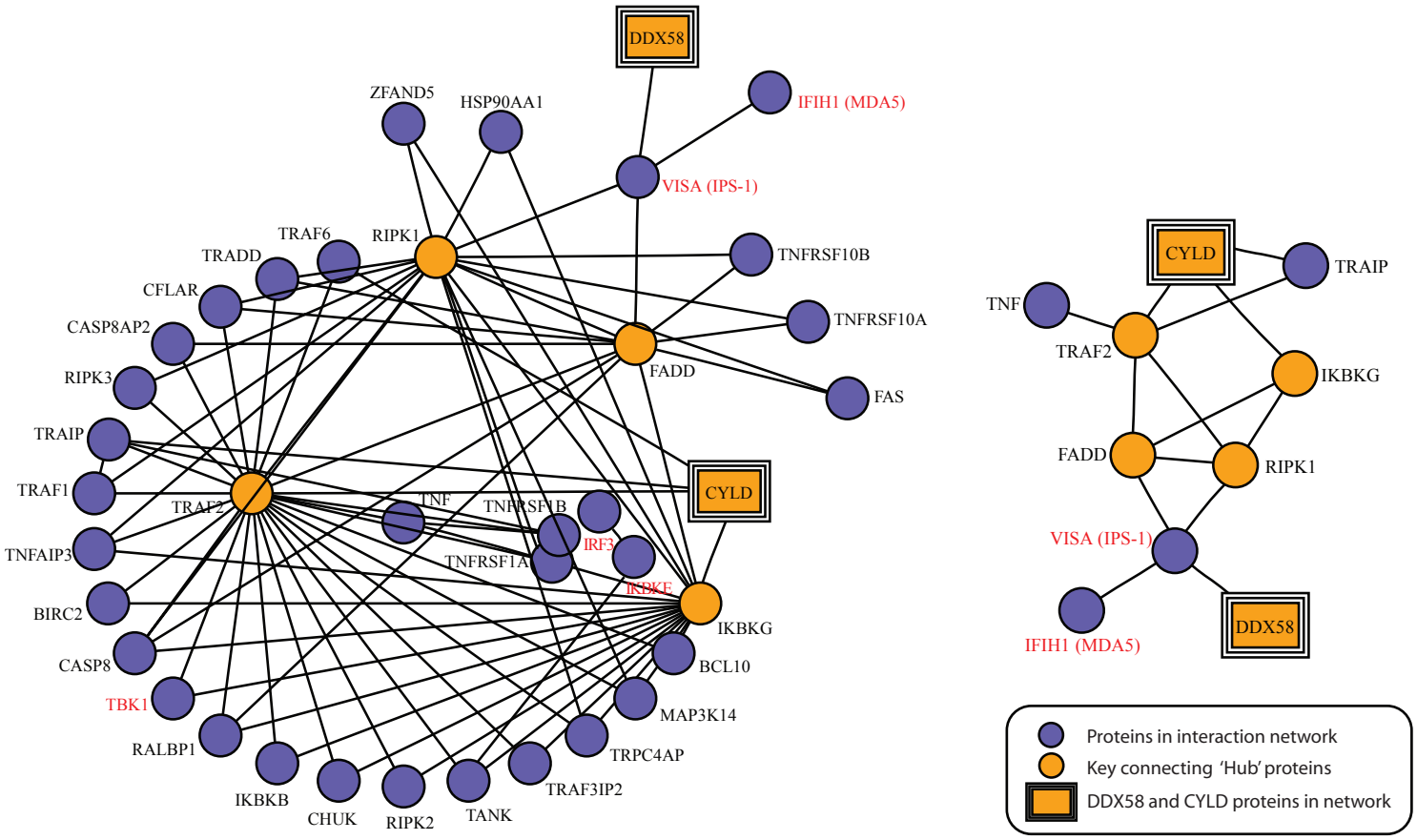
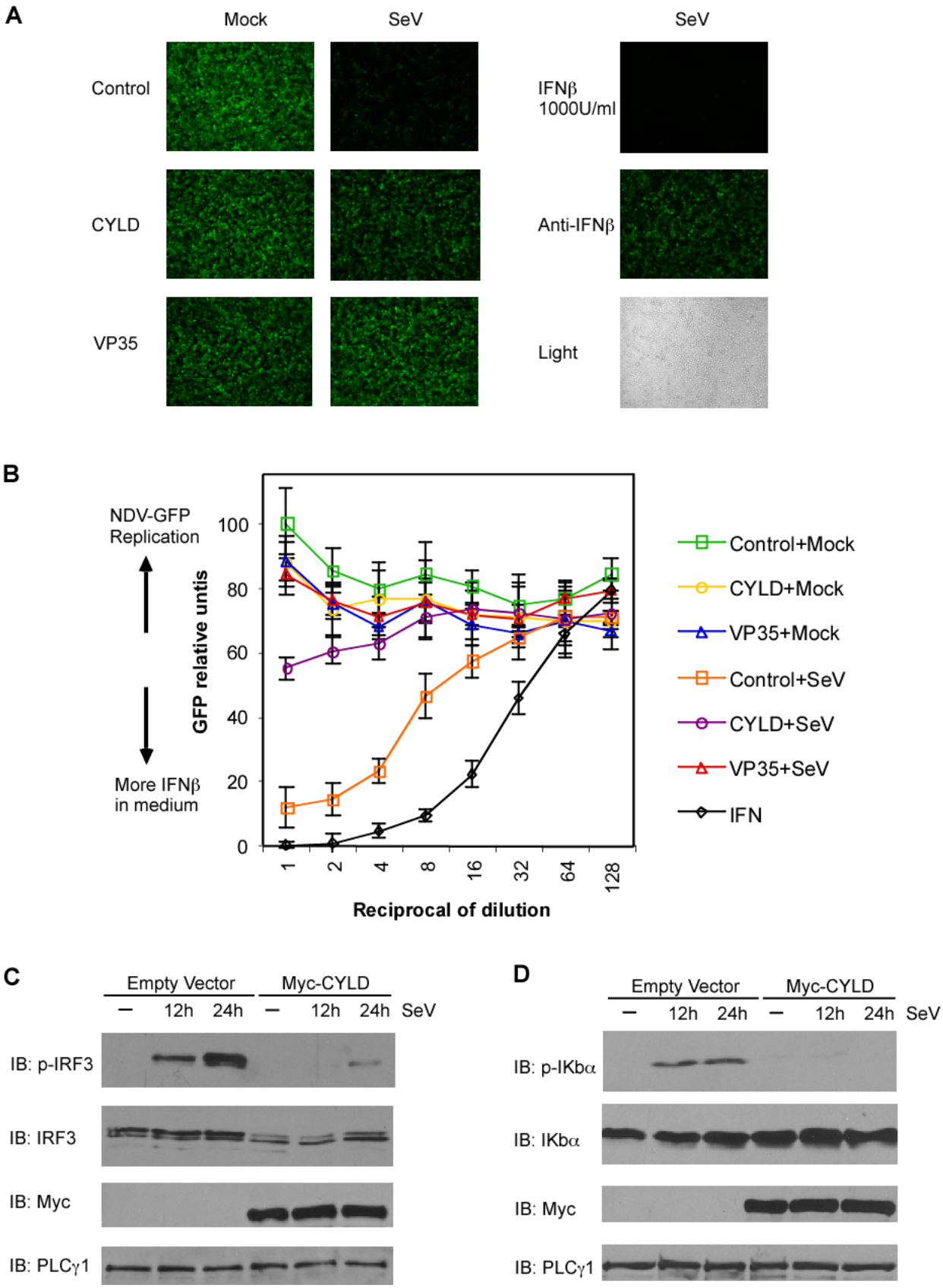
