Expanded View Figures

Figure EV1. ORF6 targets to LDs.

A HeLa cells were transfected with Strep tagged SARS-CoV-2 proteins for 12 h and treated with 200 μM OA for another 12 h, then stained with anti-Flag (red). LDs were labeled with BODIPY-493/503 (green). Cells were imaged by confocal microscopy. Scale bar represents 10 μm.

B HeLa cells were transfected with ORF6-GFP or GFP-ORF6 for 12 h and treated with 200 μM OA for another 12 h. LDs were labeled with LipidTOX Deep Red (red). Cells were imaged by confocal microscopy. Scale bar represents 10 μm.

C HeLa cells were transfected with ORF6-GFP or GFP-ORF6 for 12 h and treated or untreated with 200 μM OA for another 12 h. Cell viability was analyzed. Eight biological replicates were performed. Two-tailed Unpaired Student's *t*-test, ns means no significance. Error bars represent the mean ± SD.

D HeLa cells were transfected with Flag tagged ORF6 of different coronavirus strains and treated with 200 μM OA for another 12 h, then stained with anti-Flag (red). LDs were labeled with BODIPY-493/503 (green). Cells were imaged by confocal microscopy. Scale bar represents 10 μm.

E Colocalization of ORF6 and LDs (Pearson's Coefficient), $n = 20$ cells, three independent experiments. Two-tailed Unpaired Student's *t*-test, $^{*}P < 0.05$, $^{**}P < 0.01$, ns means no significance. Error bars represent the mean ± SD.

F HEK293T cells were transfected with ORF6-Flag or its mutants and treated or untreated with 0.1 mM DSS for 30 min. Cell lysates were analyzed via WB.

G Luciferase assays were performed as described in methods. Three biological replicates were performed. Two-tailed Unpaired Student's *t*-test, $^{****}P < 0.0001$. Error bars represent the mean ± SD.

Source data are available online for this figure.

