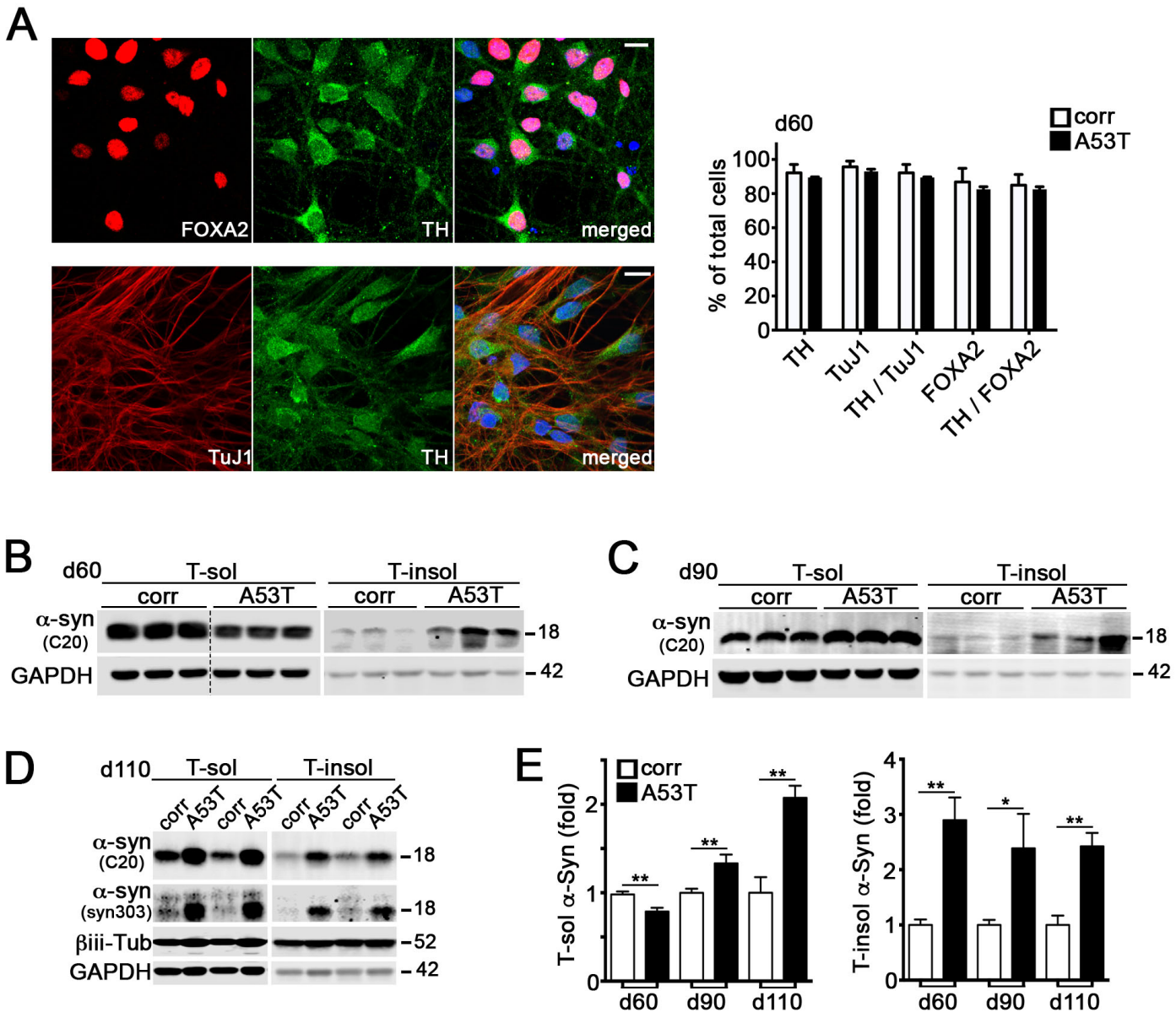
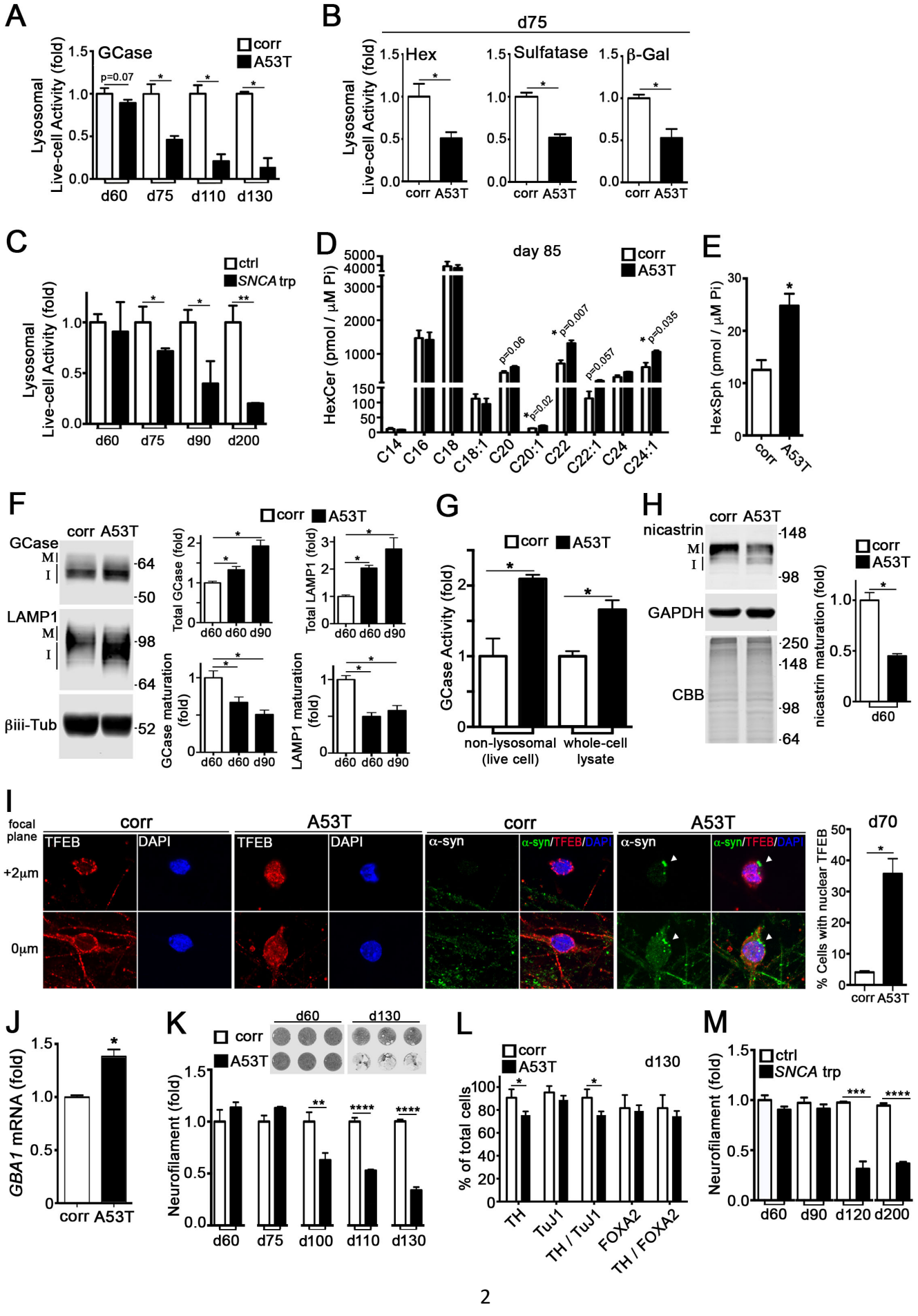
Supplementary material for Cuddy et al, *Stress-induced cellular clearance is mediated by ykt6 and disrupted by a-synuclein.*

