Supplementary information

Methods

Cell Culture and transfection

HeLa cells stably expressing vMIA were described previously (Arnoult et al, 2004). HeLa or HEK293 cells were cultured in DMEM supplemented with 10% calf serum and antibiotics. Transient transfection of HEK293 cells was carried out using the calcium phosphate precipitation method whereas HeLa cells were transiently transfected using FuGENE 6 (Roche Applied Science).

Plasmids

The plasmid for the expression of FLAG-MAVS was provided by Dr. D. Vitour (Institut Pasteur, Paris) plasmids for Myc-Drp1, Myc-OPA1 were from Dr C. Blackstone, plasmids for vMIA and vMIAΔTM were from Dr. V. Goldmacher, the plasmid for Myc-Fis1 was from Dr R.J. Youle and plasmids for Myc-Mfn1 and Myc-Mfn2 were provided by Dr. S.J. Martin. The plasmid for STING-HA was provided by Dr. G.N. Barber.

Viral infection

SeV H4 and WT strains were described previously (Strahle et al, 2006). Cells were infected with SeV at $50x10^6$ pfu/ml. Briefly, cells in 12-well (for western blot analysis) or 24-well (for luciferase assays) plates were washed once with 1X PBS and incubated with 0.4 or 0.2 ml of

Sendai virus diluted in serum-free DMEM respectively. Two hours later, an equal volume of DMEM supplemented with 20% calf serum and antibiotics was added to the cells. Cells were returned to the 37°C incubator until analysis.

Immunofluorescence microscopy

Cells grown in LabTek chambers were fixed for 10 min in 4% paraformaldehyde followed by permeabilization with 0.15% Triton X-100 in phosphate-buffered saline for 15 min. The cells were then incubated for 1 hr in blocking buffer (2% bovine serum albumin (BSA) in phosphate buffered saline) followed by incubation overnight with a mouse monoclonal anti-cytochrome c (BD Biosciences Pharmingen, clone 6H2.B4) (1:800 in blocking buffer) or a mouse anti-Protein Disulphide Isomerase (PDI, abcam, clone RL90) (1:400) for mitochondria or ER staining respectively. For both ER and mitochondrial staining, the anti-PDI and a rabbit polyclonal anti-TOM20 (Santa Cruz Biotechnology, FL-145) (1:400) were used. Next, cells were washed three times for 10 min each in blocking buffer, then incubated for 2 hr with Alexa Fluor anti-mouse and anti-rabbit secondary antibodies (Molecular Probes). Images were acquired using a Leica SP2 confocal microscope through a x63 oil fluorescence objective.

In some experiments signal intensities from each channel were reconstructed by plotting pixel values of each channel along lines drawn through optical sections. Multichannel images were separated into single channels and exported to the ImageJ software. Measurements of the pixel intensities were made along the lines shown in the respective picture.

Immunoprecipitation

For immunoprecipitation of endogenous proteins, HeLa cells cultured in 100 mm plates were

lysed in buffer B (Tris 50mM pH 7.5, NaCl 140mM, EDTA 5mM, glycerol 5% and CHAPS 1%) supplemented with the protease inhibitor mixture Complete, and the lysates cleared at 10,000xg for 15 min at 4°C. 500µl of lysate was incubated with a specific antibody (5µg/ml) or control IgG, at room temperature for immunoprecipitation.

For immunoprecipitation of overexpressed proteins, transfected HEK293 cells cultured in 6 well plates were lysed in buffer C (Tris 50mM pH7.5, NaCl 150mM, 2mM EDTA, Triton 1% and NP40 1%) supplemented with the protease inhibitor mixture Complete, and the lysates cleared at 10,000xg for 15 min at 4°C. 500µl of lysate was incubated with anti-tag antibody (1µg/ml) or control IgG, at room temperature for immunoprecipitation.

The antibodies used for immunoprecipitation were as follows: a rabbit polyclonal anti-Cardif/MAVS (Alexis Biochemicals, AT107), a rabbit polyclonal anti-Mfn1 provided by Dr. M. Rojo (Rojo et al, 2002), a mouse monoclonal anti-FLAG (Sigma Aldrich, clone M2) and a mouse monoclonal anti-myc (Sigma-Aldrich, clone 9E10). After 1 hr, 20µl of equilibrated protein G-magnetic beads (Ademtech SA) was added. Immunoprecipitation was carried out for another hour. The beads were then washed 3 times with Buffer B or C and immune complexes were resolved by SDS-PAGE.

RNA interference

RNAi against Drp1, Fis1, OPA1 and Mfn1 was performed using the sh-activated gene silencing system with shRNA constructs as described previously (Lee et al, 2004). One day after the transfection with the shRNA constructs, HeLa or HEK293 cells were grown for 2 days in DMEM containing 300µg/ml or 600µg/ml hygromycin B respectively, followed by 3-4 days in DMEM containing 50µg/ml or 100µg/ml hygromycin B for the selection of transfectants.

siRNA oligos raised against RIG-I, MAVS, Drp1, Fis1, OPA1 and Mfn1 at a final

concentration of 20 nM were transfected in HeLa cells using oligofectamine according to the manufacturer's instructions. On the third days, cells were either harvested or transfected for luciferase assays next infected. siRNAs and control siRNA were purchased in Ambion. The sequence of the siRNA oligos are as follows (only the sense strands are shown):

RIG-I siRNAa: GCAGGAUUCGAUGAGAUUGtt

RIG-I siRNAb: GGAAGAGGUGCAGUAUAUUtt

MAVS siRNA1: CCGUUUGCUGAAGACAAGAtt

MAVS siRNA2: CCACCUUGAUGCCUGUGAAtt

Drp1 siRNA: GGCUAGCCAGAGAAUUACCtt

Fis1 siRNA: GCGGGAUUACGUCUUCUACtt

OPA1 siRNA: GUUAUCAGUCUGAGCCAGGtt

Mfn1 siRNA: GGAUCACAUUUUGUUGAAGtt

Mitochondria and ER fraction isolation

HeLa cells were harvested in isotonic buffer D (210mM mannitol, 70mM sucrose, 1mM EDTA and 10mM HEPES; (pH 7.5), supplemented with the protease inhibitor mixture Complete (Roche Molecular Biochemicals). Cells were broken by ten passages through a 25-gauge needle fitted onto a 5 ml syringe, and the suspension was then centrifuged at 2000 g at 4°C to remove nuclei and unbroken cells. This procedure was repeated until nearly all of the cells were broken. Mitochondria were obtained by centrifugation at 15,000g for 10 min, and post mitochondrial supernatant was used for purification of ER fractions. Mitochondria pellet was resuspended in buffer D, and was layered on discontinuous sucrose gradients consisting of 1.2 M and 1.6 M sucrose and banded by centrifugation at 27,000g for 2 hr. Mitochondria fraction was washed in buffer D and collected and pelleted by centrifugation at 15,000g for 10 min. Purified mitochondria were resuspended in PBS and used for immunoblot analysis. To

