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Supplemental Information

Reversible Conformational Conversion of α-Synuclein into Toxic Assemblies by Glucosylceramide

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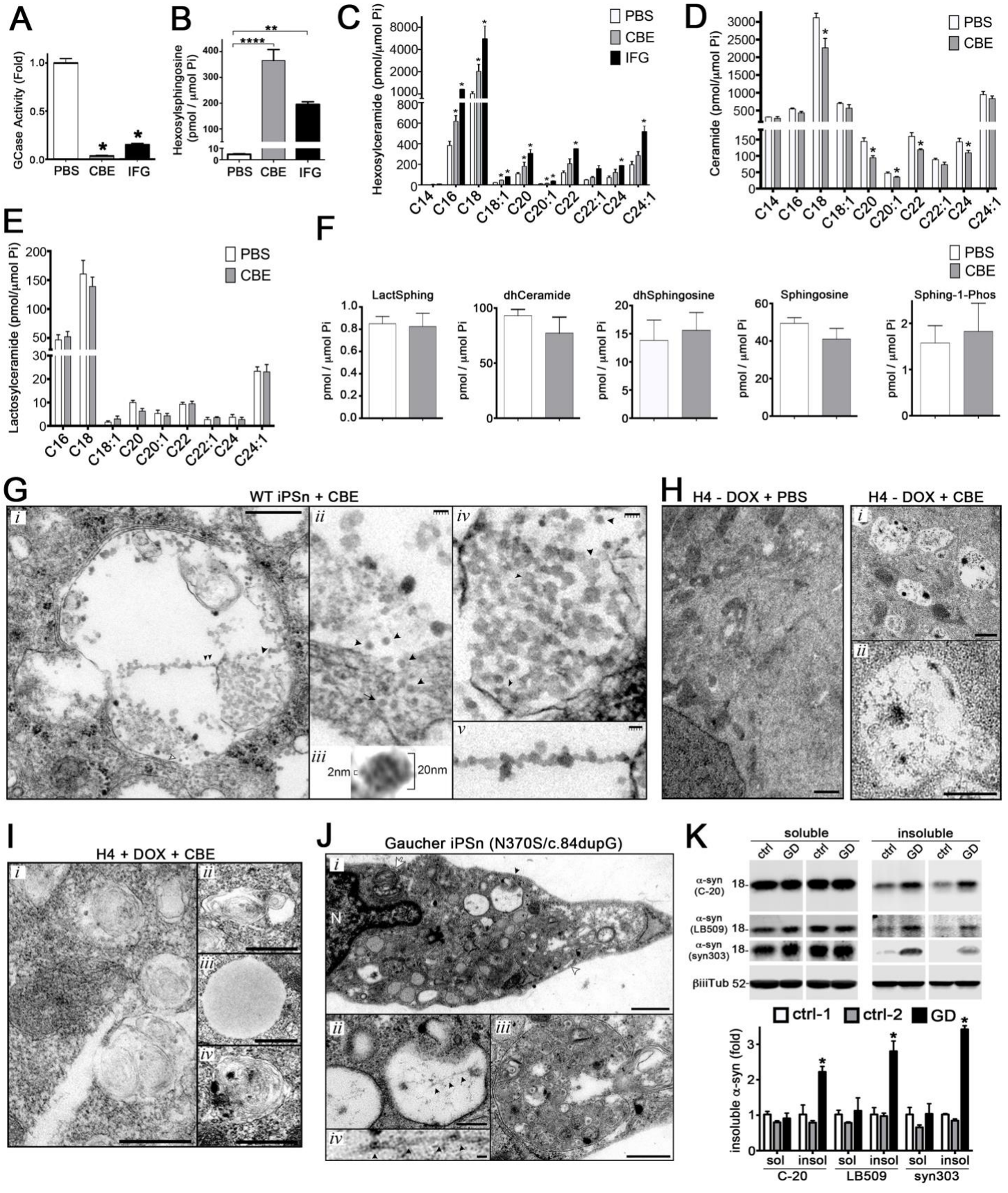
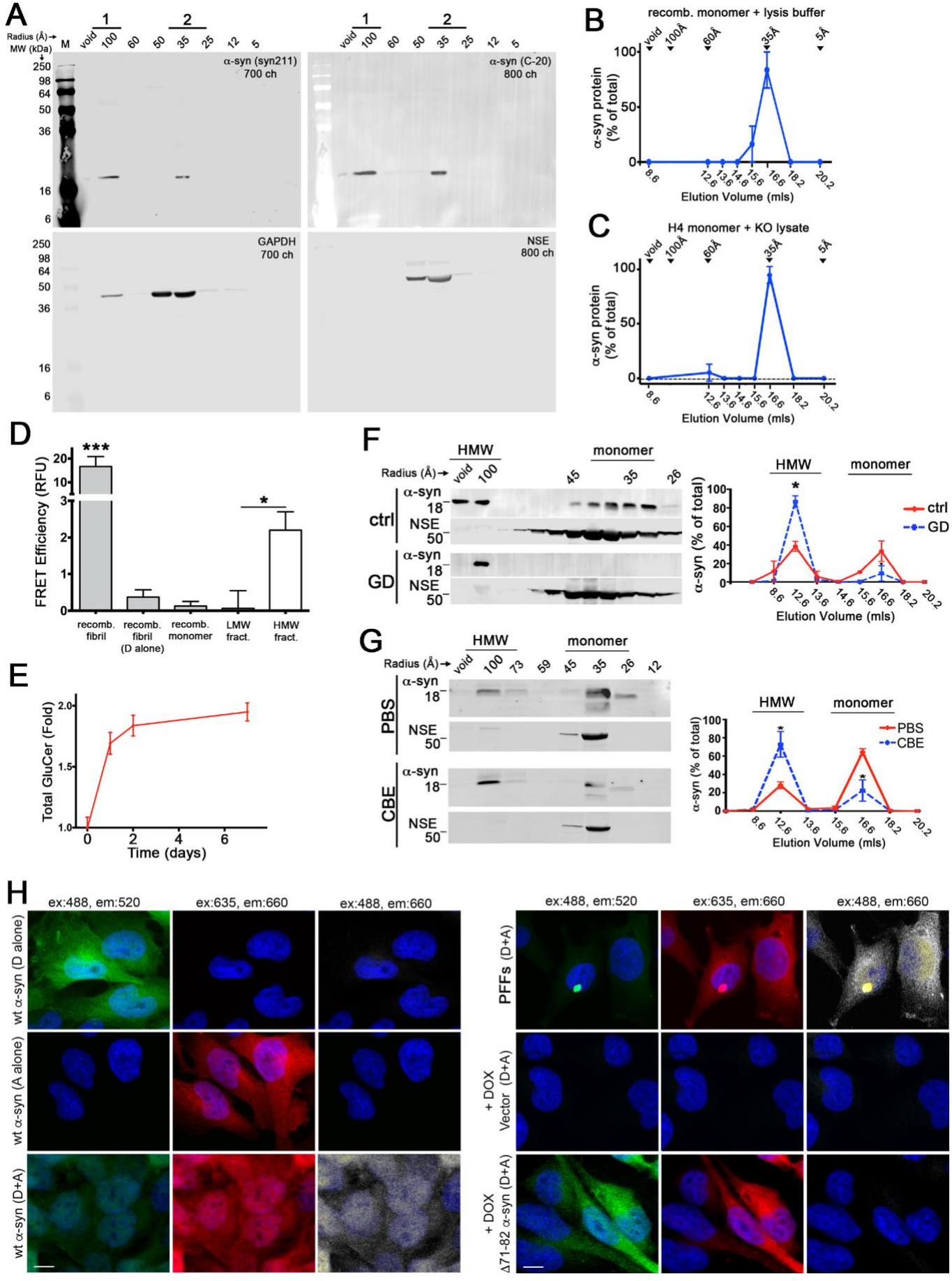
Zunke et al, Supplemental Figures and Figure Legends