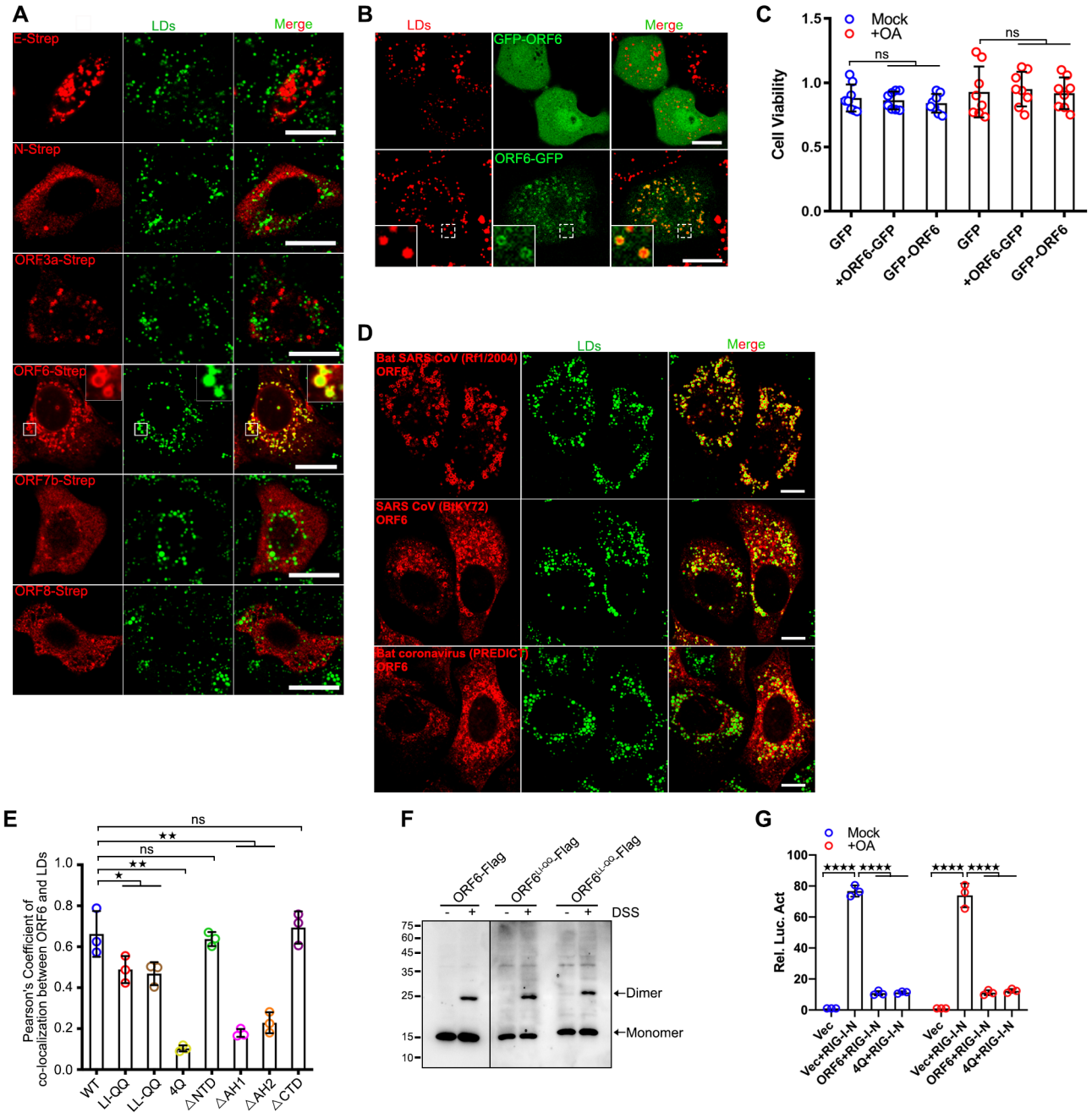
A
LDs
Merge
E-Strep
N-Strep
ORF3a-Strep
ORF6-Strep
ORF7b-Strep
ORF8-Strep
B
LDs
Merge
GFP-ORF6
ORF6-GFP
C
Mock
+OA
Cell Viability
ns
GFP
+ORF6-GFP
GFP-ORF6
D
LDs
Merge
Bat SARS CoV (Rf1/2004) ORF6
SARS CoV (BJKY72) ORF6
Bat coronavirus (PREDICT) ORF6
E
Pearson's Coefficient of co-localization between ORF6 and LDs
WT
LI-QQ
LL-QQ
4Q
ΔNTD
ΔAH1
ΔAH2
ΔCTD
F
ORF6-Flag
DSS
Dimer
Monomer
G
Mock
+OA
Rel. Luc. Act
Vec
Vec+RIG-I-N
ORF6+RIG-I-N
4Q+RIG-I-N

Figure EV1.

Figure EV2. ORF6 promotes LD biogenesis.

A Cells expressing the ORF6^{4Q}-Flag were treated with 200 μM OA for indicated times, then fixed and stained with anti-Flag (green). LDs were labeled with LipidTOX Deep Red (red). The nuclei of cells were stained with DAPI. Cells were imaged by confocal microscopy. Scale bar represents 10 μm.

B, C The interactions of GST tagged ORF6 or the mutants with the His tagged DGAT1 and His tagged DGAT2 were analyzed by GST pull-down assay.

D Vero-E6 cells were infected or non-infected with SARS-CoV-2 for 24 h. Cell lysates were analyzed via WB.

E HeLa cells were transfected with gradient of ORF6-Flag for 24 h. Protein levels of endogenous DGAT1, DGAT2, and ATGL were analyzed via WB.

F HeLa cells were transfected with gradient of ORF6-Flag for 24 h. RNA levels of *dgat1* and *dgat2* were analyzed via QPCR, three independent experiments. Two-tailed Unpaired Student's *t*-test, ns means no significance. Error bars represent the mean ± SD.