isolate ER fractions, postmitochondrial supernatant described above was layered on discontinuous sucrose gradients consisting of 1.3 M, 1.5 M and 2.0 M sucrose, and banded by centrifugation at 100,000g for 70 min. The ER fraction at the interface between the supernatant and the 1.3 M sucrose was collected, and pelleted by centrifugation at 100,000g for 45 min. The ER membranes were resuspended in PBS and were used for immunoblot analysis.

Statistical analyses

Data were compared using Student's t-test. Differences were considered to be significant if $P<0.05$.

Arnoult D, Bartle LM, Skaletskaya A, Poncet D, Zamzami N, Park PU, Sharpe J, Youle RJ, Goldmacher VS (2004) Cytomegalovirus cell death suppressor vMIA blocks Bax- but not Bak-mediated apoptosis by binding and sequestering Bax at mitochondria. *Proc Natl Acad Sci U S A* **101**(21)**:** 7988-7993

Lee YJ, Jeong SY, Karbowski M, Smith CL, Youle RJ (2004) Roles of the mammalian mitochondrial fission and fusion mediators Fis1, Drp1, and Opa1 in apoptosis. *Mol Biol Cell* **15**(11)**:** 5001-5011

Rojo M, Legros F, Chateau D, Lombes A (2002) Membrane topology and mitochondrial targeting of mitofusins, ubiquitous mammalian homologs of the transmembrane GTPase Fzo. *J Cell Sci* **115**(Pt 8)**:** 1663-1674

Strahle L, Garcin D, Kolakofsky D (2006) Sendai virus defective-interfering genomes and the activation of interferon-beta. *Virology* **351**(1)**:** 101-111

Fig S1. RLR activation leads to a selective degradation of the higher isoform of MAVS. (A) HeLa cells were infected either with SeV H4 or SeV WT and at different time points after infection, MAVS, p-IRF3, IRF3, p-IκBα and IκBα were analyzed in cell extracts by immunoblotting. Actin was used as a protein loading control. (B) HEK293 were infected with SeV H4 and at different time points after infection the same proteins as in (A) were analyzed in cell extracts by immunoblotting. (C) HeLa cells were transfected or not with poly (I:C) (1μg/ml) in the presence of zVAD-fmk (50μM), 8 hr later, MAVS, p-IRF3, IRF3, p-IκBα and IκBα were studied in cell extracts by immunoblotting. An asterisk (*) indicates a probable nonspecific protein band.

Fig S2. Knock down of RIG-I prevents mitochondrial elongation following infection. (A) Control siRNA or siRNA targeting RIG-I were transfected into HeLa cells. The efficiency of RIG-I silencing was confirmed by immunoblotting. MAVS expression pattern was also assessed. Actin was used as a protein loading control. To measure IFNβ induction and NF-κB activation, IFNβ-luc or NF-κB-luc reporter plasmids were transfected into the RNAi cells, which were then infected with SeV WT or SeV H4 for 9 hr. (B) Control siRNA or siRNA targeting RIG-I were transfected into HeLa cells. Next mitochondrial morphology was quantified in cells either non-infected or infected with SeV WT or SeV H4 for 9 or 18 hr. Data represent the means ±S.D. of 3 independent experiments, with 300 cells per condition.

Fig S3. MAVS is expressed as 2 main isoforms. Specificity of the anti-MAVS used in this study. (A) Control siRNA or siRNA targeting MAVS were transfected into HeLa cells. The efficiency of MAVS silencing was confirmed by immunoblotting using 2 different antibodies raised against MAVS. Left panel, a mouse monoclonal anti-Cardif/MAVS (Alexis

Biochemicals, clone Adri-1) (1:2000); right panel, a rabbit polyclonal anti-Cardif/MAVS (Alexis Biochemicals, AT107) (1:2000). The 2 main isoforms of MAVS are knocked down in MAVS siRNA-transfected cells. (B) MAVS was immunoprecipitated in extracts from HeLa cells with a rabbit polyclonal anti-Cardif/MAVS (Alexis Biochemicals, AT107) and the immune complexes were resolved by SDS-PAGE. MAVS was detected with the mouse monoclonal anti-Cardif/MAVS.

Fig S4. Knockdown of Fis1 or Drp1 expression induces mitochondrial fusion whereas knockdown of OPA1 or Mfn1 promotes fission. HeLa cells were transfected with constructs containing shRNA of the target sequence of Fis1, Drp1, OPA1, Mfn1 or control, and the transfectants were selected by growth in media containing hygromycin B. (A) Total cell lysates from the Drp1 RNAi cells, Fis1 RNAi cells, OPA1 RNAi, Mfn1 RNAi cells and control RNAi cells were prepared, and the expression levels of Drp1, Fis1, OPA1 and Mfn1 were analyzed by immunoblotting. Actin level was analyzed for a loading control. (B) Mitochondria of control RNAi cells, Drp1 RNAi cells, Fis1 RNAi cells, OPA1 RNAi cells or Mfn1 RNAi cells were visualized with anti-cytochrome c and analyzed by confocal microscopy. (C) Percentage of cell population with normal, fragmented or fused mitochondria in control RNAi, Drp1 RNAi, Fis1 RNAi, OPA1 RNAi or Mfn1 RNAi culture. 300 cells in several fields were counted in each experiment. Data represent the mean ± SD of 3 independent experiments.

