Appendix: Glycosphingolipid Metabolic Reprogramming Drives Neural Differentiation

Contains:

Appendix methods

Cell lines and lentiviral infections

HeLa cells were obtained from the American Tissue Type Collection (ATTC, USA); E14 mESCs were provided by the laboratory of Maurizio D'Esposito, (IGB-CNR, Naples, Italy). HeLa cells were grown in Dulbecco's modified Eagle's medium (Gibco) supplemented with 4.5 g/L glucose, 2 mM L-glutamine, 100 U/mL penicillin and streptomycin, and 10% (v/v) foetal calf serum (FCS). mESCs were cultured feeder-free either in gelatin-coated culture vessels or on poly-D-lysine/ laminin-coated glass slides. Standard gelatin coating was performed using a filter sterilised 0.1% (w/v) gelatin solution (Sigma-Aldrich, Germany) in sterile phosphate-buffered saline (PBS) for at least 30 min at room temperature. Glass slides were coated by incubation with 0.01 mg/mL poly-D-lysine (Sigma-Aldrich, USA) for 30 min at 37 °C, followed by two brief washes in PBS. Subsequently, the slides were incubated for 16 h in 2 µg/mL laminin (Sigma-Aldrich, Germany) in PBS at room temperature. Undifferentiated stem cells were maintained in expansion medium consisting of Glasgow minimal essential medium (Sigma-Aldrich, Germany) supplemented with 2 mM glutamine (Life Technologies,

Germany), 1 mM sodium pyruvate (Life Technologies, Germany), 100 µM non-essential amino acids (Life Technologies, Germany), 10% (v/v) FBS (Euroclone, Italy), 0.05 mM 2-mercaptoethanol (Sigma-Aldrich, USA), 100 U/mL penicillin/streptomycin (Life Technologies, Germany), and 1,000 U/mL leukaemia inhibitory factor (LIF; Millipore, USA). The expansion medium was replaced every 24 h, and the cells were split every second day. For neural differentiation according to (Fico et al., 2008), 10^3 cells/cm^2 were seeded on either gelatin or poly-D-lysine/ laminin-coated glass slides (see above) and maintained for 24 h in expansion medium. Differentiation was induced by LIF deprivation in LIF-free knock-out Dulbecco's minimal essential medium (Life Technologies, USA) supplemented with 15% knock-out serum replacement (Life Technologies, USA), 2 mM glutamine (Life Technologies, USA), 100 U/mL penicillin/streptomycin, and 0.1 mM 2-mercaptoethanol (Sigma-Aldrich, USA). During the differentiation, the medium was replaced every day, and the cells were fixed for immunostaining or processed for biochemical procedures on days 0 and 13, or processed for RNA extraction on days 0, 8, and 13 (day 0, undifferentiated cells before LIF deprivation). All of the cells were maintained in a humidified incubator in 5% CO2 and 95% air at 37 °C.

Whole brains from C57BL/6 mice for chromatin immunoprecipitation experiments were provided by the laboratory of Maurizio D'Esposito, (IGB-CNR, Naples, Italy).

Two different shRNA lentiviral vectors (OriGene, USA) were used to silence AUTS2 in E14 cells (E14 shAUTS2), with scrambled sh lentiviral vector was used as control. All of the cells were maintained in culture for no longer than 3 months after thawing. Appendix Table S1 lists the sequences of the shRNA constructs used in this study.

Constructs and plasmids

The promoterless and enhancerless pGL3-Basic (Cat. N° E1751) plasmid expressing the *firefly* luciferase and the pRL-CMV plasmid (Cat. N° E2261) expressing the *Renilla* luciferase were purchased from Promega (USA). To generate human GM3S promoter-luciferase reporter constructs, seven fragments were amplified by PCR from genomic DNA purified from HeLa cells (Zeng et al., 1998). For sequential deletion analysis of the GM3S promoter a series of forward primers (Appendix Table S2) were used with the common GM3SBglIIRev reverse primer to generate the various fragments analysed. The PCR-generated promoter fragments were digested with the SacI and BglII restriction enzymes (New England Biolabs, USA) and cloned into the respective sites of the pGL3-basic plasmid. All of the GM3S promoter-luciferase constructs were sequenced before use in transfection experiments. The pCMV6-AUTS2-DDK-Myc plasmid, that expressed the human AUTS2 tagged protein (NM_015570), was purchased from OriGene (Cat. N° RC218943; USA).

Immunofluorescence

HeLa or mESC cells were grown on 24-mm coverslips. At the time of observation, the cells were washed with PBS, fixed in 4% paraformaldehyde for 10 min, and quenched with 50 mM NH_4Cl in PBS. The cells were permeabilised for 10 min with 0.3% Triton X-100 in PBS and blocked in PBS containing 5% FCS and 0.05% Triton X-100 for 1 h. The cells were then incubated with primary antibodies (Appendix Table S3) for 1 h at room temperature and washed three times with 0.1% Triton X-100 in PBS. The appropriate isotype-matched, AlexaFluor-conjugated secondary antibodies (Invitrogen, USA) were used, and coverslips were mounted with Mowiol (Calbiochem, Germany).

G-STED nanoscopy

HeLa cells transiently transfected with pCMV6-AUTS2-DDK-Myc plasmid for 24 h were fixed in 4% paraformaldehyde for 10 min, and quenched with 50 mM NH4Cl

in PBS. The cells were permeabilised for 10 min with 0.3% Triton X-100 in PBS and blocked in PBS containing 5% FCS and 0.05% Triton X-100 for 1 h. Cells were then incubated with anti-FLAG primary antibody (Appendix Table S3) for 1 h at room temperature and washed three times with 0.1% Triton X-100 in PBS. The AlexaFluor-488 conjugated anti-mouse secondary antibody (Invitrogen, USA) was used, and coverslips were mounted with ProLong agent (Thermo Fisher).

