Virology 444 (2013) 41–44

Contents lists available at ScienceDirect

Virology

journal homepage: www.elsevier.com/locate/yviro

Rotavirus NSP1 inhibits interferon induced non-canonical NFκB activation by interacting with TNF receptor associated factor 2

Parikshit Bagchi [1], Rahul Bhowmick, Satabdi Nandi, Mukti Kant Nayak, Mamta Chawla-Sarkar *

Division of Virology, National Institute of Cholera and Enteric Diseases, P-33, C.I.T. Road Scheme-XM, Beliaghata, Kolkata 700010, West Bengal, India

ARTICLE INFO

Article history:
Received 3 May 2013
Returned to author for revisions
25 May 2013
Accepted 3 July 2013
Available online 23 July 2013

Keywords:
Rotavirus
NSP1
TRAF2
NFκB

ABSTRACT

TNF receptor associated factor 2 (TRAF2) plays a very important role in cellular innate immune as well as inflammatory responses. Previous studies have reported TRAF2 mediated regulation of TNF and Interferon (IFN) induced canonical and non-canonical activation of NFκB. In this study, we show that rotavirus NSP1 targets TRAF2 to regulate IFN induced non-canonical NFκB activation. Here we found that rotavirus Non-Structural Protein-1 (NSP1) interacts with TRAF2 and degrades it in a proteasome dependent manner. C-terminal part of NSP1 was sufficient for interacting with TRAF2 but it alone could not degrade TRAF2. This inhibition of interferon mediated non-canonical NFκB activation by NSP1 may modulate inflammatory cytokine production after rotavirus infection to help the virus propagation.

Introduction

Rotavirus (RV), the major cause of severe infantile gastroenteritis, is icosahedral in structure with three concentric layers of proteins and a genome of 11 dsRNA segments (Estes and Kapikian, 2007). The virus contains six structural proteins (VP1-4, VP6-7) which forms the virion, it also encodes six non-structural proteins (NSP1-6). NSP1, a RNA binding protein (Estes and Kapikian, 2007), is the only rotavirus protein implicated in evasion of innate immune response by counteracting induction of interferon (IFN) and delaying early cellular apoptosis by activation of PI3K/Akt pathway (Feng et al., 2009; Barro and Patton, 2007; Bagchi et al., 2010, 2013). Among group A rotaviruses, NSP1 is highly variable (Hua et al., 1993), except for a conserved N-terminus cystein rich motif (C-X2-C-X8-C-X2-C-X3-H-X-C-X2-C-X-5-C) which is a putative zinc finger motif. Since this region is conserved, it has been postulated that it may have an important role in function of the protein. C-terminus of NSP1 has an IRF3 binding site and has been shown to involve in IRF3 degradation (Barro and Patton, 2007). However both N-terminal and C-terminal part of NSP1 were shown to be important for PI3K/Akt activation (Bagchi et al., 2013). Here in this study we are focusing on another role of RV NSP1 to counteract host immunity. TRAFs (TNF receptor associated factors) function as adapter molecules for TNF superfamily members and regulate downstream signaling events like NFκB and AP-1 activation (Hsu et al., 1996). TRAF proteins play critical role in inflammatory processes, programmed cell death and interferon responses (Hacker et al., 2006; Saha et al., 2006; Chuan et al., 2008). E3 ubiquitin ligase family protein A20 interacts with TRAF2 (TNF receptor associated factor 2) and degrades it (Li et al., 2009). Based on microarray analysis, induction of A20 in different wild type RV strains (SA11, Wa, A5-13) was observed (Bagchi et al., 2012). So we tried to analize TRAF2 degradation in RV infected cells.

Material and methods

Reagents

Proteasomal inhibitor MG132 and interferon β were purchased from Sigma–Aldrich (St. Louis, MO, USA) and Invivogen (San Diego, CA, USA) and used at a concentration of 5 μM and 1000 U/ml respectively. siRNA for TRAF2 and control siRNA was from Ambion (NY, USA).