G Gene Ontology (GO) enrichment analysis was performed on the human interacting proteins of SARS-CoV-2 ORF6 protein, *P*-values calculated by hypergeometric test and a false discovery rate was used to account for multiple hypothesis testing. The top GO term was shown in the graph.

Source data are available online for this figure.

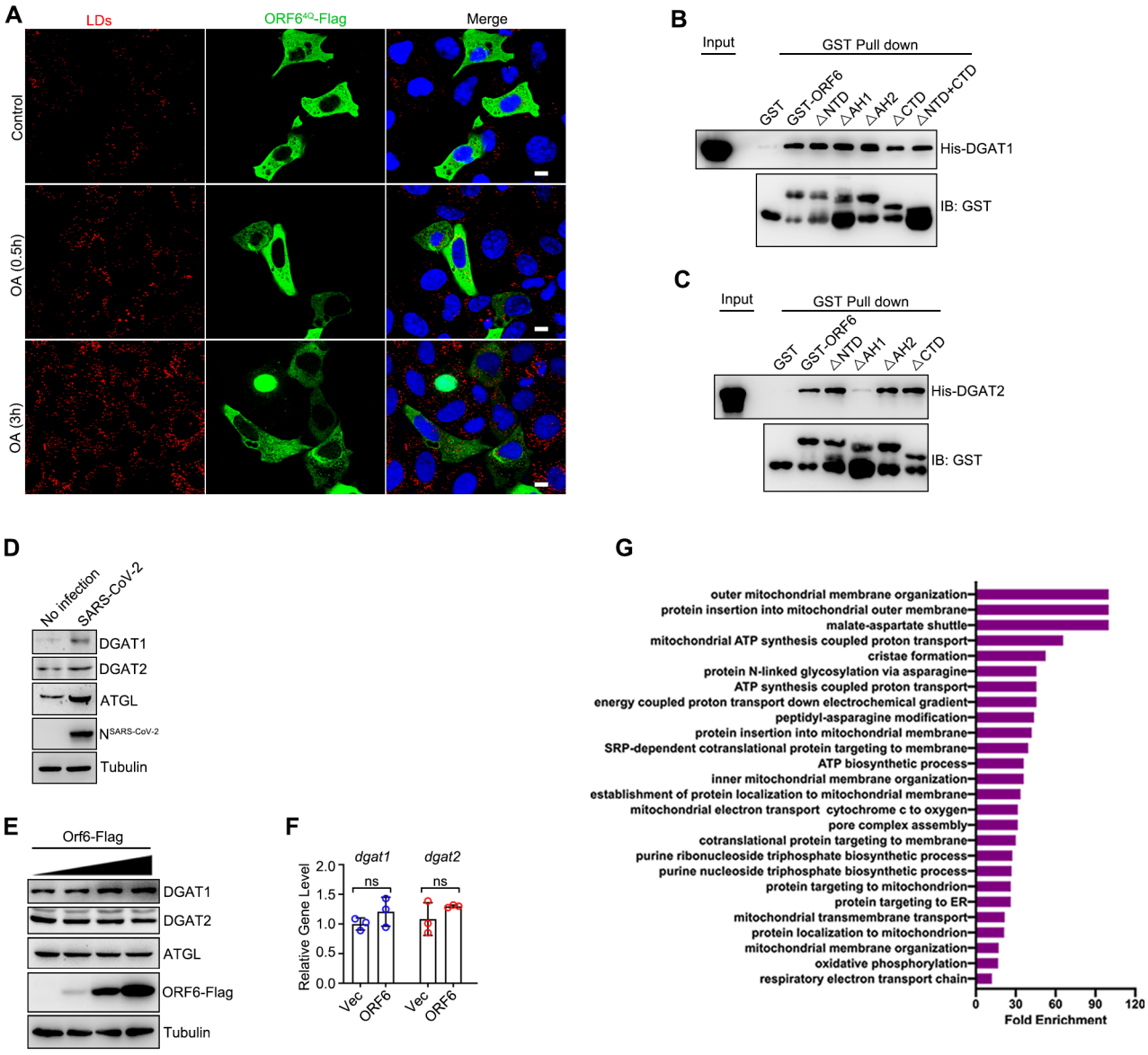
A
LDs
ORF6^4Q-Flag
Merge
Control
OA (0.5h)
OA (3h)
B
Input
GST Pull down
GST
GST-ORF6
△NTD
△AH1
△AH2
△CTD
△NTD+CTD
His-DGAT1
IB: GST
C
Input
GST Pull down
GST
GST-ORF6
△NTD
△AH1
△AH2
△CTD
His-DGAT2
IB: GST
D
No infection
SARS-CoV-2
DGAT1
DGAT2
ATGL
N^SARS-CoV-2
Tubulin
E
Orf6-Flag
DGAT1
DGAT2
ATGL
ORF6-Flag
Tubulin
F
dgat1
dgat2
ns
ns
Relative Gene Level
2.0
1.5
1.0
0.5
0.0
Vec
ORF6
Vec
ORF6
G
outer mitochondrial membrane organization
protein insertion into mitochondrial outer membrane
malate-aspartate shuttle
mitochondrial ATP synthesis coupled proton transport
cristae formation
protein N-linked glycosylation via asparagine
ATP synthesis coupled proton transport
energy coupled proton transport down electrochemical gradient
peptidyl-asparagine modification
protein insertion into mitochondrial membrane
SRP-dependent cotranslational protein targeting to membrane
ATP biosynthetic process
inner mitochondrial membrane organization
establishment of protein localization to mitochondrial membrane
mitochondrial electron transport cytochrome c to oxygen
pore complex assembly
cotranslational protein targeting to membrane
purine ribonucleoside triphosphate biosynthetic process
purine nucleoside triphosphate biosynthetic process
protein targeting to mitochondrion
protein targeting to ER
mitochondrial transmembrane transport
protein localization to mitochondrion
mitochondrial membrane organization
oxidative phosphorylation
respiratory electron transport chain
0
30
60
90
120
Fold Enrichment