Fig S5. Mitochondrial dynamics modulate RLR signaling pathway. (A) Control siRNA or siRNA targeting effectors of the mitochondrial fission/fusion machinery were transfected into HeLa cells. The efficiency of silencing was confirmed by immunoblotting 72 hr after

transfection. Actin was used as a protein loading control. (B) Percentage of cell population with normal, fragmented or fused mitochondria in control RNAi, Drp1 RNAi, Fis1 RNAi, OPA1 RNAi or Mfn1 RNAi culture. 300 cells in several fields were counted in each experiment. Data represent the mean ± SD of 2 independent experiments. (C) IFNβ-luc or NF-κB-luc reporter plasmids were transfected into the control cells or Drp1-, Fis1-, OPA1- or Mfn1-depleted cells, which were then infected with SeV H4 for 9 hr then IFNβ induction and NF-κB activation were assessed. *** $P<0.001$, ** $0.001<P<0.01$, * $0.01<P<0.05$. (D) HeLa cells were transfected either with control, Drp1, Fis1, OPA1 or Mfn1 siRNA. 72 hr later, MAVS was immunoprecipitated (IP) from cell extracts and the association with STING in each condition was examined by immunoblotting (IB). * denotes IgG light chain.

Fig S6. Mitochondrial dynamics specifically regulate MAVS-mediated signaling. Plasmids for the expression of TBK1 or MAVS were co-transfected with IFNβ-luc reporter plasmid into the control HeLa cells or Drp1-, Fis1-, OPA1- or Mfn1-depleted HeLa cells. 18 hr later, IFNβ induction was assessed. *** $P<0.001$, ** $0.001<P<0.01$, * $0.01<P<0.05$, ns not significant $P>0.05$.

Fig S7. RLR activation promotes mitochondria/ER tethering. HeLa cells were infected or not with SeV H4. After 18 hr, mitochondria and ER morphology was examined by immunofluorescence. The graphs represent the mitochondria/ER fluorescence distribution detected in the indicated cell sections. The line scans plot the intensity of mitochondria and ER. As assessed in the images (overlay) and linescans, SeV H4 infection increases mitochondria/ER co-distribution.

Fig S8. STING is an ER protein regulated by type I IFNs. (A) HeLa cells were transfected with STING-HA, next co-localization with mitochondria or ER was examined by immunofluorescence. Boxed areas are enlarged on the right. (B) Mitochondria and ER were purified. Then, the presence of MAVS and STING in each organelle was studied by immunoblotting. Control antibodies indicate accuracy of purification (calreticulin: ER, Hsp60: mitochondria). ° indicates a band from a previous immunoblot. (C) HeLa cells were infected with SeV H4 and at different time points after infection, RIG-I, MAVS, p-IRF3, IRF3, STING, p-IκBα and IκBα were analyzed in cell extracts by immunoblotting. Actin was used as a protein loading control. An asterisk (*) indicates a probable nonspecific protein band. (D) HeLa cells were treated for 18 or 24 hr with IFNα (2000 U/ml) next the up-regulation of RIG-I and STING was examined by immunoblotting.

Fig S9. MAVS, STING and Mfn1 form a tri-complex. HEK293 cells were transfected with the indicated constructs. 24 hr later, cell extracts and immunoprecipitates (IP) were analyzed by immunoblotting (IB). ° indicates a band from a previous immunoblot.

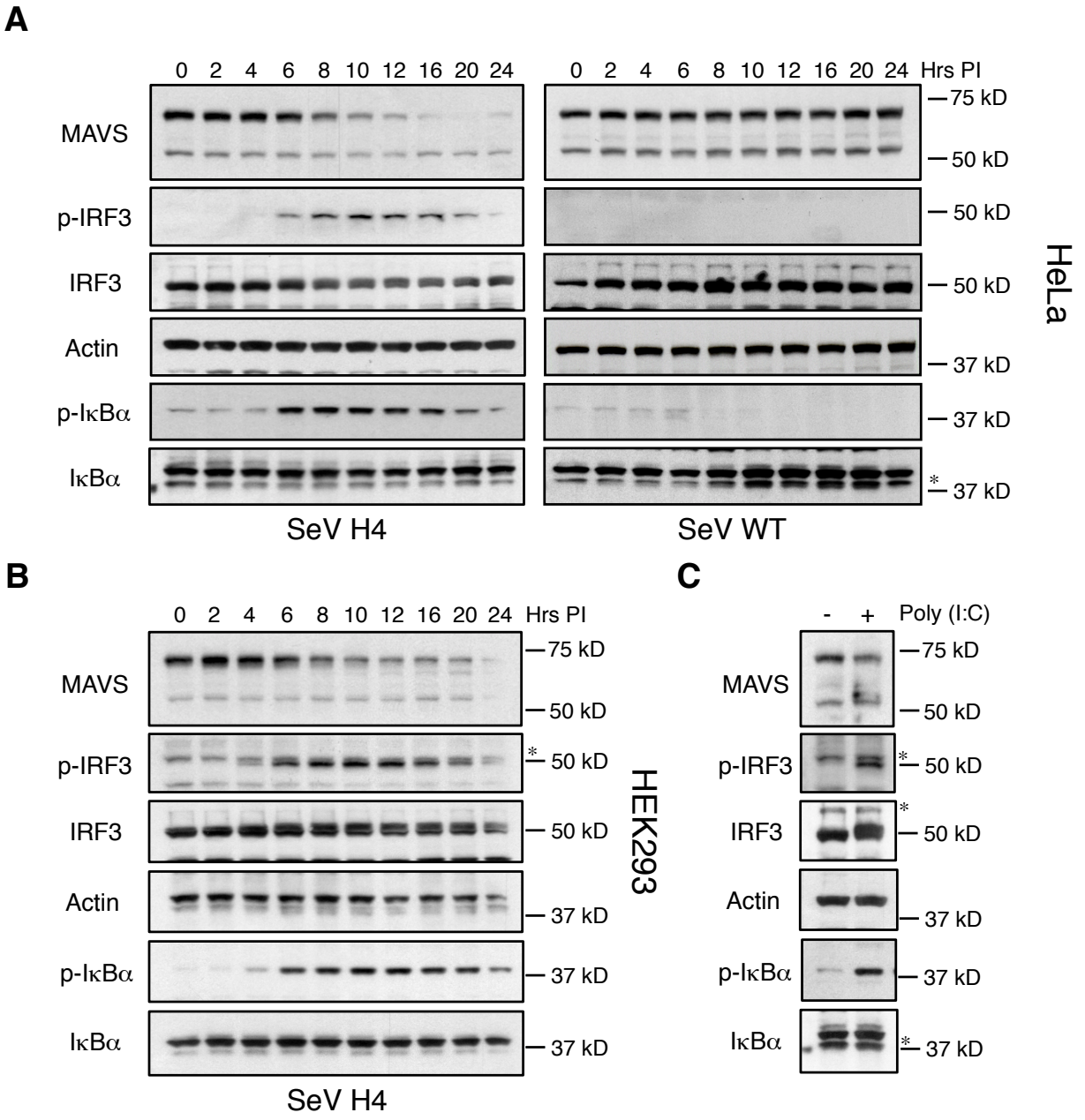
A
0 2 4 6 8 10 12 16 20 24
0 2 4 6 8 10 12 16 20 24 Hrs PI
MAVS
p-IRF3
IRF3
Actin
p-IκBα
IκBα
75 kD
50 kD
50 kD
50 kD
37 kD
37 kD
*
37 kD
SeV H4
SeV WT
HeLa
B
0 2 4 6 8 10 12 16 20 24 Hrs PI
MAVS
p-IRF3
IRF3
Actin
p-IκBα
IκBα
75 kD
50 kD
*
50 kD
50 kD
37 kD
37 kD
37 kD
SeV H4
HEK293
C
- + Poly (I:C)
MAVS
p-IRF3
IRF3
Actin
p-IκBα
IκBα
75 kD
50 kD
*
50 kD
*
50 kD
37 kD
37 kD
*
37 kD

