

## Supplemental File

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#### 1. Growth of Coriell LCL cell lines.

Epstein-Barr virus-immortalized lymphoblasts were obtained from the Coriell Biorepository (Camden, NJ) and cultured according to the information on the Coriell website (cells are seeded at at least 300,000/ml and split before they reach 1 million/mL). Cells in ~100 mL suspension in culture medium were pelleted by centrifugation and resuspended in 35 mL of Dulbecco's PBS (DPBS, Gibco 14190-144). The cells were pelleted, the supernatant was removed with a pipet, and the pellet was resuspended again in 35 mL of DPBS. Cells were pelleted, and the supernatant was removed with a pipet. The cells were resuspended in 7-10 mL of DPBS. The cells were pelleted, and the supernatant was removed with a pipette. The cells were resuspended in DPBS to give about 50 million/mL. Aliquots (0.2 mL) were transferred to polypropylene tubes (Eppendorf), and 0.8 mL of DPBS was added per tube. Tubes were transferred to a -80°C freezer.

#### 2. Isolation of CD3+ T-lymphocytes from venous blood.

These cells were isolated from ~0.5 mL of venous blood collected into standard K2EDTA blood collection tubes. T-cells were isolated using Whole Blood CD3 Microbeads, (Cat. 130-090-874) and Whole Blood Column kits (Cat. 130-093-545) together with a QuadroMACS magnet (all from Miltenyi Biotec) according to the manufacturer's instructions. After elution from the column, cells were pelleted by centrifugation at 300xg for 10 min at 4°C. The supernatant was removed by pipette and the pellet was resuspended in 5 mL of cold DPBS. After centrifugation and supernatant removal, cells were resuspended in 1 mL of cold DPBS. After centrifugation, 0.8 mL of supernatant was discarded with a pipette and another 0.1-0.2 mL of supernatant was transferred to a new polypropylene tube (Eppendorf) (saved to be used as the blank in the protein assay, see below). The cells were resuspended in the remaining ~0.1 mL of supernatant, and a small aliquot was submitted to cell counting using a haemocytometer after Trypan blue staining. Cell viability was typically >95%. The tube of cells was transferred to the -80°C freezer. FACS analysis on initial batches of pre-frozen cells using an anti-CD3 antibody (Miltenyi Biotec) showed that the T cells were ~85-90% pure.

#### 3. LC-MS/MS GALC assay with T-lymphocytes.

Materials. GALC substrate (GALC-S) (galactosyl-ceramide containing a heptanoyl fatty acyl chain) and internal standard (GALC-IS) (ceramide containing a pentadeuterated heptanoyl fatty acyl chain) were obtained from PerkinElmer. All LC solvents were

obtained from Fisher (Optima grade). Sodium taurocholate and sodium oleate were from Carbosynth (Berkshire, UK). Recombinant human GALC was from R&D Systems (Minneapolis, MN).

Assay Cocktail. The assay cocktail contained 150  $\mu$ M GALC-S, 5  $\mu$ M GALC-IS, 2.5 g/L sodium taurocholate, and 750  $\mu$ M sodium oleate in citrate phosphate buffer. The latter was made as follows. In a beaker we placed 25 mL of 0.4 M  $\text{Na}_2\text{HPO}_4$  in Milli-Q water (EMD Millipore). Citric acid (0.4 M in Milli-Q water) was added until the pH dropped to 4.20 (pH meter calibrated with pH 4 and 7 buffers). Milli-Q water was added to bring the total volume to 50 mL, which was then stored at 4°C. Stock solutions of GALC-S, GALC-IS, sodium taurocholate and sodium oleate in methanol were added to a glass tube, and the solvent was completely removed in a centrifugal concentrator under vacuum. The residue was dissolved in citrate phosphate buffer. Assay cocktail was made fresh before each use.