Figure EV2.

Figure EV3. ORF6 interacts with BAP31 and USE1.

A GFP or GFP tagged ER membrane proteins were co-expressed in HEK293T cells with ORF6-Flag. Protein interactions were analyzed by immunoprecipitation with anti-GFP beads and immunoblotting analysis.

B Vector were co-expressed with mCheey-RAMP4. Cells were treated with 200 μM OA for 12 h, and then fixed. The ER was visualized with mCheey-RAMP4 (red). LDs were labeled with LipidTOX Deep Red (white). Cells were imaged by confocal microscopy. Scale bar represents 10 μm.

C Cells co-expressing the ORF6-Flag with mCheery tagged RAMP4 were transfected with BAP31 or USE1 siRNA for 36 h and treated with 200 μM OA for 12 h, then fixed and stained with anti-Flag (green). LDs were labeled with LipidTOX Deep Red (blue). Cells were imaged by confocal microscopy. Scale bar represents 10 μm. Quantification of average number of LD-ER contacts per LD. 12 cells (Negative) and 25 cells (BAP31/USE1 KD) from three independent experiments were calculated. Two-tailed Unpaired Student's *t*-test, $^{****}P < 0.0001$, ns means no significance. Error bars represent the mean ± SD.

D Cell viability was analyzed in Fig 4L, three independent experiments. Two-tailed Unpaired Student's *t*-test, ns means no significance. Error bars represent the mean ± SD.

Source data are available online for this figure.

