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Supplementary Materials for

Ubiquitination of STING at lysine 224 controls IRF3 activation

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The PDF file includes:

Other Supplementary Material for this manuscript includes the following:
(available at immunology.sciencemag.org/cgi/content/full/2/11/eaah7119/DC1)

Materials and Methods

Cell culture, reagents and antibodies

Primary wild type, *Sting*$^{-/-}$ and *Mul1*$^{-/-}$ MEFs were prepared as described (Ishikawa and Barber, 2008). HEK293T cells, Vero cell and CRL-5800 cells were purchased from the ATCC. Platinum-E cells were purchased from Cell Biolabs. AmphoPack 293 cells and hTERT-BJ1 cells were purchased from Clontech. EA.hy926 cells were kindly provided by Blossom Damania. MEFs, HEK293T cells, Vero cell, AmphoPack 293 cells, Platinum-E cells and EA.hy926 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS and 1% penicillin/streptomycin. CRL-5800 cells were cultured in RPMI 1640 supplemented with 10% FBS and 1% penicillin/streptomycin. hTERT-BJ1 cells were cultured in a 4:1 ratio of DMEM: Medium 199 with 10% FBS, 4mM L-glutamine and 1mM sodium pyruvate. All cells were maintained at 37°C in a 5% CO_2 tissue culture incubator subject to routine cleaning and decontamination. Brefeldin A, MG132 and lactacystin were purchased from Sigma. Poly I:C was purchased from American Bioscience. Interferon stimulatory DNA (ISD, 90-mer), used as dsDNA in this study was obtained from Sigma and reconstituted in ddH_2O at 2 mg/ml, denatured at 70°C for 30 min, and allowed to anneal at room temperature before use. HSV-1 (KOS strain) was purchased from ATCC. HSV-1 γ34.5 was kindly provided by Bernard Roizman. Anti-STING rabbit polyclonal antibody was prepared as described previously (Ishikawa and Barber, 2008). Other antibodies used in the study were obtained from following sources: anti-Ubiquitin (Santa Cruz, sc-8017), anti-β-actin (Sigma, A5441), anti-HA (Sigma, H9658), anti-HA (Biolegend, 923501), anti-FLAG (Sigma, F1804); anti-FLAG (Sigma, F7425); anti-calreticulin (Abcam, ab14234); anti-IRF3 (Cell Signaling, 4302); anti-IRF3 (Santa Cruz, sc-9082); anti-phospho-IRF3 (Cell Signaling, 4947); anti-TBK1(Abcam, ab40676); anti-phospho-TBK1 (Cell Signaling, 5483); anti-p65 (Cell Signaling, 8242); anti-phospho-p65 (Cell Signaling, 3033); anti-p38 (Cell Signaling 9212); anti-phospho-p38 (Cell Signaling, 4511); anti-MUL1 (Abcam, ab84067, for mouse MUL1); anti-MUL1 (LS-c344409, LSBio, for mouse MUL1); anti-MUL1 (Sigma, SAB2702071, for human MUL1). Mouse and human Mul1 siRNA SMART pool were purchased from Dharmacon (LU-050675, LU-007062). The sequences of each individual siRNAs in the pool are list as following: Mouse #5: GAACCGAACUACCCACCUU, #6: GGACUAACACUGUGCCCUU, #7: CGAAGGAGCUGUGCGGUCU, #8: UAGAGACCGU-GUACGAGAA. Human #9: GGAGCUGUGCGGUCUGUUA, #10: GGCAUGCAGUACUAU-CUAA, #11: UAACAGCCAGUUUGUGGAA, #12: GUACAACAGCUAAUAGUUU.

DNA transfection and RNA interference

Plasmids, poly I:C or dsDNA transfection into 293T or MEF cells were carried out using Lipofectamine 2000 with Opti-MEM (Invitrogen) according to the manufacturer's instruction. dsDNA or plasmids were transfected into hTERT-BJ1 cells using Lipofectamine 2000 or Lipofectamine LTX respectively. siRNA transfections were performed using Lipofectamine RNAiMAX (Invitrogen) with Opti-MEM.

Immunoblotting, immunoprecipitation, and ubiquitination assay

Cells were boiled in SDS-sample buffer (60mM Tris.HCl pH6.8, 2% SDS, 10% glycerol, 5% β-mercaptoethanol, 0.01% bromophenol blue) and separated by SDS-PAGE and then transferred to PVDF membrane (Millipore). The membrane was blocked in PBS-T (PBS with 0.1% Tween20) with 5% Blotting-Grade Blocker (Bio-Rad) and then incubated with primary antibody at 4°C overnight. The membrane was washed three times using PBS-T buffer, and then incubated with

HRP-conjugated anti-rabbit or mouse IgG (Promega). The membrane was developed with Super Signal West Pico or Femto (Thermo) and then exposed to CL-XPosure film (Thermo). For immunoprecipitation, whole cell lysate were generated by lysing cells in RIPA buffer (50mM Tris.HCl pH7.6, 150mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS) with protease and phosphatase inhibitors (PMSF, Aprotinin, Leupeptin, Pepstatin (Gbiosciences), and Phosphatase inhibitor cocktail A (Santa Cruz Biotechnology)) at 4°C followed by centrifugation. The lysates were incubated with indicated antibodies overnight at 4°C and then added with 30 µl protein G beads for another 2 hours. The beads were washed three times with RIPA buffer followed by addition of 2× sample buffer and boiling. For detection of STING ubiquitination, a similar IP procedure was performed except including an additional stringent wash using RIPA buffer containing 2M urea to remove unspecific binding of other ubiquitinated proteins.

Virus infections and plaque assay

MEFs were seeded in 24-well plates and the next day washed with DMEM followed by infection with HSV-1 virus diluted in 100 µl DMEM at MOI of 0.1 or 1. Cells were incubated with virus for 1 hour at 37°C with gentle shaking every 15 minutes. After removal of the virus-containing media, the cells were washed with PBS and replenished with complete DMEM containing 10% FBS and antibiotics. Virally infected cells were then incubated at 37°C for an additional 24 hours post-infection and supernatants were collected for virus titration. To determine HSV-1 titers, the supernatants were diluted with DMEM at a 10-fold serial dilution and then infected Vero cells (ATCC) in 12-well plates as described above. After 1 hour incubation and washed with PBS, the cells were layered with complete DEME containing 1% low melting agarose gel. The cells were incubated at 37°C for another 48 hours, allowing for cell growth and plaque formation. Agarose gels were removed by heating in a microwaver and cells were fixed with a solution containing 0.1% crystal violet and 30% methanol. Plaque in appropriate wells were counted and viral titers were calculated and presented as pfu/ml.

Immunostaining and confocal microscopy

The cells were seeded on poly-D-Lysine coated round coverslips (BD bioscience) and the next day transfected with indicated plasmids or dsDNA using Lipofectamine 2000. The cells were fixed with 4% paraformaldehyde (PFA) in PBS for 15 min and permeabilized with 0.2% Triton X-100 in PBS for 5 min. After blocking with 1% bovine serum albumin (BSA) in PBS-T for 30 min, the coverslips were incubated with appropriate primary antibodies in PBS-T containing 1% BSA. After washing three times with PBS, the coverslips were incubated with Alexa Fluor 488-goat anti-rabbit IgG (for STING, IRF3, p65), Alexa Fluor 488-goat anti-mouse IgG (for HA), or Alexa Fluor-647 goat anti-chicken IgG (for Calreticulin) (Invitrogen). After washing three times with PBS, the coverslips were mounted onto the glass slides with ProLong Gold anti-fade reagent (Invitrogen). Images were acquired under LSM510 confocal microscope (Zeiss) or SP5 confocal microscope (Leica). For the quantification of STING, IRF3 or p65 translocation, translocated cells were counted from 100 cells in each sample and presented as a percentage of total cells. The data shown in the manuscript are representatives of at least of two independent experiments which had same outcome.

Reconstitution of *Sting*$^{-/-}$ MEFs and CRL-5800 cells

pBabe-puro plasmids expressing human STING or STING mutants were transfected into Platinum-E retroviral packing cells using Lipofectamine 2000. Two days post transfection, the

supernatants containing packed retrovirus were collected and centrifuged to get rid of floating cell debris. *Sting*$^{-/-}$ MEFs were incubated with the supernatants in the presence of 10 μg/ml of polybrene (SIGMA) for 8 hours and then replenished with fresh medium. After another two days, puromycin (SIGMA) was added to the culture medium at a final concentration of 2 μg/ml to remove uninfected cells. Reconstitution of CRL-5800 cells was similar to that of MEFs except that the retrovirus was packed in the AmphoPack 293 cells.

***In vivo* ^{32}P labeling**

Sting$^{-/-}$ MEFs were reconstituted with pBabe-puro, pBabe-hSTING or pBabe-hK224R as described above and cultured in phosphate free DMEM (Invitrogen) with 10% FBS for 2 hours. After that cells were incubated with 0.2 mCi/ml ^{32}P labeled orthophosphate (Perkin Elmer) for 30 min and then transfected with 5 μg/ml dsDNA for 9 hours. The cells were lysed in RIPA buffer with protease and phosphatase inhibitors and immunoprecipited with anti-STING antibody as described in "Immunoblotting, immunoprecipitations, and ubiquitination assays" part. After washing five times with RIPA buffer, the beads were boiled in 2×SDS-sample buffer and separated in a 7.5% acrylamide gel. The gel was fixed in fix solution (40% methanol, 10% acetic acid), dried and then exposed to BioMax Light Film (Kodak) for 6 days at -80°C.

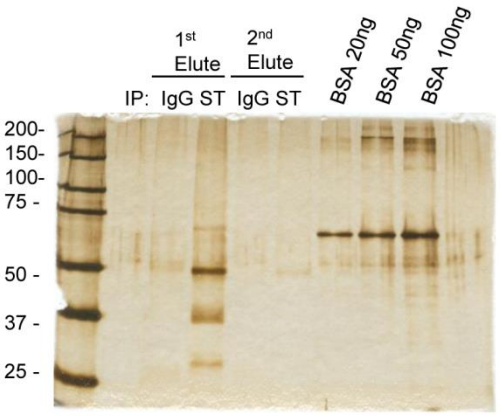
ELISA

IFNβ concentration in the supernatants from transfected MEFs were measured using an ELISA kit (PBL Interferon Source) according to the manufacturer's instructions. Values are calculated from a standard curve derived from recombinant IFNβ provided in the kit and presented as pg/ml±SD. Relative phosphor-p65 and total p65 level in the cell lysate were measured using an NF-κB p65 ELISA kit (Abcam) according to the manufacturer's instructions and data were presented as p65 (S536) / p65 (total) ratio.

GST fusion protein purification

MUL1 and MUL1-H319A coding sequence was cloned into pGEX-6P-1 vector and then transformed into E.coli BL21(DE3). Bacterial were cultured in LB medium with 0.2 mM IPTG for 3 hours before sonication in lysis buffer (20 mM Tris.Cl, pH 7.5, 1mM EDTA, 1% Triton X-100. 5mM DTT, protease inhibitor cocktail). Fusion proteins were precipitated with glutathione agarose (Pierce) overnight and washed with lysis buffer for five times before elution with high salt elution buffer (20 mM reduced L-glutathione, 100 mM Tris.Cl pH 7.5, 120 mM NaCl).

A



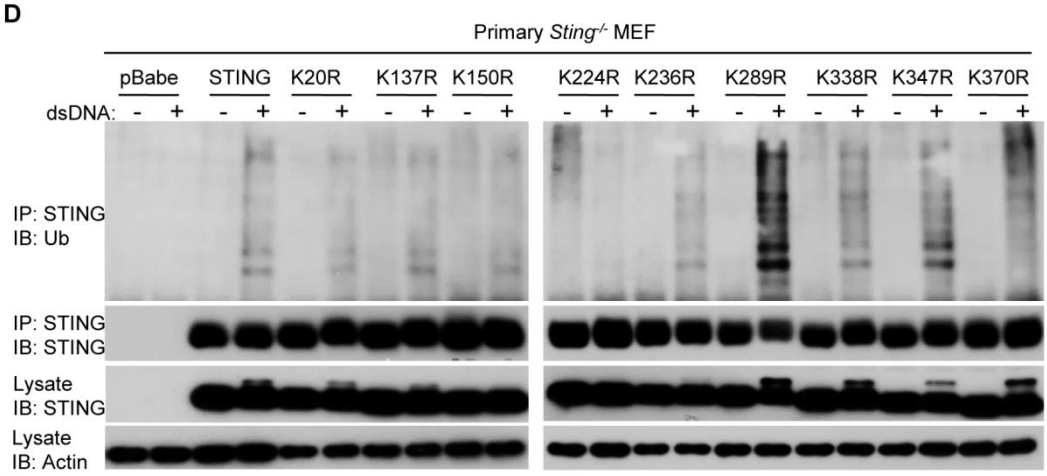
B

Score	Expectation	Peptide Sequence	
59.54	0.000041	K.LPQQTGDHAGI**K**DR.V + GlyGly (K)	K236
58.73	0.000047	K.LPQQTGDHAGIKDR.V	
56.53	0.000054	R.FLD**K**LPQQTGDHAGIK.D + GlyGly (K)	K224
50.78	0.00026	K.GNFNVAHGLAWSYYIGYLR.L	
46.68	0.00087	K.EEVTVGSLK.T	
46.67	0.0008	R.QEE**K**EEVTVGSLK.T + GlyGly (K)	K338

C

Score	Expectation	Peptide Sequence	
88.3	4.4E-8	R.LIFAG**K**QLEDGR.T + GlyGly (K)	K48
73.28	0.0000015	K.TITLEVEPSDTIENVK.A	
71.02	8.5E-7	K.ESTLHLVLR.L	
63.75	0.0000067	R.TLSDYNIQ**K**ESTLHLVLR.L + GlyGly (K)	K63

D



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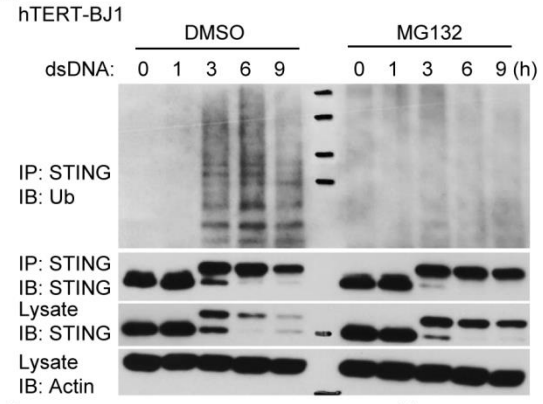

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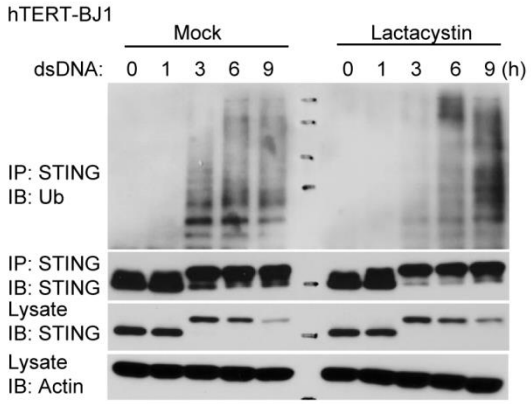


G

Ubiquitin:	6	11	27	29	33	48	63
WT	K	K	K	K	K	K	K
K48	R	R	R	R	R	K	R
K63	R	R	R	R	R	R	K

H

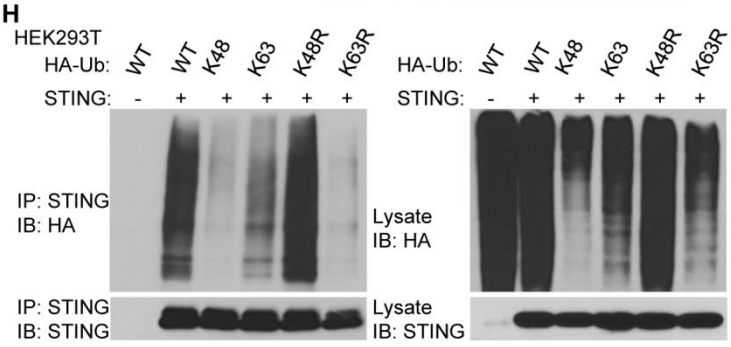


Fig. S1. STING is ubiquitinated on lysine 224 with K63-linked polyubiquitin chains. (**A**) Ubiquitinated STING proteins from HEK293T cells were purified as described in Method and Material. Purified ubiquitinated STING proteins were visualized in the gel with silver staining. ST, anti-STING antibody. (**B** and **C**) Ubiquitinated lysine residues of STING (**B**) and the polyubiquitin chain types (**C**) identified by mass spectrometry. (**D**) Primary Sting$^{-/-}$ MEFs reconstituted with hSTING or its mutants were transfected with or without dsDNA (4 μg/ml) for 6 hours. Cell lysates were immunoprecipitated with anti-STING antibody and then immunoblotted with the indicated antibodies. (**E** and **F**) MG132 (10 μM) (**E**) or Lactacystin (20 μM) (**F**) pre-treated hTERT-BJ1 cells were transfected with dsDNA (4 μg/ml) for the indicated time periods. Cell lysates were immunoprecipitated with anti-STING antibody and then immunoblotted with the indicated antibodies. (**G**) Schematic presentation of Ub and its mutants. (**H**) HEK293T cells were transfected with STING along with HA-tagged Ub or its mutants for 30 hours. Cell lysates were immunoprecipitated with anti-STING antibody and then immunoblotted with the indicated antibodies. Each panel of data is representative of at least two independent experiments which had the same outcome.

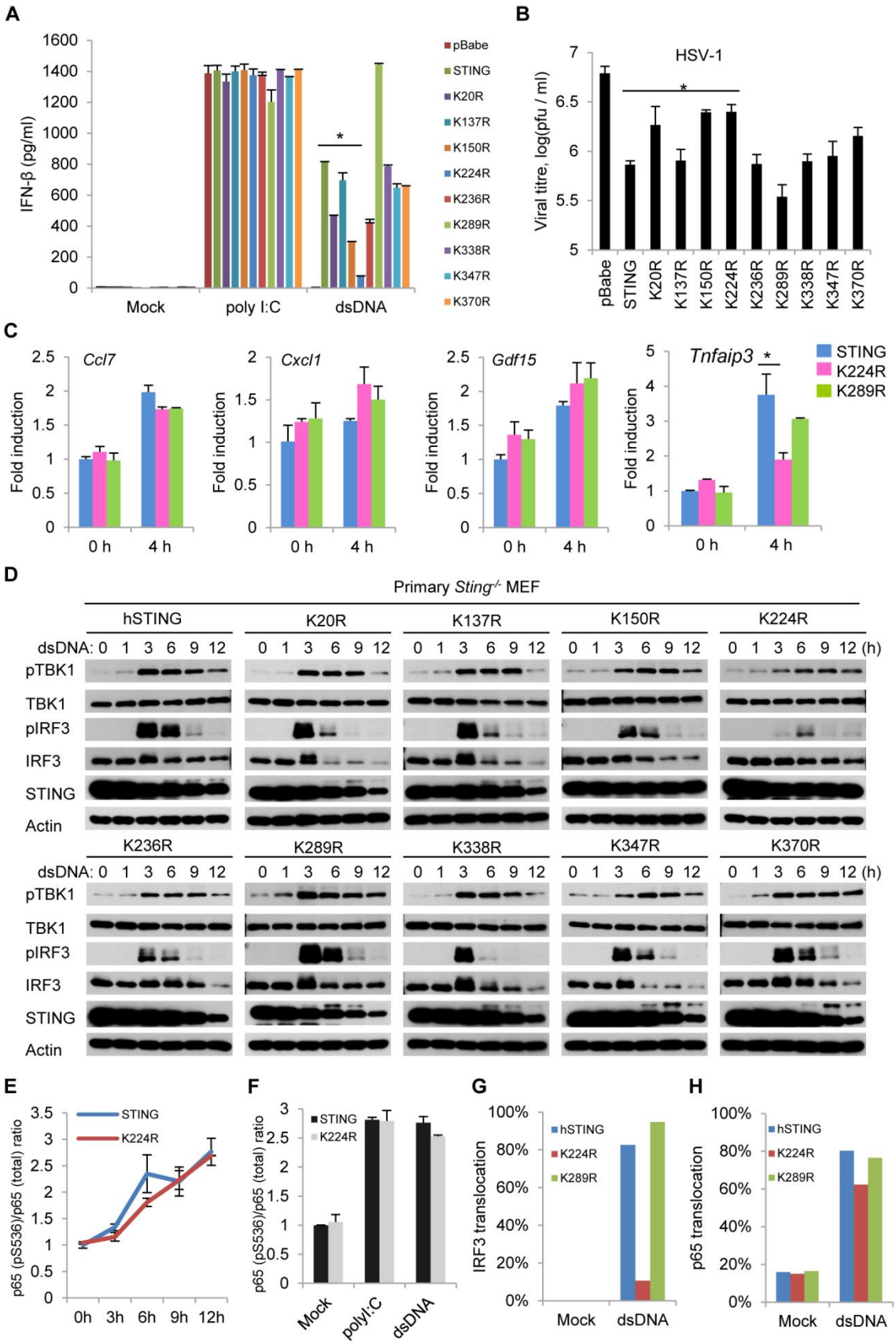

A
IFN-β (pg/ml)
1600
1400
1200
1000
800
600
400
200
0
Mock
poly I:C
dsDNA
*
pBabe
STING
K20R
K137R
K150R
K224R
K236R
K289R
K338R
K347R
K370R
B
HSV-1
Viral titre, log(pfu / ml)
7
6.5
6
5.5
5
*
pBabe
STING
K20R
K137R
K150R
K224R
K236R
K289R
K338R
K347R
K370R
C
Ccl7
Cxcl1
Gdf15
Tnfaip3
Fold induction
0 h
4 h
STING
K224R
K289R
D
Primary Sting-/- MEF
hSTING
K20R
K137R
K150R
K224R
K236R
K289R
K338R
K347R
K370R
dsDNA: 0 1 3 6 9 12 (h)
pTBK1
TBK1
pIRF3
IRF3
STING
Actin
E
p65 (pS536)/p65 (total) ratio
STING
K224R
0h 3h 6h 9h 12h
F
p65 (pS536)/p65 (total) ratio
STING
K224R
Mock
poly I:C
dsDNA
G
IRF3 translocation
hSTING
K224R
K289R
Mock
dsDNA
H
p65 translocation
hSTING
K224R
K289R
Mock
dsDNA

Fig. S2. Ubiquitination on lysine 224 is essential for STING activity. (**A**) Primary *Sting*$^{-/-}$ MEFs reconstituted with hSTING or its variants were transfected with dsDNA (4 μg/ml) or poly I:C (4 μg/ml) for 16 hours, and IFNβ production was measured by ELISA. (**B**) Reconstituted *Sting*$^{-/-}$ MEFs were infected with HSV-1 (MOI = 0.1) for 24 hours, and viral titer were measured by plaque assay. (**C**) Reconstituted *Sting*$^{-/-}$ MEFs were transfected with dsDNA (4 μg/ml) for 4 hours. Total RNA was purified and real-time PCR was carried out with indicated gene probes. (**D**) Reconstituted *Sting*$^{-/-}$ MEFs were transfected with dsDNA (4 μg/ml) for the indicated time periods, and cell lysates were immunoblotted with the indicated antibodies. (**E**) Primary *Sting*$^{-/-}$ MEFs reconstituted with hSTING or K224R were transfected with dsDNA (4 μg/ml) for the indicated time, and phospho-p65 and total p65 level was measured by ELISA and presented as p65 (S536) / p65 (total) ratio. (**F**) Primary *Sting*$^{-/-}$ MEFs reconstituted with hSTING or K224R were transfected with lipo-only, polyI:C (1 μg/ml) or dsDNA (4 μg/ml) for 12 hours, and phospho-p65 and total p65 level was measured by ELISA and presented as p65 (S536) / p65 (total) ratio. (**G** and **H**) Quantification of the IRF3 nucleus translocation in Fig. 2F (**G**) and p65 nucleus translocation in Fig. 2G (**H**). Each panel of data is representative of at least two independent experiments which had the same outcome. Data were presented as average ± SD of duplicated (A, C, E, and F) and triplicated (B) samples from each group. *P* value was determined by Student's t test, $^{*}P<0.05$, was considered statistically significant difference between two groups.

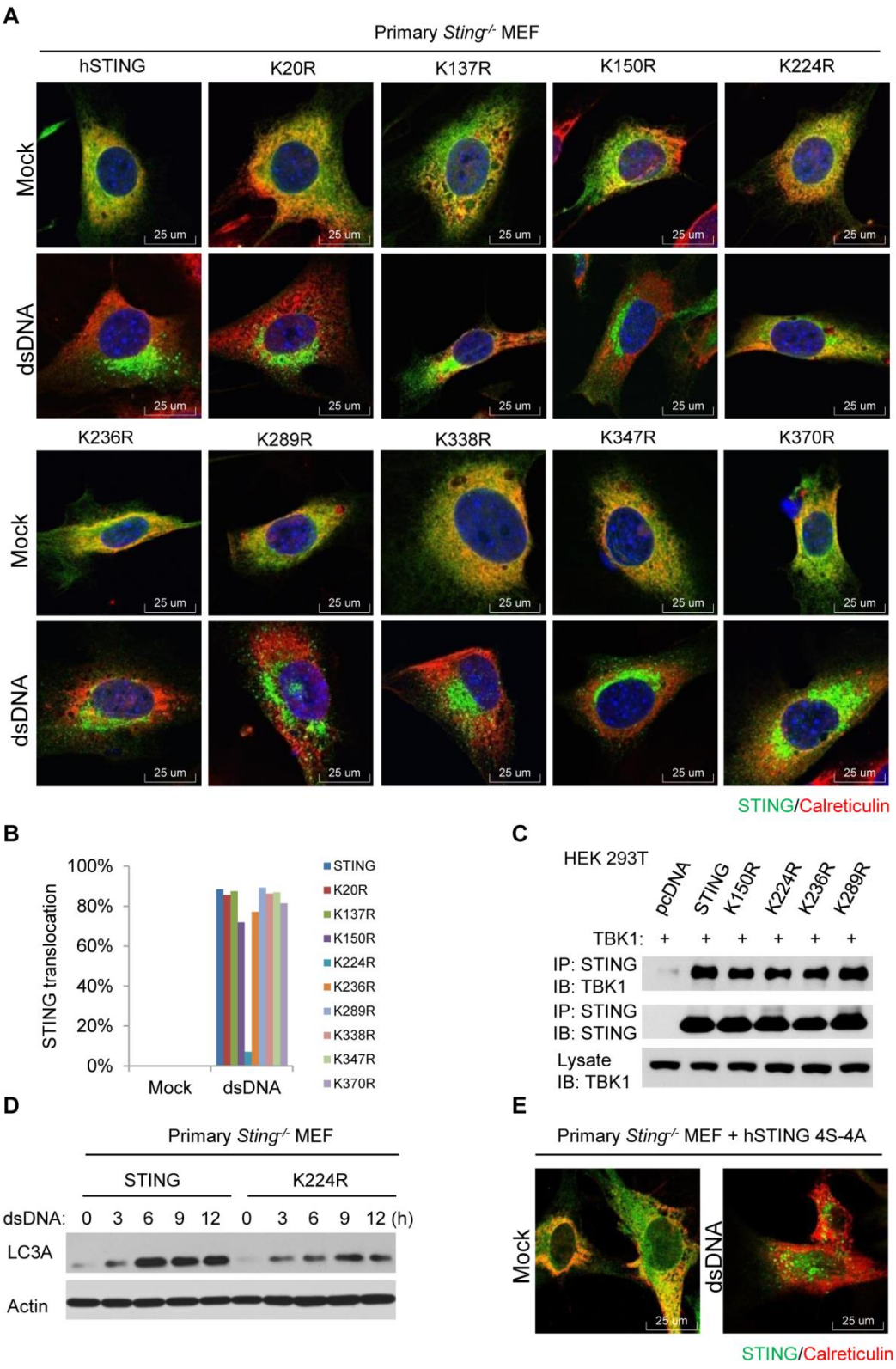


Fig. S3. Ubiquitination on lysine 224 is required for STING translocation. (**A**) Reconstituted *Sting*$^{-/-}$ MEFs were transfected with dsDNA (4 μg/ml) for 9 hours and then stained with

indicated antibodies. (**B**) Quantification of STING translocation in (**A**). (**C**) HEK293T cells were transfected with TBK1 along with STING or its mutants for 24 hours. Cell lysates were immunoprecipitated with anti-STING antibody and immunoblotted with the indicated antibodies. (**D**) Reconstituted Sting$^{-/-}$ MEFs were transfected with dsDNA (4 μg/ml) for the indicated time periods, and cell lysates were immunoblotted with indicated antibodies. (**E**) Primary *Sting*$^{-/-}$ MEFs reconstituted with hSTING 4S-4A (S345/358/366/379A) were transfected with dsDNA (4 μg/ml) for 9 hours, and then stained with indicated antibodies. Each panel of data is representative of at least two independent experiments which had the same outcome.

