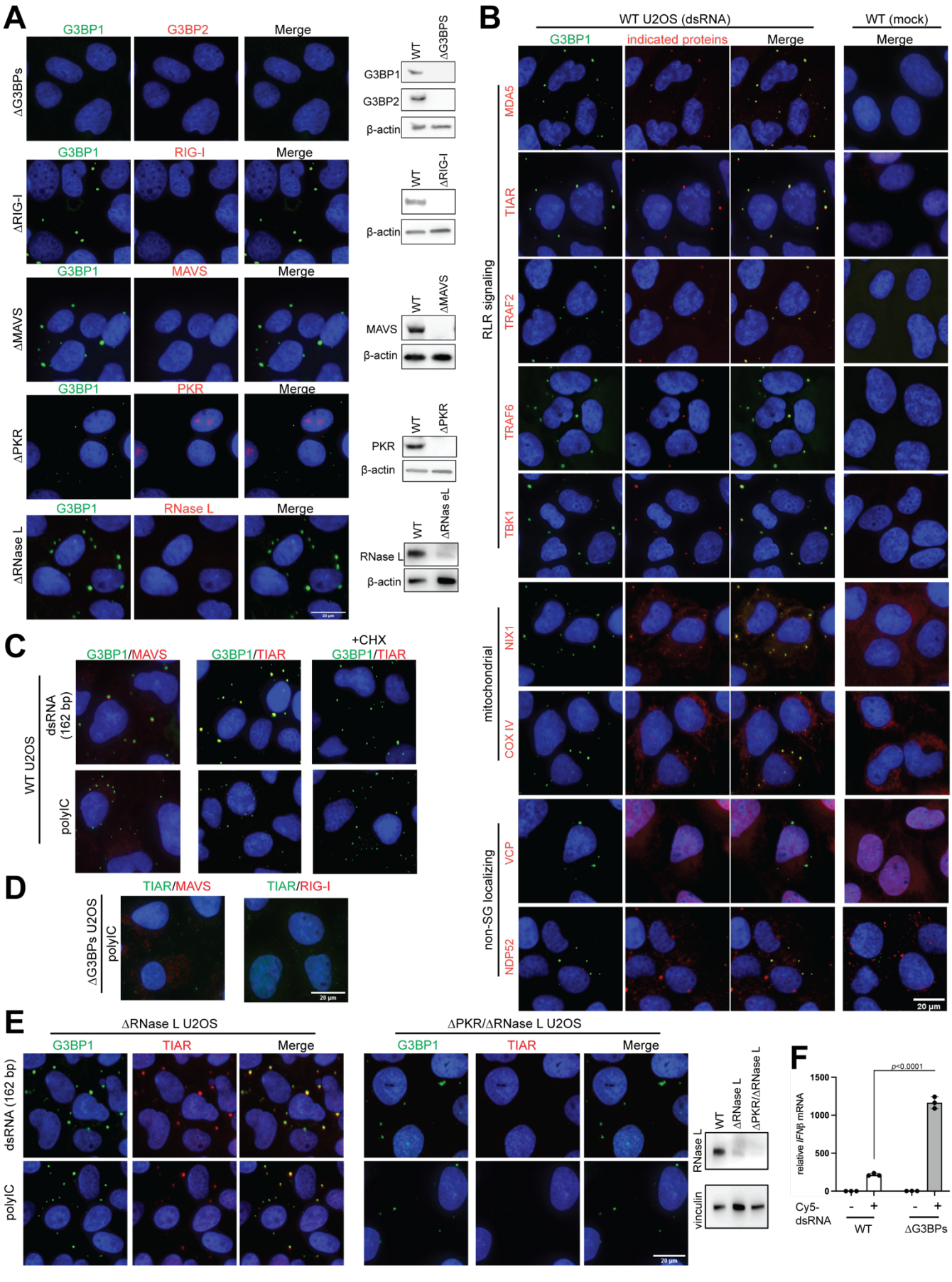
A
G3BP1
G3BP2
Merge
ΔG3BPs
RIG-I
ΔRIG-I
MAVS
ΔMAVS
PKR
ΔPKR
RNase L
ΔRNase L
WT
ΔG3BPS
ΔRIG-I
ΔMAVS
ΔPKR
ΔRNas eL
β-actin
B
WT U2OS (dsRNA)
WT (mock)
indicated proteins
RLR signaling
MDA5
TIAR
TRAF2
TRAF6
TBK1
mitochondrial
NIX1
COX IV
non-SG localizing
VCP
NDP52
20 μm
C
G3BP1/MAVS
G3BP1/TIAR
+CHX
WT U2OS
dsRNA (162 bp)
polyIC
D
TIAR/MAVS
TIAR/RIG-I
ΔG3BPs U2OS
E
ΔRNase L U2OS
ΔPKR/ΔRNase L U2OS
ΔPKR/ΔRNase L
vinculin
F
p<0.0001
relative IFNβ mRNA
1500
1000
500
0
Cy5-dsRNA
ΔG3BPs

Figure S1. dsRNA-triggered SGs are enriched for innate immune signaling molecules in the RLR pathway. Related to Figure 1.

- **A.** Validation of antibodies for G3BP1, RIG-I, MAVS, PKR and RNase L using individual knockout U2OS cells. Cells were transfected with 162 bp dsRNA (500 ng/ml) and imaged at 6 hr post-dsRNA as in Figure 1B.
- **B.** Immunofluorescence analysis of MDA5, TIAR, TRAF2, TRAF6, TBK1, NIX and COXIV (red) with G3BP1 (green) at 6 hr post-dsRNA in U2OS cells. VCP and NDP52 were also shown as examples of proteins not colocalizing with SGs.
- **C.** Immunofluorescence analysis of MAVS and TIAR (red) with G3BP1 (green) in U2OS cells in the presence and absence of cyclohexamide (CHX). Cells were stimulated with 162 bp dsRNA (500 ng/ml) or polyIC (500 ng/ml) and then treated with CHX 10 µg/mL for 6 hrs prior to imaging. All images are shown merged with the green and red fluorescent channel.
- **D.** Immunofluorescence image (merged) of MAVS and TIAR (red) with G3BP1 (green) in ΔG3BPs U2OS cells. Cells were stimulated with polyIC (500 ng/ml) for 6 hrs prior to imaging.
- **E.** Immunofluorescence image (merged) of TIAR (red) with G3BP1 (green) in ΔRNase L and ΔPKRΔRNase L U2OS cells. Cells were stimulated with 162 bp dsRNA (500 ng/ml) or polyIC (500 ng/ml) for 6 hrs prior to imaging.
- **F.** Antiviral signaling in U2OS cells (WT vs ΔG3BPs), as measured by the level of *IFNβ* mRNAs. Cells were transfected with 162 bp dsRNA 3'-labeled with Cy5 (500 ng/ml) for 6 hrs. 162 bp dsRNA activates the RLR pathway more efficiently in ΔG3BPs cells than in WT cells, regardless of the presence of 3'-Cy5 label.

