Supplementary information

Supplemental materials and methods

Cell culture and reagents. All NSCLC cell lines were purchased from ATCC. NCI-H661, NCI-H520 and NCI-H596 and were grown in RPMI 1640 medium (Invitrogen) supplemented with 10% foetal bovine serum, Hepes, NaPy, 100 U/ml penicillin/streptomycin and 2 mM glutamine. SK-MES-1 cells were grown in RPMI 1640 medium supplemented with 10% FBS, Neaa, NaPy, 100 U/ml penicillin/streptomycin and 2 mM glutamine. SW900 cells were grown in Leibovitz's L-15 medium supplemented with 10% FBS, 100 U/ml penicillin/streptomycin and 2 mM glutamine**.** HEK293-empty vector and HEK293-TLR3-HA cells (Invivogen) were grown in DMEM medium (Invitrogen Life Sciences) supplemented with 10% FBS and penicillin/streptomycin. Ha-Tag monoclonal antibody was from Invivogen. Synthetic TNFRI siRNA (SI00301945) was from Qiagen.

Electron microscopy. Cells were fixed by adding 4% glutaraldehyde in cell culture medium for 15 minutes at 4°C. Supernatants were collected and treated separately. Fixation was completed with 4% glutaraldehyde–0.2 M Na-cacodylate/HCl buffer at pH 7.4 for 30 minutes at 4°C. After fixation, cells were washed 3 times with a 0.2 M Na-cacodylate/HCl buffer at pH 7.4 and 0.4 M saccharose solution for 30 minutes. Samples were placed in 2% osmium tetroxide–0.3 M Na-cacodylate/HCl buffer at pH 7.4 for 45 minutes at 4°C, dehydrated in a graded series of ethyl alcohol and embedded in Epon resin. Ultrathin sections were cut with a RMX-MTL Ultramicrotome (Ventana), contrasted with methanolic uranyl acetate and lead citrate, and examined using a JEOL 1200 EX transmission electron microscope equipped with SIS MEGAVIEW II CCD camera (Olympus) and SIS AnalySIS software (Olympus).

Size-exclusion chromatography. NCI-H1703 cells were stimulated with 100 µg/ml poly(I:C) for 0,5 or 2 h, washed twice in cold phosphate-buffered saline, and lysed in CHAPS-containing lysis buffer (14 mM CHAPS, 150 mM NaCl, and 20 mM Tris-Hcl [pH 7.4], plus complete protease inhibitors). Lysates were loaded onto a Superdex-200 column previously equilibrated in CHAPS lysis buffer. Proteins were eluted at 800 µl/min. Fractions were maintained at 4°C and precipitated using TCA 5%.

Luciferase reporter assay. For stable generation of cell lines expressing the luciferase NF-κB and ISRE reporters, cells were transduced with luciferase NF-κB or ISRE reporter lentiviruses (SABiosciences) according to the manufacturer's recommendations, and transduced cells were selected with puromycin (2 µg/ml). NCI-H1703 and NCI-H292 cells stably expressing the luciferase NF-κB or ISRE reporter were seeded at 1.104 cells per well in 96-wells plates, cultured for 24h and then treated with Poly(I:C) for indicated times. Luciferase activity was measured with a luminometer (Tecan) by adding luciferin substrate (Steady-Glo luciferase assay, Promega) according to the manufacturer's recommendations.