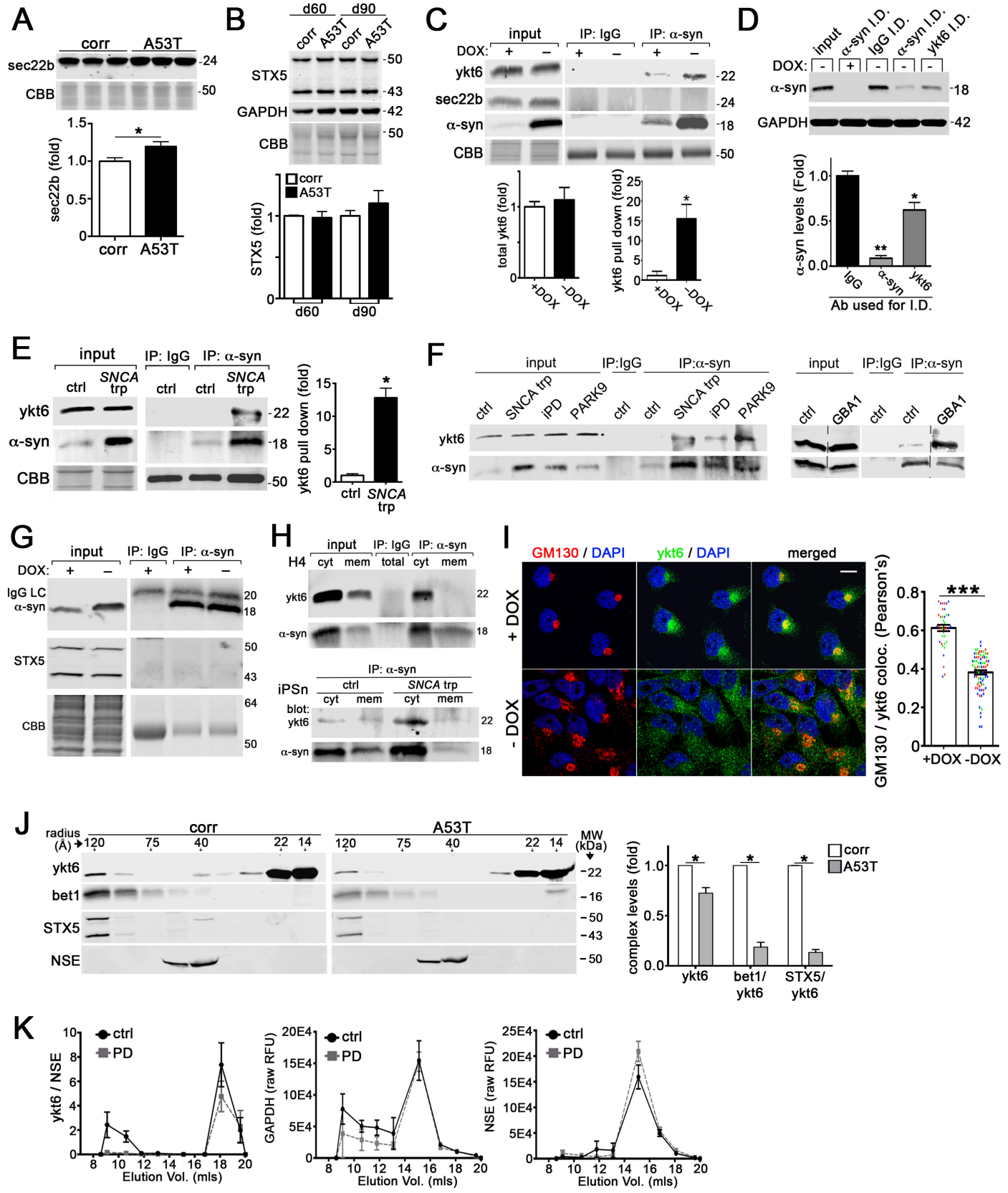
Supplemental Figures



Supplemental Figure 1 linked to Figure 1. Analysis of a-syn solubility over time in cultured midbrain DA neurons from A53T PD patients. A) Characterization of iPSn cultures by immunocytochemistry indicates efficient differentiation of midbrain dopamine neurons, as indicated by the midbrain marker FOXA2 and tyrosine hydroxylase (TH). Tuj1 is an antibody against neuron-specific b-iii-tubulin. Nuclei are shown in blue. Scale bar, 10μm. Shown is a representative image from A53T iPSn at day 60. Culture populations were quantified to the right at day 60. **B**) Sequential extraction / western blot of day 60 neurons extracted from A53T a-syn neurons, compared to isogenic corrected lines (corr). Neurons were extracted in 1% Triton X-100 (T-sol), followed by 2% SDS (T-insol). GAPDH was used as a loading control. The dotted line indicates cropped out lanes of samples from the same blot. The white space indicates two separate blots. 