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Supplemental Information

Altered Differentiation Potential of Gaucher's Disease iPSC Neuronal Progenitors due to Wnt/β-Catenin Downregulation

Ola Awad, Leelamma M. Panicker, Rania M. Deranieh, Manasa P. Srikanth, Robert A. Brown, Antanina Voit, Tejasvi Peesay, Tea Soon Park, Elias T. Zambidis, and Ricardo A. Feldman

SUPPLEMENTAL INFORMATION

SUPPLEMENTAL FIGURES AND TABLES

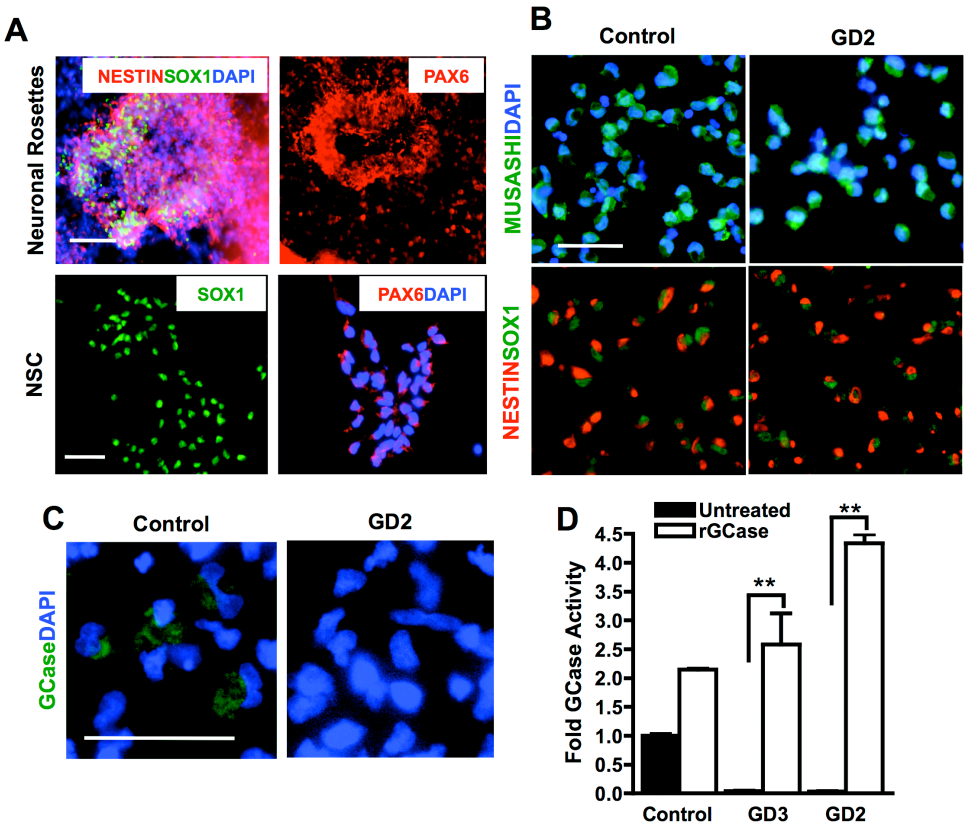

Figure S1. Characterization of iPSCs-derived neuronal progenitor cells (NPCs). A) Top panel, representative immunofluorescence images of control iPSC neuronal rosettes stained for neuronal stem cell markers SOX1, NESTIN and PAX6 using specific antibodies. Nuclei were labeled with DAPI (blue). Magnification, 10x; scale bar, 100 μm. Lower panel, immunofluorescence staining for SOX1 (green) and PAX6 (red) in NSCs, which were picked and expanded from neuronal rosettes. Magnification, 20x; scale bar, 50 μm. B) Representative immunofluorescence images of control and GD2 NPCs labeled with antibodies against MUSASHI (green), NESTIN (red) and SOX1 (green) as indicated. Nuclei were labeled with DAPI (blue). Magnification, 20x; scale bar, 50 μm. C) Immunofluorescence images of GCase expression (green) in control and GD2 NPCs. Nuclei were labeled with DAPI (blue). Magnification, 40x; scale bar, 50 μm. D) GCase enzyme activity assayed in protein lysates from control, GD2, and GD3 NPCs. Cells were either left untreated, or were treated with 0.24 U/ml rGCase for 5 days. Data represent fold-GCase activity relative to untreated control, as measured by fluorescence plate reader in duplicate wells (compiled data are repeats from two GD2 and two GD3 patients). Bars represent average $\pm$ SEM. **p<0.005 between indicated groups as assessed by One-way ANOVA.