Cell culture and virus infection

Human intestinal epithelial (HT29) and human embryonic kidney epithelial (HEK 293T) cell lines were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum and 1% antibiotic–antimycotic solution (Invitrogen, Carlsbad, CA).

* Corresponding author. Fax: +91 33 2370 5066.
E-mail addresses: chawlam70@gmail.com, sarkarmc@icmr.org.in (M. Chawla-Sarkar).
[1] Current address: Department of Cell and Developmental Biology, University of Michigan Medical School, Ann Arbor, MI 48103, USA.

0042-6822/$ - see front matter © 2013 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.virol.2013.07.003

Cells were maintained in 5% CO_2 at 37 °C humidified incubator. The bovine rotavirus wild type A5-13 and NSP1 mutant A5-16 strains (Taniguchi et al., 1996) (accession numbers D38148; D38149) were used in the study. For infection, viruses were activated with acetylated trypsin (10 μg/ml) at 37 °C for 30 min, diluted as per required multiplicity of infection (MOI) and added to the cells for adsorption (45 min) at 37 °C, followed by washing 3X with media to remove unbound virus. Infection was continued in fresh medium. MOI 2 of virus was used for HT29 cells in the study. The time of virus removal was taken as 0 h post infection (hpi) for all experiments. At different time points cells were freeze-thawed for cell lysis. Extracted and purified viral preparations were titrated by plaque assay (Bagchi et. al., 2010).

Gel electrophoresis and immunoblot analyses

Gel electrophoresis and immunoblot assay was performed as previously described (Bagchi et. al., 2010). TRAF2 antibody, A20 antibody and Anti-His antibody was from Santa Cruz Biotechnology, CA. GAPDH, NFκB p52 antibody and fluorescent tagged secondary antibodies were purchased from Cell Signaling Inc, Devers, MA and Invitrogen, USA respectively. RV NSP3 and NSP1 antibody were raised in our lab. Blots were scanned and quantitated using GelDoc XR system and Quantity One® software version 4.6.3 (BioRad, Hercules, CA).

Cloning

C-terminal His-tagged full length NSP1 construct was made by cloning NSP1 in pcDNA6 vector (Invitrogen, USA) as previously described (Bagchi et. al., 2010). pcDNSP1N and pcDNSP1C was developed by cloning N terminal (1–100 amino acid) and C terminal (101 amino acid to end) NSP1 in pcDNA6 vector respectively. Full length TRAF2 was cloned in pFLAG vector (Sigma, MO) to make FLAG tagged TRAF2 construct.

Nuclear protein extraction

HEK 293T cells were transfected with respective DNA constructs or siRNAs by Lipofectamine method (Invitrogen, USA) and either treated or mock treated with interferon β and nuclear extracts were prepared with ProteoJET Cytoplasmic and Nuclear Protein Extraction Kit (Fermentas Life Science, USA) for NFκB p52 translocation studies.

Immunoprecipitation

Immunoprecipitation experiments were performed as described previously (Bagchi et. al., 2010).

Immunofluorescence microscopy

Cos7 cells were co-transfected either with Flag-TRAF2, pcDNSP1 or Flag-TRAF2, pcDNA and after 16 hours cells were fixed with paraformaldehyde (4% w/v in PBS) for 10 min at RT and permeabilized with 0.1% triton X-100 for 20 min at 4 °C and probed with specific primary (anti-FLAG and anti NSP1) at 1:200 dilution and fluorescent tagged secondary antibodies (anti-mouse FITC, anti-rabbit Rhodamine) at 1:100 dilution. After 5X PBS wash, slides were mounted with Vectashield-DAPI (Vector laboratories, Burlingame, CA, USA) and examined under fluorescence-microscope (Carl Zeiss, Gottingen, Germany). Excitation and emission detection for each fluor was performed sequentially to avoid cross-talk.

Statistical analysis

Data are expressed as mean ± standard deviations of at least three independent experiments ($n \geq 3$). In all tests, $p < 0.05$ was considered statistically significant.