Data are presented in means ± SD. *p* values were calculated using two-tailed unpaired Student's t test (ns, $p>0.05$). All data are representative of two to three independent experiments. Images were taken from representative field of view.

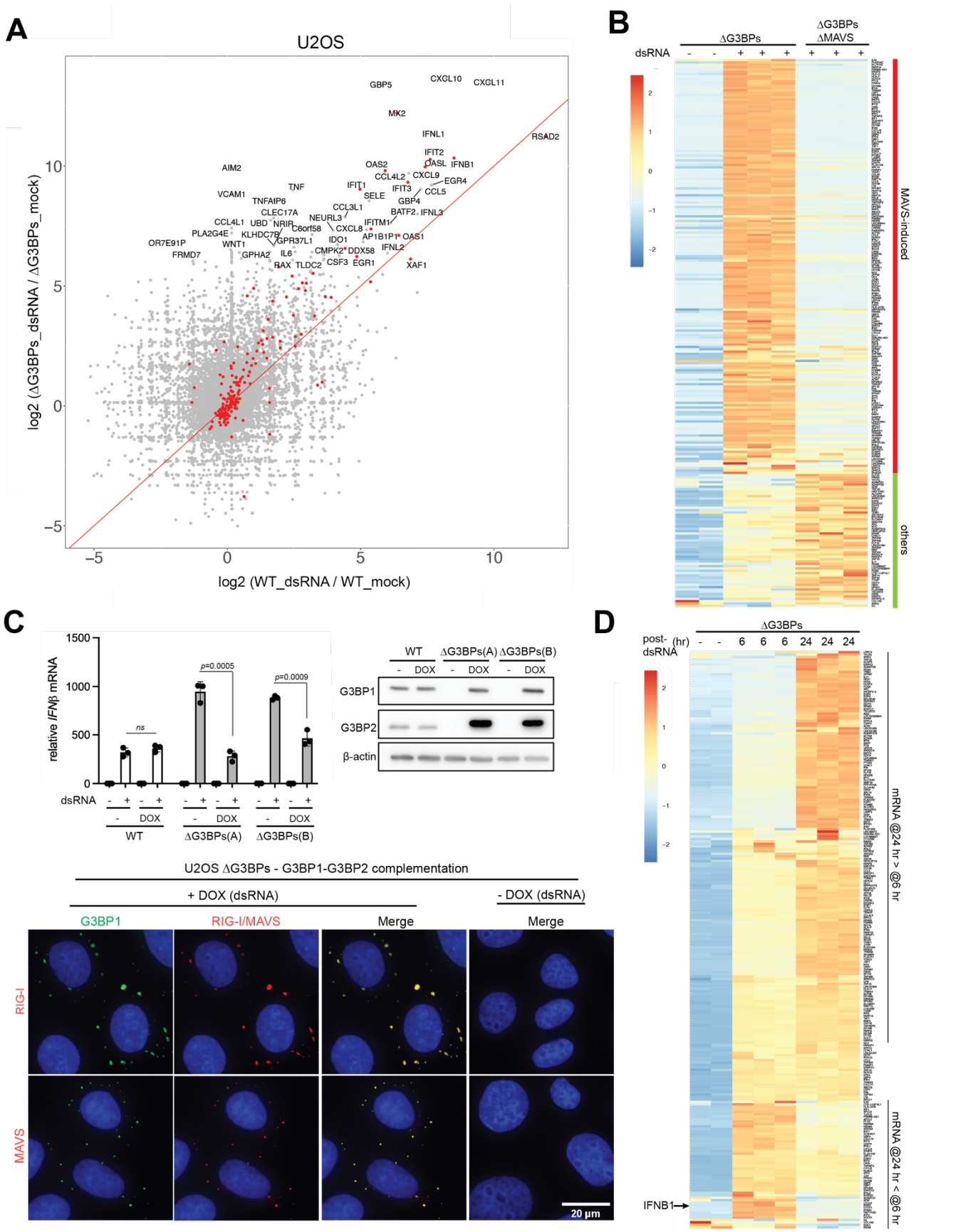

Figure S2. RLR signaling is hyperactive in SG-deficient ΔG3BPs U2OS cells. Related to Figure 1.

A. Scatter plot of log2-fold change (lfc2) upon dsRNA stimulation for 6 hrs. Lfc2 values in U2OS WT cells were plotted against those in ΔG3BPs cells. Differentially expressed genes with lfc2 > 6 in ΔG3BP cells are labeled. Genes annotated as "type I interferon production" (GO:0032606) and "response to type I interferon" (GO:0034340) are colored red.

B. Heat map of z-scores comparing the levels of mRNA in U2OS cells (ΔG3BPs vs. ΔG3BPs/ΔMAVS cells with or without dsRNA stimulation for 6 hrs). Genes showing lfc2 >2 (with $p\_adj<0.05$) upon dsRNA stimulation in WT cells were shown.

C. G3BPs complementation assay. Relative levels of *IFNβ* mRNA in U2OSΔG3BPs cells with and without complementation of G3BP1/2, which were expressed under the control of a doxycycline (DOX)-inducible promoter. Two separate clones (A and B) were treated with DOX (1 μg/ml) for 24 hrs and transfected with 162 bp dsRNA (500 ng/ml) for 6 hrs prior to analysis. Bottom: IF analysis of SGs with and without DOX induction.

D. Heat map of z-scores comparing the levels of mRNA in U2OSΔG3BPs cells at 6 and 24 hr post-dsRNA; indicated genes were from (B) and were re-ordered by hierarchical clustering.

Data are presented in means ± SD. *p* values were calculated using two-tailed unpaired Student's t test (ns, $p>0.05$). All data are representative of two to three independent experiments. Images were taken from representative field of view. Raw data for the heatmaps can be found in the supplemental files (data 2 and 3).