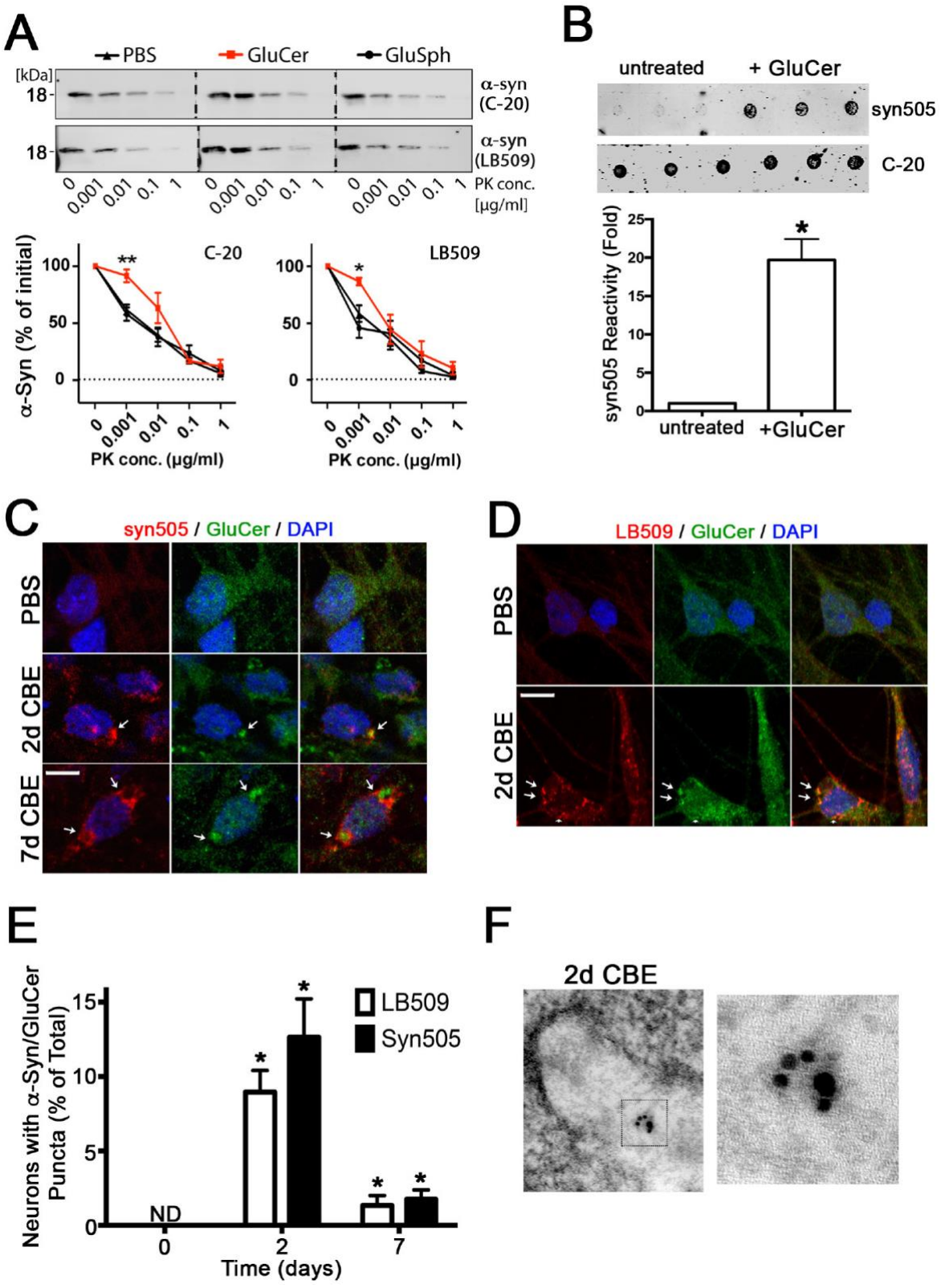


Supplemental Figure 1 linked to Figure 1. Lipid accumulation and pathological analysis of iPSn treated with GCase inhibitors and GD patient neurons. A) iPS midbrain cultures from healthy controls were treated with 50 μM conduritol-β-epoxide (CBE) or isofagomine (IFG) for 7 days. GCase activity was measured from whole cell lysates from neurons treated with CBE or IFG, using an artificial GCase substrate that fluoresces upon cleavage, 4-MU-Gluc (n=4). **B**) Hexosylsphingosine species were measured by high performance liquid chromatography-tandem mass spectrometry (HPLC-MS/MS) after CBE or IFG treatment and expressed as picomole substrate / micromole of inorganic phosphate (Pi). **C**) HPLC-MS/MS measurement of hexosylceramide species separated by N-acyl fatty acid chain length after CBE or IFG treatment, expressed as pmol / μmol Pi (n=4). Hexosylsphingosine and hexosylceramides include both glucosyl and galactosyl species, however glucosylceramides make up ca. 90% of the total hexosylceramide species in neuronal cultures. **D, E**) HPLC-MS/MS measurement of ceramide or lactosylceramide species separated by N-acyl fatty acid chain length. **F**) HPLC-MS/MS measurement of sphingolipids related to the glucosylceramide metabolic pathway, demonstrating specificity of CBE at the utilized time and concentration. **G**) 2d CBE-treated neurons demonstrate accumulation of annular structures within vacuoles (i, black arrow head) that occasionally coalesce in a linear manner (i, double arrow head) and surround disrupted regions of the vacuole (i, white arrow head). Higher magnification of structures indicated by the white arrow (ii), a single annular structure of 20nm in diameter with the appearance of a 2nm pore (iii), higher magnification of annular structures (iv), and linear alignment (v). Scale bars= 500nm (i), 50nm with 10nm ticks (ii, iv, v). **H**) Left EM of H4 cells expressing α-syn treated with PBS. Scale bar=100nm. Right, H4 cells treated with CBE for 5 days showing fibrillogranular inclusions. Scale bars= 500 nm. **I**) CBE-treated H4 cells depleted of α-syn through doxycycline addition demonstrate membrane accumulations of multilamellar structure (ii, iv) or electron lucent vacuoles (iii) in the absence of fibrillar material. Scale bars= 500 nm. **J**) EM analysis of GD patient neurons (N370S / c.84dupG) showing enlarged vacuoles containing multiple vesicular bodies (white arrow head in panel (i), close up shown in panel (iii)), multilamellar body (white arrow in panel (i)), and a disrupted vacuole containing fibrillar material (black arrow head in panel (i), close up shown in panel (ii) and fibrillar material shown in (iv). Scale bar= 1 μm (i), **K**) Elevated insoluble α-syn in GD iPSn. Sequential extraction of GD iPS midbrain neurons (expressing N370S / c.84dupG mutations) by 1% Triton X-100 (soluble), then 2% SDS buffer (insoluble) followed by western blot analysis. GD samples were compared to two separate iPS control lines previously characterized (Mazzulli et al., 2016a). The blots were quantified below (n=4). For all blots, the molecular weight is shown in kilodaltons. All quantifications represent the mean +/- SEM. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.