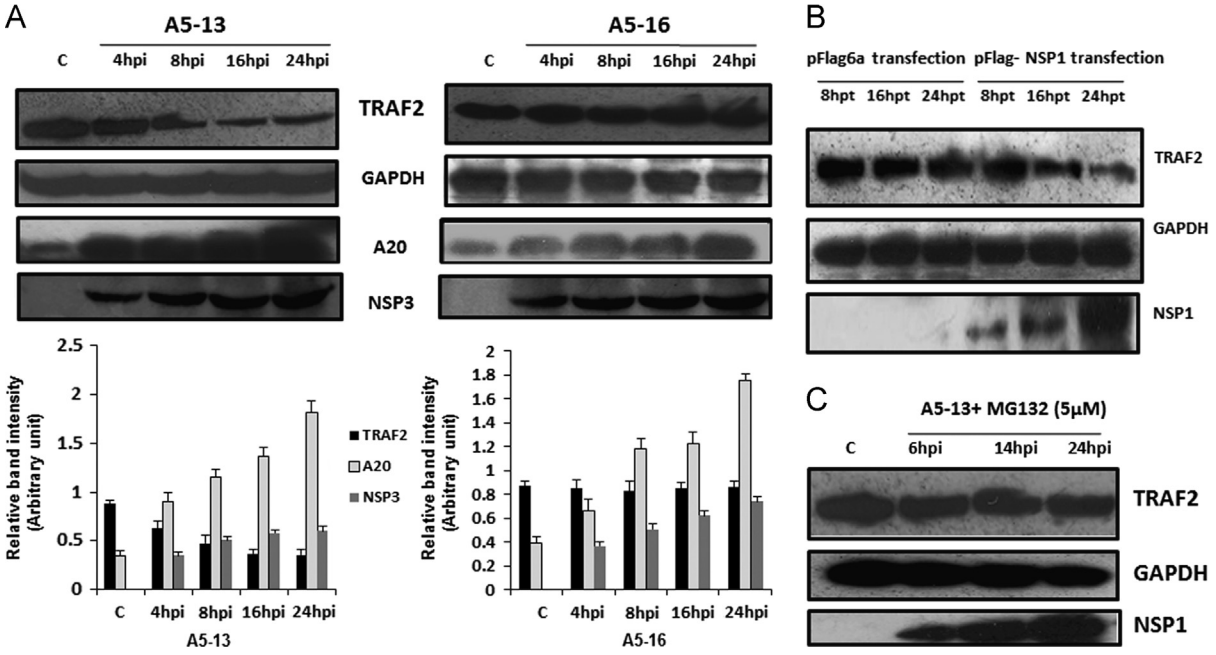

Fig. 1. Rotavirus NSP1 degrades TRAF2 in a proteasome dependent manner. (A) HT29 cells were mock infected or infected with either A5-13 or A5-16 at MOI 2 and cell lysates were taken at indicated time points for western blotting with TRAF2 antibody and A20 antibody. Blots were reprobed with GAPDH as loading control and RV NSP3 antibody as a marker of RV infection. The band intensity of TRAF2, A20 and NSP3 were normalized against GAPDH. The data represent the means ± the standard deviation. (B) HEK293 cells were transfected with either empty vector or with pFlag-NSP1 and cell lysates were taken at indicated time after transfection and western blotted with TRAF2 antibody. Blots were reprobed with GAPDH as loading control and NSP1. (C) HT29 cells were mock infected or infected with A5-13 in presence of proteasomal inhibitor MG132. At indicated time after infection cell lysates were western blotted with TRAF2 antibody. Blots were reprobed with GAPDH as loading control and NSP1. All experiments are representative of three independent experiments.