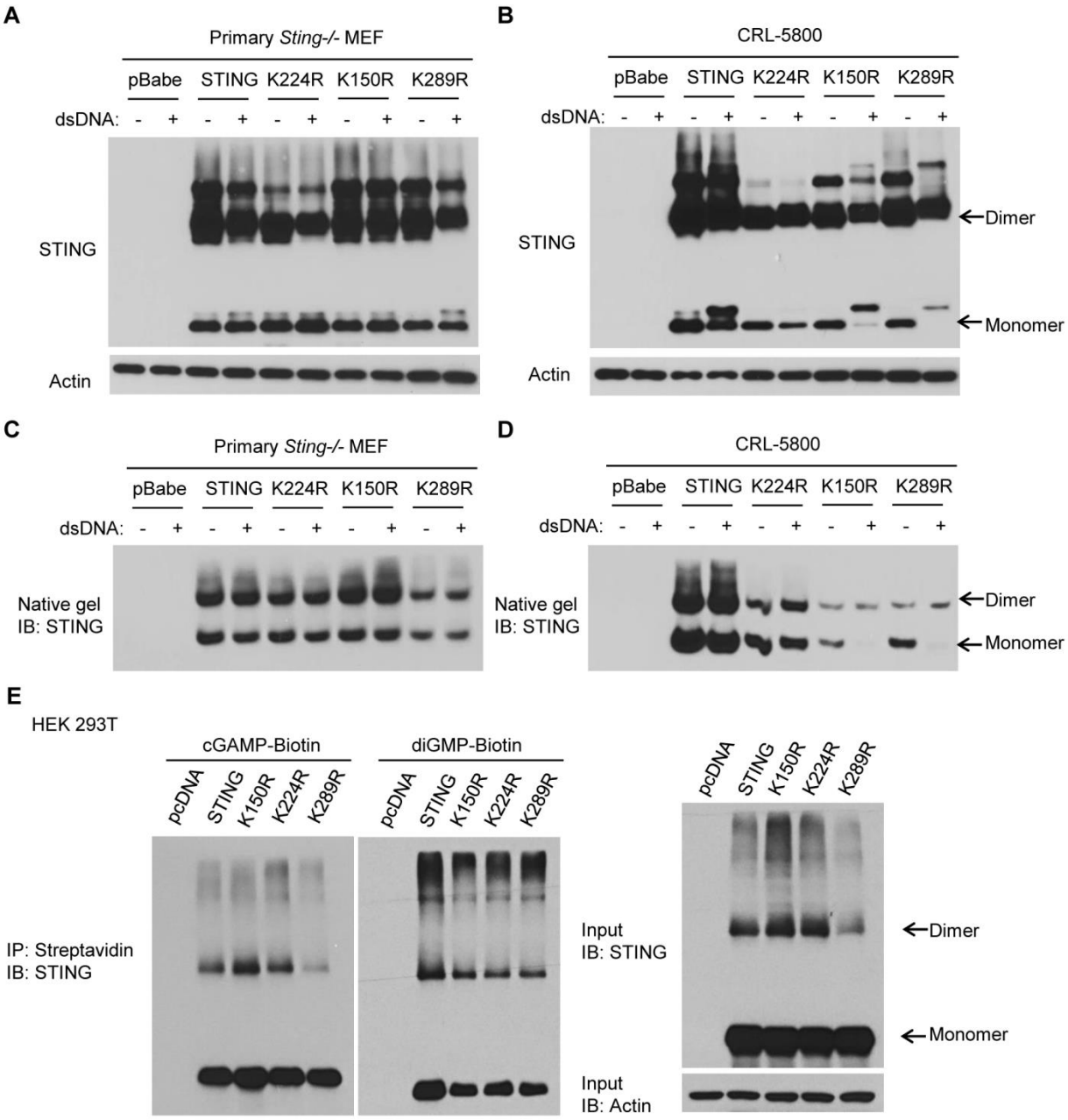


Fig. S4. K224R mutation does not affect STING dimer formation or its interaction with CDNs. (**A** and **B**) Primary *Sting*$^{-/-}$ MEFs (**A**) or CRL-5800 cells (**B**) reconstituted with hSTING or its mutants were transfected with dsDNA (4 μg/ml) for 6 hours and then treated with DSS (2 mM) for 30 min. Cell lysates were then immunoblotted with indicated antibodies. (**C** and **D**) Primary *Sting*$^{-/-}$ MEFs (**C**) or CRL-5800 cells (**D**) reconstituted with hSTING or its mutants were transfected with dsDNA (4 μg/ml) for 6 hours, and cell lysates were analyzed by native PAGE with anti-STING antibody. (**E**) HEK293T cells were transfected with STING or its mutants for 24 hours, and cell lysates were incubated with biotin-labeled cGAMP or diGMP (2 μM) followed with UV crosslinking (1J/cm^2). Crosslinked lysates were precipitated with streptavidin

agrose and then immunoblotted with indicated antibodies. Each panel of data is representative of at least two independent experiments which had the same outcome.

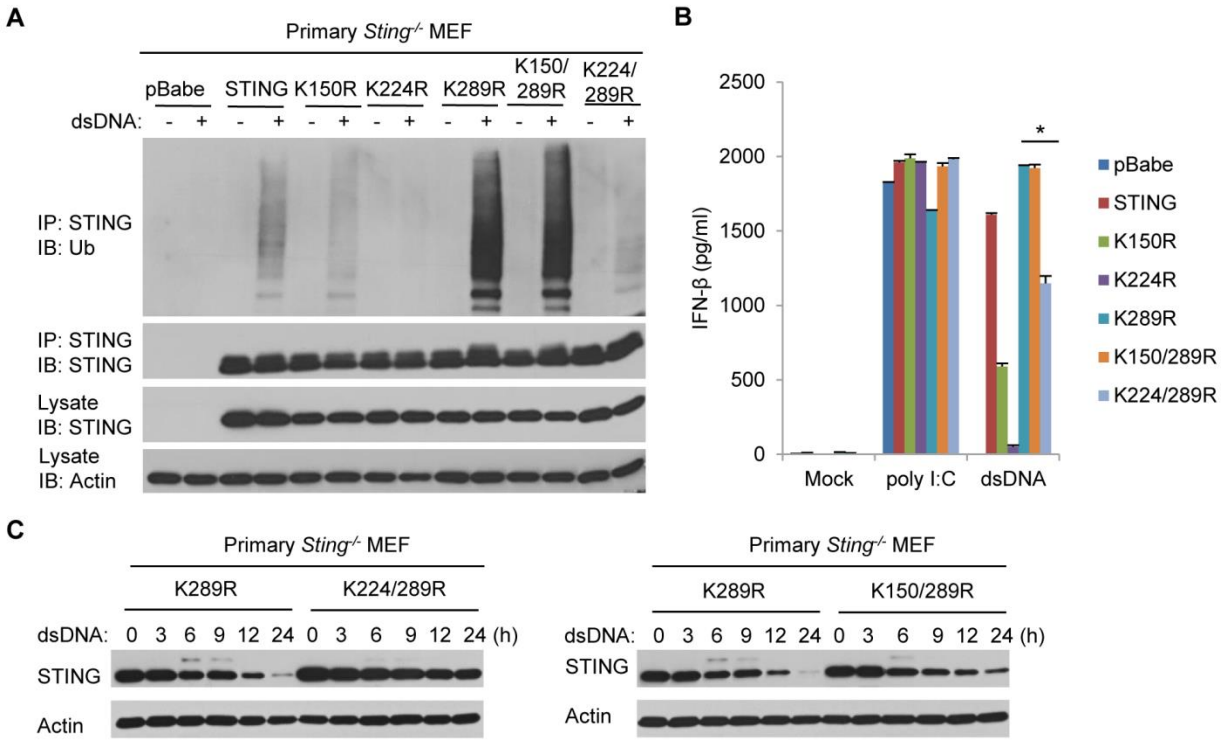


Fig. S5. Hyperactivity of STING K289R is caused by increased ubiquitination on K224. (**A**) Reconstituted *Sting*$^{-/-}$ MEFs were transfected with or without dsDNA (4 μg/ml) for 6 hours. Cell lysates were immunoprecipitated with anti-STING antibody and then immunoblotted with the indicated antibodies. (**B**) Reconstituted *Sting*$^{-/-}$ MEFs were transfected with dsDNA (4 μg/ml) or poly I:C (4 μg/ml) for 16 hours, and IFN-β production was measured by ELISA. (**C**) Reconstituted *Sting*$^{-/-}$ MEFs were transfected with dsDNA (4 μg/ml) for the indicated time periods, and cell lysates were immunoblotted with indicated antibodies. Each panel of data is representative of at least two independent experiments which had the same outcome. Data in (B) were presented as average ± SD of duplicated samples from each group. *P* value was determined by Student's t test, $^{*}P<0.05$, statistically significant difference between two groups.

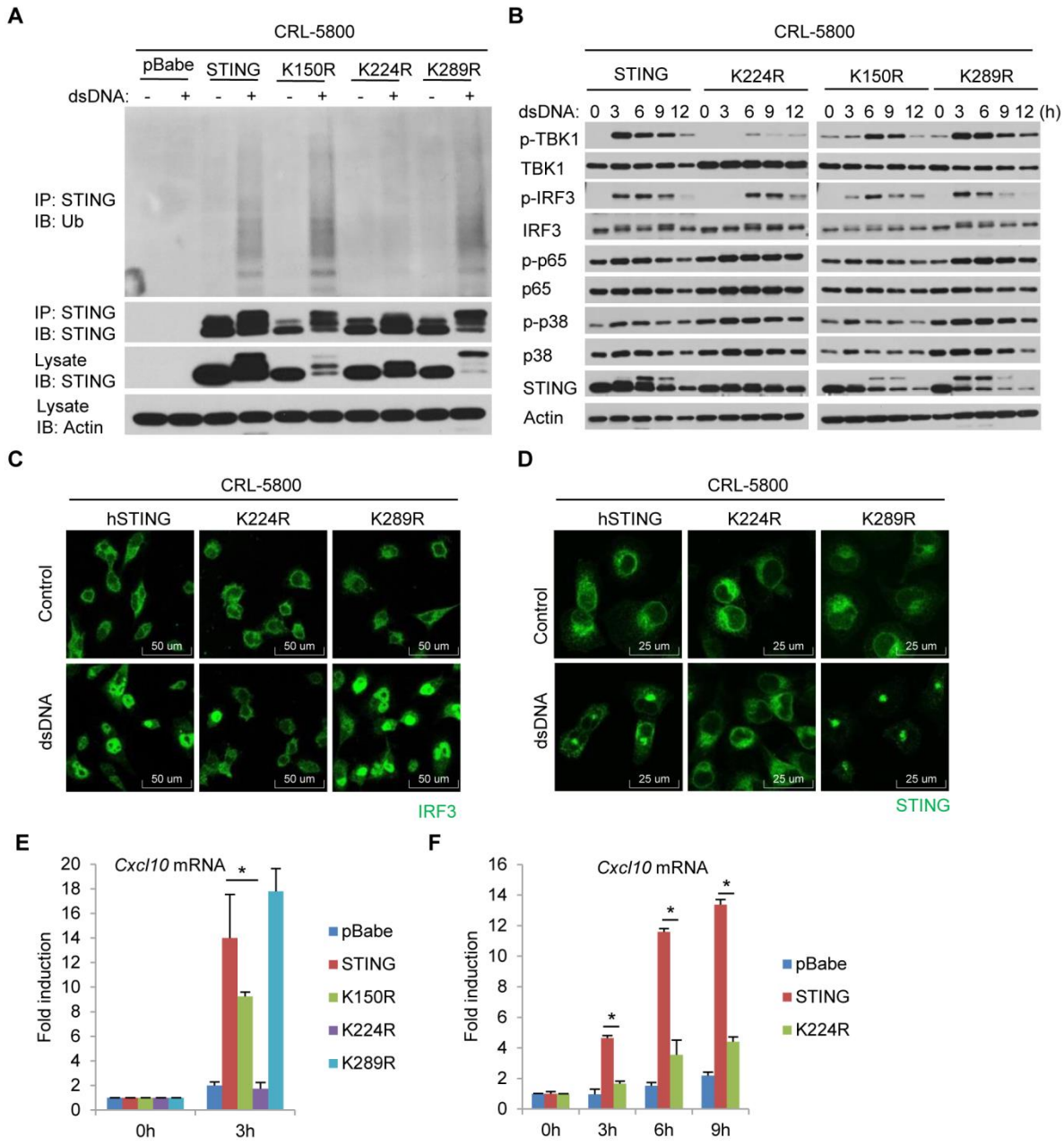
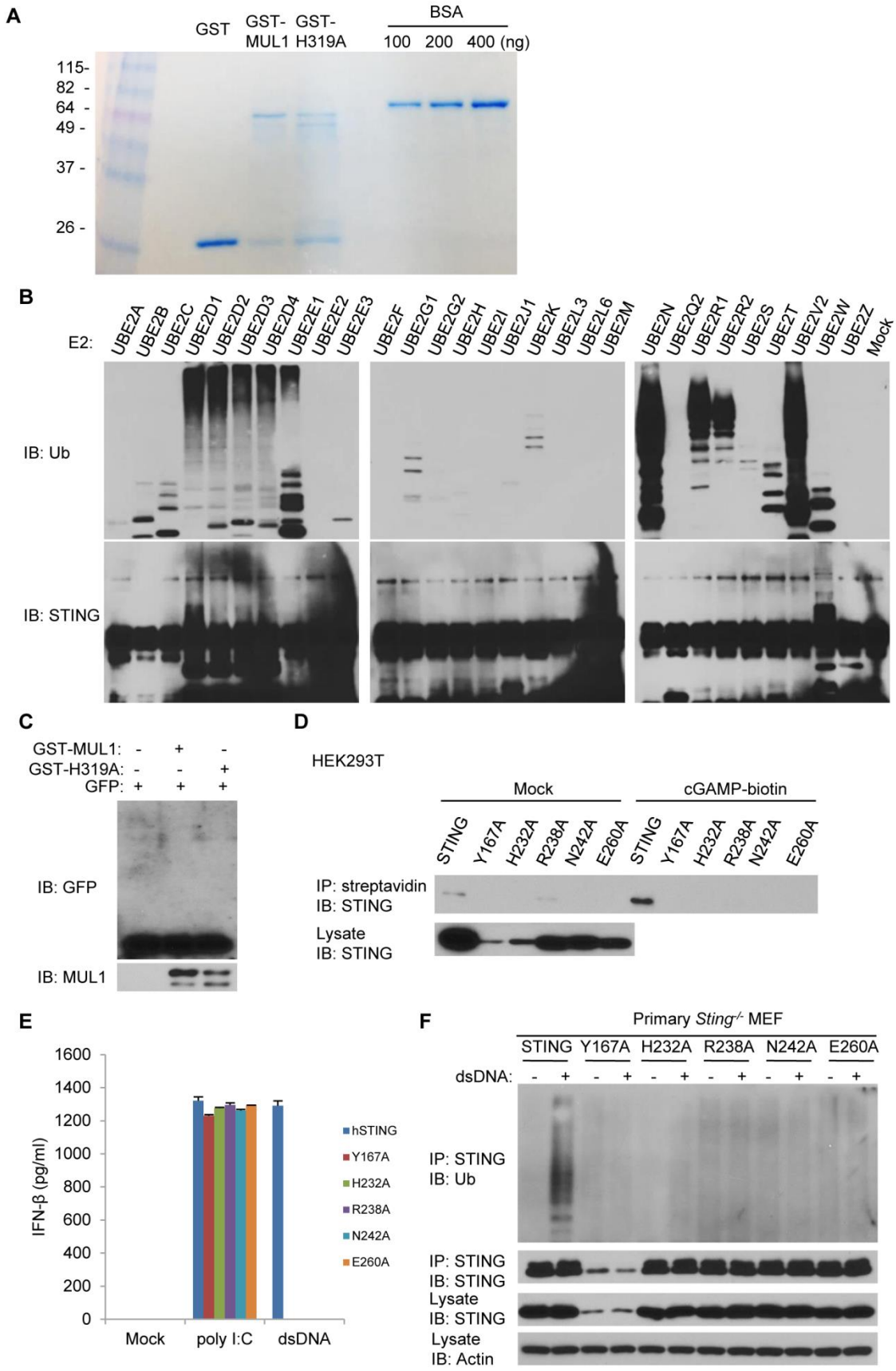


Fig. S6. Ubiquitination on K224 is essential for STING activity in human cells. (**A**) CRL-5800 cells were reconstituted with empty vector, hSTING or its mutants using retroviruses. The cells were transfected with dsDNA (4 μg/ml) for 3 hours. Cell lysates were immunoprecipitated with anti-STING antibody and then analyzed by immunoblot with the indicated antibodies. (**B**) Reconstituted CRL-5800 cells were transfected with dsDNA (4 μg/ml) for the indicated time periods, and cell lysates were immunoblotted with the indicated antibodies. (**C** and **D**) Reconstituted CRL-5800 cells were transfected with dsDNA (4 μg/ml) for 6 hours (**C**) or 3 hours (**D**), and then stained with indicated antibodies and imaged by confocal microscopy. (**E**) Reconstituted CRL-5800 cells were transfected with dsDNA (4 μg/ml) for 3 hours, and induction of *Cxcl10* mRNAs was measured by real-time PCR. (**F**) Reconstituted CRL-5800 cells were transfected with dsDNA (4 μg/ml) for the indicated time periods, and induction of *Cxcl10*

mRNAs was measured by real-time e PCR. Each panel of data is representative of at least two independent experiments which had the same outcome. Data in (E) and (F) were presented as average ± SD of duplicated samples from each group. *P* value was determined by Student's t test, $*P<0.05$, statistically significant difference between two groups.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GST
GST-MUL1
GST-H319A
BSA
100 200 400 (ng)
115-
82 -
64 -
49 -
37 -
26 -
B
E2:
UBE2A UBE2B UBE2C UBE2D1 UBE2D2 UBE2D3 UBE2D4 UBE2E1 UBE2E2 UBE2E3 UBE2F UBE2G1 UBE2G2 UBE2H UBE2I UBE2J1 UBE2K UBE2L3 UBE2L6 UBE2M UBE2N UBE2Q2 UBE2R1 UBE2R2 UBE2S UBE2T UBE2V2 UBE2W UBE2Z Mock
IB: Ub
IB: STING
C
GST-MUL1: - + -
GST-H319A: - - +
GFP: + + +
IB: GFP
IB: MUL1
D
HEK293T
Mock
cGAMP-biotin
STING Y167A H232A R238A N242A E260A STING Y167A H232A R238A N242A E260A
IP: streptavidin
IB: STING
Lysate
IB: STING
E
IFN-β (pg/ml)
1600
1400
1200
1000
800
600
400
200
0
Mock
poly I:C
dsDNA
hSTING
Y167A
H232A
R238A
N242A
E260A
F
Primary Sting-/- MEF
STING Y167A H232A R238A N242A E260A
dsDNA: - + - + - + - + - + - +
IP: STING
IB: Ub
IP: STING
IB: STING
Lysate
IB: STING
Lysate
IB: Actin

Fig. S7. MUL1 ubiquitinates STING in vitro. (**A**) GST-tagged MUL1 and MUL1 H319A were purified from *E.Coli* BL21(DE3) and were visualized in the gel with Coomassie Brilliant Blue staining. (**B**) Purified E1, ubiquitin, STING (152-379 aa) and GST-MUL1 proteins were mixed with the indicated E2 proteins and incubated at 37°C for 2 hours. The mixture was then analyzed by immunoblot with the indicated antibodies. (**C**) Purified E1, E2, ubiquitin, GFP, GST-MUL1 or GST-MUL1 H319A proteins were mixed together and incubated at 37°C for 2 hours. The mixture was then analyzed by immunoblot with the indicated antibodies. (**D**) HEK293T cells were transfected with indicated STING variants for 24 hours and lysed with TNE buffer. Cell lysates were incubated with or without biotin-labeled cGAMP (2 μM) at 4°C for 30 min followed with streptavidin beads precipitation and then immunobloted with indicated antibodies. (**E**) Reconstituted *Sting*$^{-/-}$ MEFs were transfected with dsDNA (4 μg/ml) for 16 hr, and IFNβ production was measured by ELISA. (**F**) Reconstituted *Sting*$^{-/-}$ MEFs were transfected with or without dsDNA (4 μg/ml) for 6 hours. Cell lysates were immunoprecipitated with anti-STING antibody and then immunoblotted with the indicated antibodies. Each panel of data is representative of at least two independent experiments which had the same outcome. Data in (E) were presented as average ± SD of duplicated samples from each group.

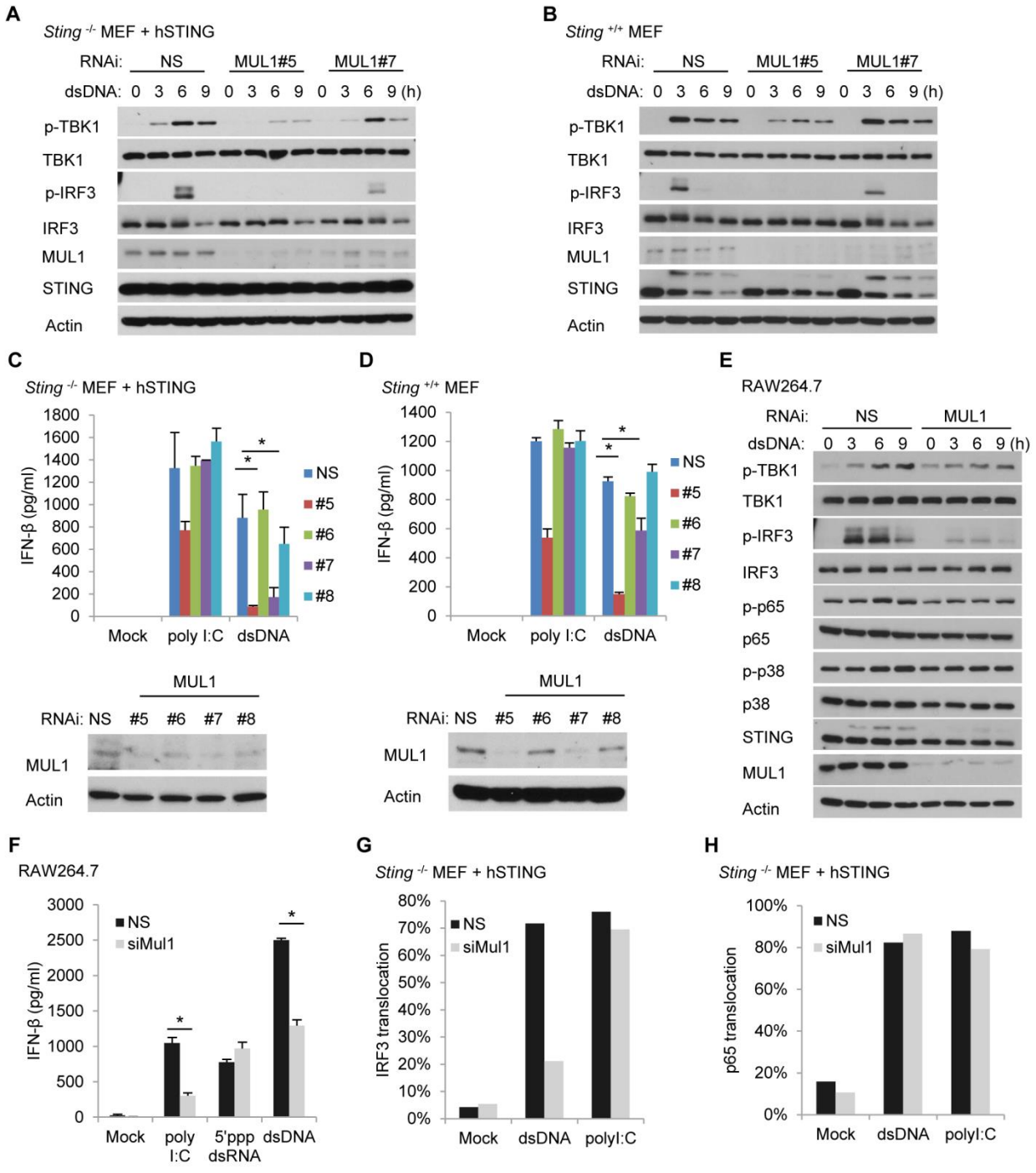


Fig. S8. MUL1 regulates dsDNA-induced STING-dependent innate response. (**A** and **B**) siRNA-treated reconstituted *Sting*$^{-/-}$ MEFs (**A**) or wild-type MEFs (**B**) were transfected with dsDNA (4 μg/ml) for the indicated time periods, and cell lysates were immunoblotted with indicated antibodies. NS, nonspecific siRNA. (**C** and **D**) siRNA-treated reconstituted *Sting*$^{-/-}$ MEFs (**C**) or wild-type MEFs (**D**) were transfected with poly I:C (1 μg/ml), or dsDNA (4 μg/ml) for 16 hours, and IFNβ production was measured by ELISA. (**E**) siRNA-treated

reconstituted RAW 264.7 cells were transfected with dsDNA (4 μg/ml) for the indicated time periods, and cell lysates were immunoblotted with indicated antibodies. (**F**) siRNA-treated reconstituted RAW 264.7 cells were transfected with poly I:C (1 μg/ml), 5'ppp dsRNA (1 μg/ml), or dsDNA (4 μg/ml) for 16 hours, and IFNβ production was measured by ELISA. (**G** and **H**) Quantification of the IRF3 nucleus translocation in Fig. 6H (**G**) and p65 nucleus translocation in Fig. 6I (**H**). Each panel of data is a representative of at least two independent experiments which had the same outcome. Data in (C), (D), and (F) were presented as average ± SD of duplicated samples from each group. *P* value was determined by Student's t test, $^{*}P<0.05$, statistically significant difference between two groups.

