Molecular Immunology 58 (2014) 27–31

Contents lists available at ScienceDirect

Molecular Immunology

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

TRAF6-mediated ubiquitination of NEMO requires p62/sequestosome-1☆☆

Tiziana Zotti[a,1], Ivan Scudiero[a,1], Pio Settembre[a,b], Angela Ferravante[a], Pellegrino Mazzone[a,b], Luca D'Andrea[a,b], Carla Reale[a], Pasquale Vito[b,c,*], Romania Stilo[a,b]

[a] Biogem, Via Camporeale, 83031 Ariano Irpino, Italy
[b] Dipartimento di Scienze e Tecnologie, Università degli Studi del Sannio, Via Port'Arsa 11, 82100 Benevento, Italy
[c] College of Arts and Sciences, Qatar University, P.O. Box 2713, Doha, Qatar

ARTICLE INFO

Article history:
Received 3 July 2013
Received in revised form
27 September 2013
Accepted 21 October 2013
Available online 20 November 2013

Keywords:
TRAF6
p62/sequestosome
Ubiquitin
NF-κB
IL-1β
TNFα

ABSTRACT

The atypical protein kinase C-interacting protein p62/sequestosome-1 (p62) has emerged as a crucial molecule in a variety of cellular functions due to its involvement in various signaling mechanisms. p62 has been implicated in the activation of NF-κB in TNFα-stimulated cells and has been shown to be activated in response to interleukin-1β (IL-1β). Here we demonstrate that p62 interacts with NEMO, the regulatory subunit of the complex responsible for activation of NF-κB transcription factor. Depletion of p62 obtained through a short interfering RNA targeting p62 mRNA abrogated TRAF6 capacity to promote NEMO ubiquitination and severely impairs NF-κB activation following IL-1β stimulation.

Together, these results indicate that p62 is an important intermediary in the NF-κB activation pathways implemented through non-degradative ubiquitination events.

1. Introduction

NF-κB is an inducible and ubiquitously expressed transcription factor for genes involved in a variety of biological processes, including immune and inflammatory responses, cell survival, cell adhesion, differentiation, and cell growth (Baltimore, 2009; Hayden and Ghosh, 2008). In most resting cells, NF-κB is retained in the cytoplasm by binding to the inhibitory I-κB, which masks the nuclear localization sequences of NF-κB. (Baltimore, 2009; Hayden and Ghosh, 2008). In the canonical pathway, NF-κB is activated in response to a wide variety of stimuli that promote phosphorylation of I-κBα. Phosphorylated I-κBs are degraded after being conjugated with K48-linked ubiquitin chains, which releases NF-κB and allows its translocation to the nucleus to catalyze transcription of target genes. Thus, phosphorylation followed by degradative ubiquitination of I-kBα is a critical step in the pathway controlling NF-κB activation, and this step is catalyzed by an I-kBα kinase (IKK) complex consisting of the two kinases IKKα, IKKβ and the regulatory subunit IKKγ (also called NEMO) (Bonizzi and Karin, 2004). In recent years, the importance of polyubiquitin as a signaling molecule has come to the forefront of biochemical signaling research. In fact, it is now accepted that one way through which specific stimuli regulate activation of NF-κB is implemented through the non-degradative ubiquitination of the regulatory subunit NEMO (Chiu et al., 2009; Sun, 2011). The pathway that involves non-degradative ubiquitination of NEMO is particularly used by the TRAF family of adapter proteins, which has been shown to play an important role in several growth factor and cytokine signaling pathway, such as mitogen-activated protein kinases NF-κB and PI3K/Akt, in response to microbial, growth factor and cytokine stimuli (Deng et al., 2000; Geetha et al., 2005; Zotti et al., 2011). All TRAF proteins, except TRAF1, also contain a N-terminal RING domain common to E3 ubiquitin ligases, followed by several zinc fingers (Ha et al., 2009; Zotti et al., 2012). Within the TRAF family, TRAF6 has been shown to undergo lysine-63 (K63)-linked auto-ubiquitination (Deng et al., 2000), and to facilitate a diversity of signaling pathways by catalyzing K63 linked ubiquitination of specific substrates

☆☆ This work was in part supported by the Telethon grant GGP08125B.
* Corresponding author at: Dipartimento di Scienze e Tecnologie, Università degli Studi del Sannio, Via Port'Arsa 11, 82100 Benevento, Italy. Tel.: +39 3899385896.
E-mail addresses: vito@unisannio.it, vito@qu.edu.qa (P. Vito).
[1] These authors contributed equally to this work.