Figure S1

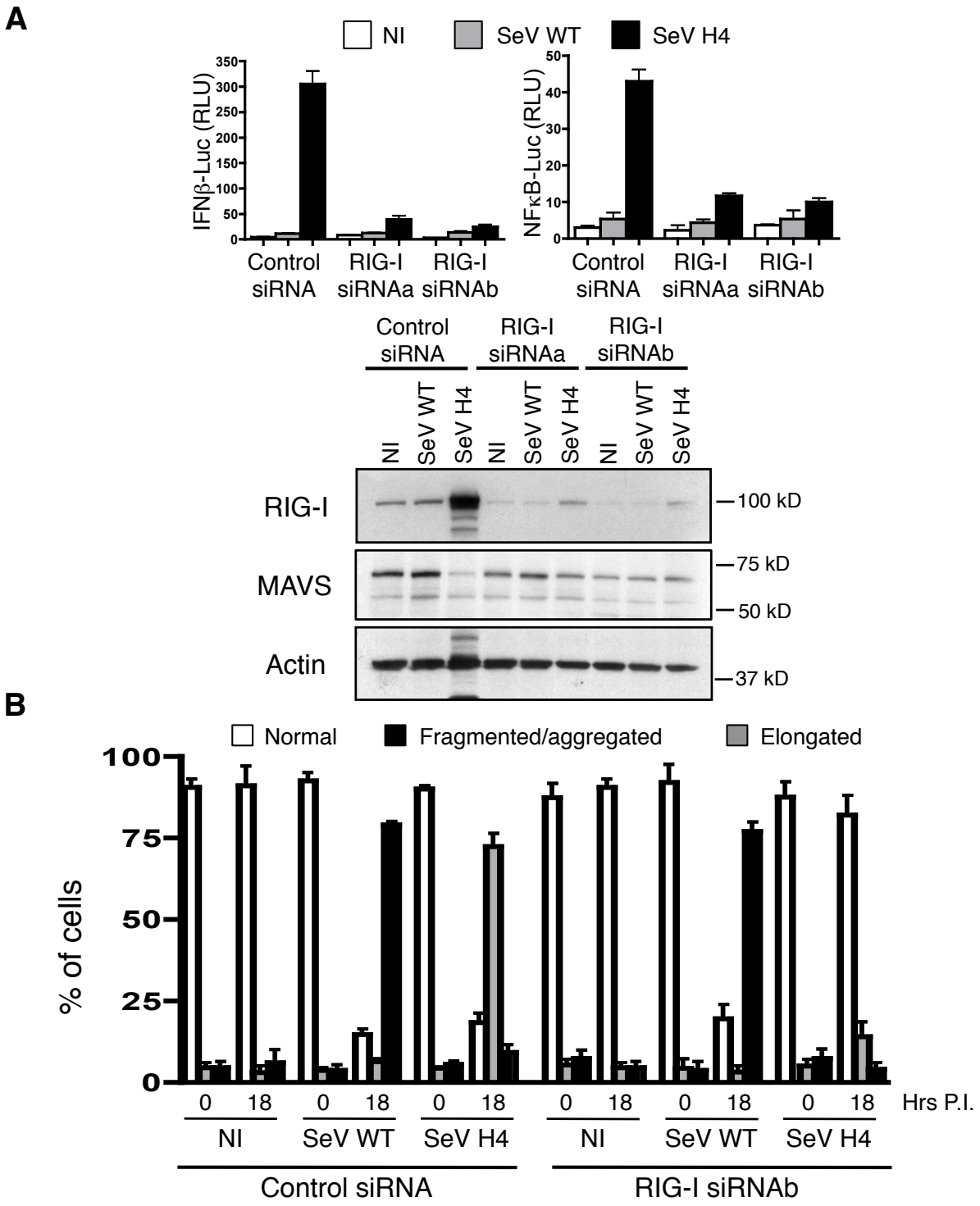
A
NI
SeV WT
SeV H4
IFNβ-Luc (RLU)
NFκB-Luc (RLU)
Control siRNA
RIG-I siRNAa
RIG-I siRNAb
RIG-I
MAVS
Actin
100 kD
75 kD
50 kD
37 kD
B
Normal
Fragmented/aggregated
Elongated
% of cells
Hrs P.I.
Control siRNA
RIG-I siRNAb