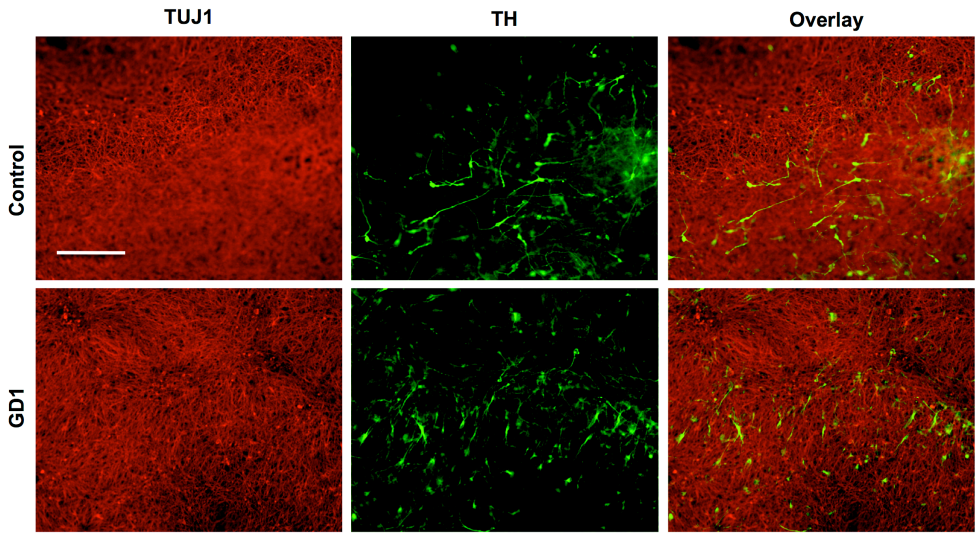

Figure S2. Dopaminergic differentiation of non-neuronopathic GD1 NPCs. Representative immunofluorescence images from control and GD1 dopaminergic differentiation cultures. Neurons were co-labeled with anti-TUJ1 (red) and anti-tyrosine hydroxylase (TH, green) antibodies. Last panel shows the overlay of both markers. Magnification 20x; scale bar, 100 μm.

