Expanded View Figures

Figure EV1. UNC13D inhibits signaling induced by cytoplasmic DNA, but not RNA.

A–L Quantification of the relative expression of relevant proteins in Fig 1A–L. $n = 3$ biological replicates.

M–O UNC13D stable knockdown (KD) HT29 cells and their control cells were transfected with plasmid DNA (1 μg/ml) (M and O) or infected with SeV (N) for the indicated time. Protein levels were detected by immunoblotting with anti-UNC13D, -p-TBK1, -p-IRF3 or -GAPDH antibody (M and N). IFN-β mRNA levels were measured by qPCR (O). $n = 3$ technical replicates.

P UNC13D knockout (KO) HeLa cells and their control cells were infected with GFP-HSV for 18 or 24 h. GFP expression was examined via whole cell lysis (WCL) with immunoblotting (IB). The relevant band intensities were quantified using ImageJ. $n = 3$ biological replicates.

Data information: Statistically significant differences were determined using ANOVA. Data are shown as the mean ± s.d. ns, not significant, $P > 0.05$; $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

Source data are available online for this figure.

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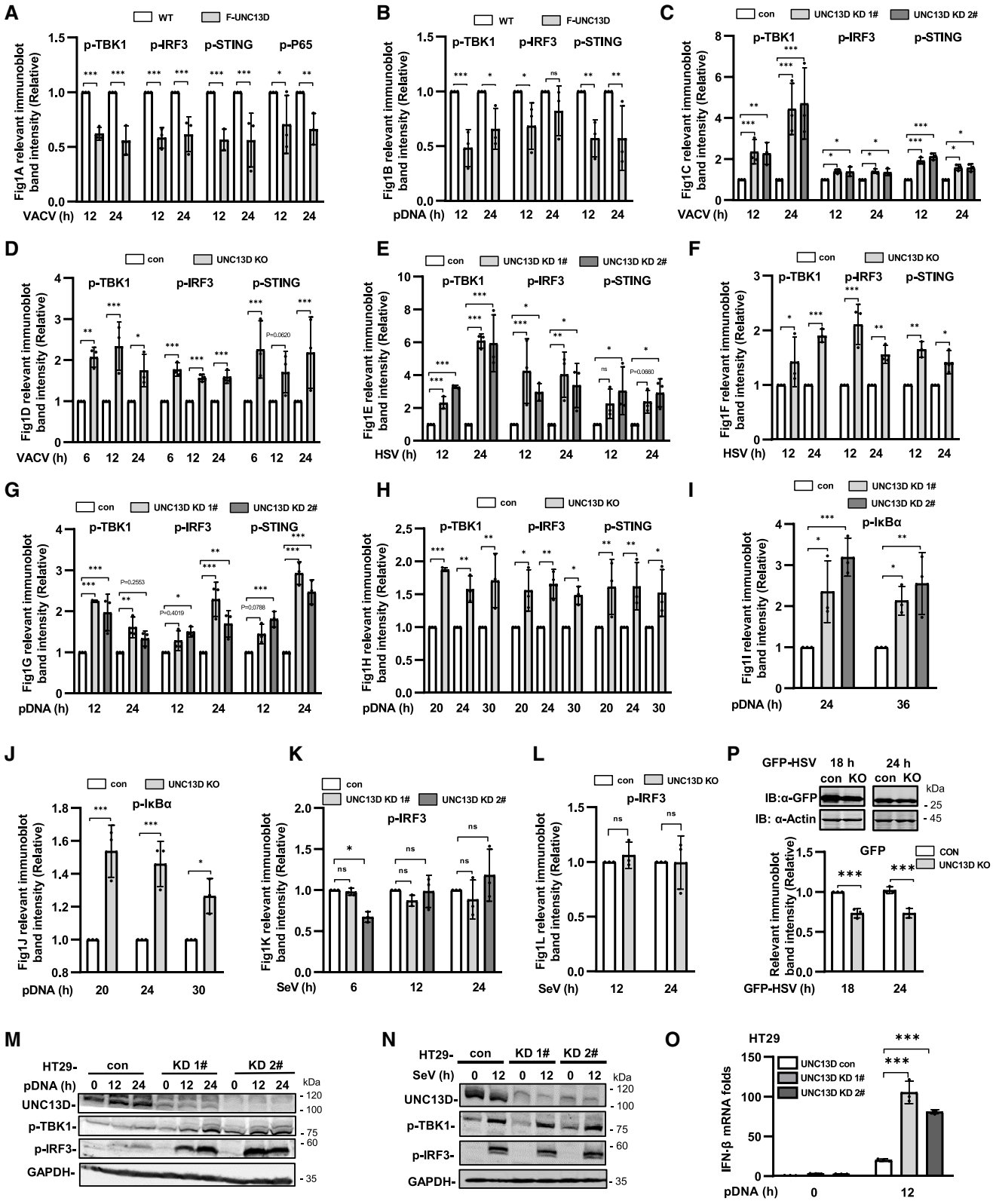
A
WT
F-UNC13D
p-TBK1
p-IRF3
p-STING
p-P65
Fig1A relevant immunoblot band intensity (Relative)
VACV (h) 12 24 12 24 12 24 12 24
B
WT
F-UNC13D
p-TBK1
p-IRF3
p-STING
Fig1B relevant immunoblot band intensity (Relative)
pDNA (h) 12 24 12 24 12 24
C
con
UNC13D KD 1#
UNC13D KD 2#
p-TBK1
p-IRF3
p-STING
Fig1C relevant immunoblot band intensity (Relative)
VACV (h) 12 24 12 24 12 24
D
con
UNC13D KO
p-TBK1
