Stem Cell Reports, Volume *12*

Supplemental Information

Modeling G2019S-LRRK2 Sporadic Parkinson's Disease in 3D Midbrain Organoids

Hongwon Kim, Hyeok Ju Park, Hwan Choi, Yujung Chang, Hanseul Park, Jaein Shin, Junyeop Kim, Christopher J. Lengner, Yong Kyu Lee, and Jongpil Kim

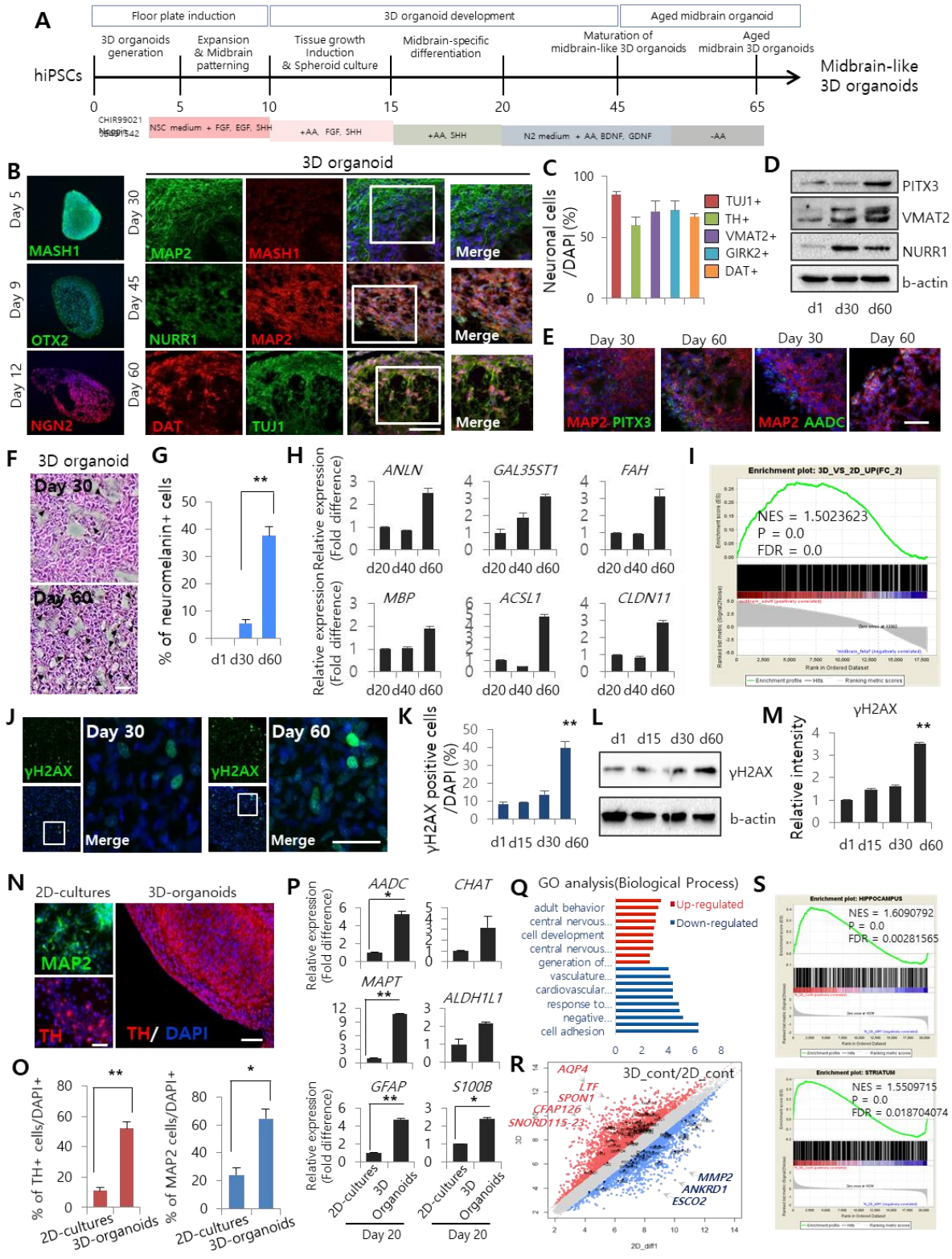
A
Floor plate induction
3D organoid development
Aged midbrain organoid
3D organoids generation
Expansion & Midbrain patterning
Tissue growth Induction & Spheroid culture
Midbrain-specific differentiation
Maturation of midbrain-like 3D organoids
Aged midbrain 3D organoids
hiPSCs
Midbrain-like 3D organoids
0
5
10
15
20
45
65
CHIR99021
NSC medium + FGF, EGF, SHH
+AA, FGF, SHH
+AA, SHH
N2 medium + AA, BDNF, GDNF
-AA
B
3D organoid
Day 5
Day 9
Day 12
Day 30
Day 45
Day 60
MASH1
OTX2
NGN2
MAP2
NURR1
DAT
MASH1
MAP2
TUJ1
Merge
C
Neuronal cells /DAPI (%)
TUJ1+
TH+
VMAT2+
GIRK2+
DAT+
D
PITX3
VMAT2
NURR1
b-actin
d1 d30 d60
E
Day 30
Day 60
MAP2 PITX3
MAP2 AADC
F
3D organoid
Day 30
Day 60
G
% of neuromelanin+ cells
d1 d30 d60
H
Relative expression (Fold difference)
ANLN
GAL35ST1
FAH
MBP
ACSL1
CLDN11
d20 d40 d60
I
Enrichment plot: 3D_VS_2D_UP(FC_2)
NES = 1.5023623
P = 0.0
FDR = 0.0
J
γH2AX
Day 30
Day 60
Merge
K
γH2AX positive cells /DAPI (%)
d1 d15 d30 d60
L
d1 d15 d30 d60
γH2AX
b-actin
M
γH2AX
Relative intensity
N
2D-cultures
3D-organoids
MAP2
TH
TH/ DAPI
O
% of TH+ cells/DAPI+
% of MAP2 cells/DAPI+
2D-cultures
3D-organoids
P
Relative expression (Fold difference)
AADC
CHAT
MAPT
ALDH1L1
GFAP
S100B
2D-cultures
3D Organoids
Day 20
Q
GO analysis(Biological Process)
Up-regulated
Down-regulated
adult behavior
central nervous...