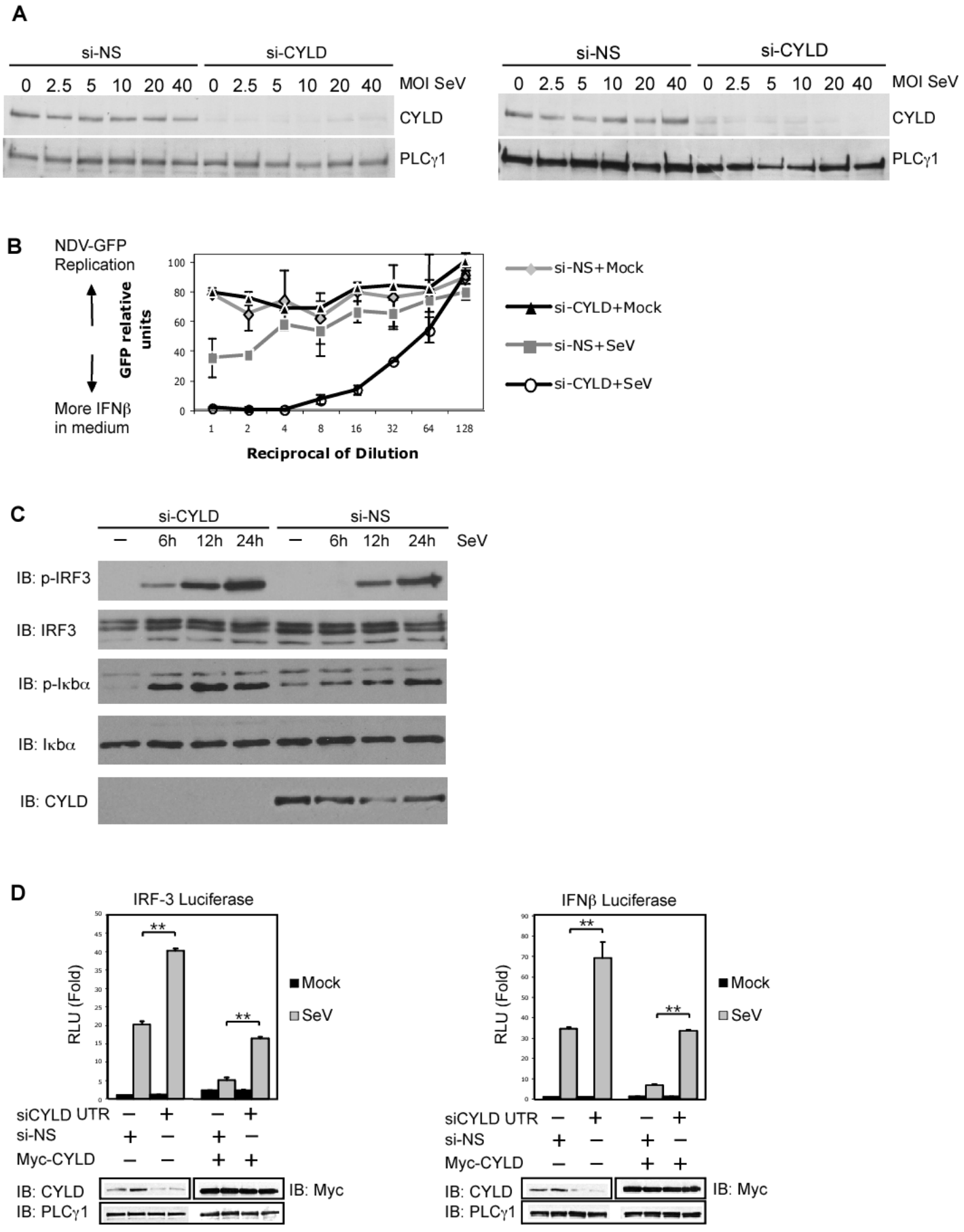
Supplementary Figure 1