p-IRF3
p-STING
P=0.0620
Fig1D relevant immunoblot band intensity (Relative)
VACV (h) 6 12 24 6 12 24 6 12 24
E
con
UNC13D KD 1#
UNC13D KD 2#
p-TBK1
p-IRF3
p-STING
P=0.0690
Fig1E relevant immunoblot band intensity (Relative)
HSV (h) 12 24 12 24 12 24
F
con
UNC13D KO
p-TBK1
p-IRF3
p-STING
Fig1F relevant immunoblot band intensity (Relative)
HSV (h) 12 24 12 24 12 24
G
con
UNC13D KD 1#
UNC13D KD 2#
p-TBK1
p-IRF3
p-STING
P=0.2553
P=0.4019
P=0.0788
Fig1G relevant immunoblot band intensity (Relative)
pDNA (h) 12 24 12 24 12 24
H
con
UNC13D KO
p-TBK1
p-IRF3
p-STING
Fig1H relevant immunoblot band intensity (Relative)
pDNA (h) 20 24 30 20 24 30 20 24 30
I
con
UNC13D KD 1#
UNC13D KD 2#
p-IκBα
Fig1I relevant immunoblot band intensity (Relative)
pDNA (h) 24 36
J
con
UNC13D KO
p-IκBα
Fig1J relevant immunoblot band intensity (Relative)
pDNA (h) 20 24 30
K
con
UNC13D KD 1#
UNC13D KD 2#
p-IRF3
Fig1K relevant immunoblot band intensity (Relative)
SeV (h) 6 12 24
L
con
UNC13D KO
p-IRF3
Fig1L relevant immunoblot band intensity (Relative)
SeV (h) 12 24
P
GFP-HSV 18 h 24 h
con KO con KO
kDa
IB:α-GFP
IB: α-Actin
25
45
GFP
CON
UNC13D KO
Relative immunoblot band intensity (Relative)
GFP-HSV (h) 18 24
M
HT29-
con
KD 1#
KD 2#
pDNA (h) 0 12 24 0 12 24 0 12 24
kDa
UNC13D-
p-TBK1-
p-IRF3-
GAPDH-
120
100
75
60
35
N
HT29-
con
KD 1#
KD 2#
SeV (h) 0 12 0 12 0 12
kDa
UNC13D-
p-TBK1-
p-IRF3-
GAPDH-
120
100
75
60
35
O
HT29
UNC13D con
UNC13D KD 1#
UNC13D KD 2#
IFN-β mRNA folds
pDNA (h) 0 12

Figure EV1.

Figure EV2. UNC13B has no effect on interferon-β induced by viruses, UNC13D inhibits STING signaling.