Results

To delineate the mechanism by which RV may degrade TRAF2, HT29 cells were either mock infected or infected with wild type bovine RV strain A5-13 (MOI 2) or its isogenic NSP1 mutant strain A5-16 (MOI 2). Time course study showed TRAF2 degradation in A5-13 infected cells from 4hpi whereas in A5-16 infected cells, there was no TRAF2 degradation however A20 is induced by both strains (Fig. 1A). Other RV strains with functional NSP1 such as SA11(simian) and OSU (porcine) also resulted in degradation of TRAF2 suggesting it to be a strain independent phenomenon (Supplementary Fig. S1). To study whether in addition to NSP1, other RV proteins also play a role in TRAF2 degradation, HEK293 cells were transfected with either empty vector or pcDNSP1 and cell lysates were prepared at increasing time points. Immunoblotting for TRAF2 revealed time dependent decrease in TRAF2 expression suggesting that in absence of other viral proteins, NSP1 alone is sufficient for TRAF2 degradation (Fig. 1B). Since NSP1 has been reported to have putative E3 ubiquitin ligase like activity (Graff et al., 2007), TRAF2 degradation was analyzed in presence of proteasomal inhibitor MG132. In HT29 cells infected with A5-13 strain in presence of MG132 (5 μM), TRAF2 degradation was not observed, suggesting it to be proteasome dependent activity (Fig. 1C).

To investigate whether NSP1 interacted with TRAF2, first HT29 cells were infected with A5-13 and cell lysates were immunoprecipitated with TRAF2 antibody or rabbit serum as control. As shown in Fig. 2A, NSP1 co-immunoprecipitated with TRAF2. Interaction of NSP1 with TRAF2 was further confirmed when NSP1 (His-tagged, cloned in pcDNA6 vector, Invitrogen) co-immunoprecipitated with TRAF2 (Flag tagged, cloned in pFLAG vector, Sigma) in HEK 293 cells overexpressing these proteins (Fig. 2B). Co-localization of NSP1 and TRAF2 was also observed by immunofluorescence microscopy (Fig. 2C). Once interaction between the two proteins was confirmed, region of NSP1 (N-terminal or C-terminal) responsible for this interaction was analyzed. HEK293 cells were co-transfected with either Flag-TRAF2 and pcDNSP1N (N-terminal 1–100 amino acids) or Flag-TRAF2 and pcDNSP1C (C-terminal 101-end) and immunoprecipitated with either rabbit serum as control or with anti-His antibody. As shown in Fig. 2C, C-terminal region of NSP1 co-immunoprecipitated with TRAF2, whereas no interaction between NSP1N and TRAF2 was observed. To analyze whether C-terminal NSP1 was sufficient for TRAF2 degradation, HEK293 cells were transfected with either empty vector or full length pcDNSP1 or pcDNSP1N or pcDNSP1C and after 24 h cell lysates were immunoblotted with TRAF2 antibody. Unlike interaction studies (Fig. 2C), N-terminal or C-terminal region of NSP1 could not degrade TRAF2 (Fig. 2D) whereas TRAF2 degradation was observed in NSP1 (full length) expressing cells (Fig. 2D). This suggested that NSP1-C terminal region may bind to TRAF2, but N-terminal region is also required for degradation. This is consistent with previous reports on NSP1 mediated degradation of IRF3. It can be hypothesized that NSP1C binds to TRAF2 which is ubiquitinylated by putative E3 ubiquitin ligase activity of NSP1-N terminal domain followed by proteasomal degradation.

Once the TRAF2 degradation by NSP1 was confirmed, its significance during rotavirus infection was analyzed. TRAF2 plays an important role in IFN mediated non-canonical NFκB activation by facilitating p65-p52 interaction leading to their nuclear translocation (Chuan et al., 2008), HEK293 cells were transfected with either empty vector or Flag-NSP1 or siTRAF2 or control siRNA