A
Radius (Å)→
MW (kDa)
M
void
100
60
50
35
25
12
5
α-syn (syn211)
700 ch
α-syn (C-20)
800 ch
GAPDH
700 ch
NSE
800 ch
B
recomb. monomer + lysis buffer
α-syn protein (% of total)
Elution Volume (mls)
C
H4 monomer + KO lysate
D
FRET Efficiency (RFU)
recomb. fibril
recomb. fibril (D alone)
recomb. monomer
LMW fract.
HMW fract.
E
Total GluCer (Fold)
Time (days)
F
HMW
monomer
ctrl
GD
α-syn
NSE
α-syn (% of total)
G
PBS
CBE
H
ex:488, em:520
ex:635, em:660
ex:488, em:660
wt α-syn (D alone)
wt α-syn (A alone)
wt α-syn (D+A)
PFFs (D+A)
+ DOX Vector (D+A)
+ DOX Δ71-82 α-syn (D+A)

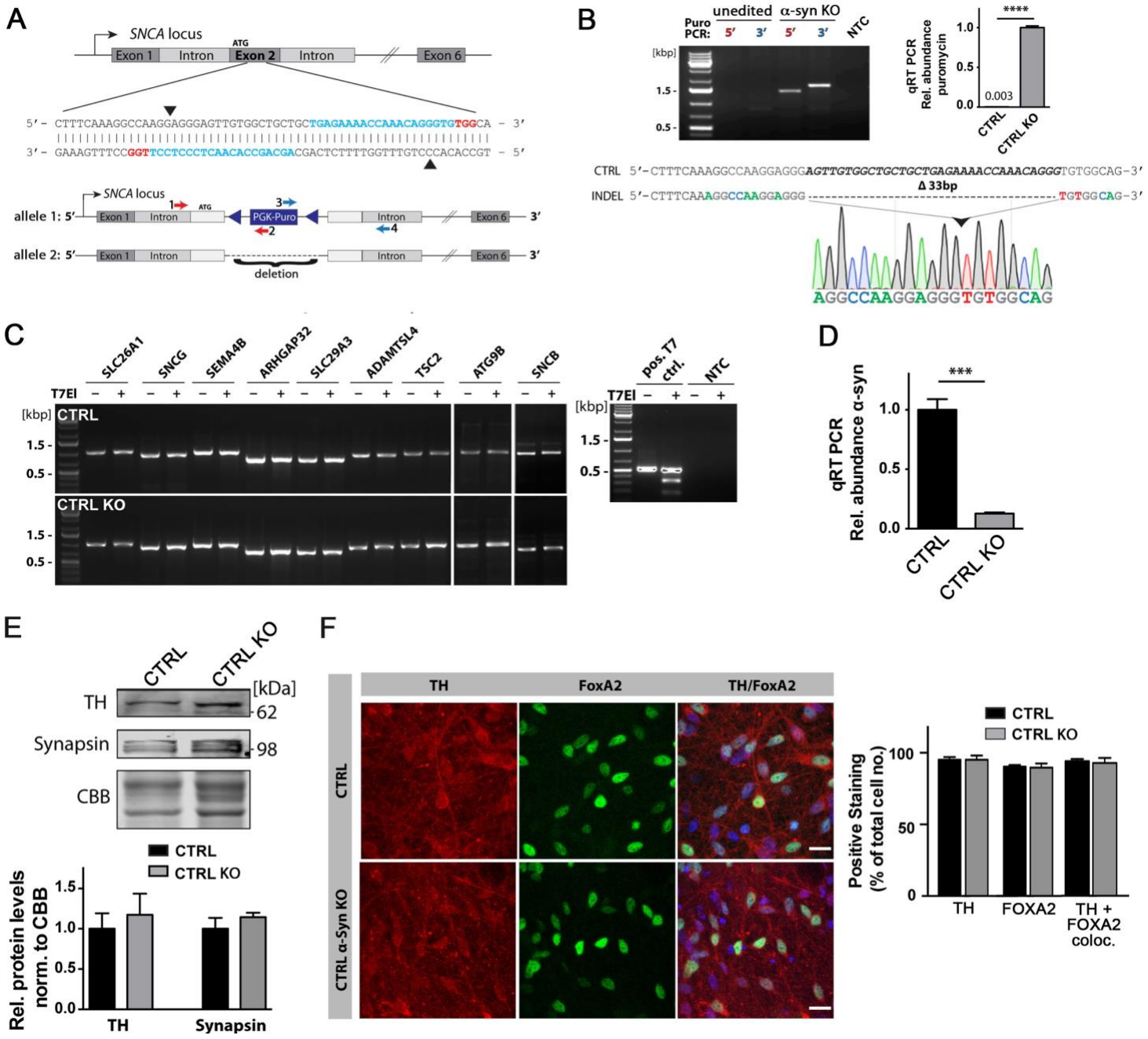
Supplemental Figure 2 Linked to Figures 1 and 2. Measurement of distinct α-syn conformers in human iPSn and H4 cells. **A**) 1% Triton X-100, 100,000 x g soluble neuronal lysates from control midbrain neurons were analyzed by size exclusion chromatography (SEC) followed by SDS-PAGE / western blot of the collected fractions using two anti- α-syn antibodies (syn211 (mouse) or C-20 (rabbit)). The blot was developed using anti-mouse Alexa 680 (700 channel) or anti-rabbit IRdye800 (800 channel) secondary antibodies on the same blot. The horizontal marker indicates molecular radius in angstroms (Å) obtained from the native SEC fractions, while the vertical marker indicates molecular weight (MW) in kilodaltons (kDa) obtained by SDS-PAGE of each fraction. Two distinct elution peaks were observed, one at ca. 100 Å (form 1) and the other at 35 Å (form 2), consistent with a HMW and monomeric form of α-syn, respectively. The 35 Å-sized monomeric form has been previously documented from extensively characterized (Weinreb et al., 1996). The elution of GAPDH and neuron specific enolase (NSE) was used as a loading control, as well as to determine if the extraction / analysis method affects protein structure of these well-defined proteins. GAPDH elution peak is at ca. 49 Å, corresponding to a 145 kDa globular protein, consistent with its known tetrameric structure (36 kDa x 4) under non-denaturing conditions. The NSE elution peak is ca. 40 