A Diagram of UNC13 family members.
B qPCR analysis of UNC13 mRNA levels in wild-type HeLa, HT29, HEK293T and A549 cells. $n = 3$ technical replicates.
C qPCR analysis of UNC13B mRNA levels in UNC13B stable knockdown (KD) HeLa cells. $n = 3$ technical replicates.
D–G UNC13B stable knockdown (KD) HeLa cells and control HeLa cells were infected with VACV (D) or SeV (E and G), or transfected with plasmid DNA (F) (1 μg/ml), for 12 h or for the indicated time. Protein levels were detected by immunoblotting with anti-p-TBK1, -p-IRF3 or -GAPDH antibody (D and E). IFN-β mRNA levels were measured by qPCR (F and G). $n = 3$ technical replicates.
H–N Quantification of the relative expression of relevant proteins in Fig 2B–H. $n = 3$ biological replicates.
O, P UNC13D knockout (KO) HeLa cells and HeLa cells stably expressing FLAG-tagged UNC13D (F-UNC13D) and control HeLa cells were treated with cGAMP (100 nM) for 45 min or SeV for 12 h. Confocal microscopy was used to determine the localization of endogenous STING (red), ER marker Calnexin (green) and nuclei (blue) (O). Scale bars: 15 μm. The colocalization of STING and calnexin was analyzed using ImageJ (P). $n = 6$ biological replicates.

Data information: Statistically significant differences were determined using Student's *t*-test (L–N) or ANOVA (C, F–K, P). Data are shown as the mean ± s.d. ns, not significant, $P > 0.05$; $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

Source data are available online for this figure.

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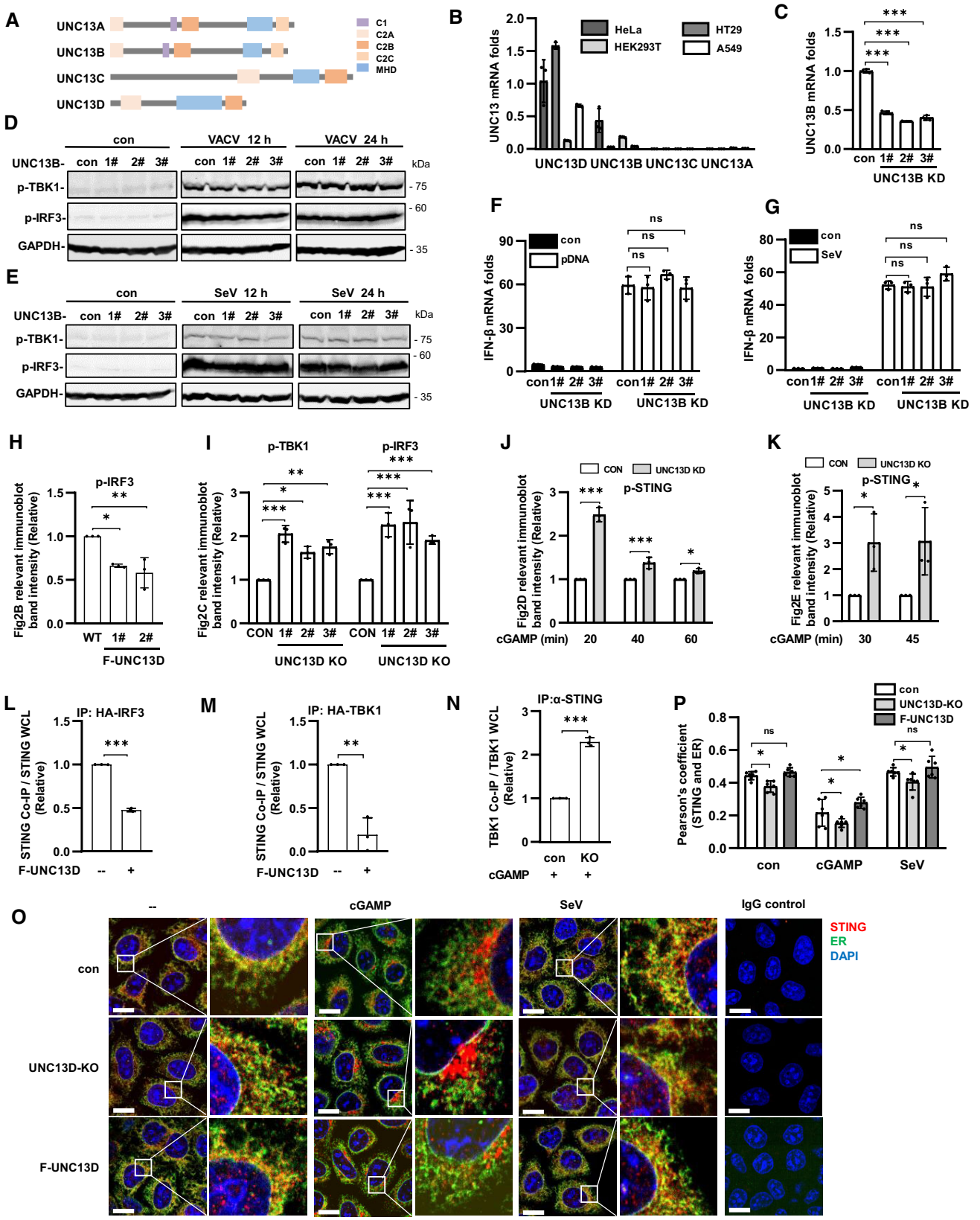
A
UNC13A
UNC13B
UNC13C
UNC13D
C1
C2A
C2B
C2C
MHD
B
UNC13 mRNA folds
HeLa
HEK293T
HT29
A549
UNC13D UNC13B UNC13C UNC13A
C
UNC13B mRNA folds
UNC13B KD
D
con
VACV 12 h
VACV 24 h
UNC13B-
p-TBK1-
p-IRF3-
GAPDH-
kDa
E
SeV 12 h
SeV 24 h
F
IFN-β mRNA folds
pDNA
G
SeV
H
Fig2B relevant immunoblot band intensity (Relative)
p-IRF3
F-UNC13D
I
p-TBK1
p-IRF3
Fig2C relevant immunoblot band intensity (Relative)
UNC13D KO
J
CON
UNC13D KD
p-STING
Fig2D relevant immunoblot band intensity (Relative)
cGAMP (min)
K
UNC13D KO
Fig2E relevant immunoblot band intensity (Relative)
L
IP: HA-IRF3
STING Co-IP / STING WCL (Relative)
M
IP: HA-TBK1
N
IP:α-STING
TBK1 Co-IP / TBK1 WCL (Relative)
cGAMP
P
Pearson's coefficient (STING and ER)
O
--
cGAMP
SeV
IgG control
STING
ER
DAPI
UNC13D-KO