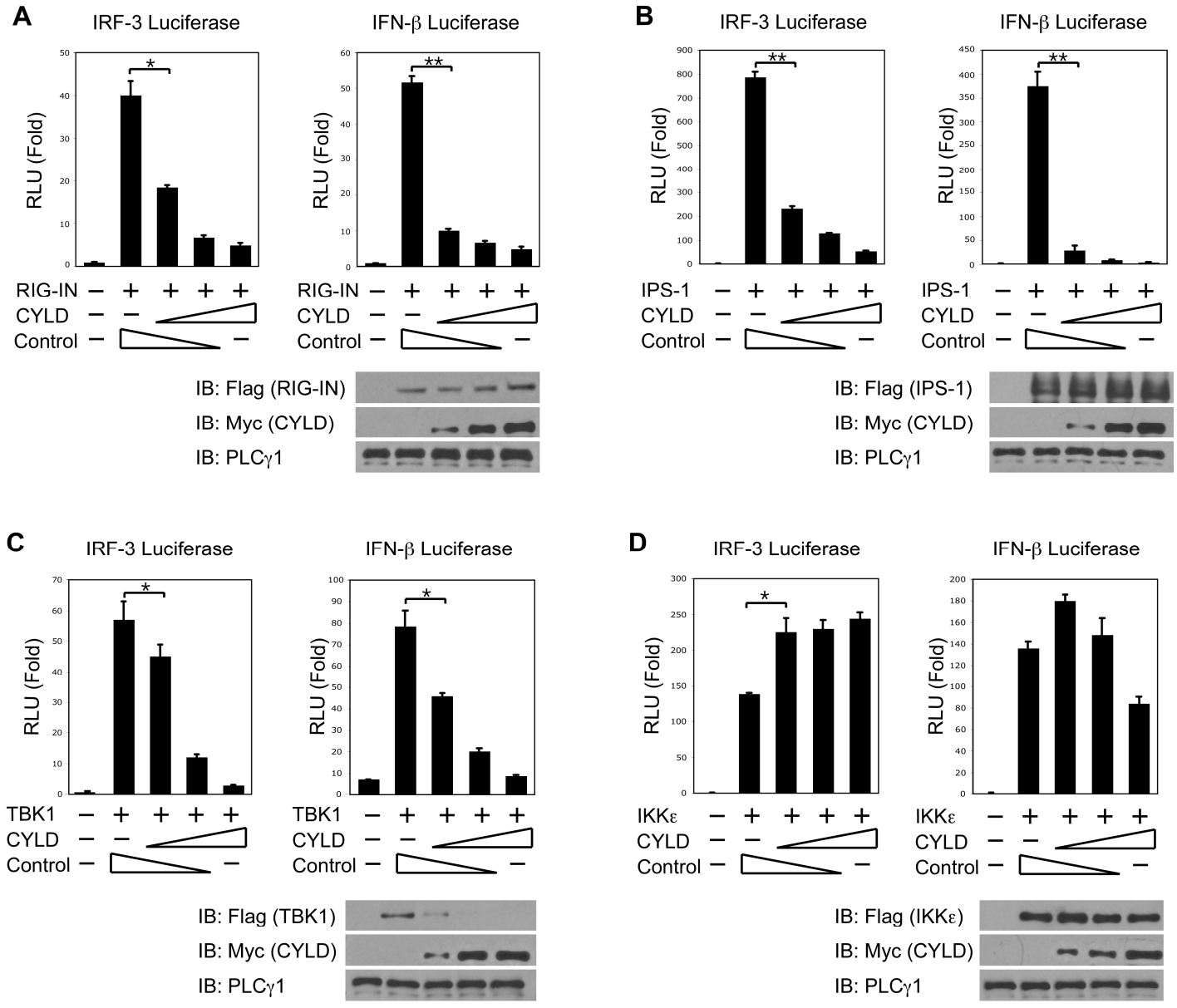
Supplementary Figure 2



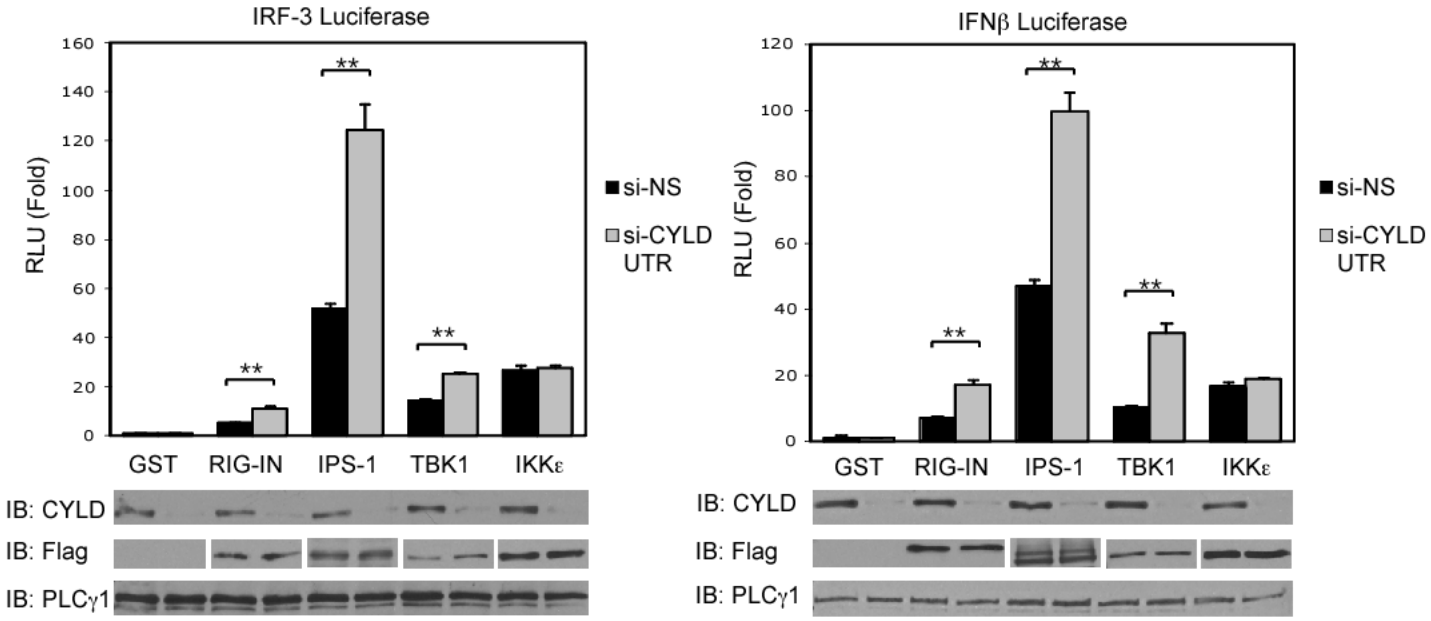
Supplementary Figure 3



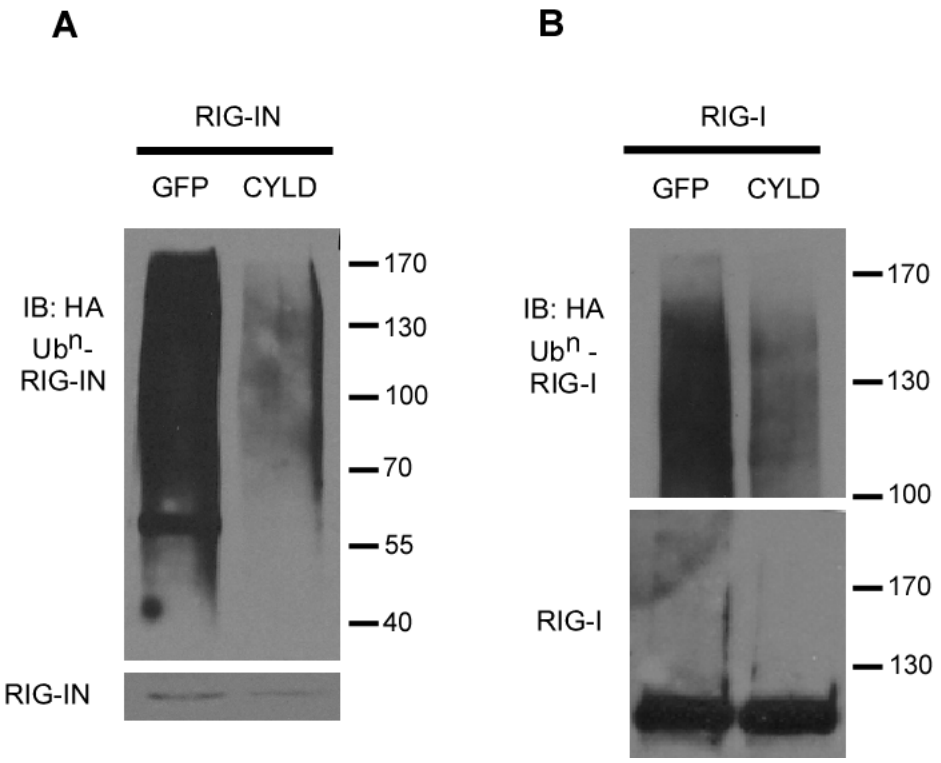
Supplementary Figure 4



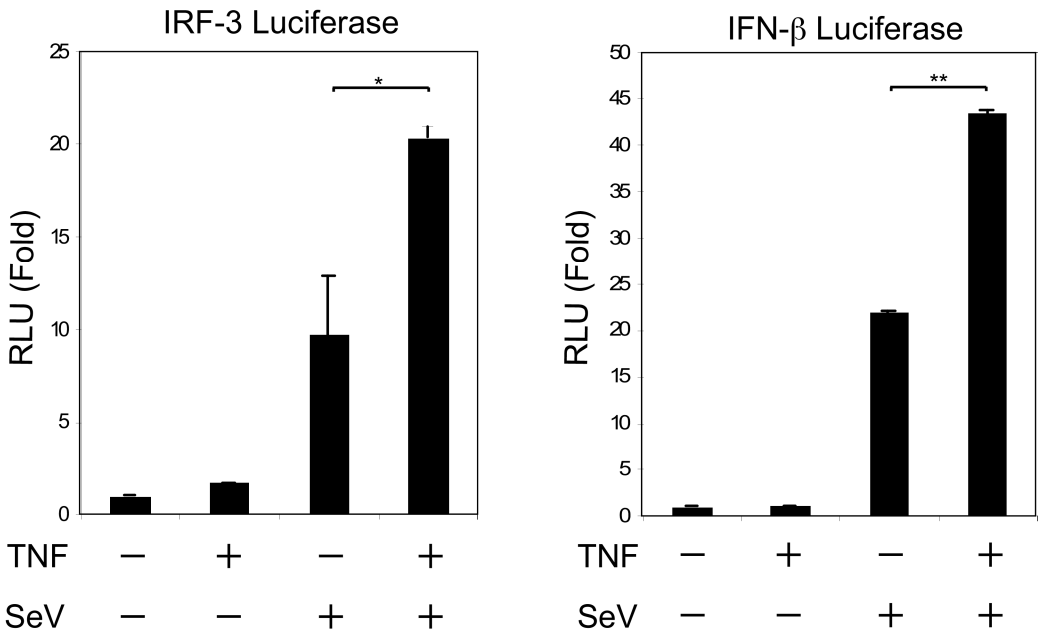
Supplementary Figure 5



Supplementary Figure 6



Supplementary Figure 7



Supplementary Figure Legends

Supplementary Figure 1

CYLD interacts with RIG-I

Protein interaction network analysis identifies connections between CYLD and DDX58 (triple-bordered rectangles) via important 'hub' proteins, FADD, RIPK1, TRAF2 and IKBKG (IKKγ) (orange circles). VISA (IPS-1), IFIH1 (MDA5), IRF3 and IRF3 kinases, TBK1 and IKBKE (IKKε) are highlighted in red. A simplified network is shown on the right.

Supplementary Figure 2

CYLD inhibits SeV induced IFNβ production and phosphorylation of IRF3 and IκBα.

(A) After UV irradiation, a series of twofold dilutions of conditioned media from mock- or SeV-infected transfectants was overlaid onto Vero cells in a 96-well plate in triplicates. 