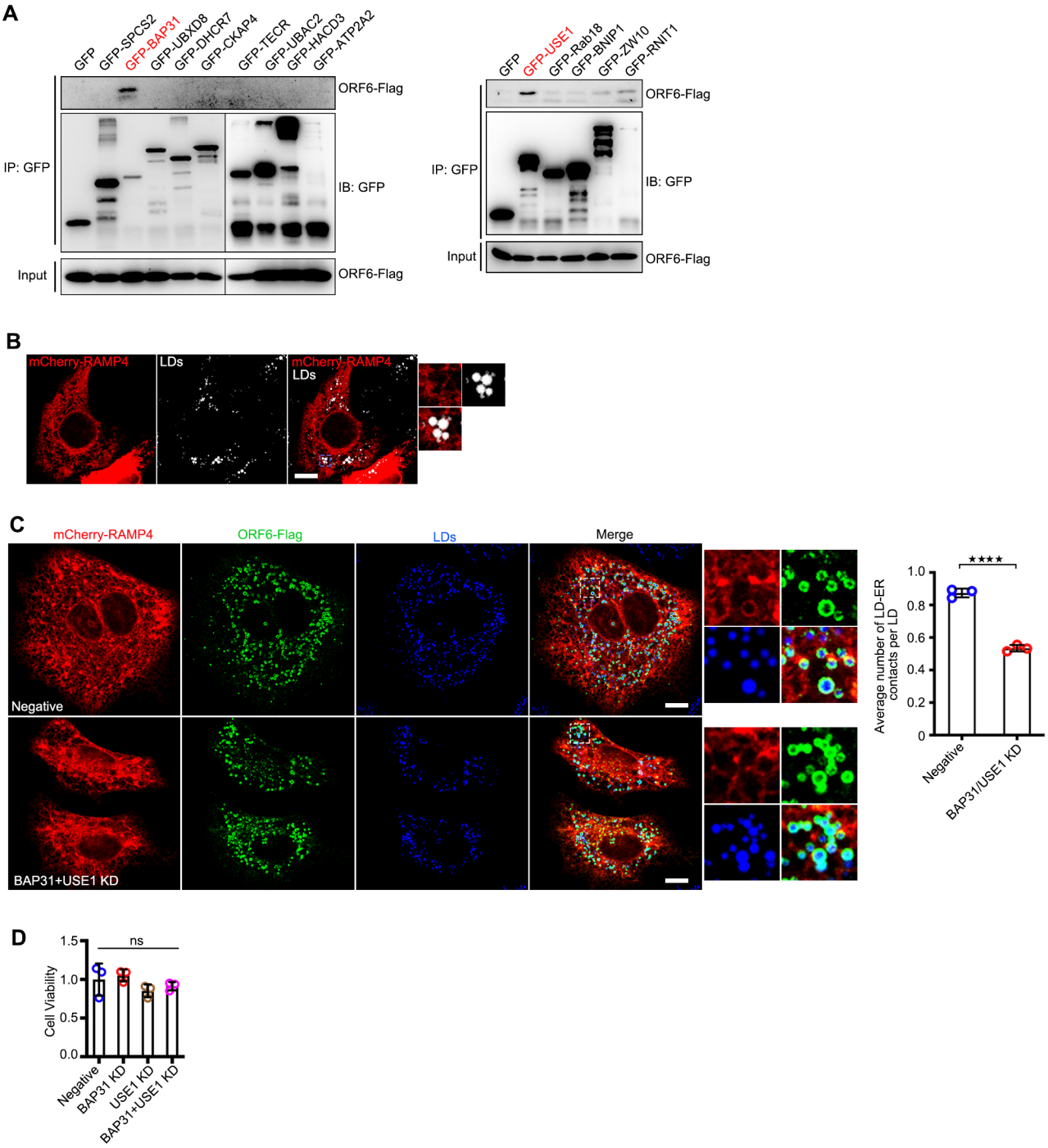
A
GFP
GFP-SPCS2
GFP-BAP31
GFP-UBXD8
GFP-DHCR7
GFP-CKAP4
GFP-TECR
GFP-UBAC2
GFP-HACD3
GFP-ATP2A2
ORF6-Flag
IP: GFP
IB: GFP
Input
ORF6-Flag
GFP
GFP-USE1
GFP-Rab18
GFP-BNIP1
GFP-ZW10
GFP-RNIT1
ORF6-Flag
IP: GFP
IB: GFP
Input
ORF6-Flag
B
mCherry-RAMP4
LDs
mCherry-RAMP4
LDs
C
mCherry-RAMP4
ORF6-Flag
LDs
Merge
Negative
BAP31+USE1 KD
Average number of LD-ER contacts per LD
****
Negative
BAP31/USE1 KD
D
ns
Cell Viability
Negative
BAP31 KD
USE1 KD
BAP31+USE1 KD

Figure EV3.

