Supplemental Figures

Figure S1. CCCP induces autophagy in HCT116 cells

HCT116-Bax (-) parental cells (A) or the daughter cell line stably expressing GFP-LC3 (B-D) were treated with CCCP at various doses (A-B) or at 30 μm (C-D) in the absence or the presence of E64D (10 μM)/pepstatin A (10 μM) or CQ (10 μM)(D) for 16 hrs. The total lysates were subjected to western blot analysis with anti-LC3 (A), or anti-GFP (B). Cells were imaged for the formation of GFP-LC3 puncta (C) and the number of GFP-LC3 puncta per cell (mean+SD) was quantified. Scale bar: 20 μm

Figure S2. CCCP induces autophagy in MEF

(**A-C**). Wild type, Atg5-deficient and Atg7-deficient MEF were infected with Ad-GFP-LC3 overnight and then treated with vehicle control or CCCP (30 μM, A or indicated doses, B-C) for 6 hrs before being analyzed for the number of GFP-LC3 puncta per cell (mean+SD) with fluorescence microscopy and LC3-II formation with immunoblot analysis using anti-GFP. Scale bar: 20 μm. (**D-E**). Wild type MEF stably expressing GFP-LC3 (GFP-LC3-MEF) were treated with CCCP (30 μM) for the indicated time and analyzed for the number GFP-LC3 puncta per cell (mean+SD) (D) and LC3-II formation (E). (**F**). GFP-LC3-MEFs were treated with CCCP (30 μM) for 16 hrs, fixed and stained with anti-LAMP2. GFP-LC3 dots were colocalized with LAMP2 signals. Scale bar: 20 μm. (**G-H**). GFP-LC3-MEFs were treated with CCCP (30 μm) in the presence or absence of E64D (10 μM)/pepstatin A (10 μM) or CQ (10 μM) for 16 hours. The number of GFP-LC3 dots per cell (mean+SD) was quantified (G) and the total lysates were subjected to immunoblot analysis with anti-LC3 (H).

Figure S3. CCCP promotes a functional autophagy

(**A**). HeLa cells were transfected with the tandem GFP-mRFP-LC3 constructs for 24 hrs. The cells were either untreated or treated with CCCP (30 μM) for 6 hrs followed by fluorescence microscopy. Signals from both the GFP channel and the RFP channels were recorded and overlaid. Scale bar: 20 μm. (**B**) The number of total LC3 dots, LC3 dots with RFP signals only (mRFP dots) and LC3 dots with both GFP and RFP signals (yellow dots) in each cell were quantified (mean+SD). mRFP dots represented those LC3 dots that were localized in the degradative autolysosome where the GFP signals were preferentially quenched by the acidic environment.

Figure S4. Expression of Parkin in cell lines

Various cell lines were treated with or without CCCP (20 μM) for 6 hrs and total cell lysates were subjected to western blot analysis using anti-Parkin and anti-β-actin antibodies.

Figure S5. CCCP can induce an increase in p62/SQSTM1 puncta but not its translocation to the mitochondria in MEF expressing little Parkin

Wild type MEF transfected with EGFP-p62/SQSTM1 (green) were treated with CCCP (30 μm) for 6 hours and then fixed for immunostaining with anti-Tom 20 (red). Scale bar: 20 μm. An increase in p62 puncta could be observed in response to CCCP treatment, but very few of p62 puncta were colocalized with Tom20-positive mitochondria because the expression of Parkin in MEF was below the detectable level (Figure S4).

Figure S6. CCCP induces Parkin translocation and mitochondria elimination in 293 cells

(**A**). HEK 293 cells expressing mCherry-Parkin were treated with CCCP (20 μM) for 16 hrs. Cells were fixed and immunostained forTom20. Colocalization of Parkin with Tom20 could be seen in CCCP-treated cells. Many Parkin-positive cells also lost Tom20 staining. Scale bar: 20 μm. (**B-C**) The percentage of cells with Parkin translocation to the mitochondria (B) and the percentage of cells with less or no mitochondria in Parkin-negative and Parkin-positive populations (C) were quantified (mean+SD).

Figure S7. CCCP-induced LC3 punctation depends on ROS in HeLa cells

GFP-LC3-HeLa cells were treated with CCCP (20 μM) in the presence or absence of NAC (10 mM) for 16 hrs. Cells were fixed and examined by confocal microscopy. The number of GFP-LC3 puncta (mean+SD) was quantified. $p<0.01$, one way ANOVA. Scale bar: 20 μm.

Figure S8. Deletion of Nix affects rapamycin-induced GFP-LC3 punctation

Nix +/+ and Nix -/- MEF cells were infected with adenovirus GFP-LC3 (100 viral particle per cell) for overnight and the cells were either nontreated or treated with rapamycin (1 μM) for 6 hrs followed by fluorescence microscopy. The numbers of GFP-LC3 dots per cell (mea±SD) were quantified.