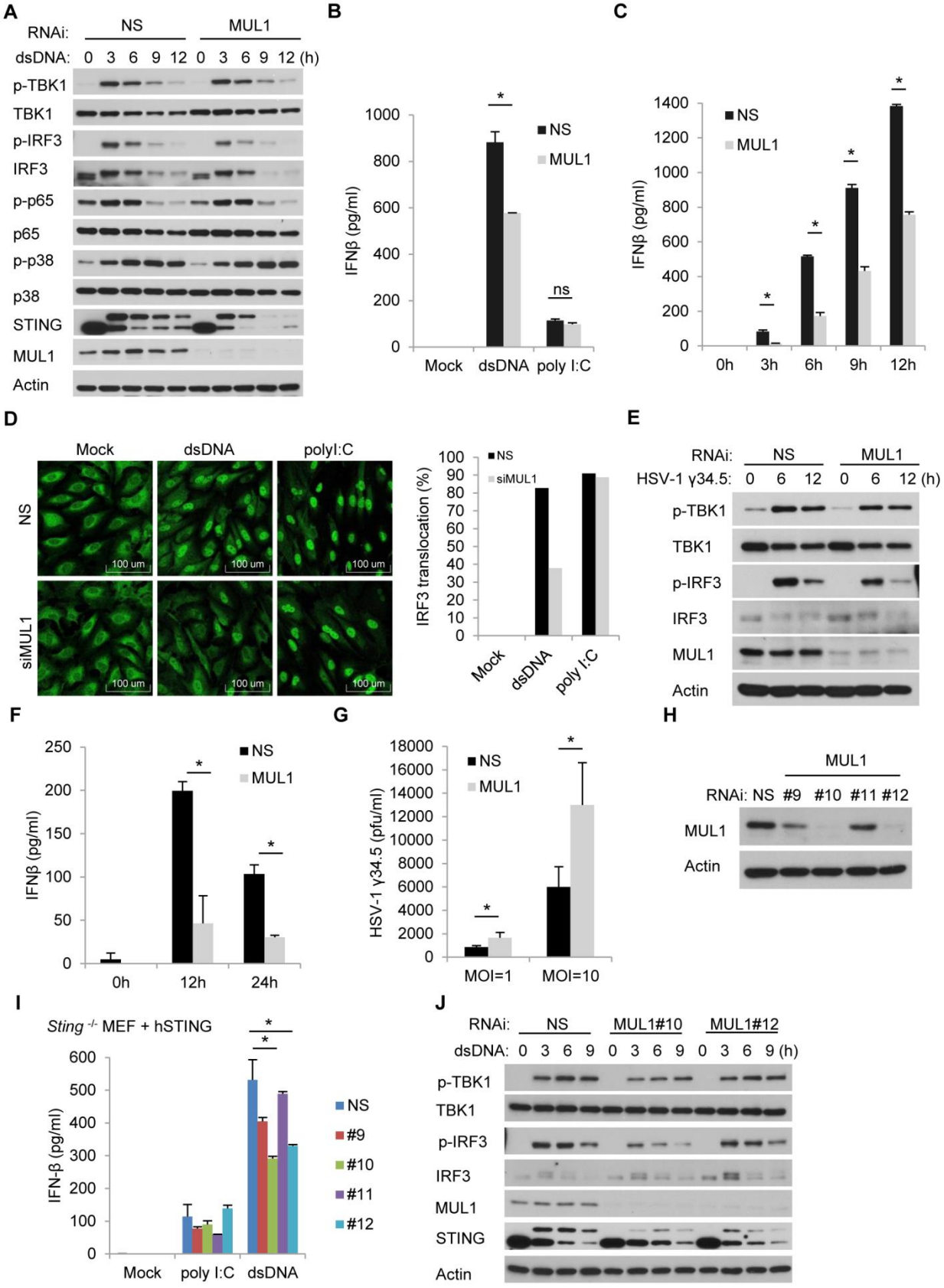

A
RNAi: NS MUL1
dsDNA: 0 3 6 9 12 0 3 6 9 12 (h)
p-TBK1
TBK1
p-IRF3
IRF3
p-p65
p65
p-p38
p38
STING
MUL1
Actin
B
IFNβ (pg/ml)
NS
MUL1
ns
Mock dsDNA poly I:C
C
IFNβ (pg/ml)
NS
MUL1
0h 3h 6h 9h 12h
D
Mock dsDNA polyI:C
NS
siMUL1
100 um
IRF3 translocation (%)
NS
siMUL1
Mock dsDNA poly I:C
E
RNAi: NS MUL1
HSV-1 γ34.5: 0 6 12 0 6 12 (h)
p-TBK1
TBK1
p-IRF3
IRF3
MUL1
Actin
F
IFNβ (pg/ml)
NS
MUL1
0h 12h 24h
G
HSV-1 γ34.5 (pfu/ml)
NS
MUL1
MOI=1 MOI=10
H
MUL1
RNAi: NS #9 #10 #11 #12
MUL1
Actin
I
Sting -/- MEF + hSTING
IFN-β (pg/ml)
NS
#9
#10
#11
#12
Mock poly I:C dsDNA
J
RNAi: NS MUL1#10 MUL1#12
dsDNA: 0 3 6 9 0 3 6 9 0 3 6 9 (h)
p-TBK1
TBK1
p-IRF3
IRF3
MUL1
STING
Actin

Fig. S9. MUL1 partially regulates STING activity in human cells. (**A**) siRNA-treated EA.hy926 cells were transfected with dsDNA (4 μg/ml) for the indicated time periods, and cell lysates were immunoblotted with indicated antibodies. NS, nonspecific siRNA. (**B**) siRNA-treated EA.hy926 cells were transfected with dsDNA (4 μg/ml) or poly I:C (1 μg/ml) for 16 hours, and IFNβ production was measured by ELISA. (**C**) siRNA-treated EA.hy926 cells were transfected with dsDNA (4 μg/ml) for the indicated time periods, and IFNβ production was measured by ELISA. (**D**) siRNA-treated EA.hy926 ells were transfected with dsDNA (4 μg/ml) or poly I:C (4 μg/ml) for 3 hours, and then stained with anti-IRF3 antibody. Cells with nuclear IRF3 staining were counted as a percentage of total cells (5 fields of cells counted per sample). (**E**) siRNA-treated EA.hy926 cells were infected with HSV-1 γ34.5 (MOI=10) for the indicated time periods, and cell lysates were immunoblotted with indicated antibodies. (**F**) siRNA-treated EA.hy926 cells were infected with HSV-1 γ34.5 (MOI=10) for the indicated time periods, and IFNβ production was measured by ELISA. (**G**) siRNA-treated EA.hy926 cells were infected with HSV-1 γ34.5 (MOI=1 or 10) for 24 hours, and viral titer was measure by plaque assay. (**H**) EA.hy926 cells were transfected with NS siRNA or four individual Mul1 siRNAs for 72 hours, and cell lysates were immunoblotted with indicated antibodies. (**I**) siRNA-treated EA.hy926 cells were transfected with dsDNA (4 μg/ml) or poly I:C (1 μg/ml) for 16 hours, and IFN β production was measured by ELISA. (**J**) siRNA-treated EA.hy926 cells were transfected with dsDNA (4 μg/ml) for the indicated time periods, and cell lysates were immunoblotted with indicated antibodies. Each panel of data is representative of at least two independent experiments which had the same outcome. Data were presented as average ± SD of duplicated (B, C, F, and I) and triplicated (G) samples from each group. *P* value was determined by Student's t test, **P* <0.05, statistically significant difference between two groups; ns, not significant.

Fig. S10. Entire Western blots

Fig. 1A

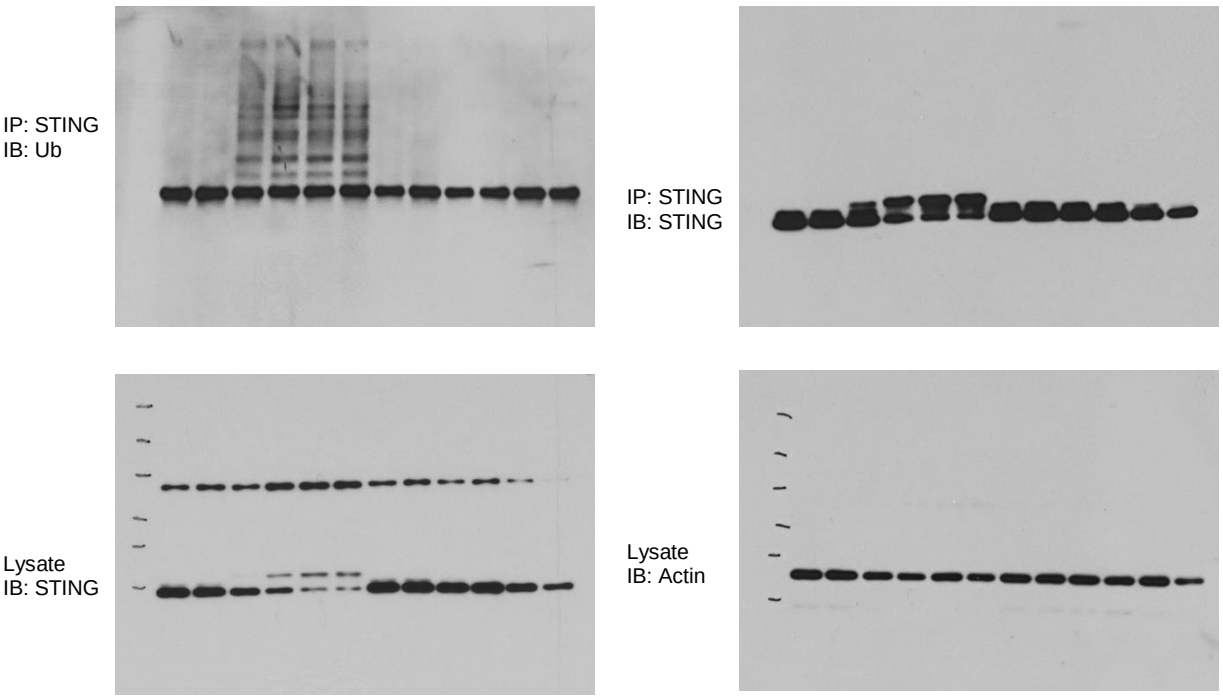


Fig. 1B

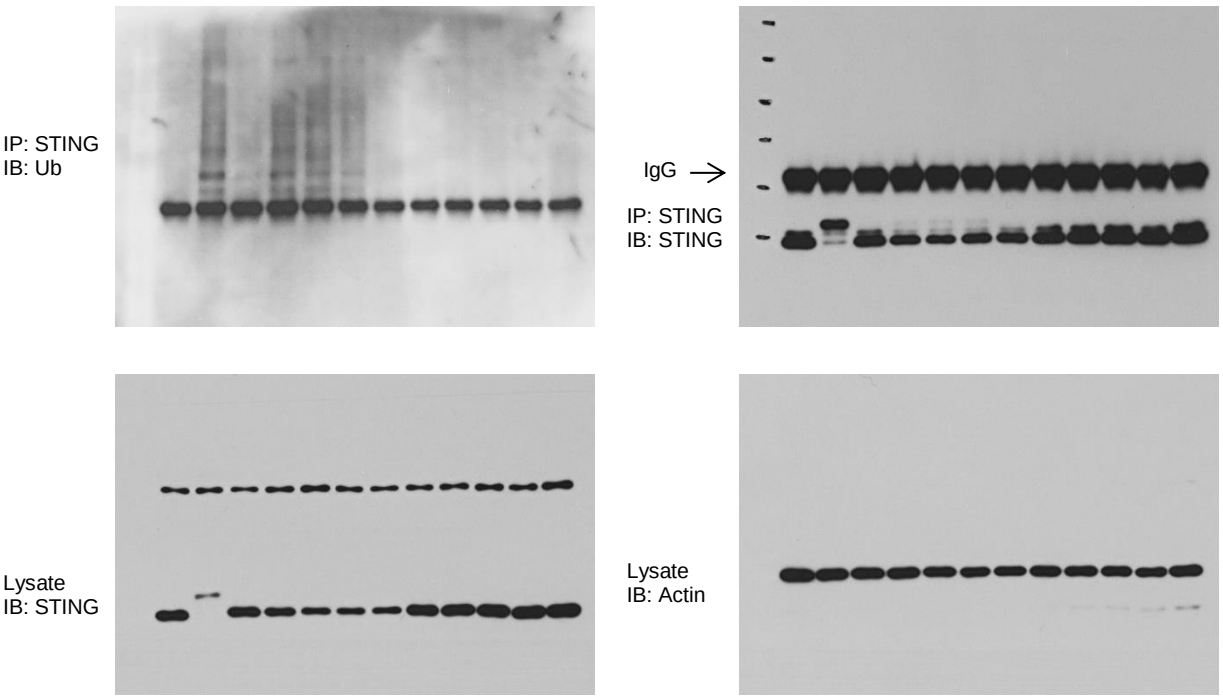


Fig. 1C

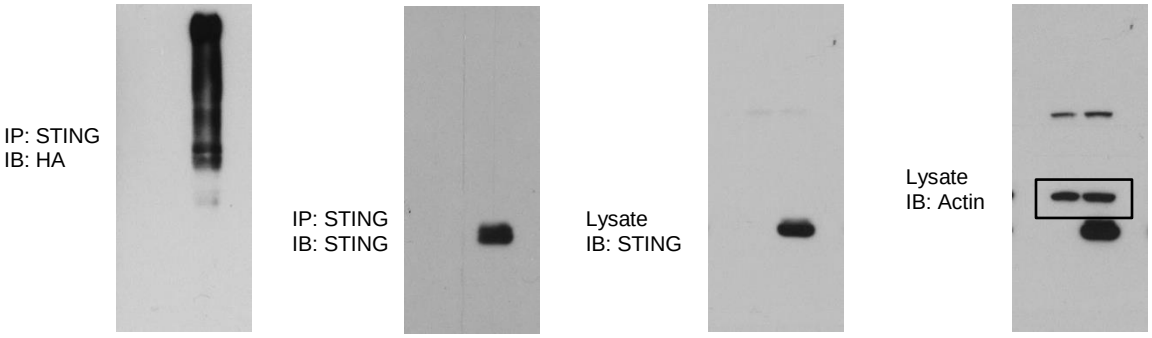


Fig. 1E

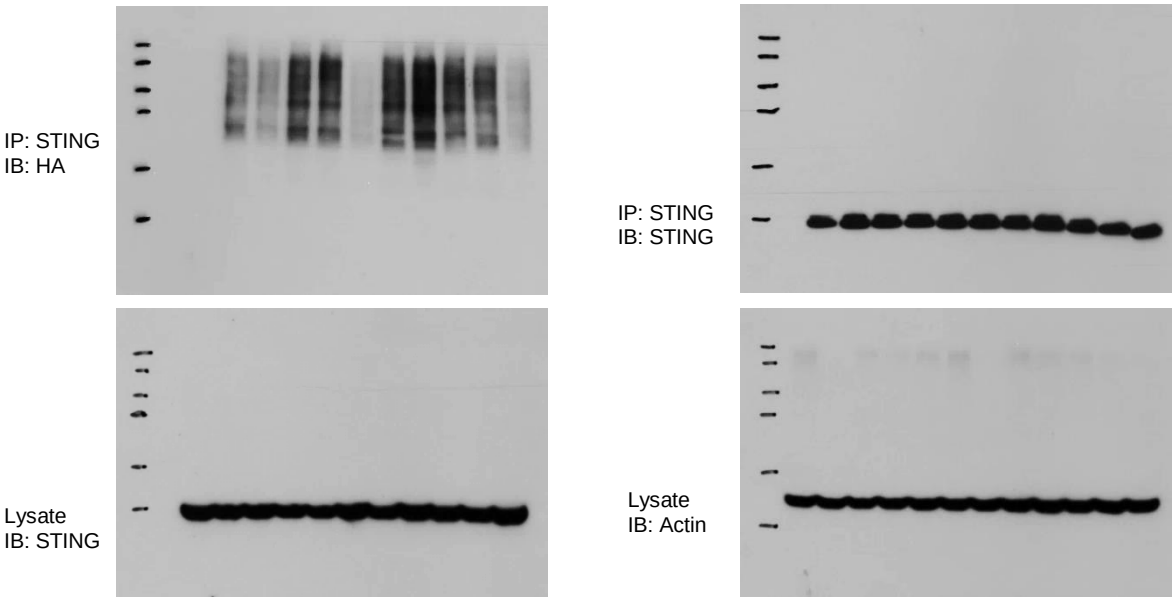


Fig. 1F

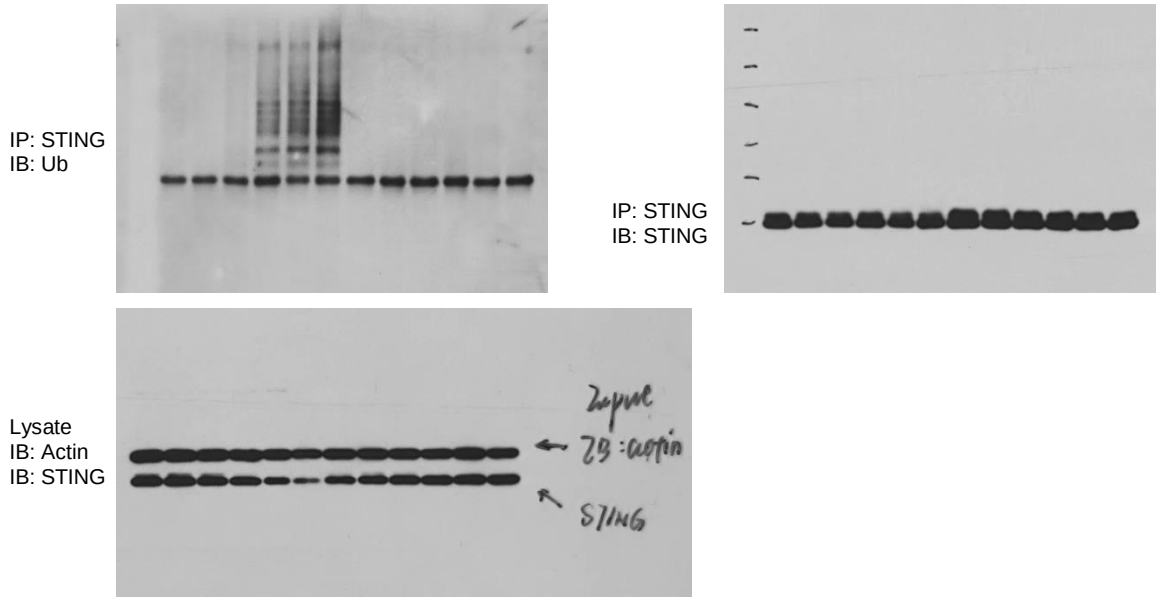
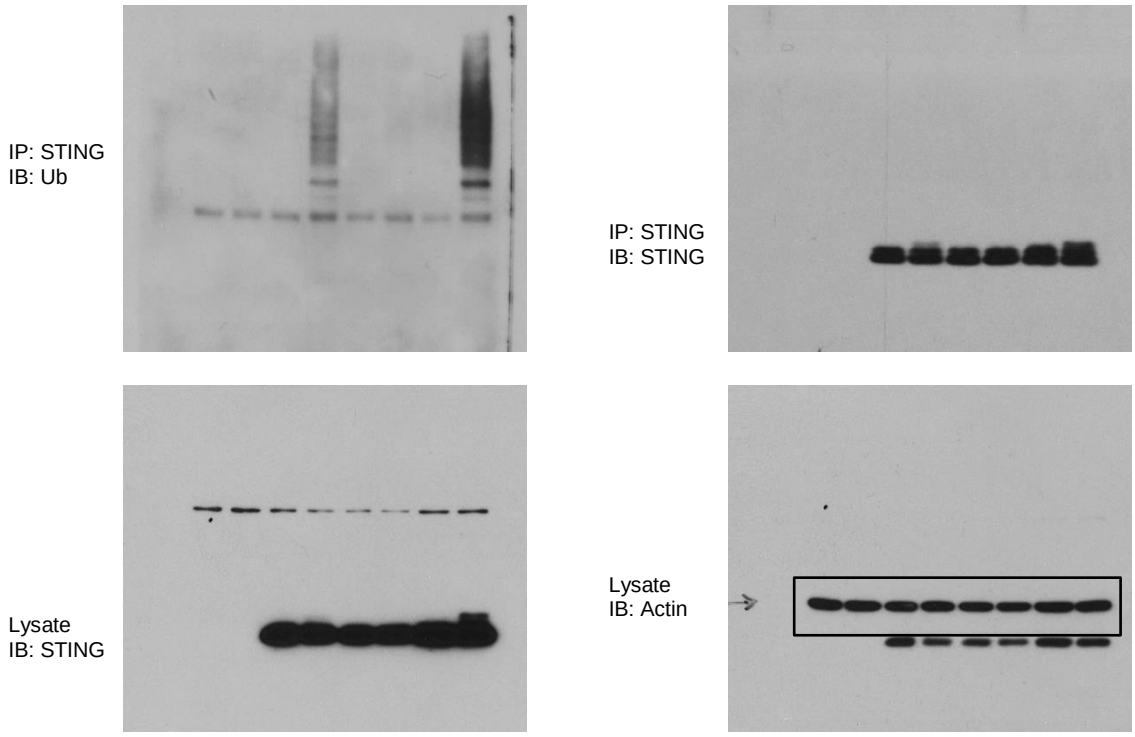


Fig. 1G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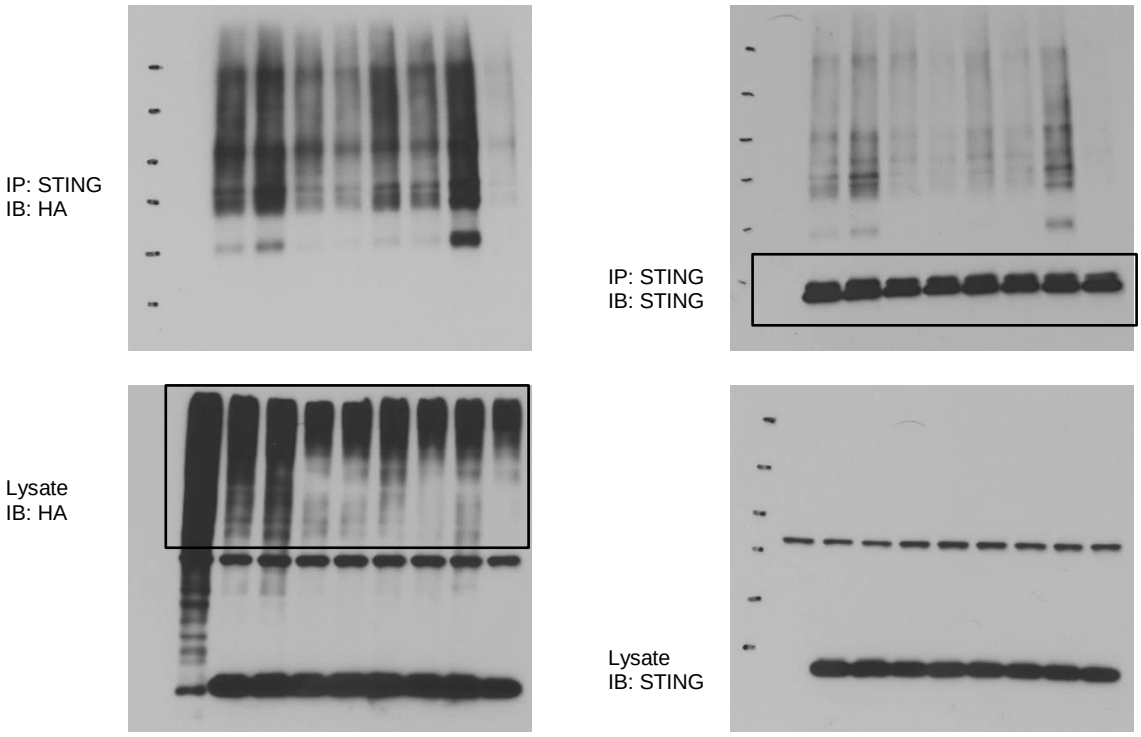
IB: Ub
IP: STING
IB: STING
Lysate
IB: STING
Lysate
IB: Actin

Fig. 1I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IB: HA
IP: STING
IB: STING
Lysate
IB: HA
Lysate
IB: STING