Figure EV2.

Figure EV3. UNC13D impairs STING oligomerization.

A–F (A, E) Quantification of the relative expression of STING from co-immunoprecipitation (IP) results shown in Fig 3A and E. $n = 3$ biological replicates. (B–D, F) Quantification of the colocalization of relevant proteins in Fig 3B–D and F. $n = 3$ biological replicates.

G–I Quantification of the relative expression of relevant proteins in Fig 3H–J. $n = 3$ biological replicates.

J–L UNC13D stable knockdown (KD) (J) or knockout (KO) (K and L) HeLa cells and control HeLa cells were stimulated with cGAMP for the indicated time. Cell lysates were separated by native (top) (J and K), non-reducing (top) (L) or SDS (bottom) PAGE and analyzed by immunoblotting (IB) with anti-UNC13D, -p-IRF3, -STING or -tubulin antibody.

M HEK293T cells were transfected with HA-tagged UNC13D, VN/VC-STING or vector for 24 h, followed by stimulation with cGAMP (100 nM) (or unstimulated) for 30 min. Cell lysates were separated by native (top) or SDS (bottom) PAGE and analyzed by immunoblotting (IB) with anti-HA, -STING or -GAPDH antibody.

Data information: Statistically significant differences were determined using ANOVA. Data are shown as the mean ± s.d. ns, not significant, $P > 0.05$; *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

Source data are available online for this figure.