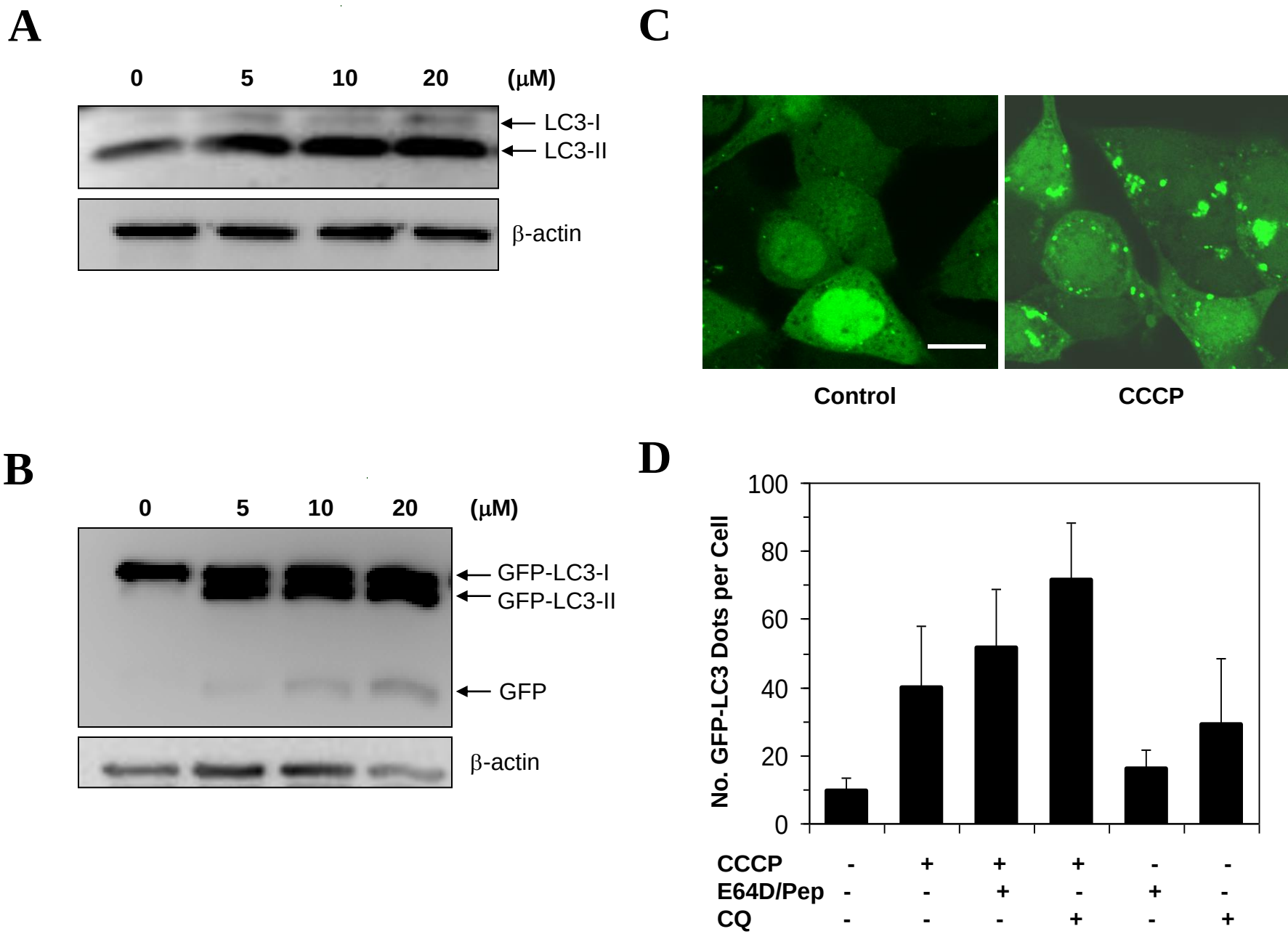

A
0
5
10
20
(μM)
LC3-I
LC3-II
β-actin
B
0
5
10
20
(μM)
GFP-LC3-I
GFP-LC3-II
GFP
β-actin
C
Control
CCCP
D
No. GFP-LC3 Dots per Cell
100
80
60
40
20
0
CCCP - + + + - -
E64D/Pep - - + - + -
CQ - - - + - +