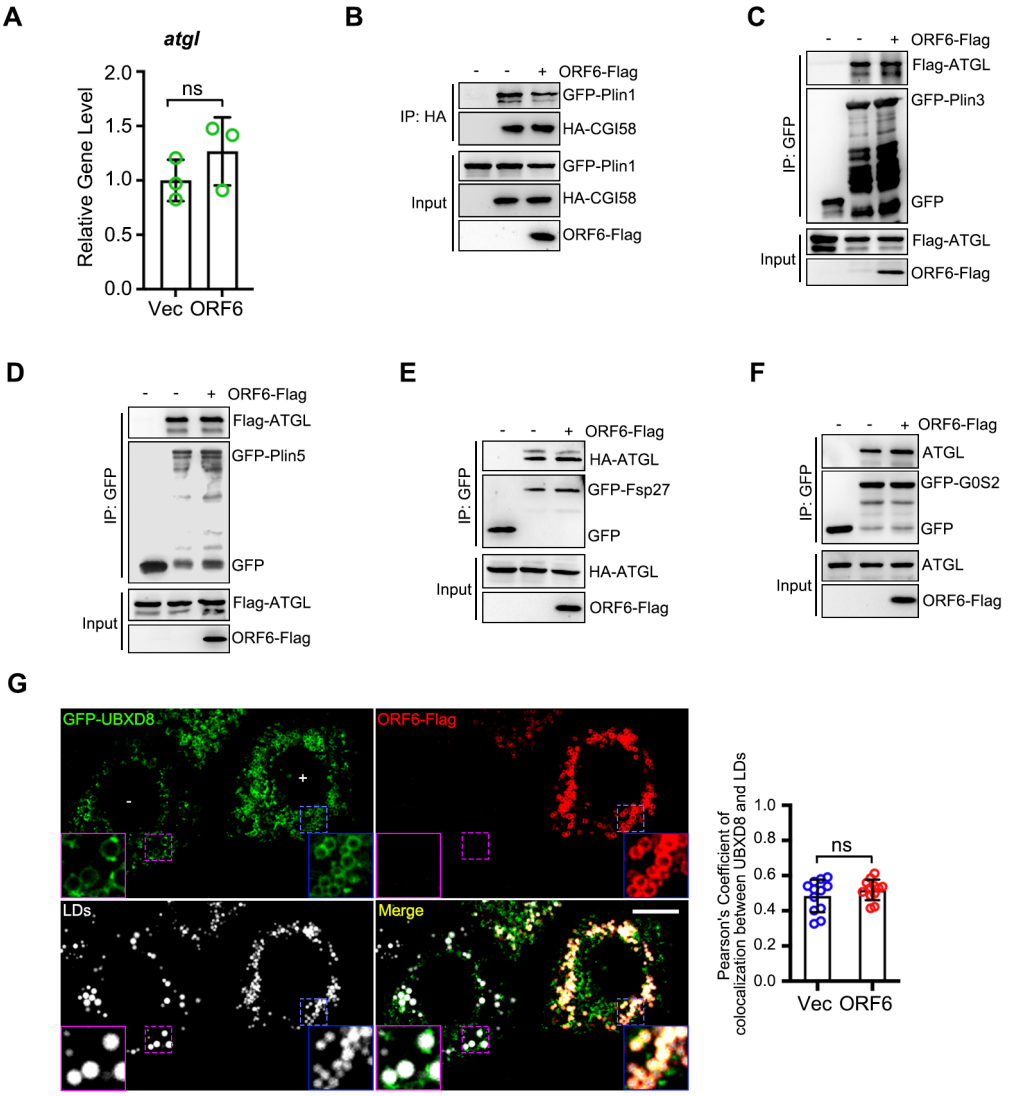

Figure EV4. ORF6 enhances lipolysis.