For STED, cells were imaged with a 100x, NA 1.4 oil objective on a Leica SP5 gated STED microscope. Alexa Fluor 488 labelled probes were excited with the 488 nm wavelength of a pulsed white light (WL) laser (80 MHz) and depleted with a CW 592 nm STED laser. All images were acquired in 2D STED mode, i.e. with only lateral resolution improvement.

Deconvolution of STED data was carried out using the STED module in Huygens Professional Deconvolution software (version 14.10; Scientific Volume Imaging). This software contains a theoretical estimation of the STED PSF that is based on values that are calculated from the metadata of the acquired image.

Electron microscopy

HeLa cells transiently transfected with pCMV6-AUTS2-DKK-Myc for 24 h were fixed with 4% paraformaldehyde and 0.05% gluteraldehyde for 30 min at room temperature (Mironov et al., 2001). The cells were permeabilised for 10 min with 0.3% Triton X-100 in PBS and blocked in PBS containing 5% FCS and 0.05% Triton X-100 for 1 h. Cells were than incubated with anti-Myc or anti-FLAG antibodies (Appendix Table S3) in blocking solution (PBS containing 1% BSA and 50 mM NH_4Cl) at 4°C for overnight, followed by incubation with the specific secondary antibodies labeled with nanogold in the same solution for 2 h at room temperature. Transfected cells were labeled using the nanogold enhancer (Nanoprobes) according to the manufacturer's instructions. The samples were then epon embedded and ultra-thin sections (50-70 nm) were prepared. The

samples were then examined under electron microscope (Tecnai-12; FEI) and pictures acquired by a Veletta CCD digital camera.

Flow cytometric analysis

E14-mESCs in their undifferentiated state or induced to neurons were subjected to trypsin digestion and resuspended in PBS containing 2% FBS. For cell-surface GSL staining, resuspended cells were incubated with optimal concentrations of the primary antibodies and fluorescent-labeled secondary antibodies and analyzed by FACSCanto (BD Biosciences). Isotype control antibodies were used as a negative control. Viable cells were gated, and GSL expressions were further analyzed in the gated region. Antibodies used are described in Appendix Table S3.

Isolation of ShTxB and ChTxB double-positive cells

HeLa cells (2×10^7) in suspension were incubated with fluorescent ShTxB and ChTxB in culture medium for 30 min at 4 °C, and then washed twice with PBS and sorted using a flow cytometer (FACSAria) with the DIVA software (Becton Dickinson, USA). The double-positive cells isolated were kept in culture for five days and stained again with fluorescent ShTxB and ChTxB as described above.

siRNA treatments and transfections

The siRNAs for human FAPP2 (NM_001197026), GCS (NM_003358), B4GALT5/LCS (NM_004776), SIAT9/GM3S (AY152815.2), A4GALT/Gb3S (NM_017436), COL4A3BP/CERT (NM_001130105.1), SMS1 (NM_147156.3), AUTS2 (NM_015570.3), CTDSP2 (NM_005730.3), p300 (NM_001429.3), HDAC1 (NM_004964.2), HDAC2 (NM_001527.3), B3GALNT1/Gb4S (NM_033168.2), B3GALT5/Gb5S (NM_001278650.1), GBGT1/ForssmanS (NM_001282632.1) and the mouse ortholog form of GM3S, ST3GAL5/mGM3S

(NM_011375.3) (Appendix Table S1) were obtained from Dharmacon (USA). HeLa cells were plated at 30% confluence in 24-well plates on 24-mm coverslips, or in 12-well plates, and transfected with 120 pmol of siRNAs with Oligofectamine (Invitrogen, USA), according to the manufacturer instructions. At 72 h after the initial treatment with the siRNAs, the cells were processed for the different experiments. The silencing efficiencies were evaluated either by Western blot or by qPCR using specific primers (Appendix Table S4). For the silencing of murine GM3S in differentiating mESCs, three boosting of murine GM3S siRNA (120 pmol) were performed at day 3, 6 and 9 of the neuronal differentiation by using the RNAiMAX transfecting reagent (Invitrogen, USA) according the manufacturer instructions. For pCMV6-AUTS2-DKK-Myc transfection, HeLa cells were plated at 50% confluence in 24-well, 12-well or 6-well plates and transfected, for 24 h with 0.3 µg/well to 2 µg/well of plasmid, with LTX-Lipofectamine (Invitrogen, USA), according to manufacturer instructions. The 5′-flanking fragments of *hST3GalV* gene (encoding GM3S) in pGL3, pGL3-Basic and pRL-CMV were transfected into HeLa cells using TransIT-LT1 Transfection Reagent (Mirus Bio LLC, USA), according to the manufacturer instructions.

RNA extraction and real-time PCR

Total RNA was isolated from HeLa cells and mESCs using RNeasy Mini kits (Qiagen, Germany) according to the manufacturer instructions. The yield and the integrity of the RNA were determined using a spectrophotometer (NanoDrop 2000c; Thermo Scientific, USA), by TAE agarose gel electrophoresis, and with an Agilent 2100 Bioanalyser (Agilent Technologies, USA). RNA (1 µg) was reverse transcribed using QuantiTect Reverse Transcription kits (Qiagen, Germany) according to the manufacturer instructions and subjected to real-time qPCR with gene-specific primers (Appendix Table S4) in the presence of LightCycler® 480 SYBR Green I Master Mix (Roche, Switzerland) on a LightCycler® 480 II detection system (Roche, Switzerland). Relative abundance of mRNA was calculated by normalization to hypoxanthine

phosphoribosyltransferase, for the mouse cells, and the homologous hypoxanthine phosphoribosyltransferase -1 for human cells.