Fig. 2E

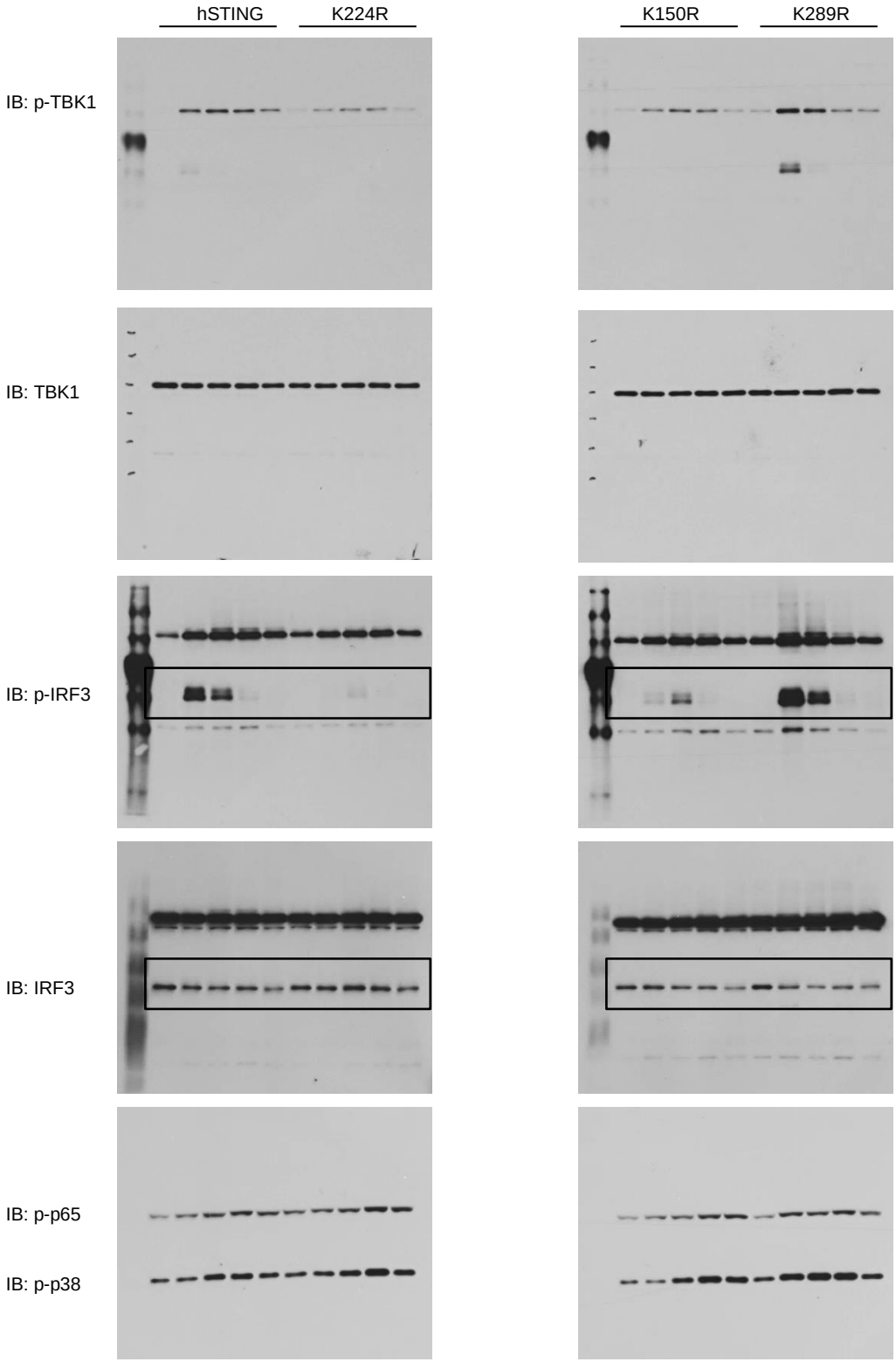

hSTING
K224R
K150R
K289R
IB: p-TBK1
IB: TBK1
IB: p-IRF3
IB: IRF3
IB: p-p65
IB: p-p38

Fig. 2E

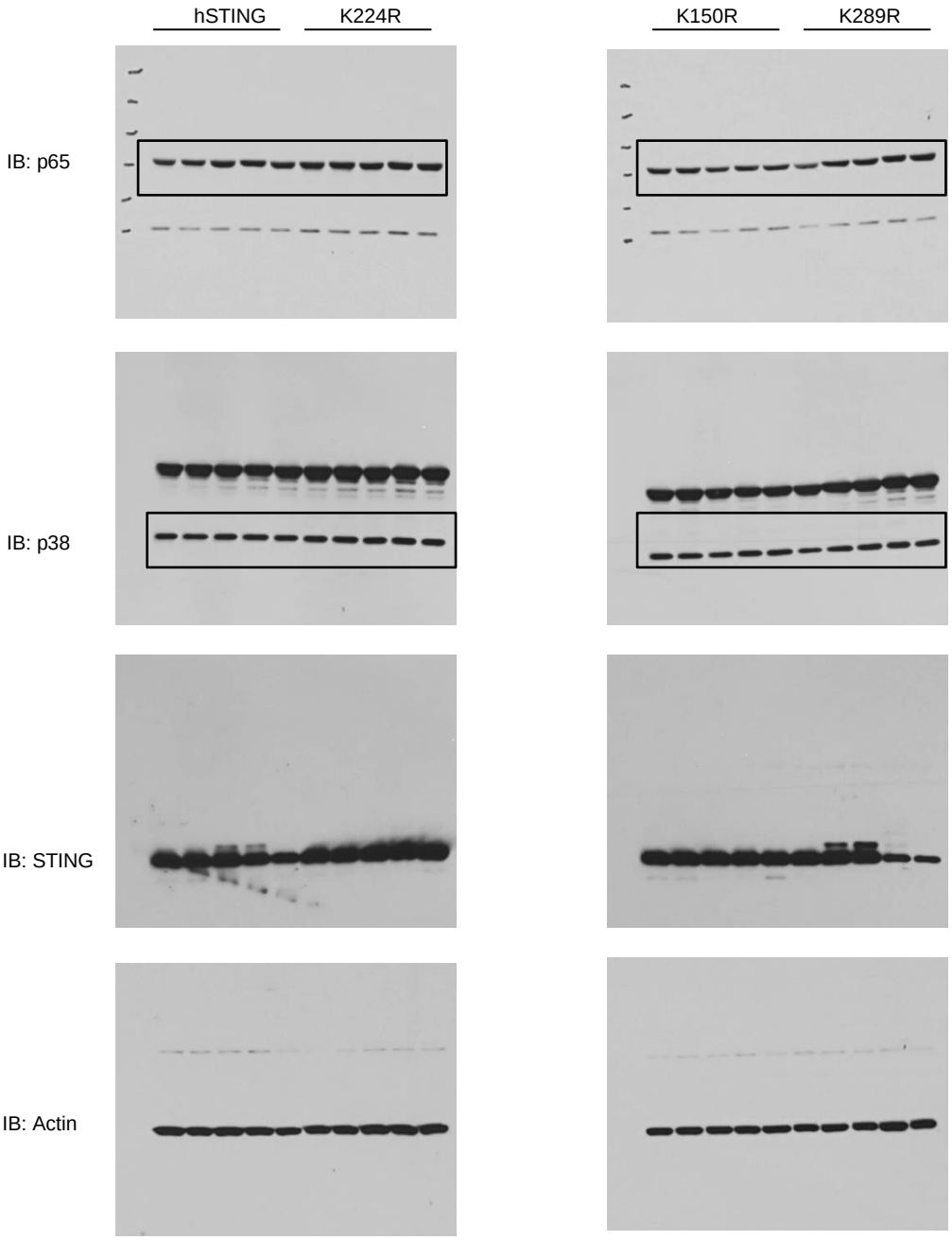
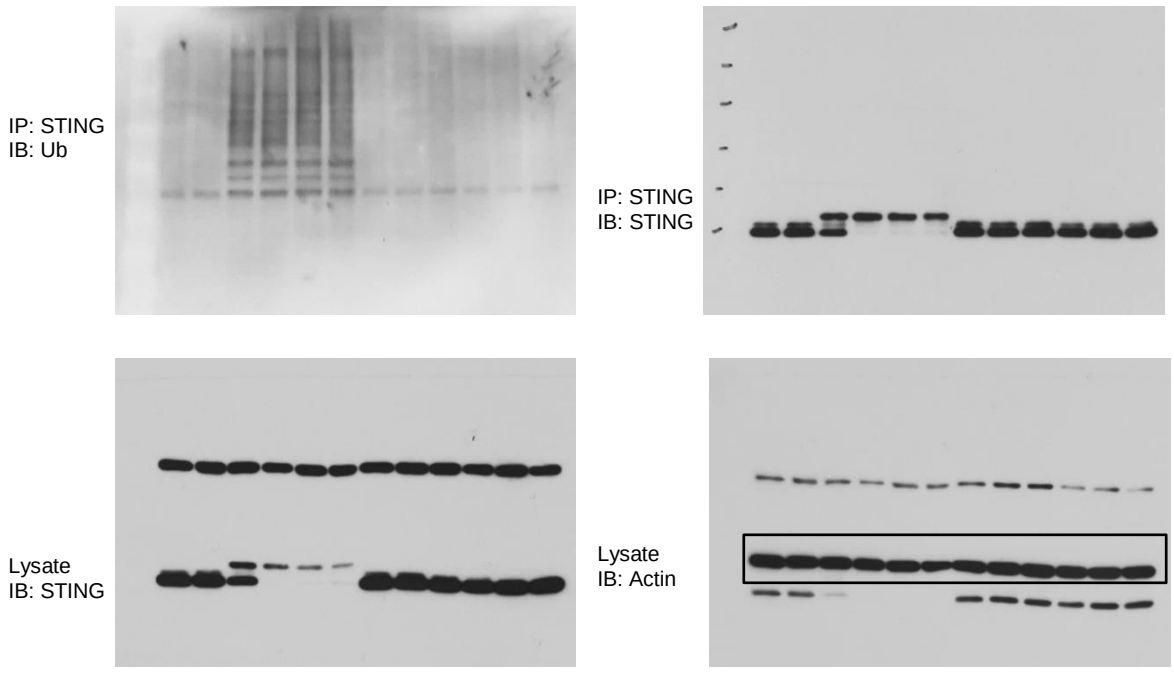


Fig. 3D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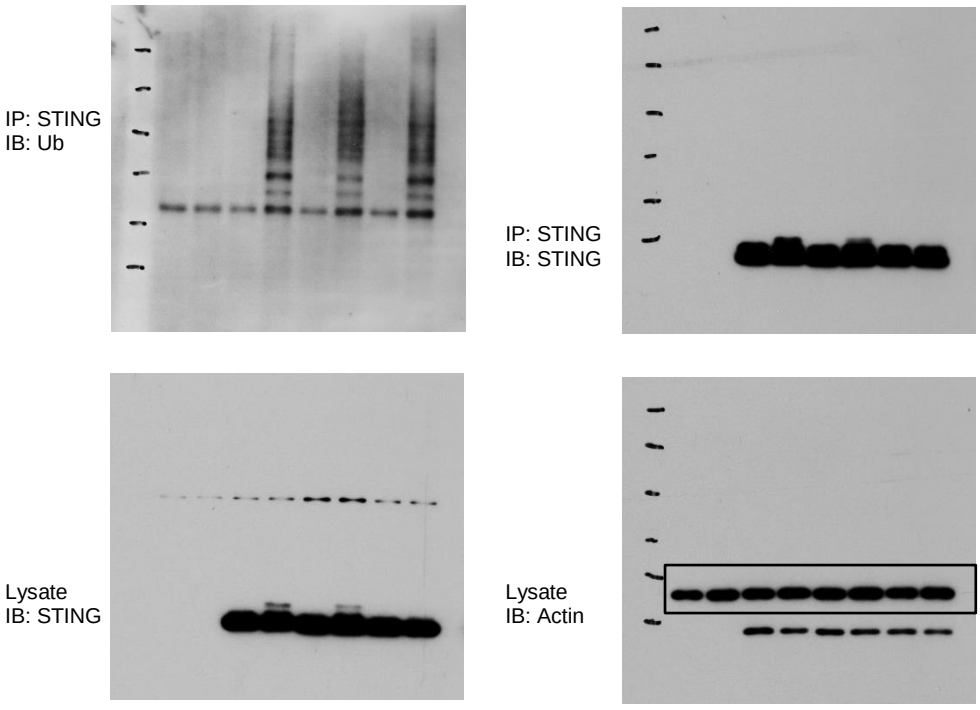
IB: Ub
IP: STING
IB: STING
Lysate
IB: STING
Lysate
IB: Actin

Fig. 3E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IB: STING
Lysate
IB: STING
Lysate
IB: Actin

Fig. 3F

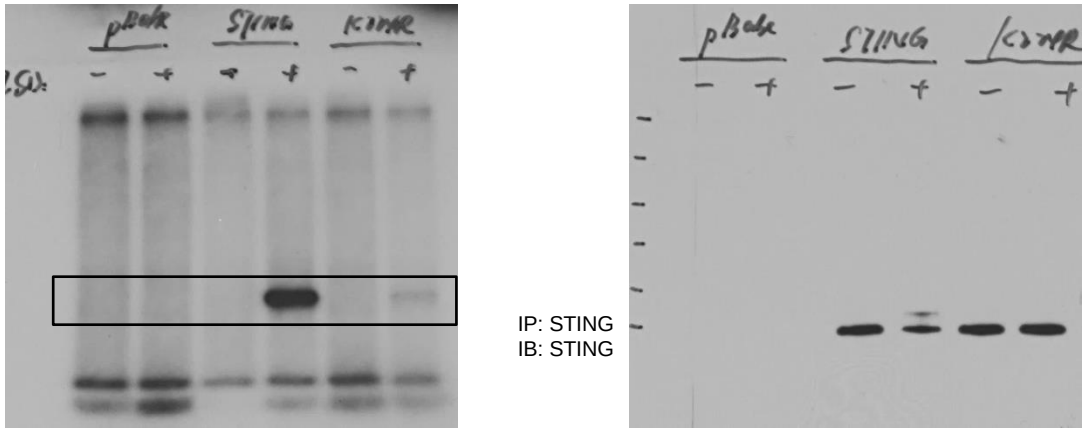

^{32}P
IP: STING
IB: STING

Fig. 3G

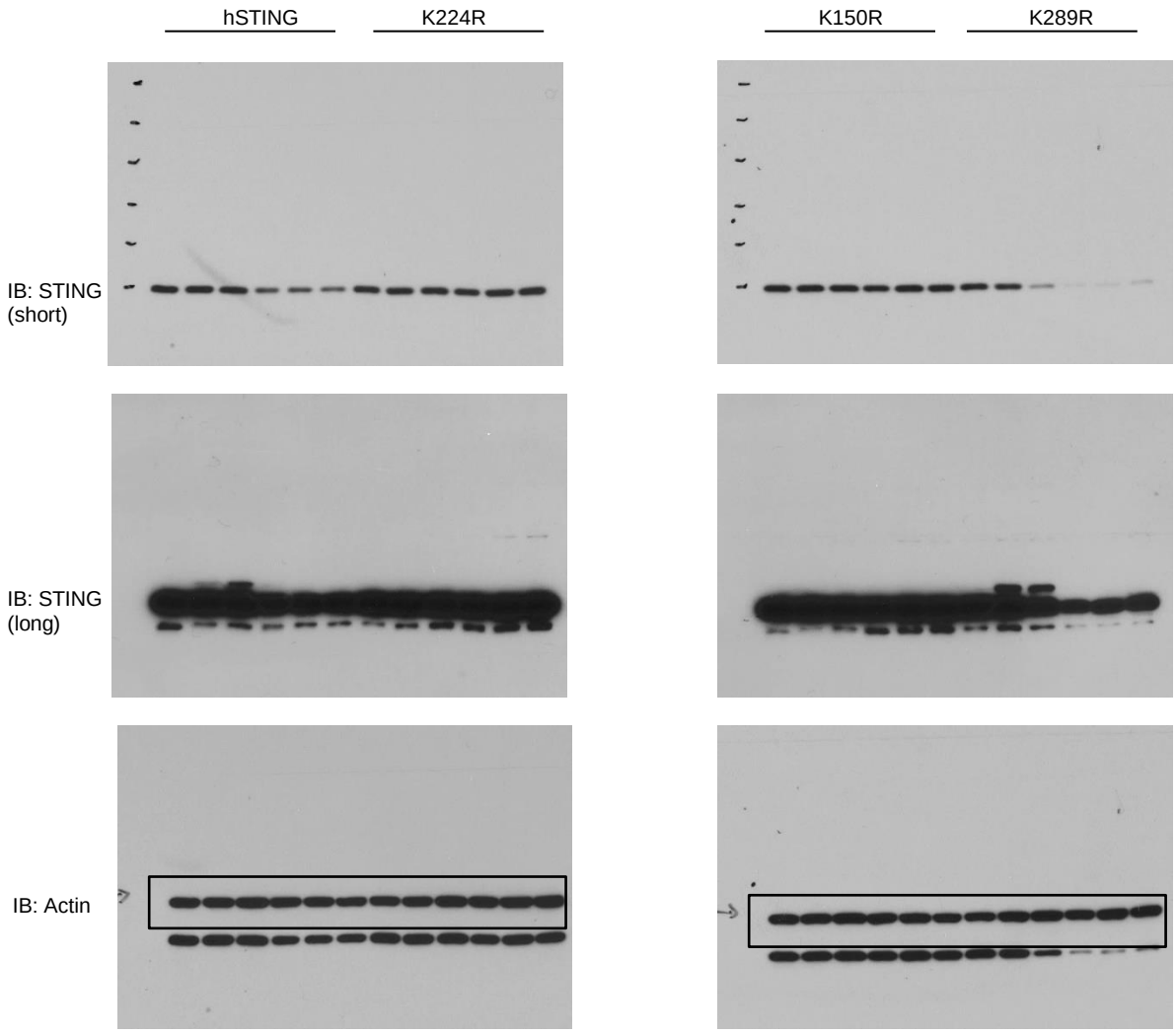
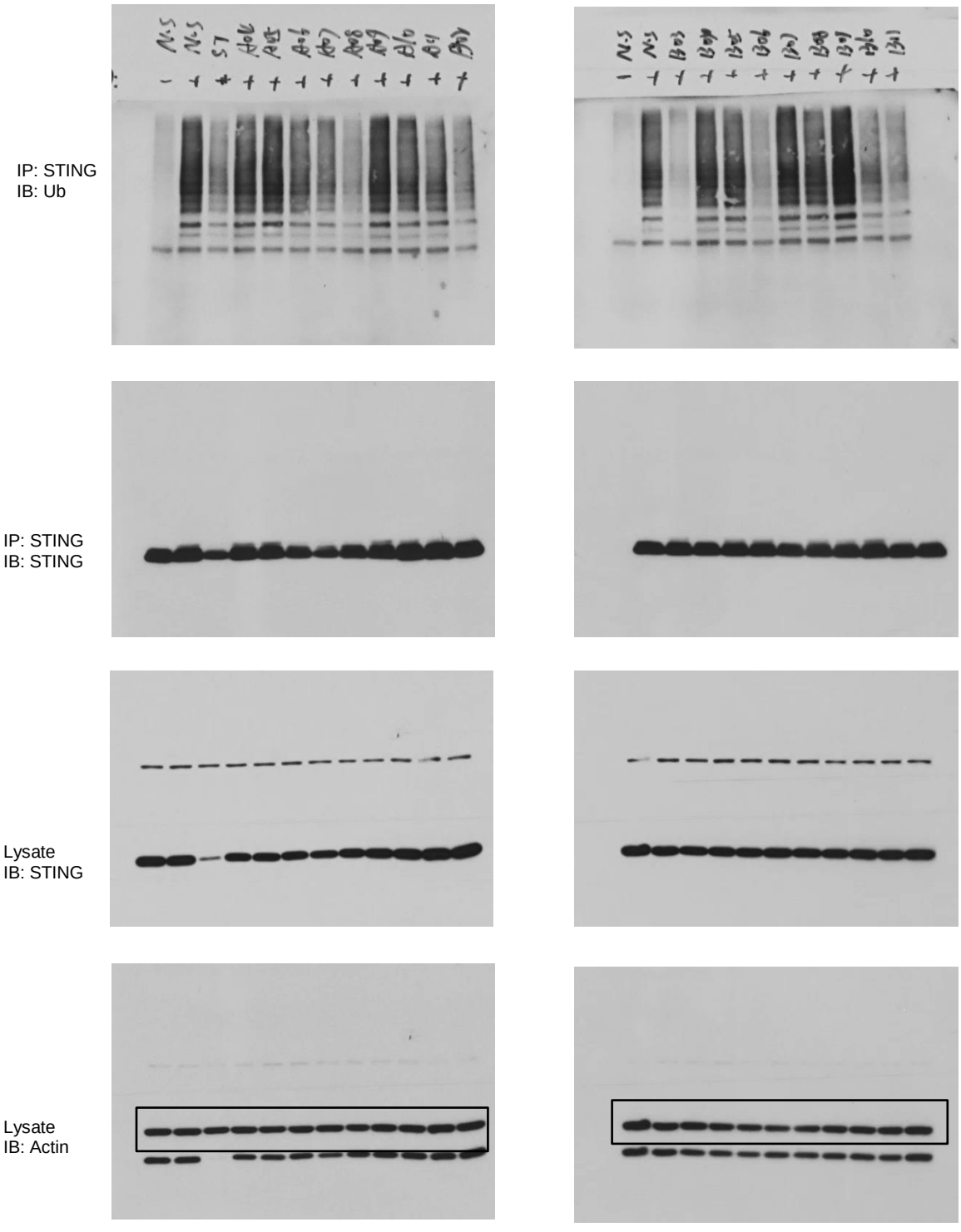

hSTING
K224R
K150R
K289R
IB: STING (short)
IB: STING (long)
IB: Actin

Fig. 4B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IB: Ub
IP: STING
IB: STING
Lysate
IB: STING
Lysate
IB: Actin

Fig. 4E

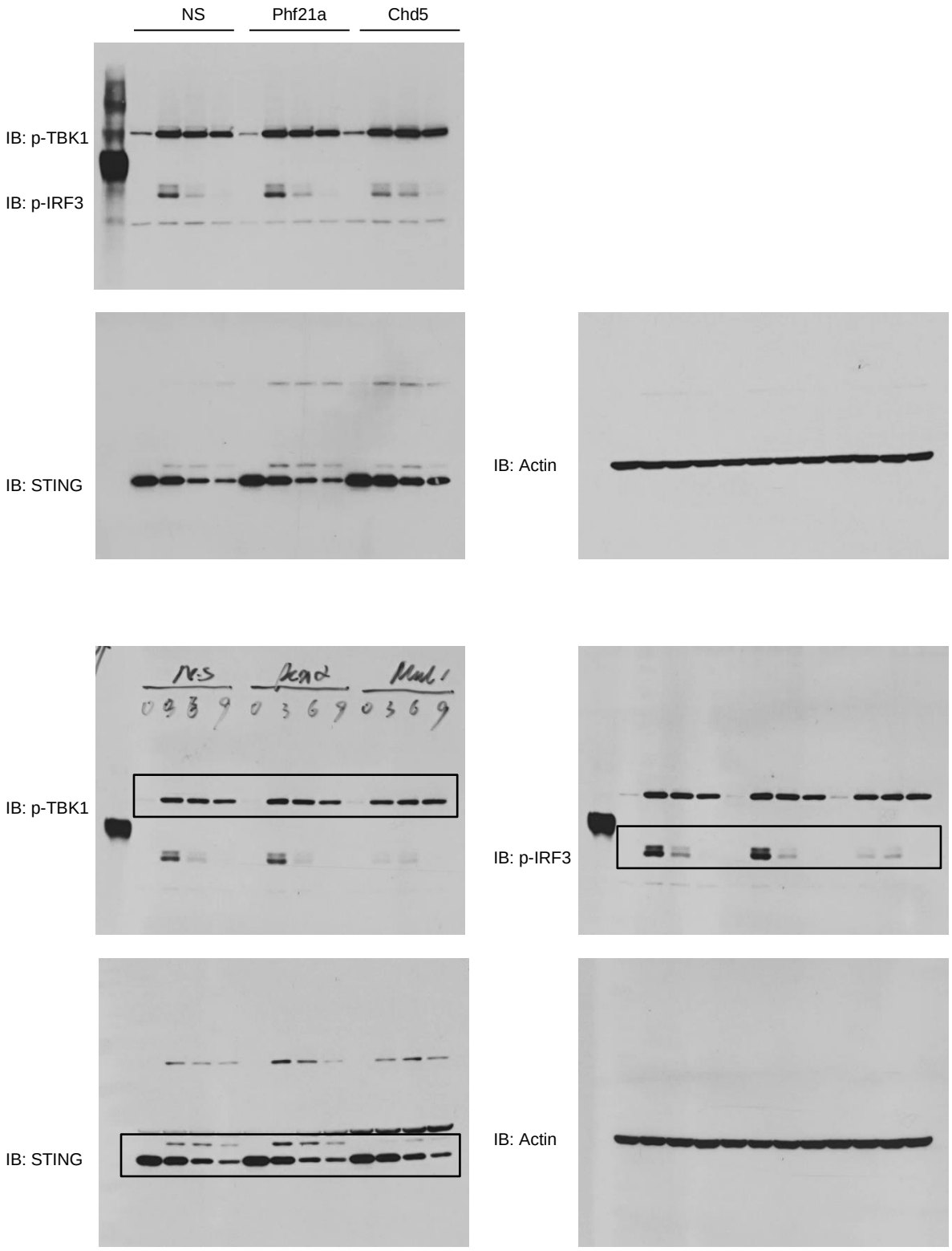

NS
Phf21a
Chd5
IB: p-TBK1
IB: p-IRF3
IB: STING
IB: Actin
IB: p-TBK1
IB: p-IRF3
IB: STING
IB: Actin

Fig. 4E

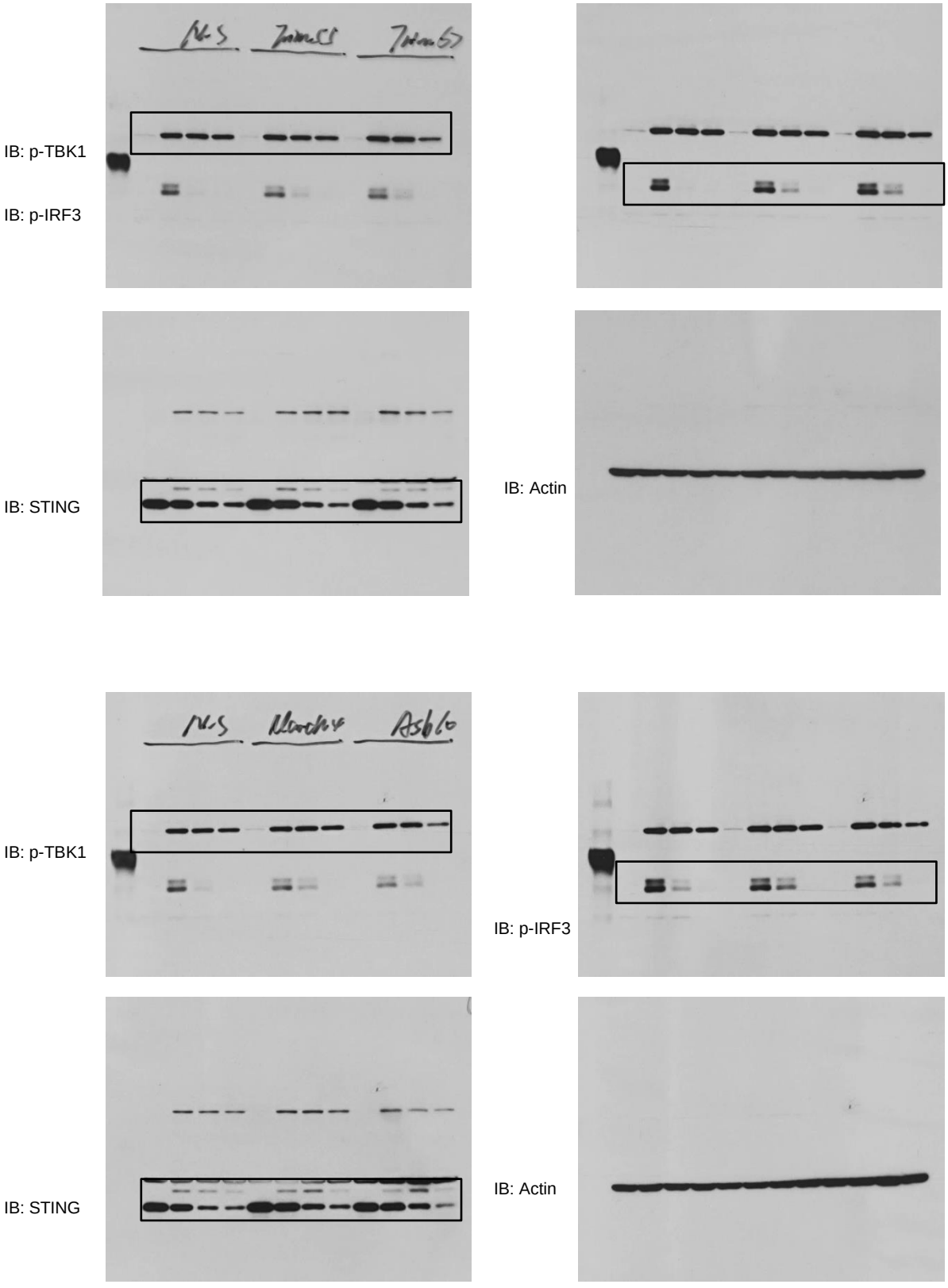

IB: p-TBK1
IB: p-IRF3
IB: STING
IB: Actin
IB: p-TBK1
IB: p-IRF3
IB: STING
IB: Actin