Figure S2

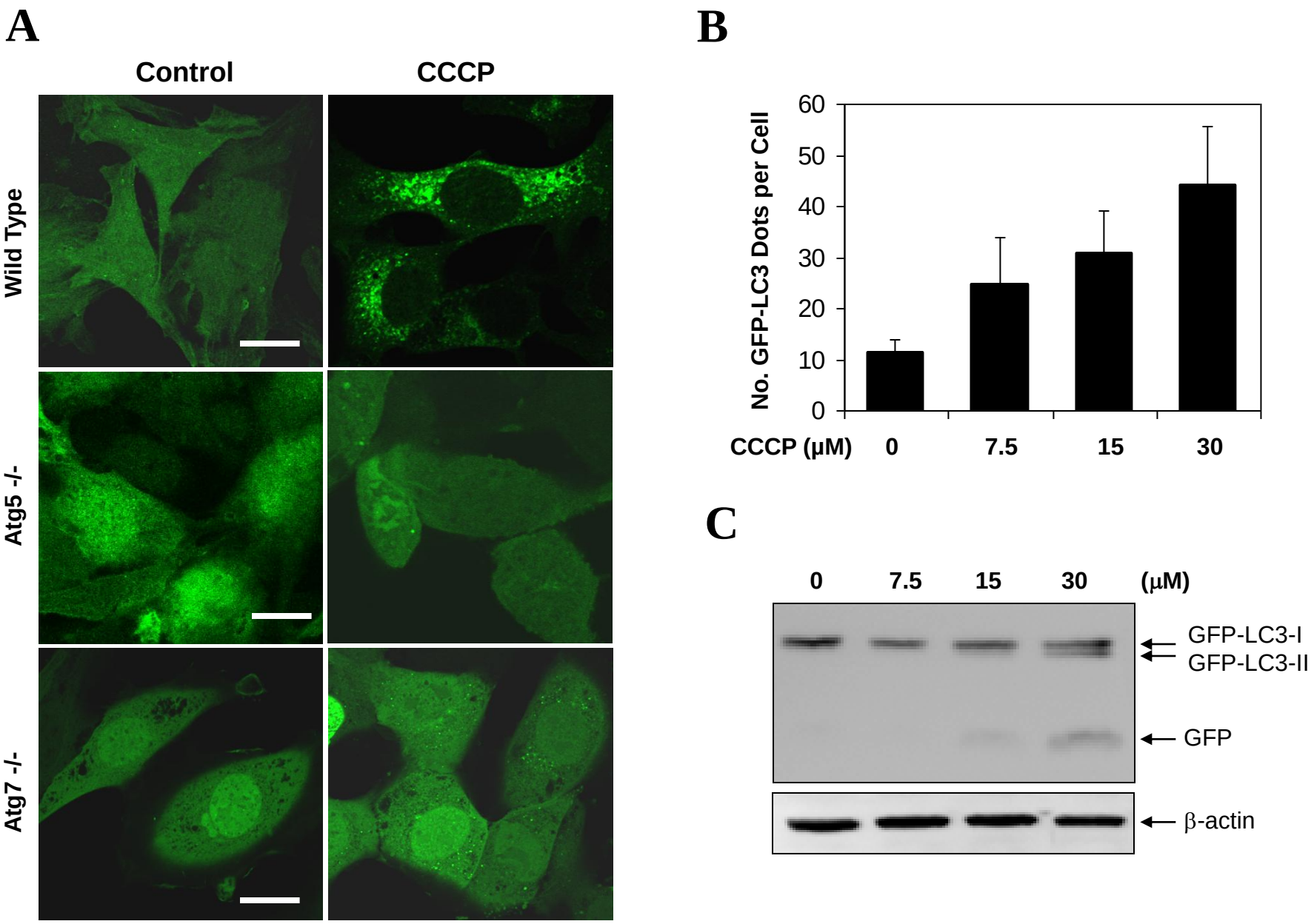
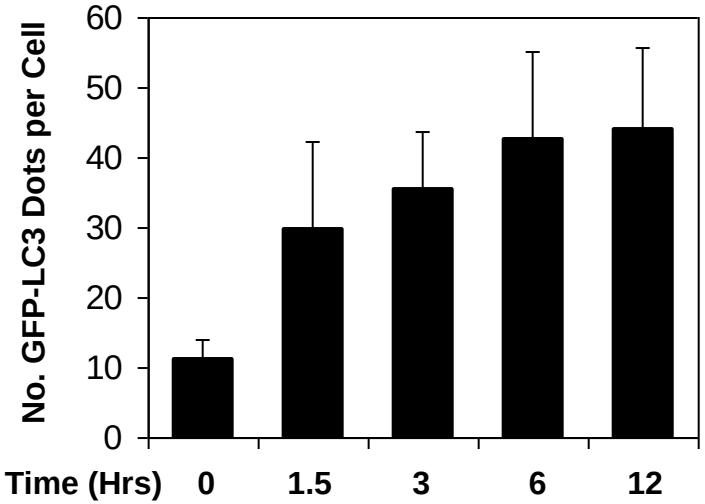
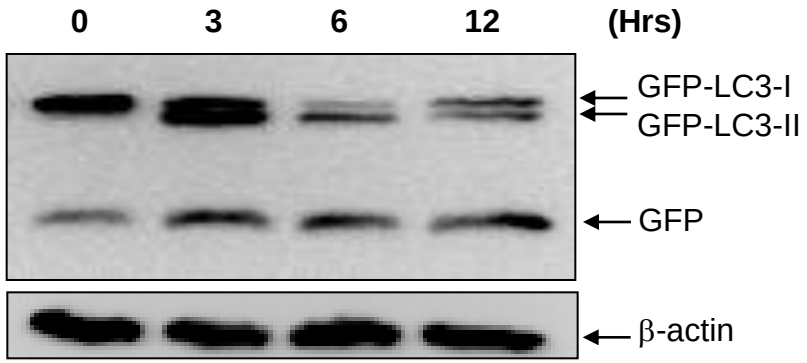

A
Control
CCCP
Wild Type
Atg5 -/-
Atg7 -/-
B
No. GFP-LC3 Dots per Cell
60
50
40
30
20
10
0
CCCP (μM)
0
7.5
15
30
C
0
7.5
15
30
(μM)
GFP-LC3-I
GFP-LC3-II
GFP
β-actin