Å, corresponding to a 92 kDa globular protein, and is consistent with its previously documented native dimer structure (47 kDa x 2) under non-denaturing conditions. These data indicate that the extraction procedure and SEC analysis method do not change native protein structure of these proteins. **B)** Recombinant α-syn was purified from transformed *E. coli*, mixed with the same lysis buffer as used in panel A, and analyzed by SEC. The protein remains monomeric under these conditions, indicating that *in vitro* oligomerization does not occur from the lysis buffer or during the SEC analysis (n=3). **C**) Monomeric α-syn was isolated from human H4 cells and mixed with a midbrain neuronal lysate that does not contain α-syn (*SNCA* knock-out line, see supplemental figure 4 for details). The lysate spiked with monomeric α -syn was analyzed by SEC as described above, and α-syn was quantified as percent of total (n=3). Nearly all of the isolated monomeric α-syn remained monomeric after incubation with neuronal lysates, indicating that HMW α-syn does not occur from *in vitro* oligomerization or interaction with other cellular constituents during the lysis procedure. **D**) Analysis of FRET efficiency, using syn211-conjugated Alexa 488 or 647 antibodies, of recombinant α-syn fibrils or isolated fractions containing HMW or LMW α-syn. HMW fractions yielded significant FRET indicating close proximity of the two antibodies that may occur from a self-associated species (n=5). Recombinant monomers or fibrils mixed with donor alone (D) were used as negative controls. Low efficiency FRET of LMW fractions indicate a monomeric state of the protein. **E**) Measurement of total GluCer species in iPSn at different times after CBE addition, performed as in figure S1 (n=3). **F**) SEC / western blot analysis (C-20) of soluble lysates extracted from Gaucher patient iPS midbrain neurons (expressing N370S / c.84dupG mutations) compared to a healthy control. Right, quantification of α-syn forms detected in each fraction by SEC / western blot (n=3). **G**)