Fig. 5A

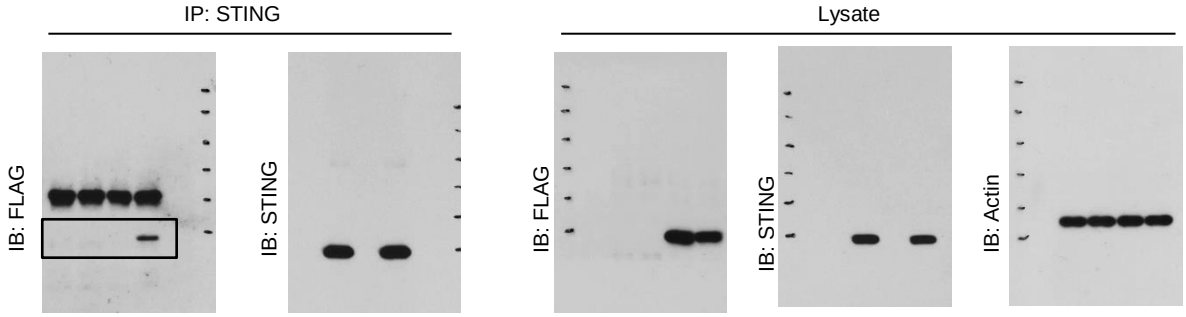


Fig. 5B

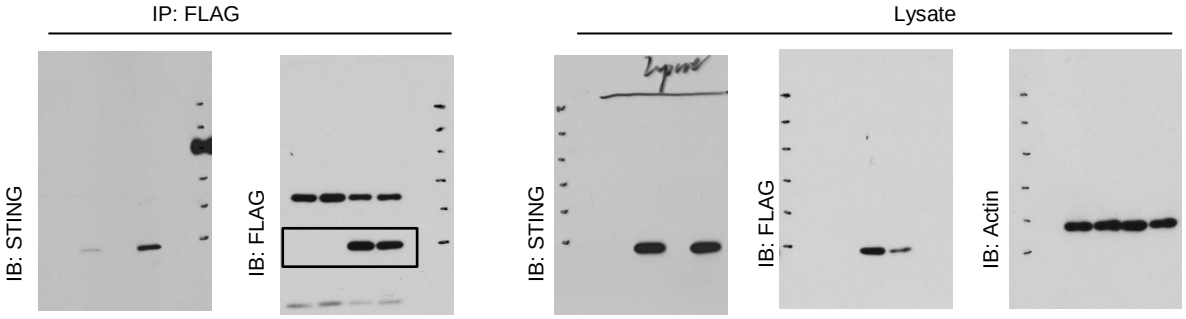


Fig. 5C

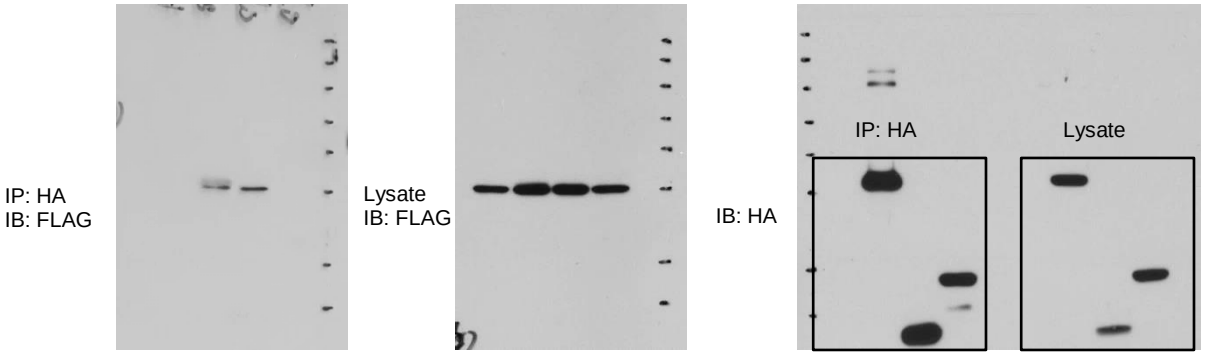


Fig. 5D

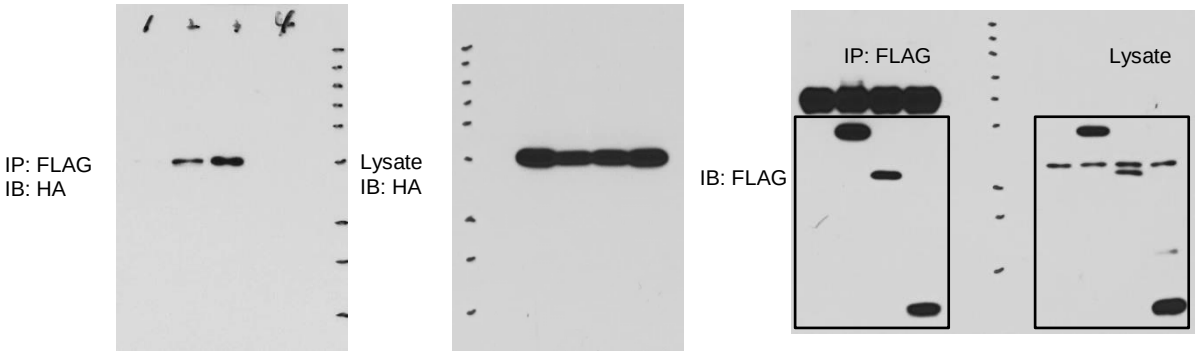


Fig. 5E

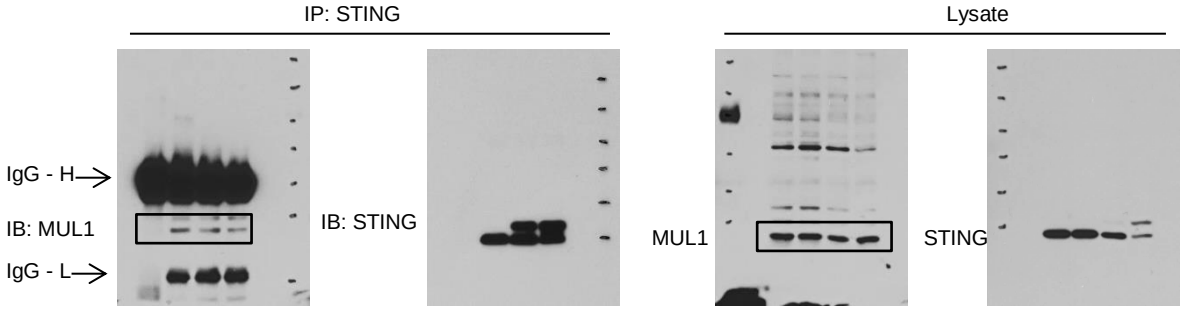


Fig. 5F

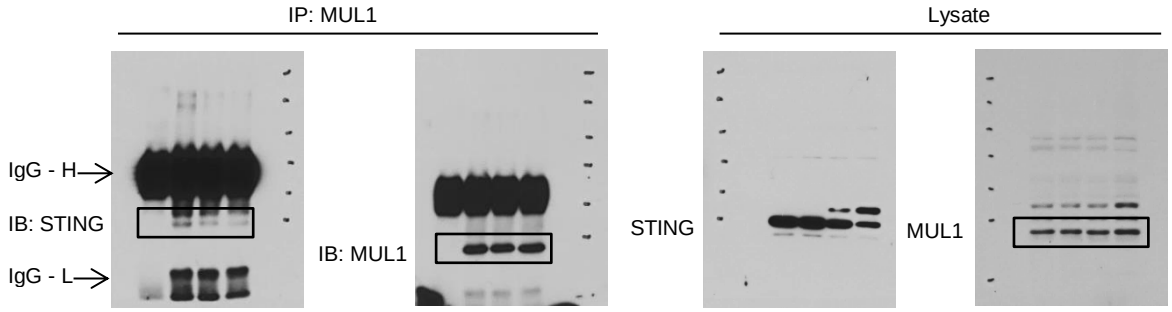


Fig. 5H

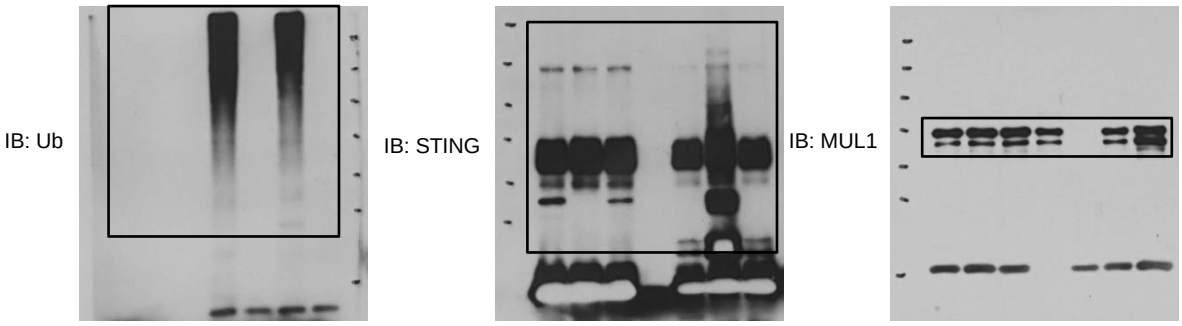


Fig. 5I

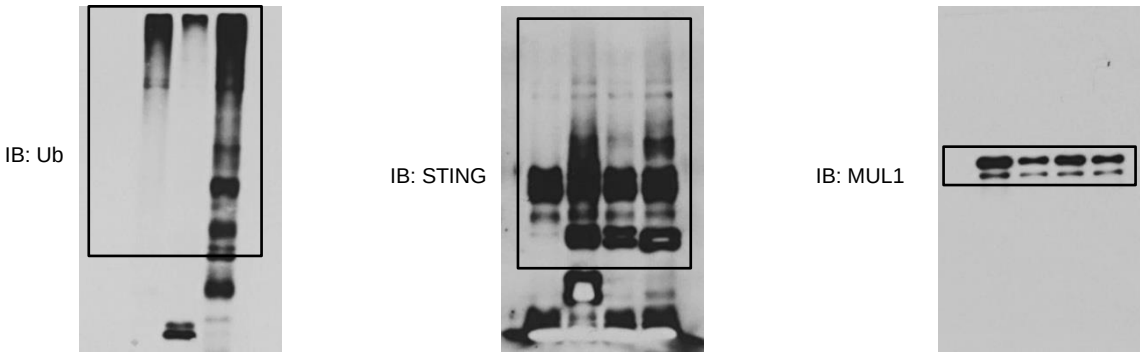


Fig. 6A

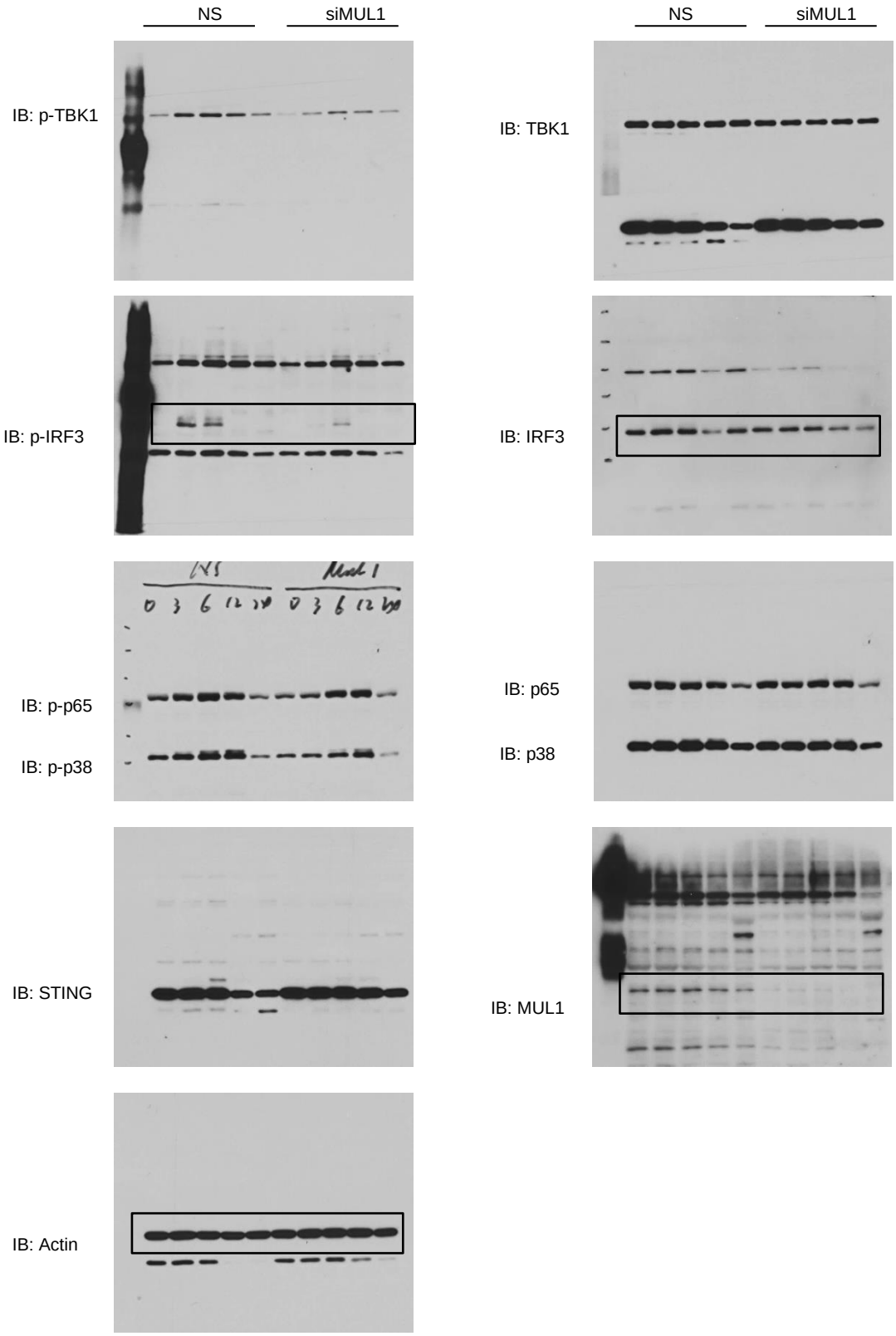

NS
siMUL1
IB: p-TBK1
IB: TBK1
IB: p-IRF3
IB: IRF3
IB: p-p65
IB: p-p38
IB: p65
IB: p38
IB: STING
IB: MUL1
IB: Actin

Fig. 6B

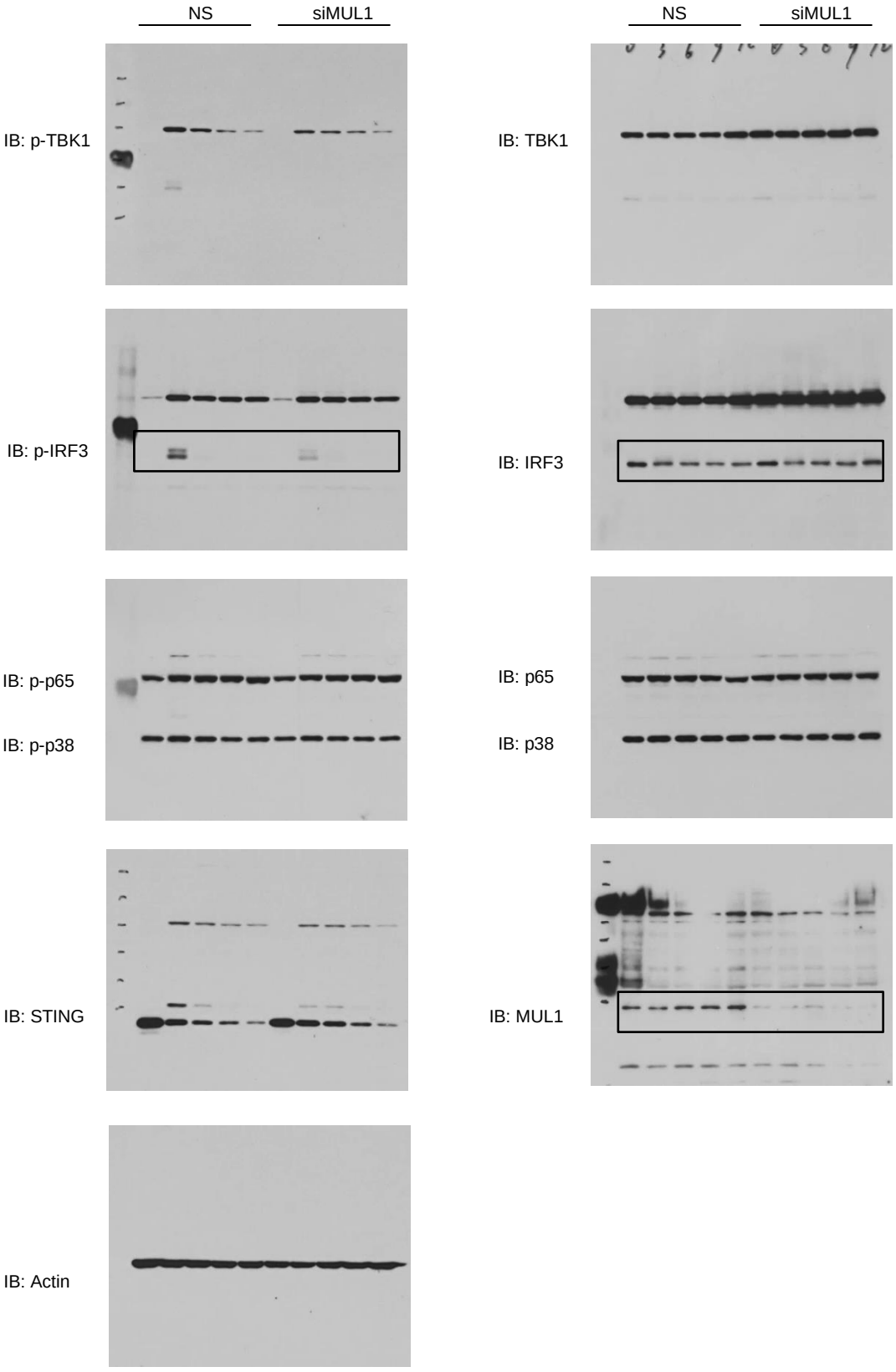
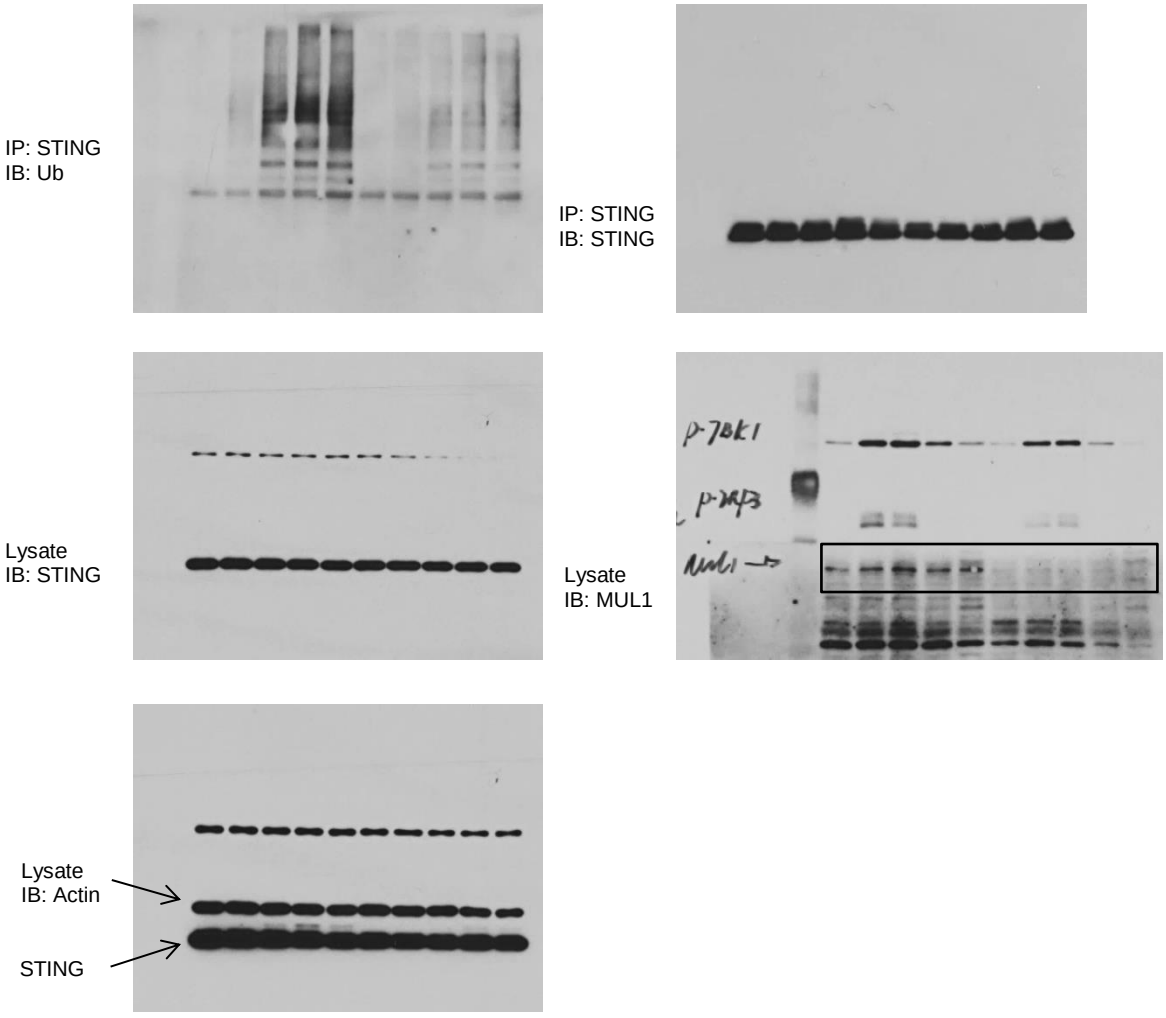

NS
siMUL1
NS
siMUL1
IB: p-TBK1
IB: TBK1
IB: p-IRF3
IB: IRF3
IB: p-p65
IB: p-p38
IB: p65
IB: p38
IB: STING
IB: MUL1
IB: Actin

Fig. 6C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IB: Ub
IP: STING
IB: STING
Lysate
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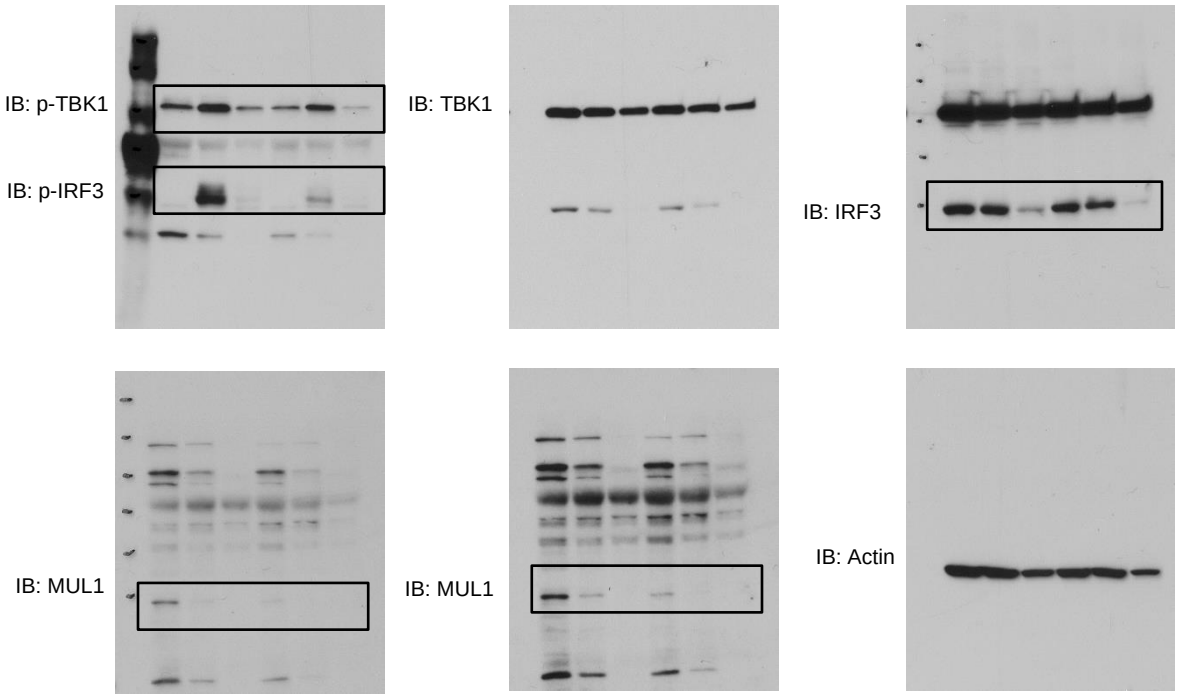
Lysate
IB: MUL1
p-70k1
p-4EBP3
Lysate
IB: Actin
STING

