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Supplemental information

Coronavirus subverts ER-phagy by hijacking FAM134B and ATL3 into p62 condensates to facilitate viral replication

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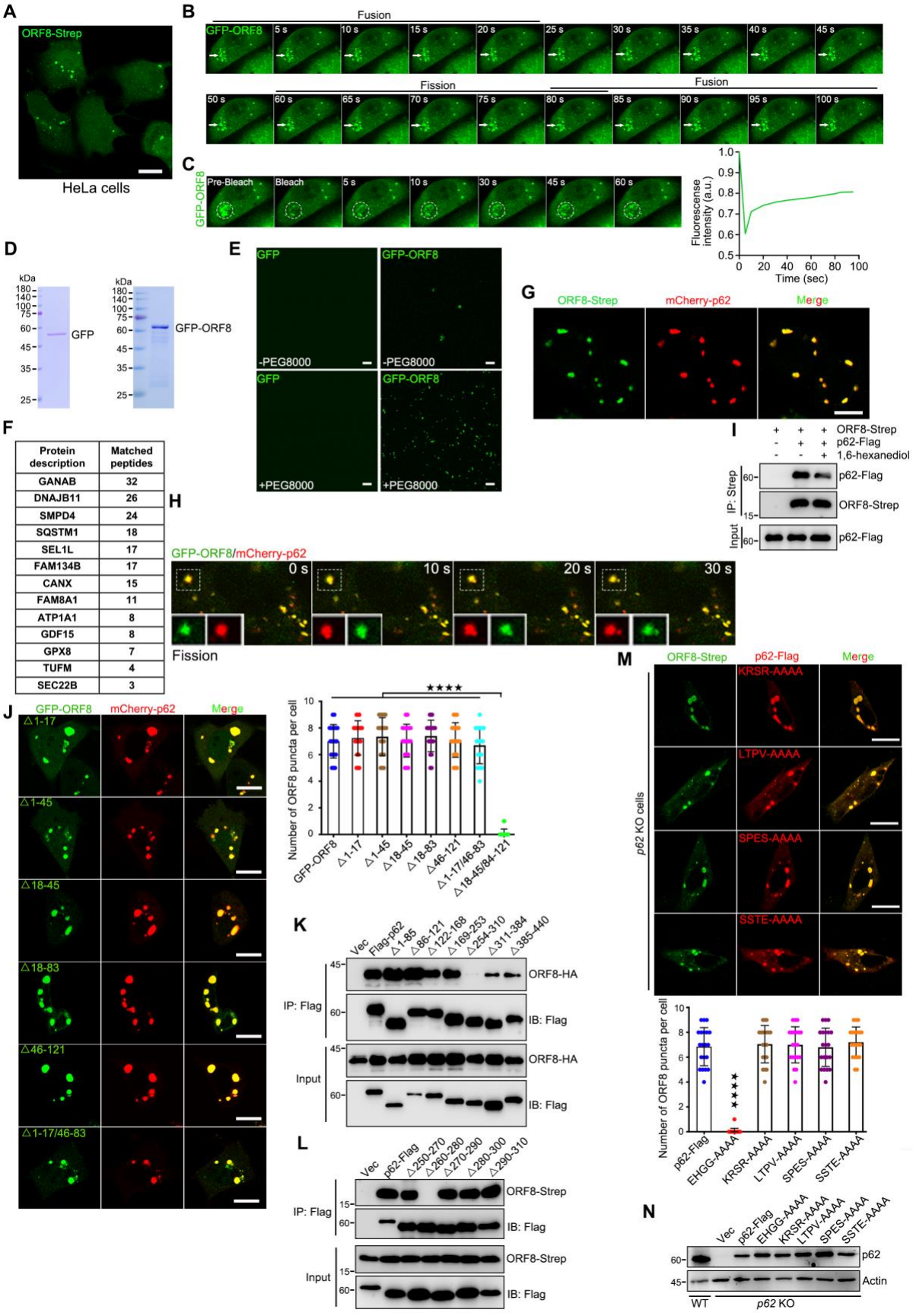
A
ORF8-Strep
HeLa cells
B
Fusion
GFP-ORF8
5 s
10 s
15 s
20 s
25 s
30 s
35 s
40 s
45 s
Fission
Fusion
50 s
60 s
65 s
70 s
75 s
80 s
85 s
90 s
95 s
100 s
C
GFP-ORF8
Pre-Bleach
Bleach
5 s
10 s
30 s
45 s
60 s
Fluorescense intensity (a.u.)
Time (sec)
D
kDa
GFP
GFP-ORF8
E
GFP
GFP-ORF8
-PEG8000
+PEG8000
F
Protein description | Matched peptides
GANAB | 32
DNAJB11 | 26
SMPD4 | 24
SQSTM1 | 18
SEL1L | 17
FAM134B | 17
CANX | 15
FAM8A1 | 11
ATP1A1 | 8
GDF15 | 8
GPX8 | 7
TUFM | 4
SEC22B | 3
G
ORF8-Strep
mCherry-p62
Merge
H
GFP-ORF8/mCherry-p62
0 s
10 s
20 s
30 s
Fission
I
ORF8-Strep
p62-Flag
1,6-hexanediol
IP: Strep
Input
J
GFP-ORF8
mCherry-p62
Merge
△1-17
△1-45
△18-45
△18-83
△46-121
△1-17/46-83
Number of ORF8 puncta per cell
GFP-ORF8
△1-17
△1-45
△18-45
△18-83
△46-121
△1-17/46-83
△18-45/84-121
K
Vec
Flag-p62
△1-85
△86-121
△122-168
△169-253
△254-310
△311-384
△385-440
IP: Flag
Input
ORF8-HA
IB: Flag
L
Vec
p62-Flag
△250-270
△260-280
△270-290
△280-300
△290-310
ORF8-Strep
M
ORF8-Strep
p62-Flag
Merge
KRSR-AAAA
LTPV-AAAA
SPES-AAAA
SSTE-AAAA
p62 KO cells
Number of ORF8 puncta per cell
p62-Flag
EHGG-AAAA
KRSR-AAAA
LTPV-AAAA
SPES-AAAA
SSTE-AAAA
N
Vec
p62-Flag
EHGG-AAAA
KRSR-AAAA
LTPV-AAAA
SPES-AAAA
SSTE-AAAA
p62
Actin
WT
p62 KO