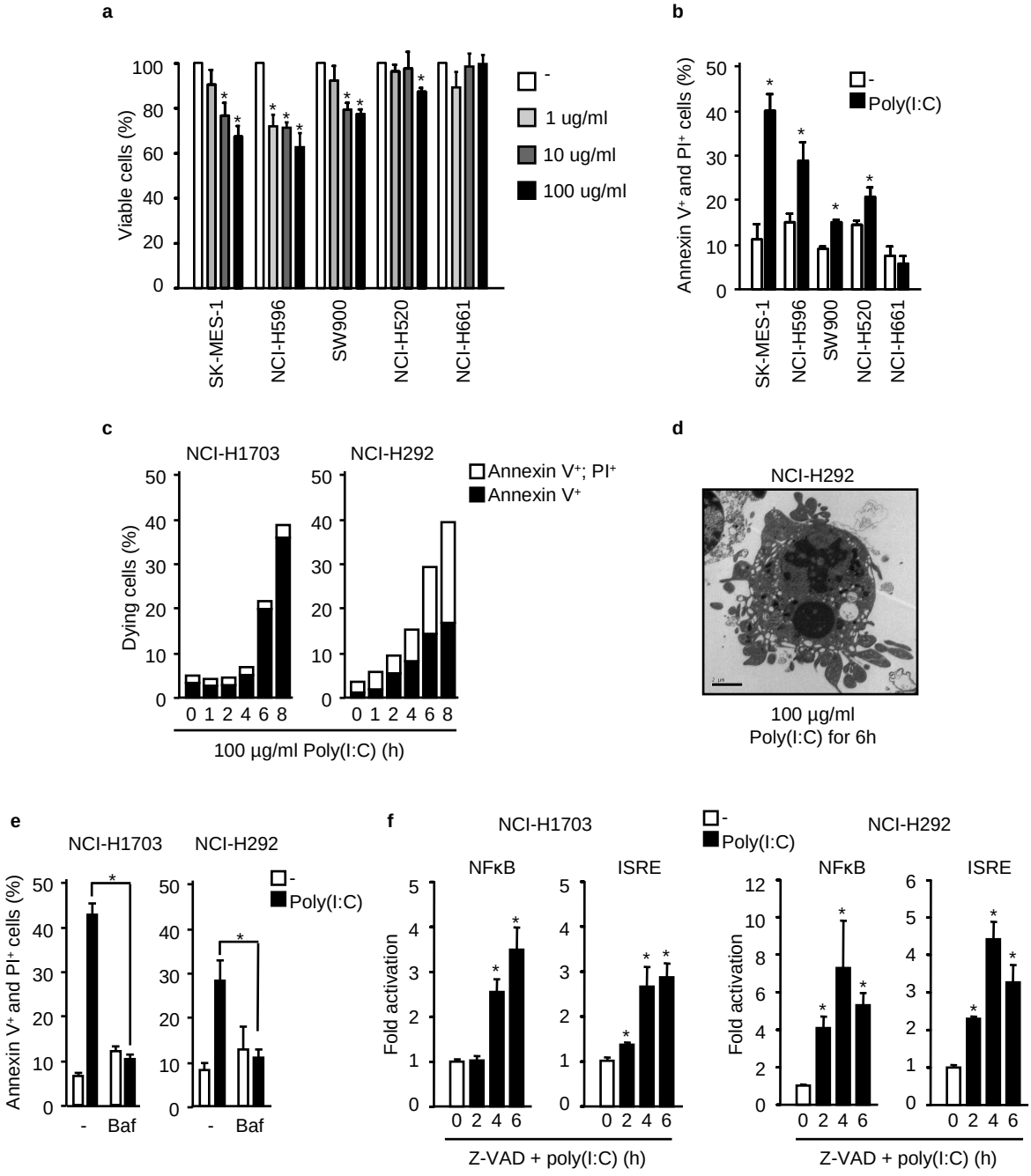

Figure S1. Poly(I:C) decreases NSCLC cells survival and induces apoptosis through TLR3/TRIF pathway.
(a) Various NSCLC cell lines were treated for 24h with Poly(I:C) at the indicated concentrations, and viable cells were numbered with MTS assay and expressed as % of untreated cells. *, $p<0.05$ compared with untreated cells; error bars represent s.e.m. of three independent experiments performed in triplicate.
(b) Percentage of annexin V positive $^{(+)}$ and PI^+ NSCLC cells treated for 24h with 100 µg/ml Poly(I:C). *, $p<0.05$ compared with untreated cells; error bars represent s.e.m. of three independent experiments.
(c) Percentage of annexin V+ and PI+ NCI-H1703 and NCI-H292 cells treated with 100 µg/ml Poly(I:C) for the indicated times. A representative experiment is shown.
(d) Representative picture taken by TEM of NCI-H292 cultured for 6h with 100 µg/ml Poly(I:C) showing typical features of apoptosis. Scale bar, 2 µm.
(e) Percentage of annexin V+ and PI+ NCI-H1703 and NCI-H292 cells incubated for 1h with 50 nM bafilomycin-A1 (Baf) and then exposed to 100 µg/ml Poly(I:C) for 24h. *, $p<0.05$; error bars represent s.e.m. of three independent experiments.