A QPCR analysis of RNA level of *atgl* in vector and ORF6-Flag expressed cells, three independent experiments. Two-tailed Unpaired Student's *t*-test, ns means no significance. Error bars represent the mean ± SD.

B–F The effect of ORF6-Flag on the interactions of GFP-Plin1 with HA-CGI58, or GFP-Plin3 with Flag-ATGL, or GFP-Plin5 with Flag-ATGL, or GFP-Fsp27 with HA-ATGL, or G0S2-GFP with ATGL were analyzed by immunoprecipitation.

G HeLa cells were transfected with GFP-UBXD8 and vector or ORF6-Flag for 12 h and treated with 200 μM OA for 12 h, then fixed and stained with anti-Flag (red). LDs were labeled with LipidTOX Deep Red (white). Cells were imaged by confocal microscopy. Scale bar represents 10 μm. Blue ROIs indicate cells expressing ORF6 and purple ROIs indicate the cells without ORF6 expression. Colocalization of UBXD8 and LDs (Pearson's Coefficient), $n = 12$ cells, two independent experiments. Two-tailed Unpaired Student's *t*-test, ns means no significance. Error bars represent the mean ± SD.

Source data are available online for this figure.

Figure EV5. ORF6 links LDs to mitochondria.

A HeLa-Parkin cells were transfected with vector or ORF6-Flag for 24 h, or treated with 10 μM CCCP for 6 h. Cell lysates were analyzed via WB.

B Representative transmission electron micrograph showing the close contact between LD, ER and mitochondria in ORF6 expressed HeLa cells treated with 200 μM OA for 12 h. LD, lipid droplets. ER, endoplasmic reticulum. M, mitochondria. Scale bars 500 nm.

C Representative transmission electron micrograph of ORF6-Flag stable expressing or wild-type HeLa cells. Red arrows mark the contact sites between LDs and mitochondria. LD, lipid droplets. M, mitochondria.

D Cos7 cells expressing ORF6^{4Q}-Flag or ORF6$^{\Delta AH1}$-Flag were treated with 200 μM OA for 12 h, and then were fixed and stained with anti-Flag (white) and anti-Tom20 (red). Tom20 represents mitochondria marker. LDs were labeled with BODIPY-493/503 (green). Cells were imaged by confocal microscopy. Scale bar represents 10 μm.

E Cells were transfected with Negative or si-ORF6 for 24 h, and then further transfected with ORF6-Strep, or ORF3a-Strep, or ORF7a-Strep, or ORF7b-Strep, or ORF8-Strep, or ORF9b-Strep, or ORF9c-Strep, or ORF10-Strep for 24 h. Cell lysates were analyzed via WB.

F Cells were transfected with Negative or si-ORF6 for 24 h, and then further transfected with ORF6-Flag (RNAi resistant), or ORF6^{4Q}-Flag (RNAi resistant) for 24 h. Cell lysates were analyzed via WB.

G Vero-E6 cells were transfected with Negative or si-ORF6 for 24 h, and then further transfected with ORF6-Flag (RNAi resistant), or ORF6^{4Q}-Flag (RNAi resistant) for 12 h. Cells were further infected with SARS-CoV-2 for 24 h. Viral RNA level was determined by RT-qPCR, three independent replicates. Two-tailed Unpaired Student's *t*-test, $*P < 0.05$. ns means no significance. Error bars represent the mean ± SD.

H–J Purified GST protein or GST-ORF6 or its mutants were incubated with purified His tagged MTX1, or MTX2, or SAMM50, and analyzed the interactions by GST pull-down.

K Quantification of LD number in (Fig 7F). 25 cells (Negative) and 50 cells (Triple KD) from three independent experiments were calculated. Two-tailed Unpaired Student's *t*-test, ns means no significance. Error bars represent the mean ± SD.