Assay. Into a polypropylene, deep-well, 96-well plate we added 30  $\mu$ L of assay cocktail and 10  $\mu$ L of cell lysate containing 2  $\mu$ g of cell protein (protein determined as described in Section 5 below). The plate was sealed with a Micro Mat (Sigma Z374938) and placed in an orbital shaker incubator (200 rpm, 37°C) for 16 h. Reactions were quenched by adding 0.2 mL of methanol and mixed by pipetting up and down ~20-times. The plate was centrifuged at 1000 x g for 5 min at room temperature, and 0.2 mL of supernatant was transferred to a polypropylene, 96-well autosampler plate. The plate was wrapped with aluminum foil and placed in the autosampler of the LC-MS/MS and cooled to 8°C.

LC-MS/MS. LC-MS/MS was carried out on a Waters Xevo TQ instrument operating in positive ion mode. The LC column was a Waters ACQUITY CSH C18 1.7  $\mu$ m, 2.1 x 50 mm (Cat. 186005296) equipped with a Waters guard column (Cat. 18605303). Mobile phase A was water with 0.1% formic acid, and mobile phase B was acetonitrile/methanol (1/1) with 0.1% formic acid. The solvent program was: 0-1.49 min, linear gradient from 50 to 100% B, then 1.49-1.50 min to 50% B, then hold from 1.50-2.0 min at 50% B for column re-equilibration. Ten  $\mu$ L of sample was injected per run.

#### MS/MS instrument settings.

Waters Xevo TQ ESI Settings:

Polarity	ES+
Capillary (kV)	3.50
Cone (V)	26.00
Extractor (V)	3.00
Source Temperature (°C)	150
Desolvation Temperature (°C)	500
Cone Gas Flow (L/Hr)	30
Desolvation Gas Flow (L/Hr)	1000
Collision Gas Flow (mL/Min)	0.15
LM 1 Resolution	2.9
HM 1 Resolution	15.0
LM 2 Resolution	2.8
HM 2 Resolution	14.7

Multiple Reaction Monitoring Settings:

Reaction	Dwell (s)	ConeV	Col.Energy	Compound
1: 412.38 > 264.27	0.106	16.0	20.0	GALC-P
2: 417.41 > 264.27	0.106	16.0	20.0	GALC-IS
3: 574.43 > 264.20	0.106	16.0	20.0	GALC-S

#### Autosampler.

The weak needle wash was water/acetonitrile (90/10) with 0.1% formic acid. The strong needle wash was acetonitrile with 0.1% formic acid. Injection was 10  $\mu$ L, full loop.

Monitoring of GALC-S was optional. There was a GALC-P peak where GALC-S eluted due to thermal breakage of GALC-S to GALC-P in the ESI source.

#### **4. BCA protein assay.**

The concentration of protein in cell lysates was measured with the BCA kit from ThermoFisher (Cat. 23252) using bovine serum albumin as a standard. A calibration curve was prepared using bovine serum albumin at 0, 62.5, 125, 250, and 500  $\mu$ g/mL. Assays were carried out with 9  $\mu$ L of bovine serum albumin standard of cell lysate per well. Volumes of kit components were as added as described in the manufacturer's instructions. Plates were read in a plate reader with a filter appropriate for measuring the absorbance at 570 nm.

#### **5. Calculation of the analytical range.**

The analytical range of the LC-MS/MS GALC assay was the enzyme-dependent assay response observed with the HIGH quality control sample (GM13793-HIGH) divided by the assay response from all enzyme-independent processes. The latter included the following contributions: 1) Assay response from any GALC-P present in GALC-S as an impurity; 2) Any assay response from the cell lysate unrelated to GALC-P; 3) Any GALC-P formed from GALC-S by non-enzymatic breakdown during the incubation period; 4) Any assay response due to GALC-P formed by one or more enzymes in the cell lysate other than GALC; 5) Any GALC-P assay response from the buffer.

When buffer alone was analyzed by LC-MS/MS, there was no discernable GALC-P MRM peak at the LC retention time of GALC-P, thus contribution 5 was 0. When GALC-S in buffer without cell lysate was incubated, the GALC-P assay response was 64-122 ion counts (Table 1, main text). When this sample was not incubated the GALC-P assay response was in the same range as the incubated sample, showing that contributions 1 and 3 were insignificant. When cell lysate was incubated in the absence of GALC-S, the GALC-P assay response was 8-10 ion counts (Table 1), showing that contribution 2 was not a factor. When GALC-S was incubated with a lysate of cells that lack GALC (GM06805-LOW) the GALC-P assay response was 880-1129 ion counts (contribution 4). Thus, the entire GALC-independent assay response was dominated by contribution 4 (one or more non-GALC enzymes in the cell lysate). When GALC-S was incubated with the GM13793-HIGH lysate, GALC-P ion counts were 143,518-154,801 ion counts (Table 1).