(f) NCI-H1703 and NCI-H292 cells stably expressing luciferase NF-κB and ISRE reporters were treated 1h with 20 µM Z-VAD, exposed to 100 µg/ml Poly(I:C) for the indicated times, and the luciferase activity was measured. *, $p<0.05$ compared with untreated cells; error bars represent s.e.m. of at least three independent experiments performed in triplicate.

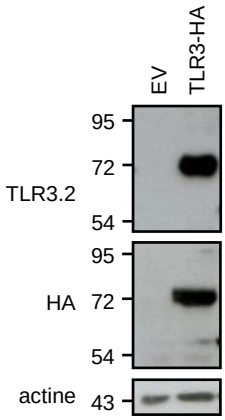

Figure S2. TLR3.2 monoclonal antibody recognizes specifically human TLR3.
HEK293-empty vector (EV) and HEK293-TLR3-HA (TLR3-HA) cells (Invivogen) were lysed and immunoblotted with our TLR3.2 mAb or with a HA-Tag mAb .

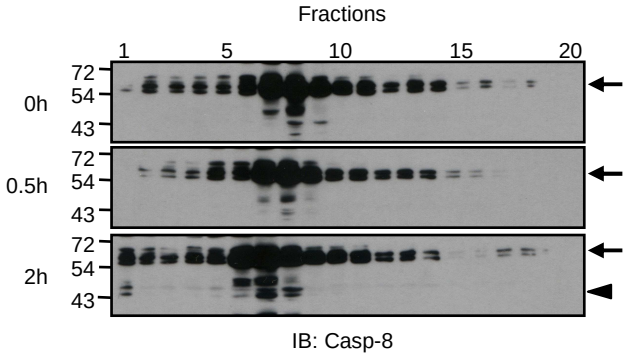

Figure S3. Caspase-8 exhibits high apparent molecular weights after poly(I:C) stimulation.
NCI-H1703 cells were stimulated with 100 µg/ml poly(I:C) for the indicated times, and subsequently lysed in CHAPS lysis buffer. After fractionation on a S200 gel filtration column, caspase-8 was immunoblotted. Arrows correspond to full-length proteins, while arrowheads indicate cleaved fragments.