3 biological replicates are shown. **C**) Samples were analyzed as in A, after 90 days post-differentiation. **D**) Samples were analyzed as in A, after 110 days post-differentiation. A-syn was detected with both C-20 (total) and syn303 (preferentially detects oxidized a-syn) anti-a-syn antibodies. **E**) Quantification of soluble and insoluble a-syn blots at different times post-differentiation. For all quantifications, values are the mean +/- SEM, *p<0.05, **p<0.01, Student's T-test.

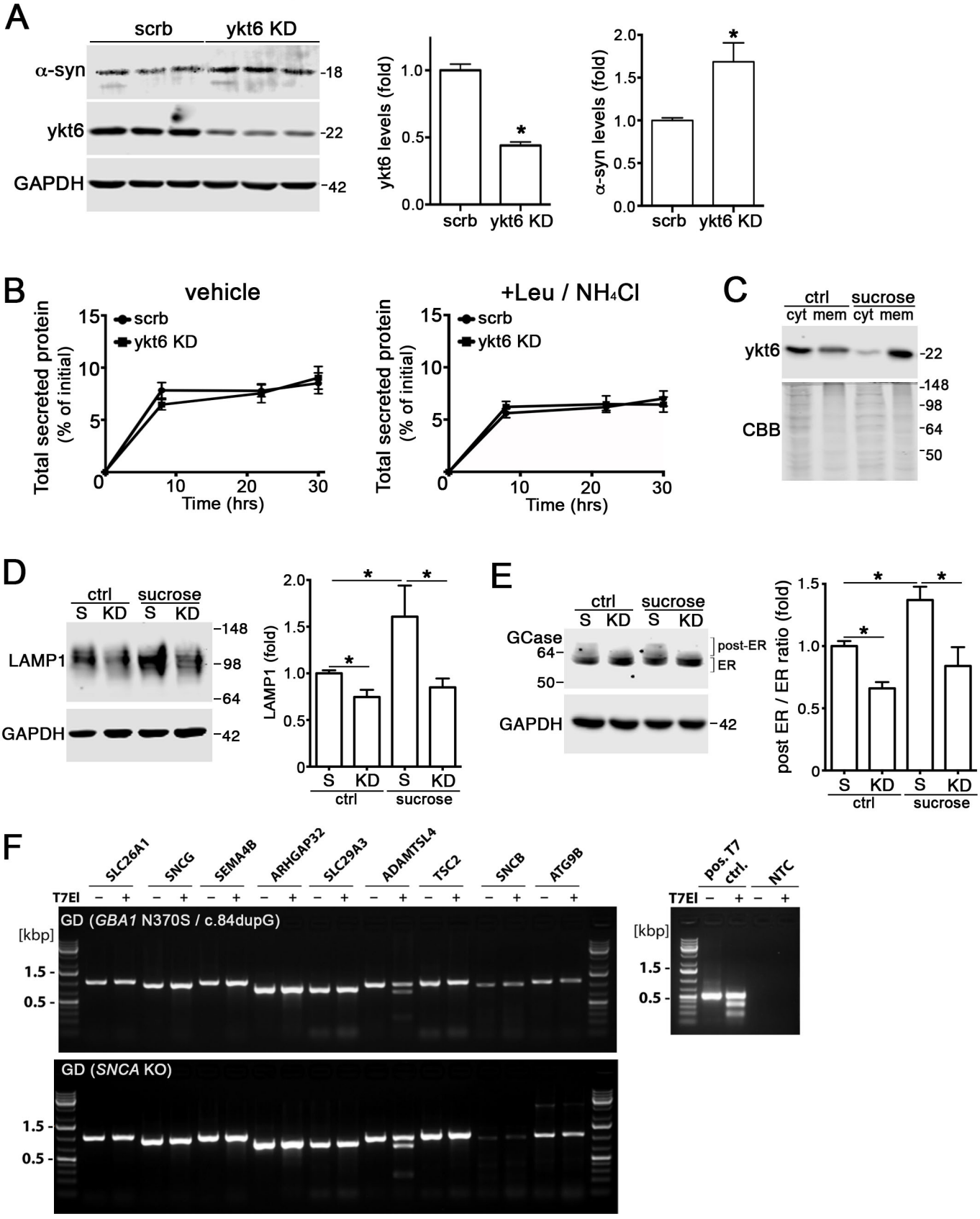

A
GCase
corr
A53T
Lysosomal Live-cell Activity (fold)
p=0.07
d60 d75 d110 d130
B
d75
Hex
Sulfatase
β-Gal
corr A53T
C
ctrl
SNCA trp
d60 d75 d90 d200
D
day 85
HexCer (pmol / μM Pi)
p=0.06
p=0.02
p=0.007
p=0.057
p=0.035
C14 C16 C18 C18:1 C20 C20:1 C22 C22:1 C24 C24:1
E
HexSph (pmol / μM Pi)
corr A53T
F
GCase
LAMP1
βiii-Tub
Total GCase (fold)
Total LAMP1 (fold)
GCase maturation (fold)
LAMP1 maturation (fold)
d60 d60 d90
G
GCase Activity (fold)
non-lysosomal (live cell)
whole-cell lysate
H
nicastrin
GAPDH
CBB
nicastrin maturation (fold)
d60
I
focal plane
+2μm
0μm
TFEB
DAPI
α-syn
α-syn/TFEB/DAPI
d70
% Cells with nuclear TFEB
J
GBA1 mRNA (fold)
K
d60
d130
Neurofilament (fold)
d60 d75 d100 d110 d130
L
d130
% of total cells
TH
TuJ1
TH / TuJ1
FOXA2
TH / FOXA2
M
Neurofilament (fold)
d60 d90 d120 d200

