for GALC activity in vivo it may indirectly enhance GALC activity by, for example, aiding lipid accessibility at the bilayer surface. The GALC–SapA complex structure reveals new details of how saposins bind to lysosomal hydrolases. However, it is not the first SapA dimer structure and comparison with the previous structure of a human SapA lipoprotein disc reveals significant differences in dimer assembly\textsuperscript{22}. Interestingly, the conformation of the SapA monomers in these structures are essentially identical (RMSD 0.86 Å\textsuperscript{2} over 80 C\textsubscript{α} atoms, with 79.7\% sequence identity), adopting the same hinge angle upon dimer formation (Supplementary Fig. 6a). Despite this similarity, the relative orientations of the monomers within the dimer differs between these structures (Supplementary Fig. 6b). In our structure the monomers are head-to-head, with all termini together at one end, while in the published lipoprotein disc structure the monomers adopt a head-to-tail orientation. However, molecular dynamics studies of the previous lipoprotein disc structure and subsequent work monitoring the dynamics of these particles shows there is significant plasticity in the arrangement of the SapA molecules as their relative orientations are only determined via binding to LDAO (Supplementary Fig. 6c)\textsuperscript{2,24,34}. In complex with GALC, the SapA dimer possesses direct protein–protein contacts between saposin monomers, including extensive hydrophobic interactions where the termini come together and additional contacts near the interface with GALC (Supplementary Fig. 6d). Comparison of the different SapA dimer structures shows that the GALC-interacting surface identified in our structure is surface exposed in the lipoprotein disc structure. Docking of GALC onto the surface of the lipoprotein disc structure reveals this SapA dimer to be compatible with GALC binding (Supplementary Fig. 7a). In this alternative arrangement the opening on the surface of the SapA dimer is less hydrophobic and, due to the loss of interactions with the second SapA chain, the substrate channel is more open and exposed to surrounding solvent (Supplementary Fig. 7b, c). Although there are no steric clashes that would prevent this complex forming, the tighter SapA dimer and enclosed hydrophobic channel identified in our complex provides a more protected environment from the hydrophilic solvent. We therefore propose that our structure, possessing a large buried surface area, an enclosed hydrophobic channel and an obvious mechanism for substrate presentation to GALC, represents the bona fide interaction that stimulates SapA-mediated lipid catabolism in the lysosome.

As both open and closed forms of SapA have been described we considered whether the closed, monomeric conformation could be compatible with binding to GALC. Closed SapA can be docked onto the open form of SapA via either the terminal helices or the central two helices (Supplementary Fig. 8a). Only this second alignment yields an interface that maintains any of the critical interactions identified in our pulldowns (Supplementary Fig. 8b). This arrangement would block binding of a second SapA and GALC, resulting in a 1:1 complex possessing a buried surface area of 669 Å\textsuperscript{2} (compared with 1366 Å\textsuperscript{2} for the tetramer) that is not predicted by ePISA to form a stable complex (CSS = 0)\textsuperscript{35}. Thus, although there is no steric hindrance to binding of closed SapA to GALC it is likely to be an extremely weak interaction, below the detection limit of our assays, if it exists at all.

Previous work has identified the pathogenic mechanisms underlying specific disease-causing mutations of GALC, including protein misfolding and active site defects\textsuperscript{28,36}. Two mutations responsible for causing Krabbe disease, E215K and P302R, have been shown to traffic correctly in cells, identifying that they are correctly folded, and retain enzymatic activity\textsuperscript{36–39}. These two mutations lie on the GALC surface adjacent to the SapA interaction site (Supplementary Fig. 9). Although these residues do not form direct interactions with SapA residues, their proximity to the binding site and their mutation to bulky, charged sidechains suggests these mutations may alter the surface shape and surface charge of GALC, interfering with binding to SapA. Thus, an inability to bind their cognate saposin may represent a new pathologic mechanism for sphingolipidoses.