SEC / western blot (C-20) of soluble lysates extracted from H4 cells, treated with phosphate buffered saline (PBS) or 50 μM CBE for 5 days. Neuron specific enolase (NSE) was used as a loading control. Right, Quantification of α-syn forms from SEC fractions that eluted between 8.6 and 20 mls followed by western blot (n=3, values are the mean +/- SEM, *p<0.05). Student's t-test was used for statistical analysis. Note that fractions were collected and combined slightly differently in panel F compared to A and G, which resulted in broader elution peaks in panel F. **H**) FRET analysis in fixed H4 cells using syn211-Alexa 488 and 647 antibodies. FRET signal (ex=488, em=660) was not detected in samples incubated with donor alone (D) or acceptor alone (A), but was detected with D + A were combined. Pre-formed fibrils (PFFs) were used to measure the maximal response of the FRET signal in detecting polymerized forms of the protein. H4 cells depleted of WT α-syn (+ DOX) were used to monitor antibody specificity, while +DOX and infected with lentivirus expressing Δ71-82 α-syn, which cannot polymerize, was used to demonstrate the FRET assay can only detect self-associated forms containing multiple copies of α-syn. Scale bars = 10 μm.



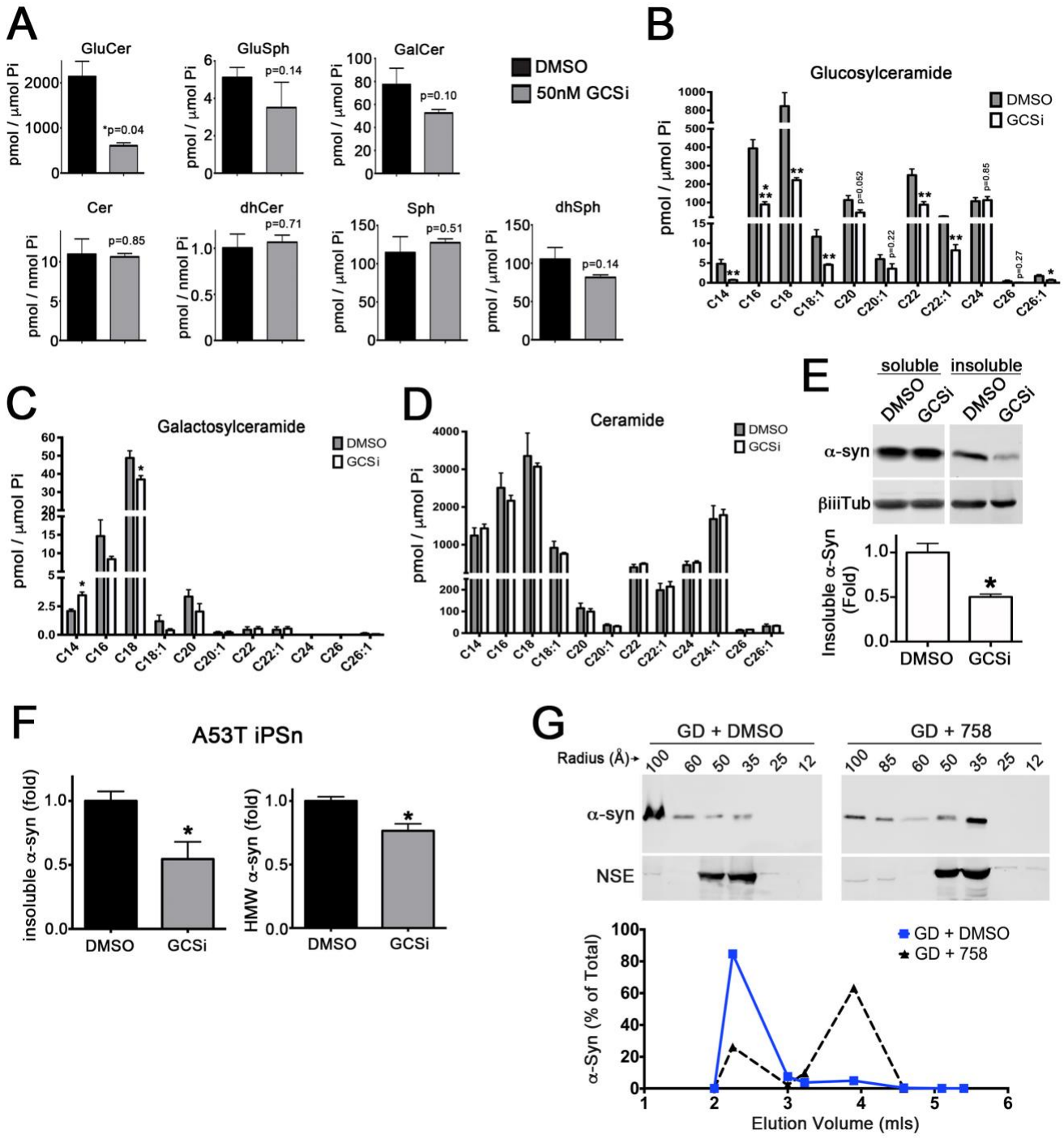
Supplemental Figure 3 linked to Figure 3 and 4. Conversion of α-syn species by GluCer *in vitro* and in neurons. **A**) GluCer directly converts α-syn into PK resistant species. Recombinant purified α-syn (*E. coli* derived) was incubated with GluCer or GluSph at pH 5.5 with sample agitation, followed by PK digest and western blot. (n=3, *p< 0.05, **p<0.01, GluCer compared to GluSph and PBS). **B**) Native dot blot analysis of recombinant α-syn

mixed with GluCer using the same conditions as in A) showing increased reactivity with syn505. Three replicates are shown and quantified below. C-20 was used to detect total α-syn. **C**) Immunostaining analysis of iPSn treated with 50 μM CBE for 2 or 7 days, with syn505 (red) and anti-GluCer antibodies (green). Nuclei are stained with DAPI in blue. **D**) iPSn were analyzed for α-syn-GluCer colocalization using LB509 and anti-GluCer after 2 days of CBE treatment. **E**) Quantification of neurons containing α-syn / GluCer colocalized puncta (n=3). **F**) Immuno-electron microscopy analysis of 2-day CBE treated cells using syn505 (6 nm gold secondary) and anti-GluCer (10 nm gold secondary). For all quantifications, values are the mean +/- SEM, *p<0.05, **p<0.01. Student's t-test (B) or ANOVA with Tukey's post-hoc test (A, E) were used for statistical analysis.