Supplemental Figure 2 linked to Figure 1. Temporal analysis of lysosomal dysfunction and neuron degeneration in PD patient midbrain neurons. A) Lysosomal β-glucocerebrosidase (GCase) activity was assessed within lysosomes of living midbrain DA neurons derived from an A53T PD patient iPSn using a compartment specific assay as described (Mazzulli et al., 2016a) at different days post-differentiation, indicating an age-dependent decline in activity compared to isogenic control lines (corr) (n=4). Lysosomal activity was defined as activity that is responsive to the lysosomal inhibitor, Bafilomycin A1 (BafA1). **B**) Lysosomal activity of hexosaminidase, sulfatase, and beta-galactosidase was done as in A. **C**) Lysosomal GCase activity was assessed within lysosomes of living midbrain DA neurons of a *SNCA* trp patient and compared to a healthy control line, as in A. **D)** Quantification of GCase substrates, hexosylceramide species, separated by acyl fatty acid chain lengths, in A53T and corr iPSn, normalized to inorganic phosphate (Pi). **E)** Analysis of hexosylsphingosine levels at 85 days post-differentiation. (n=3). **F**) Analysis of lysosomal protein levels and maturation by western blot of day 60 and 90 lysates (M, mature; I, Immature). βiii-Tubulin, GAPDH or Coomassie brilliant blue (CBB) were used as loading controls. Total levels were normalized to βiii-Tubulin, while maturation was assessed by calculating M / I ratios. Values are expressed as fold changed compared to corr at day 60 (n=3). **G)** Non-lysosomal activity (ie, not BafA1 responsive) was measured in living corr or A53T neurons, as well as *in vitro* using whole-cell lysates at day 75. **H**) Nicastrin maturation was analyzed by western blot and quantified to the right (n=3). **I**) Confocal immunofluorescence analysis of TFEB (red) and a-syn (green) in A53T and corr cultures at d70, demonstrating nuclear translocation of TFEB within cells that accumulate cell body a-syn inclusions (white arrows). Two focal planes are shown, 0 and +2 μm, to demonstrate that nuclei (DAPI, blue) colocalize with TFEB in A53T neurons. **J**) Measurement of a TFEB target, *GBA1* mRNA, in d70 cultures, normalized to beta-actin mRNA (n=3). **K**) Neuron degeneration was assessed over time in A53T vs. corrected iPSn by quantification of neurofilaments and normalized to total cell volume (n=4). A representative immunostaining result is shown above the graph. **L)** Toxicity analysis was done at day 130 by counting TH/Tuj1/FOXA2 immunoreactive cells and normalized to nuclei (DAPI). **M**) Neuron degeneration was assessed as in K, in *SNCA* trp vs control iPSn. For all quantifications, values are the mean +/- SEM, *p<0.05, **p<0.01, ****p<0.0001, Student's T-test.



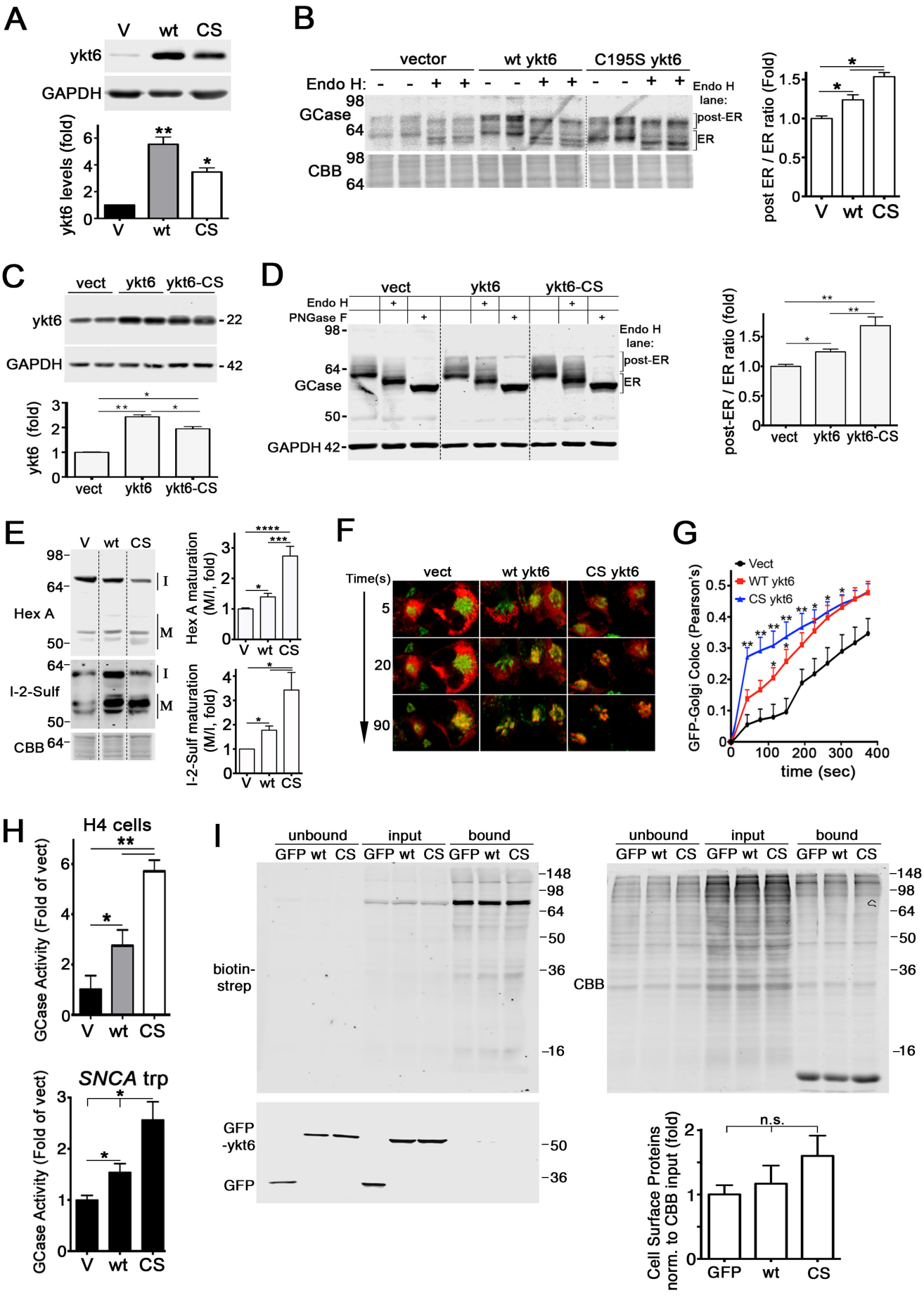
Supplemental Figure 3 linked to Figure 1. a-Synuclein forms a complex with ykt6 and disrupts its Golgi localization. A) Western blot analysis of sec22b in A53T or isogenic corrected (corr) iPSn at day 60 (3 replicates shown). Quantification is on the right (n=6). Coomassie brilliant blue (CBB) was used as a loading control. **B**) Western blot analysis of STX5 in A53T or corr iPSn at day 60 and 90. CBB and GAPDH were used as loading