Figure S2

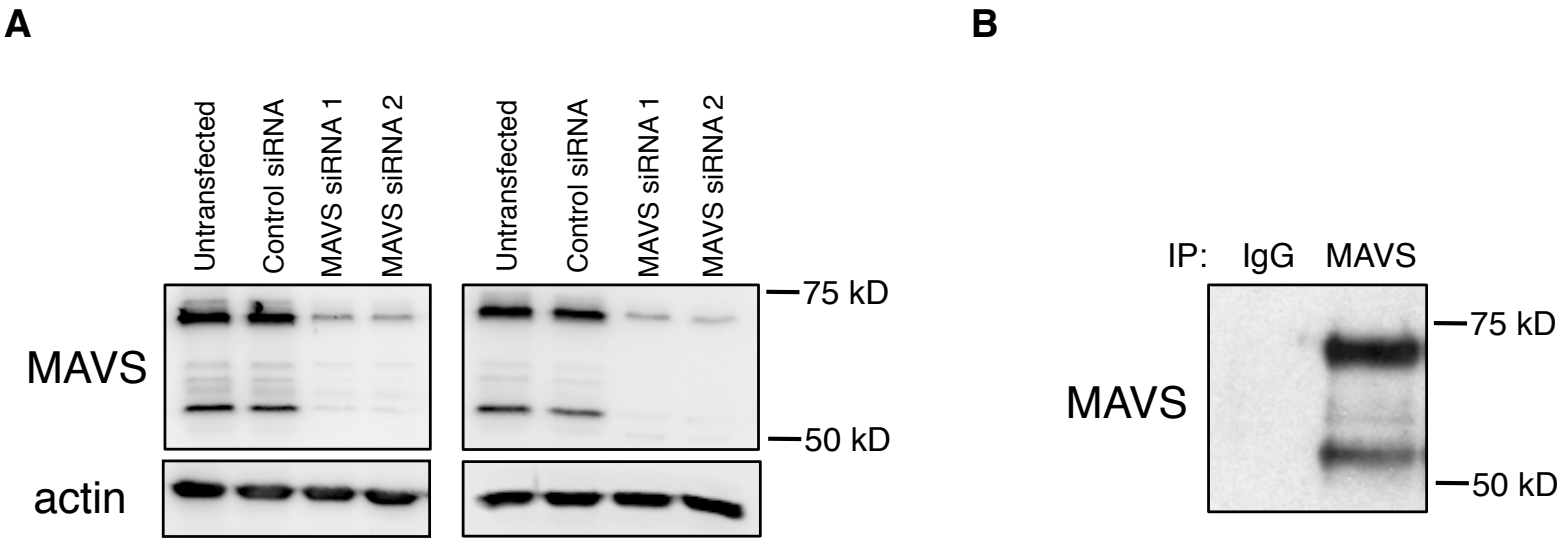
A
Untransfected
Control siRNA
MAVS siRNA 1
MAVS siRNA 2
Untransfected
Control siRNA
MAVS siRNA 1
MAVS siRNA 2
MAVS
actin
75 kD
50 kD
B
IP: IgG MAVS
MAVS
75 kD
50 kD

Figure S3

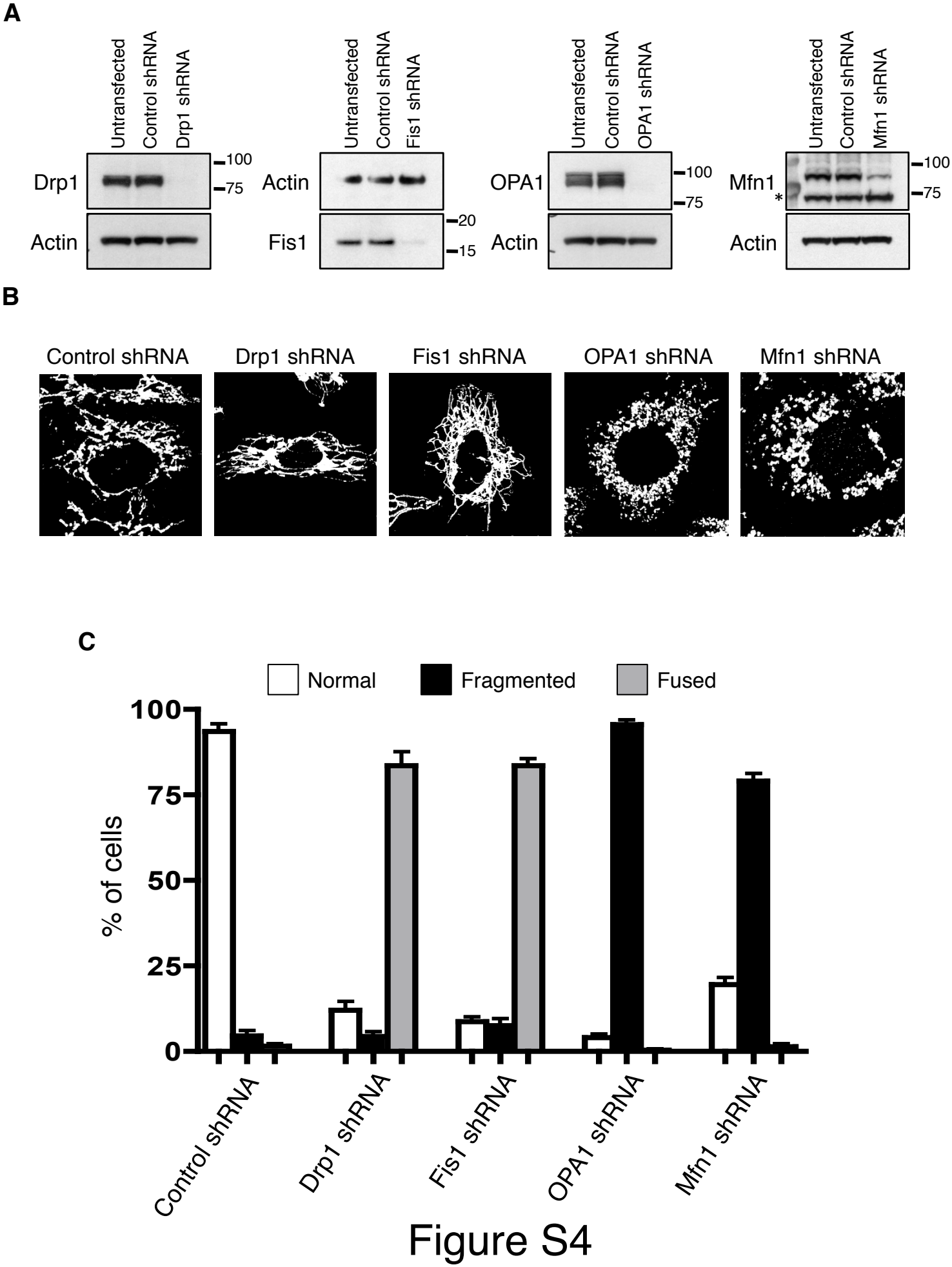
A
Untransfected
Control shRNA
Drp1 shRNA
Drp1
Actin
100
75
Fis1 shRNA
Actin
Fis1
20
15
OPA1 shRNA
OPA1
Mfn1 shRNA
Mfn1
*
B
Control shRNA
Drp1 shRNA
Fis1 shRNA
OPA1 shRNA
Mfn1 shRNA
C
Normal
Fragmented
Fused
% of cells
100
75
50
25
0