Source data are available online for this figure.

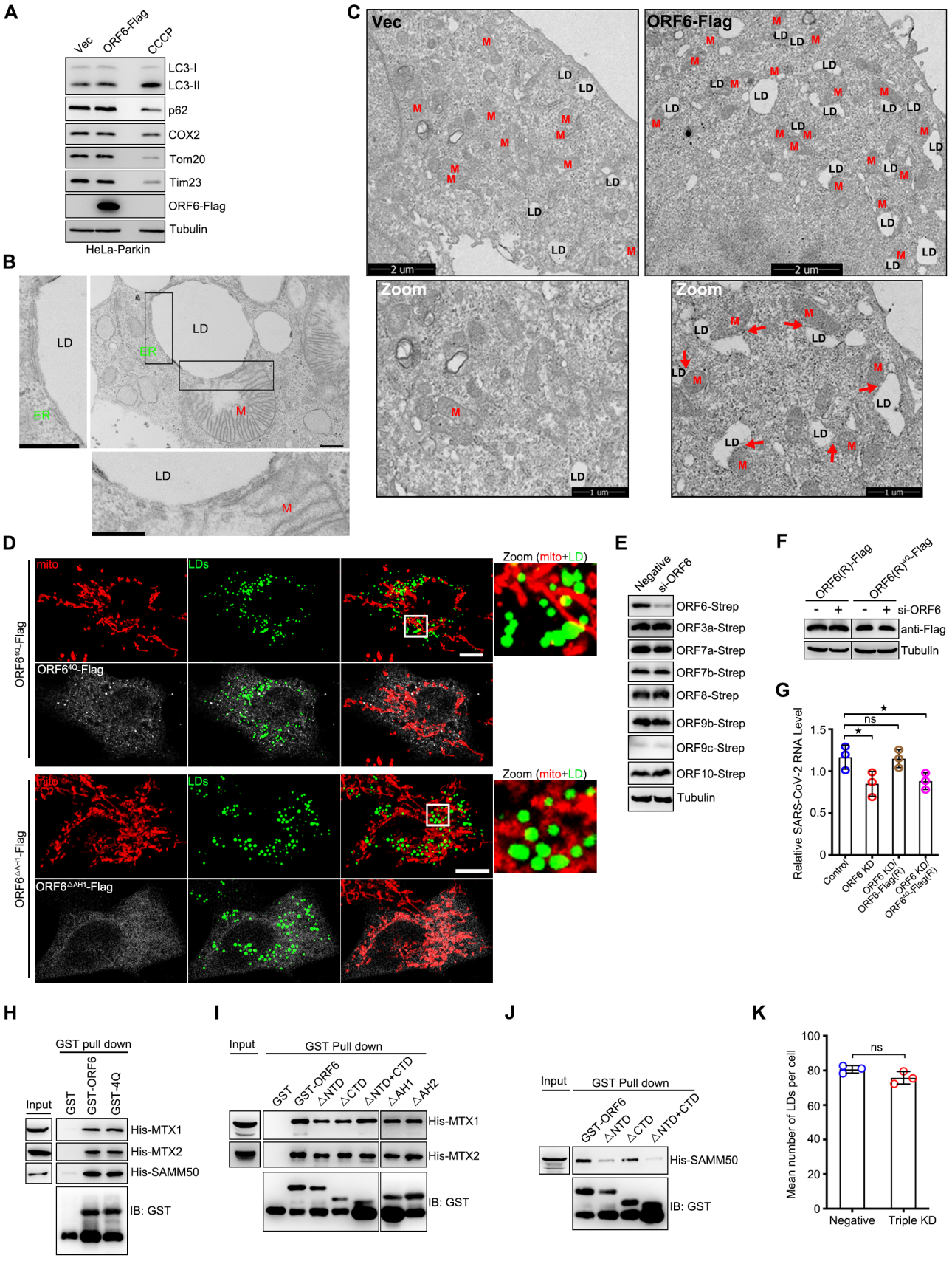

Figure EV5.