cell development
central nervous...
generation of...
vasculature...
cardiovascular...
response to...
negative...
cell adhesion
R
3D_cont/2D_cont
AQP4
LTF
SPON1
CFAP126
SNORD115-23
MMP2
ANKRD1
ESCO2
2D_diff1
S
Enrichment plot: HIPPOCAMPUS
NES = 1.6090792
P = 0.0
FDR = 0.00281565
Enrichment plot: STRIATUM
NES = 1.5509715
P = 0.0
FDR = 0.018704074

Figure S1. Related to Figure 1. (A) Schematic of organizing midbrain 3D organoids from hiPSCs. Timeline of various differentiation protocols for generating efficacious and optimal 3D organoids. (B) Representative immunofluorescence images of the stages of 3D organoid generation for 2 months. Scale bar = 100 μm. (C) The percentage of TUJ1-, TH-, VMAT2-, DAT- and GIRK2-positive midbrain dopaminergic neurons in midbrain 3D organoids at day 60. (D) Western blot analysis of AADC, PITX3, VMAT2 and NURR1 in immunoprecipitates of midbrain 3D organoids. (E) Representative images for mature dopaminergic neuron marker, PITX3 and AADC at 30 and 60 days. (F) Fontana-masson staining showing neuromelanin positive cells in midbrain 3D organoids at 30 and 60 days. Scale bar=50 μm. (G) Quantification of the number of neuromelanin-positive cells in midbrain 3D organoids. Data represent the mean ± SEM. *ANOVA*, *p<0.05, **p<0.01 (n=3 per sample). (H) Age-associated genes *ANLN*, *GAL35ST1*, *FAH*, *MBP*, *ACSL1* and *CLDN11* were increased in midbrain 3D organoids at 60 days. (I) Gene set enrichment analysis of the microarray data showing the considerable enrichment of human aged brain compared to fetal brain (GSM2639572 and GSM175904, http://www.brain-map.org). (J) Immunostaining of phosphorylation of histone H2AX (γH2AX) in midbrain 3D organoids at day 30 and 60. Scale bar =50 μm. (K) The percentage of gamma-H2AX/DAPI-positive cells in midbrain 3D organoids. Data represent the mean ± SEM. *ANOVA*, *p<0.05, **p<0.01 (n=3 per sample). (L and M) Western blot analysis shows an increase in gamma-H2AX levels at day 60. Data represent the mean ± SEM. *ANOVA*, *p<0.05, **p<0.01. (N) Representative immunofluorescence images of MAP2- and TH-positive dopaminergic neurons in 3D cultures and 2D cultures. Scale bar=100 μm. (O) Quantification of the number of TH- and MAP2-positive cells among all DAPI-positive cells in 3D and 2D cultures at 20 days. Data represent the mean ± SEM. *ANOVA*, *p<0.05, **p<0.01 (n=3 per sample). (P) Real-time qPCR analysis of the expression of the neuronal markers *AADC*, *CHAT* and *MAPT* and the astrocyte markers *GFAP*, *S100B* and *ALDH1L1* at 20 days. Data represent the mean ± SEM. *ANOVA*, *p<0.05, **p<0.01 (n=3 per sample). (Q) Scatter plot of the microarray expression data comparing midbrain 3D organoids and 2D cultures. (R) Gene ontology analysis for biological processes in midbrain 3D organoids and 2D cultures. (S) Gene set enrichment analysis of the microarray data from midbrain 3D organoids compared to 2D cultures showing the considerable enrichment of human brain tissues, cerebral cortex and striatum.