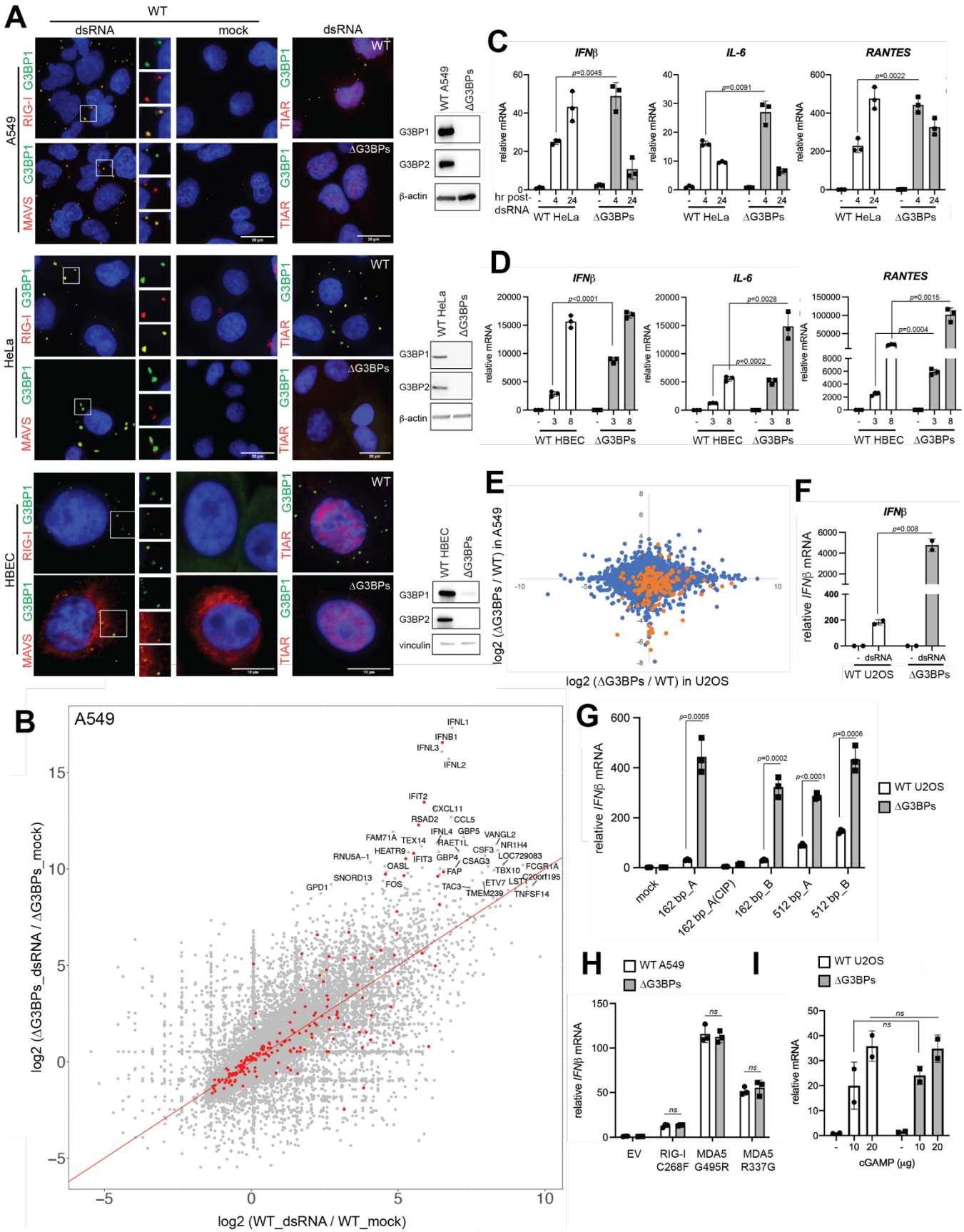

Figure S3. G3BPs suppress RLR signaling in both A549 and HeLa cells. Related to Figure 1.

A. Immunofluorescence images (merged) of RIG-I (red), MAVS (red), TIAR (red) and G3BP1 (green) in A549, HeLa and HBEC cells. Insets show individual fluorescent images of representative foci. A549 and HeLa cells were transfected with 162 bp dsRNA containing 5'ppp (500 ng/ml) for 6 hrs prior to imaging. HBEC cells were transfected with 162 bp dsRNA containing 5'ppp (200 ng/ml) for 4 hrs prior to imaging. Nuclei were stained with Hoechst 3342.

B. Scatter plot of log2-fold change (lfc2) upon dsRNA stimulation (500 ng/mL) for 4 hrs. Lfc2 values in WT A549 were plotted against those in ΔG3BPs cells. Differentially expressed genes with lfc2 > 9 in ΔG3BP cells were labeled. Genes annotated as "type I interferon production" (GO:0032606) and "response to type I interferon" (GO:0034340) were colored red.

C. Antiviral signaling in HeLa cells (WT vs ΔG3BPs), as measured by the level of *IFNβ* (left), *IL-6* (middle), and *RANTES* (right) mRNAs. Cells were transfected with 162 bp dsRNA with 5'ppp (500 ng/ml) for 4 or 24 hrs.

D. Antiviral signaling in HBEC cells (WT vs ΔG3BPs), as measured by the level of *IFNβ* (left), *IL-6* (middle), and *RANTES* (right) mRNAs. Cells were transfected with 162 bp dsRNA with 5'ppp (100 ng/ml) for 3 or 8 hrs.

E. Trancriptome comparison between WT vs. ΔG3BPs in the basal state (without dsRNA stimuli). Lfc2 changes from WT to ΔG3BPs in A549 cells (y-axis) were plotted against those in U2OS cells (x-axis). Averaged normalized read counts from two biological repeats were used. Each data point indicates individual gene. Genes shown in Figure 1B were highlighted in orange color.

F. Antiviral signaling in U2OS cells (WT vs ΔG3BPs) in response to dsRNA electroporation, as measured by the level of *IFNβ* mRNAs at 8 hr post-electroporation.

G. Antiviral signaling in U2OS cells (WT vs ΔG3BPs) upon transfection of 162 bp or 512 bp dsRNAs (500 ng/ml) with two different sequences (A and B, see Table S3), as measured by the level of *IFNβ* mRNAs at 6 hr transfection. 162 bp_A was also treated with calf intestinal phosphatase (CIP) to remove 5'ppp, which abrogated antiviral signaling.