The analytical range was thus obtained as:

$$(148,719 - 1,013)/(1,013) = 149.$$

#### **6. Studies with inhibitors.**

Inhibitor structures are shown in Supplemental Figure 1. The compounds N1-AC and N1-C4 up to 250  $\mu$ M did not inhibit GALC activity with either cell line GM06805-LOW and GM13793-HIGH. AGF, N2-C9, and N2-C4 at 250  $\mu$ M inhibited GALC by 60-80% with little or no effect on the activity in GM06805-LOW cells. On the other hand, DGJ up to 250  $\mu$ M inhibited the activity in GM06805-LOW by ~53% with little to no effect on GALC in GM13793-HIGH. This differential inhibition supported that the small amount of conversion of GALC-S to GALC-P in GM06805-LOW cells was due to one or more enzymes other than GALC (such as GLB1). Inhibitors were not used in subsequent studies since the level of GALC activity in GM06805-LOW null cells was considered to be sufficiently close to zero. We also studied the effect of the buffer. In sodium acetate, the activity measured in GM06805-LOW cells was 5.2 % of the activity seen in GM13793-HIGH cells. By contrast, in citrate phosphate buffer GM06805-LOW cells gave 0.4% the activity seen with GM13793-HIGH cells. These results together with the inhibitor studies showed that the enzyme that gave measurable activity in GM06805-LOW cells was not GALC (possibly GLB1). Proof that it was GLB1 was not obtained since the interfering enzyme(s) contributed only a trace amount of activity on the GALC substrate, and thus little would be gained by establishing its identity. The result also shows that the buffer composition had a large effect on the substrate specificity of enzymes in the cell lysates capable of cleaving GALC-S.

## 7. Synthesis of [ $^3$ H]galactosylceramide.

Galactosylceramide (2.5 mg, C16:0 fatty acyl chain, Avanti Polar Lipids Cat. 860521P) was dissolved in 0.6 mL of tetrahydrofuran. Then, 0.6 mL of 10 mM potassium phosphate, pH 7.0 containing 150 Units of galactose oxidase (Worthington Cat. LS004522) was added. This mixture was stirred in a capped vial overnight at room temperature. Analysis of a 10  $\mu$ L aliquot by thin layer chromatography on a silica plate with 10% methanol in chloroform and staining with iodine showed almost complete conversion of starting material to the aldehyde. The latter was extracted by adding chloroform/methanol (4/1), and the organic solvent was evaporated to give a crude residue, which was used in the next step.

[ $^3$ H]NaBH<sub>4</sub> (American Radiolabeled Chemicals, 250 Ci/mole, 5 mCi) was dissolved in 0.27 mL of 10 mM aqueous NaOH. The aldehyde was dissolved in 0.22 mL of tetrahydrofuran. An aliquot of [ $^3$ H]NaBH<sub>4</sub> solution (16  $\mu$ L) was added, and the mixture was stirred in a capped vial overnight at room temperature. Unlabeled NaBH<sub>4</sub> (5  $\mu$ L of 100 mg/mL in 1 mM NaOH) was added and the mixture was stirred for 1 h. In a fume hood we added 1 M acetic acid in water until the mixture was acidified (pH paper). Then 1.5 mL of chloroform/methanol (2/1) was added and the mixture was stirred briefly on a vortex mixture. After the solvent layers separated, the lower phase was transferred to a new tube and the remaining aqueous layer was extracted again as above. The solvent was removed from the combined extracts and the residue was dissolved in a small volume of 1% methanol in chloroform and applied to a silica column (1 x 1.5 cm). The column was washed with 4-5 mL of 4% methanol in chloroform, and then the product was eluted with 6% methanol in chloroform. Product-containing fractions were identified by silica thin layer chromatography as above. The specific radioactivity was estimated to be ~5 Ci/mole assuming 100% conversion of starting lipid to product.