Luciferase assay

5′-flanking regions of the *hST3GalV* gene encoding GM3S, were cloned upstream of to the firefly luciferase reporter gene in pGL3 plasmid (pGL3-1600/+15, pGL3-847/+15, pGL3-575/+15, pGL3-432/+15, pGL3-324/+15, pGL3-177/+15, pGL3-83/+15), and were transfected (2 μg) into HeLa cells. pGL3-Basic (2 μg) was transfected as the negative control. To normalise for the efficiency of transfection, these cells were simultaneously co-transfected with 50 ng of pRL-CMV, constitutively expressing *Renilla* luciferase. To minimize the effects due to the different degrees of transfection the co-trasfected cells carrying the same construct were detached by trypsinization 16 h later, re-plated in multiple wells. Finally, the cells were transfected with siRNAs and/or treated with exogenous lipids. At 72 h after silencing, the cells were washed in PBS and lysed, 20 μl of cell extract was processed using the Dual-Luciferase Reporter assay system (Promega, USA), according to the manufacturer instructions. The emitted light was detected using a GloMax 96 Microplate Luminometer (Promega, USA), and the luciferase activity was quantified accordingly.

Immunoblotting

Proteins were detected using the primary antibodies listed in the Appendix Table S3. The signals were visualized using a goat anti-rabbit or goat anti-mouse IgG-HRP secondary antibody (Santa Cruz, USA) at a 1:10,000 dilution.

Chromatin immunoprecipitation

HeLa cells (5×10^6) transfected with the pCMV6-AUTS2-DKK-Myc plasmid or silenced for AUTS2 or Gb3S were fixed with 1% formaldehyde. Alternatively,

whole mouse brains or mESCs at day 0 or 13 of the neuronal differentiation were fixed as specified above. After cross-linking, crude nuclear extracts were isolated and subjected to sonication, which resulted in 200 bp to 500 bp DNA fragments. After immunoprecipitation with the antibodies listed in the Appendix Table S3, the immunocomplexes were purified by co-precipitation with protein A-Sepharose (GE Healthcare, UK). Species matched IgG were used as the negative control. The amount of recovered DNA was determined, and the quantification of chromatin-immunoprecipitated DNA fragments was performed using qRT-PCR with the primers listed in Appendix Table S5. The enrichment of the DNA was calculated in terms of % input = $2^{-\Delta Ct} \times 100$, where ΔCt (threshold cycle) was determined by $Ct_{IP\ sample} - Ct_{Input}$, and 100 refers to the input being 1% of the chromatin amount exposed to the immunoprecipitation.

Measurement of cellular sphingolipid content

HeLa cells were pulse-labelled in serum-free 2% fatty-acid-free BSA in Dulbecco's modified Eagle's medium, at a final concentration of 1 μCi/mL [3H]-sphingosine, for 2 h. After 24 h chase in complete medium cells were harvested and processed for lipid extraction. Lipids were spotted onto silica-gel high performance-TLC (HPTLC) plates (Merck, Germany) (D'Angelo et al., 2007), and resolved with a mixture of chloroform: methanol: water (65:25:4 v/v/v). To visualise the unlabelled standards (i.e., Cer, GlcCer, LacCer, Gb3, SM and GM3) the TLC plates were placed in a sealed tank saturated with iodine vapour, while the radiolabelled lipids were analysed using a RITA® TLC Analyser (Raytest, Germany), and quantified using GINA® (Raytest, Germany) software analysis.

Drugs and exogenous glycosphingolipid treatments

For SAHA treatment, HeLa cells were treated with 5 μM SAHA (Sigma-Aldrich, USA) for 16 h in complete medium. For treatment with inhibitors of GSL synthesis, E14-mESCs were treated with NB-DNJ 25 μM, Fumonisin B1 25 μM,

PPMP 5 μM or Myriocin 2,5 μM for the whole duration of the differentiation. 10 or 25 μM exogenous GSLs (Matreya LLC, USA) were dissolved in methanol, and incubated for 16 h in complete medium for HeLa cells, or for the whole duration of the differentiation in the case of E14-mESCs.

Appendix Tables:

Human gene	Accession number	siRNA sequence
FAPP2	NM_001197026	• GAGAUAGACUGCAGCAUAUUU • GAAUUGAUGUGGGAACUUUUU • GAAAUCAACCUGUAAUACUUU • CCUAAGAAAUCCAACAGAAUU
CERT	NM_001130105	• GAAGAUGACUUUCCUACAAUU • GAAGUUGGCUGAAAUGGAAUU • GCGAGAGUAUCCUAAAUUUUU • UCAAAGGGAUAAAGUGGUAUU
GCS	NM_003358	• GAUAUGAAGUUGCAAAGUAUU • GCGAAUCCAUGACAAUAUAUU • GGACCAAACUACGAAUUAAUU • GAUGCUAGAUUGUUUAUAGUU
LCS	NM_004776	• GUGAAAUUGGAAUGGAUUAUU • GCUUAACAGUGGAACAAUUUU • GGAAAGUGAUCGCAACUAUUU
GM3S	NM_003896	• CAAUGGCGCUGUUAUUUGAUU • GACCAUGCAUAAUGUGACAUU • CGGAAGUUCUCCAGUAAAGUU • AGGAAUACUGCACGGAUUAUU
Gb3S	NM_017436	• AGAAAGGGCAGCUCUAUAAUU • GGACACGGACUUCAUUGUUUU • UGAAAGGGCUUCCGGGUGGUU • GCACUCAUGUGGAAGUUCGUU
AUTS2	NM_015570	• UGACAGAGAUAGAGAUGUAUU • AGACUCAUCUGUUAGUAAAUU • GAAAGGCUCAGUGAUAGUUUU • CACAUAAGCUGGACUUUGGUU
CTDSP2	NM_005730	• GAAAGACCCUCAUCCUGGAUU • CAACAAUGCUGACUUCAUAUU • CCACUGAGCUCGCUGCGUAUU • GGCCUUAUGUGGAUGAGUUUU
SMS1	NM_147156	• CUACACUCCCAGUACCUGGUU • CACACUAUGGCCAAUCAGCUU
HDAC1	NM_004964	• CGCCAAGUGUGUGGAAUUUUU • CGAAUCCGCAUGACUCAUAUU • CUCAUAAUUUGCUGCUCAAUU
HDAC2	NM_001527	• CAAAUACUAUGCUGUCAAUUU

