Supplementary materials

NADPH oxidase promotes Parkinsonian phenotypes by impairing autophagic flux in an mTORC1-independent fashion in a cellular model of Parkinson's disease

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Supplementary figure legends

Supplementary Figure S1. (**a**) SHSY-5Y cells were treated or untreated with 0.5 μM rotenone for 6 h. Some cells were preincubated with 5 μM gp91 ds for 1 h followed by 6 hour of rotenone treatment. As control, some cells were treated with 5 μM gp91 ds for 7 h or hydrogen peroxide (H_2O_2, 200 μM) for 15 min. ROS generation was measured by monitoring DCF-DA fluorescence intensity. DCF fluorescence is represented in RGB color scale using imageJ. (**b**) MTT assay was performed to measure cell-survivability in cells treated as (**a**). Bar diagram indicates quantitative analysis of at least 3 biological replicates. Results are represented as means of SE (SEM). $^{*\#}$,$p < 0.05$ versus all groups, unless otherwise indicated. N.S. indicates non-significant. (**c**) SHSY-5Y cells were treated or untreated with 0.5 μM rotenone for 6 h. Some cells were preincubated with 5 μM gp91 ds for 1 h or 5 mM NAC for 2 h followed by 6 hour of rotenone treatment. DMSO was used as control (vehicle). Cell lysates were analyzed by immunoblotting with antibodies as indicated. GAPDH was used as a loading control.

Supplementary Figure S2. (**a**) SHSY-5Y cells were treated or untreated with 0.5 μM rotenone for 24 h. Some cells were preincubated with 5 μM gp91 ds for 1 h followed by 6 hour of rotenone treatment. As control, some cells were treated with 5 μM gp91 ds for 25 h. Cells were labelled with endogenous beta-actin (green) and cathepsin D (red) for immunofluorescence. Representative cells are shown. The punctate structures indicate lysosomal localization of cathepsin D. Nucleus was labeled with DAPI (blue). (**b**) Cells were transfected with GFP-RFP-LC3 construct and incubated for 24 h, followed by treatment as indicated in (**a**). Live cell imaging was performed using confocal microscope. Bafilomycin was used as a positive control of autophagic flux blocker. Representative cells are shown where yellow pixels indicate colocalization of GFP and RFP in the merged images. Nucleus is indicated with DAPI (blue) staining. (**c**) MTT assay was performed to measure cell-survivability in cells treated as (**a**). DMSO was used as a control (vehicle).

Bar diagrams indicate quantitative analysis of at least 3 biological replicates. Results are represented as means of SE (SEM). N.S. indicates non-significant.

Supplementary Figure S3. (**a**) SHSY-5Y cells were treated or untreated with 10 μM rotenone for 24 h. Some cells were preincubated with 5 μM gp91 ds for 1 h followed by 24 hour of rotenone treatment. As control, some cells were treated with 5 μM gp91 ds for 25 h or hydrogen peroxide (H_2O_2, 200 μM) for 15 min. ROS generation was measured by monitoring DCF fluorescence intensity. DCF fluorescence is represented in RGB color scale using imageJ. (**b**) Lysates from cells treated as in (**a**) were analyzed by using immunoblot assay with the antibodies as indicated. (**c**) MTT assay was performed to measure cell-survivability in cells treated as (**a**). DMSO was used as a control (vehicle).

GAPDH was detected as a loading control for all immunoblots. Bar diagrams indicate quantitative analysis of at least 3 biological replicates. Results are represented as means of SE (SEM). $^{*}p < 0.05$ versus all groups, unless otherwise indicated. N.S. indicates non-significant.

Supplementary tables

Table ST1.

Sequences of oligos used in real-time qPCR analysis.

Gene name	Forward oligos	Reverse oligos
GAPDH	TGCACCACCAACTGCTTAGC	GGCATGGACTGTGGTCATGAG
SQSTM1	AAGCTGCCTTGTACCCAC	CGCTCCGATGTCATAGTTCTTG
MAP1LC3B	AGCAGCATCCAACCAAAATC	CTGTGTCCGTTCACCAACAG