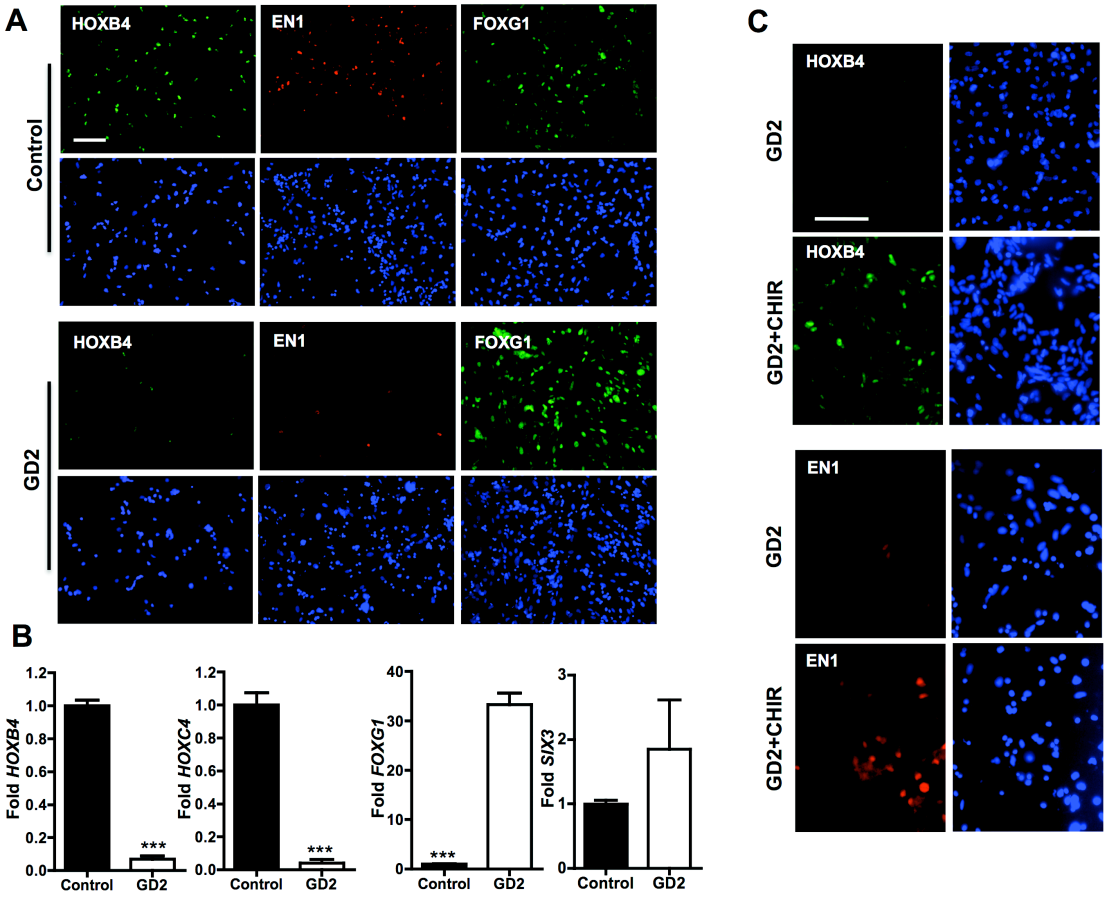

Figure S3. Analysis of anterior-posterior marker expression in control and GD2 NPCs. A) Representative immunofluorescence images from control (Top panel) and GD2 (lower panel) NPCs that were labeled with either anti-HOXB4, anti-EN1 or anti-FOXG1 antibodies as indicated. Also shown are the corresponding DAPI-labeled nuclei for each panel. Magnification 20x; scale bar, 100 μm. B) qRT-PCR analysis of the hindbrain/spinal cord markers *HOXB4* and *HOXC4*, and the forebrain markers *FOXG1* and *SIX3* in control and GD2 NPCs. Data represent fold-change relative to control. *p<0.0001 between control and GD2 as assessed by unpaired Student's t-test. Error bars ±SEM, n = 3 per group (compiled data from two GD2 patients). C) Representative immunofluorescence images from GD2 NPCs that were labeled with either anti-HOXB4 (top 4 panels) or anti-EN1 (lower 4 panels) antibodies. Before immunofluorescence staining, the cultures were either left untreated or were treated with 3μM CHIR for ten days as indicated. Also shown are the corresponding DAPI-labeled nuclei. Magnification 20x; scale bar, 100 μm.