controls. Quantification is on the right. **C)** Co-IP of a-syn and ykt6 in inducible H4 cells expressing wt a-syn (tet-off) (n=2). **D**) a-syn/ykt6 interactions were confirmed by analyzing immunodepleted (I.D.) fractions of H4 lysates, showing that a-syn can be immunodepleted by ykt6 antibodies (n=3). **E**) Co-IP analysis in PD patient *SNCA* trp iPSn at day 80 (n=3). **F**) Co-IP analysis of PD patient iPSn derived from idiopathic PD (iPD), or PD patients harboring a *GBA1* mutation (N370S / wt), *SNCA* trp or *PARK9* mutations (from ref (Mazzulli et al., 2016b)). **G**) Inducible H4 cells were used for co-IP of a-syn followed by western blot of STX5. **H**) Extracts from H4 cells were separated into cytosolic or membrane fractions followed by co-IP for a-syn / western blot for ykt6, demonstrating that ykt6 interacts with a-syn in cytosolic fractions. Below, co-IP of cytosol or membrane fractions from PD iPSn expressing *SNCA* trp, as done in panel F. **I)** Immunofluorescence analysis of H4 cells of Golgi (GM130, red) or ykt6 (green), showing that a-syn accumulation (- DOX) results in a more diffuse ykt6 staining (n=3 culture wells, data points represent individual cells). **J)** Gel filtration / western blot analysis of extracts from isogenic corrected (corr) or A53T PD iPSn at day 75. Complexes eluted at ca. 120A were quantified on the right. NSE indicates equal column loading. **K**) Quantifications of SEC/western blot analysis of post-mortem brain lysates, corresponding to the blots shown in Figure 1E (n=3). For all quantifications, values are the mean +/- SEM, *p<0.05, **p<0.01, ***p<0.001.