Figure S4

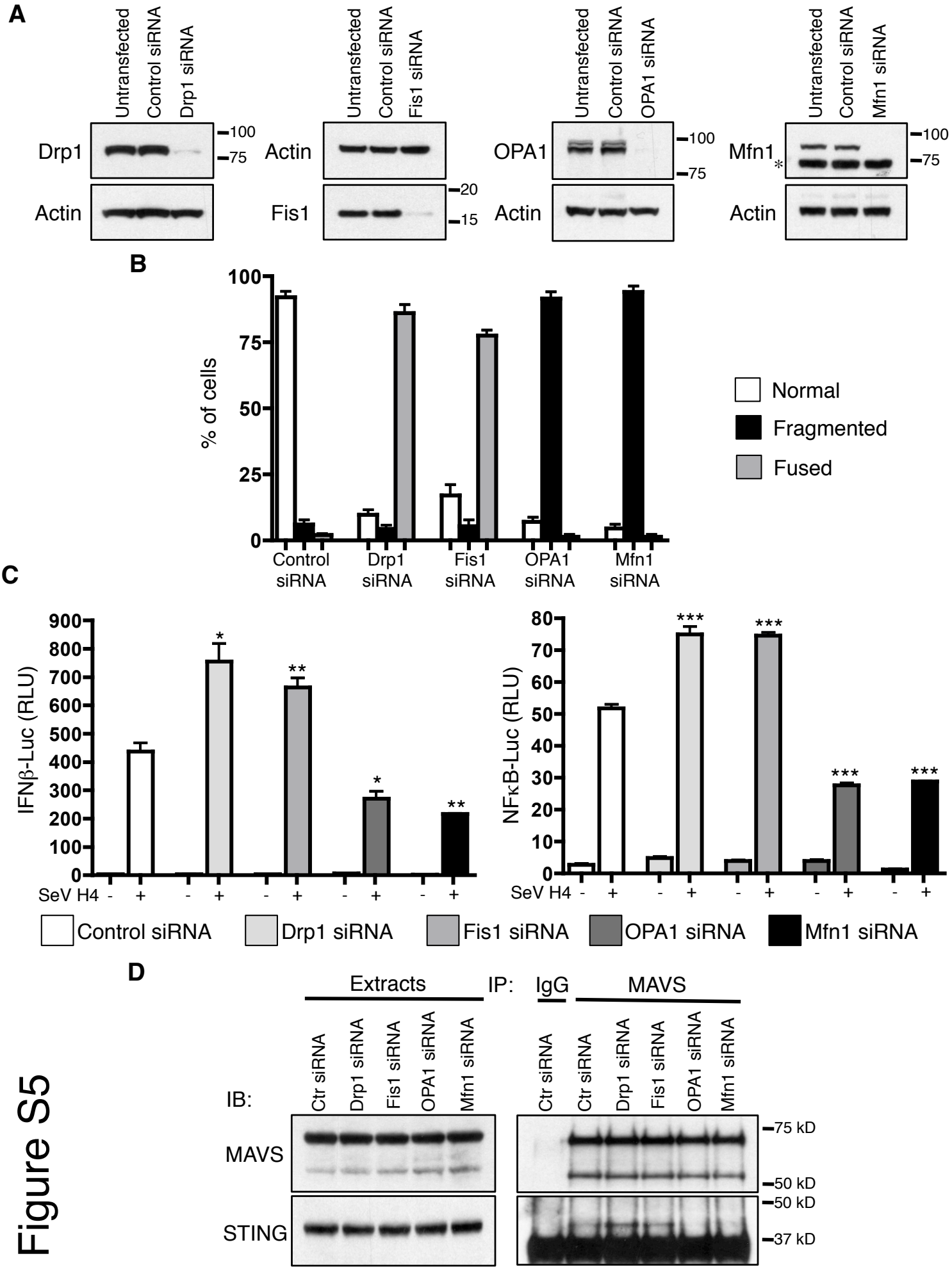
A
Untransfected
Control siRNA
Drp1 siRNA
Drp1
Actin
100
75
Fis1 siRNA
Fis1
20
15
OPA1 siRNA
OPA1
Mfn1 siRNA
Mfn1
*
B
% of cells
Normal
Fragmented
Fused
Control siRNA
Drp1 siRNA
Fis1 siRNA
OPA1 siRNA
Mfn1 siRNA
C
IFNβ-Luc (RLU)
NFκB-Luc (RLU)
SeV H4
D
Extracts
IP:
IgG
MAVS
Ctr siRNA
IB:
MAVS
STING
75 kD
50 kD
37 kD