Figure S1. SARS-CoV-2 ORF8 interacts with p62. Related to Figure 1.

(**A**) Cells were transfected with ORF8-Strep for 24 h, then stained with anti-Strep antibody and imaged by confocal microscopy. Scar bar represents 10 μm.

(**B**) GFP-ORF8 puncta undergo fusion and fission, images are shown at indicated timepoints after imaging.

(**C**) HeLa cells with exogenous expression of GFP-ORF8 undergo liquid-like behavior--fluorescence recovery after photobleaching (FRAP), images are shown before and at indicated timepoints after bleaching. Time 0 indicates the time of photobleaching. Quantification of fluorescence intensity recovery of GFP-ORF8 in the bleached droplet.

(**D**) GFP and GFP-ORF8 were purified from *Escherichia Coli* BL21 and analyzed via Coomassie Blue.

(**E**) *In vitro* phase separation assay of GFP or GFP-ORF8. Fluorescence images of 10 μM GST-GFP or 10 μM GST-GFP-ORF8 in phase separation assay buffer with or without 10% PEG8000. Representative images of three independent experiments are shown. Scar bar, 10 μm.

(**F**) HEK293T expressing ORF8-Flag cells were harvested and subjected to Flag IP, followed by MS to identify ORF8-binding proteins.

(**G**) Cells were co-transfected with ORF8-Strep and mCherry-p62 for 24 h, then stained with anti-Strep antibody and imaged by confocal microscopy. Scar bar represents 10 μm.

(**H**) HeLa cells with exogenous expression of GFP-ORF8 and mCherry-p62 undergo fission, images are shown at indicated timepoints after imaging.

(**I**) Analysis of interaction between ORF8 and p62 under 1,6-hexanediol treatment by co-IP assay with Strep antibody.

(**J**) Analysis of colocalization of GFP-ORF8 or deleted mutants with mCherry-p62 by confocal microscopy. Scar bar represents 10 μm.

(**K and L**) Analysis of interaction of p62-Flag or deleted mutants with ORF8-Strep using co-IP assay.

(**M**) *p62* KO cells were transfected with indicated plasmids, followed by analysis of the colocalization of p62-Flag and point mutants with ORF8-Strep with anti-Flag and anti-Strep antibodies by confocal microscopy. Scar bar represents 10 μm.

(**N**) Lysates from (**M**) were analyzed via WB.