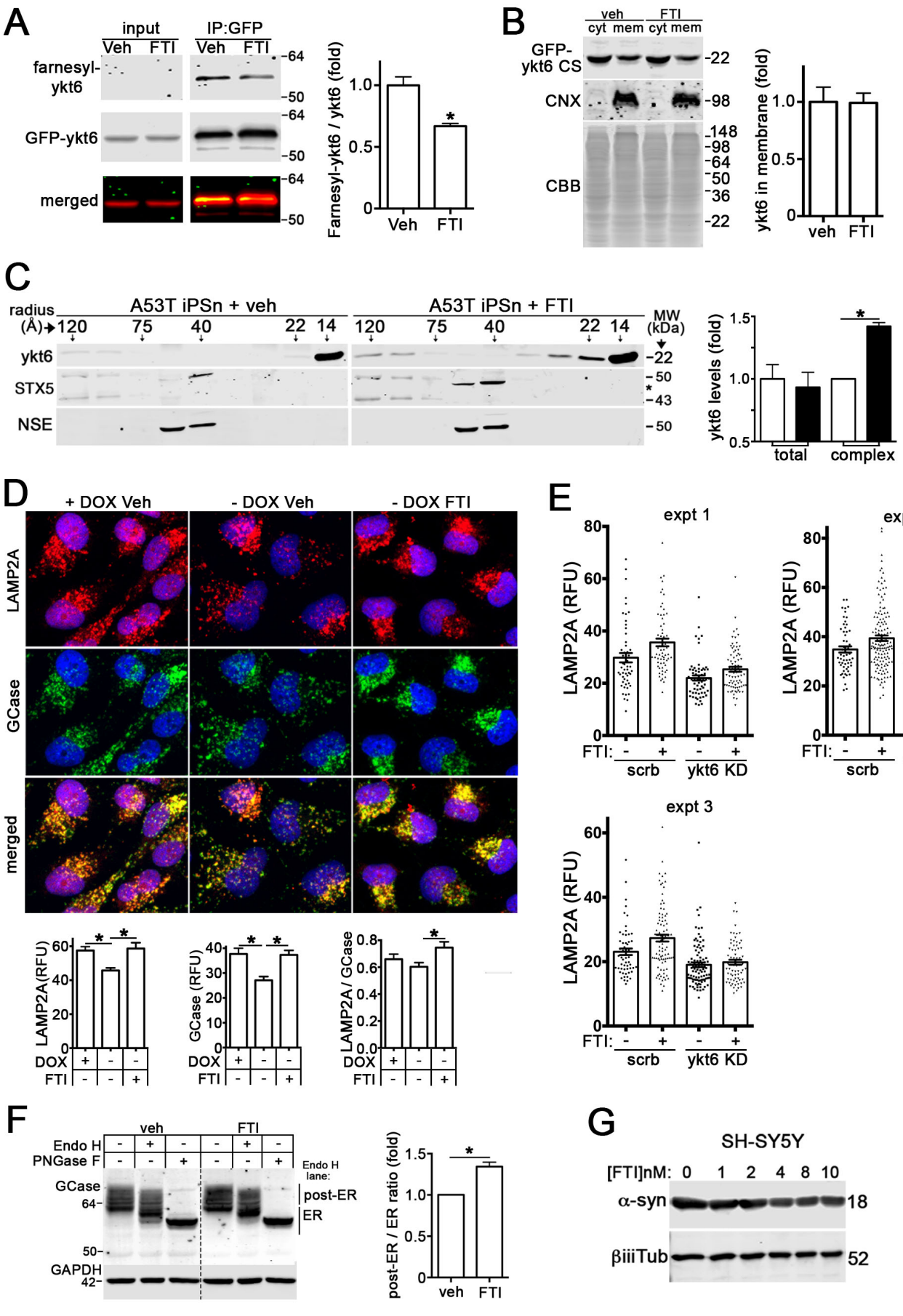


Supplemental Figure 4 linked to figures 2 and 3. The effects of ykt6 knock-down on the lysosomal system and characterization of GD-*SNCA* KO iPSCs. A) Western blot analysis of SH-SY5Y cells indicates that ykt6 KD results

in a-syn accumulation at 10 days post-differentiation, subsequent to lysosomal dysfunction. **B**) Protein secretion was measured by radioactive pulse-chase from the same culture wells that correspond to proteolysis data of figure 2H. **C**) Control iPSn were treated with 50mM sucrose for 7 days followed by membrane shift analysis of ykt6. **D**) Control iPSn were infected with scrambled (S) or ykt6 shRNA (KD) and treated with or without sucrose as done in panel B. LAMP1 levels were analyzed by western blot. GAPDH is a loading control (n=4). **E**) Cultures were treated as described in panel C, and analyzed for GCase maturation by western blot analysis (n=4). For all quantifications, values are the mean +/- SEM, *p<0.05. **F**) Analysis of off-target edits by CRISPR/Cas9 in *SNCA* knock out (KO) Gaucher disease iPSCs. The targeting strategy has been previously published (Zunke et al., 2018). Genomic sequences showing the closest homology to *SNCA* were analyzed for mis-matched mutations by T7 digestion, and compared to the parental GD line. PCR amplicons were analyzed by agarose gel electrophoresis stained with ethidium bromide. For all quantifications, values are the mean +/-SEM, *p<0.05. Student's T-test was used in panel A, while ANOVA with Tukey's post-hoc test was used in panels D and E.


A
V wt CS
ykt6
GAPDH
ykt6 levels (fold)
B
vector wt ykt6 C195S ykt6
Endo H:
Endo H lane:
post-ER
ER
GCase
CBB
post ER / ER ratio (Fold)
C
vect ykt6 ykt6-CS
ykt6
GAPDH
ykt6 (fold)
D
vect ykt6 ykt6-CS
Endo H
PNGase F
Endo H lane:
post-ER
ER
GCase
GAPDH
post-ER / ER ratio (fold)
E
V wt CS
Hex A
I-2-Sulf
CBB
Hex A maturation (M/I, fold)
I-2-Sulf maturation (M/I, fold)
F
Time(s)
vect wt ykt6 CS ykt6
5
20
90
G
Vect
WT ykt6
CS ykt6
GFP-Golgi Coloc (Pearson's)
time (sec)
H
H4 cells
GCase Activity (Fold of vect)
SNCA trp
I
unbound input bound
GFP wt CS
biotin-strep
CBB
GFP -ykt6
GFP
Cell Surface Proteins norm. to CBB input (fold)
n.s.

Supplemental Figure 5 linked to Figure 4. Enhancement of hydrolase trafficking and lysosomal activity by ykt6 wt or CS overexpression. A) H4 cells expressing a-syn were transfected to express ykt6 wild-type or ykt6 CS, followed by western blot analysis (n=3). **B**) Maturation of GCase was analyzed as in figure 4B (n=3). **C**) Overexpression of ykt6 wild-type or farnesyl-deficient mutant C195S (CS) in SH-SY5Y wt a-syn cells by lentiviral infection was assessed by western blot at 5 days post-infection (n=6). **D**) Maturation of GCase in SH-SY5Y wt a-syn cells expressing ykt6 constructs was assessed by endoglycosidase H or PNGase F digest followed by western blot for GCase (8E4). Quantification is shown on the right (n=6). **E**) Western blot analysis of lysosomal hydrolases hexosaminidase A and Iduronate-2-sulfatase reveals that ykt6 wt and CS enhance the levels of mature enzymes in *SNCA* trp iPSn. (n=3). **F**) ER-Golgi trafficking was directly assessed in H4 cells by live-cell microscopy using a RFP reporter fusion that can be induced to move out of the ER upon addition of a ligand (see methods). **G**) Upon induction, movement of the reporter into the Golgi was analyzed by co-localization analysis with a live-cell Golgi marker (green) at different times after induction. Initial Golgi entry was analyzed by integrating the area under the curve between 0- 200 seconds (n=4). **H**) Live-cell lysosomal GCase activity was measured as in Figure S2A in transfected H4 cells at 3 days post transfection (left) or in PD patient iPSn at 6 weeks post-infection (right). **I)** Analysis of cell surface proteins in SH-SY5Y wt a-syn cells, infected with GFP control, wild-type ykt6 (wt) or CS mutant, was done by cell-surface biotinylation, followed by pull-down of biotinylated proteins with streptavidin beads. Total cell surface proteins were analyzed by SDS-PAGE and detected with streptavidin-conjugated secondaries. CBB staining shows the total protein. Quantification is shown below (n=6). For all quantifications, values are the mean +/- SEM, *p<0.05, **p<0.01. N.S, not significant.