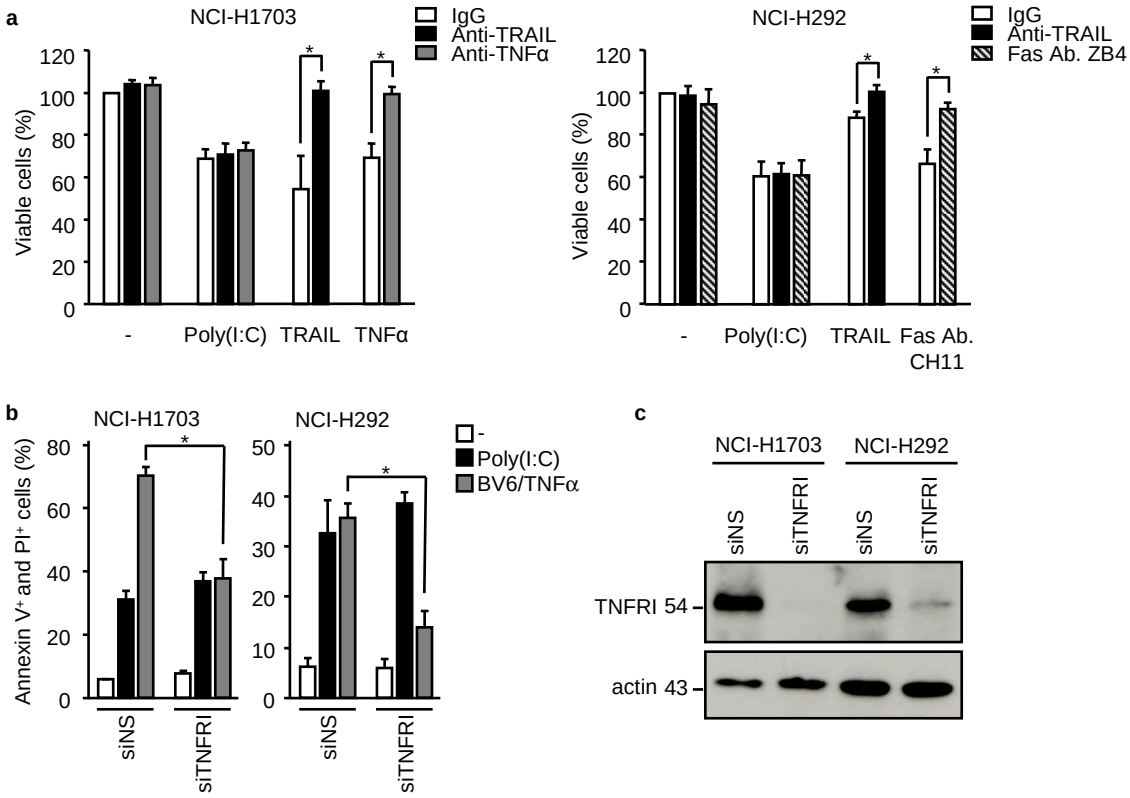

Figure S4. TLR3 activation decreases cell viability independent of classical death receptors.
(a) NCI-H1703 and NCI-H292 cells were pretreated 2 h with 10 µg/ml control IgG or antibodies blocking TRAIL, TNFα or Fas (clone ZB4), and then exposed for 24 h to 100 µg/ml Poly(I:C) , 100 ng/ml TRAIL, 50 ng/ml TNFα, or 200 ng/ml anti-Fas antibody clone CH11. Viable cells were numbered with MTS assay and expressed as % of cells treated with control IgG. *, $p<0.05$; error bars represent s.e.m. of three independent experiments performed in triplicate.
(b) NCI-H1703 and NCI-H292 cells were transfected with siRNA targeting TNFRI for 72h, exposed for 6h to 100 µg/ml Poly(I:C) or the combination BV6/TNFα, and the percentage of annexin V+ and PI+ cells was measured. *, $p<0.05$ compared with siNS-transfected cells treated with the combination BV6/TNFα; error bars represent s.e.m. of three independent experiments.
(c) siNS or siTNFRI-transfected NCI-H1703 and NCI-H292 cells were lysed, and then immunoblotted as indicated.

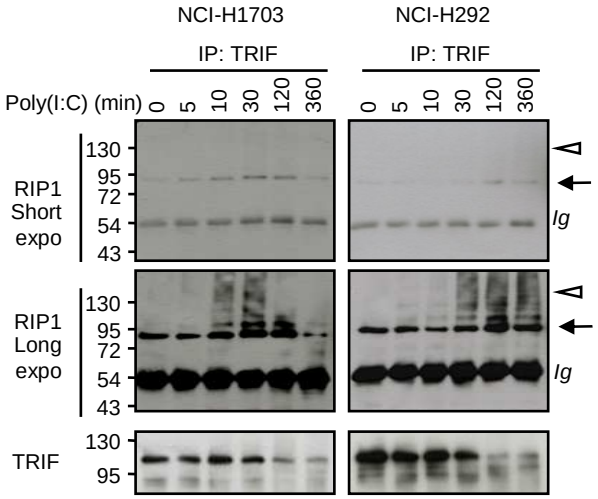

Figure S5. RIP1 associates with TRIF after TLR3 activation.
NCI-H1703 and NCI-H292 cells were stimulated with 100 µg/ml Poly(I:C) for the indicated times, and TRIF immunoprecipitates (IP) were analyzed by immunoblot. Arrows and open arrowheads point to full length and modified RIP1, respectively. Ig, denotes immunoglobulin heavy chain.