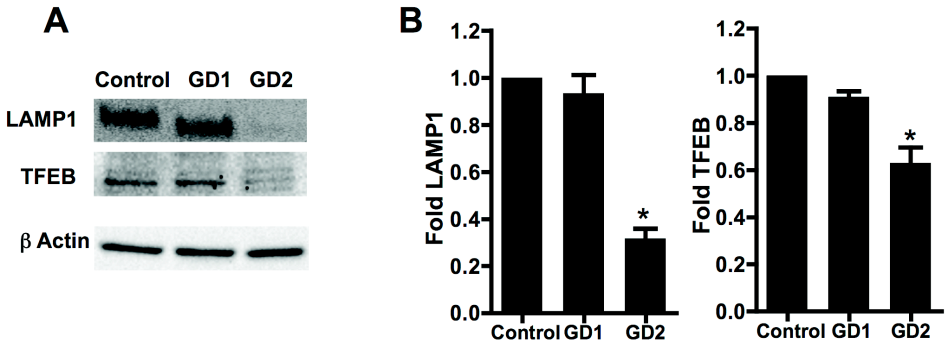

Figure S4. Lysosomal alterations in neuronopathic GD NPCs. A) Western blot showing LAMP1 and TFEB levels in control, GD1, and GD2 NPCs. β-actin was used as a loading control. B) Bar graphs represent quantitation of LAMP1 (left panel) and TFEB (right panel) levels in GD NPCs relative to control NPCs. Data represent average ±SEM, n = 3 per group (compiled data are from repeats from two GD2 and one GD1 patient). *$p<0.05$ between GD2 and both control and GD1 as assessed by One-way ANOVA.