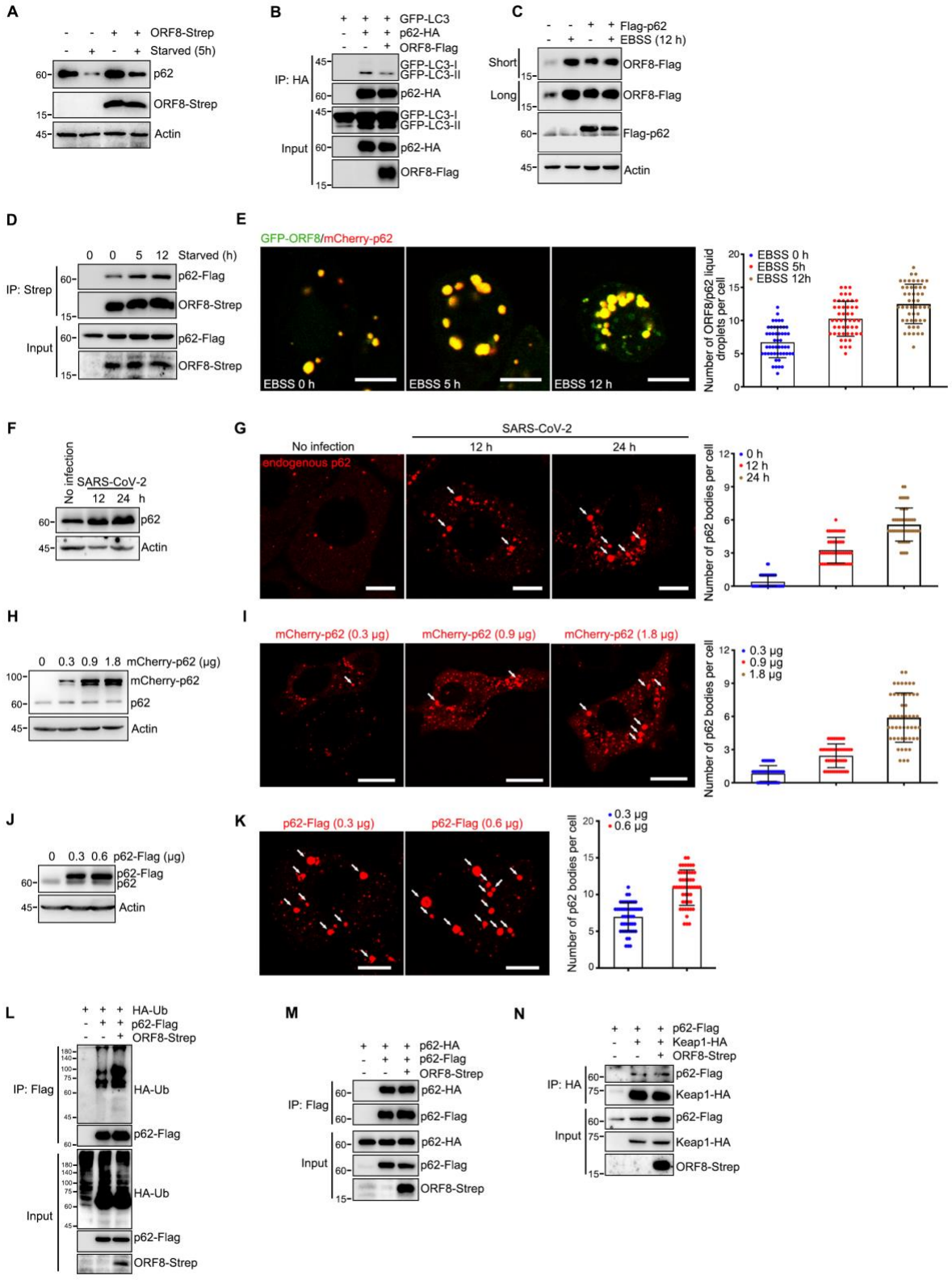
A
ORF8-Strep
Starved (5h)
p62
ORF8-Strep
Actin
B
GFP-LC3
p62-HA
ORF8-Flag
IP: HA
GFP-LC3-I
GFP-LC3-II
p62-HA
Input
GFP-LC3-I
GFP-LC3-II
p62-HA
ORF8-Flag
C
Flag-p62
EBSS (12 h)
Short
Long
ORF8-Flag
ORF8-Flag
Flag-p62
Actin
D
Starved (h)
IP: Strep
p62-Flag
ORF8-Strep
Input
p62-Flag
ORF8-Strep
E
GFP-ORF8/mCherry-p62
EBSS 0 h
EBSS 5 h
EBSS 12 h
Number of ORF8/p62 liquid droplets per cell
EBSS 0 h
EBSS 5h
EBSS 12h
F
No infection
SARS-CoV-2
12 24 h
p62
Actin
G
SARS-CoV-2
No infection
12 h
24 h
endogenous p62
Number of p62 bodies per cell
0 h
12 h
24 h
H
mCherry-p62 (μg)
mCherry-p62
p62
Actin
I
mCherry-p62 (0.3 μg)
mCherry-p62 (0.9 μg)
mCherry-p62 (1.8 μg)
Number of p62 bodies per cell
0.3 μg
0.9 μg
1.8 μg
J
p62-Flag (μg)
p62-Flag
p62
Actin
K
p62-Flag (0.3 μg)
p62-Flag (0.6 μg)
Number of p62 bodies per cell
0.3 μg
0.6 μg
L
HA-Ub
p62-Flag
ORF8-Strep
IP: Flag
HA-Ub
p62-Flag
Input
HA-Ub
p62-Flag
ORF8-Strep
M
p62-HA
p62-Flag
ORF8-Strep
IP: Flag
p62-HA
p62-Flag
Input
p62-HA
p62-Flag
ORF8-Strep
N
p62-Flag
Keap1-HA
ORF8-Strep
IP: HA
p62-Flag
Keap1-HA
Input
p62-Flag
Keap1-HA
ORF8-Strep