A
input
IP:GFP
Veh
FTI
farnesyl-ykt6
GFP-ykt6
merged
Farnesyl-ykt6 / ykt6 (fold)
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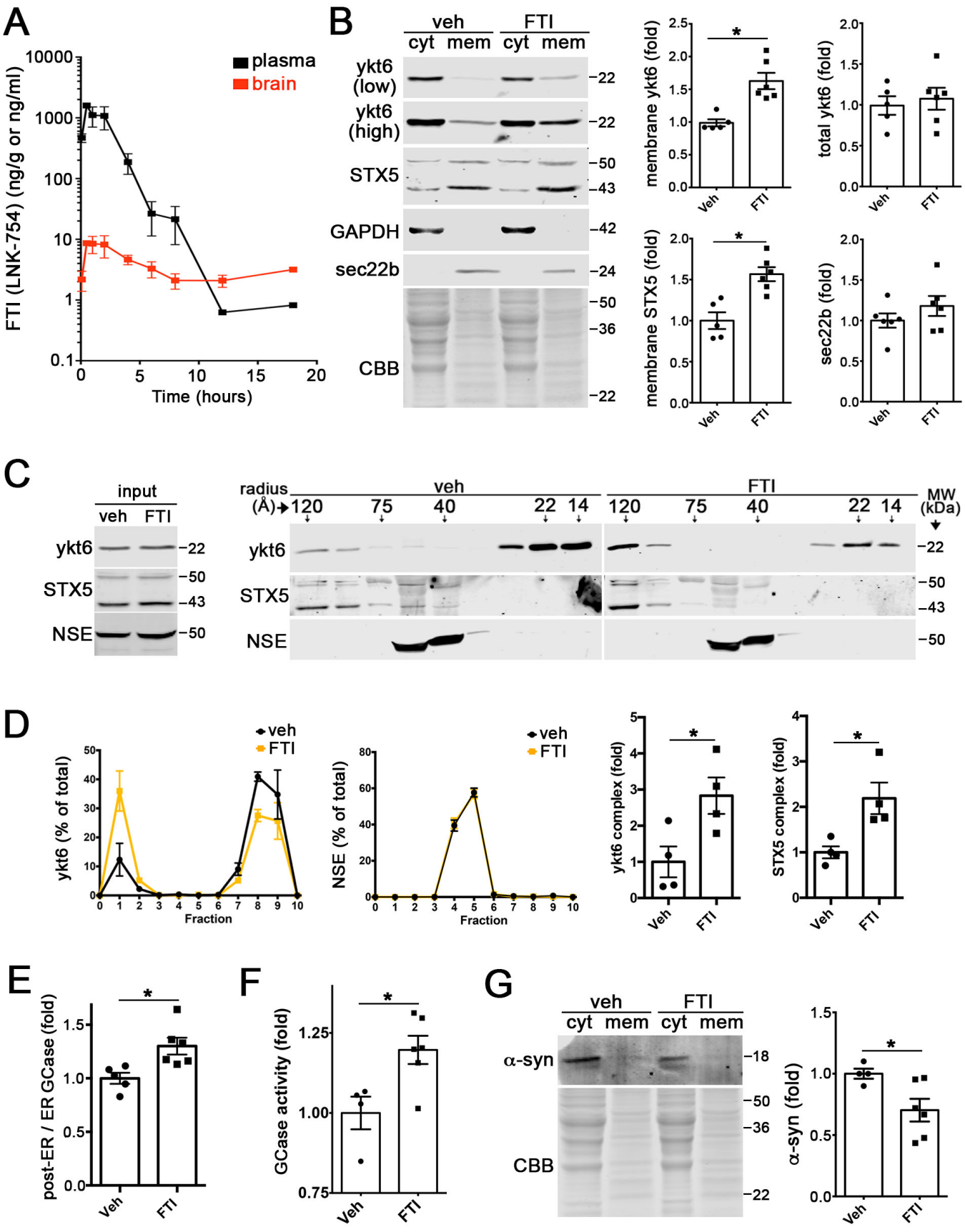
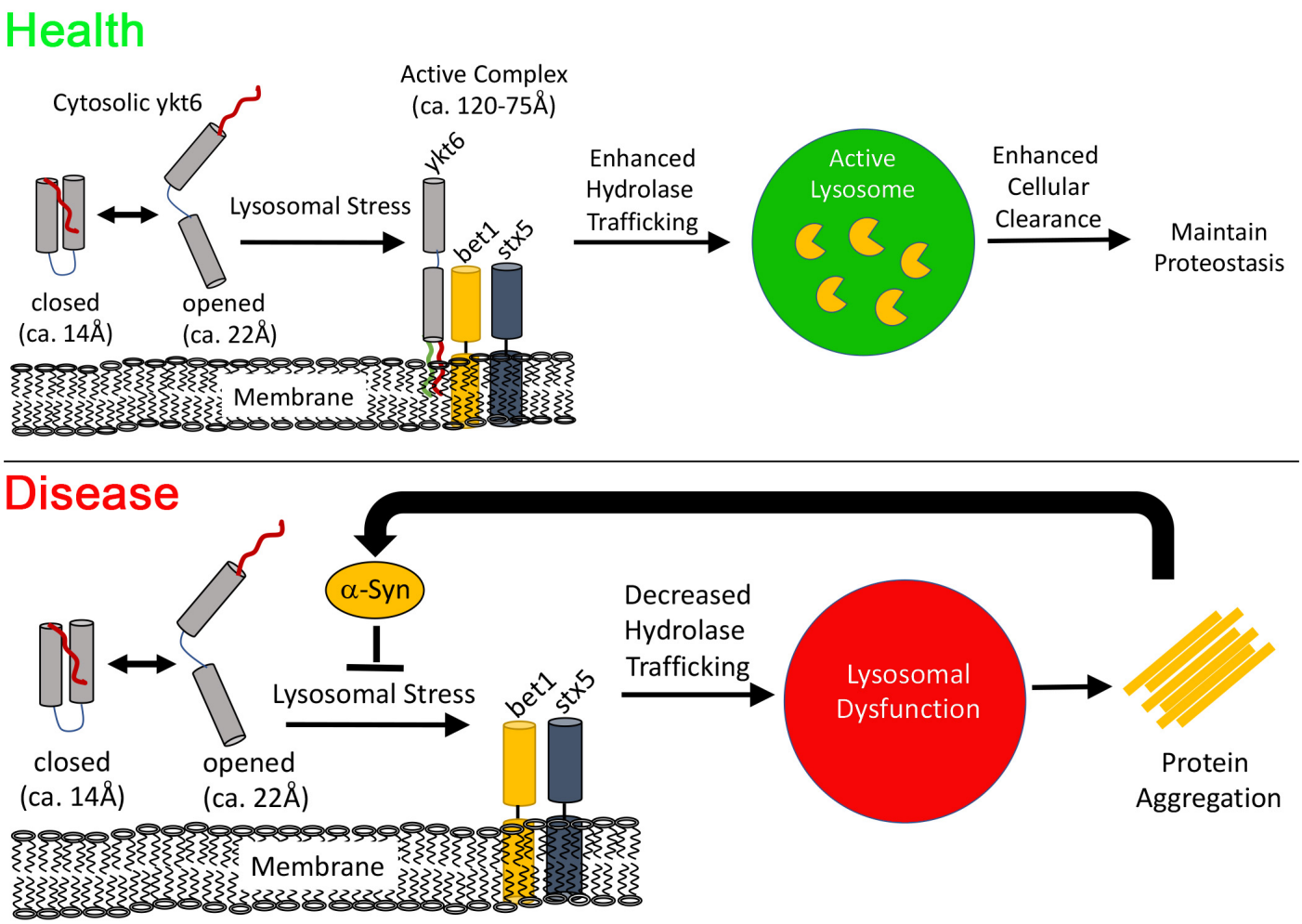
veh
FTI
cyt mem cyt mem
GFP-ykt6 CS
CNX
CBB
ykt6 in membrane (fold)
C
radius (Å)
A53T iPSn + veh
A53T iPSn + FTI
MW (kDa)
ykt6
STX5
NSE
ykt6 levels (fold)
total
complex
D
+ DOX Veh
- DOX Veh
- DOX FTI
LAMP2A
GCase
merged
LAMP2A(RFU)
GCase (RFU)
LAMP2A/ GCase
DOX
FTI
E
expt 1
expt 2
expt 3
LAMP2A (RFU)
scrb
ykt6 KD
F
Endo H
PNGase F
GCase
GAPDH
post-ER
ER
post-ER / ER ratio (fold)
G
SH-SY5Y
[FTI]nM: 0 1 2 4 8 10
α-syn
βiiiTub