Fig. 6K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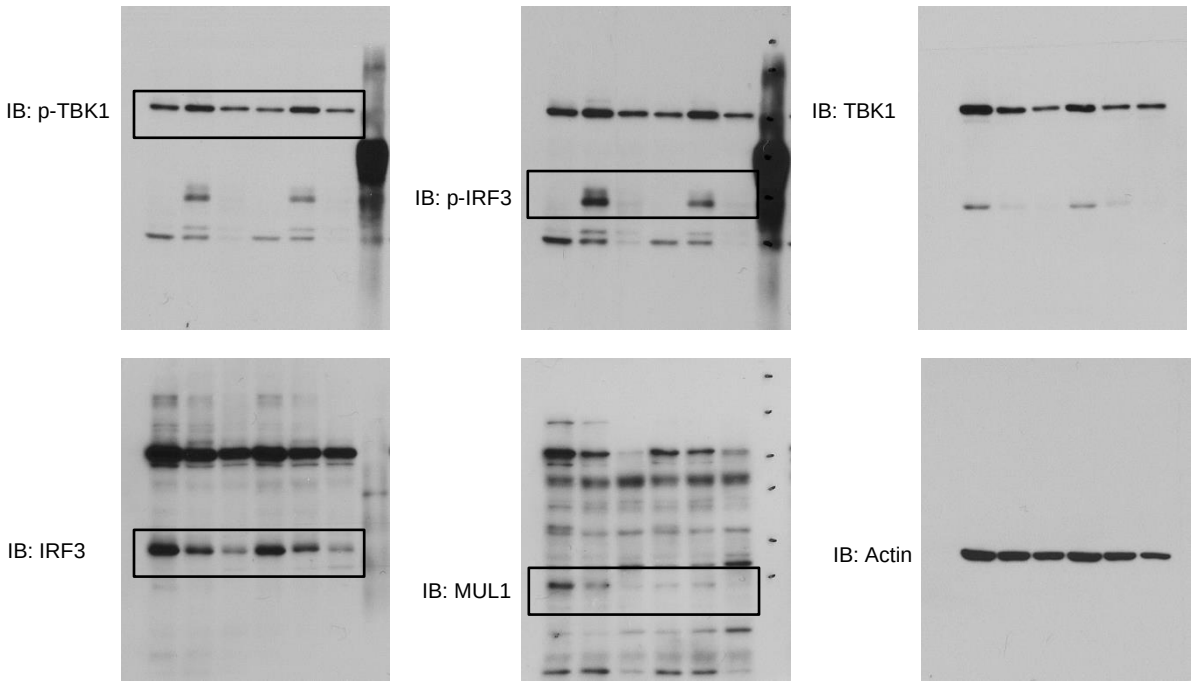
IB: MUL1
IB: MUL1
IB: Actin

Fig. 6L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IB: Actin

Fig. 7A

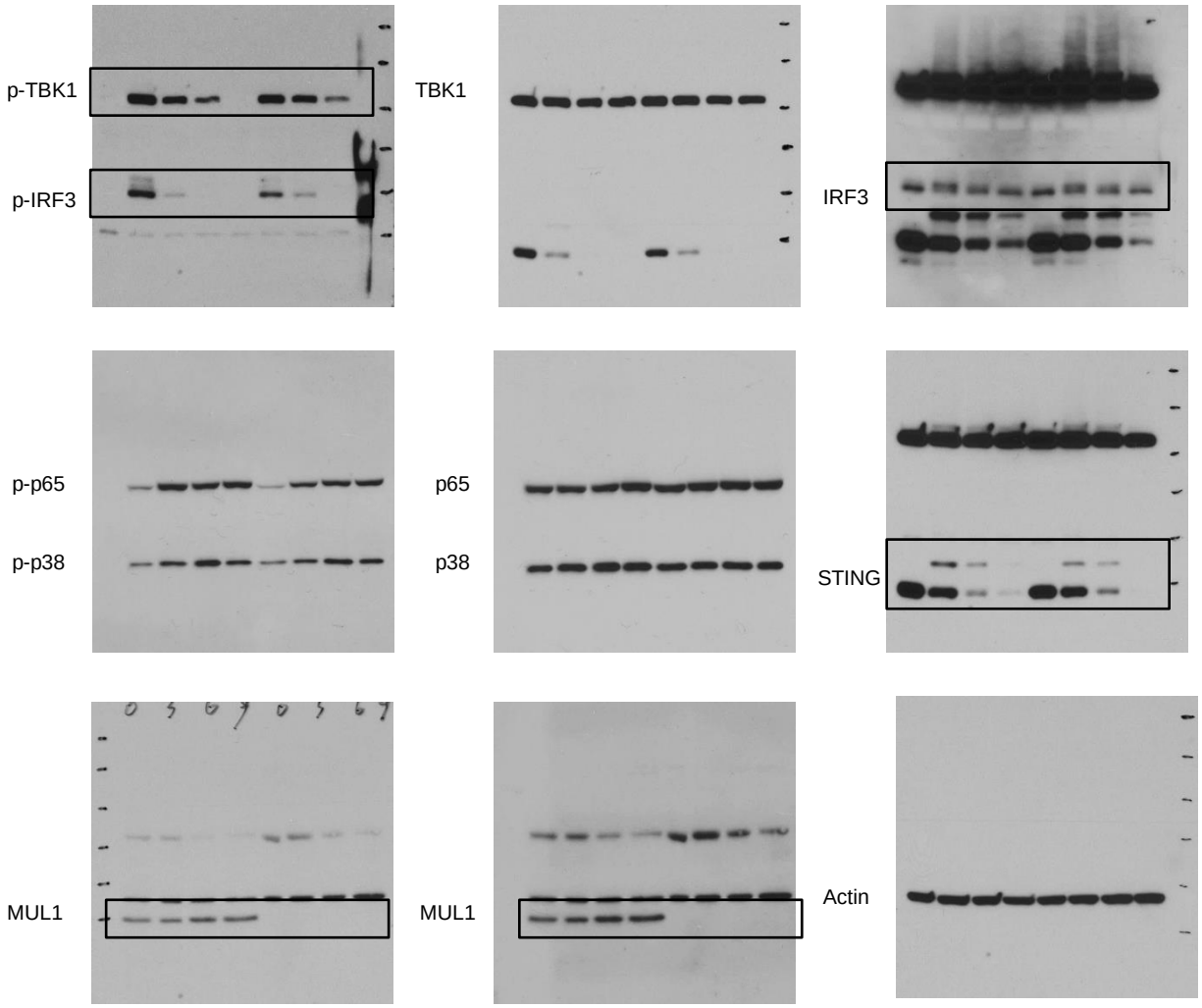
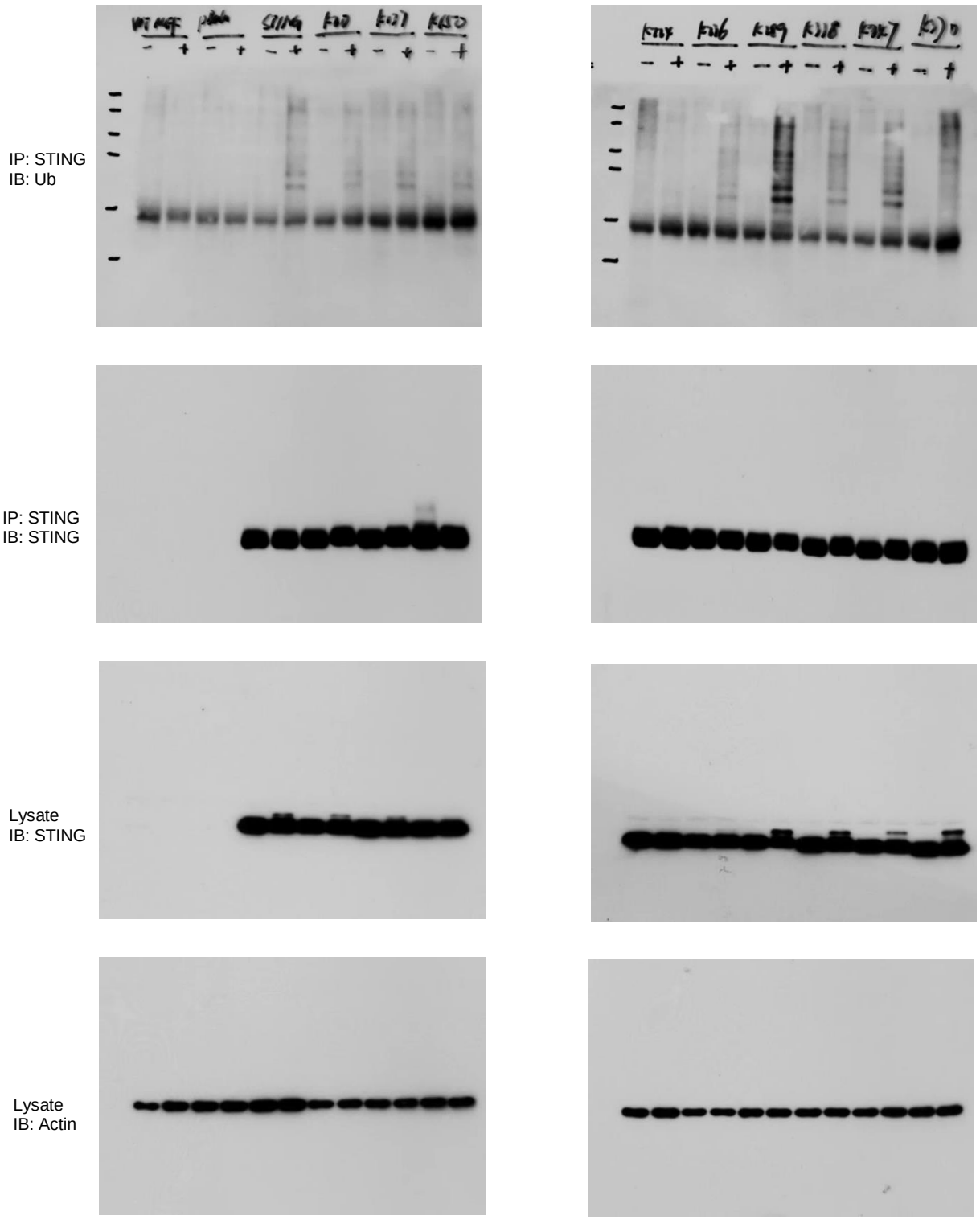

p-TBK1
p-IRF3
TBK1
IRF3
p-p65
p-p38
p65
p38
STING
MUL1
MUL1
Actin

Fig. S1D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IB: Ub
IP: STING
IB: STING
Lysate
IB: STING
Lysate
IB: Actin