Figure S2. ORF8 inhibits Autophagic degradation of p62. Related to Figure 1.

(**A**) HeLa cells were transfected with vector or ORF8-Strep and starved for 5 h. Endogenous p62 was analyzed via WB.

(**B**) Analysis of interaction between GFP-LC3 and p62-HA with ORF8-Flag overexpression by co-IP assay.

(**C**) HeLa cells stably expressing ORF8-Flag were transfected with vector or Flag-p62 and then starved for 5 h. Endogenous protein level of p62 was analyzed via WB.

(**D**) Analysis of interaction between p62-Flag and ORF8-Strep under starvation for indicated time by co-IP assay.

(**E**) HeLa cells were transfected with GFP-ORF8 and mCherry-p62 and starved in EBSS for indicated time. Cells were analyzed via confocal microscopy. Scar bar, 10 μm. The large size (>1 μm) of p62 bodies were counted from 50 cells of three independent experiments.

(**F and G**) Vero-E6 cells were non-infected or infected with SARS-CoV-2 for indicated time. Endogenous p62 was analyzed via WB (**F**) and confocal microscopy (**G**) with anti-p62 antibody. Scar bar, 10 μm. The large size (>1 μm) of p62 bodies were counted from 50 cells of three independent experiments.

(**H and I**) HeLa cells were transfected with gradient mCherry-p62 and analyzed via WB (**H**) and confocal microscopy (**I**). Scar bar, 10 μm. The large size (>1 μm) of p62 bodies were counted from 50 cells of three independent experiments.

(**J and K**) HeLa cells were transfected with gradient p62-Flag and analyzed via WB (**J**) and confocal microscopy (**K**) with anti-Flag antibody. Scar bar, 10 μm. The large size (>1 μm) of p62 bodies were counted from 50 cells of three independent experiments.

(**L-N**) Analysis of interaction between p62-Flag and HA-Ub (**L**), p62-Flag and p62-HA (**M**), p62-Flag and Keap1-HA (**N**) under ORF8-Strep overexpression by co-IP assay.