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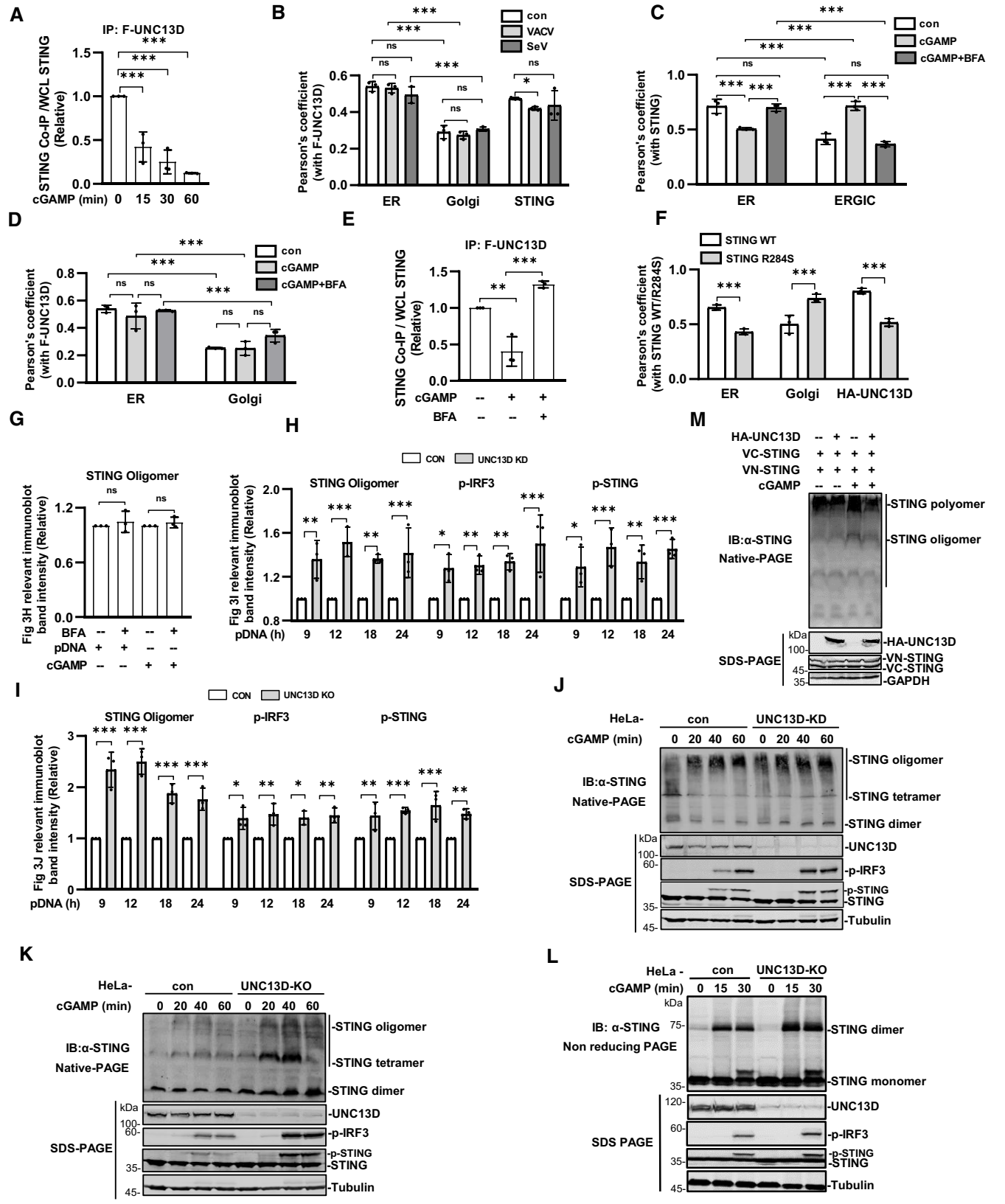
A
IP: F-UNC13D
STING Co-IP /WCL STING (Relative)
cGAMP (min) 0 15 30 60
B
con
VACV
SeV
Pearson's coefficient (with F-UNC13D)
ER
Golgi
STING
C
con
cGAMP
cGAMP+BFA
Pearson's coefficient (with STING)
ER
ERGIC
D
con
cGAMP
cGAMP+BFA
Pearson's coefficient (with F-UNC13D)
ER
Golgi
E
IP: F-UNC13D
STING Co-IP / WCL STING (Relative)
cGAMP
BFA
F
STING WT
STING R284S
Pearson's coefficient (with STING WT/R284S)
ER
Golgi
HA-UNC13D
G
STING Oligomer
Fig 3H relevant immunoblot band intensity (Relative)
BFA
pDNA
cGAMP
H
CON
UNC13D KD
STING Oligomer
p-IRF3
p-STING
Fig 3I relevant immunoblot band intensity (Relative)
pDNA (h) 9 12 18 24
M
HA-UNC13D
VC-STING
VN-STING
cGAMP
STING polyomer
STING oligomer
IB:α-STING
Native-PAGE
SDS-PAGE
kDa
HA-UNC13D
VN-STING
VC-STING
GAPDH
I
CON
UNC13D KO
STING Oligomer
p-IRF3
p-STING
Fig 3J relevant immunoblot band intensity (Relative)
pDNA (h) 9 12 18 24
J
HeLa-
con
UNC13D-KD
cGAMP (min) 0 20 40 60
STING oligomer
STING tetramer
STING dimer
IB:α-STING
Native-PAGE
SDS-PAGE
UNC13D
p-IRF3
p-STING
STING
Tubulin
K
HeLa-
con
UNC13D-KO
cGAMP (min) 0 20 40 60
STING oligomer
STING tetramer
STING dimer
IB:α-STING
Native-PAGE
SDS-PAGE
UNC13D
p-IRF3
p-STING
STING
Tubulin
L
HeLa -
con
UNC13D-KO
cGAMP (min) 0 15 30
IB: α-STING
Non reducing PAGE
STING dimer
STING monomer
SDS PAGE
UNC13D
p-IRF3
p-STING
STING
Tubulin