Figure S5

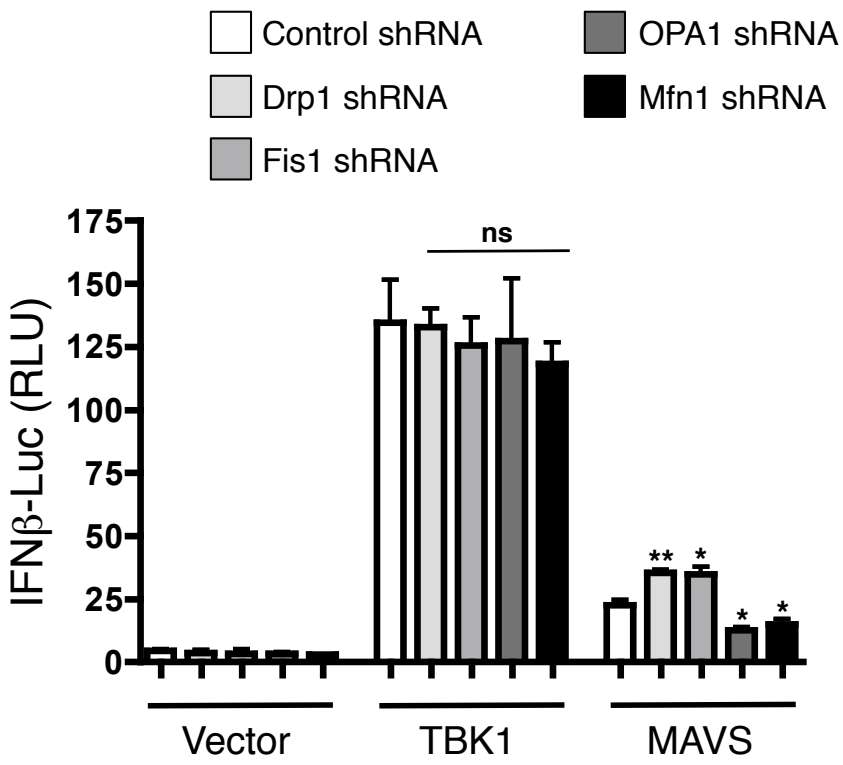
Control shRNA
Drp1 shRNA
Fis1 shRNA
OPA1 shRNA
Mfn1 shRNA
IFNβ-Luc (RLU)
175
150
125
100
75
50
25
0
ns
**
*
*
*
Vector
TBK1
MAVS

Figure S6

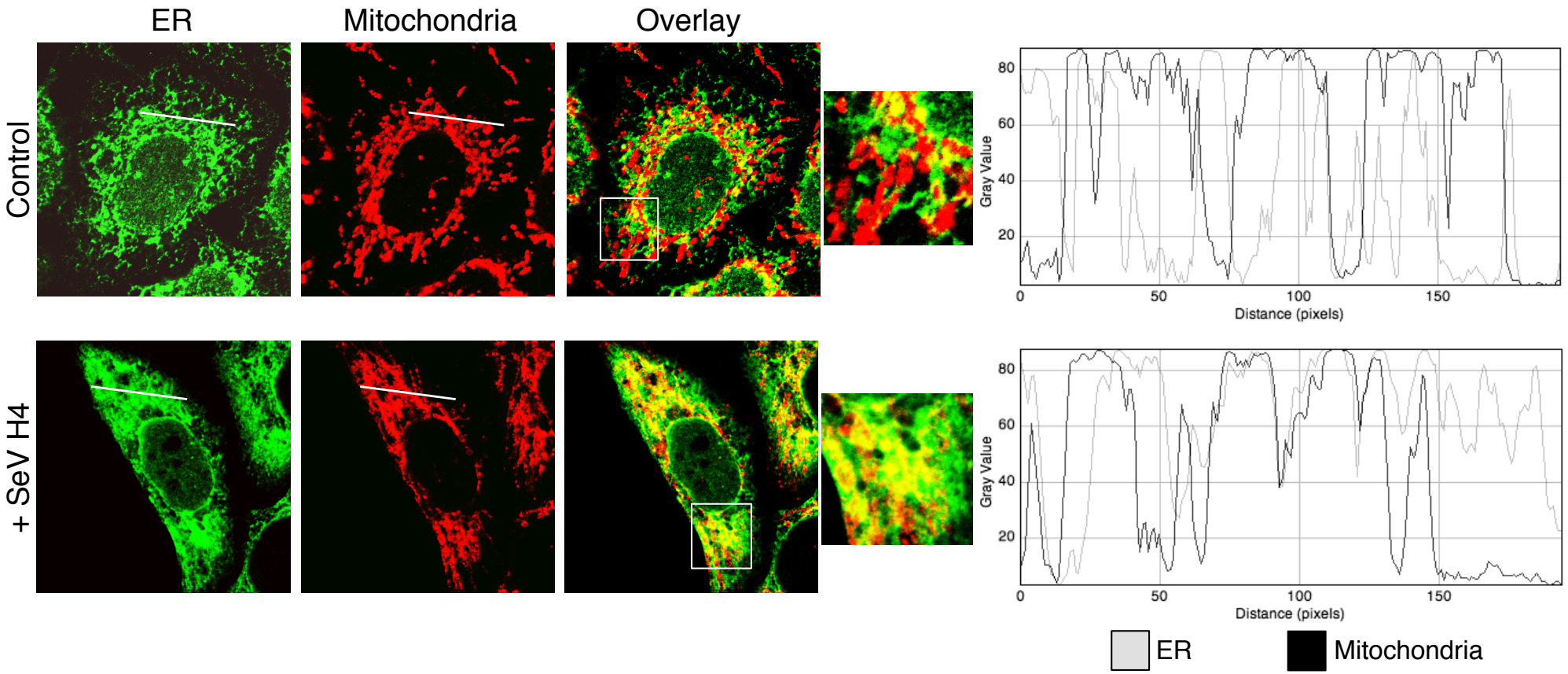
ER
Mitochondria
Overlay
Control
+ SeV H4
Gray Value
Distance (pixels)
0
50
100
150
20
40
60
80
ER
Mitochondria