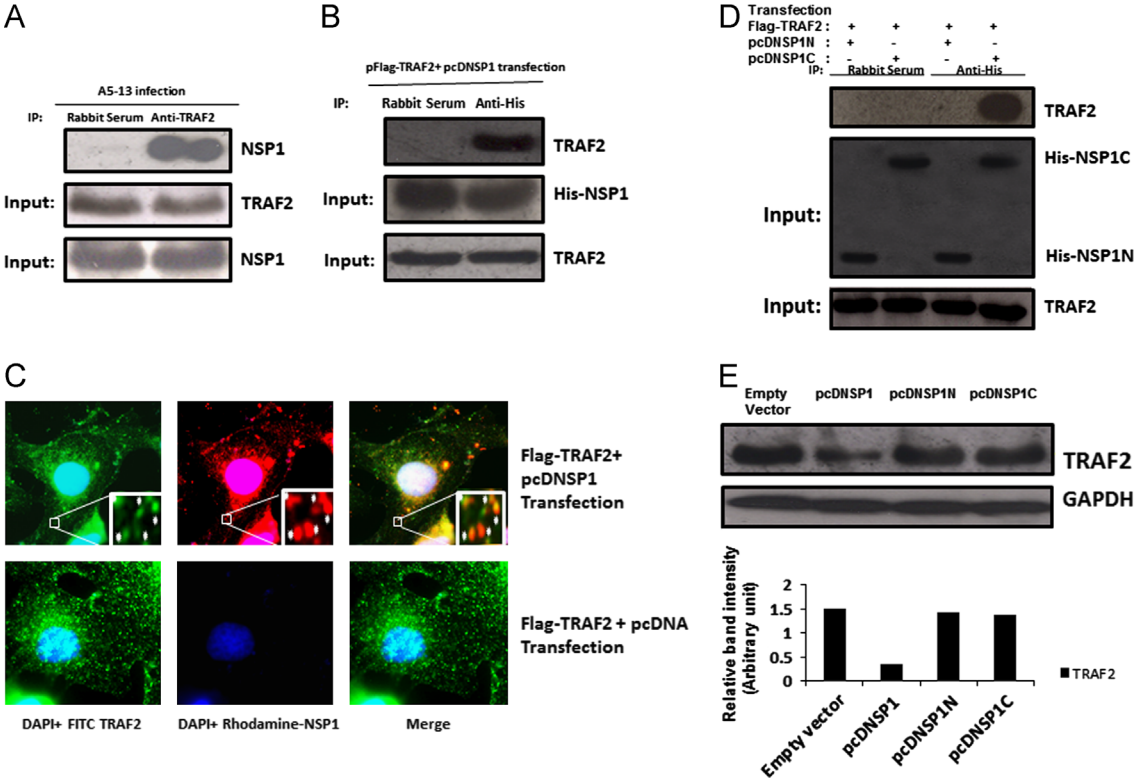

Fig. 2. Rotavirus NSP1 interacts with TRAF2 and causes its degradation. (A) HT29 cells were infected with A5-13, immunoprecipitated with either rabbit serum or with TRAF2 antibody (1:500 dilution) and western blotted with raised antibody against RV NSP1 antibody which showed positive interaction between NSP1 and TRAF2. (B) HEK 293 cells were co-transfected with pFlag-TRAF2, pcDNSP1, immunoprecipitated with either rabbit serum or with Anti-His antibody (1:500 dilution) and western blotted with TRAF2 antibody which further confirms positive interaction between NSP1 and TRAF2. (C) Immunofluorescence microscopy data showed clear co-localization of TRAF2 with NSP1. Nucleus was stained with DAPI. (D) HEK 293 cells were either co-transfected with Flag-TRAF2, pcDNSP1N or Flag-TRAF2, pcDNSP1C, immunoprecipitated with either rabbit serum or with Anti-His antibody (1:500 dilution) and western blotted with TRAF2 antibody which indicated that TRAF2 interacts with C-terminal part of NSP1. (E) HEK293 cells were either transfected with empty vector or pcDNSP1 or pcDNSP1N or pcDNSP1C and cell lysates were western blotted with TRAF2. Blots were reprobed with GAPDH as loading control. The band intensity of TRAF2 was normalized against GAPDH. All experiments are representative of three independent experiments.