H. Antiviral signaling in A549 cells (WT vs ΔG3BPs) after transient transfection (1 μg) of plasmids expressing constitutively active gain-of-function (GOF) mutant RIG-I (C268F) or MDA5 (G495R or R337G). Cells were analyzed 24 hrs post plasmid transfection.

I. Antiviral signaling in U2OS cells (WT vs ΔG3BPs) in response to 2′3′-Cyclic GMP-AMP (cGAMP, 10 or 20 μg), as measured by the level of *IFNβ* mRNAs at 6 hr post-treatment.

Data are presented in means ± SD. *p* values were calculated using two-tailed unpaired Student's t test (ns, $p>0.05$). RNA-seq data were confirmed by RT-qPCR analysis of select few genes in three independent experiments. All other data are representative of three independent experiments. Images were taken from representative field of view.

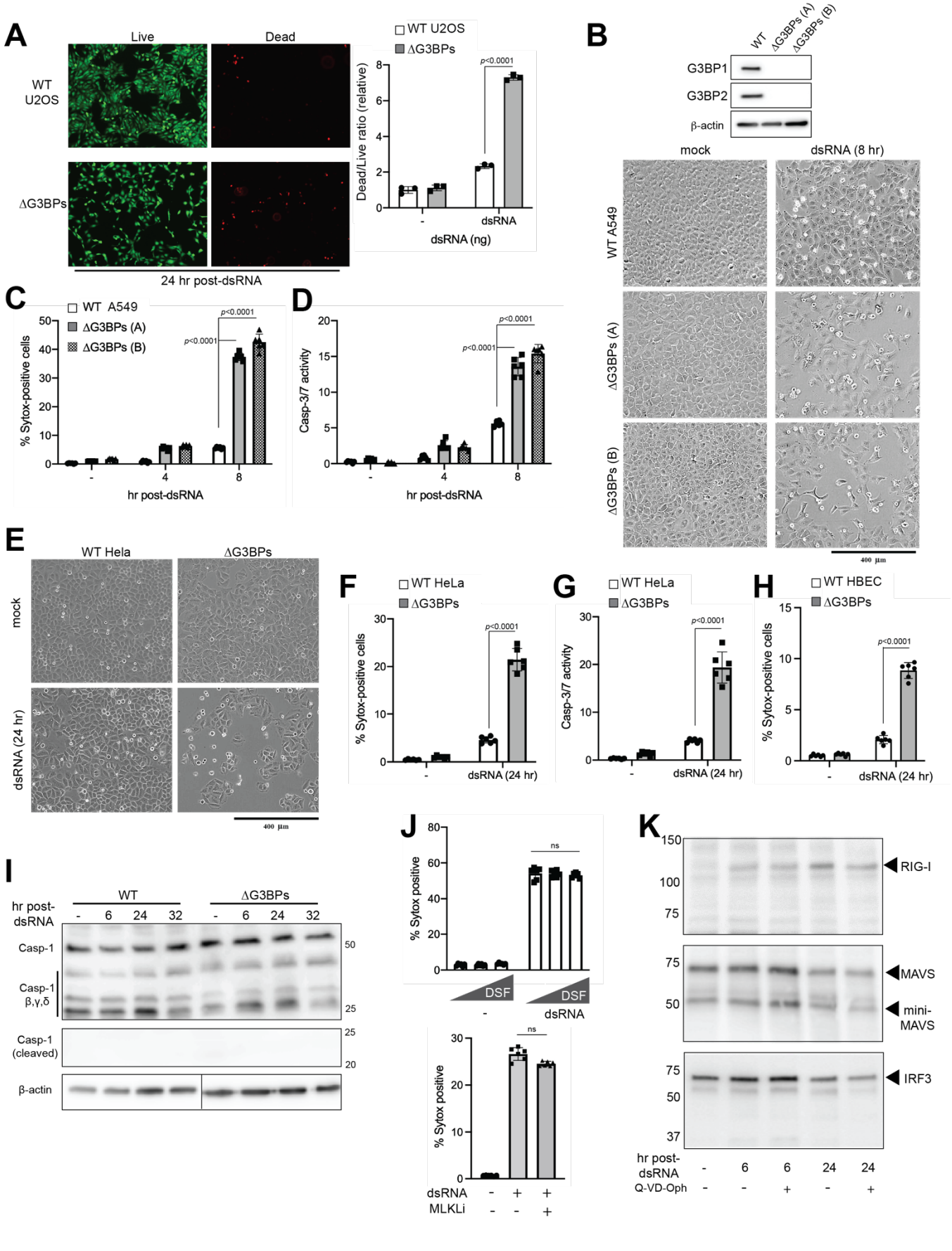
A
Live
Dead
WT U2OS
ΔG3BPs
24 hr post-dsRNA
WT U2OS
ΔG3BPs
Dead/Live ratio (relative)
p<0.0001
dsRNA
dsRNA (ng)
B
WT
ΔG3BPs (A)
ΔG3BPs (B)
G3BP1
G3BP2
β-actin
mock
dsRNA (8 hr)
WT A549
ΔG3BPs (A)
ΔG3BPs (B)
400 μm
C
WT A549
ΔG3BPs (A)
ΔG3BPs (B)
% Sytox-positive cells
p<0.0001
hr post-dsRNA
D
Casp-3/7 activity
p<0.0001
hr post-dsRNA
E
WT Hela
ΔG3BPs
mock
dsRNA (24 hr)
400 μm
F
WT HeLa
ΔG3BPs
% Sytox-positive cells
p<0.0001
dsRNA (24 hr)
G
WT HeLa
ΔG3BPs
Casp-3/7 activity
p<0.0001
dsRNA (24 hr)
H
WT HBEC
ΔG3BPs
% Sytox-positive cells
p<0.0001
dsRNA (24 hr)
I
WT
ΔG3BPs
hr post-dsRNA
- 6 24 32 - 6 24 32
Casp-1
Casp-1 β,γ,δ
Casp-1 (cleaved)
β-actin
50
25
25
20
J
% Sytox positive
ns
DSF
DSF
-
dsRNA
% Sytox positive
ns
dsRNA - + +
MLKLi - - +
K
RIG-I
MAVS
mini-MAVS
IRF3
hr post-dsRNA - 6 6 24 24
Q-VD-Oph - - + - +

Figure S4. SG deficiency results in more pronounced apoptosis in U2OS, A549 and HeLa cells upon dsRNA stimulation. Related to Figure 4.