Fig. S1E

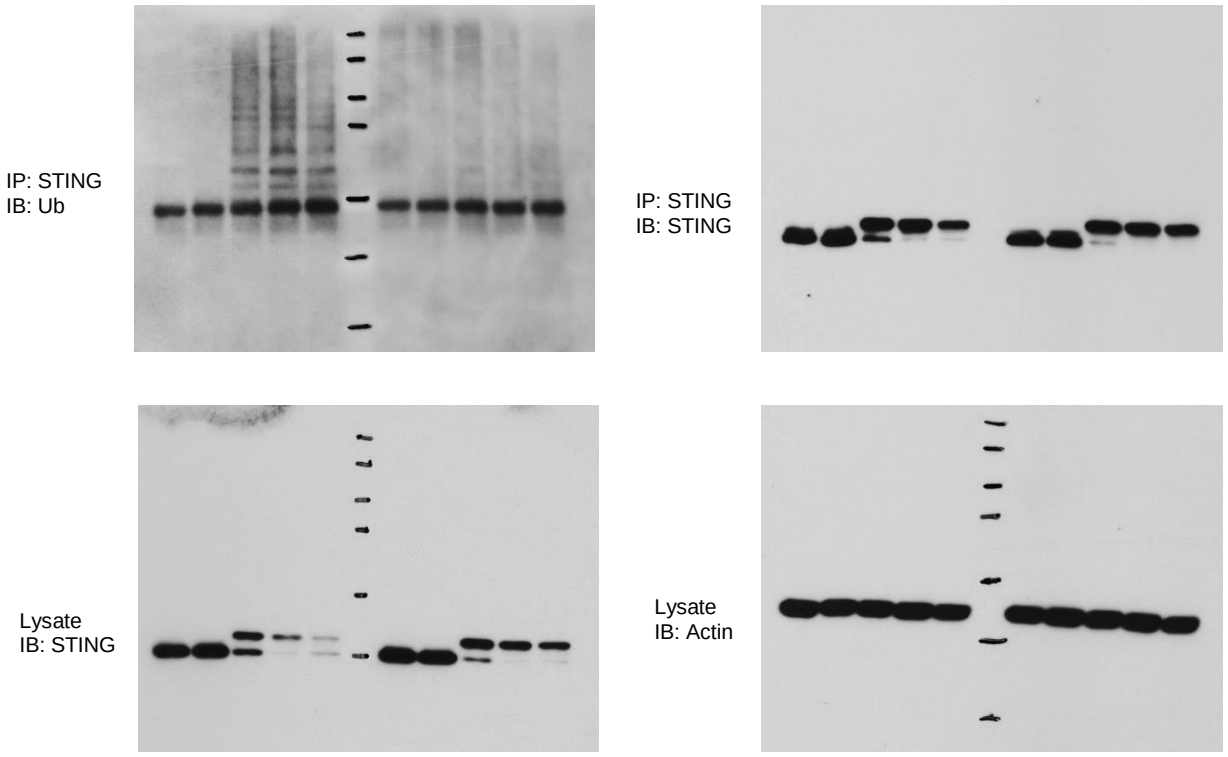


Fig. S1F

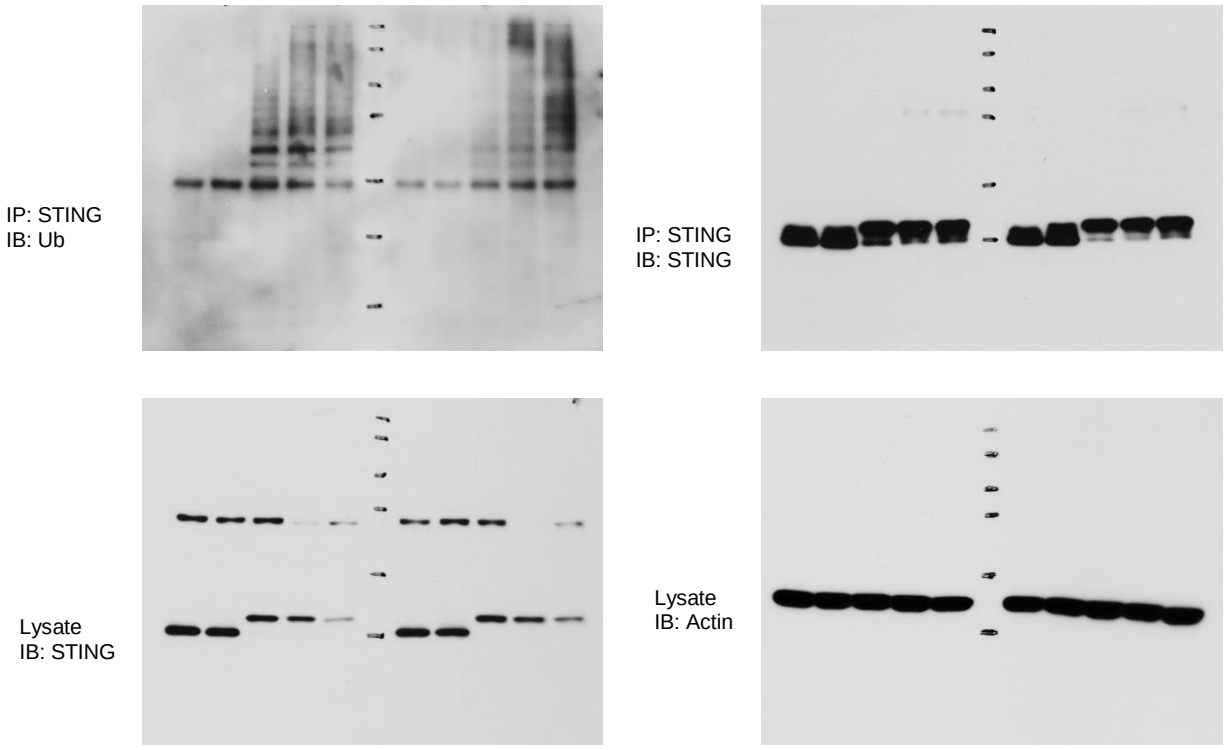


Fig. S1G

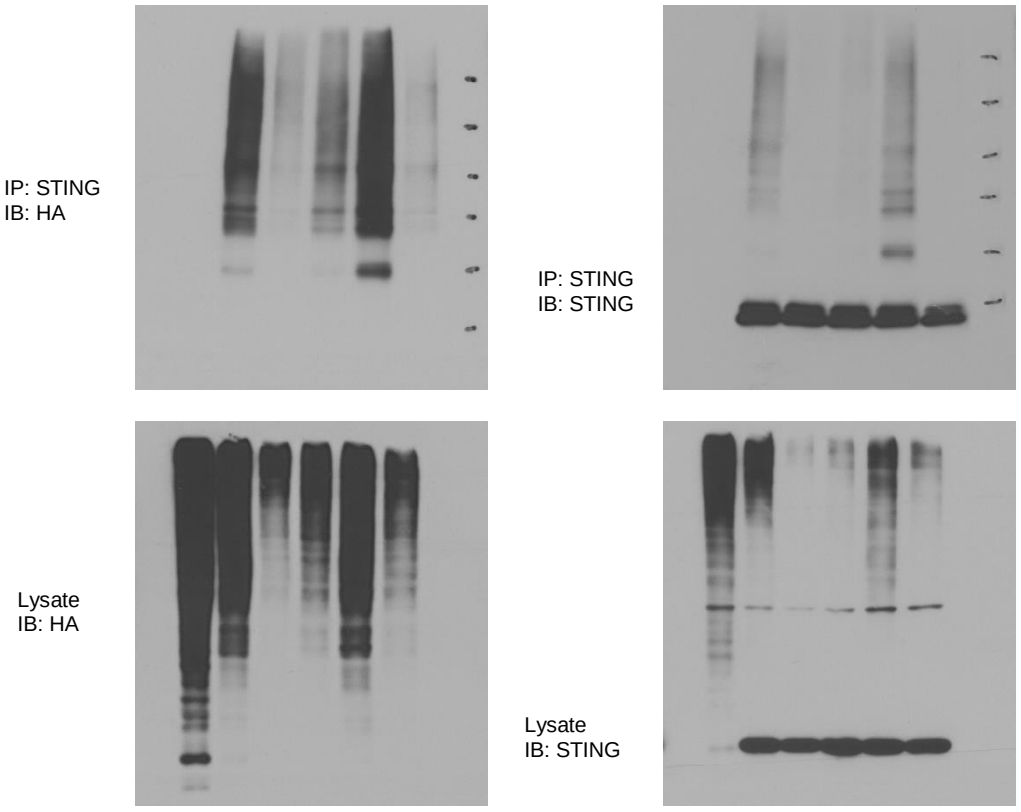


Fig. S2D

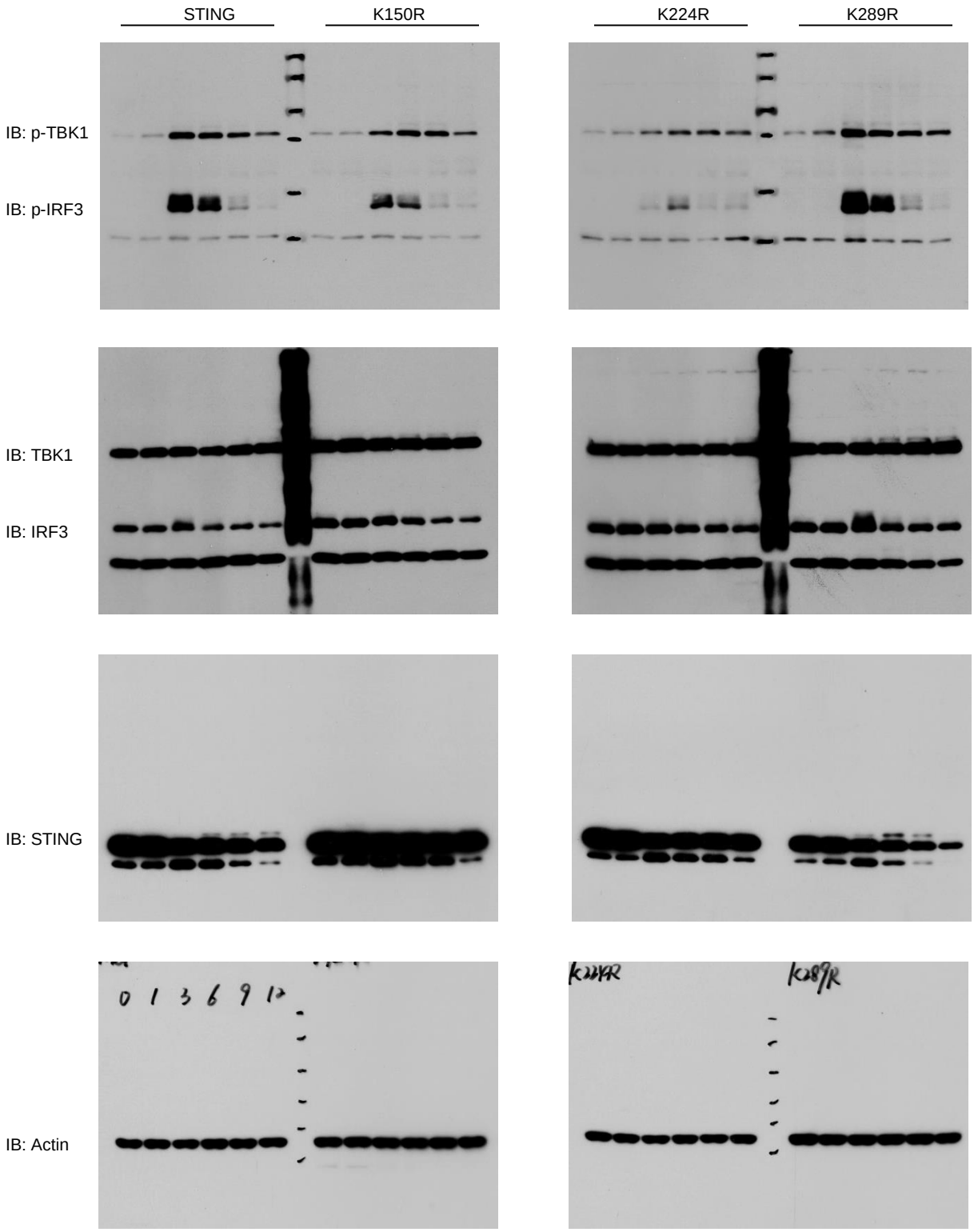

STING
K150R
K224R
K289R
IB: p-TBK1
IB: p-IRF3
IB: TBK1
IB: IRF3
IB: STING
IB: Actin
0 1 3 6 9 12

Fig. S2D

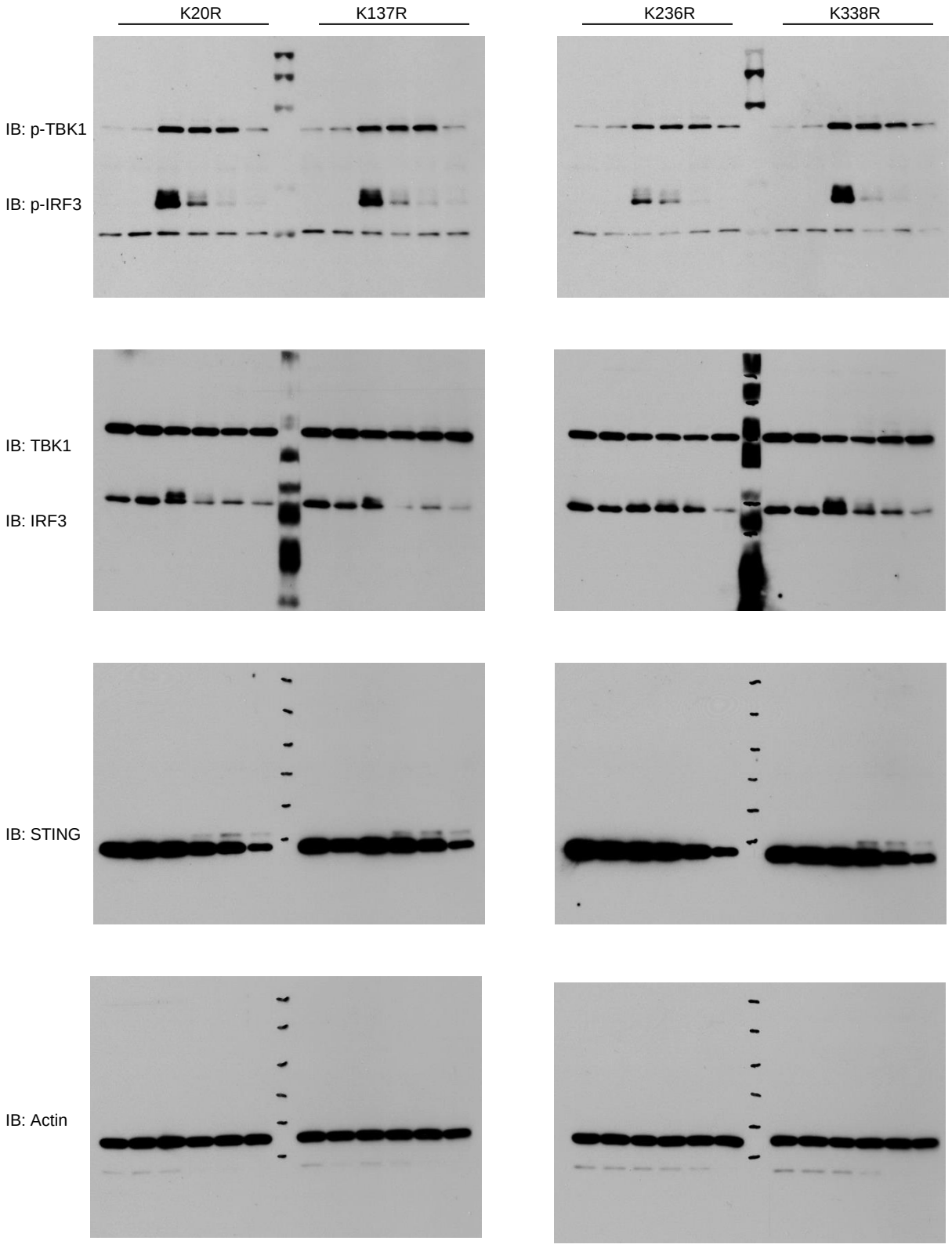

K20R
K137R
K236R
K338R
IB: p-TBK1
IB: p-IRF3
IB: TBK1
IB: IRF3
IB: STING
IB: Actin

Fig. S2D

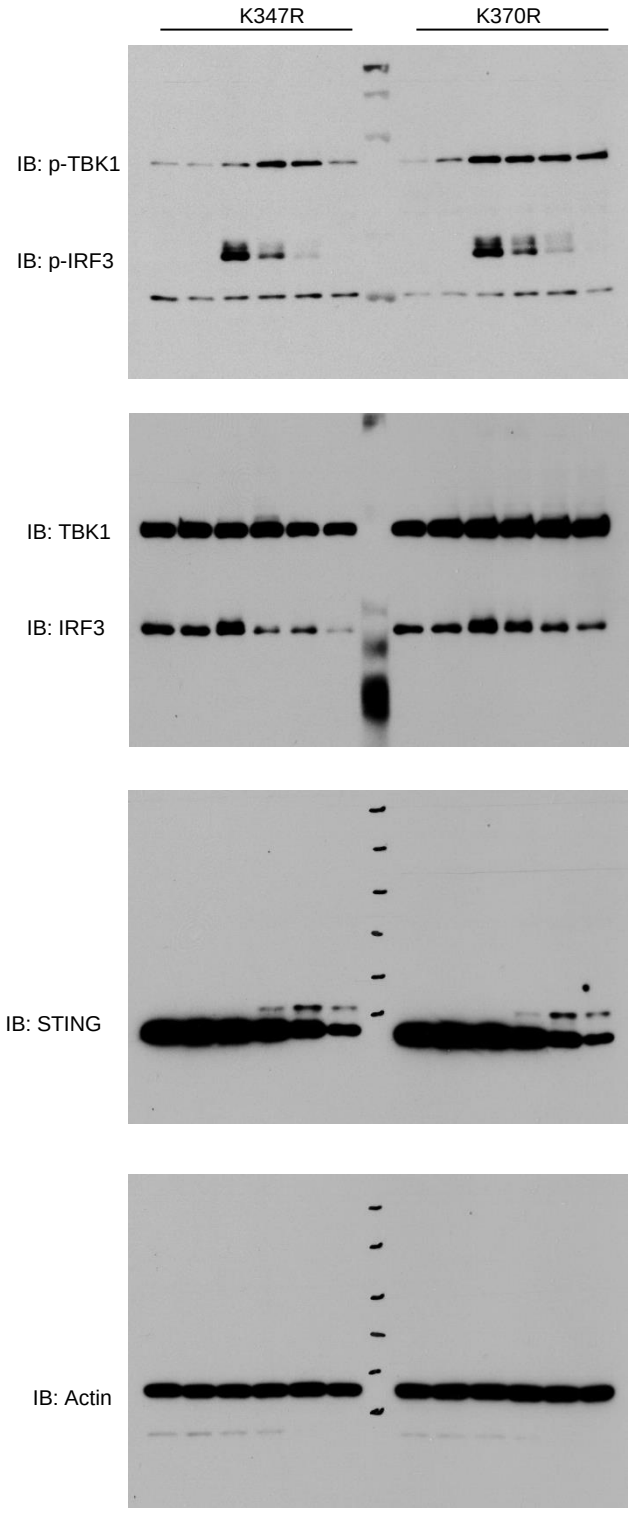

K347R
K370R
IB: p-TBK1
IB: p-IRF3
IB: TBK1
IB: IRF3
IB: STING
IB: Actin

Fig. S3C

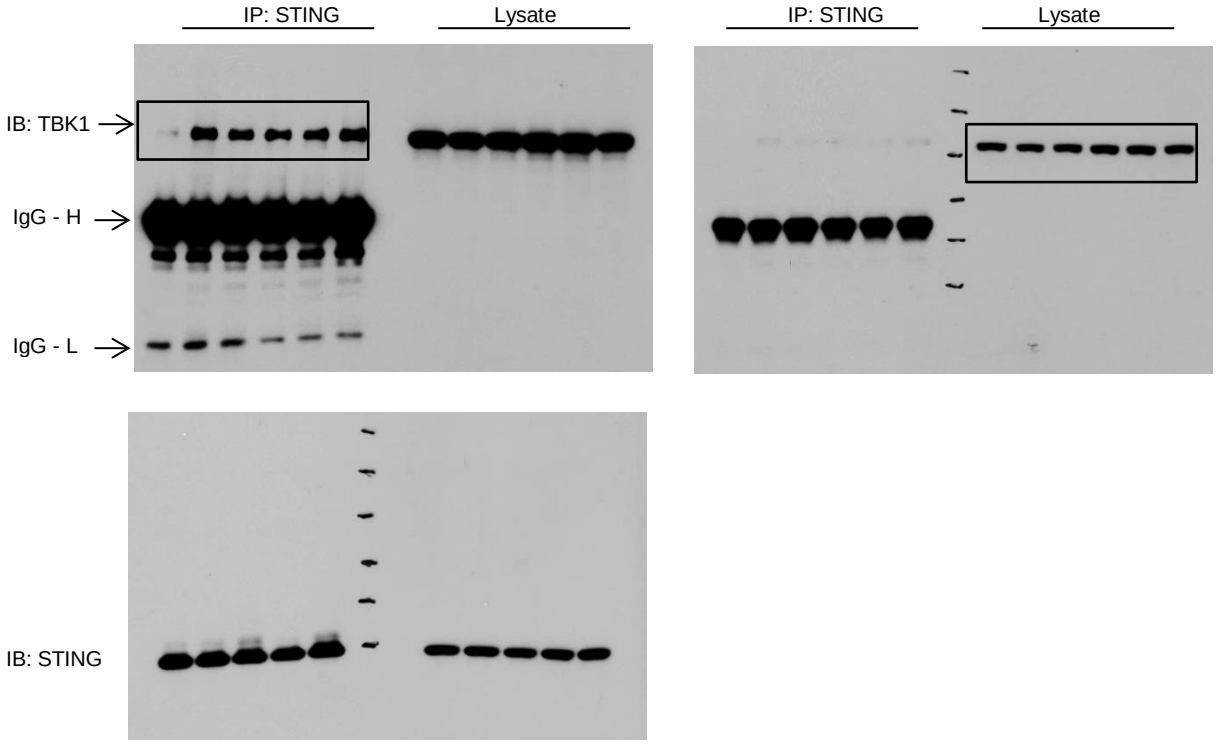


Fig. S3D

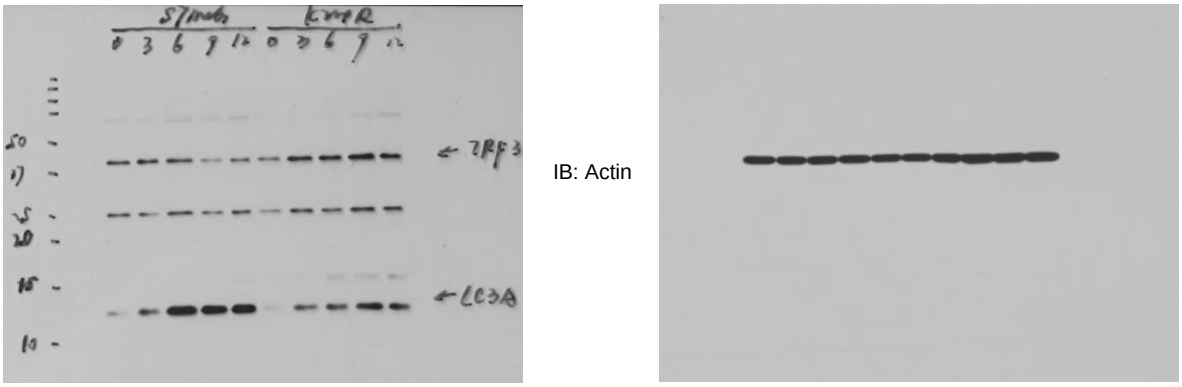


Fig. S4A

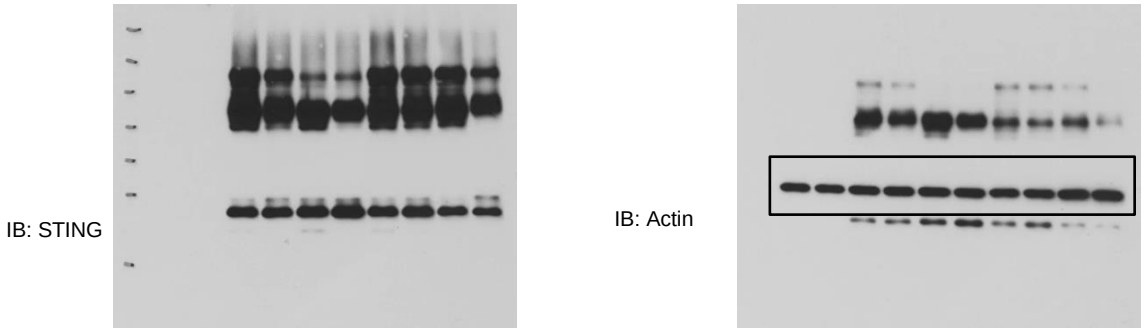


Fig. S4B

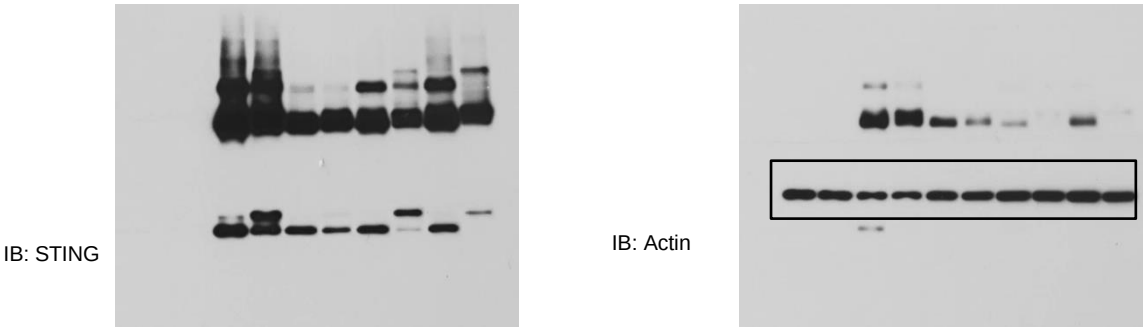


Fig. S4C

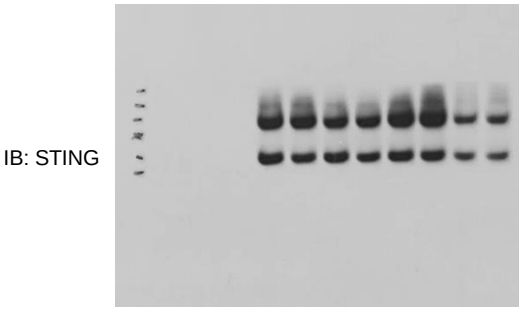


Fig. S4D

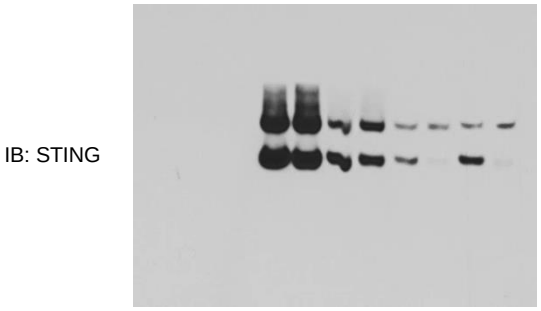


Fig. S4E

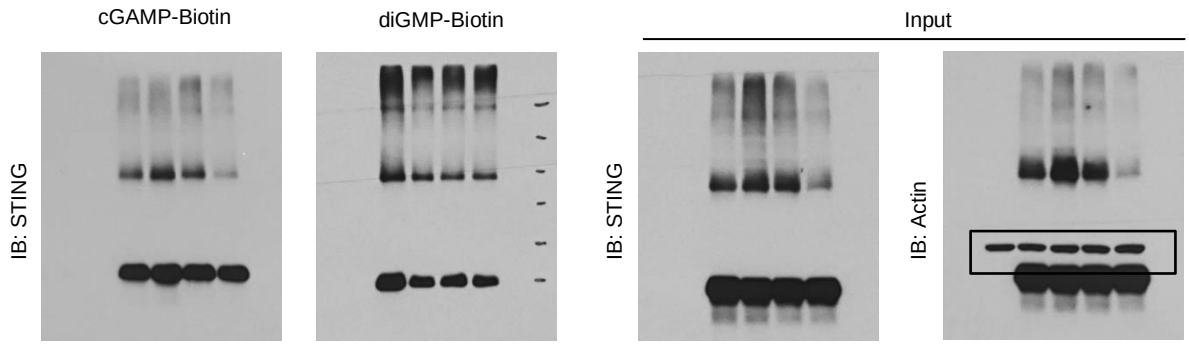


Fig. S5A

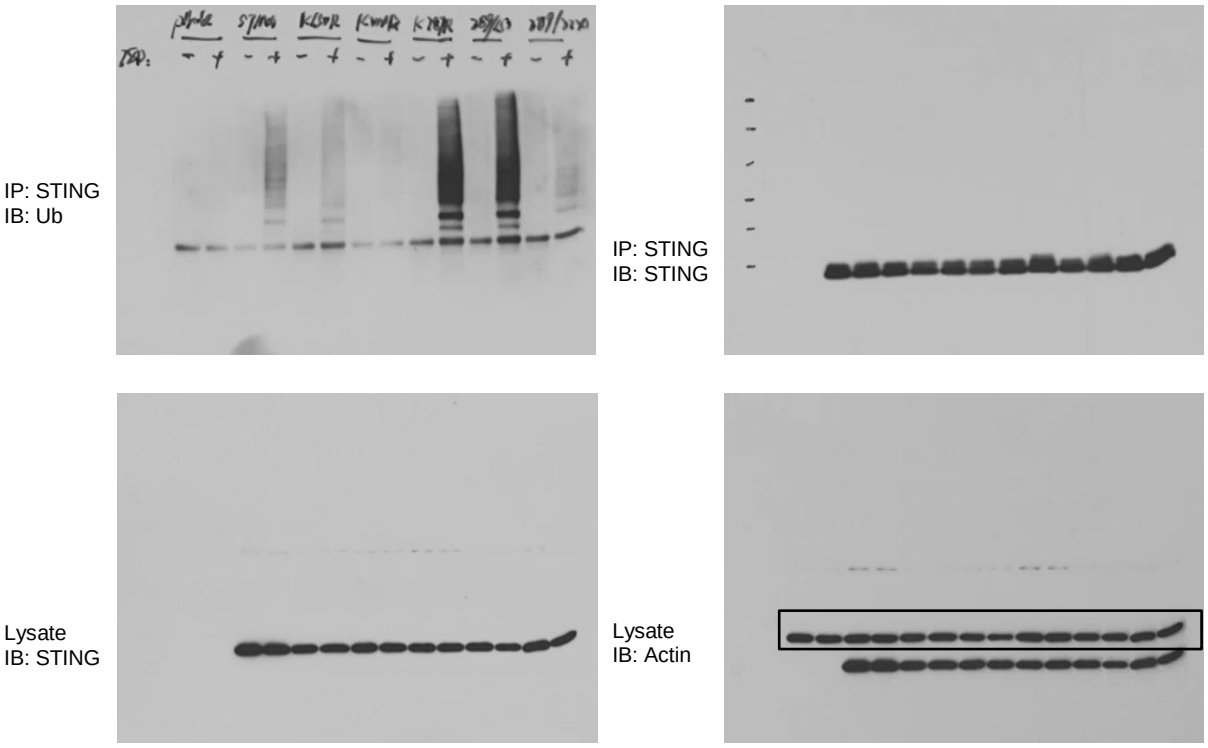


Fig. S5C

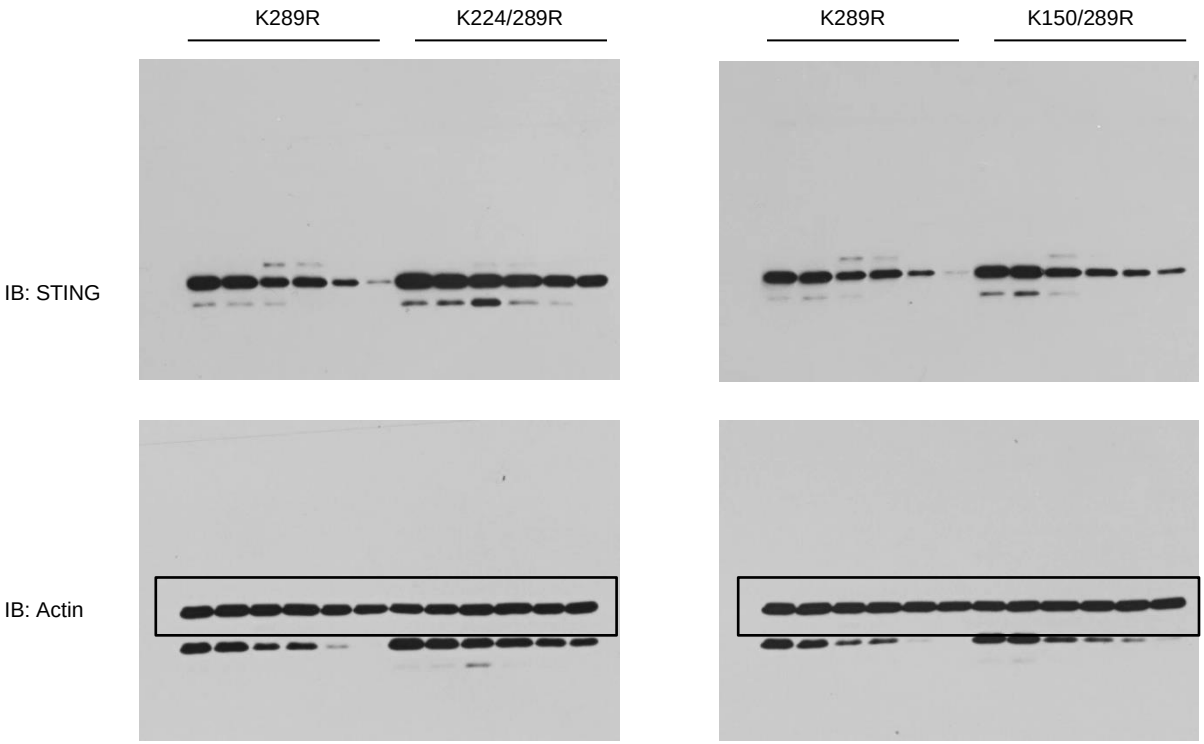


Fig. S6A

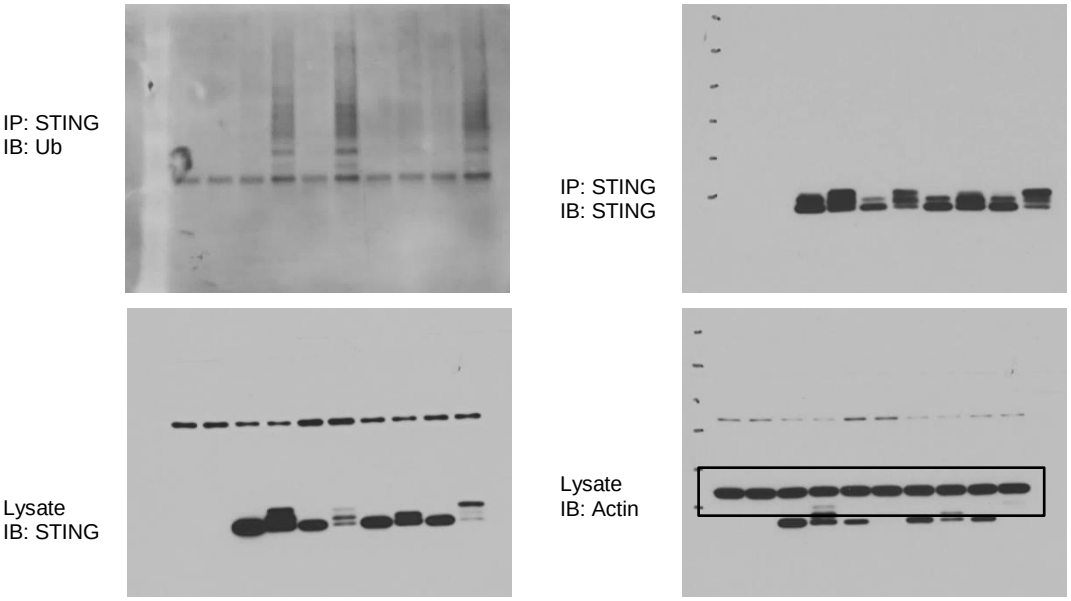


Fig. S6B

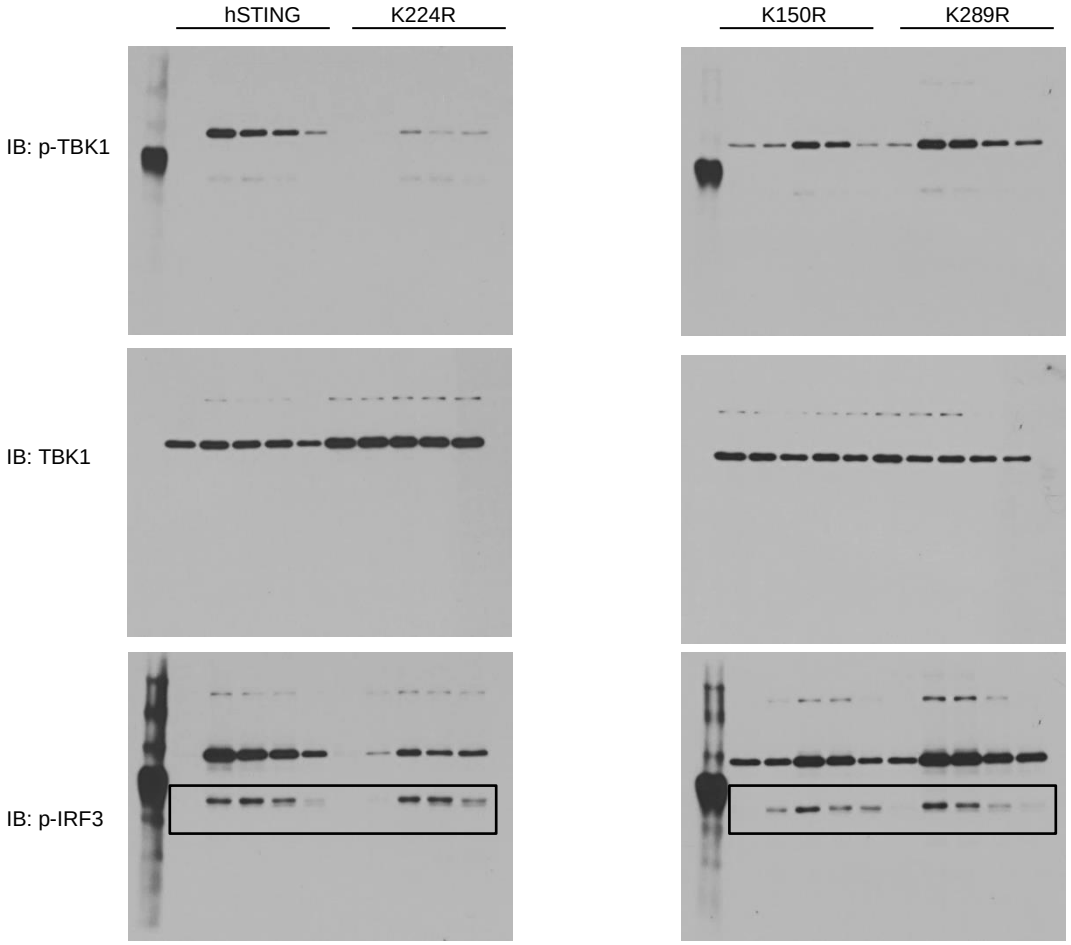


Fig. S6B

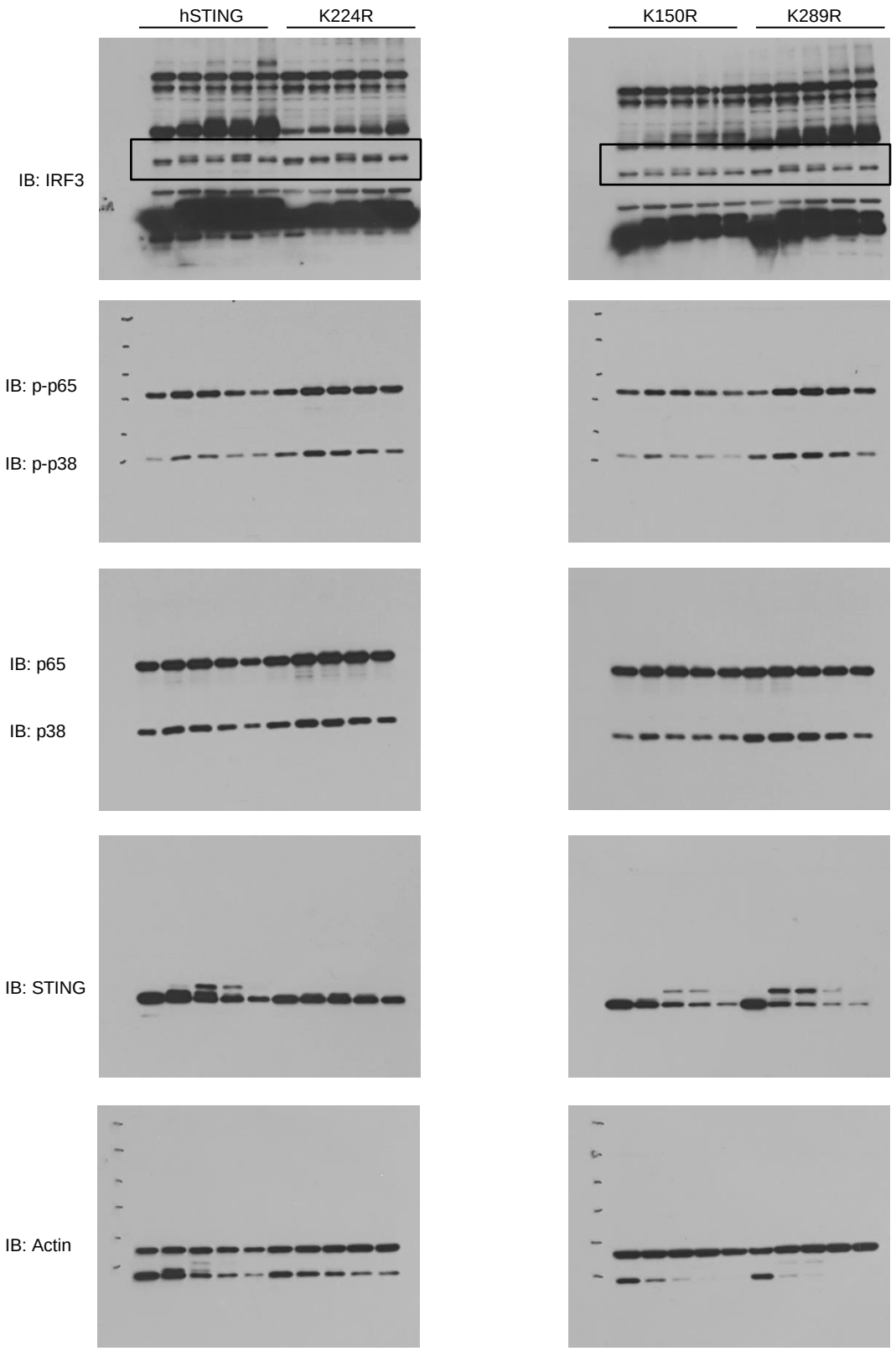

hSTING
K224R
K150R
K289R
IB: IRF3
IB: p-p65
IB: p-p38
IB: p65
IB: p38
IB: STING
IB: Actin