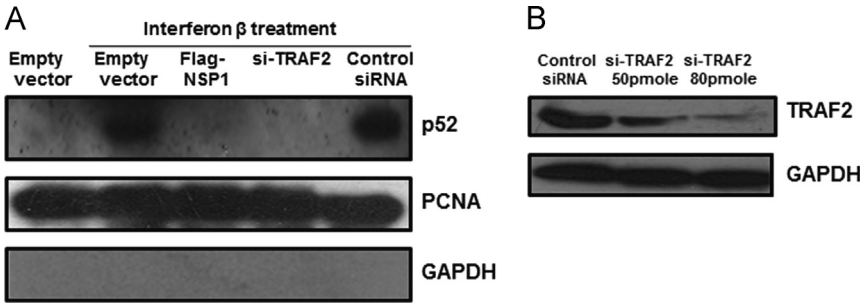

Fig. 3. NSP1 inhibits interferon mediated NFκB p52 activation. (A) HEK293 cells were either transfected with empty vector or Flag-NSP1 or TRAF2 siRNA (80 pmole) or control siRNA in presence or absence interferon β. Western blot of nuclear extract with NFκB p52 antibody was performed to detect nuclear localized p52. Blot was reprobed with PCNA as loading control and with GAPDH to inquire about the presence of any cytosolic fraction. (B) Functionality of TRAF2 siRNA was checked using two concentration (50 pmole and 80 pmole). Blots were reprobed with GAPDH as loading control. All experiments are representative of three independent experiments.

individually and treated with interferon β (1000 U/ml) for 30 min In addition IFN β untreated and empty vector transfected HEK293 cell was also analyzed. Nuclear lysates were immunoblotted with anti p52 showed that RV NSP1 inhibits the IFN β mediated nuclear translocation of NFκB p52 (Fig. 3A). Down regulation of TRAF2 by siRNA also resulted in inhibition of interferon induced NFκB p52 nuclear translocation (Fig. 3A, B). No degradation of TRAF2 was observed in either empty vector or control siRNA transfected cells.

Conclusion

Many other viruses have also been shown to interact with TRAF2 to regulate NFκB activation (Choi et al., 2006; Park et al., 2002; Yang et al., 2008; Song & Kang, 2010). Previous studies have shown that RV NSP1 antagonizes IFN by degradation of IRFs and RIG1 (Graff et al., 2007; Qin et al., 2011), however still significant amount of IFNβ is induced in RV infected cells (Bagchi et al., 2010). Here we report that RV NSP1 not only interacts with, but also degrades TRAF2 resulting in inhibition of interferon induced non-canonical NFκB activation which may antagonize virus induced cellular cytokine responses to facilitate virus propagation (Toby, 2009). Overall this study highlights one of the many strategies by which RV NSP1 counteracts host response.

Acknowledgments

The study was supported by Financial Assistance for Intramural Projects from Indian Council of Medical Research (ICMR), New Delhi. P. Bagchi (SRF), S. Nandi (SRF) and R. Bhowmick (JRF) are supported by fellowships from CSIR, Govt. of India and M. K. Nayak is supported by ICMR, Govt. of India.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.virol.2013.07.003.

References

Bagchi, P., Dutta, D., Chattopadhyay, S., Mukherjee, A., Halder, U.C., Sarkar, S., Kobayashi, N., Komoto, S., Taniguchi, K., Chawla-Sarkar, M., 2010. Rotavirus non-structural protein-1 suppresses virus induced cellular apoptosis to facilitate viral growth by activating the cell-survival pathways during early stages of infection. J. Virol. 84, 6834–6845.

Bagchi, P., Nandi, S., Nayak, M.K., Chawla-Sarkar, M., 2013. Molecular mechanism behind rotavirus NSP1 mediated PI3kinase activation: interaction between NSP1 and p85 subunit of PI3K. J. Virol. 87, 2358–2362.

Bagchi, P., Nandi, S., Chattopadhyay, S., Bhowmick, R., Halder, U.C., Kobayashi, N., Chawla-Sarkar, M., 2012. Identification of common host genes involved in pathogenesis of different rotavirus strains: an attempt to recognize probable antiviral targets. Virus Res. 169, 144–153.

Barro, M., Patton, J.T., 2007. Rotavirus NSP1 inhibits expression of type I interferon by antagonizing the function of interferon regulatory factors IRF3, IRF5, and IRF7. J. Virol. 81, 4473–4481.

Choi, S.H., Park, K.J., Ahn, B.Y., Jung, G., Lai, M.M., Hwang, S.B., 2006. Hepatitis C virus nonstructural 5B protein regulates tumor necrosis factor alpha signaling through effects on cellular Ikappa B kinase. Mol. Cell Biol. 26, 3048–3059.