- **A.** Cell death in WT vs. ΔG3BPs U2OS cells in response to 162 bp dsRNA transfection (500 ng/ml). Cells were stained with the LIVE/DEAD® Viability/Cytotoxicity stain (Thermo) 24 hr post-dsRNA and were imaged using a Nikon Eclipse TS2R inverted microscope (left). The ratio of dead-to-live cells were determined by the ratio of the DEAD stain intensity (red) to the LIVE stain intensity (green) as quantitated by ImageJ (right).
- **B-D.** Cell death in WT vs. ΔG3BPs A549 cells in response to 162 bp dsRNA transfection (500 ng/ml). Two independent clones of ΔG3BPs were analyzed. Cell death was measured at 8 hr post-dsRNA by brightfield microscopy (B), Sytox uptake (C), and caspase 3/7 activity assay (D).
- **E-G.** Cell death in WT vs. ΔG3BPs HeLa cells in response to 162 bp dsRNA transfection (500 ng/ml). Cell death was measured at 24 hr post-dsRNA by brightfield microscopy (E), Sytox uptake (F), and caspase 3/7 activity assay (G).
- **H.** Cell death in WT vs. ΔG3BPs HBEC cells in response to 162 bp dsRNA transfection (100 ng/ml). Cell death was measured at 8 hr post-dsRNA by Sytox uptake.
- **I.** Caspase-1 cleavage analysis of WT and ΔG3BPs U2OS cells. Cells were transfected with 162 bp dsRNA (500 ng/ml) and were analyzed at indicated time. No caspase-1 cleavage was observed in either WT or ΔG3BPs cells upon dsRNA transfection.
- **J.** Effect of the gasdermin D inhibitor (disulfiram, DFS) and MLKL inhibitor (necrosulfonamide, MLKLi) on dsRNA-induced cell death in U2OSΔG3BPs. Cells were pre-treated with DMSO, disulfiram (10 or 25 μM) or MLKLi (1 μM) and transfected with 162 bp dsRNA (500 ng/ml).
- **K.** Analysis of RIG-I, MAVS or IRF3 cleavage upon dsRNA stimulation. U2OSΔG3BPs cells were transfected with 162 bp dsRNA (500 ng/ml) in the presence or absence of pan-caspase inhibitor (Q-VD-OPh, 10 μM). Cells were harvested at indicated time points and analyzed by SDS-PAGE.

Data are presented in means ± SD. *p* values were calculated using two-tailed unpaired Student's t test (ns, $p>0.05$). All data are representative of two to three independent experiments.

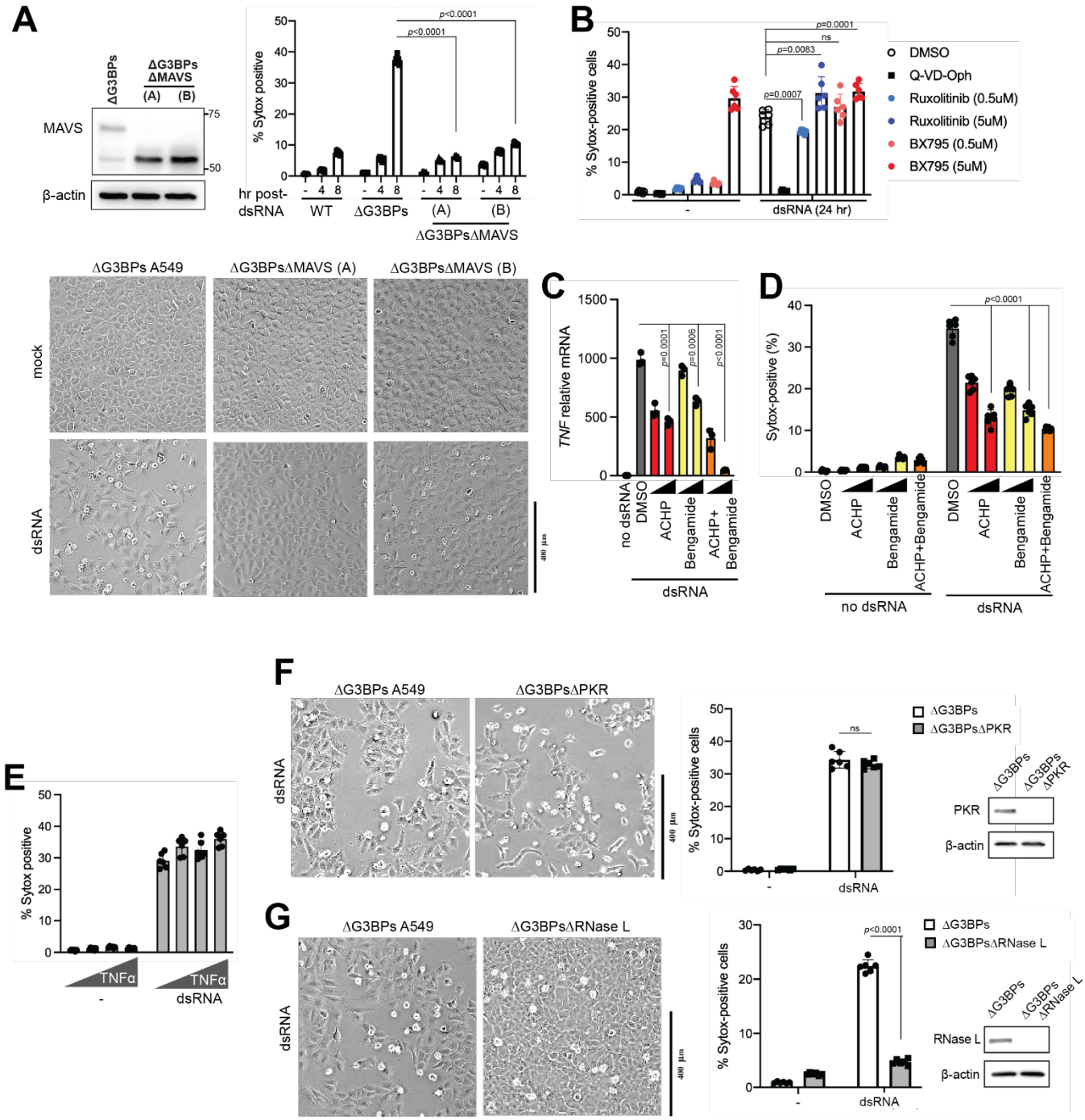

Figure S5. Apoptosis in SG-deficient ΔG3BPs cells is dependent on MAVS. Related to Figure 5.

