Supplementary Materials

Figure titles and legends

Figure S1: Impaired CCCP-induced TOM20 degradation in *PARK2* KO neurons

A) *PARK2* KO and control neurons were untreated or treated with vehicle (DMSO) or 10 μM CCCP for 24, 48, 72, and 96 hrs and immunofluorescence stained for TOM20 (red) and Hoechst (blue) to visualize mitochondria and nuclei as indicated. Scale bar: 10 μm. **B)** No significant differences in total cell numbers were observed upon CCCP or vehicle (0.1% DMSO) exposure although there was a clear trend towards fewer cells after 72 and 96 hrs. **C)** Quantification of TOM20+ mitochondrial area normalized to number of Hoechst+ nuclei showed that the mitochondrial area in control neurons was significantly reduced after 48, 72, and 96 hrs of CCCP treatment. In contrast, mitochondria were retained in the *PARK2* KO neurons. Vehicle (0.1% DMSO) did not affect area of TOM20 immunoreactivity. Data presented as mean±SEM, n=9 technical replicates, data from 3 independent differentiations, Significant differences are indicated by $^{**}p < 0.01$, $^{***}p < 0.001$ (control vs. *PARK2* KO), $^{\#}p < 0.05$ (untreated control vs. CCCP-treated control), one-way ANOVA followed by Dunnett's post hoc test for multiple comparisons

Figure S2: USP30 inhibition as a strategy to enhance mitophagy

A) USP30 opposes parkin-mediated ubiquitination. **B)** Western blotting and densitometric analysis demonstrating comparable levels of USP30 protein expression in *PARK2* KO and control neurons. Representative blots of two independent experiments.

Figure S3: Lactate dehydrogenase (LDH) release did not reveal cytotoxic effects of USP30 inhibitors (+/-CCCP)

Differentiated neurons were treated with with 0 µM (untreated), 0.75 µM, 1.5 µM, 3 µM, 6 µM of USP30i-37 and USP30i-3 inhibitors added 4 h prior to CCCP (10 µM, 48 h). Necrotic cell death was assessed by LDH analysis of conditioned media from **A)** control neurons treated with USP30i-37, **B)** *PARK2* KO neurons treated with USP30i-37, **C)** control neurons treated with USP30i-3, and **D)** *PARK2* KO neurons treated with USP30i-3. No significant cytotoxicity was observed after treatment in either cell line, but there was a tendency to increase with increasing dose. Data presented as mean ± SEM, n=9-15 technical replicates, data from 3 independent differentiations.

Figure S4: Immunofluorescence staining showed that 3 µM USP30 inhibitors did not affect the numbers of nuclei (DAPI), mature neurons (MAP2), or dopaminergic neurons (TH) (+/-CCCP)

Healthy control and *PARK2* KO neurons were treated with 0 µM (untreated), 0.75 µM, 1.5 µM, 3 µM, 6 µM of USP30i-37 and USP30i-3. Cells were fixed and stained for DAPI (blue), MAP2 (red), and TH (green). **A)** Representative immunoflorescence pictures of DAPI+, MAP2+, and TH+ cells in healthy control and *PARK2* KO iPSC-derived neurons. Scale bars: 200 µm. **B-E)** Quantification of total cell count in **B, C)** healthy control and **D, E)** *PARK2* KO neurons. The toxicity of USP30 compounds (decreased cell number and compromised cell morphology) increased in both cell lines at the highest concentrations. Data presented as mean±SEM, n=9 technical replicates, data from 3 independent differentiations. Significant differences are indicated by $^{*,\#}p < 0.05$, $^{**,\#\#}p < 0.01$, $^{***}p < 0.001$, one-way ANOVA followed by Dunnett's post hoc test for multiple comparisons.

Figure S5: Morphological assessment of nuclei in control and *PARK2* KO neurons after treatment with USP30i-37 and USP30i-3.