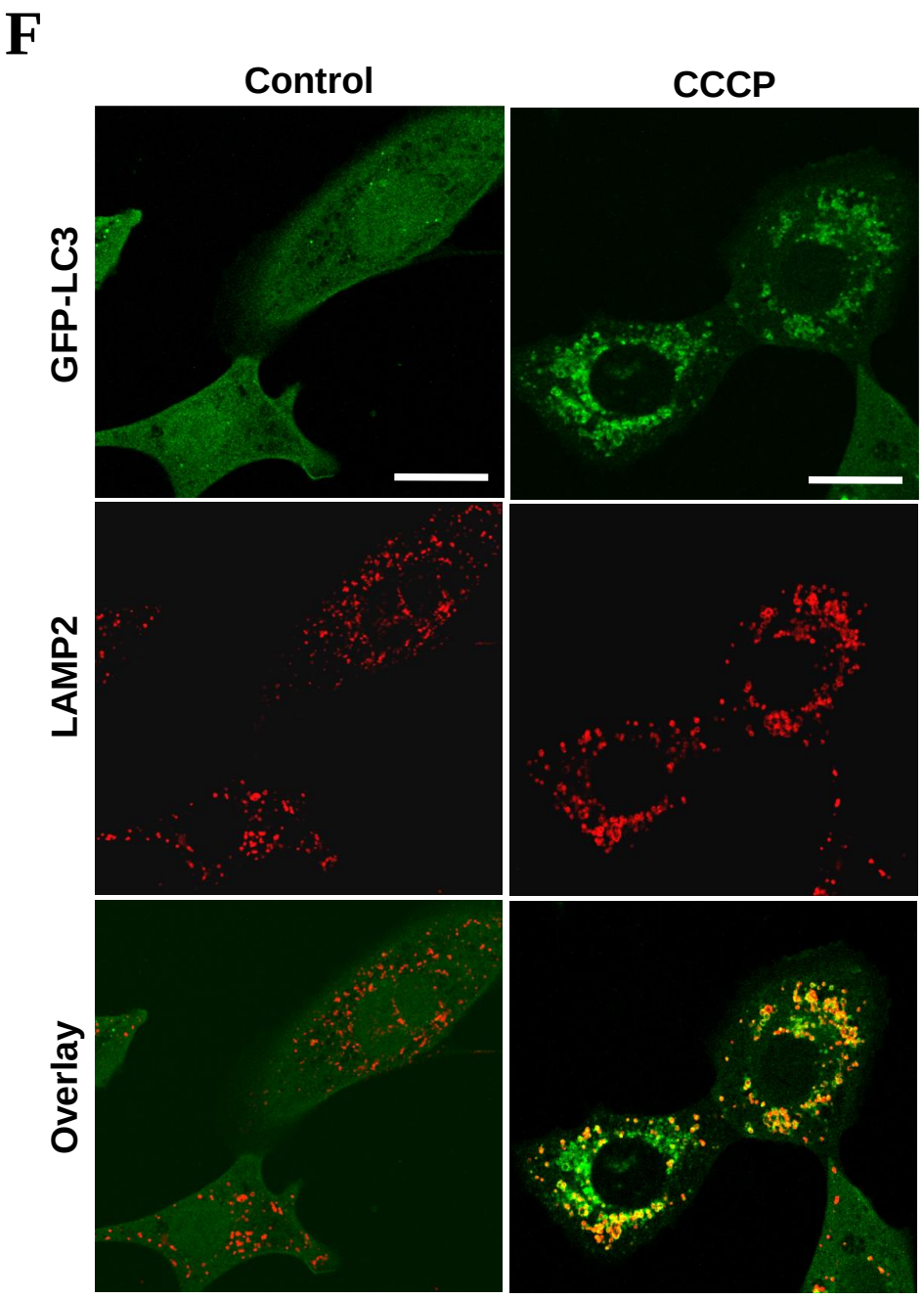
Figure S2

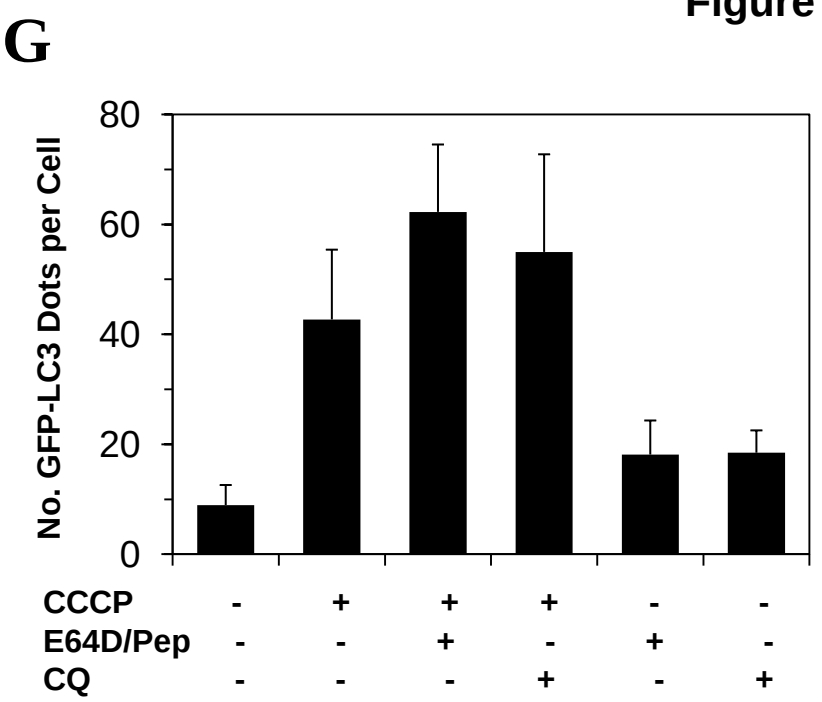
D


No. GFP-LC3 Dots per Cell
60
50
40
30
20
10
0
Time (Hrs)
0
1.5
3
6
12

E


0
3
6
12
(Hrs)
GFP-LC3-I
GFP-LC3-II
GFP
β-actin