24 h later, cells were infected with NDV-GFP (MOI of 6), and virus replication was examined by fluorescence microscopy. Shown are the results obtained with conditioned media from cells transfected with 2ug CYLD, Ebola virus VP35, or bacterial GST as a negative control. Green fluorescence indicates NDV-GFP replication, i.e., low IFN level in the conditioned media. Conversely, lack of fluorescence indicates no NDV-GFP replication, i.e., high IFN level in the conditioned media. The SeV-infected plus anti-IFNβ panel shows that neutralizing IFNβ antibody rescues NDV-GFP replication. Panels shown are from Vero cells treated with a 16-fold dilution of the conditioned medium.

(B) GFP expression in Vero cells infected with NDV-GFP virus after treatment with twofold dilutions of conditioned media from cells transfected with CYLD or controls. Shown are the results obtained with conditioned media from cells transfected with 2ug CYLD, Ebola virus

VP35, or bacterial GST as a negative control. Higher levels of GFP expression indicate lower levels of IFNβ level in the media.

(C) 293 EBNA cells were transfected for 24 hours with 1μg CYLD or empty vector negative control, and infected with 10 MOI of SeV for 12 or 24 hours. Lysates were immunoblotted for phospho-IRF3 and total IRF3, as well as for Myc-CYLD and PLCγ1 as a loading control.

(D) 293 EBNA cells were transfected for 24 hours with 2.5μg CYLD or empty vector negative control, and infected with 10 MOI of SeV for 12 or 24 hours. Lysates were immunoblotted for phospho-IκBα and total IκBα, as well as for Myc-CYLD and PLCγ1 as a loading control.

Supplementary Figure 3

CYLD knockdown enhances SeV induced IFNβ production and phosphorylation of IRF3 and IκBα.

(A) Lysates corresponding to the samples in Figure 2D were immunoblotted with anti-CYLD to demonstrate knockdown of endogenous CYLD. Blots were stripped and re-probed with anti-PLCγ1 to monitor for equivalent loading.

(B) GFP expression in Vero cells infected with NDV-GFP virus after treatment with twofold dilutions of conditioned media from cells transfected for 48 hours with siRNA duplexes that are non-silencing (si-NS) or against CYLD (si-CYLD) and infected with SeV for 24 hours at 20 MOI. Higher levels of GFP expression indicate lower levels of IFNβ level in the media.