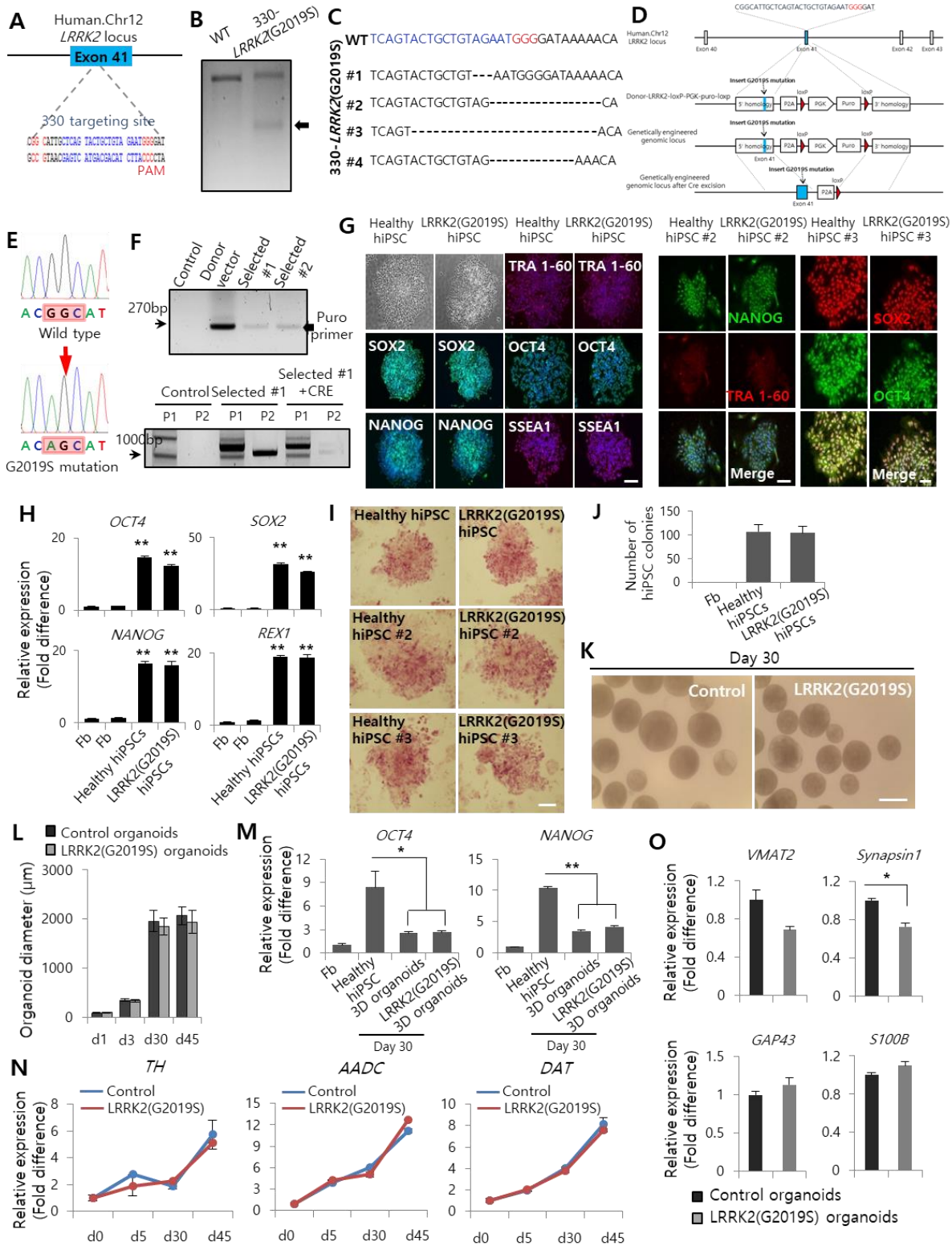
A
Human.Chr12
LRRK2 locus
Exon 41
330 targeting site
PAM
B
WT
330-LRRK2(G2019S)
C
330-LRRK2(G2019S)
WT TCAGTACTGCTGTAGAATGGGGATAAAAACA
#1 TCAGTACTGCTGT---AATGGGGATAAAAACA
#2 TCAGTACTGCTGTAG----------CA
#3 TCAGT----------ACA
#4 TCAGTACTGCTGTAG----------AAACA
D
Human.Chr12 LRRK2 locus
Exon 40
Exon 41
Exon 42
Exon 43
Insert G2019S mutation
Donor-LRRK2-loxP-PGK-puro-loxp
5' homolog
P2A
loxP
PGK
Puro
3' homolog
Genetically engineered genomic locus
Genetically engineered genomic locus after Cre excision
E
A C G G C A T
Wild type
A C A G C A T
G2019S mutation
F
Control
Donor vector
Selected #1
Selected #2
270bp
Puro primer
Control
Selected #1
Selected #1 +CRE
P1
P2
1000bp
G
Healthy hiPSC
LRRK2(G2019S) hiPSC
Healthy hiPSC #2
LRRK2(G2019S) hiPSC #2
Healthy hiPSC #3
LRRK2(G2019S) hiPSC #3
TRA 1-60
SOX2
OCT4
NANOG
SSEA1
Merge
H
OCT4
SOX2
NANOG
REX1
Relative expression (Fold difference)
Fb
Healthy hiPSCs
LRRK2(G2019S) hiPSCs
I
Healthy hiPSC
LRRK2(G2019S) hiPSC
Healthy hiPSC #2
LRRK2(G2019S) hiPSC #2
Healthy hiPSC #3
LRRK2(G2019S) hiPSC #3
J
Number of hiPSC colonies
Fb
Healthy hiPSCs
LRRK2(G2019S) hiPSCs
K
Day 30
Control
LRRK2(G2019S)
L
Control organoids
LRRK2(G2019S) organoids
Organoid diameter (μm)
d1
d3
d30
d45
M
OCT4
NANOG
Relative expression (Fold difference)
Fb
Healthy hiPSC
3D organoids
LRRK2(G2019S) 3D organoids
Day 30
O
VMAT2
Synapsin1
GAP43
S100B
Relative expression (Fold difference)
Control organoids
LRRK2(G2019S) organoids
N
TH
AADC
DAT
Control
LRRK2(G2019S)
Relative expression (Fold difference)
d0
d5
d30
d45

Figure S2. Related to Figure 2. (A) The sequence of Cas9 targeting a locus within the human *LRRK2* gene. The single-guide RNA targeting site is marked in blue, and the 5'-NGG protospacer adjacent motif site is labeled in red. The arrow indicates the *LRRK2*-G2019S mutation site. (B) SURVEYOR mutation detection assay gel showing modification of the Cas9 targeting site by the single-guide RNA. (C) Representative sequences of indels in the human *LRRK2* gene targeted by CRISPR-Cas9. Deletions