Differentiated neurons were treated with with 0 μM (untreated), 0.75 μM, 1.5 μM, 3 μM, 6 μM of USP30i-37 and USP30i-3 inhibitors added 4 h prior to CCCP (10 μM, 48 h). Nucelar morphology was assessed by quantification of **(A-B)** fragmented nuclei in control and *PARK2* KO neurons treated with **A)** USP30i-37 and **B)** USP30i-3, and **(C-D)** pycnotic nuclei in control and *PARK2* KO neurons treated with **C)** USP30i-37 and **D)** USP30i-3. Data presented as mean±SEM, n=9 technical replicates, data from 3 independent differentiations. Significant differences are indicated by *·$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ns: non significant, one-way ANOVA followed by Dunnett's post hoc test for multiple comparisons.

Figure S1

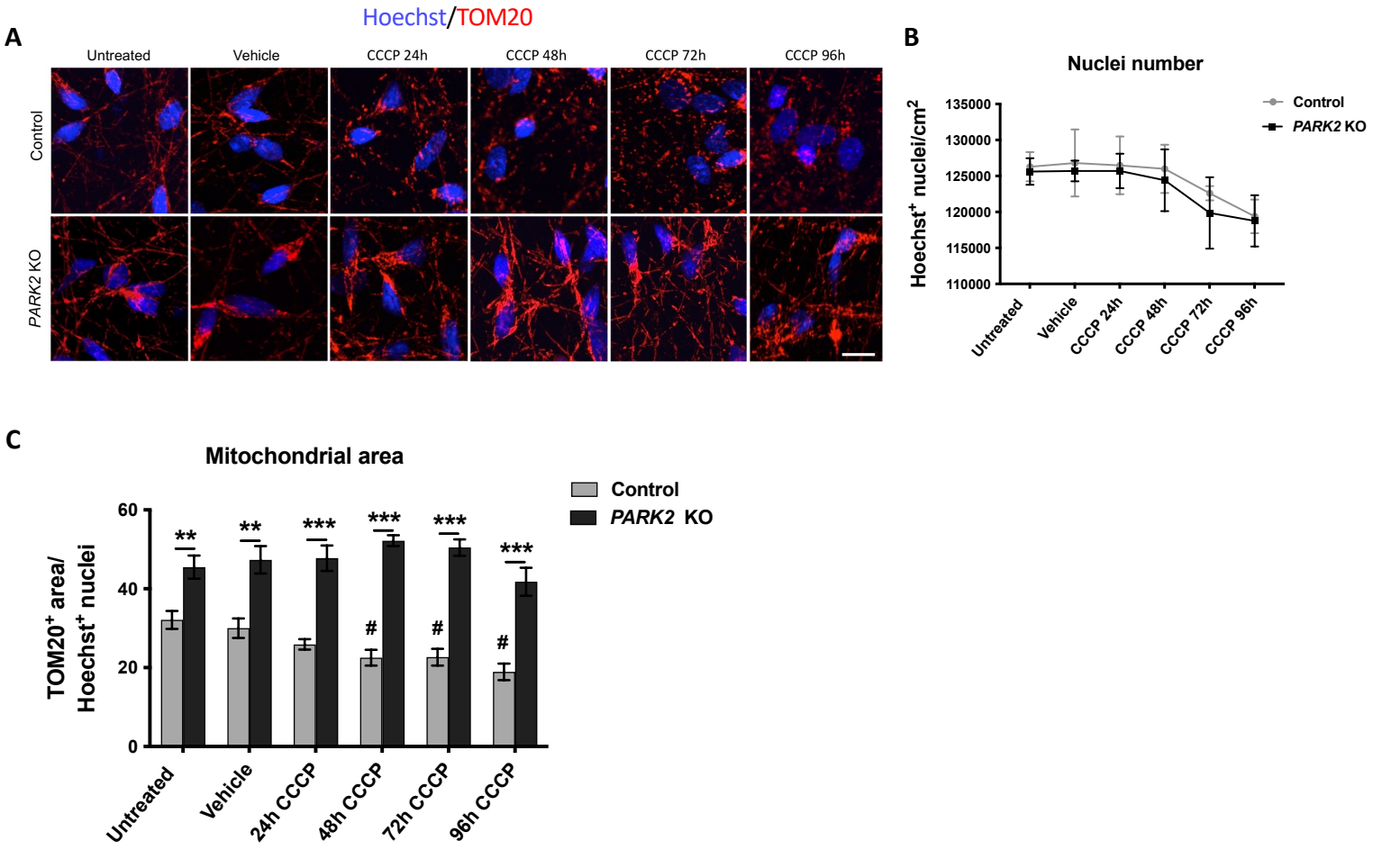
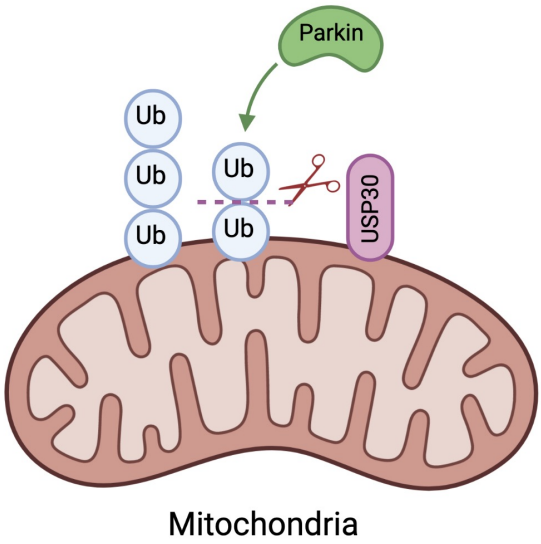