Our structure reveals how saposins and hydrolytic enzymes can interact and can be compared with the recent structures of acid sphingomyelinase (ASM), which possesses an intramolecular saposin-like domain\textsuperscript{40–42}. Although the enzymatic portion of ASM is not structurally similar to GALC, the saposin-like region adopts a similar conformation to SapA in the GALC–SapA complex, allowing a reliable overlay to be made based on this portion (Supplementary Fig. 10a). This comparison reveals that the catalytic domains are in similar positions relative to the saposin (or saposin-like domain) and that the enzyme active sites are adjacent to the saposin, although not directly overlaid (Supplementary Fig. 10b). However, there are significant differences between these structures and this may reflect the different mechanisms by which the saposin domains facilitate lipid processing. Specifically, SapA is thought to function as a ‘solubiliser’, an assertion strongly supported by both the GALC–SapA structure presented here and the SapA lipoprotein disc structure\textsuperscript{22}. ASM, on the other hand, is likely to function via a ‘lipase’ mechanism. Specifically, ASM has been shown to bind tightly to negatively charged membranes and the saposin domain is likely to play a critical role in orienting the active site towards the membrane\textsuperscript{11,43}. Dimerisation of the saposin-like domain would not be required in this ‘lipase’ model of ASM activity, and indeed we observe that steric hindrance would prevent formation of a SapA-like dimer by the ASM saposin-like domain. We therefore conclude that the mechanisms used by saposins to facilitate lipid presentation will influence the stoichiometry and topology of their association with their cognate hydrolases.
Methods

Protein expression and purification. His-tagged murine GALC (mGALC) was expressed using our stably transfected HEK 293 T cell line and purified from conditioned medium using nickel-affinity chromatography (Ni-NTA agarose resin) in phosphate-buffered saline pH 7.4\textsuperscript{28,31}. Untagged murine SapA (mSapA) was expressed in Escherichia coli Origami (DE3) cells and purified as follows. Cleared lysate was heat-treated (100 °C, 30 min), precipitated proteins were cleared by centrifugation (40,000 g, 30 min) and supernatant containing mSapA was dialysed overnight in the presence of 20 μg mL\textsuperscript{−1} DNase against anion exchange buffer (50 mM Tris pH 7.4, 25 mM NaCl). mSapA was further purified by anion exchange chromatography (HiTrap Q Sepharose column) followed by size-exclusion chromatography (HiLoad 6/60 Superdex 75 column) in 50 mM Tris pH 7.4, 150 mM NaCl\textsuperscript{28,31}. mSapA mutants were made by site-directed mutagenesis and purified exactly as for wild-type. Purified mGALC was concentrated to 5–15 mg mL\textsuperscript{−1} and stored in phosphate-buffered saline pH 7.4 at 4 °C. Purified mSapA was concentrated to 8.0 mg mL\textsuperscript{−1} and stored in 50 mM Tris pH 7.4, 150 mM NaCl at 4 °C.

Protein complex crystallisation. mGALC was mixed with magnetic Ni-NTA agarose beads under saturating conditions (30 μL beads, 75 μg GALC per experiment) and incubated with mixing (90 min, 4 °C). Loaded beads were transferred to a flat bottomed 96-well plate and washed twice in 200 μL reservoir solution supplemented with 20% (v/v) glycerol and 0.1% LDAO complex and purified mGALC were combined to give a final SapA:GALC molar ratio of 2:1 and incubated at room temperature for 15 min. This scaled and merged using AIMLESS the xia2 automated data processing pipeline\textsuperscript{44}. Diffraction data were indexed and integrated using DIALS and recorded at Diamond Light Source beamline I04-1 on a Pilatus 6 M detector. Data were processed and merged using SCALA and scaled and merged using AIMLESS the xia2 automated data processing pipeline\textsuperscript{44}. The structure was refined using autobuster\textsuperscript{49}, over-fitting of the data being minimised by the use of local structure similarity restraints\textsuperscript{50} to the high-resolution structure of mouse GALC (PDB ID 4CCE\textsuperscript{28}) and between the NCS-related GALC and SapA monomers. Model geometry was evaluated with MolProbity throughout the refinement process\textsuperscript{51}. Final refinement statistics are presented in Table 1. Structural figures were rendered using PyMOL (Schrodinger LLC).

Structure analysis. The analysis of buried surface area and interface interactions in the GALC–SapA structure was carried out using the ePISA service at the European Bioinformatics Institute, EBI\textsuperscript{35}. Hydrogen bonding representations were created using LIGPLOT + implementing DMIPL0T to generate schematic diagrams of protein–protein interactions\textsuperscript{32}. Structure-based alignments were carried out using SMX superposition implemented within COOT\textsuperscript{52}. Multiple sequence alignments were carried out using the MUSCLE service at the EBI\textsuperscript{35}. The figure of saposin sequence alignment and conservation was produced using ALINE\textsuperscript{53}. For the electrostatic potential calculations partial charges were assigned using PDB2PQR\textsuperscript{54}, which uses PROPKA\textsuperscript{55} to determine protein pKa values. Electrostatic surfaces were calculated using APBS\textsuperscript{58}.

Data availability. The atomic coordinates and structure factors for the GALC–SapA complex have been deposited in the Protein Data Bank under accession code 5NXB. Other data are available from the corresponding author upon reasonable request.

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