are indicated with a dash. (D) Schematic representation of the donor vector including the *LRRK2*-G2019S mutation site. Enlarged sequence represents CRISPR-Cas9-induced targeting site and genetically engineered genomic locus in exon 41 of *LRRK2* locus. (E) Sanger sequencing showing the G2019S mutation site in exon 41 of the *LRRK2* gene after the introduction of the Cas9 *LRRK2*-targeting and donor plasmids. (F) Representative images of PCR products using puromycin-specific primers to amplify puromycin-resistant hiPSC colonies. A CRE-transduced hiPSC colony was confirmed by the appropriate primers. (G) Representative immunofluorescence images of colonies positive for the pluripotency markers SOX2, NANOG, TRA1-60, OCT4, and SSEA1 among healthy and LRRK2-G2019S-targeted hiPSCs. Passage matched control and targeted iPSC lines was analyzed in the experiments. Scale bar=100 μm. (H) Quantitative RT-PCR analysis of healthy hiPSCs and LRRK2-G2019S hiPSCs for the pluripotency markers *OCT4*, *SOX2*, *NANOG*, and *REX1*. Data represent the mean ± SEM. *ANOVA* (n=3 per sample). (I) AP staining of healthy hiPSCs and LRRK2-G2019S hiPSCs. Scale bar=100 μm. (J) Quantification of the number of alkaline phosphatase-positive clones among healthy hiPSCs and LRRK2-G2019S hiPSCs. (K) Bright-field images of control and LRRK2-G2019S 3D organoids. Scale bar=1 mm. (L) Measurement of the size of control and LRRK2-G2019S 3D organoids at 45 days. (M) Real-time qPCR analysis of pluripotency marker (*OCT4* and *NANOG*) expression in control and LRRK2-G2019S 3D organoids at 30 days. Data represent the mean ± SEM. *ANOVA*, *p<0.05, **p<0.01 (n=3 per sample). (N) Relative quantification of the dopaminergic neuronal markers *TH*, *AADC*, and *DAT* in control and LRRK2-G2019S 3D organoids using qRT-PCR. (O) Quantitative RT-PCR analysis of control and LRRK2-G2019S 3D organoids for neuronal markers (*VMAT2*, *Synapsin1*, and *GAP43*) and an astrocyte marker (*S100B*) at 60 days. Data represent the mean ± SEM. *ANOVA*, *p<0.05, **p<0.01 (n=3 per sample).