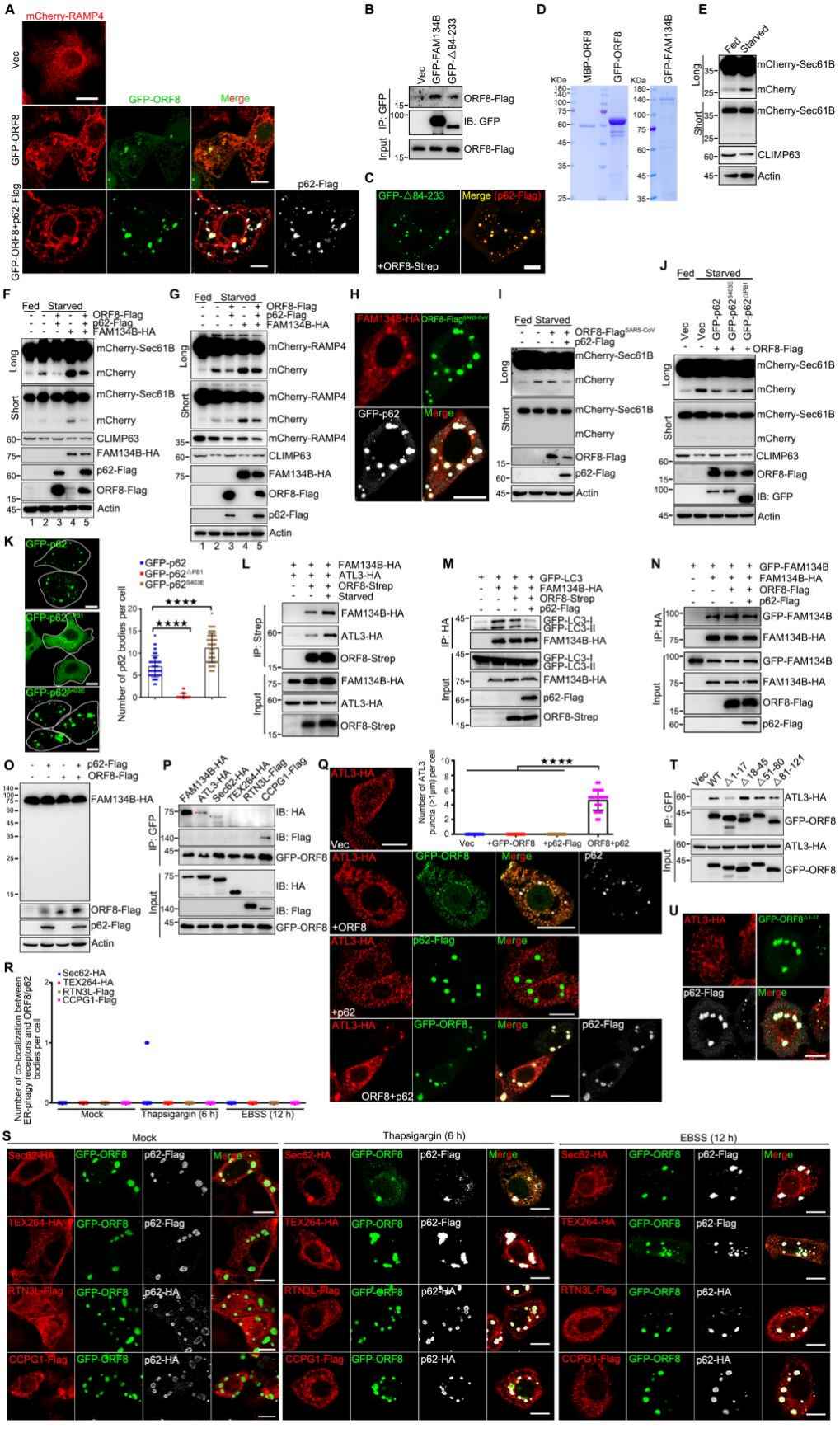
A
mCherry-RAMP4
Vec
GFP-ORF8
Merge
GFP-ORF8
p62-Flag
GFP-ORF8+p62-Flag
B
Vec
GFP-FAM134B
GFP-△84-233
IP: GFP
ORF8-Flag
IB: GFP
Input
ORF8-Flag
C
GFP-△84-233
Merge (p62-Flag)
+ORF8-Strep
D
MBP-ORF8
GFP-ORF8
GFP-FAM134B
KDa
E
Fed
Starved
Long
Short
mCherry-Sec61B
mCherry
CLIMP63
Actin
F
Fed Starved
ORF8-Flag
p62-Flag
FAM134B-HA
mCherry-Sec61B
mCherry
CLIMP63
FAM134B-HA
p62-Flag
ORF8-Flag
Actin
1 2 3 4 5
G
Fed Starved
ORF8-Flag
p62-Flag
FAM134B-HA
mCherry-RAMP4
mCherry
CLIMP63
FAM134B-HA
ORF8-Flag
p62-Flag
Actin
1 2 3 4 5
H
FAM134B-HA
ORF8-Flag
GFP-p62
Merge
I
Fed Starved
ORF8-Flag
p62-Flag
mCherry-Sec61B
mCherry
ORF8-Flag
p62-Flag
Actin
J
Fed Starved
Vec
GFP-p62
ORF8-Flag
mCherry-Sec61B
mCherry
CLIMP63
ORF8-Flag
IB: GFP
Actin
K
GFP-p62
Number of p62 bodies per cell
****
L
FAM134B-HA
ATL3-HA
ORF8-Strep
Starved
IP: Strep
Input
M
GFP-LC3
FAM134B-HA
ORF8-Strep
p62-Flag
IP: HA
GFP-LC3-I
GFP-LC3-II
N
GFP-FAM134B
FAM134B-HA
ORF8-Flag
p62-Flag
O
p62-Flag
ORF8-Flag
FAM134B-HA
P
IP: GFP
IB: HA
IB: Flag
GFP-ORF8
Input
Q
ATL3-HA
Vec
+ORF8
+p62
ORF8+p62
GFP-ORF8
p62
p62-Flag
Merge
Number of ATL3 puncta (>1μm) per cell
T
Vec
WT
△1-17
△18-45
△51-80
△81-121
IP: GFP
Input
U
GFP-ORF8△1-17
R
Sec62-HA
TEX264-HA
RTN3L-Flag
CCPG1-Flag
Number of co-localization between ER-phagy receptors and ORF8/p62 bodies per cell
Mock
Thapsigargin (6 h)
EBSS (12 h)
S
Mock
Thapsigargin (6 h)
EBSS (12 h)
Sec62-HA
TEX264-HA
RTN3L-Flag
CCPG1-Flag
GFP-ORF8
p62-Flag
p62-HA
Merge