(C) 293 EBNA cells were transfected for 48 hours with siRNA duplexes that are non-silencing (si-NS) or against CYLD (si-CYLD), and then infected with 10 MOI of SeV for 6, 12, or 24 hours. Lysates were immunoblotted for phospho-IRF3 and total IRF3, phospho-IκBα and total IκBα, as well as for endogenous CYLD.

(D) 293 EBNA cells were transfected with siRNA duplexes targeting the 3'UTR of CYLD (siCYLD UTR) or non-silencing (si-NS) duplexes; with 1μg of CYLD or an empty vector control; and with luciferase reporter constructs. The cells were infected with 20 MOI of Sendai virus for 24 hours and luciferase assays were performed. The results are expressed as mean ± s.d. (n=3; * indicates $p<0.05$, ** indicates $p<0.01$ by Student's *t* test). Lysates from the luciferase experiments were blotted for expression of endogenous or Myc-CYLD, and PLCγ1 as a loading control.

Supplementary Figure 4

Functional interactions of CYLD with IPS-1, TBK1 and IKKε

IRF-3 and IFNβ promoter activity in 293 EBNA cells co-transfected with **(A)** 50 ng of RIG-I CARD domain (RIG-IN) and 0.25, 0.5, or 1 ug of CYLD; **(B)** 50 ng of IPS-1, and 0.5, 1, or 2 ug of CYLD; **(C)** 50 ng of TBK1, and 0.5, 1, or 2 ug of CYLD, **(D)** 100 ng of IKKε, and 0.5, 1, or 2 ug of CYLD. The results are expressed as mean ± s.d. (n=3, * indicates $p<0.05$, ** indicates $p<0.01$). Lysates from the luciferase experiments were blotted for expression of Myc-CYLD and Flag-tagged RIG-IN, IPS-1, TBK1 or IKKε; and PLCγ1 as a loading control.

Supplementary Figure 5

CYLD knockdown augments IFNβ pathway activation by overexpression of RIG-IN, IPS-1, and TBK1.

293 EBNA cells were transfected with siRNA duplexes targeting the 3'UTR of CYLD (si-CYLD UTR) or non-silencing (si-NS) duplexes; together with 200ng each of RIG-IN, IPS-1, TBK1 or IKKε; and with luciferase reporter constructs. Luciferase assays were performed 24

hours after transfection. The results are expressed as mean ± s.d. (n=3, ** indicates $p<0.01$). Lysates from the luciferase experiments were blotted for expression of endogenous CYLD and Flag-tagged RIG-IN, IPS-1, TBK1 or IKKε; and PLCγ1 as a loading control.

Supplementary Figure 6:

CYLD deubiquitinates RIG-I in a cell-free assay.

293 EBNA derivative cells were co-transfected with FLAG-RIG-I or FLAG-RIG-I CARD domain (RIG-IN) and HA-K63-ubiquitin. FLAG immunoprecipitation was performed in order to obtain ubiquitinated substrates for cell-free DUB assay. Separate plates of cells were transfected with FLAG-GFP or FLAG-CYLD for 24 hours before FLAG immunoprecipitation in the absence of protease inhibitors and purified proteins obtained by elution with FLAG peptide. Ubiquitinated RIG-I and RIG-IN attached to the M2 FLAG-beads were mixed with either purified GFP or CYLD to determine if CYLD can remove ubiquitin chains directly from these proteins. The top panels in A and B were immunoblotted with anti-HA to detect ubiquitin and reprobed with RIG-I antibody to detect RIG-IN (A) or RIG-I (B) as a loading control.

Supplementary Figure 7:

TNF potentiates the IFN response induced by SeV infection.

Luciferase assays were performed to quantify activation of IRF-3 and IFNβ promoter reporters in cells stimulated with TNF for 24 hours prior to infection with SeV (MOI 2.5) for 16 hours. The results are expressed as mean ± s.d. (n=3, * indicates $p<0.05$, ** indicates $p<0.01$).

Supplementary Materials and Methods

Constructing human protein interaction network

The network was constructed by iteratively connecting interacting proteins, with protein interaction data obtained from the Human Protein Reference Database (HPRD) (PMID: 16381900) and Biomolecular Interaction Network Database (BIND) (PMID:12519993). The network uses graph theoretical representations in which components (gene products) are depicted as nodes and interactions between components as edges. Graph layout descriptions were written in the Dot language (Gansner and North, 2000), which implements a multi-dimensional scaling heuristic and uses an iterative solver (Newton-Raphson algorithm) that searches for low-energy configurations to optimize the graph layout when creating a virtual physical model (Spring model) (Kamada and Kawai, 1989).

Cell lines and viruses

The 293 EBNA cell line was obtained from Invitrogen Life Technologies. A derivative of the 293 EBNA cell line with an NF-κB-GFP reporter was previously described (Yang et al., 2005). Vero cells and 293 derivative cell lines were cultured in DMEM (Invitrogen Life Technologies) with 10% bovine calf serum and 15 μg/ml gentamycin. Sendai virus Cantell (SeV) and a Newcastle disease virus expressing green fluorescent protein (NDV-GFP), described previously (Park et al., 2003) were grown in 10-day-old embryonated chicken eggs for 2 days at 37°C.