Figure EV3.

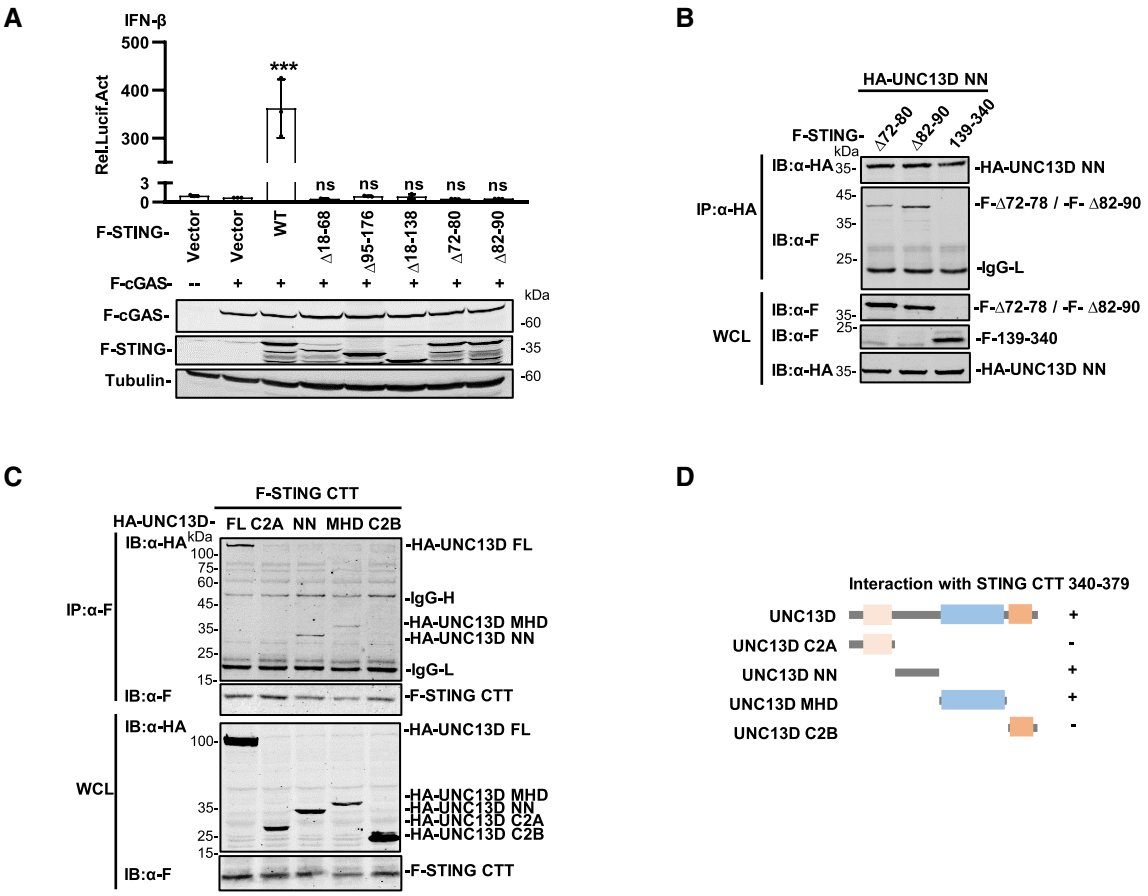

Figure EV4. Domain mapping of the STING-UNC13D interaction.

A HEK293T cells were transfected for 24 h with the IFN-β-luciferase reporter plasmid, pRL-TK and the other indicated plasmids, followed by dual-luciferase reporter assays. The expression of plasmids was examined by western blotting with anti-Flag or -tubulin antibody. $n = 3$ technical replicates.

B, C HEK293T cells were transfected with the indicated plasmids (5 μg each) for 24 h. Whole cell lysates (WCL) were examined, and cell lysates were immunoprecipitated (IP) with the indicated antibodies, followed by immunoblotting (IB) with anti-Flag or -HA antibody.

D Diagram of domain mapping for STING C-terminal tail (CTT) (aa 340–379)-UNC13D truncation interactions.

Data information: Statistically significant differences were determined using ANOVA. Data are shown as the mean ± s.d. ns, not significant, $P > 0.05$; ***$P < 0.001$.
Source data are available online for this figure.

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Figure EV5. UNC13D dysfunction enhances basal levels of IFN-β, IL6 and TNFα, H-151 impairs the recruitment of TBK1 to STING.

A, B qPCR analysis of IFN-β, IL6, UNC13D or TNFα mRNA levels in UNC13D stable knockdown (KD) HT29 (A) and THP1 cells (B) without stimulation. $n = 3$ technical replicates.

C Construction of UNC13D knockout HeLa cells stably expressing FLAG-tagged wild-type UNC13D (UNC13D RE) and FHL3 mutation (FHL3 mut), UNC13D knockout HeLa cells with STING knockout cells (STING KO) and UNC13D knockout HeLa cells with cGAS stable knockdown (cGAS KD) cells. Cell lysates were subjected to immunoblotting (IB) with anti-UNC13D, -Flag, -cGAS, -STING or -actin antibody.