A
Hoechst/TOM20
Untreated
Vehicle
CCCP 24h
CCCP 48h
CCCP 72h
CCCP 96h
Control
PARK2 KO
B
Nuclei number
Hoechst+ nuclei/cm2
135000
130000
125000
120000
115000
110000
Control
PARK2 KO
Untreated
Vehicle
CCCP 24h
CCCP 48h
CCCP 72h
CCCP 96h
C
Mitochondrial area
TOM20+ area/ Hoechst+ nuclei
60
40
20
0
Control
PARK2 KO
**
**
***
***
***
***
#
#
#
Untreated
Vehicle
24h CCCP
48h CCCP
72h CCCP
96h CCCP

Figure S2

A


Parkin
Ub
Ub
Ub
Ub
Ub
USP30
Mitochondria

B

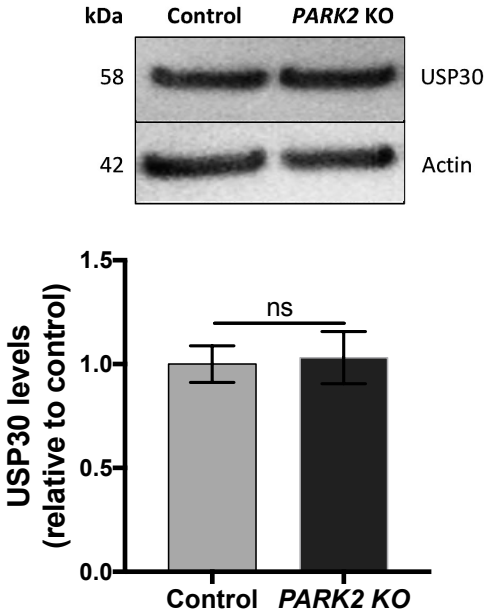
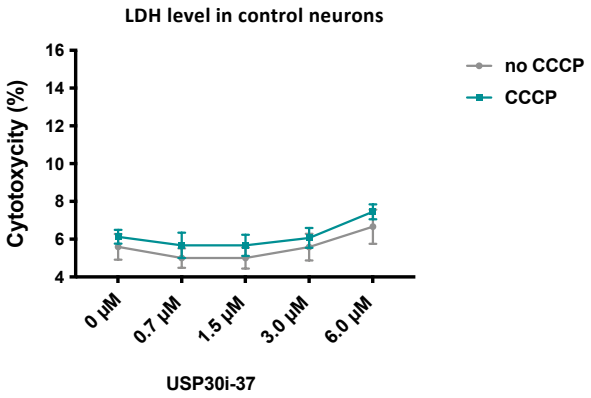
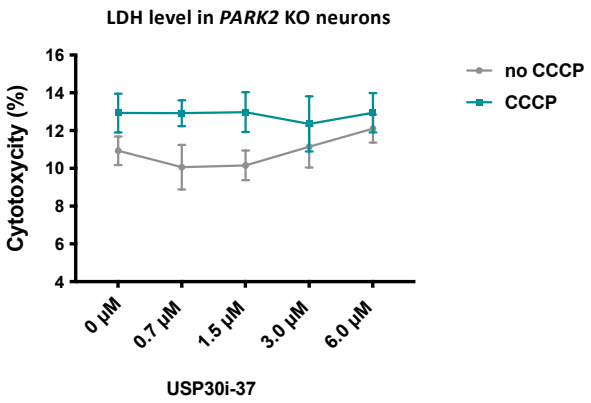