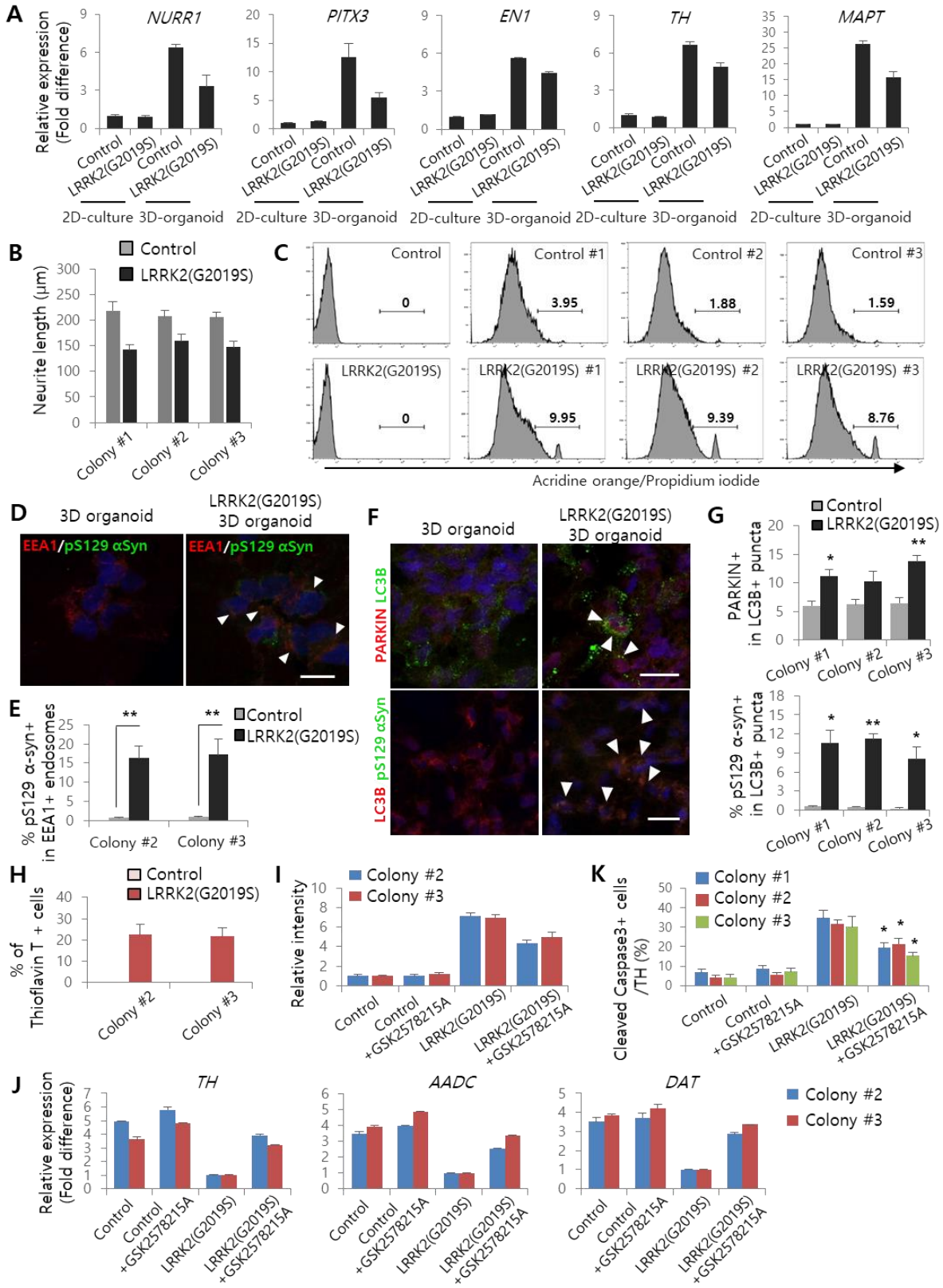
A
NURR1
PITX3
EN1
TH
MAPT
Relative expression (Fold difference)
Control
LRRK2(G2019S)
2D-culture
3D-organoid
B
Control
LRRK2(G2019S)
Neurite length (μm)
Colony #1
Colony #2
Colony #3
C
Control
Control #1
Control #2
Control #3
LRRK2(G2019S)
LRRK2(G2019S) #1
LRRK2(G2019S) #2
LRRK2(G2019S) #3
0
3.95
1.88
1.59
9.95
9.39
8.76
Acridine orange/Propidium iodide
D
3D organoid
LRRK2(G2019S) 3D organoid
EEA1/pS129 αSyn
E
% pS129 α-syn+ in EEA1+ endosomes
F
PARKIN LC3B
LC3B pS129 αSyn
G
PARKIN+ in LC3B+ puncta
% pS129 α-syn+ in LC3B+ puncta
H
% of Thioflavin T + cells
I
Colony #2
Colony #3
Relative intensity
Control
Control +GSK2578215A
LRRK2(G2019S)
LRRK2(G2019S) +GSK2578215A
K
Colony #1
Colony #2
Colony #3
Cleaved Caspase3+ cells /TH (%)
J
TH
AADC
DAT

Figure S3. Related to Figure2 and Figure 3. (A) Relative quantification of the mature neuronal marker *NURR1*, *PITX3*, *EN1*, *TH* and *MAPT* in control and LRRK2-G2019S 3D organoids compared to control and LRRK2-G2019S 2D cultures. Data represent the mean ± SEM. *ANOVA* (n=3 per sample). (B) Measurement of the neurite length of control and LRRK2-G2019S expressing dopaminergic neurons in 3D organoids from each 3 individuals. Data represent mean ± SEM (n=5 per sample). (C) Fluorescence-activated cell sorting analysis of acridine orange/propidium idodide-positive cells from control and LRRK2-G2019S mutant 3D organoids. (D) Representative images showing colocalization analysis of EEA1-positive endosomes with pS129-α-synuclein-positive puncta. Scale bar=20 μm. (E) The percentage of pS129-α-synuclein- and EEA1-positive puncta among all EEA1-positive puncta from each 2 individuals. Data represent the mean ± SEM. *ANOVA*, *p<0.05, **p<0.01 (n=6 per sample). (F) Representative images showing colocalization