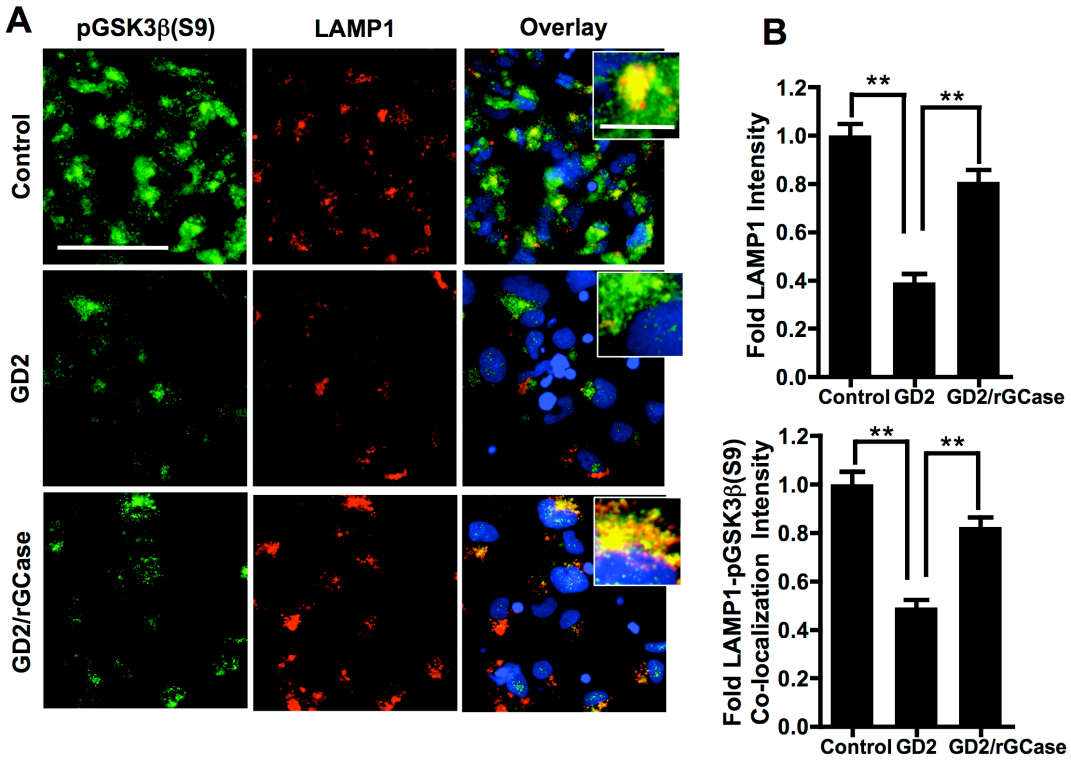

Figure S5. Decreased pGSK3 β (S9) association with the lysosomes in neuronopathic GD NPCs.
A) Representative immunofluorescence images for pGSK3β(S9) (green), LAMP1 (red), and DAPI (blue) staining in control and GD2 NPCs with and without rGCase treatment for 5 days. The overlay panels show co-localization of pGSK3β(S9) signal with LAMP1-labeled lysosomes. Magnification 40x; scale bar, 75 μm. Inset is an enlargement of an area in each overlay panel showing co-localization of pGSK3β(S9) with LAMP1 in control NPCs, and in untreated or rGCase-treated GD2 NPCs. Magnification 40x; scale bar, 25 μm. B) Top graph, quantification of LAMP1 fluorescence signal intensity in GD2 NPCs with and without rGCase treatment. Data presented as fold-signal intensity relative to control NPCs. Bottom graph, LAMP1/pGSK3β(S9) co-localization fluorescence signal intensity in GD2 NPCs with and without rGCase treatment. Data presented as fold-signal intensity relative to control NPCs. Average fluorescence intensity was measured in >100 cells/group assayed in at least 4 independent high-power fields. n = 3 per group (compiled data are repeats from one GD2 patient). Bars represent average $\pm$ SEM. **p<0.005 between GD2 and both control and GD2/rGCase as assessed by One-way ANOVA.

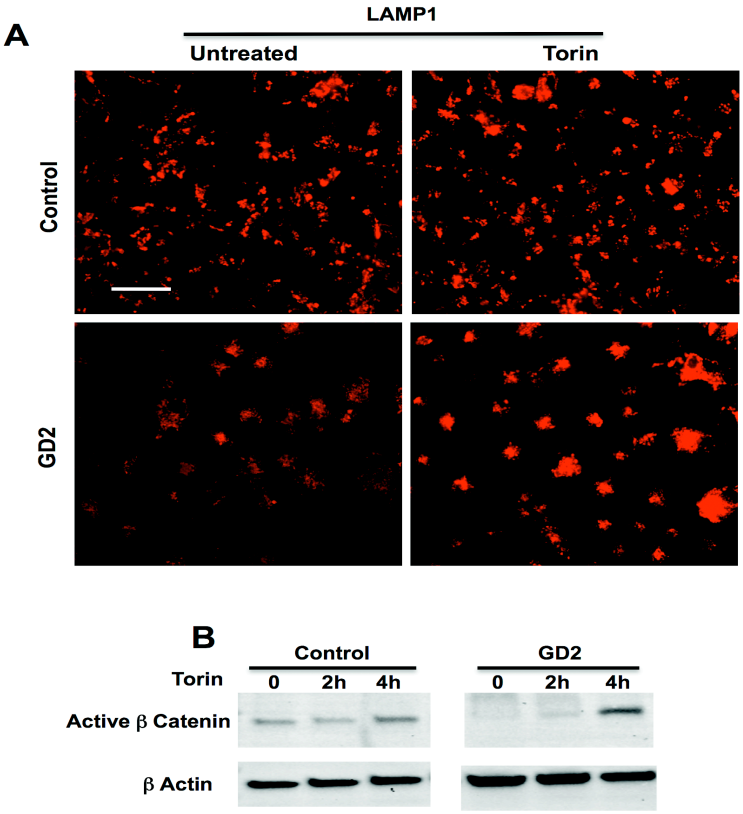

Figure S6. Pharmacological mTOR inhibition increases active β-catenin level in neuronopathic GD NPCs. A) Representative immunofluorescence images from control and GD2 NPCs that were labeled with anti-LAMP1 antibodies (red) to visualize the lysosomes. Cells were either left untreated or were treated with 1 μM Torin for 4 hours. Magnification 20x; scale bar, 100 μm. B) Western blot showing active β-catenin level in control and GD2 NPCs that were either left untreated, or were treated with 1 μM Torin for 2 and 4 hours as indicated. β-actin was used as a loading control.

Table S1. Primary antibodies used for Immunofluorescence staining.