## 8. Radiometric assay of GALC.

Leukocytes were human peripheral blood mononuclear cells obtained from StemCell, Technologies (Vancouver, BC, Cat. 70025). Cells (1.5 million) were homogenized in Milli-Q water on ice using a small Teflon tissue grinder. The concentration

of protein in the lysate was determined by the modified Lowry assay using bovine serum albumin as a standard. The lysate was diluted with Milli-Q water to give 1 mg/mL protein and used immediately in the GALC assay described below.

Stock solutions of sodium taurocholate in methanol, sodium oleate in methanol, unlabeled galactosylceramide in methanol/chloroform and [<sup>3</sup>H]galactosylceramide (~5 Ci/mole) in chloroform/methanol were mixed in a glass tube, and solvent was completely removed in a centrifugal concentrator under vacuum. To the residue was added 1 mL of citrate phosphate buffer (0.1 M in phosphate), pH 4.2, and the solution was mixed on vortex mixer for ~30 s. The solution contained 150 nmole of [<sup>3</sup>H]galactosylceramide (1000 dpm per nmole), 1 mg of sodium taurocholate, and 0.1 mg of sodium oleate.

Aliquots of the above assay cocktail (0.2 mL) were distributed into 4 glass tubes. To tubes 1 and 2 we added 0.05 mL of Milli-Q water (control), and to tubes 3 and 4 we added 0.05 mL of leukocyte lysate (50 µg of protein) in water. All tubes were sealed with Parafilm, and placed in a shaker/incubator at 37°C for 3 hr. Tubes were placed on ice, and 0.1 mL of D-galactose in water (0.5 mg/mL) was added followed by 1.5 mL of chloroform/methanol (2/1). The tubes were mixed on a vortexer and then centrifuged at 1000 x g to allow separation of the solvent layers (i.e., to make sure the layers were clarified). A 0.5 mL aliquot of the water layer (upper phase) was mixed with scintillation fluid, and the sample was submitted to scintillation counting (20 min per sample, counted 4 times). Data is shown in the Table below.

Sample	dpm	mean dpm	GALC activity (nmole/hr/mg)
Control 1	47, 50, 47, 50	48.5	
Control 2	49, 50, 53, 55	51.8	
Cell lysate 1	406, 410, 398, 410	406	2.3
Cell lysate 2	439,428,429,436	433	2.5

## 9. Measurement of psychosine in dried blood spots.

At least 2 mg of d5-psychosine (Avanti Polar Lipids) was weighed into a glass vial on a 0.01 mg analytical balance, and HPLC-grade methanol was added to give a 1 mg/mL solution. The concentration of d5-psychosine was determined by quantitative <sup>1</sup>H-NMR in CD<sub>3</sub>OD using N,N-dimethylformamide as an internal standard. A 10 nM stock of this internal standard in methanol was prepared by serial dilution (Hamilton glass syringes). The stock solution was stored in a glass vial with a Teflon-septum screw cap at -80°C.

We added 250 µL of 10 nM d5-psychosine in methanol to a 3 mm DBS punch in a 96-well, deep-well, polypropylene plate. The plate was sealed with sealing film (Sigma Micro Mat, Cat. Z374938), and shaken at 200 rpm in an orbital shaker at 37°C for 2 h. The plate was centrifuged for 5 min at 1000 x g at room temperature. Without disturbing the punch, 200 µL of supernatant was transferred to a new 96-well polypropylene plate, and the solvent was evaporated with a jet of nitrogen at room temperature. For convenience, the plate could be stored at -20°C prior to LC-MSMS. Just prior to LC-MSMS, 100 µL of LC mobile phase A was added to each well, and the well contents mixed by pipetting up and down at least ten times with a Pipetman.