kDa
Control
PARK2 KO
58
USP30
42
Actin
USP30 levels (relative to control)
1.5
1.0
0.5
0.0
ns
Control
PARK2 KO

Figure S3

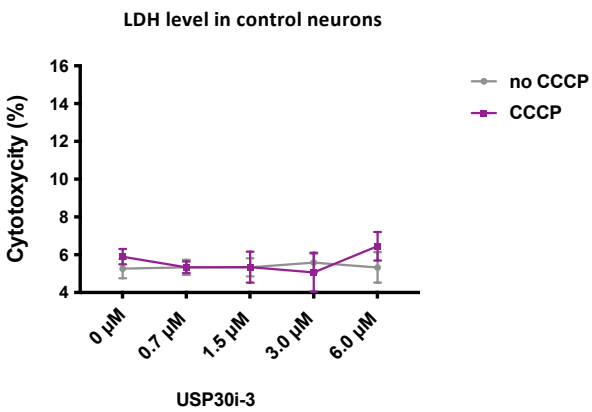
A



B



C



D

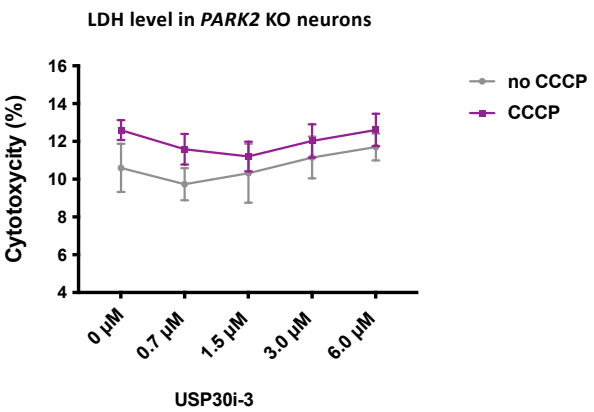
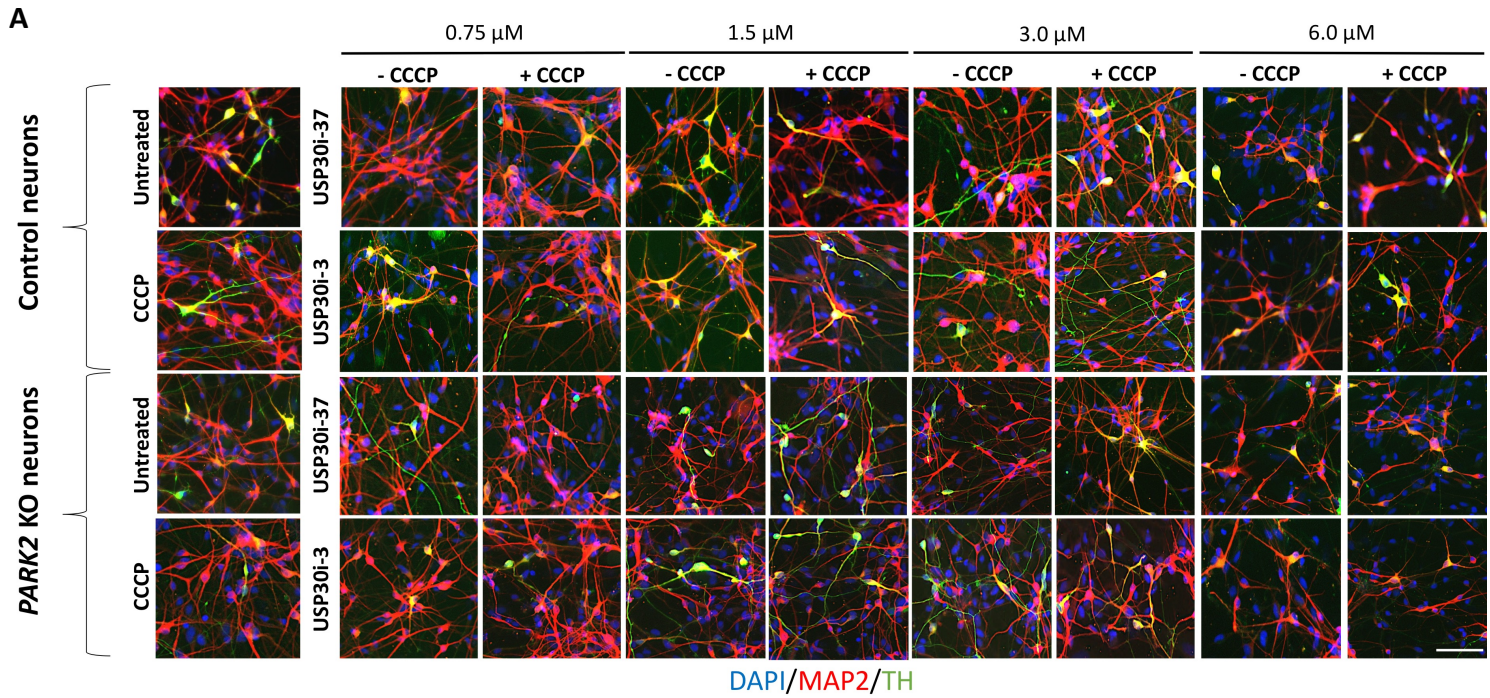
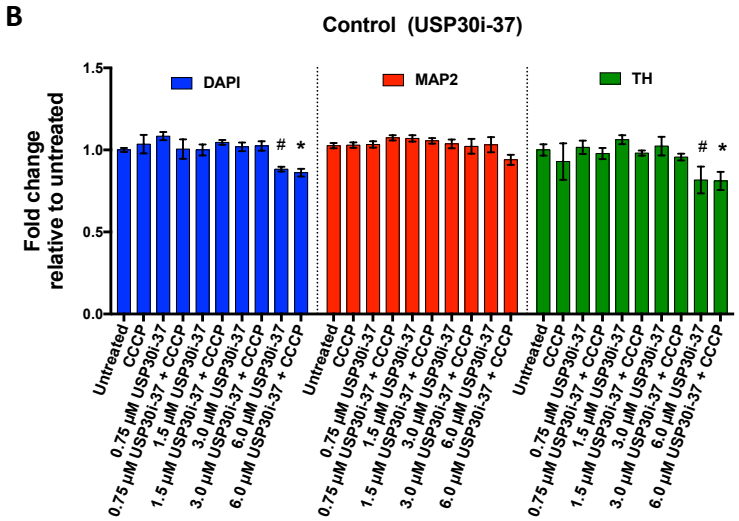
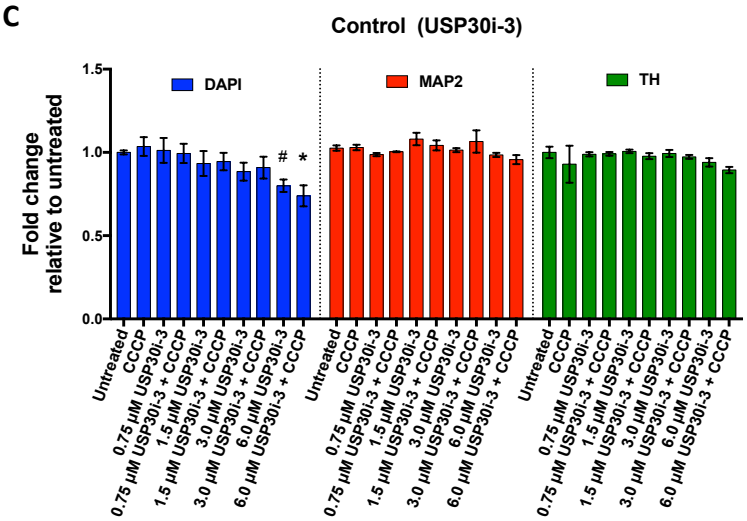
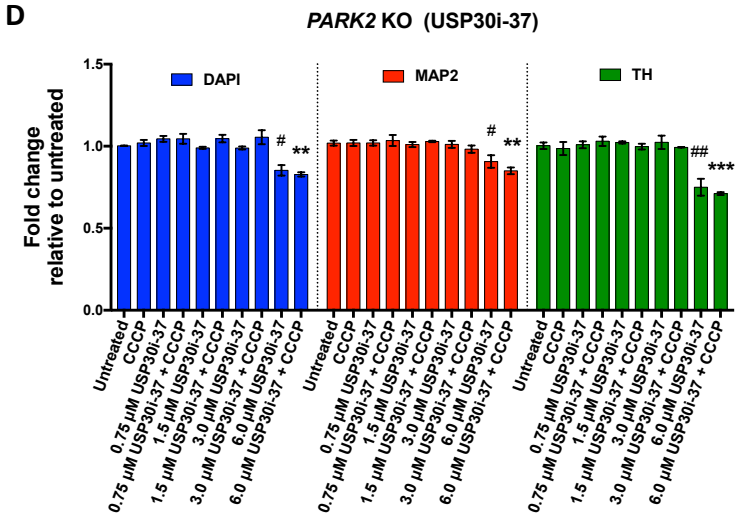