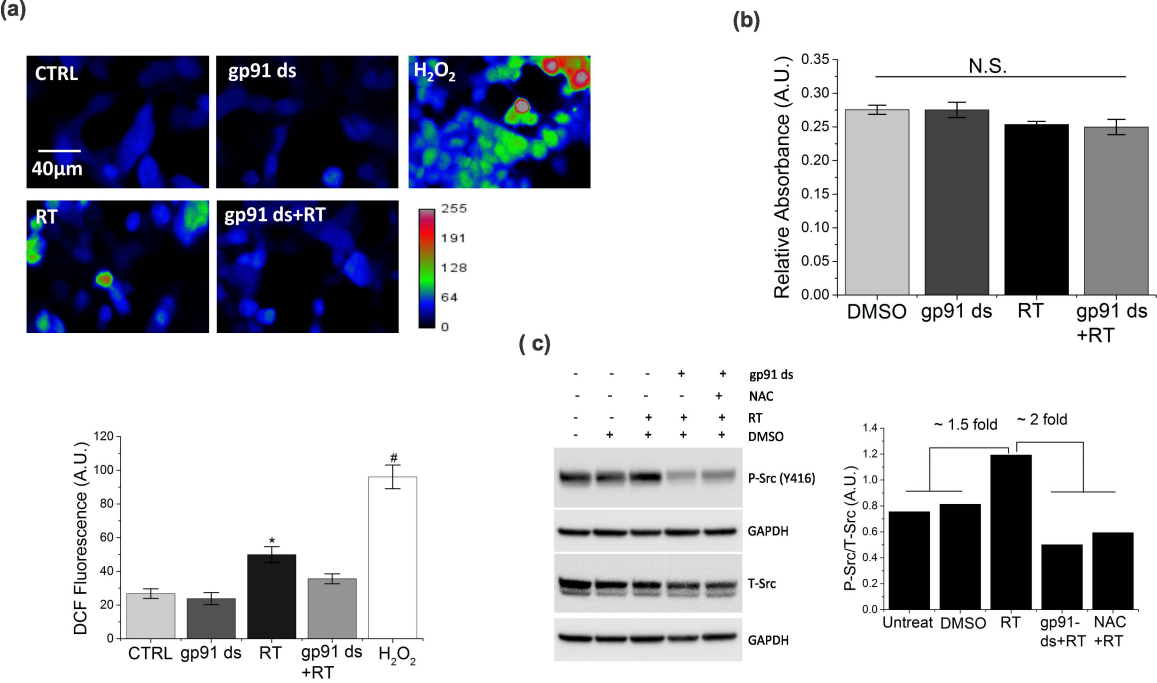
Table ST2.