LC-MS/MS was carried out on a Waters Acquity UPLC system with a Waters UPLC column (UPLC BEH amide, 1.7  $\mu$ m, 2.1 mm x 50 mm, part no. 186004800) and a guard column (UPLC BEH amide VanGuard 1.7  $\mu$ m, 2.1 mm x 5 mm, part no. 186004799). The flow rate was 0.3 mL/min, and the column temperature was 40°C. Elution started with 99.5% mobile phase A (0.1% ammonium hydroxide, 2 mM ammonium acetate in 95% acetonitrile/5% water, ammonium hydroxide and ammonium acetate based on total volume of acetonitrile + water, 0.1% ammonium hydroxide is 0.5 mL of concentrated aqueous ammonium hydroxide per 500 mL acetonitrile/water) and 0.5% of mobile phase B (0.1% ammonium hydroxide, 2 mM ammonium acetate in water) to 95% mobile phase A over 4.5 min (linear gradient). This was followed by a linear gradient to 80% mobile phase A from 4.5 min to 5 min. From 5 to 6 min the solvent was held at 80% mobile phase A. At 6.01 min the solvent was switched back to initial conditions (99.5% mobile phase A). Acetonitrile and water were from Fisher, Optima Grade. When making the mobile phase A, the ammonium acetate was completely dissolved in water before adding acetonitrile. When stored at 4°C for up to two weeks in tightly capped bottles, there was very little shift in the psychosine UPLC retention time (for example 3.27 to 3.31 min). If the retention time changed, new batches of mobile phases were made.

MSMS was carried out by SRM in positive mode (Waters Xevo TQS) with Mass Lynx software (Version 4.1). ESI source, SRM, and autosampler parameters are given below. Typical chromatograms are in Supplemental Figure 3.

Dried blood spots spiked with various amounts of psychosine were prepared and analyzed by LC-MS/MS as quality control standards. Non-deuterated psychosine (Matreya, Ca. 1305) stock solutions (10 and 100 nM in methanol) were prepared as for d5-psychosine stock solutions (see above). To a glass vial was added 0.25 mL of 10 nM or 100 nM psychosine in methanol, and solvent was removed in a vacuum centrifuge (Speed-Vac). To the residue was added 0.25 mL of fresh blood drawn from a healthy adult into a K2EDTA blood collection tube. The vial was capped (Teflon-rubber septum screw cap) and mixed on a vortex mixer for 3 min at room temperature. Blood was mixed up and down a few times with a Pipettor just before pipetting 50  $\mu$ L onto filter paper on newborn screening cards (Whatmann 903 paper). The filter papers were allowed to dry for 4 h at room temperature. Dried blood spots were stored in a container with desiccant at -20°C. LC-MS/MS studies with these spiked dried blood spots and with a solution of psychosine in methanol (not added to a dried blood) were used to show that the extraction yield of psychosine from the dried blood spot was ~70% using the procedure with methanol and d5-psychosine given above. Some dried blood spots were spiked with glucosyl-psychosine (Avanti Polar Lipids) to establish the identity of this analyte in LC-MS/MS runs (Supplemental Figure 3).

### Waters TQS micro MS/MS Settings

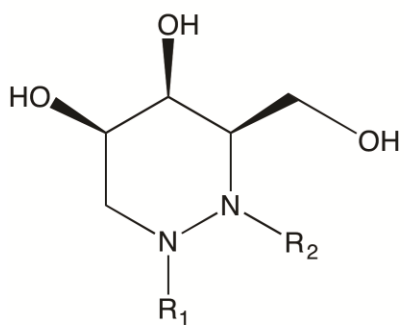
Parameter (units)	XEVO-TQS
Capillary (kV)	3.5
Cone (V)	20
Source Temperature (°C)	150
Desolvation Temperature (°C)	600
Cone Gas Flow (L/Hr)	50
Desolvation Gas Flow (L/hour)	1200
Collision Gas Flow (mL/Min)	ON
LM 1 Resolution	12
HM 1 Resolution	15
Ion Energy 1	-0.3
MS Mode Collision Energy	21
MSMS Mode Collision Energy	21
MS Mode Entrance	1
MS Mode Exit	1
LM 2 Resolution	12.6
HM 2 Resolution	14.9
Gain	1
Collision Gas	Argon

Analyte	dwel (s)	SRM transition(m/z)	Cone Voltage	Collision evergy(eV)
Psychosine	0.163	462.30 > 282.20	20	21
d5-Psychosine	0.163	467.35 > 287.35	20	21

#### Autosampler:

The strong needle wash was isopropanol/methanol/water (47.5/47.5/5). The weak needle wash was 95% acetonitrile/5% water. Extension loop size was 10 µL, full loop injected, overfill factor 1.5 times. The autosampler temperature was 15°C.