A. Cell death in ΔG3BPs and ΔG3BPsΔMAVS A549 as measured by Sytox uptake (top) and bright field microscopy (bottom) at 8 hr post-dsRNA. Cells were transfected with 162 bp dsRNA as in Figure 1B.

B. Cell death in U2OSΔG3BPs cells in the presence of an inhibitor for TBK1 (BX795) or JAK (Ruxolitinib). Pan-caspase inhibitor Q-VD-Oph (10 μM) was used for comparison. Cells were pre-treated with the indicated inhibitor 30 min prior to dsRNA transfection (500 ng/ml). Cell death was measured by Sytox uptake at 24 hr post-dsRNA.

C. Role of NF-kB on TNF induction. U2OSΔG3BPs cells were pre-treated with NF-kB inhibitors (bengamide B and ACHP, 1 or 5 μM, individually or in combination) for 1 hr in FBS-free media.

Cells were then supplemented with FBS (10%) and were transfected with dsRNA (500 ng/ml). Cells were harvested 6 hr post-dsRNA and *TNF* mRNA level was analyzed by RT-qPCR.

D. Cell death in U2OSΔG3BPs in the presence of an inhibitor for NF-kB (bengamide B and ACHP, 1 or 5 μM, individually or 5 μM in combination). Cells were treated as in (C) and cell death was measured by Sytox uptake at 24 hr post-dsRNA.

E. Cell death in U2OSΔG3BPs cells in response to an increasing concentration of TNFα (10, 20, 50 ng/ml). Cell death was measured by Sytox uptake at 24 hr post-treatment.

F. Cell death in ΔG3BPs and ΔG3BPsΔPKR A549 cells in response to dsRNA. Cell death was measured by brightfield microscopy (left) and Sytox uptake (right) at 8 hr post-dsRNA.

G. Cell death in ΔG3BPs and ΔG3BPsΔRNase L A549 cells in response to dsRNA. Cell death was measured by brightfield (left) and Sytox uptake (right) at 8 hr post-dsRNA.

Data are presented in means ± SD. *p* values were calculated using two-tailed unpaired Student's t test (ns, $p>0.05$). All data are representative of three independent experiments.

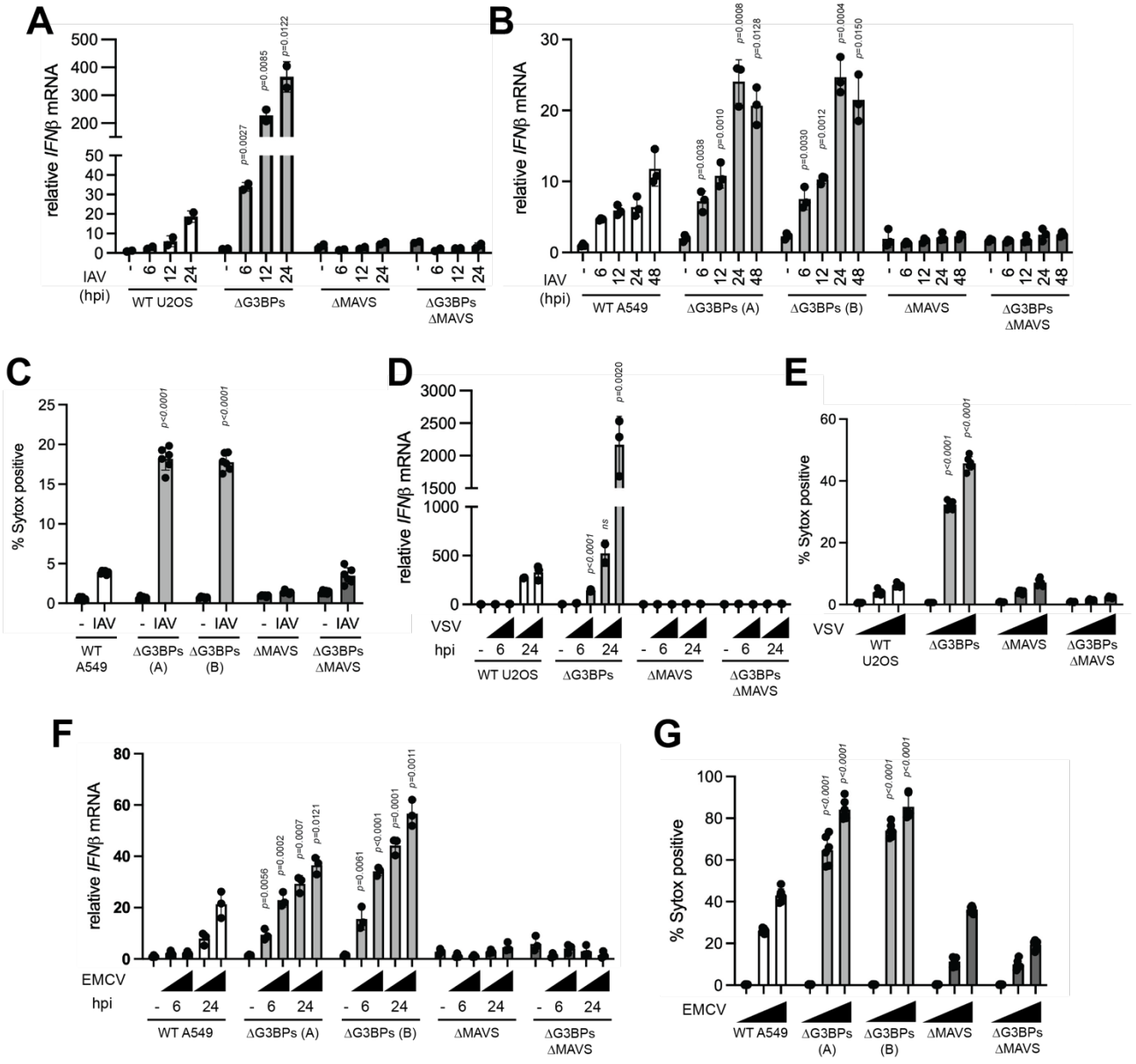

Figure S6. SGs suppress immune response and cell death during viral infection. Related to Figure 6.