Figure S4


A
0.75 µM
1.5 µM
3.0 µM
6.0 µM
- CCCP
+ CCCP
Control neurons
PARK2 KO neurons
Untreated
CCCP
USP30i-37
USP30i-3
DAPI/MAP2/TH


B
Control (USP30i-37)
DAPI
MAP2
TH
Fold change relative to untreated
Untreated
CCCP
0.75 µM USP30i-37
0.75 µM USP30i-37 + CCCP
1.5 µM USP30i-37
1.5 µM USP30i-37 + CCCP
3.0 µM USP30i-37
3.0 µM USP30i-37 + CCCP
6.0 µM USP30i-37
6.0 µM USP30i-37 + CCCP


C
Control (USP30i-3)
DAPI
MAP2
TH
Fold change relative to untreated
Untreated
CCCP
0.75 µM USP30i-3
0.75 µM USP30i-3 + CCCP
1.5 µM USP30i-3
1.5 µM USP30i-3 + CCCP
3.0 µM USP30i-3
3.0 µM USP30i-3 + CCCP
6.0 µM USP30i-3
6.0 µM USP30i-3 + CCCP


D
PARK2 KO (USP30i-37)
DAPI
MAP2
TH
Fold change relative to untreated
Untreated
CCCP
0.75 µM USP30i-37
0.75 µM USP30i-37 + CCCP
1.5 µM USP30i-37
1.5 µM USP30i-37 + CCCP
3.0 µM USP30i-37
3.0 µM USP30i-37 + CCCP
6.0 µM USP30i-37
6.0 µM USP30i-37 + CCCP