		• CUCAUUAUCUGGUGAUAGAUU • CAGUGAUGAGUAUAUCAAAUU
p300	NM_001429	• GGACTACCCTATCAAGTAAUU
Gb4S	NM_033168.2	• GAUAUGAGGUUCUUACAUU • CAGGUUAUCCUCUAAUUGA • GUCGGGAUCUGUUUGAAUU • GUGCCAAGGAUCUAUGAAA
Gb5S	NM_001278650.1	• GCAAGUGGUUUGUCAGUAA • CGAGUCAGGUGUACAAUGU • GGACAGGUACCCACCAUUC • GAGCGUCCCAUACAUUAAA
ForssmanS	NM_001282632.1	• CCACUGAACCUGACCAUUG • GGGUGUAUCUUGAGAACUG • UAUGAGCGCAGGCGUGUUU • GUAUAUGAGUUUACUAGGG
Mouse gene	**Accession number**	**siRNA sequence**
GM3S	NM_011375.3	• CGGAAGUUCUCCAGUAAAGUU
Mouse gene	**Accession number**	**29mer shRNA sequence**
AUTS2	NM_177047	• shA-ACCTCAGCCGAAGAGGACATCATTGATGG • shB-AAGCGGAGGTCTCGATCACAGCGAGACCG
Scrambled-sh	—	• scr-sh-GCACTACCAGAGCTAACTCAGATAGTACT

Appendix Table S1: SiRNA and shRNA sequences used in this study to silence the expression of the indicated human and mouse genes.

GM3S promoter	Primer sequence
–1600GM3SsacIForward	ATCGAGCTCAACAGACCCAGACTTGAGAGAGTCC
–847GM3SsacIForward	ATCGAGCTCGCTCCCAATATCCTCACGTTAGAAC
–575GM3SsacIForward	ATCGAGCTCACTCAGAAGGAGATCACAGGCAC
–432GM3SsacIForward	ATCGAGCTCACGTTCAGCTGTGTCCAAGAAATAA
–324GM3SsacIForward	ATCGAGCTCCATTTCCTCTCCAGAACCAAGGG
–177GM3SsacIForward	ATCGAGCTCATCGTGCTCTCCGATTGGCCG
–83GM3SsacIForward	ATCGAGCTCAGCTGAATGGGCGCGAGACG
GM3SbglIIReverse	AAGAGATCTTGCTCCGCAAACTAATGAGGGGGC

Appendix Table S2: PCR primers used for the cloning of different the GM3S promoter fragments into the pGL3-basic plasmid.

	USAGE					
Antibody/Toxins	**FACS**	**IF**	**WB**	**ChIP**	**Source**	**Provider**
ChTxB-Alexa Fluor 488	—	1 : 1000	—	—	—	Invitrogen C22841
ShTxB-CY3	—	1 : 1000	—	—	—	Dr. Ludger Johannes lab
anti-FLAG	—	1 : 400	1 : 2000	5µg/1000µg nuclear lysate	mouse	Sigma F1804
anti-Ring1b	—	1 : 400	—	—	mouse	MBL D139-3
anti-AUTS2	—	1 : 200	1 : 400	—	rabbit	Sigma HPA000390
phospho(Ser10)-His-H3	—	1 : 200	—	—	rabbit	Millipore 09-797
anti-H3K27me3	—	1 : 400	—	2µg/100µg nuclear lysate	mouse	Abcam ab6002
anti-H3K4me3	—	1 : 400	—	2µg/100µg nuclear lysate	rabbit	Abcam ab8580
anti-H3K27Ac	—	1 : 500	—	2µg/100µg nuclear lysate	rabbit	Abcam ab4729
anti-H3K9/14Ac	—	—	—	2µg/100µg nuclear lysate	rabbit	Millipore 06-599
anti-H4Ac	—	—	—	2µg/100µg nuclear lysate	rabbit	Millipore 06-866
anti-H2AK119Ub	—	—	—	1µl/100µg nuclear lysate	rabbit	CST 8240
anti-Tuj-1	—	1 : 400	1 : 1000	—	mouse	Covance MMS-435P
anti-GFAP	—	1 : 300	—	—	rabbit	Dako Cytomation Z0334
anti-TH	—	1 : 200	—	—	rabbit	Millipore AB 152
anti-5-HT	—	1 : 200	—	—	rabbit	Sigma S-5545
anti-PCGF5	—	1 : 400	—	—	mouse	Abcam ab76724
anti-Nanog	—	—	1 : 400	—	rabbit	Millipore SC1000