D Quantification of the relative expression of relevant proteins in Fig 5D. $n = 3$ biological replicates.

E, F Wild-type HeLa cells were pretreated with H-151 (2 μM) for 2 h before stimulation with cGAMP (100 nM) for 30 min, or they were infected with SeV for 6 h, or they were untreated (left). Protein levels were detected by immunoblotting with anti-p-TBK1, -p-IRF3, -STING, -p-IκBα, -tubulin or -actin antibody (E). The relative expression of relevant proteins was analyzed using ImageJ (F). $n = 3$ biological replicates.

G, H Wild-type HeLa cells were pretreated with H-151 (2 μM) for 2 h before stimulation with cGAMP (100 nM) for 30 min, or they were untreated. Cell localization of ER marker calnexin (green), Golgi marker GRASP65 (green), STING (red) and nuclei (blue) were examined via confocal microscopy. Scale bar: 10 μm (G). The colocalization of relevant proteins was analyzed using ImageJ (H). $n = 3$ biological replicates.

I, J Wild-type HeLa cells were pretreated with H-151 (2 μM) for 2 h before stimulation with cGAMP (100 nM) for 30 min, or they were untreated (left). Cell lysates were separated by native (top) or SDS (bottom) PAGE and analyzed by immunoblotting (IB) with anti-p-TBK1, -p-IRF3, -STING or -tubulin antibody (I). The relative expression of relevant proteins was analyzed using ImageJ (J). $n = 3$ biological replicates.

K, L HEK293T cells were pretreated with H-151 (2 μM) or left untreated and transfected with the indicated plasmids (5 μg each) for 24 h. Whole cell lysates (WCL) were examined, and cell lysates were immunoprecipitated (IP) with IgG and anti-FLAG antibodies, followed by immunoblotting (IB) with anti-Flag or -HA antibody. Quantification of the relative expression of STING from co-immunoprecipitation (IP) experiments (L). $n = 3$ biological replicates.