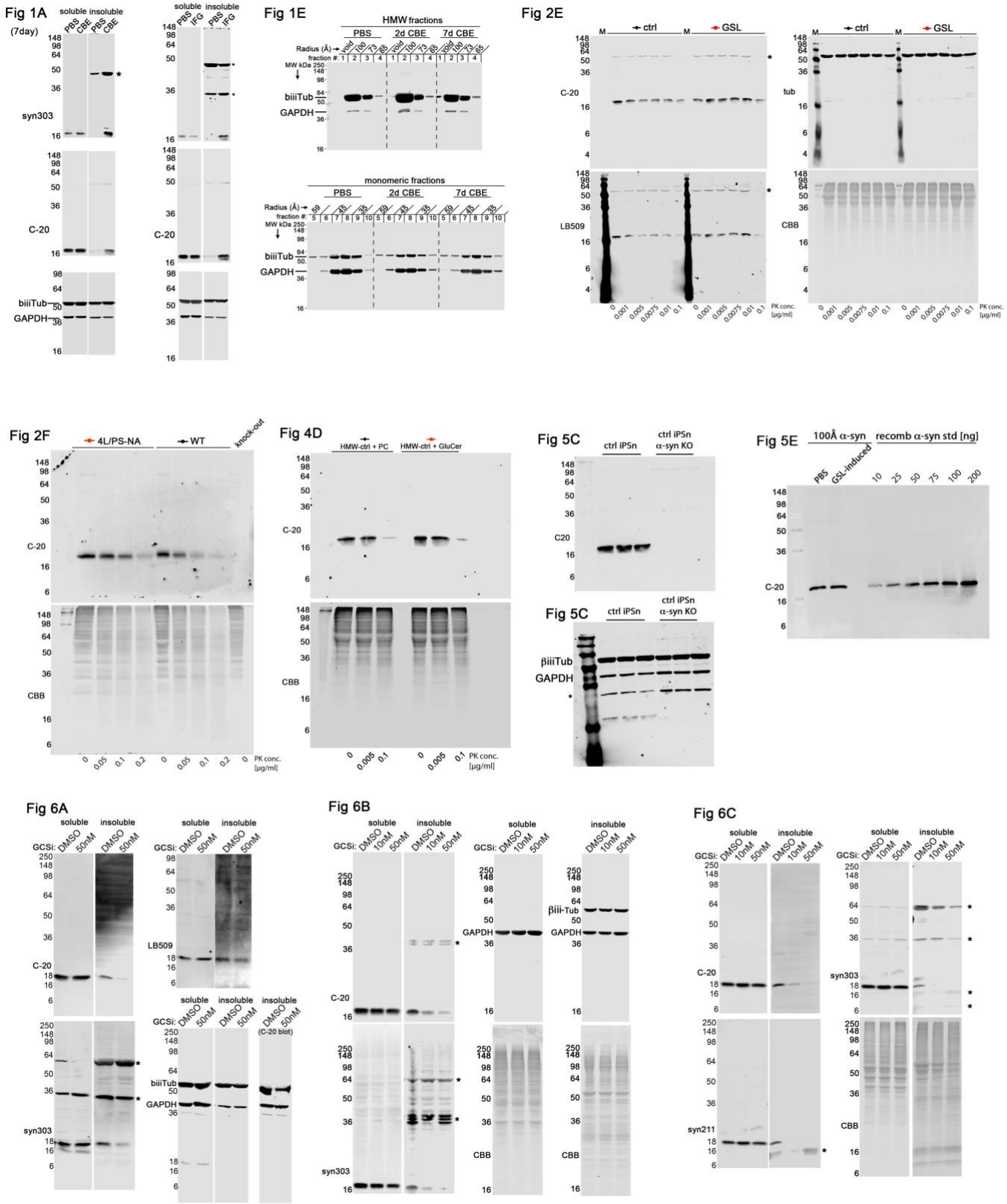
Supplemental Figure 4 linked to Figure 5. Generation of α-syn knock-out lines through CRISPR-Cas9 mediated genome editing. A) We utilized a CRISPR Cas9 double nicking approach by designing two RNA-guided Cas9-containing plasmids along with a homologous recombination plasmid to interrupt the *SNCA* gene at exon 2 with a puromycin expression cassette driven by the phosphoglycerate kinase (PGK) promoter. Red or Blue arrows indicate the diagnostic PCR primers used to detect PGK-puromycin insertion shown in panel B. Red sequence represents protospacer adjacent motif (PAM) sites while blue sequence represents guide RNA annealing sites. **B**) Analysis of puromycin selected iPSC lines demonstrated specific insertion into one allele, while the other allele exhibited a 33bp deletion that occurred from a Cas9-induced insertion-deletion (INDEL). **C**) Analysis of off-target effects by the T7 endonuclease assay indicated that the genome editing strategy was specific. The top 9

genes showing homology to *SNCA* were analyzed, demonstrating lack of modification by the CRISPR constructs and specific disruption of *SNCA*. NTC, non-template control. **D**) Quantitative analysis of α-syn mRNA by RT-PCR revealed a 90% depletion of α-syn transcript in KO lines. **E**) Western blot analysis of lysates extracted from differentiated α-syn KO iPSC neurons indicated equivalent differentiation efficiencies compared to the parental unedited line, showing equal protein levels of the synaptic marker synapsin, and catecholamine marker tyrosine hydroxylase (TH). Coomassie brilliant blue (CBB) was used to indicate gel loading. **F**) Immunofluorescence analysis of day 60 midbrain neurons indicated identical percentages of neurons co-expressing midbrain specific FOXA2 and TH in parental vs. KO lines. For all quantifications, values are the mean +/- SEM, n=3. ***$p<0.001$, ****$p<0.0001$; Student's t-test.