Yang, Chuan He, Murti, A., Pfeffer, S.R., Fan, M., Du, Z., Pfeffer, L.M., 2008. The role of TRAF2 binding to the type I Interferon receptor in alternative NFκB activation and antiviral response. J. Biol. Chem. 283, 14309–14316.

Estes, M.K., Kapikian, A.Z., 2007. Rotavirus. In: Knite, D.M., Howley, P.N., Griffin, D.E., Lamb, R.A., Martin, M.A., Roizman, B., Straus, E.E. (Eds.), Fields Virology, 5th ed. Lippincott Williams and Wilkins, Philadelphia, pp. 1917–1974.

Feng, N., Sen, A., Nguyen, H., Vo, P., Hoshino, Y., Deal, E.M., Greenberg, H.B., 2009. Variation in antagonism of the interferon response to rotavirus NSP1 results in differential infectivity in mouse embryonic fibroblasts. J. Virol. 83, 6987–6994.

Graff, J.W., Ewen, J., Ettayebi, K., Hardy, M.E., 2007. Zinc-binding domain of rotavirus NSP1 is required for proteasome-dependent degradation of IRF3 and autoregulatory NSP1 stability. J. Gen. Virol. 88, 613–620.

Hacker, H., Redecke, V., Blagoev, B., Kratchmarova, I., Hsu, L.C., Wang, G.G., Kamps, M.P., Raz, E., Wagner, H., Hacker, G., Mann, M., Karin, M., 2006. Specificity in Toll-like receptor signalling through distinct effector functions of TRAF3 and TRAF6. Nature 439, 204–207.

Hsu, H., Shu, H.B., Pan, M.G., Goeddel, D.V., 1996. TRADD-TRAF2 and TRADD-FADD interactions define two distinct TNF receptor 1 signal transduction pathways. Cell 84, 299–308.

Hua, J., Mansell, E.A., Patton, J.T., 1993. Comparative analysis of the rotavirus NS53 gene: conservation of basic and cysteine-rich regions in the protein and possible stem-loop structures in the RNA. Virology 196, 372–378.

Li, L., Soetandyo, N., Wang, Q., Ye, Y., 2009. The zinc finger protein A20 targets TRAF2 to the lysosomes for degradation. Biochim. Biophys. Acta 1793, 346–353.

Park, K.J., Choi, S.H., Lee, S.Y., Hwang, S.B., Lai, M.M., 2002. Nonstructural 5A protein of hepatitis C virus modulates tumor necrosis factor alpha-stimulated nuclear factor kappa B activation. J. Biol. Chem. 277, 13122–13128.

Qin, L., Ren, L., Zhou, Z., Lei, X., Chen, L., Xue, Q., Liu, X., Wang, J., Hung, T., 2011. Rotavirus nonstructural protein 1 antagonizes innate immune response by interacting with retinoic acid inducible gene I. J. Virol. 8, 526.

Saha, S.K., Pietras, E.M., He, J.Q., Kang, J.R., Liu, S.Y., Oganesyan, G., Shahangian, A., Zarnegar, B., Shiba, T.L., Wang, Y., Cheng, G., 2006. Regulation of antiviral responses by a direct and specific interaction between TRAF3 and Cardif. EMBO J. 25, 3257–3263.

Song, Y.J., Kang, M.S., 2010. Roles of TRAF2 and TRAF3 in Epstein–Barr virus latent membrane protein 1-induced alternative NF-kappaB activation. Virus Genes 41, 174–180.

Taniguchi, K., Kojima, K., Urasawa, S., 1996. Nondefective rotavirus mutants with an NSP1 gene which has a deletion of 500 nucleotides, including a cysteine-rich zinc finger motif-encoding region (nucleotides 156–248), or which has a nonsense codon at nucleotides 153–155. J. Virol. 70, 4125–4130.

Toby, Lawrence, 2009. The nuclear factor NF-kB pathway in inflammation. Cold Spring Harbor Perspect. Biol. 1, a001651.