Antibodies and reagents

Antibodies were obtained from the following sources: anti-Myc (Clone 9E10, Santa Cruz Biotechnology), anti-FLAG (M2, Sigma), anti-HA (Clone 12CA5, Roche), anti-tubulin (B7,

Santa Cruz Biotechnology), anti-CYLD pAb (Reiley et al., 2004) (generous gift of Dr. Shao-Cong Sun, MD Anderson Cancer Center, Houston, TX), anti-PLCγ1 pAb (generous gift of the late Dr. Paul Leibson, Mayo Clinic, Rochester, MN), anti-CYLD mAb (Clone E4, Santa Cruz Biotechnology); and anti-RIG-I mAb (clone 1C3, generated by Estanis Nistal-Villan and Dr. Adolfo Garcia-Sastre with the assistance of the Mount Sinai Hybridoma Shared Research Facility); anti-phospho-IRF3 mAb (clone 4D4G, Cell Signaling) and anti-IRF3 pAb (Cell Signaling); anti-phospho-IκBα (Clone 5A5, Cell Signaling); and anti-IκBα (Clone 6A920, Imgenenex). Recombinant human TNFα was purchased from Peprotech.

Expression constructs

Full-length CYLD was amplified by Pfu Turbo DNA polymerase (Stratagene) from a human spleen cDNA library. Epitope-tagged CYLD, or the irrelevant GST control protein, were sub-cloned into the vector pEAK10. The negative control plasmid encoding FLAG-tagged A20ZF1-4 consists of the first four zinc fingers of A20 (residues 383-655) fused N-terminally to the FLAG eiptope. The plasmids encoding FLAG-tagged RIG-I, the RIG-I N-terminal CARD domain, VP35 (Cardenas et al., 2006) and FLAG-IPS-1 (Komuro and Horvath, 2006) have been described previously. Plasmids encoding FLAG-tagged TBK-1 and IKKε (Sharma et al., 2003) were kindly provided by John Hiscott (McGill University, Montreal, Canada). The p55C1BLuc luciferase reporter with IRF binding sites was kindly provided by Dr. Takeshi Fujita (Kyoto University, Kyoto, Japan). The IFNβ reporter p110IFNβluc was kindly provided by Dr. Dimitris Thanos (Biomedical Sciences Research Center 'Al. Fleming', Athens, Greece). pEF-HA-K63-Ub encoding a HA-tagged ubiquitin mutant with a single lysine at position 63 was generously provided by Dr. James Chen (UT-Southwestern, Dallas, TX). Transfections were performed using calcium phosphate precipitation.

RNAi-mediated knockdown

siRNA duplexes (ON-TARGET*plus* SMARTpool) targeting *CYLD* and non-silencing control duplexes were purchased from Dharmacon. To target the 3' UTR of *CYLD*, a duplex with the sense sequence 5'-CUACUUAGCUGAAGGGUAAUU-3' was purchased from Dharmacon. 293 EBNA cells were transfected with 100 pmol of si-NS or si-CYLD using the DharmaFECT 1 reagent according to the manufacturer's instructions. For luciferase experiments, reporter plasmids and pRL-null (Promega) were co-transfected with the RNA duplexes for 48 hours followed by SeV infection at the indicated MOI for an additional 16-24 hours. Culture supernatants were harvested for IFN bioassays and the cells lysed for luciferase assays.

Luciferase assays

$0.5x10^6$ 293 EBNA cells were plated in a 6-well plate and transfected 24 h later. 10 ng of the pRL-null (Promega) and 500 ng of the p55C1BLuc or p110IFNβluc reporter construct were used per well, along with expression plasmids as indicated. The cells were lysed 36 h after transfection. When infecting with SeV, medium was changed and virus added 36 h after transfection, and cells were lysed 24 h later. All luciferase assays were performed in triplicate using the Dual-Luciferase Reporter Assay System (Promega) according to the manufacturer's instructions.

Western blotting

Cells were gently lysed in ice-cold buffer containing 1% Triton X-100, 20 mM Tris, pH 7.5, 40 mM NaCl, 5 mM EDTA, 30 mM NaF, 30 mM $Na_4P_2O_7$ and supplemented with Protease Inhibitor Cocktail Set V (EMD Biosciences.) Protein concentrations in the extract were

determined using the bicinchoninic acid (BCA) protein assay kit (Pierce) and equivalent amounts of protein were resolved on SDS-PAGE gels. Western blotting was performed using standard methodologies.

Co-immunoprecipitation

Transfected 293 EBNA cells were lysed in 1% Triton buffer as described above. Lysates were clarified by centrifugation at 10,000 x *g* at 4°C and incubated with Protein G sepharose beads (Amersham Biosciences) with agitation for 2 h at 4°C. The beads were removed by centrifugation at 8,000 x *g* for 2 minutes and the lysate was incubated with anti-FLAG M2 agarose beads (Sigma-Aldrich) with agitation for either 2 h or 16 h at 4°C. Beads were collected by centrifugation at 8,000 x *g* for 2 minutes, and washed 5 times with 1% Triton lysis buffer containing 140 mM, 250 mM or 500 mM NaCl. Beads were then washed once with protein elution buffer containing 0.1% Triton X-100, 150 mM NaCl, 20 mM Tris pH 7.5, 0.2 mM EDTA, and 15% glycerol. The proteins were eluted twice for 30 minutes each, by competition using 100 ug/ml FLAG peptide (Sigma-Aldrich) in protein elution buffer. Lysates were resolved on SDS-PAGE and analyzed by immunoblotting.