Antibody	Working Dilution	Supplier/ Catalog Number
SOX1	1:200	Millipore AB15766
NESTIN	1:200	BD Transduction Laboratories 611658
PAX6	1:100	Millipore MAB5552
MUSASHI1	1:200	eBioscience, 14-9896-82
GBA	1:100	Sigma Aldrich WH0002629M1
TH	1:100	Novus Biologicals NB300-109
TH	1:100	Sigma Aldrich T2928
PITX3	1:200	Millipore AB5722
VMAT2	1:100	Millipore AB1598
LMX1	1:100	Millipore AB10533
FOX2A	1:100	Santa Cruz sc-6554
GIRK2	1:250	Millipore AB5200
MAP2	1:200	Millipore MAB3418
TUJ1	1:200	Neuromics MO15013
LAMP1	1:200	DSHB H4A3
ENGRAIL1 (EN1)	1:10	DHSB 4G11
HOXB4	1:10	DHSB I12
FOXG1	1:500	Abcam, ab 18259
β-Catenin	1:200	Santa Cruz Biotech sc-7199
Non-phospho (active) β-catenin	1:100	Cell Signaling mAb 8814
Phospho-GSK-3β (Ser9)	1:200	Cell Signaling mAb 9323

Table S2. Sequence of qRT-PCR primers used in this study.

AADC-F	5′-GGGACCACAACATGCTGCTC-3′
AADC-R	5′-CCACTCCATTCAGAAGGTGCC-3′
LMX1B-F	5′-AACTGTACTGCAAACAAGACTACC-3′
LMX1B-R	5′-TTCATGTCCCCATCTTCATCCTC-3′
Nurr1-F	5′-CGGACAGCAGTCCTCCATTAAGGT-3′
Nurr1-R	5′-CTGAAATCGGCAGTACTGACAGCG-3′
TH-F	5-GGTTCCCAAGAAAAGTGTCAG-3′
TH-R	5′-GGTGTAGACCTCCTTCCAG-3′
DBH-F	5′-GTGCTACATTAAGGAGCTTCCAAAG-3′
DBH-R	5′-GGCCTCATTGCCCTTGGT-3′
VMAT2-F	5′-CTTTGGAGTTGGTTTTGC-3′
VMAT2-R	5′-GCAGTTGTGATCCATGAG-3′
FOXG1-F	5′-CCCGTCAATGACTTCGCAGA-3′
FOXG1-R	5′-GTCCCGTCGTAAAACTTGGC-3′
SIX3-F	5′-ACCGGCCTCACTCCCACACA-3′
SIX3-R	5′-CGCTCGGTCCAATGGCCTGG-3′
HOXB4-F	5′-ACGTGAGCACGGTAAACCCCAA-3′
HOXB4-R	5′-ATTCCTTCTCCAGCTCCAAGACCT-3′
HOXC4-F	5′-TTCACGTTAGCACGGTGAAC-3′
HOXC4-R	5′-GACTTTGGTGTTGGGGAGTC-3′
GAPDH-F	5′-CAAGATCATCACGAATGCCTC-3′
GAPDH-R	5′-GCATGGACTGTGGTCATGAGTC-3′

SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Generation of iPSC-neuronal progenitor cells (NPCs). All the control and GD iPSC lines used in this study have been previously described (Panicker et al. 2012; Panicker et al. 2014). The GD iPSC lines were derived from two acute neuronopathic type 2 GD patients harboring the bi-allelic mutations L444P/Rec*Nci*I and W184R/D409H (GD2), two neuronopathic type 3 GD patients with L444P/L444P mutations (GD3), and one non-neuronopathic type 1 GD patient with N370S/N370S mutations (GD1). The analysis was carried out using two clones from each GD2 donor, one clone from each GD3 donor and one clone from GD1. iPSCs were cultured on irradiated MEFs as we previously described (Panicker et al. 2012). To generate neuronal progenitors from iPSCs we followed our previously described protocol (Awad et al. 2015). Briefly, iPSC-derived embryoid bodies were grown in Matrigel-coated plates (BD Biosciences, San Jose, CA) and differentiated into neuronal rosettes. Neuronal rosettes were manually picked, dissociated using StemPro® Accutase (Thermo Fisher Scientific, Halethorpe MD), and expanded in Neurobasal medium (Life Technologies, Carlsbad, CA) containing 1X (Vol/Vol) MEM non-essential amino acids (Life Technologies), 1X (Vol/Vol) GlutaMAX-I CTS (Life Technologies), 1X (Vol/Vol) B27 supplement (Life Technologies), 1X (Vol/Vol) penicillin/streptomycin, and 20 ng/mL bFGF (Stemgent, Lexington, MA). NPCs were maintained in culture at high density (>80%) with media change every other day.