Data information: Statistically significant differences were determined using Student's *t*-test (J and L) or ANOVA (A, B, D, F and H). Data are shown as the mean ± s.d. ns, not significant, $P > 0.05$; $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.
Source data are available online for this figure.

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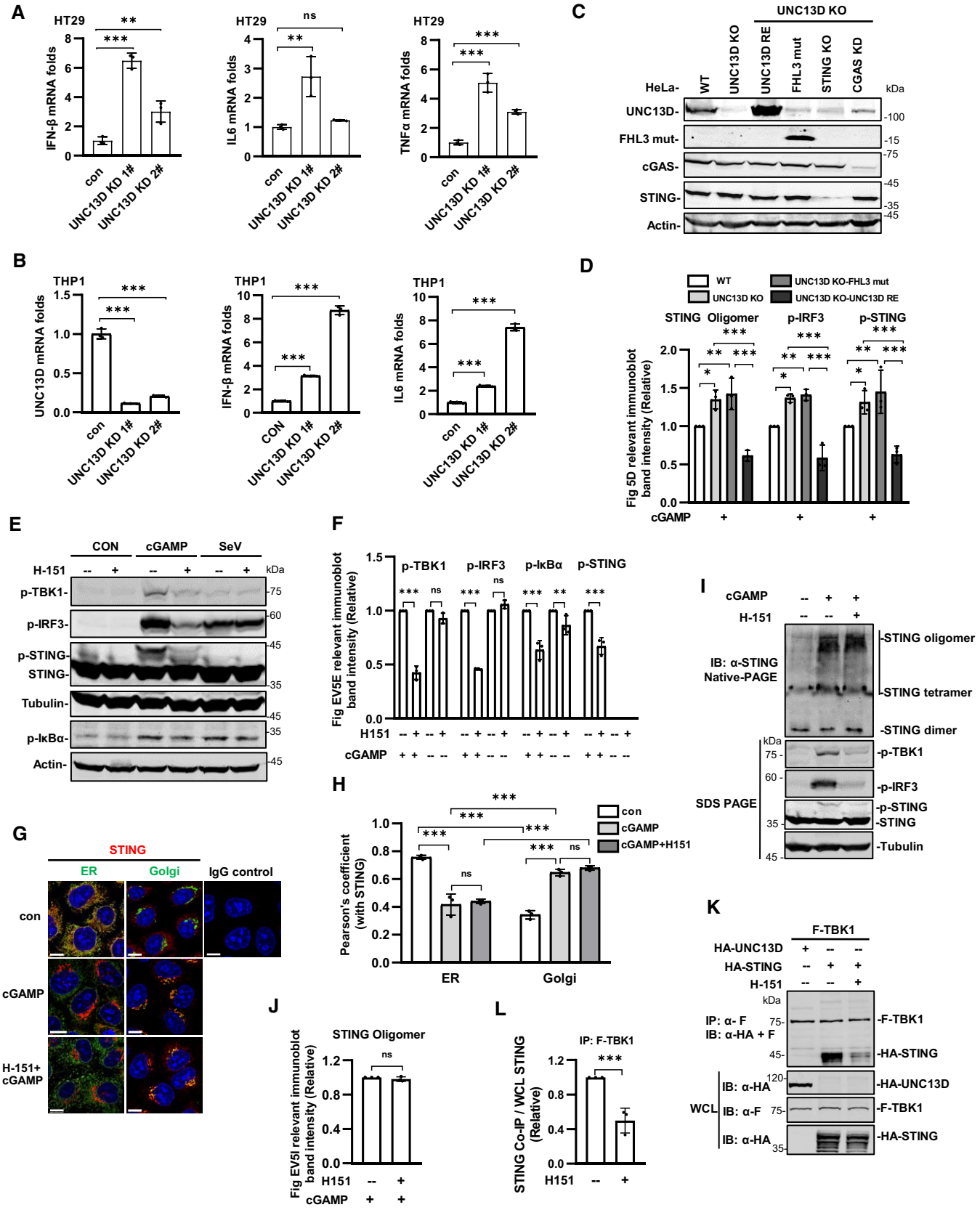

Figure EV5.

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