Figure S3. ORF8/p62 condensates inhibit ER-phagy. Related to Figure 2.

(**A**) HeLa cells expressing mCherry-RAMP4 were transfected with vector or GFP-ORF8 or GFP-ORF8 and p62-Flag for 24 h and analyzed via confocal microscopy. Scar bar represents 10 μm.

(**B**) Analysis of interaction of ORF8-Flag with GFP-FAM134B or mutant by co-IP assay.

(**C**) HeLa cells were transfected with GFP-FAM134B$^{\triangle 84\text{-}233}$, ORF8-Strep and p62-Flag. Analysis of colocalization of GFP-FAM134B$^{\triangle 84\text{-}233}$ with ORF8/p62 bodies by confocal microscopy with Flag antibody. Scar bar represents 10 μm.

(**D**) MBP-ORF8, GST-GFP-ORF8, and GST-GFP-FAM134B were purified from *Escherichia Coli* BL21 and analyzed via Coomassie Blue.

(**E**) U2OS cells expressing mCherry-Sec61B were starved in EBSS for 12 h. Lysates were analyzed via WB.

(**F**) U2OS cells expressing mCherry-Sec61B were transfected with indicated plasmids for 24 h and then starved in EBSS for 12 h. Lysates were analyzed via WB.

(**G**) U2OS cells expressing mCherry-RAMP4 were transfected with indicated plasmids for 24 h and then starved in EBSS for 12 h. Lysates were analyzed via WB.

(**H**) HeLa cells were transfected with FAM134B-HA and SARS-CoV ORF8-Flag and GFP-p62 for 24 h and analyzed via confocal microscopy with anti-HA and anti-Flag antibodies. Scar bar represents 10 μm.

(**I**) U2OS cells expressing mCherry-Sec61B were transfected with indicated plasmids for 24 h and then starved in EBSS for 12 h. Lysates were analyzed via WB.

(**J**) U2OS cells expressing mCherry-Sec61B were transfected with indicated plasmids for 24 h and then starved in EBSS for 12 h. Lysates were analyzed via WB.

(**K**) HeLa cells were transfected with GFP-p62 or mutants for 24 h and imaged by confocal microscopy. Scar bar represents 10 μm. The large size (>1 μm) of p62 bodies were counted from 50 cells of three independent experiments.

(**L**) Analysis of interaction between ORF8-Strep and FAM134B-HA or ATL3-HA with starvation treatment by co-IP assay.

(**M**) Analysis of interaction between GFP-LC3 and FAM134B-HA with ORF8 and p62 co-expression by co-IP assay.

(**N**) Analysis of self-interaction between GFP-FAM134B and FAM134B-HA with ORF8 and p62 co-expression by co-IP assay.

(**O**) U2OS cells were transfected with indicated plasmids and analyzed the specific cleavage of FAM134B via WB.

(**P**) Co-IP assay for interaction between ORF8 and ER-phagy receptors in HEK293T cells.