Fig. S7C

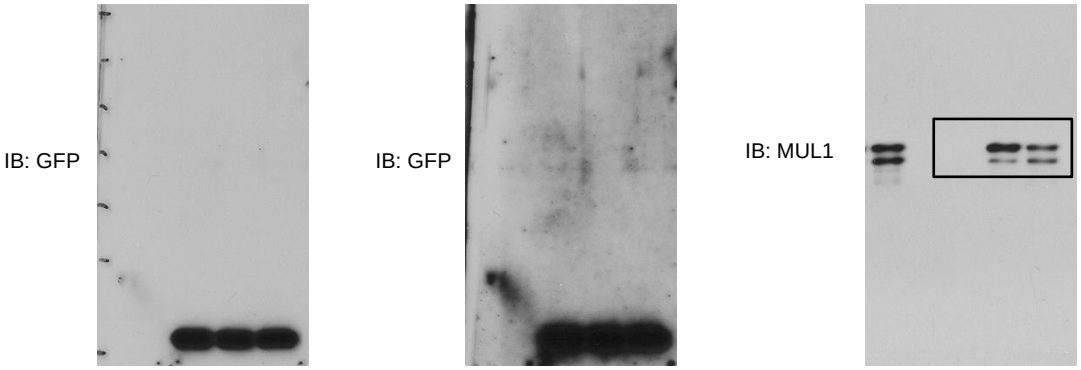


Fig. S7D

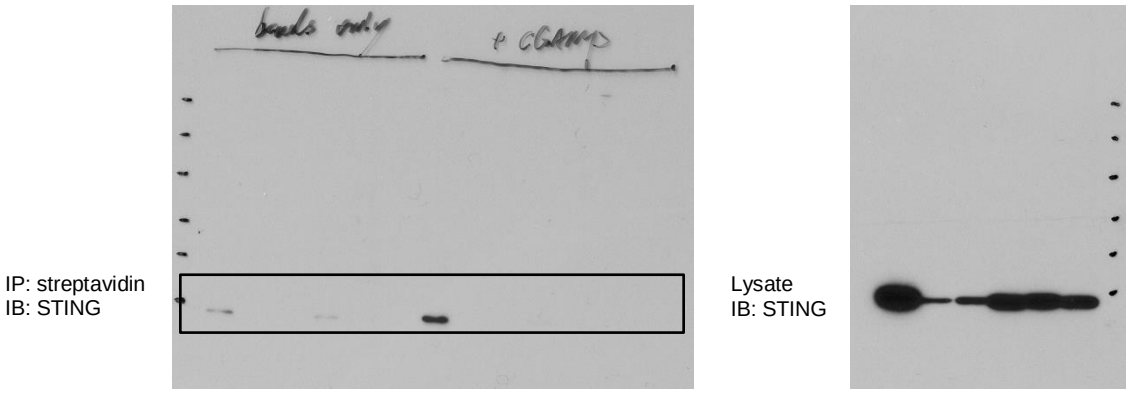


Fig. S7F

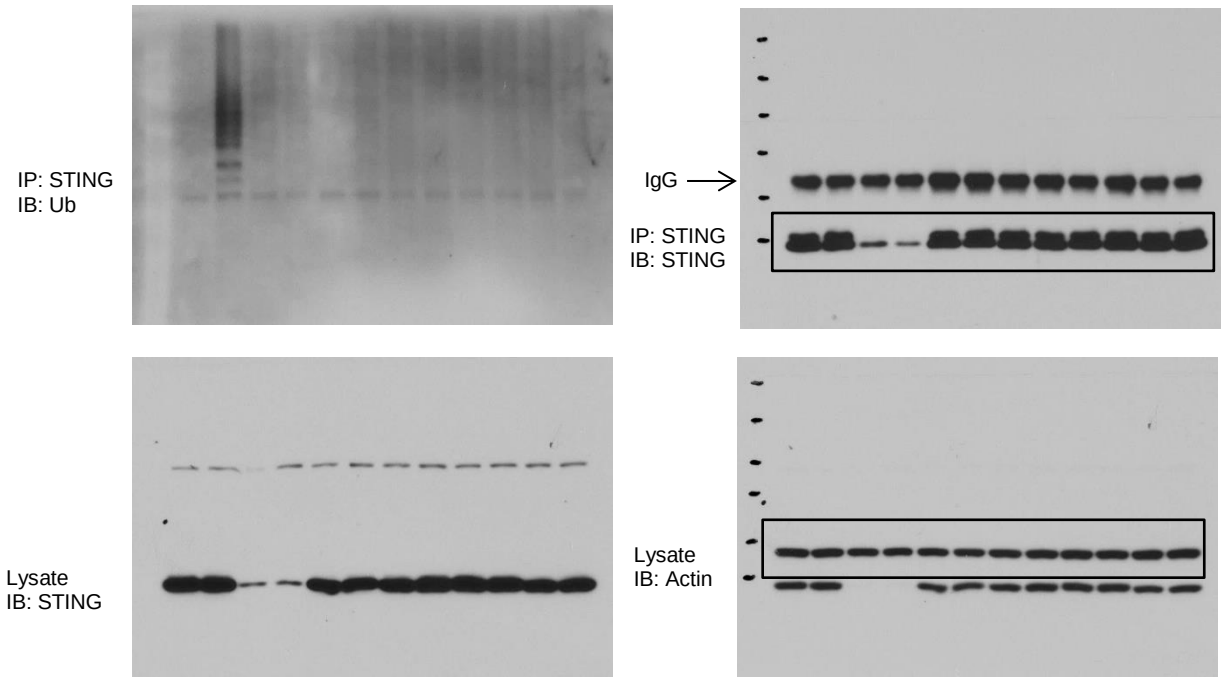


Fig. S8A

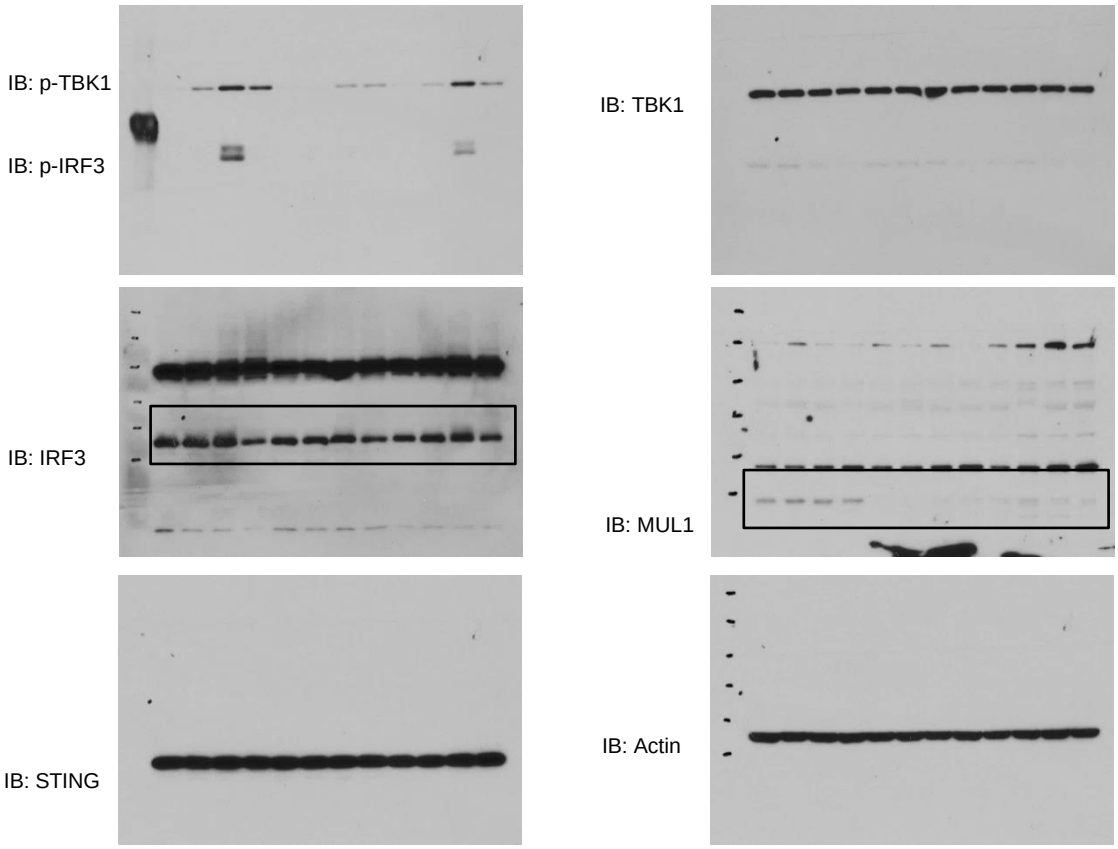


Fig. S8B

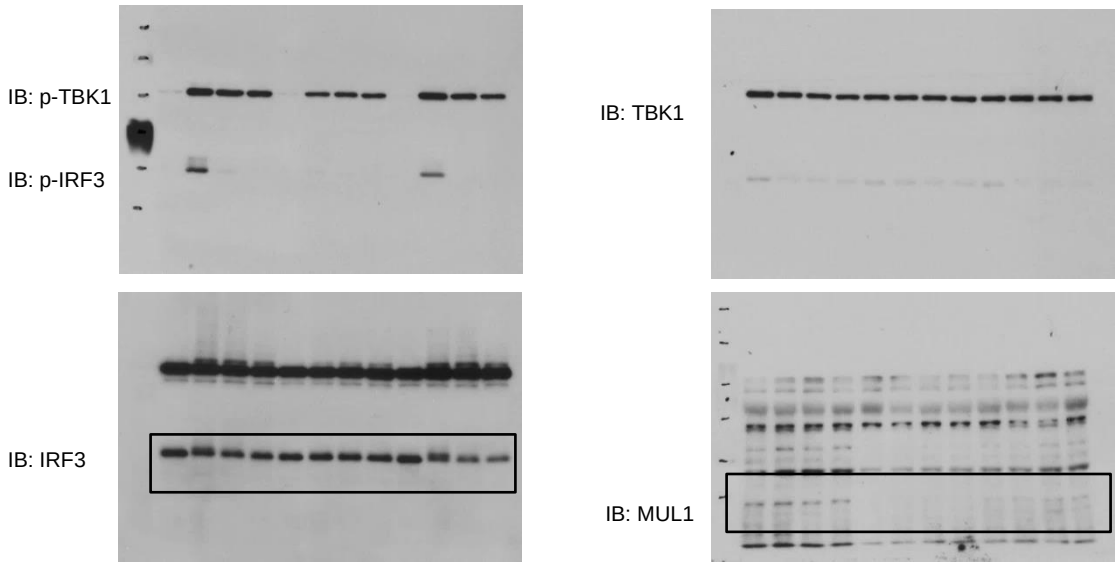


Fig. S8B

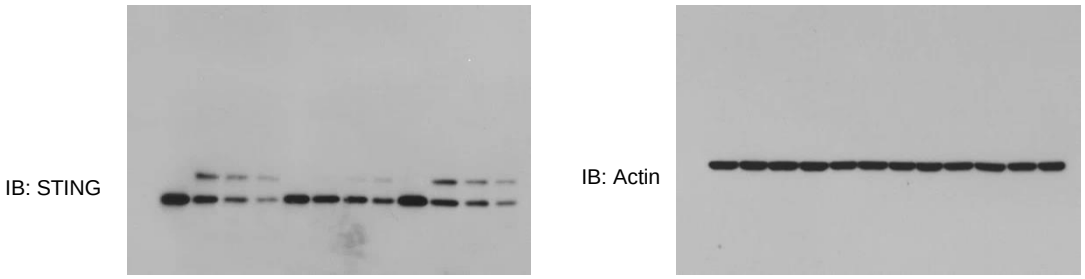


Fig. S8C

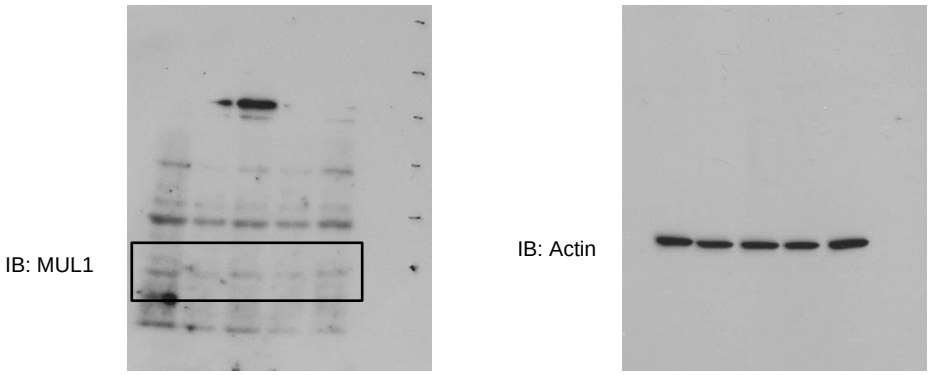


Fig. S8D

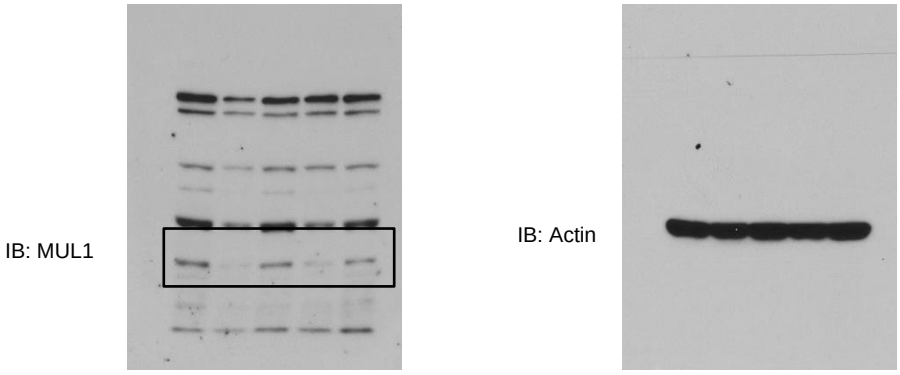


Fig. S8E

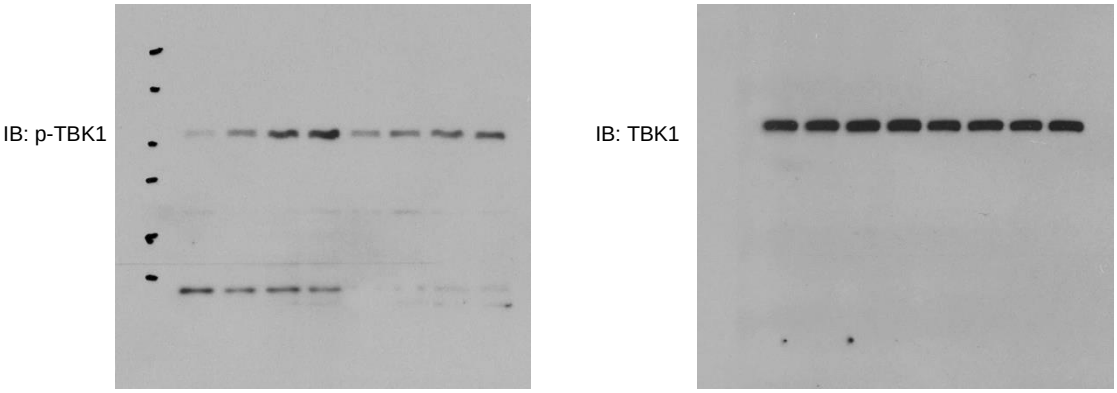
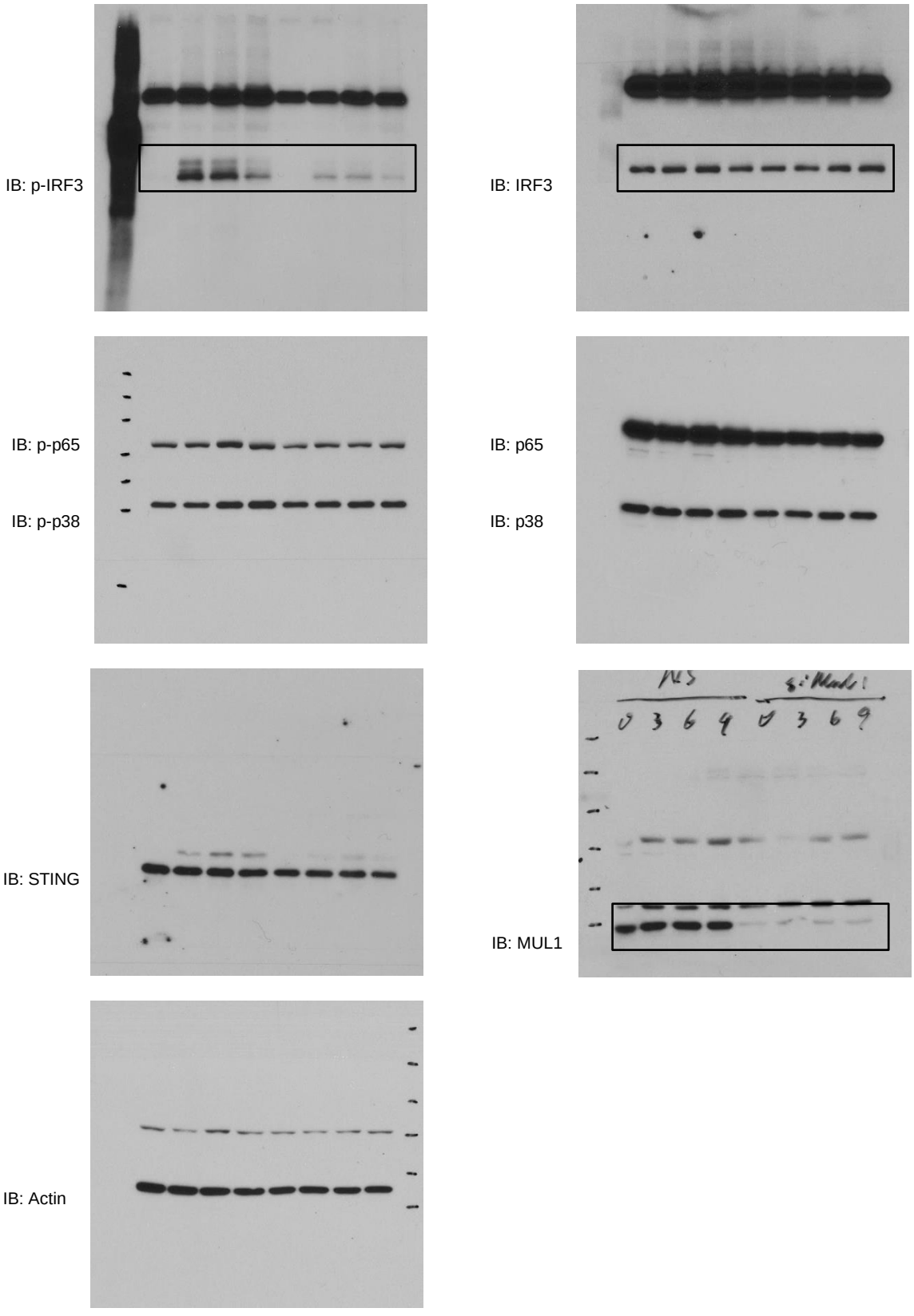


Fig. S8E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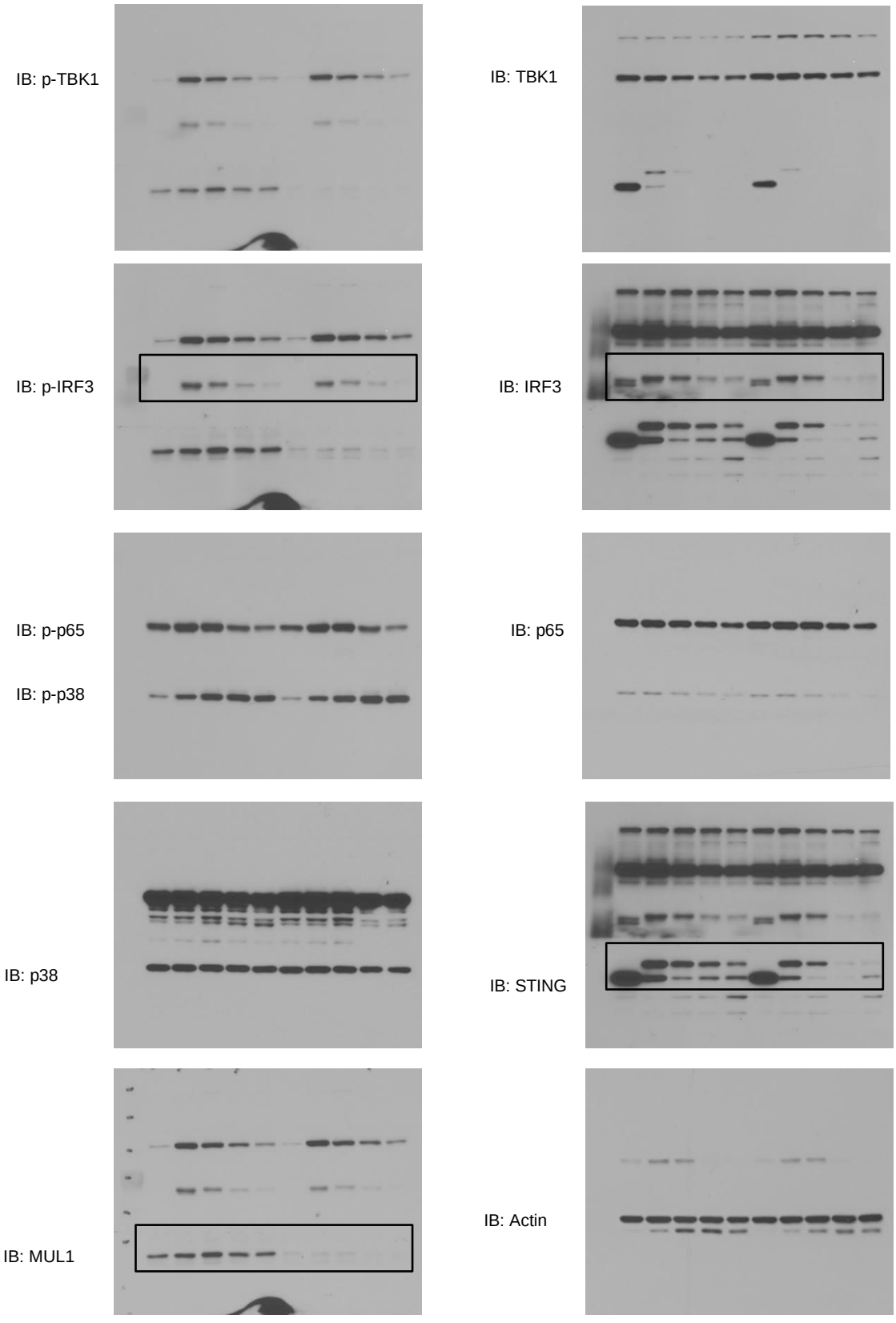
IB: IRF3
IB: p-p65
IB: p-p38
IB: p65
IB: p38
IB: STING
IB: MUL1
IB: Actin
0 3 6 9 0 3 6 9

Fig. S9A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IB: p65
IB: p38
IB: STING
IB: MUL1
IB: Actin

Fig. S9E

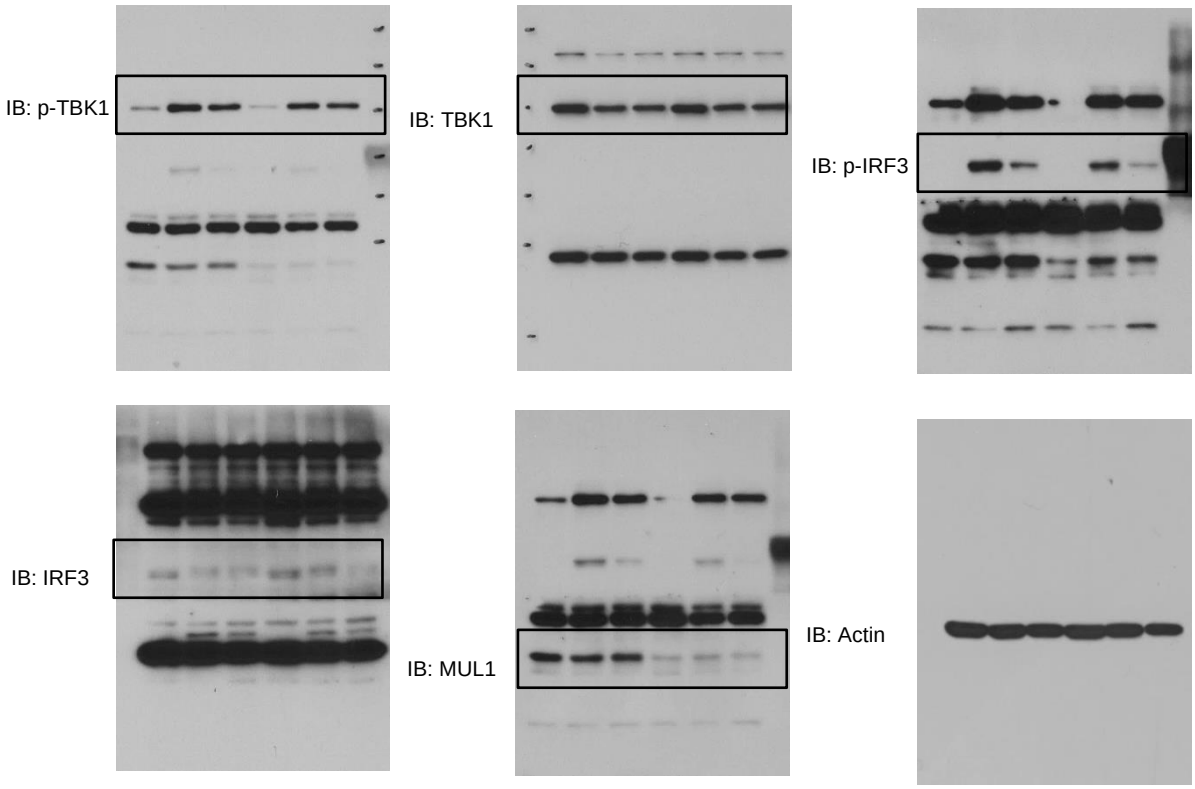

Fig. S9H

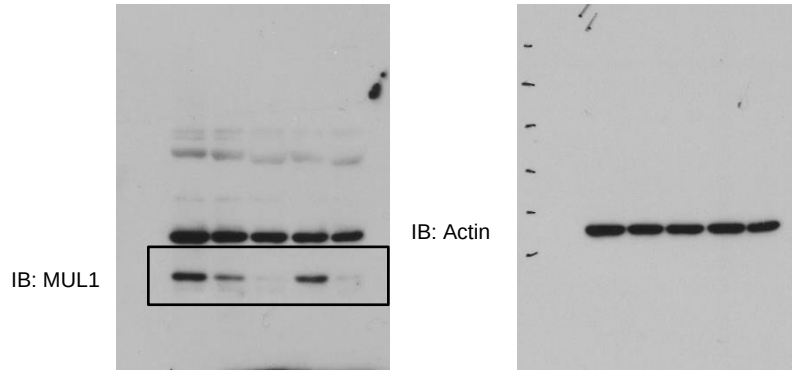

Fig. S9J