anti-Oct4	—	—	1 : 400	—	mouse	Santa Cruz sc-5279
anti-β-Actin	—	—	1 : 10000	—	rabbit	Sigma A2066
anti-MeCP2	—	1 : 500	—	—	rabbit	Sigma M9317
anti-HP1α	—	1 : 1000	—	—	mouse	Euromedex 2HP-1H5 AS
Preimmune IgGs	—	—	—	2μg/100μg or 5μg/1000μg nuclear lysate	rabbit	Millipore 12-370
anti-Forssman antigen	1 : 50	—	—	—	rat	Santa Cruz sc-23939
anti-Gb4	1 : 100	—	—	—	rabbit	Matreya LLC 1960
Anti-GT1b	1 : 100	1 : 400	—	—	mouse	Millipore MAB5608

Appendix Table S3: List of the antibodies used in this study for flow cytometry (FACS), Immunofluorescence (IF), Western Blotting (WB), and chromatin immunoprecipitation (ChIP).

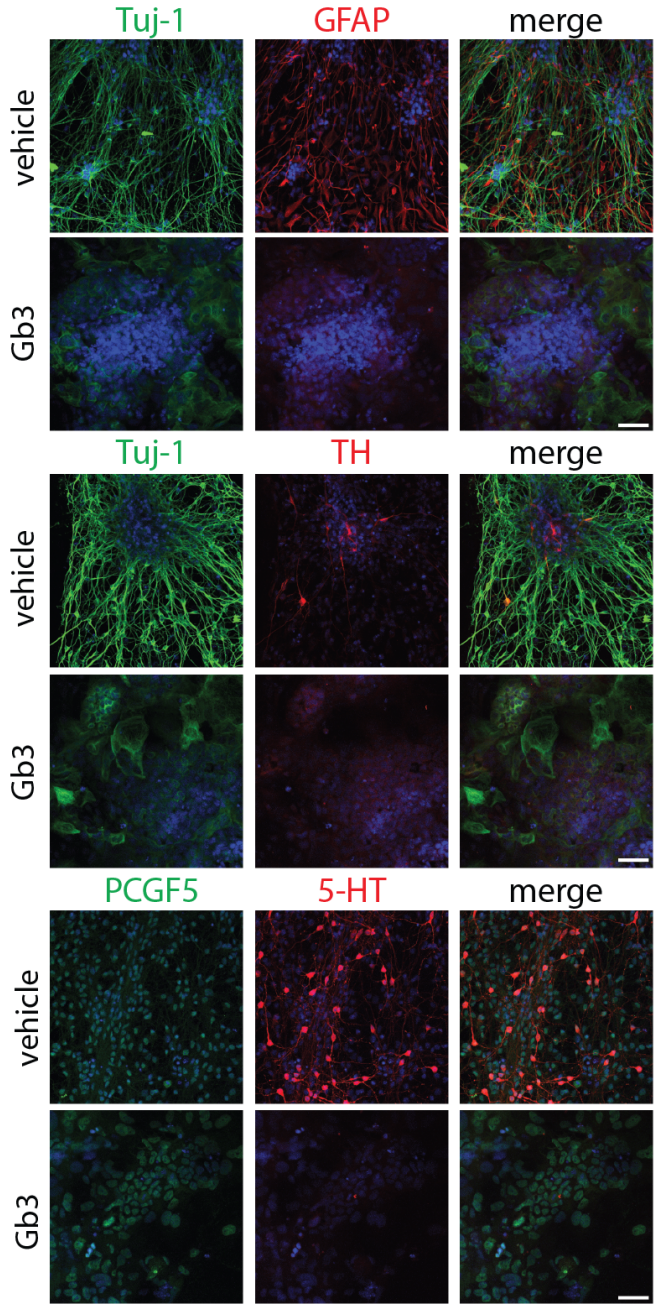
Human gene	Primer Forward	Primer Reverse
GCS	TTCGGGTTCGTCCTCTTC	GCTTGCTATAAGGCTGTTTGTC
LCS	CAATCGGTGCTCAGGTTTATG	GGTTTCACTGTGGTTCAAGTC
Gb3S	GATCCCCACCTCTCTGCAAT	TTGGACATGGTATCCCCAGA
GM3S	TGGTTATTGGAAGCGGAGG	TCTGAATATCCCTCAACTGGT
GM1S	CAACCTCACCCTAAAGACCC	AGGGACGTTGACATACACATC
FAPP2	ACATCAGGATCCGATTGAGA	ATGCACCTTCTGGATGTGTT
CERT	TGTGGATCATGACAGTGCTC	ATTTCCTGGTTTCCCTCTGG
SMS1	GCATTTCAACTGTTCTCCGAAG	GATAGACAAGCCACCTCCAG
AUTS2	TCAAGCCAGGACAGAACAG	GGAAGGAGCTGAACTATCACTG
CTDSP2	GGTGACAGAGGAAGATCAAGG	GGCACTATGAAGTCAGCATTG
HOXA5	GCAAGCTGCACATAAGTCAT	TCAGGTAACGGTTGAAGTGG
NLGN4X	ACCGAGCTCTTCAGTTGTAAC	TGAGGAACTGGTTGATTTGGA
SLC48A1	TTCTCCATCTTCCTCGTCTG	ATCTTGCATGTACATCACGTG
PGCP	TCTCATTCGATCCGTGGC	CATTTCTGCATCTTCCACCG
HDAC1	GCTCAACTATGGTCTCTACCG	AGTCATCGCTGTGGTACTTG
HDAC2	TGGCGTACAGTCAAGGAG	TGGGTCATGCGGATTCTATG
HPRT1	AGCTTGCTGGTGAAAAGGAC	GTCAAGGGCATATCCAACAAC
p300	CGAAGGACCAGACTACAGAAG	ACTGCCACGGATCATACTTG
LMO4	CACGTCCTGTTACACCAAAAG	AGTTCACTCGCAGGAATCG
ZEB1	GAGGATGACACAGGAAAGGA	GCATCTGACTCGCATTCATC
FBXW7	TGGAGAATTTTGGCTGAAGAC	TGGACTGTGTATGAAACCTGG
FAT4	TCAGCCAGTGGAGAACTTG	TGTTCCAGTTCCAGTCAAGG
ADAM17	TCAGCATTCTTGTCCATTGTG	GAACCGATGCAGAATCCATG
FLVCR1	TCACTTACCCTGAATCTGAAGG	CTGCCTTAGGACCATAGTCTG
Gb4S	TCCAGCCACCGTCAAATC	CCTTGATAGCTTTTCCCACAAC
Gb5S	ACAAACCAAGCCCAGAACC	CTCATCTTCGGGAAAGCCAT
ForssmanS	CAAGCCTCAGTGTCCTGTG	CTTCTCCCTCTTGTAGTGCAG
Mouse gene	**Primer Forward**	**Primer Reverse**
HPRT	GTAATGATCAGTCAACGGGGGAC	CAGCAAGCTTGCAACCTTAACCA
Gb3S	TCCTCAAGAACCTGCTCAAC	CACAGTGCCAAGAACTCATG
GM3S	TCAGAGCCTCAGTCAAGATTC	GATGTGTAGCCAAGACAACG
GM2S	TGGATAAACTCAACCGGCAG	GTGTCTTACGAGGATGGTGAAG
GM1S	CTGTGTCAAATTGGCTGGTG	CAGTCCTCTCCCCATTCATG
AUTS2	GCCATGACCAGCTTTGTTAC	TCTGTCGTTTCTCCGCAC
NURR1	GAGGGTCTGTGCGCTGTTTG	TGTCCGTGCGAACCACTTCT
Tuj-1	ATCAGCAAGGTGCGTGAGGAG	ATGGACAGGGTGGCGTTGTAG
Nestin	AGCAACTGGCACACCTCAAGA	CTCAGCCTCCAGCAGAGTCC
Sox2	AGGGCTGGACTGCGAACTG	TTTGCACCCCTCCCAATTC
Vimentin	CGGCTGCGAGAGAAATTGC	CCACTTTCCGTTCAAGGTCAAG
LC3S	TGAAGTCACATCTAGCATCCTG	TGGTACATCCAAATCCGTCG