(**Q**) Immunofluorescence of cells expressing ATL3-HA or ATL3-HA and GFP-ORF8 or ATL3-HA, p62-Flag and GFP-ORF8 with anti-HA and anti-Flag antibodies. Scar bar, 10 μm.

(**R**) The number of co-localization between ER-phagy receptors and ORF8/p62 bodies in each cell from (**S**) was counted from 20 cells of two independent experiments.

(**S**) HeLa cells expressing GFP-ORF8 and p62-Flag or p62-HA were transfected with ER-phagy receptors for 24 h and analyzed via confocal microscopy with anti-HA and anti-Flag antibodies. Immunofluorescence imaging displayed localization between ER-phagy receptors and ORF8/p62 puncta under different treatment. Scar bar, 10 μm.

(**T**) GFP-ORF8 truncations were expressed as indicated with ATL3-HA. Cell lysates were subjected to immunoprecipitation with GFP antibody and analyzed via WB.

(**U**) HeLa cells were transfected with GFP-ORF8 truncation with ATL3-HA and p62-Flag for 24 h, then stained with anti-HA and anti-Flag antibodies and imaged by confocal microscopy. Scar bar represents 10 μm.

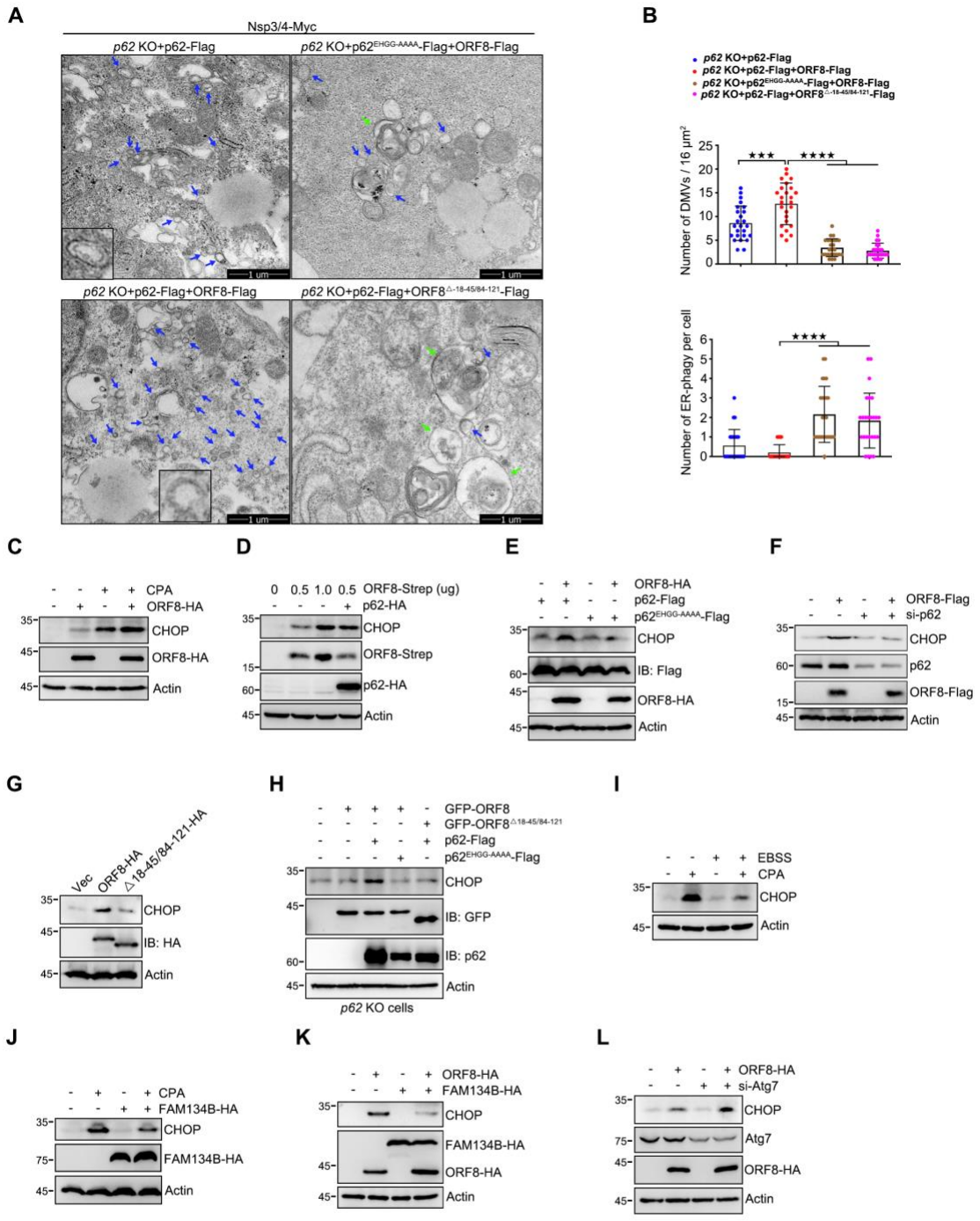
A
Nsp3/4-Myc
p62 KO+p62-Flag
p62 KO+p62EHGG-AAAA-Flag+ORF8-Flag
p62 KO+p62-Flag+ORF8-Flag
p62 KO+p62-Flag+ORF8△-18-45/84-121-Flag
1 um
B
p62 KO+p62-Flag
p62 KO+p62-Flag+ORF8-Flag
p62 KO+p62EHGG-AAAA-Flag+ORF8-Flag
p62 KO+p62-Flag+ORF8△-18-45/84-121-Flag
Number of DMVs / 16 μm2
Number of ER-phagy per cell
***
****
C
CPA
ORF8-HA
CHOP
ORF8-HA
Actin
D
0 0.5 1.0 0.5 ORF8-Strep (ug)
p62-HA
CHOP
ORF8-Strep
p62-HA
Actin
E
ORF8-HA
p62-Flag
p62EHGG-AAAA-Flag
CHOP
IB: Flag
ORF8-HA
Actin
F
ORF8-Flag
si-p62
CHOP
p62
ORF8-Flag
Actin
G
Vec
ORF8-HA
△18-45/84-121-HA
CHOP
IB: HA
Actin
H
GFP-ORF8
GFP-ORF8△18-45/84-121
p62-Flag
p62EHGG-AAAA-Flag
CHOP
IB: GFP
IB: p62
Actin
p62 KO cells
I
EBSS
CPA
CHOP
Actin
J
CPA
FAM134B-HA
CHOP
FAM134B-HA
Actin
K
ORF8-HA
FAM134B-HA
CHOP
FAM134B-HA
ORF8-HA
Actin
L
ORF8-HA
si-Atg7
CHOP
Atg7
ORF8-HA
Actin