F
Control
CCCP
GFP-LC3
LAMP2
Overlay


G
No. GFP-LC3 Dots per Cell
0
20
40
60
80
CCCP - + + + - -
E64D/Pep - - + - + -
CQ - - - + - +

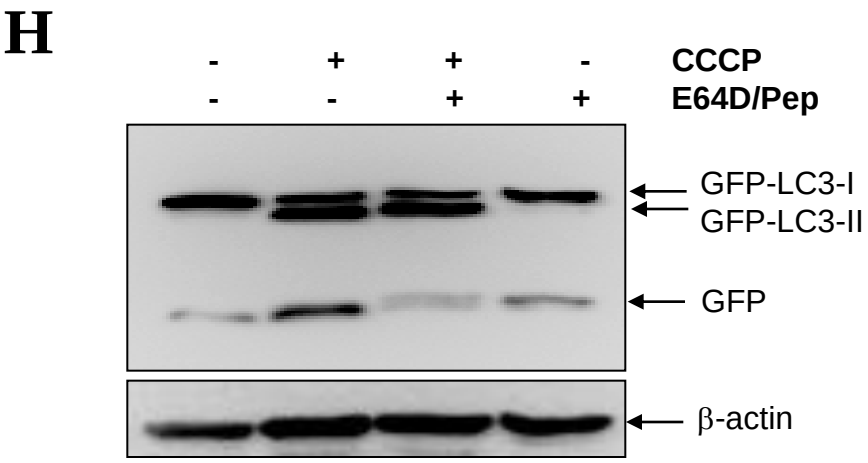
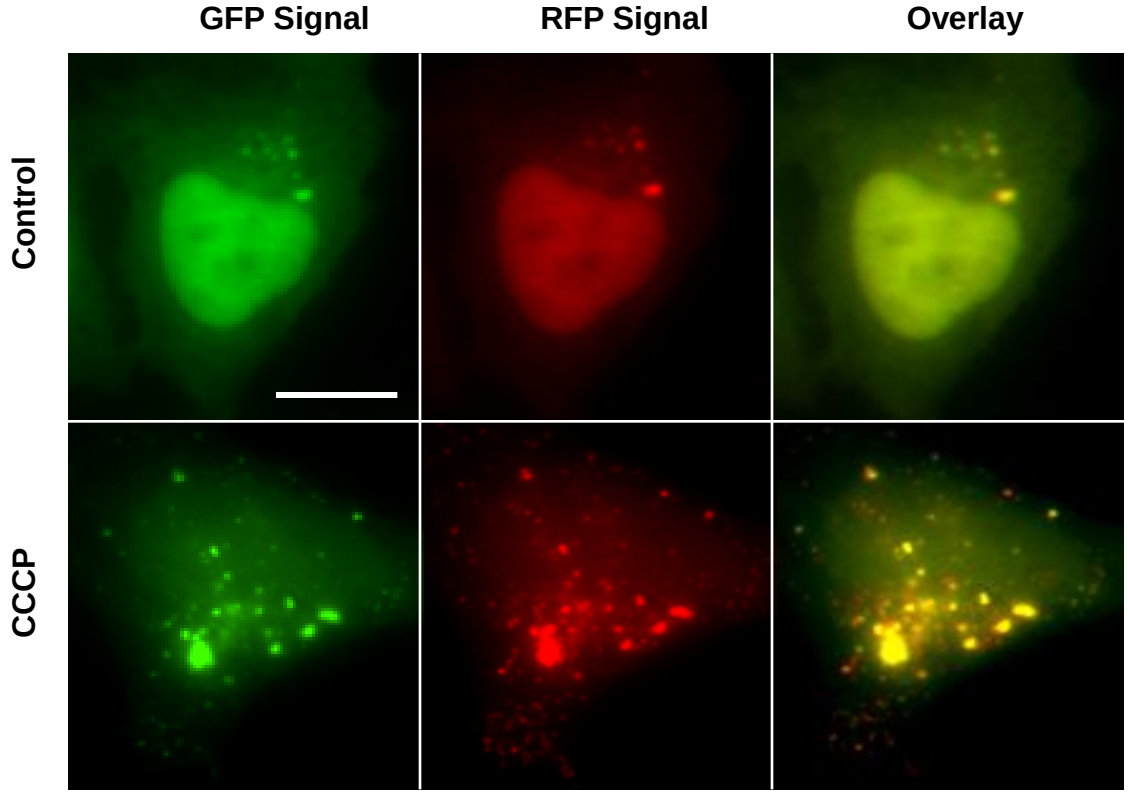