Appendix Table S4: Real Time qPCR primers used in this study for mRNA levels assessment.

		Forward Primer	Reverse Primer
GM3 synthase promoter	–1568/–1339	CCCGATATCTGGAGAACAAAGG	GGGCATTCCTGAAAATAAGAGG
	–927/–847	AAATCGCCCAGTGACAGCTT	AAGCGTTGCAAAGGAAAAGG
	–443/–310	CGCACAGTATACGTTCAGCTGTGTCC	CCCCCTTGGTTCTGGAGAGGAAATG
	–179/+15	GAAAGTTGCAGGCGCACAGT	CCCCCTTGGTTCTGGAGAGG
Murine GM3S promoter	-1500	AGTTCCCAGGCGACCATGTT	TCCTAGACCCTGAGCCAAGAG
	TSS	CGCCCCACCTACTTCTCGG	GGAGACCAATCAGAGTGCGC
	MyoD1	CCGCCTGAGCAAAGTAAATGA	GGCAACCGCTGGTTTGG
	GAPDH	TCGACAGTCAGCCGCATCT	CTAGCCTCCCGGGTTTCTCT
	Murine MyoD	TCCGAGGTCAGCTCCGAAGT	GCCATACGCGGTAGCACTTG
	Murine GAPDH	TGAATGCTGCTTCCCGAGTA	CTCAACTTTTCCGCAGCCTT
	Murine Uchl1	TGTTTCTGCTCCCGTCTCCC	ACAAACCCGAGGAGCCGAAA
	AUTS2 binding site chr10:31608364;31608595	CCTGTCTAGAAGCAGATACGAAGA	GTGATTTTAATGATGGCTCGAA
	HoxA1	Cell Signaling Technologies Cat.N°. #7707	