- **A.** Antiviral signaling upon infection with IAV$^{\Delta NS1}$ (MOI=1) in U2OS cells, as measured by *IFNβ* mRNA.
- **B.** Antiviral signaling upon infection with IAV$^{\Delta NS1}$ (MOI=0.1) in A549 cells, as measured by *IFNβ* mRNA.
- **C.** Cell death upon infection with IAV$^{\Delta NS1}$ (MOI=0.1), as measured by Sytox uptake. A549 cells were infected virus as in (B) and analyzed at 24 hpi.
- **D.** Antiviral signaling upon infection with VSV^{M51R} (MOI=0.1 or 1) in U2OS cells, as measured by *IFNβ* mRNA.
- **E.** Cell death upon infection with VSV^{M51R} (MOI=0.1 or 1), as measured by Sytox uptake. U2OS cells were infected with VSV^{M51R} as in (D) and analyzed at 24 hpi.
- **F.** Antiviral signaling upon infection with EMCV (MOI=0.01 or 0.1) in A549 cells, as measured by *IFNβ* mRNA.

G. Cell death upon infection with EMCV (MOI=0.1 or 0.3), as measured by Sytox uptake. A549 cells were infected with EMCV as in (F) and analyzed at 24 hpi.

Data are presented in means ± SD. All data are representative of at least three independent experiments. *p* values were calculated using two-tailed unpaired Student's t test (ns, $p>0.05$). Unless indicated otherwise, *p* values were calculated in comparison to WT values at equivalent conditions.

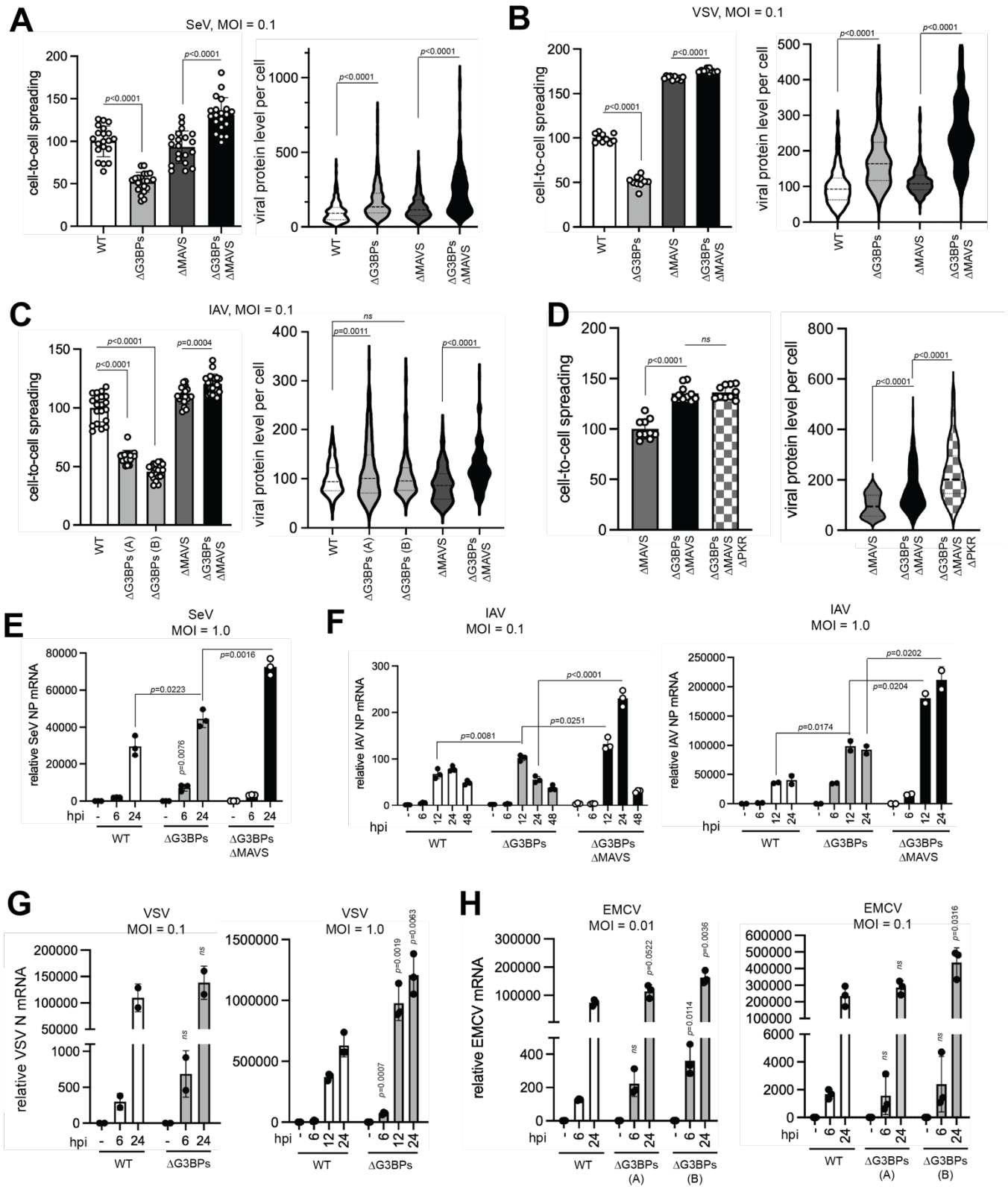

Figure S7. SGs also suppress viral replication independent of the RLR pathway. Related to Figure 6.

A. SeV cell-to-cell spreading and viral protein level per infected cell. Left: Relative cell-to-cell spreading of SeV. U2OS cells were infected with SeV (MOI=0.1), stained with anti-SeV serum 18 hpi, analyzed for number of cells above the background fluorescence (i.e. mock infection

staining) per field of view. Each data point represents a field of view (n=20). Right: Relative level of SeV protein staining in infected cells, as measured by corrected total cell fluorescence (CTCF). Each data point represents an infected cell (n=200). Data were normalized against the WT average value in both graphs.

B. Same as (A) for VSV^{M51R} (MOI=0.1) infection in U2OS cells. (Left) n=10, (Right) n=200.

C. Same as (A) for $IAV^{\Delta NS1}$ (MOI=0.1) infection in A549 cells. (Left) n=20, (Right) n=200.

D. SeV cell-to-cell spreading and viral protein level per infected cell. SeV (MOI=1) was used. Role of PKR in ΔG3BPsΔMAVS cells was analyzed in U2OS cells. (Left) n=10, (Right) n=200.