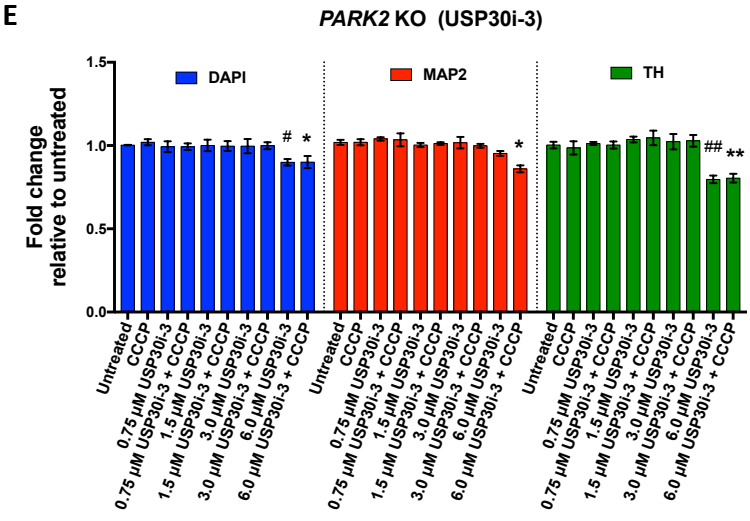
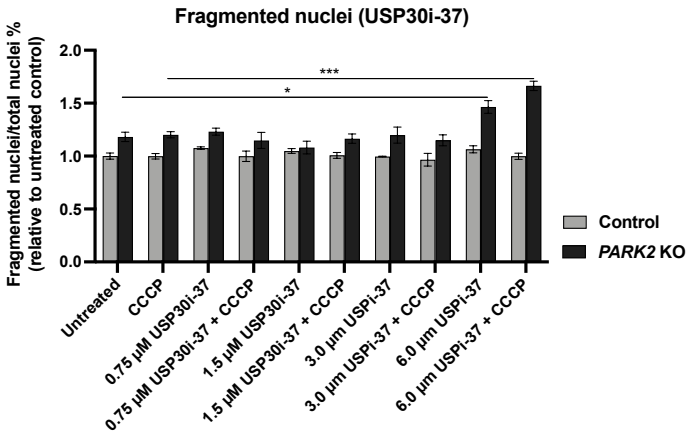
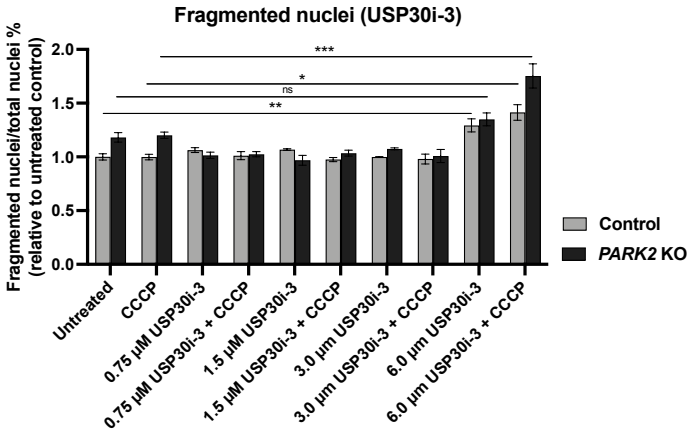

E
PARK2 KO (USP30i-3)
DAPI
MAP2
TH
Fold change relative to untreated
Untreated
CCCP
0.75 µM USP30i-3
0.75 µM USP30i-3 + CCCP
1.5 µM USP30i-3
1.5 µM USP30i-3 + CCCP
3.0 µM USP30i-3
3.0 µM USP30i-3 + CCCP
6.0 µM USP30i-3
6.0 µM USP30i-3 + CCCP

Figure S5

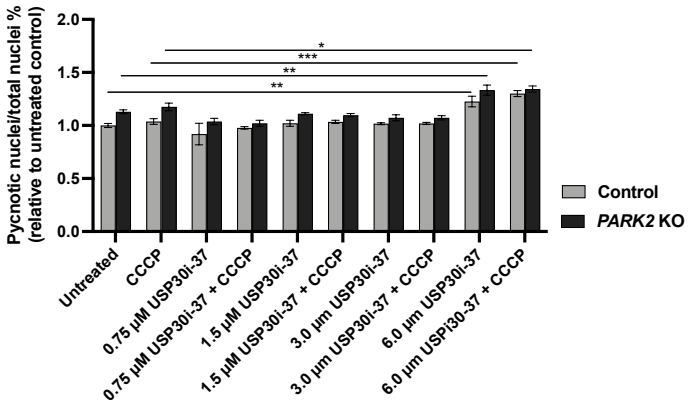
A



B



C



D