H
- + + - CCCP
- - + + E64D/Pep
GFP-LC3-I
GFP-LC3-II
GFP
β-actin

Figure S3

A


GFP Signal
RFP Signal
Overlay
Control
CCCP

B

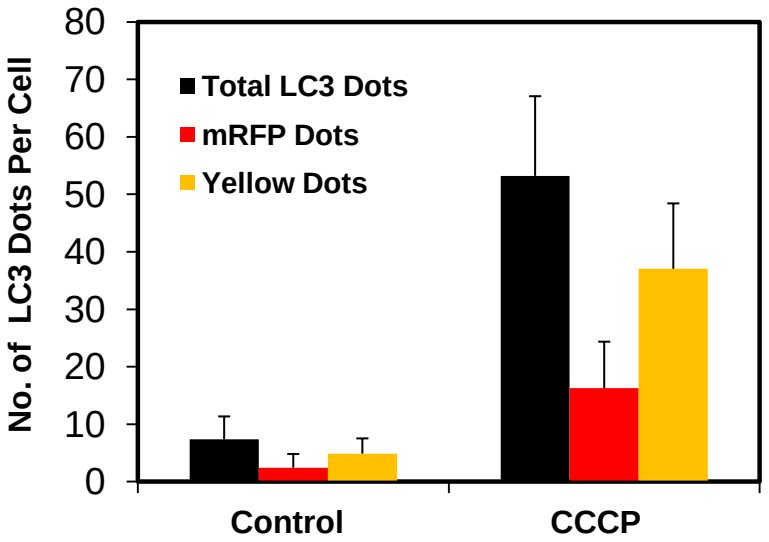

No. of LC3 Dots Per Cell
80
70
60
50
40
30
20
10
0
Total LC3 Dots
mRFP Dots
Yellow Dots
Control
CCCP