analysis of LC3B-positive autophagy with pS129-α-synuclein-positive puncta and PARKIN-positive mitophagy. Scale bar=20 μm. (G) The percentage of pS129-α-synuclein- and PARKIN-positive puncta among all LC3B-positive puncta. Data represent the mean ± SEM. *ANOVA*, *p<0.05, **p<0.01 (n=4 per sample). (H) The percentage of Thioflavin T-positive cells from 2 individuals. Data represent the mean ± SEM. *ANOVA*, **p<0.01 (n=5 per sample). (I) Relative intensity of phosphorylated α-synuclein oligomer levels in LRRK2-G2019S 3D organoids with and without the LRRK2 kinase inhibitor. Data represent the mean ± SEM. *ANOVA*, *p<0.05, **p<0.01 (n=3 per sample). (J) Relative gene expression of *TH*, *AADC* and *DAT* in LRRK2-G2019S 3D organoids with and without the LRRK2 kinase inhibitor. Data represent the mean ± SEM. *ANOVA*, *p<0.05, **p<0.01 (n=3 per sample). (K) The percentage of cleaved caspase3-positive cells from each 3 individuals. Data represent the mean ± SEM. *ANOVA*, **p<0.01 (n=5 per sample).

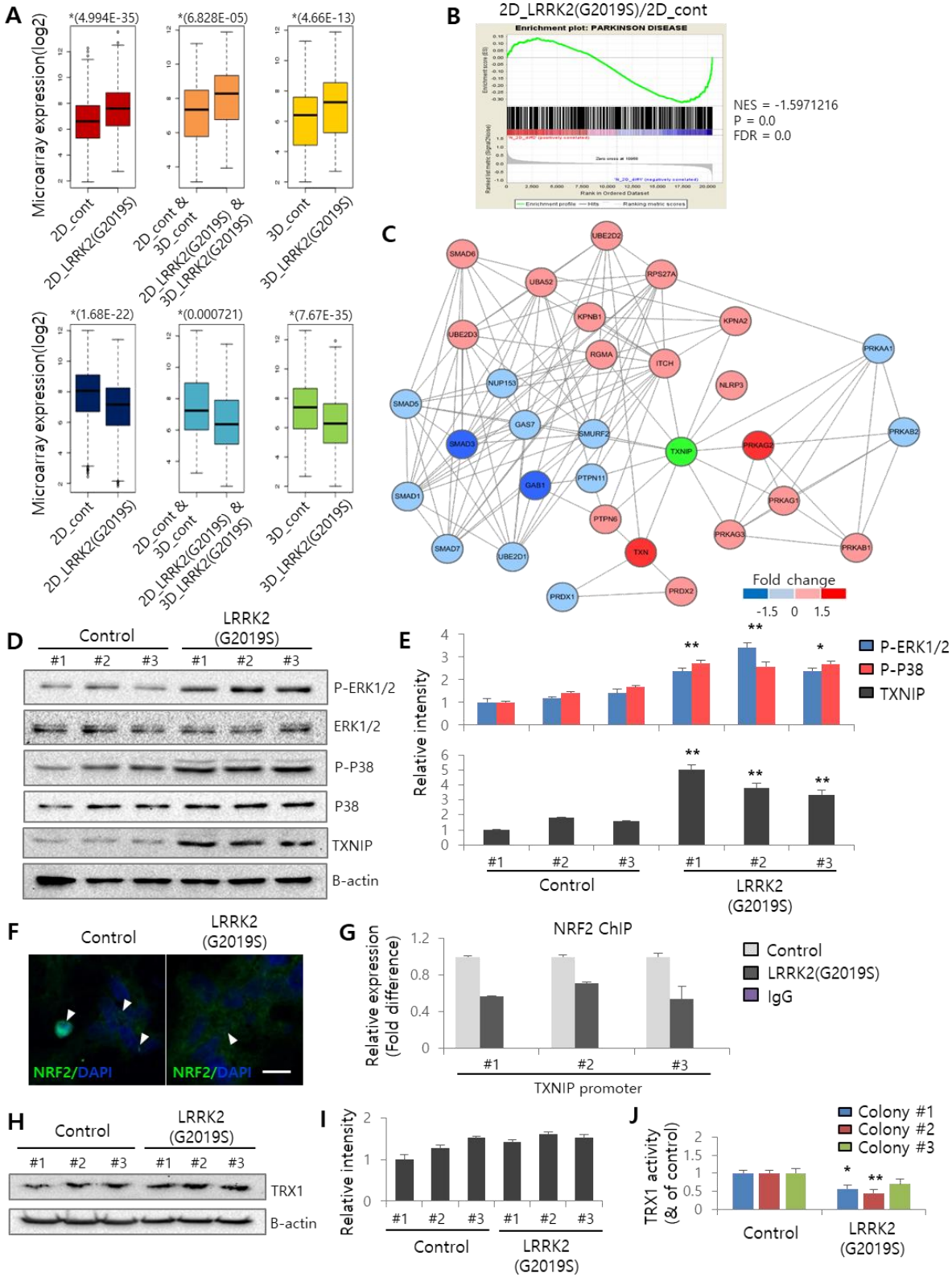
A
*(4.994E-35)
*(6.828E-05)
*(4.66E-13)
Microarray expression(log2)
2D_cont
2D_LRRK2(G2019S)
2D_cont & 3D_cont
2D_LRRK2(G2019S) & 3D_LRRK2(G2019S)
3D_cont
3D_LRRK2(G2019S)
*(1.68E-22)
*(0.000721)
*(7.67E-35)
B
2D_LRRK2(G2019S)/2D_cont
Enrichment plot: PARKINSON DISEASE
NES = -1.5971216
P = 0.0
FDR = 0.0
C
UBE2D2
SMAD6
UBA52
RPS27A
KPNB1
KPNA2
UBE2D3
RGMA
ITCH
PRKAA1
NUP153
NLRP3
SMAD5
GAS7
SMURF2
PRKAB2
SMAD3
TXNIP
PRKAG2
GAB1
PTPN11
SMAD1
PRKAG1
PTPN6
PRKAG3
SMAD7
UBE2D1
TXN
PRKAB1
PRDX1
PRDX2
Fold change
-1.5 0 1.5
D
Control
LRRK2 (G2019S)
#1 #2 #3 #1 #2 #3
P-ERK1/2
ERK1/2
P-P38
P38
TXNIP
B-actin
E
Relative intensity
P-ERK1/2
P-P38
TXNIP
Control
LRRK2 (G2019S)
F
Control
LRRK2 (G2019S)
NRF2/DAPI
NRF2/DAPI
G
NRF2 ChIP
Relative expression (Fold difference)