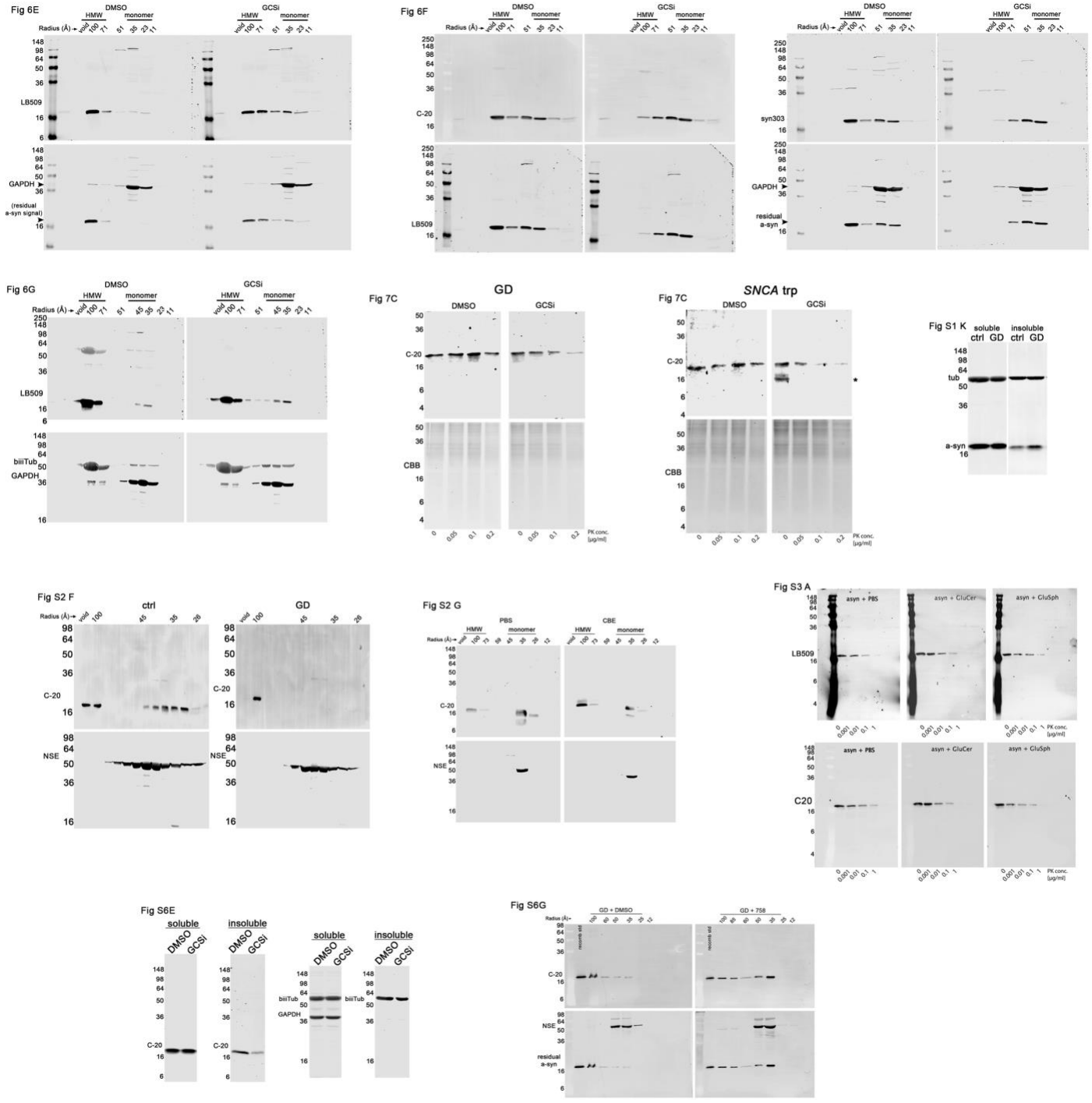


Supplemental Figure 5 linked to Figures 6 and 7. GSL reduction in Gaucher and PD patient-derived iPS midbrain neurons by GCSi or GCase activation A) Gaucher patient iPS midbrain neurons (N370S / c.84dupG) were incubated with 50nM glucosylceramide synthase inhibitor (GCSi) for 4 weeks followed by analysis of GSLs by supercritical fluid HPLC-MS/MS. Total GluCer species and other sphingolipids were quantified and expressed as pmol / µmole inorganic phosphate (Pi). **B-D**) Analysis of individual GluCer, GalCer, and ceramide species separated by N-acyl fatty acid chain length (n=4) *p<0.05, **p<0.01, ***p<0.001. Student's t-test was used for statistical analysis. **E**) Sequential extraction / western blot analysis of GD patient neurons (L444P/L444P) treated

with GCSi for 4 weeks at 50 nM. The quantification of insoluble α-syn is shown below (n=3). **F**) iPSn derived from a PD patient expressing A53T α-syn were treated with GCSi and analyzed for insoluble and HMW forms of α-syn as described in figure 6 (n=4, *p<0.05). **G**) Activation of GCase by small molecule 758 reduces HMW α-syn in GD patient-derived iPS midbrain neurons. Top, Gaucher patient iPS midbrain neurons (N370S / c.84dupG) were treated with a previously established small molecule GCase activator, 758, at 10 μM for 14 days. Soluble extracts (300 μg) were injected on a TOSOH SuperSW3000 SEC column, followed by western blot of collected fractions (C-20). Bottom, Quantification of the blots demonstrated that reducing GSLs restores HMW / monomer ratios to control levels. For all quantifications, values are the mean +/- SEM. Student's t-test was used for statistical analysis.



Supplemental Figure 6 Linked to Figures 1-6. Full-length blots corresponding to Figures 1-6. MW shown in kilodaltons. Asterisks indicate irrelevant bands (observed by secondary antibody alone or previously identified as non-specific through validation methods utilizing α-syn knock-out tissues). See corresponding figures for full explanations.



Supplemental Figure 7 linked to Figures 6-7. Full-length blots corresponding to Figures 6-7 and other SI Figures. MW shown in kilodaltons. Asterisks indicate irrelevant bands (observed by secondary antibody alone or previously identified as non-specific through validation methods utilizing α-syn knock-out tissues). See corresponding figures for full explanations.