Figure S7

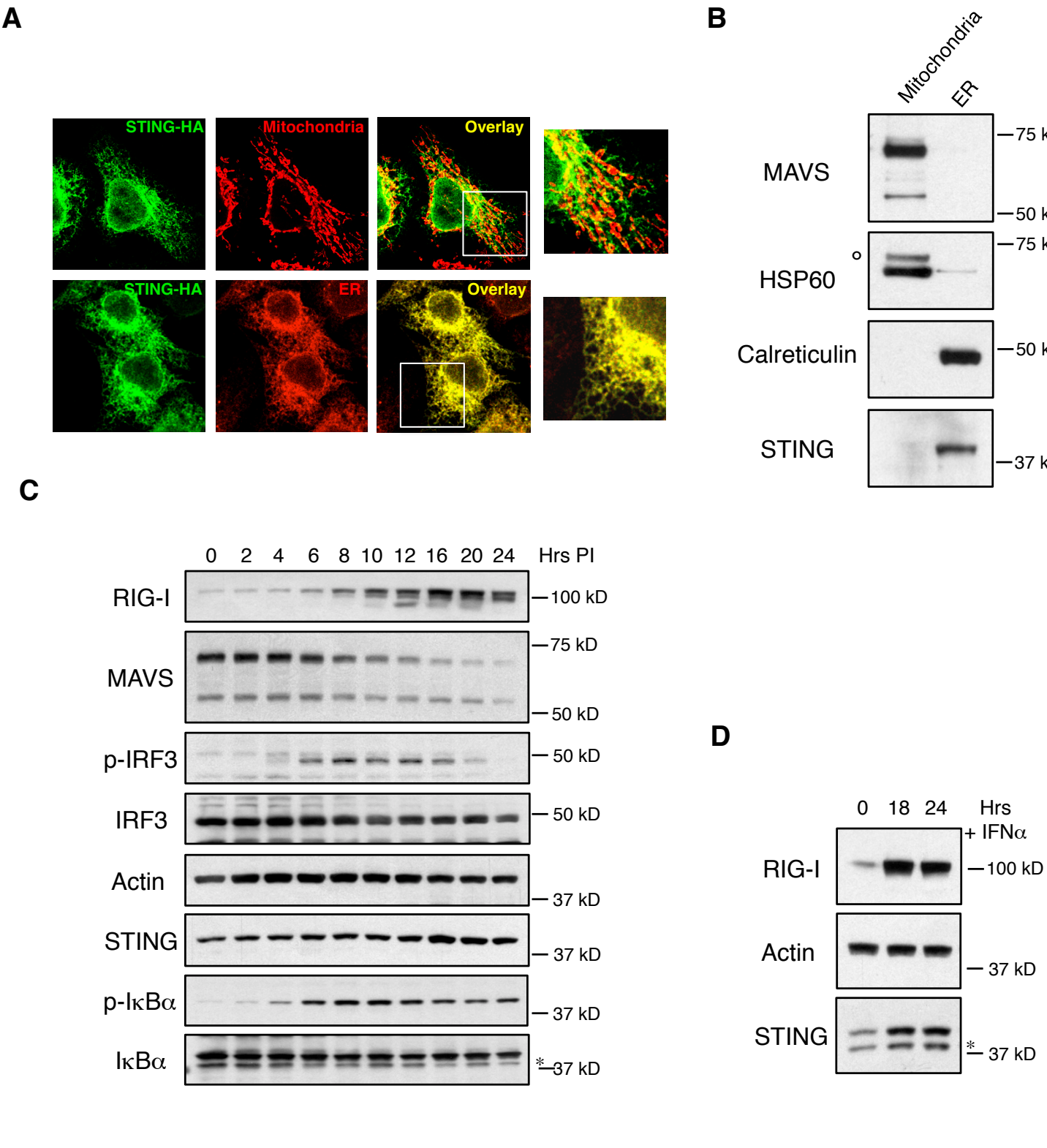
A
STING-HA
Mitochondria
Overlay
STING-HA
ER
Overlay
B
Mitochondria
ER
MAVS
75 kD
50 kD
HSP60
°
75 kD
Calreticulin
50 kD
STING
37 kD
C
0 2 4 6 8 10 12 16 20 24 Hrs PI
RIG-I
100 kD
MAVS
75 kD
50 kD
p-IRF3
50 kD
IRF3
50 kD
Actin
37 kD
STING
37 kD
p-IκBα
37 kD
IκBα
*
37 kD
D
0 18 24 Hrs
+ IFNα
RIG-I
100 kD
Actin
37 kD
STING
*
37 kD

Figure S8

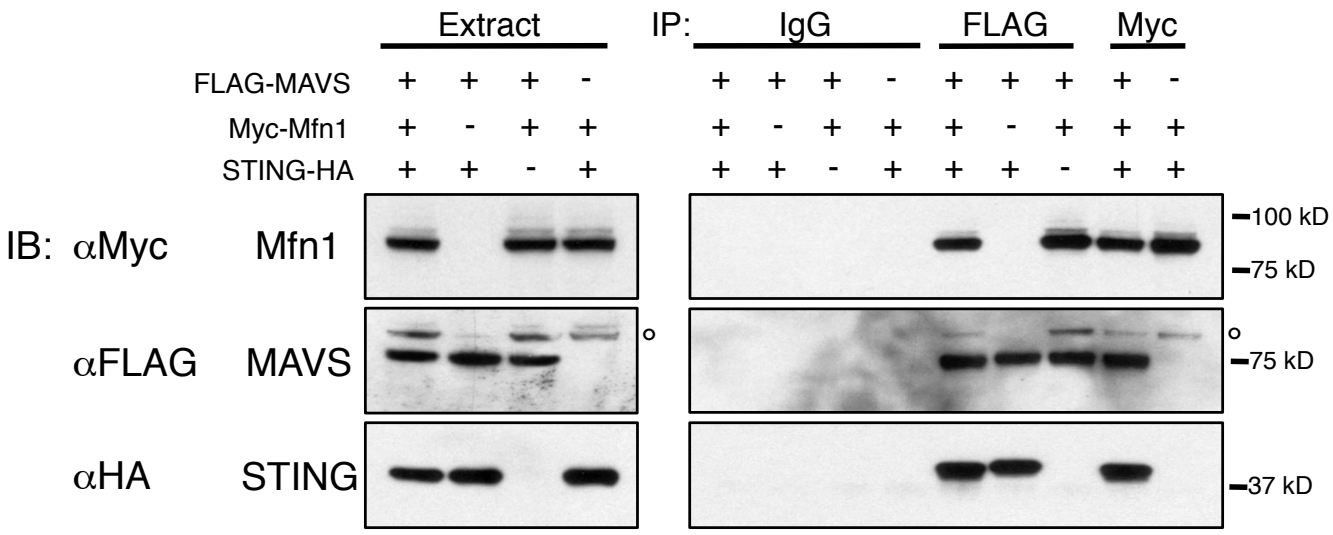
Extract
IP:
IgG
FLAG
Myc
FLAG-MAVS + + + - + + + - + + + + -
Myc-Mfn1 + - + + + - + + + - + + +
STING-HA + + - + + + - + + + - + +
IB: αMyc Mfn1
αFLAG MAVS
αHA STING
100 kD
75 kD
75 kD
37 kD
°
°

Figure S9