Control
LRRK2(G2019S)
IgG
TXNIP promoter
H
Control
LRRK2 (G2019S)
TRX1
B-actin
I
Relative intensity
Control
LRRK2 (G2019S)
J
TRX1 activity (& of control)
Colony #1
Colony #2
Colony #3
Control
LRRK2 (G2019S)

Figure S4. Related to Figure 4 and Figure 5. (A) The color of boxplots shows specific group color in Fig. 4A and yellow dotted line indicates average median of boxplots. (B) Gene set enrichment analysis of the microarray expression data from LRRK2-G2019S knock-in 2D cultures compared to wild-type 2D cultures. (C) Graph of the TXNIP protein interaction network in LRRK2-G2019S 3D organoids. Red circles indicate high expression, and blue circles represents low expression. (D and E) Western blots of P-ERK, P-P38 and TXNIP protein was determined in control and LRRK2-G2019S mutant 3D organoids from each 3 individuals. Expression of phosphorylated ERK and P38 was increased in midbrain-like 3D organoids including LRRK2-G2019S mutation. Data represent the mean ± SEM. *ANOVA*, *p<0.05, **p<0.01 (n=3 per sample). (F) Immunostaining of NRF2-positive cells in control and LRRK2-G2019S mutant 3D organoids. Scale bar=20 μm. (G) Chromatin immunoprecipitation analysis using NRF2 antibody (1:100, Cell signaling) in control and LRRK2-G2019S mutant 3D organoids from each 3 individuals. Immunoprecipitated DNA fragments was performed by real-time PCR using the human *TXNIP* promoter primers. Relative fold expression was calculated normalizing data to input. (H and I) Western blots of Thioredoxin1 protein was determined in control and LRRK2-G2019S mutant 3D organoids. (J) Thioredoxin1 activity was measured using Thioredoxin1 assay kit (Enzo life science) in control and LRRK2-G2019S mutant 3D organoids from each 3 individuals. Data represent the mean ± SEM. *ANOVA*, *p<0.05, **p<0.01 (n=4 per sample).