Dopaminergic differentiation of iPSC-NPCs. Differentiation of NPCs to dopaminergic neurons was performed as described by Swistowski et al. 2012. NPCs were plated at 50% density on culture dishes or glass cover slips coated with Poly-L-ornithine (20 μg/mL) and laminin (10 μg/mL) (Sigma-Aldrich St. Louis, MO). Dopaminergic differentiation was initiated by culturing neuronal stem cells (NSCs) for 10 days in Neurobasal medium (Life Technologies) supplemented with 1X (Vol/Vol) MEM non-essential amino acids (Life Technologies), 1X (Vol/Vol) GlutaMAX-I CTS, 1X (Vol/Vol) B27 supplement, SHH (200 ng/mL, Miltenyi Biotec, Bergisch Gladbach, Germany) and FGF8 (100 ng/mL, Miltenyi Biotec). SHH and FGF8 were then withdrawn and replaced with BDNF (20 ng/mL, R&D Systems, Minneapolis, MN) and GDNF (20 ng/mL, R&D Systems), cAMP (100 nM) (Sigma-Aldrich) and Ascorbic acid (200 μM) (Sigma-Aldrich). Neurons were maintained in culture for 3-4 weeks with media change every 2-3 days. In some experiments, 3 μM CHIR99021 (Stemgent) was added to the media during DA differentiation as indicated. For direct differentiation of DA neurons from iPSC without going through a rosette stage, we followed the protocol described by Kriks et al. (Kriks et al. 2011), which is outlined in Figure 5A. Briefly, iPSC lines were cultured on mouse embryonic fibroblast (MEF) in hES/hiPSC medium (DMEM/F12, 20% Knockout serum, L-glutamine, β-mercaptoethanol, MEM/NEAA, and 16 ng/mL bFGF). iPSC colonies were dissociated using StemPro®Accutase (Thermo Fisher Scientific). MEFs were removed by adherence to gelatin for 1 hr at 37°C. The dissociated iPSCs were then collected and counted. About 10^6 cells from each line were resuspended in MEF conditioned medium supplemented with 10 μM ROCK inhibitor (Tocris Bioscience, Ellisville, MO). Cells were then plated on Matrigel-coated plates and expanded until confluent in conditioned medium supplemented with 10 ng/mL bFGF and 10 μM ROCK inhibitor. Differentiation was induced by treatment with 100 nM LDN193189 (Stemgent), 10 μM SB431542 (Tocris), 100 ng/mL SHH, 2 μM Purmorphamine (Millipore), and 3 μM CHIR99021 (Stemgent) as outlined in Figure 5A. After 11 days, cells were passaged onto Poly-L-ornithine/laminin/fibronectin in Neurobasal medium supplemented with 20 ng/mL BDNF (R&D systems), 200 μM Ascorbic acid (Sigma), 20 ng/mL GDNF (R &D systems), 1 mM cAMP (Millipore), 2 ng/mL TGFβ3 (R&D systems), and 10 μM DAPT (Tocris). Cells were fed every other day with the same medium composition for 10-15 more days and analyzed as described in the text.

Immunofluorescence analysis. NPCs were plated on chamber slides (Thermo Fisher Scientific), glass coverslips, or glass-bottom culture dishes (MatTek, Ashland MA), and differentiated to DA neurons as described above. NPCs or DA neurons were fixed in 4% (Vol/Vol) paraformaldehyde for 15 minutes and blocked in PBS containing 8% FBS (Vol/Vol) for 1 hour. Primary antibodies or isotype controls were diluted in PBS containing 2 mg/mL saponin and incubated for 2 hours at room temperature, or at 4°C overnight, followed by 1 hour incubation with the corresponding fluorochrome-conjugated secondary antibodies. Table S1 lists all the primary antibodies that were used. The secondary antibodies were: DyLight 488- or 594-conjugated mouse or rabbit (Jackson ImmunoResearch Laboratories, West Grove, PA); and Alexa fluor 488-, or 594-conjugated mouse or rabbit (Life Technologies), all at 1:200 dilution.