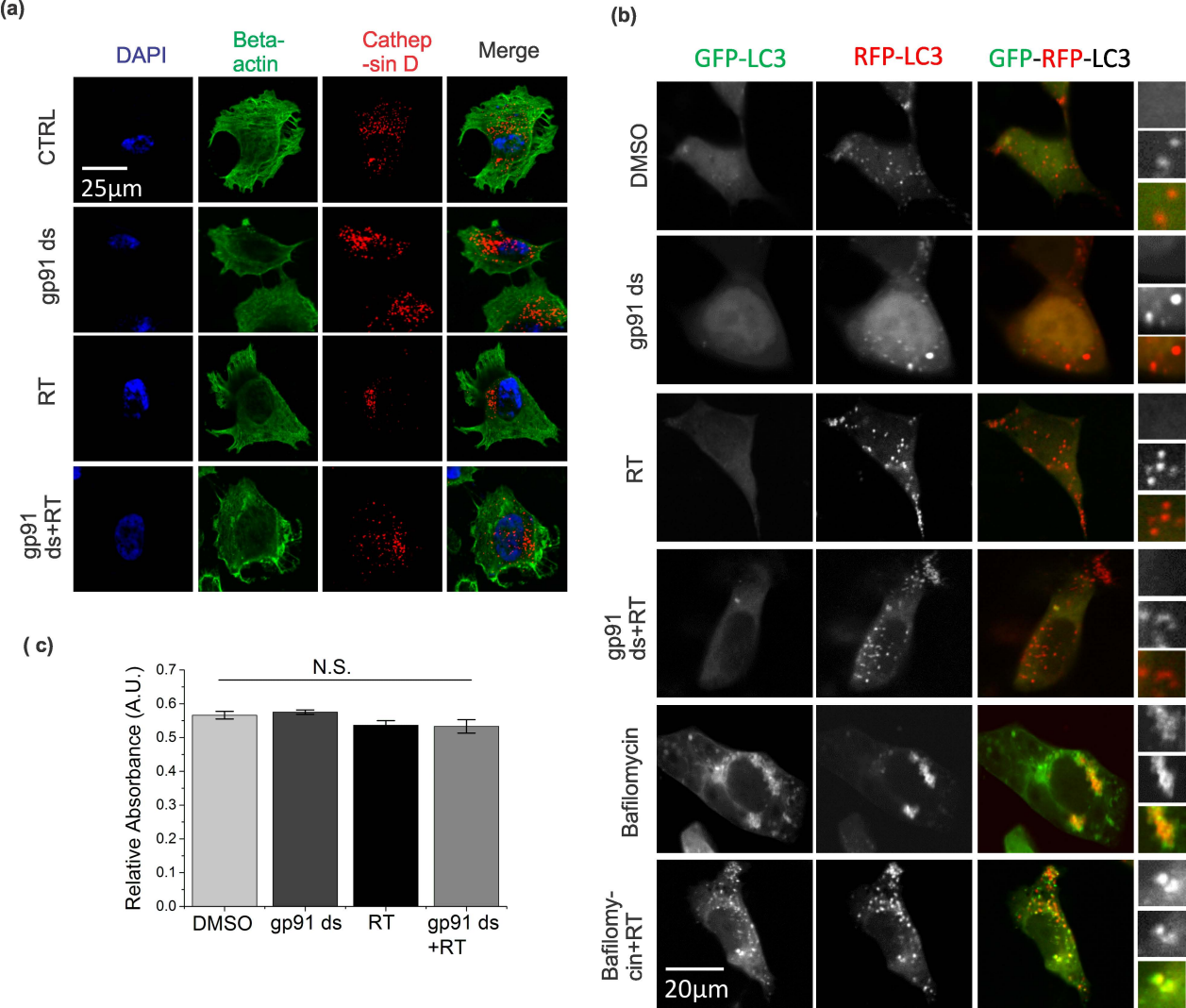
Antibodies

NAME	COMPANY	DILUTION
LAMP1	Cell signaling	1:1000
LAMP2	Santa Cruz Biotechnology	1:1000
P62	BD Bioscience	1:1000
LC3 (Immunofluorescence)	Novus Biologicals	1:100
LC3 (Immunoblot)	Cell signaling	1:1000
GAPDH	Millipore	1:2000
P-Src	Cell signaling	1:500
T-Src	Cell signaling	1:1000
P-PI3K	Cell signaling	1:300
T-PI3K	Cell signaling	1:500
P-Akt	Cell signaling	1:500
T-Akt	Cell signaling	1:1000
P-mTOR	Cell signaling	1:500
T-mTOR (Immunoblot)	Cell signaling	1:1000

T-mTOR (Immunofluorescence)	Cell signaling	1:50
P-ULK1	Cell signaling	1:1000
T-ULK1	Cell signaling	1:1000
P-S6K	Cell signaling	1:300
T-S6K	Cell signaling	1:1000
P-4E-BP1	Cell signaling	1:1000
T-4E-BP1	Cell signaling	1:1000
P-S6 (S235/236)	Cell signaling	1:1000
P-S6 (S240/244)	Cell signaling	1:1000
T-S6	Cell signaling	1:2000
P-Beclin1	PhosphoSolutions	1:50
T-Beclin1	Cell signaling	1:1000
P-AMPK	Cell signaling	1:500
T-AMPK	Cell signaling	1:1000
VPS34	Invitrogen	1:300
Beta-actin (Immunofluorescence)	Cell signaling	1: 200

Cathepsin D	Santa Cruz Biotechnology	1:100
PARP-1	Cell signaling	1:500
Total caspase-3	Cell signaling	1:1000
Cleaved caspase-3	Cell signaling	1:100


(a)
CTRL
gp91 ds
H2O2
40μm
RT
gp91 ds+RT
255
191
128
64
0
(b)
N.S.
Relative Absorbance (A.U.)
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0.00
DMSO
gp91 ds
RT
gp91 ds +RT
(c)
gp91 ds
NAC
RT
DMSO
P-Src (Y416)
GAPDH
T-Src
GAPDH
~ 1.5 fold
~ 2 fold
P-Src/T-Src (A.U.)
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Untreat
DMSO
RT
gp91-ds+RT
NAC +RT
DCF Fluorescence (A.U.)
120
100
80
60
40
20
0
*
#
CTRL
gp91 ds
RT
gp91 ds +RT
H2O2