***In vivo* and cell-free deubiquitination assay**

FLAG-tagged RIG-I, RIG-IN, IPS-1, TBK1 or IKKε were co-transfected into a 293 EBNA derivative cell line together with HA-tagged K63 ubiquitin, and either empty vector or myc-CYLD. 24 hours post-transfection cells were incubated with 25 μM MG132 (EMD Biosciences) for 4 hours and then lysed in triton lysis buffer (20 mM Tris, 40 mM NaCl, 5 mM EDTA, 50 mM NaF, 30 mM $Na_4P_20_7$, 1% Triton-x-100 pH7.4, supplemented with protease inhibitors). Triton-soluble lysates were denatured with 1% SDS at 100°C for 5 min followed by FLAG

immunoprecipitation with M2 beads. FLAG-tagged proteins were eluted with FLAG peptide, resolved by SDS-PAGE and sequentially blotted with anti-HA and anti-FLAG.

For the cell-free deubiquitination assay, CYLD enzyme and the negative control GFP were generated by transfection of a 293 EBNA derivative cell line with FLAG-CYLD or FLAG-GFP. 24 hours post-transfection, FLAG-tagged proteins were immunoaffinity-purified with M2 beads and eluted with 300 μg/ml of FLAG peptide in DUB assay buffer (20 mM Tris, 5mM $MgCl_2$, 1mM DTT, pH7.5). To generate ubiquitinated substrates, FLAG-tagged RIG-I or the N-terminal RIG-IN mutant were transfected together with HA-tagged K63-ubiquitin and immunoprecipitated 24 hours later with anti-FLAG M2 beads (Sigma). Ubiquitinated substrates attached to the M2 beads were incubated with either the purified GFP or CYLD in a final volume of 50 μl DUB assay buffer at 37°C for 2 hours. The substrates bound to the M2 beads were subsequently eluted with FLAG peptide, resolved by SDS-PAGE and blotted with anti-HA mAb to visualize the level of poly-ubiquitination.

Quantitative PCR

RNA was isolated using the RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions. Quantitative PCR (qPCR)was performed by the Mount Sinai Real-Time PCR Shared Research Facility. Briefly, cDNA was generated using 1-5 μg total RNA, 0.5 μg dT_{18}, 0.5 mM dNTPs, 50 U StrataScript (Stratagene), and manufacture-supplied buffer. Real-time PCR was performed using 1:50 dilution of cDNA, 5 mM $MgCl_2$, 0.2 mM dNTPs, SYBR Green I (Molecular Probes, Eugene, OR), 2 μM of each primer, 0.25 U PlatinumTaq DNA polymerase (Invitrogen), and manufacturer-supplied buffer. The primers used for real-time PCR are as follows:

IFNβ sense 5'-GTCAGAGTGGAAATCCTAAG-3'

anti-sense 5'-ACAGCATCTGCTGGTTGAAG-3',

β-actin sense 5'-ACTGGAACGGTGAAGGTGAC-3'

anti-sense 5'-GTGGACTTGGGAGAGGACTG-3',

rps11 sense 5'-GCCGAGACTATCTGCACTAC-3'

anti-sense 5'-ATGTCCAGCCTCAGAACTTC-3',

α-tubulin sense 5'-GCCTGGACCACAAGTTTGAC-3'

anti-sense 5'-TGAAATTCTGGGAGCATGAC-3'.

References

Gansner, E.R. and North, S.C. (2000) An open graph visualization system and its applications to software engineering. . *Software Practice and Experience* **30**, 1203-1233.

Kamada, T. and Kawai, S. (1989) An algorithm for drawing general undirected graphs. *Information Processing Letters*, **31**.

Komuro, A. and Horvath, C.M. (2006) RNA and Virus-Independent Inhibition of Antiviral Signaling by RNA Helicase LGP2. *J Virol*.

Park, M.S., Shaw, M.L., Munoz-Jordan, J., Cros, J.F., Nakaya, T., Bouvier, N., Palese, P., Garcia-Sastre, A. and Basler, C.F. (2003) Newcastle disease virus (NDV)-based assay demonstrates interferon-antagonist activity for the NDV V protein and the Nipah virus V, W, and C proteins. *J Virol*, **77**, 1501-1511.

Reiley, W., Zhang, M. and Sun, S.C. (2004) Negative regulation of JNK signaling by the tumor suppressor CYLD. *J Biol Chem*, **279**, 55161-55167.

Sharma, S., tenOever, B.R., Grandvaux, N., Zhou, G.P., Lin, R. and Hiscott, J. (2003) Triggering the interferon antiviral response through an IKK-related pathway. *Science*, **300**, 1148-1151.

Yang, M., Hase, H., Legarda-Addison, D., Varughese, L., Seed, B. and Ting, A.T. (2005) B cell maturation antigen, the receptor for a proliferation-inducing ligand and B cell-activating factor of the TNF family, induces antigen presentation in B cells. *J Immunol*, **175**, 2814-2824.