DAPI-containing mounting medium (Vectashield; Vector Laboratories, Burlingame, CA) was used to visualize cell nuclei. For some experiments, NPCs were treated with CHIR99021 (3 μM) (Stemgent) for 3 days, with proteasome inhibitor Clasto-Lactacystin β-lactone (0.5 mg/mL) (Cayman Chemical, Ann Arbor MI) for 18 hours, with Torin (1 μM) for 4 hours, or with recombinant human GCase as described below.

Recombinant GCase treatment. Recombinant human GCase (rGCase) (Cerezyme®, Genzyme, Cambridge, MA) was added to NPCs at a concentration of 0.24 U/mL for 5 days. In some experiments, rGCase was added at the EB stage and incubation was continued throughout the NPCs generation and DA differentiation period (6-8 weeks), and replenished with each media change as indicated in the text. Cerezyme was obtained from patient infusion remnants.

GCase assay. GCase enzyme activity was assayed in NPCs lysate using fluorescence-conjugated substrate, as previously described (Panicker et al. 2012; Awad et al. 2015)

Western blot analysis. Cell lysates of NPCs were prepared using RIPA buffer with protease inhibitor (Roche) and phosphatase inhibitors (Pierce) followed by sonication. Cell lysates were denatured in SDS-loading buffer at 95°C for 5 min, loaded onto a 4-20% polyacrylamide gel (Bio-Rad, Hercules CA) for electrophoresis. Gels were transferred to a PVDF membrane (Millipore). The membranes were blocked with 5% BSA and probed with the following antibodies as indicated: TFEB (Novus Biologicals, Cat No. NB100-1030), LAMP1 (DSHB, Cat. No. H4A3), non-phospho (active) β-catenin (Cell Signaling, Cat. No. 8814), phospho-GSK-3β (Ser9) (Cell Signaling, Cat. No. 9323), GSK-3β (Cell Signaling, Cat. No. 12456), or β Actin (Cell Signaling, Cat. No. 4967), followed by incubation with the corresponding HRP-conjugated secondary antibodies. Blots were developed using a chemiluminiscence kit (Thermo Fisher Scientific) and visualized using Chemi-doc system (Bio-Rad). Bands were quantitated and analyzed using ImageJ software (NIH).

Real-Time PCR. For gene expression analysis, mRNA was isolated form DA neuronal cultures at the indicated time points using RNA isolation kit (Qiagen, Germantown, MD), and cDNA was synthesized using the iScript kit (Bio-Rad). Gene expression was determined by quantitative PCR (7900 HT; Applied Biosystems, Foster City, CA) in duplicate or triplicate wells using the SYBR Green PCR Master Mix (Thermo Fisher Scientific). The relative mRNA expression of each gene tested was normalized to the values of GAPDH mRNA for each reaction, and then normalized to the mRNA levels for the corresponding genes. Table S2 lists the sequence for all primers used in this study.

Imaging. Fluorescence images were captured using an inverted Nikon Eclipse TE-2000 microscope with Nikon Imaging Systems (NIS)-Elements AR 3.0 collection software, or upright Nikon Eclipse E-800 microscope with SPOT Imaging Systems. High-resolution images were captured using Zeiss LSM-510 confocal microscope (Carl Zeiss) and an AxioCam digital microscope camera or DMi8 Leica fluorescence microscope with Leica Application Suite X software. Fluorescence intensity was measured using ImageJ software with RGB analysis plugin. For quantitation of TH expression, NPCs or iPSCs were differentiated to DA neurons as described above. Fluorescence microscopy images were acquired at 20x magnification from at least three fields. The percentage of TH expression was calculated as the number of TH positive cells divided by the number of DAPI-positive nuclei in the same vision field.