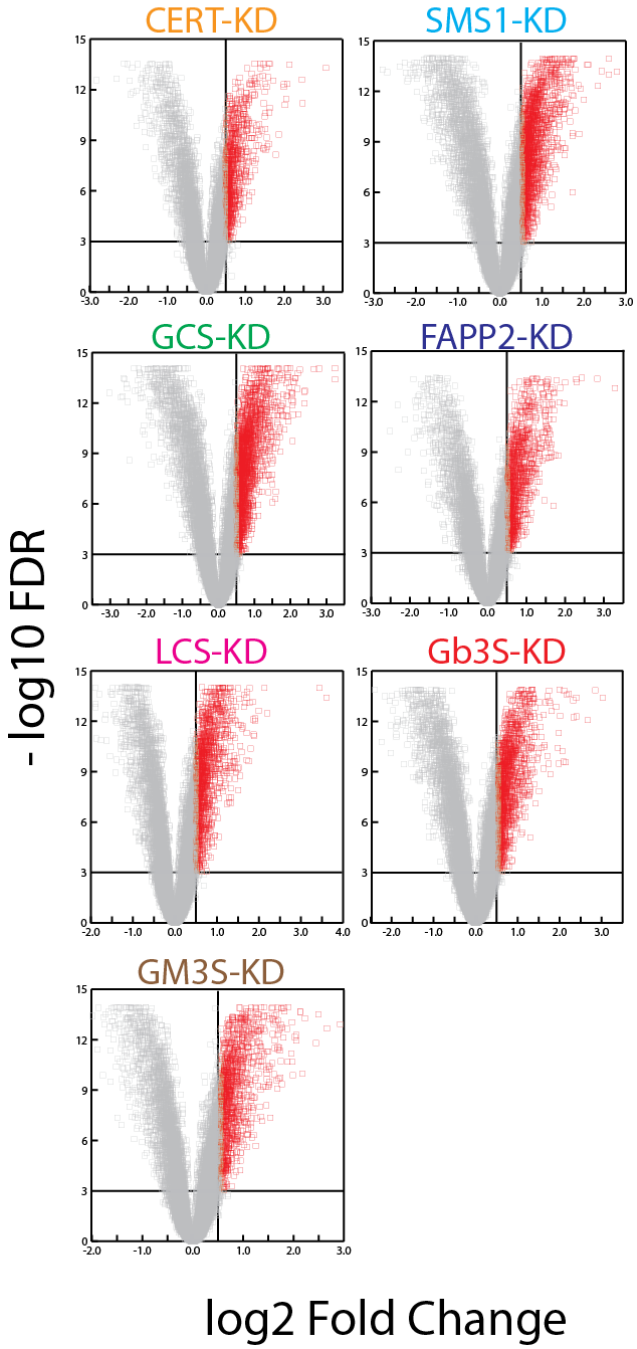
Appendix Table S5: Real Time qPCR primers used in this study for ChIP efficiencies assessment.

Appendix FigS1



Appendix FigS1. **Globosides inhibit neural differentiation**
E14-mESCs were induced to differentiate into neurons over 13 days in the presence of vehicle (methanol) or Gb3 (25 μM), fixed and analysed for the expression of specific neuronal populations markers using immunofluorescence. Tuj-1, neuron-specific class III b-tubulin; GFAP, glial fibrillary acidic protein; TH, tyrosine hydroxylase; PCGF5, polycomb group ring finger 5; 5-HT, 5-hydroxytryptamine. Bar, 100 μM.