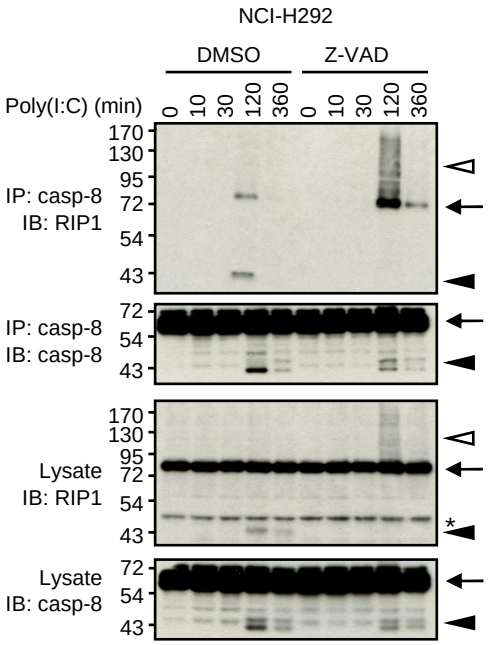

Figure S6. RIP1 cleavage is inhibited by Z-VAD pretreatment
NCI-H292 cells were incubated for 1h with 20 µM caspases inhibitor Z-VAD and then treated with 100 µg/ml Poly(I:C) for the indicated times. Caspase-8 immunoprecipitates were analysed by immunoblot. Arrows, filled arrowheads, and open arrowheads point to full length, cleaved, and modified proteins, respectively. *, indicates nonspecific band.

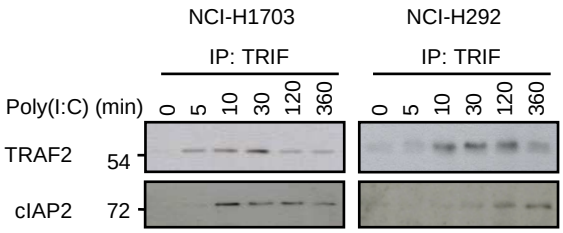

Figure S7. The ubiquitin ligases TRAF2 and cIAP2 associate with TRIF after Poly(I:C) treatment.
NCI-H1703 and NCI-H292 cells were exposed to 100 µg/ml Poly(I:C) for the indicated times, and TRIF immunoprecipitates (IP) and cell lysates were analyzed by immunoblot.

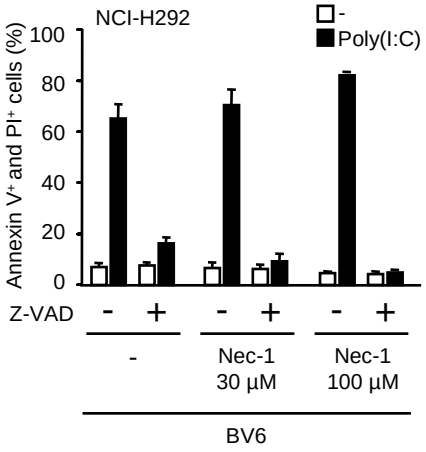

Figure S8. Cell death induced by Poly(I:C) plus Smac mimetic BV6 is not inhibited by necrostatin-1
Percentage of annexin V^+ and PI^+ NCI-H292 cells incubated for 1h with 5 µM Smac mimetic BV6 in presence or absence of 20 µM Z-VAD and/or necrostatin-1 at the indicated concentrations, and then treated with 100 µg/ml Poly(I:C) for 6h. Error bars represent s.e.m. of two independent experiments.