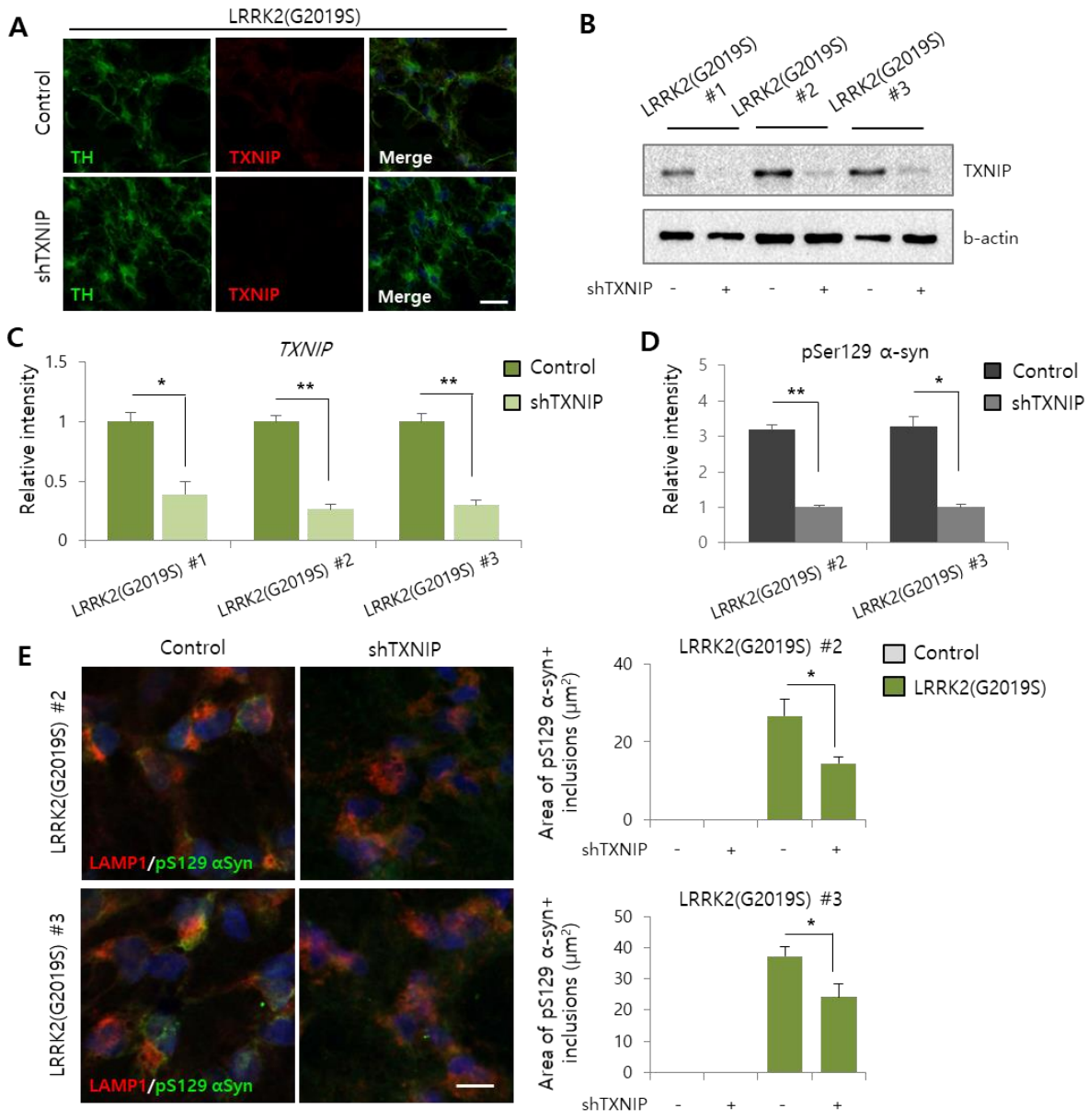
A
LRRK2(G2019S)
Control
shTXNIP
TH
TXNIP
Merge
B
LRRK2(G2019S) #1
LRRK2(G2019S) #2
LRRK2(G2019S) #3
TXNIP
b-actin
shTXNIP - + - + - +
C
TXNIP
Relative intensity
Control
shTXNIP
LRRK2(G2019S) #1
LRRK2(G2019S) #2
LRRK2(G2019S) #3
D
pSer129 α-syn
Relative intensity
Control
shTXNIP
LRRK2(G2019S) #2
LRRK2(G2019S) #3
E
Control
shTXNIP
LRRK2(G2019S) #2
LRRK2(G2019S) #3
LAMP1/pS129 αSyn
Area of pS129 α-syn+ inclusions (μm²)
Control
LRRK2(G2019S)
shTXNIP - + - +

Figure S5. Related to Figure 5. (A) Immunofluorescence analysis of TXNIP protein was decreased in sh*TXNIP* treated LRRK2-G2019S mutant 3D organoids from 3 individuals. (B and C) Western blots of TXNIP protein was determined in control and sh*TXNIP* treated LRRK2-G2019S mutant 3D organoids. Data represent the mean ± SEM. *ANOVA*, *p<0.05, **p<0.01 (n=3 per sample). (D) Relative intensity of phosphorylated α-synuclein oligomer levels in LRRK2-G2019S 3D organoids with and without *TXNIP*-shRNA. Data represent the mean ± SEM. *ANOVA*, *p<0.05, **p<0.01 (n=3 per sample). (E) Quantification of LAMP1+ and pS129-α-synuclein+ puncta showing a reduction of pS129-α-synuclein inclusions from each 2 individuals. Data represent the mean ± SEM. *ANOVA*, *p<0.05 (n=5 per sample).