Figure S4

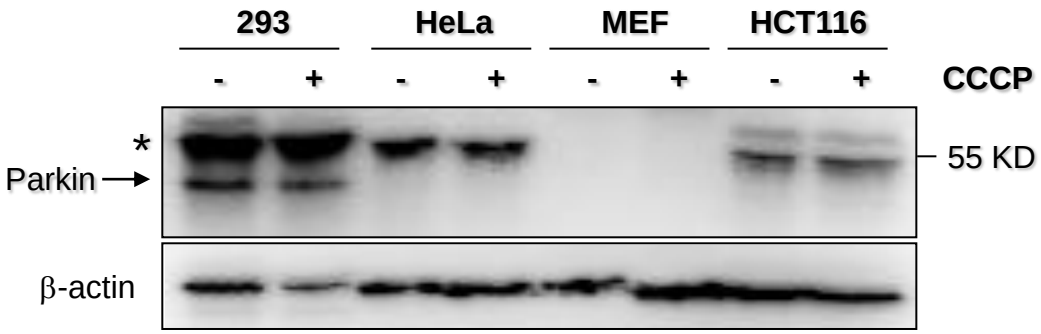


Figure S5

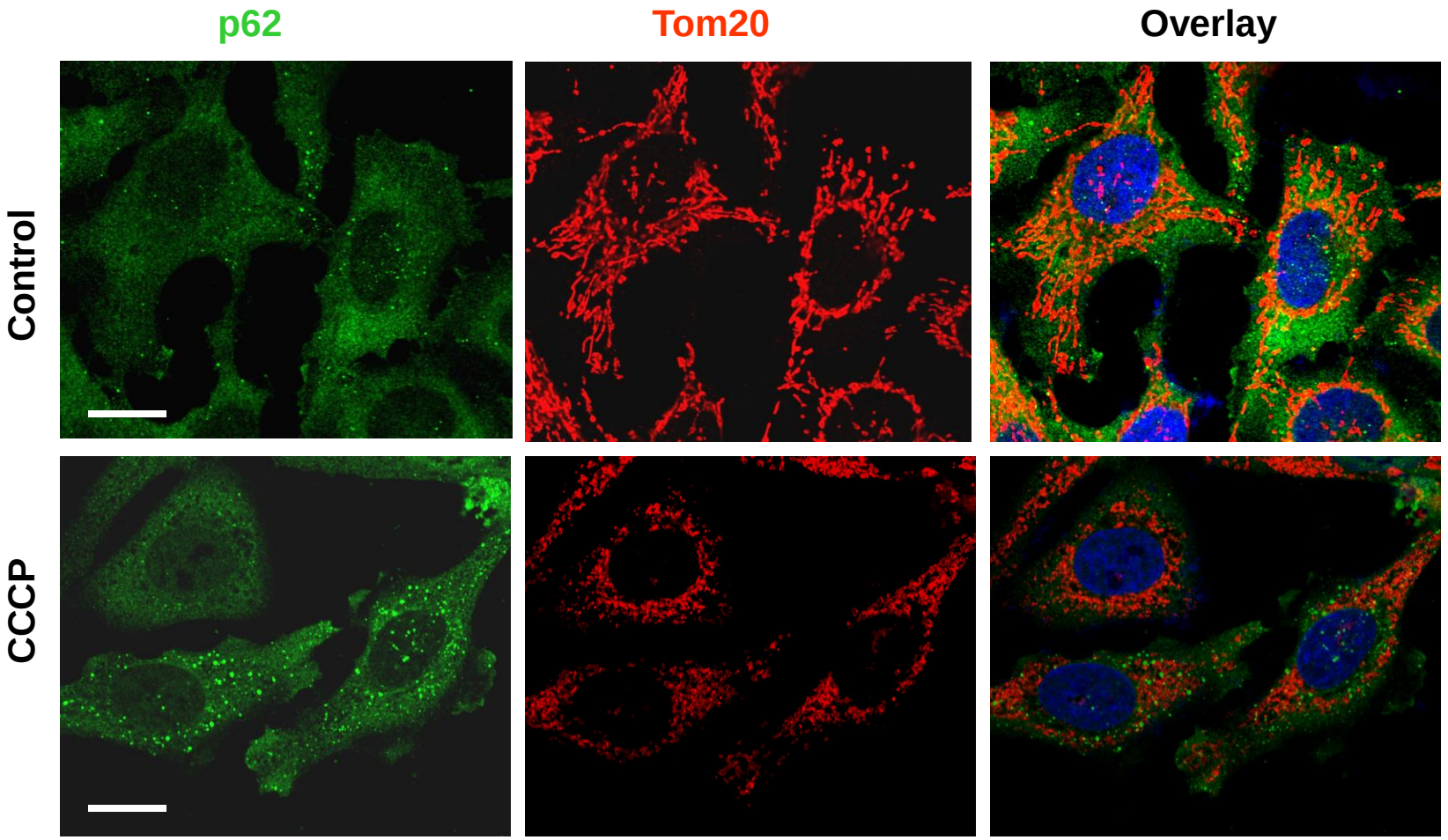


Figure S6

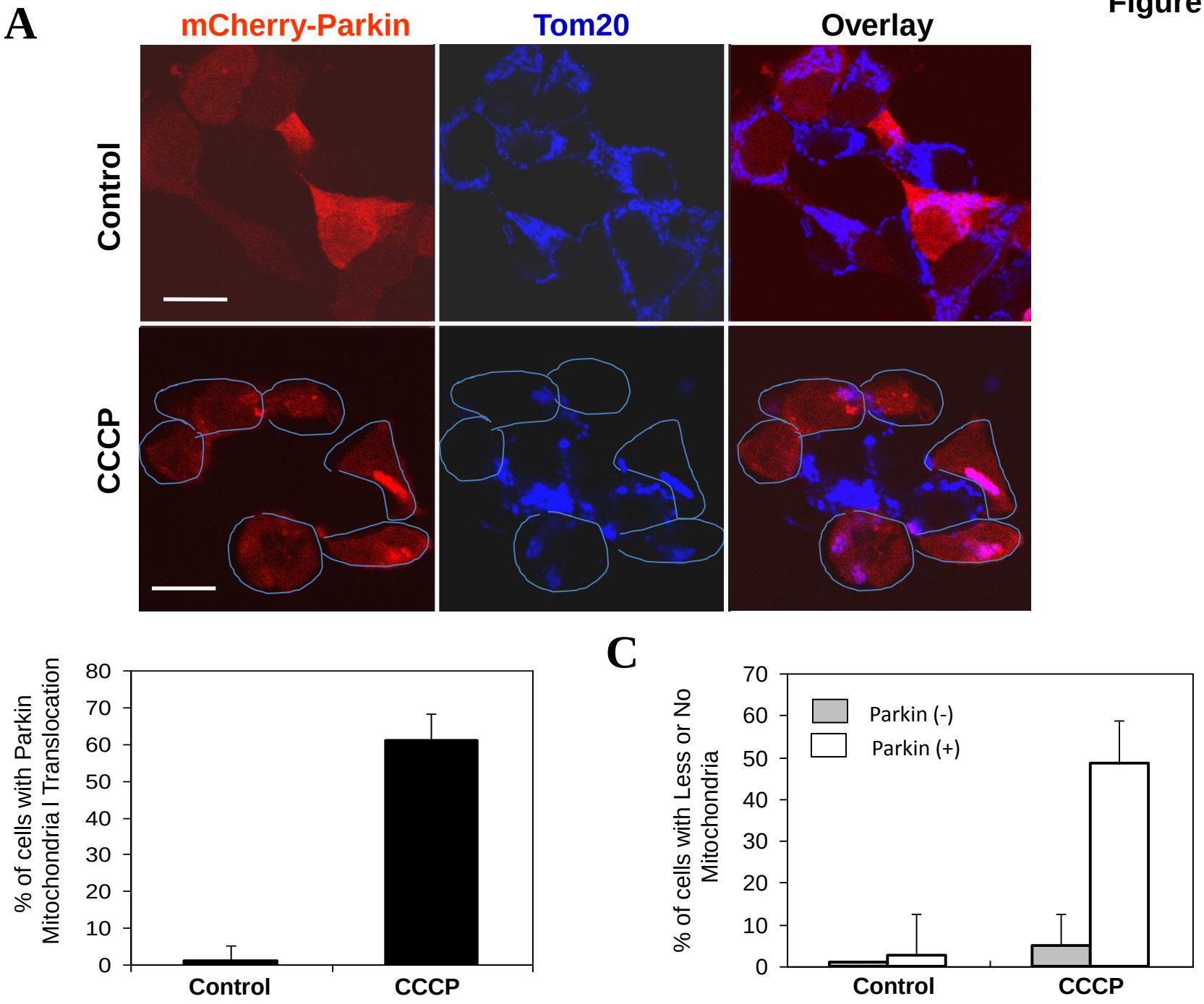
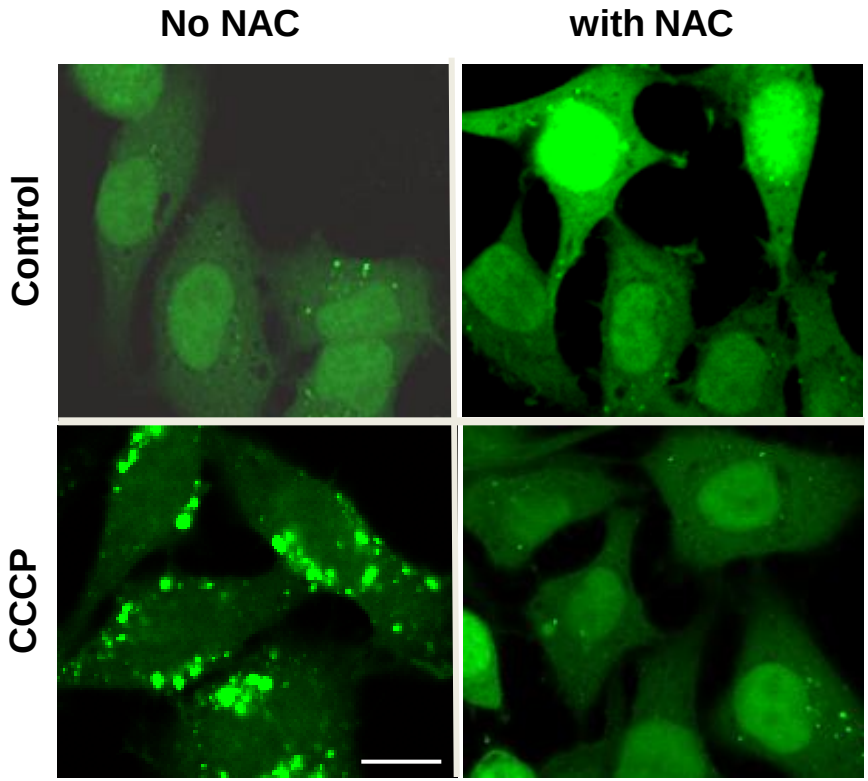

A
mCherry-Parkin
Tom20
Overlay
Control
CCCP
B
% of cells with Parkin Mitochondria l Translocation
80
70
60
50