E. Level of SeV NP mRNA during infection. U2OS cells were infected with SeV (MOI=1) and analyzed at indicated time points.

F. Level of $IAV^{\Delta NS1}$ NP mRNA during infection. Left: A549 cells were infected with $IAV^{\Delta NS1}$ (MOI=0.1) for indicated time. Right: U2OS cells were infected with $IAV^{\Delta NS1}$ (MOI=1) for indicated time.

G. Level of VSV N mRNA during infection. U2OS cells were infected with VSV^{M51R} (MOI=0.1 or MOI=1.0) for indicated time.

H. Level of EMCV mRNA during infection. A549 cells were infected with EMCV (MOI=0.01 or 0.1) for indicated time.

Data are presented in means ± SD. All data are representative of at least three independent experiments. *p* values were calculated using two-tailed unpaired Student's t test (ns, $p>0.05$). Unless indicated otherwise, *p* values were calculated in comparison to WT values at equivalent conditions.

Table S1. Primer Sequences, Related to Figures 1-2, 4-7

Name	Sequence
Human IFN-β fwd	AAACTCATGAGCAGTCTGCA
Human IFN-β rev	AGGAGATCTTCAGTTTCGGAGG
Human RANTES fwd	TACACCAGTGGCAAGTGCTC
Human RANTES rev	TGTACTCCCGAACCCATTTC
Human IL-6 fwd	GGAGACTTGCCTGGTGAAAA
Human IL-6 rev	GTCAGGGGTGGTTATTGCAT
Human TNF-α fwd	GAAGCAGGCAATCACAGAAA
Human TNF-α rev	TGAAACCGACCATAGTGGAA
Human ACTIN-β fwd	GCACAGAGCCTCGCCTT
Human ACTIN-β rev	GTTGTCGACAGCG
RT primer Sendai Virus	TGTTCTTACTAGGACAAG
Sendai Virus NP mRNA fwd	TGCCCTGGAAGATGAGTTAG
Sendai Virus NP mRNA rev	GCCTGTTGGTTTGTGGTAAG
RT primer IAVdelNS1 NP	CCAGATCGTTCGAGTCGTTTTTTTTTTTTTTTTTCTTTAATTGTC
IAVdelNS1 NP mRNA fwd	CCAGATCGTTCGAGTCGT
IAVdelNS1 NP mRNA rev	CGATCGTGCCCTCCTTTG
EMCV RNA fwd	GACGCTTGAAGACGTTGTCTTCTTA
EMCV RNA rev	CCCTACCTCACGGAATGGGGCAAAG
RT primer VSV N mRNA	CCAGATCGTTCGAGTCGTTTTTTTTTTTTTTTTTTACACGGCGATCTTGCC
VSV N mRNA fwd	CCAGATCGTTCGAGTCGT
VSV N mRNA rev	CGCGGTGGTGTTGTGTTC

Table S2. gRNA Sequences, Related to STAR Methods

Name	Sequence
G3BP1 gRNA1	GGAGAAGCCTAGTCCCCTGC
G3BP1 gRNA2	CTAGTCCCCTGCTGGTCGGG
G3BP2 gRNA1	GAGTGATGGAGTAGTTGTCC
G3BP2 gRNA2	GAAATCTACTACTCCTCCTC
PKR gRNA[1]	ATTATGAACAGTGTGCATCG
UBAP2L gRNA	CACCACGAGCGCCTCTTCAA
RIG-I gRNA	TCGTGCTCGGTGGTCATGC
MAVS gRNA	CACCGCCCGGGCCGCCGCTGAA
PKR gRNA 1[2]	TAATACATACCGTCAGAAGC
PKR gRNA 2[2]	TTATCCATGGGGAATTACAT
IRF3 gRNA 1	CACCGGACACCTCTCCGGACACCAA
IRF3 gRNA 2	AAACTTGGTGTCCGGAGAGGTGTCC
UBAP2L gRNA	GCCTGGACTACATTCCGCAA
RNase L gRNA	TTTGAGGCGAAAGACAAAGG

[1] Used by Dr. Ivanov's lab to generate U2OSΔPKR

[2] Used to generate U2OSΔG3BPsΔPKR and A549ΔG3BPsΔPKR

Table S3. dsRNA Sequences, Related to STAR Methods

Name	Sequence
162 bp_A	gggagaatgtcgaatgggtattccacagacgagaatttccgctatctcatctcgtgcttcagggccagggtgaaaatgtacatc caggtggagcctgtgctggactacctgacctttctgcctgcagaggtgaaggagcagattcagaggacagtctctccc
162 bp_B	gggagaatgaacacgattaacatcgctaagaacgacttctctgacatcgaactggctgctatcccgttcaacactctggctgac cattacggtgagcgtttagctctctccc
512 bp_A	gggagaatgtcgaatgggtattccacagacgagaatttccgctatctcatctcgtgcttcagggccagggtgaaaatgtacatc caggtggagcctgtgctggactacctgacctttctgcctgcagaggtgaaggagcagattcagaggacagtcgccacctccggg aacatgcaggcagttgaactgctgctgagcaccttggagaagggagtctggcaccttggttggactcgggaattcgtggaggcc ctccggagaaccggcagccctctggccgcccgctacatgaaccctgagctcacggacttgccctctccatcgtttgagaacgctc atgatgaatatctccaactgctgaacctccttcagcccactctggtggacaagcttctagttagagacgtcttggataagtgcatg gaggaggaactgttgacaattgaagacagaaaccggattgctgctgcagaaaacaatggaaatgaatcaggtgtaagagag cttctccc
512 bp_B	gggagaatgtggtcgaagccacttggaataagaccggcgtgcgcttgtctatatgttacttccaccacattgccgtcttttggca atgtgagggcccggaacctggccctgtcttcttgacgaacattcctaggggactttcccctctcgccaaaggaatgtaaggtctg ttgaatgtcgtgaaggaagcagttcctctggaagcttcttgaagacaaacagcgtctgtagcgacccctttgcaggcagcggaac cccccacctggtaacaggtgcctctgcggccgaaagccacgtgtataagatacacctgcaaaggcggcacaaccccagtgcca cgttgtgcgttggatagttgtggaaagagtcaaatggctttccccaagcgtattcaacaaggggctgaaggatgcccagaaggt accccactggttgggatctgatctggggcctcggtgcaggtgctttacacctgttgagtcgaggttaaaaaacgtctaggccccc tctccc

Only one strand sequence was shown.