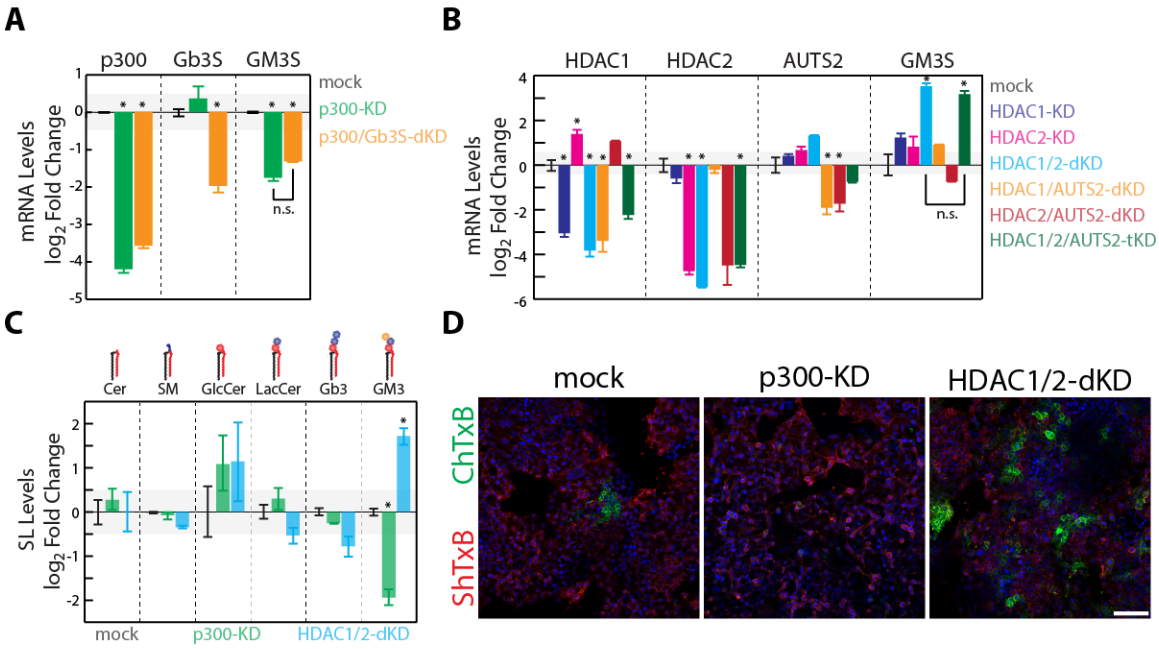
Appendix FigS2



Appendix FigS2. Transcriptome Analysis of Gb3-regulated Genes

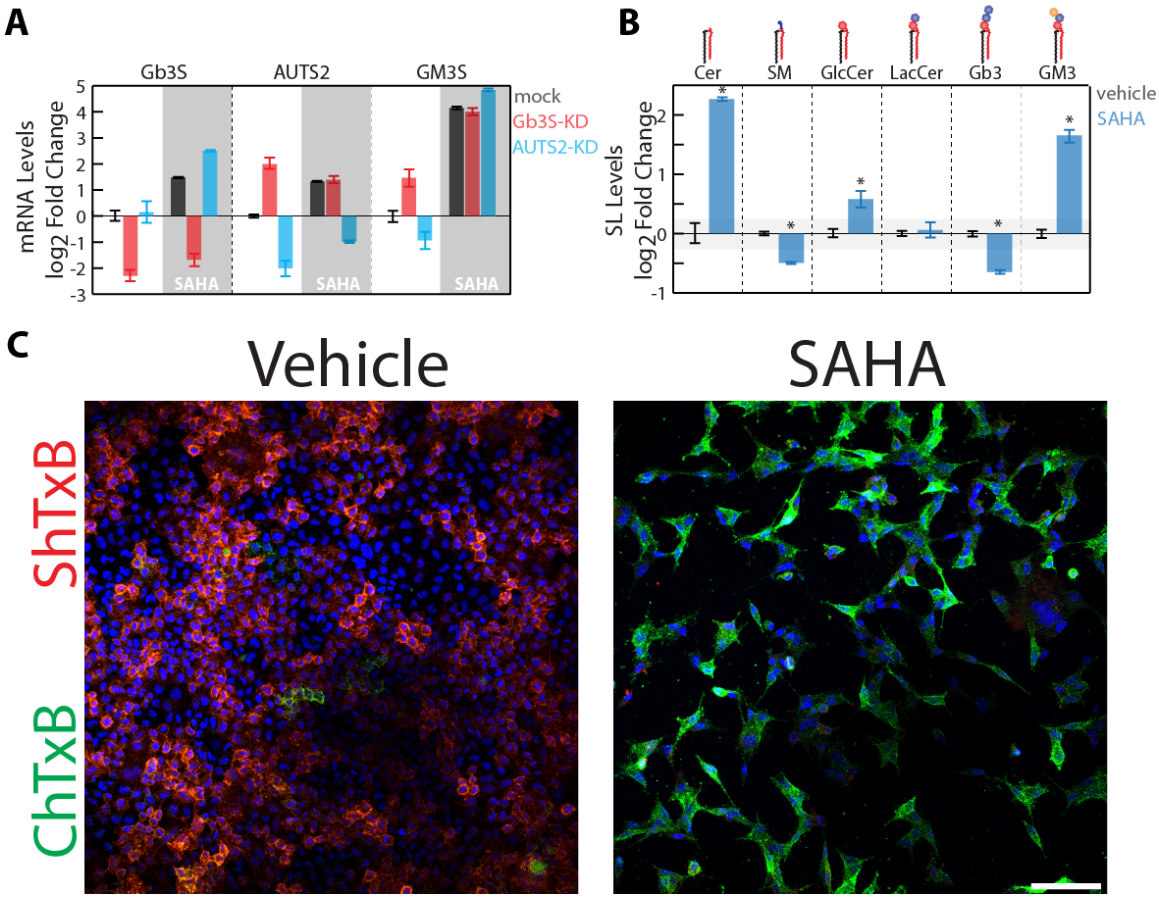
Volcano plots of data obtained from microarray analysis of HeLa cells either mock treated or silenced for expression of the indicated genes (see **Dataset EV1** for complete microarray data; this dataset has been regesterd on GEO databse with the following accession number GSE107044). Thresholds of 0.001 ($-\log_{10} = 3$) for the false discovery rate, and 1.41 ($\log_2 = 0.5$) for fold change were selected to obtain significantly upregulated genes (red) under each condition.

Appendix FigS3



Appendix FigS3. **Histone Acetylation Controls GM3S Expression.**
HeLa cells control or Gb3S-KD were silenced for p300 **(A);** HeLa cells control or AUTS2-KD were silenced for HDAC1 and/or HDAC2 **(B)**. qPCR was performed to determine knock-down efficiencies and the p300, HDAC1, HDAC2, Gb3S, AUTS2 and GM3S mRNA changes. mRNA levels are expressed as $\log_2$ fold changes to mock-treated cells. Data are means ±SD of at least three independent experiments. *, p ≤0.05. **C)** Effects of p300, HDAC1/HDAC2 silencing on SL composition (expressed as $\log_2$ fold changes to mock-treated cells). Cells were pulse labelled as in **Fig 4A**. Data are means ±SEM of at least three independent experiments. *, p ≤0.05. **D)** Effect of p300 and HDAC1/HDAC2 silencing on ChTxB and ShTxB distribution in HeLa cell populations. Cells were fixed and stained with Alexa488-ChTxB (green), Cy3-ShTxB (red), and DAPI (blue), and imaged using confocal microscopy. Bar, 100 µm.

Appendix FigS4



Appendix FigS4: Chemical Modulation of Histone Acetylation Impacts on GM3S Expression.

A) Effects of HDAC inhibitor suberoylanilide hydroxamic acid (SAHA) on Gb3–AUTS2 axis dependent GM3S expression. HeLa cells silenced for Gb3S or AUTS2 were incubated with 5 μM SAHA for 16 h. qPCR was performed to determine both the knock-down efficiency and the Gb3S, AUTS2 and GM3S mRNA changes. mRNA levels are expressed as $\log_2$ fold changes to mock-treated cells. Data are means ±SD of at least three independent experiments. *, p ≤0.05. **B)** Effects of SAHA treatment on SL composition (expressed as $\log_2$ fold changes with respect to control cells). Control and treated HeLa cells were pulse labelled as in **Fig 4A**. Data are means ±SEM of at least three independent experiments. *, p ≤0.05. **C)** Effects of SAHA treatment on ChTxB and ShTxB distribution in HeLa cell populations. The cells were fixed and stained with Alexa488-ChTxB (green), Cy3-ShTxB (red), and DAPI (blue), and imaged using confocal microscopy. Bar, 100 μm.

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