Supplemental Figure 6 linked to Figures 5 and 6. The effects of FTI treatment on ykt6 and lysosomes. A) Farnesylation of ykt6 was measured in SH-SY5Y cells expressing GFP-ykt6. Cells treated with or without FTI (LNK-754) were cultured with farnesyl-azide to label farnesyl-modified proteins, immunoprecipitated with GFP-affinity beads, and then conjugated with phosphine-biotin. Biotin was detected with streptavidin-conjugated IRDye800, and normalized to total levels of GFP-ykt6. See methods for details. The merged blot image shows total ykt6 (red) and farnesylated forms (green) (n=3). **B**) Membrane shift analysis of SH-SY5Y cells stably expressing GFP-ykt6-CS, treated with 5nM FTI. Calnexin (CNX) and Coomassie blue (CBB) were used as a loading controls. **C**) Gel filtration / western blot analysis of FTI treated A53T iPSn. Ykt6 complex levels eluting at ca. 120A were quantified on the right (n=2). **D**) Analysis of GCase localization in H4 cells treated with 5nM FTI demonstrates an increase in the levels of lysosomal marker LAMP2A and GCase (n=3). **E**) Raw quantification data of LAMP2A immunofluorescence intensity of individual cells from H4 cells treated with FTI, +/- ykt6 KD. Each data point represents LAMP2A intensity from individual cells, from 3 different culture wells. **F**) Analysis of ER-Golgi trafficking of GCase by endo H resistance after treatment with FTI in SH-SY5Y cells overexpressing wt a-syn (n=3). **G**) Western blot analysis of Triton X-100 soluble a-syn in SH-SY5Y wt a-syn cells after treatment with various concentrations of FTI for 5 days. For all quantifications, values are the mean +/- SEM, *$p<0.05$.



Supplemental Figure 7 linked to Figures 7 and 8. FTI treatment of wild-type mice enhances ykt6 SNARE complexes, lysosomal activity, and reduces a-syn. A) Pharmacokinetic analysis of FTI (LNK-754) in plasma (ng/ml) or brain (ng/g) of wild-type mice (oral gavage, 9mg/kg, single dose). **B)** Wild-type mice (C57Bl/6) were

i.p. injected daily with FTI for 14 days at 0.9mg/kg and compared to vehicle injected mice. Extracts from the cortex were analyzed for cytosolic (cyt) and membrane (mem) distribution of ykt6 (veh, n=5; FTI, n=6). **C**) Representative gel filtration / western blot analysis of cortical extracts of veh or FTI injected wild-type mice. Inputs are shown on the left. NSE indicates equal column loading. **D**) Quantification of ykt6 and STX5 complexes (ca. 120A) from gel filtration analysis in C (n=4 mice). Fraction 1 corresponds to complexes at 120A, while fractions 7-9 correspond to ykt6 monomers at 22-14 A. **E**) The levels of post ER (endo H resistant) GCase were analyzed by western blot and post ER / ER ratios were quantified from the cortex of FTI treated mice (veh, n=5; FTI, n=6). **F**) GCase activity was assessed in cortical lysates and normalized to total protein (veh, n=4; FTI, n=6). **G**) a-Syn levels were quantified from cytosolic or membrane isolated fractions from the cortex of wild-type FTI treated mice (veh, n=4; FTI, n=6). For all quantifications, values are the mean +/- SEM, *p<0.05.



Supplemental Figure 8 linked to Figures 1-8. Self-propagating protein aggregation of α-syn occurs by disabling the physiological response to lysosomal stress. Top, Under physiological conditions, human midbrain neurons can respond to lysosomal stress by activating ykt6 SNARE assembly. This homeostatic response can rebalance proteostasis by promoting hydrolase trafficking into lysosomal compartments, increasing activity, and enhancing cellular clearance. Bottom, Under conditions of pathological protein accumulation, a-syn disables the lysosomal stress response by interfering with ykt6 SNARE assembly and inducing lysosomal dysfunction. This in turn, creates a permissive environment for aggregate persistence by rendering the lysosomal system incapable of meeting the degradation requirements of the cell, eventually resulting self-propagating protein aggregation and cell death.