40
30
20
10
0
Control
CCCP
C
% of cells with Less or No Mitochondria
70
60
50
40
30
20
10
0
Parkin (-)
Parkin (+)
Control
CCCP

Figure S7

A


No NAC
with NAC
Control
CCCP

B

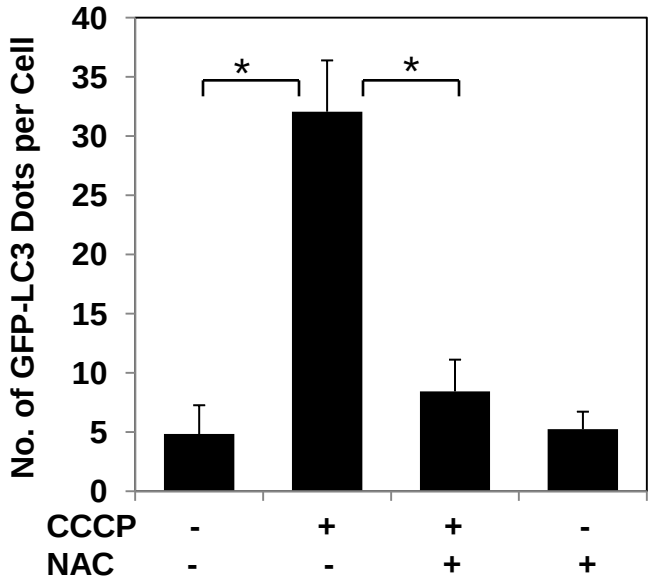

No. of GFP-LC3 Dots per Cell
40
35
30
25
20
15
10
5
0
*
*
CCCP - + + -
NAC - - + +

Figure S8