Figure S4. ORF8/p62 condensates inhibit ER-phagy, promote DMVs production, and induce ER stress. Related to Figure 3.

(**A**) *p62* KO Vero-E6 cells expressing Nsp3/4-Myc were transfected with WT or p62$^{EHGG-AAAA}$ with ORF8-Flag or ORF8$^{\triangle 18-45/84-121}$-Flag for 36 h and then analyzed via transmission electron microscopy. The DMV structures (blue arrowhead) and ER-phagy (green arrowhead) are shown.

(**B**) The number of DMV and ER-phagy in each cell was counted from 25 cells of two independent experiments. Two-tailed Unpaired Student's t-test, ***P < 0.001, ****P < 0.0001.

(**C**) Cell lysates were collected from HEK293T cells transfected with ORF8-HA and ER stress marker CHOP was analyzed via WB. CPA treatment for 12 h was used as positive control.

(**D**) HEK293T cells were transfected with dose-dependent expression of ORF8-Strep with or without p62-HA for 24 h. Lysates were analyzed via WB.

(**E**) HEK293T cells were transfected with p62-Flag or point mutant with or without ORF8-HA for 24 h. Lysates were analyzed via WB.

(**F**) HEK293T cells were transfected with control or p62 siRNA with or without ORF8-Flag. Lysates were analyzed via WB.

(**G**) HEK293T cells were transfected with ORF8-HA and deleted mutant for 24 h. Lysates were analyzed via WB.

(**H**) *p62* KO Vero-E6 cells were transfected with p62-Flag or point mutant with or without ORF8-HA or deleted mutant for 24 h. Lysates were analyzed via WB.

(**I**) HEK293T cells were treated with CPA and/or EBSS. Lysates were analyzed via WB.

(**J**) HEK293T cells were transfected with vector or FAM134B-HA with or without CPA treatment. Lysates were analyzed via WB.

(**K**) HEK293T cells were transfected with vector or FAM134B-HA with or without ORF8-HA for 24 h. Lysates were analyzed via WB.

(**L**) HEK293T cells were transfected with control or Atg7 siRNA with or without ORF8-HA. Lysates were analyzed via WB.