(a)
DAPI
Beta-actin
Cathep-sin D
Merge
CTRL
gp91 ds
RT
gp91 ds+RT
25μm
(b)
GFP-LC3
RFP-LC3
GFP-RFP-LC3
DMSO
gp91 ds
RT
gp91 ds+RT
Bafilomycin
Bafilomy-cin+RT
20μm
(c)
N.S.
Relative Absorbance (A.U.)
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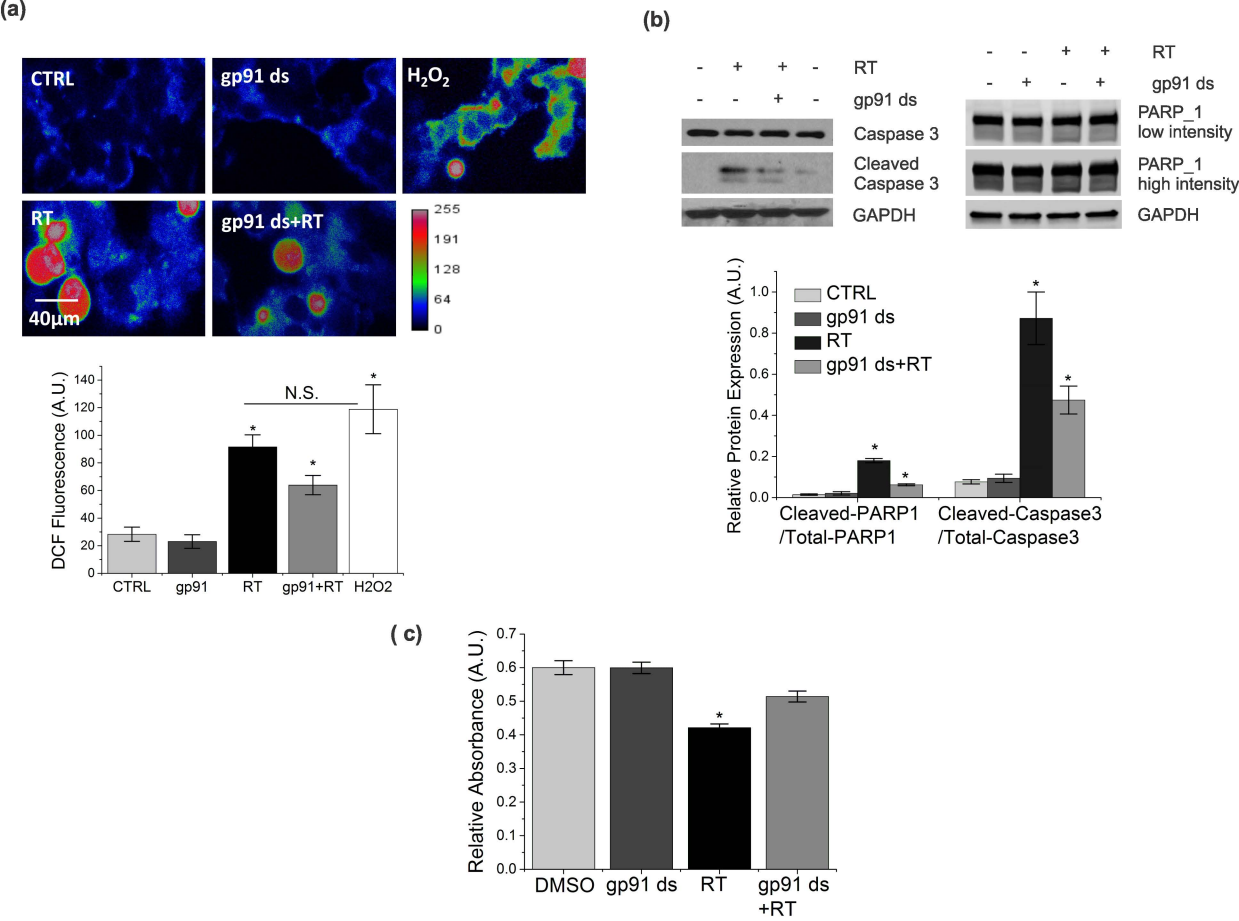
0.1
0.2
0.3
0.4
0.5
0.6
0.7
DMSO
gp91 ds
RT
gp91 ds +RT


(a)
CTRL
gp91 ds
H_2O_2
RT
gp91 ds+RT
40μm
255
191
128
64
0
DCF Fluorescence (A.U.)
N.S.
CTRL
gp91
RT
gp91+RT
H2O2
(b)
RT
gp91 ds
Caspase 3
Cleaved Caspase 3
GAPDH
PARP_1 low intensity
PARP_1 high intensity
Relative Protein Expression (A.U.)
CTRL
gp91 ds
RT
gp91 ds+RT
Cleaved-PARP1 /Total-PARP1
Cleaved-Caspase3 /Total-Caspase3
(c)
Relative Absorbance (A.U.)
DMSO
gp91 ds
RT
gp91 ds +RT