### 10. Supplemental Figure 1. Structures of the glycohydrolase inhibitors.



AGF,  $R_1 = R_2 = H$

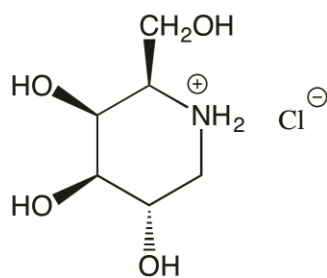
N1-Ac,  $R_1 = Ac, R_2 = H$

N1-C4,  $R_1 = (CH_2)_3CH_3, R_2 = H$

N1-C9,  $R_1 = (CH_2)_8CH_3, R_2 = H$

N2-C4,  $R_1 = H, R_2 = (CH_2)_3CH_3$

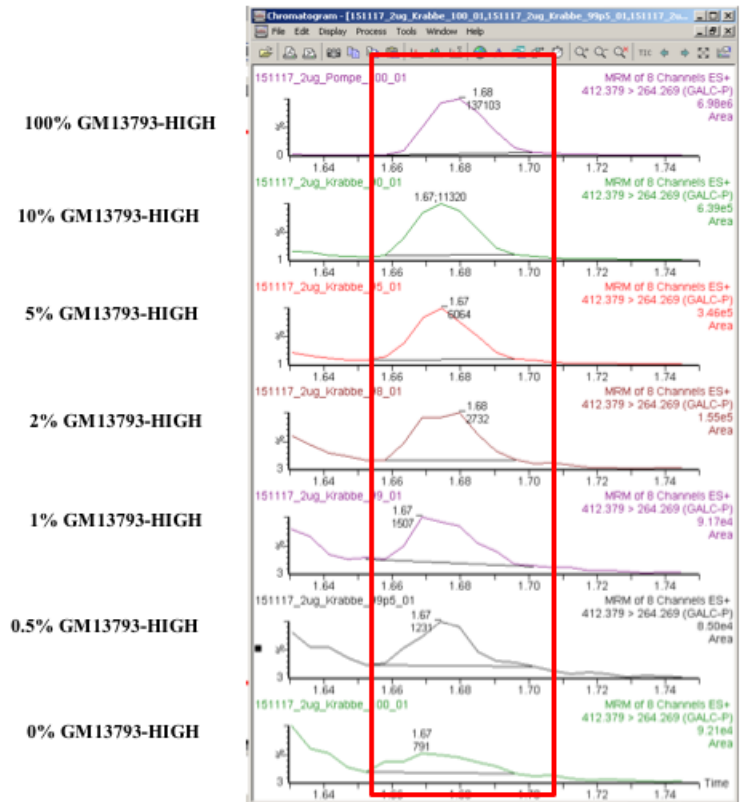
N2-C9,  $R_1 = H, R_2 = (CH_2)_8CH_3$



DGJ



11. Supplemental Figure 2. GALC-P ion chromatograms for studies with mixtures of GM13793-HIGH and GM06805-low cells.



## 12. Supplemental Figure 3. Psychosine ion chromatograms.

The peak in the red box is the psychosine peak. Glucosyl-sphingosine is the peak at 3.16 min. The identity of the peak at 3.82 min is not known and not always seen. DBS-Psy-glucosyl is a adult blood DBS spiked with 300 fmole of psychosine and 300 fmole of glucosyl-sphingosine. DBS-300fmole and DBS-30fmole are an adult DBS spiked with 300 and 30 fmole psychosine, respectively. DBS-normal is a DBS from a healthy newborn. Blank is filter paper only. DBS from patient with various onsets of Krabbe disease are also shown.