0161-5890
http://dx.doi.org/10.1016/j.molimm.2013.10.015

(Geetha et al., 2005; Kanayama et al., 2004). However, TRAF6 autoubiquitination was found not to be required for TRAF6 to ubiquitinate NEMO and promote optimal NF-κB activation following IL-1β stimulation (Walsh et al., 2008).

p62 (also known as sequestosome-1), is an adaptor protein involved in trafficking molecules to autophagy (Duran et al., 2011). In addition to these catabolic roles, p62 also regulates various signaling events, including the signal transduction pathways activated by receptors activated by tumor necrosis factor (TNF)-α, IL-1β, nerve growth factor, and RANK-L through scaffolding TRAF6 and atypical protein kinase C with these receptors (Sanz et al., 2000; Wooten et al., 2005a; Duran et al., 2004; Abbott et al., 2007).

In this study, we found that p62 interacts with and is required for TRAF6-mediated ubiquitination of NEMO and optimal IL-1β signaling.

2. Materials and methods

2.1. Cell culture, plasmids, antibodies and reagents

HEK293 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% FCS and transfected by calcium phosphate precipitation. Lentiviral vectors expressing shp62 RNAs were obtained from Sigma and used according to the manufacturer's instructions. The sequence of shp62 #3 used in this study is the following: CATTGAAGTTGATATCGATGTGGAGCACG.

Sources of antibodies and reagents were the following: anti-FLAG, anti-HA and anti-actin, Sigma; anti-NEMO, anti-ubiquitin, Santa Cruz Biotechnology; recombinant tumor necrosis factor α (TNFα,) was from Miltenyi Biotech; interleukin-1β (IL-1β) was obtained from Sigma.

2.2. Immunoblot analysis and coprecipitation

Cell lysates were made in lysis buffer (25 mM Tris–HCl pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% NP-40, 5% glycerol and a mixture of protease inhibitors). Proteins were separated by SDS-PAGE, transferred onto nitrocellulose membrane and incubated with primary antibodies followed by horseradish peroxidase conjugated secondary antibodies (Amersham Biosciences). Blots were developed using an ECL system (Pierce). For coimmunoprecipitation experiments, cells were lysed in lysis buffer and immunocomplexes were bound to protein A/G (Roche), resolved by SDS-PAGE and analyzed by immunoblot assay.

For the denaturing immunoprecipitation, 293T cells were transiently transfected at 60% confluency with retroviral vectors encoding the indicated shRNAs. Cell lysates prepared in lysis buffer (1% NP-40, 150 mM NaCl, 50 mM Tris–HCl pH 7.2, a protease inhibitors cocktail, 50 mM NaF, 1 mM NaOV, 25 mM beta-phosphoglycerate lysis) underwent anti-NEMO immmunoprecipitation for 4 hrs at 4 °C. Immunocomplexes were then dissociated in PBS containing 1% SDS at 37 °C for 1 h, diluted 10-fold and subjected to a second round of immunoprecipitation by anti-ubiquitin antibody. Immunocomplexes were then washed three times, resolved on 10% SDS-PAGE and immunoblotted as indicated.

2.3. Luciferase assay

To assess for NF-κB activation, HEK293 were transfected with the indicated plasmidic DNAs together with pNF-kB-luc (Clontech) in 6-well plates. After transfection and treatments, luciferase activity was determined with Luciferase Assay System (Promega). Plasmids expressing RSV-β-galactosidase were used in transfection mixture in order to normalize for efficiency of transfection.

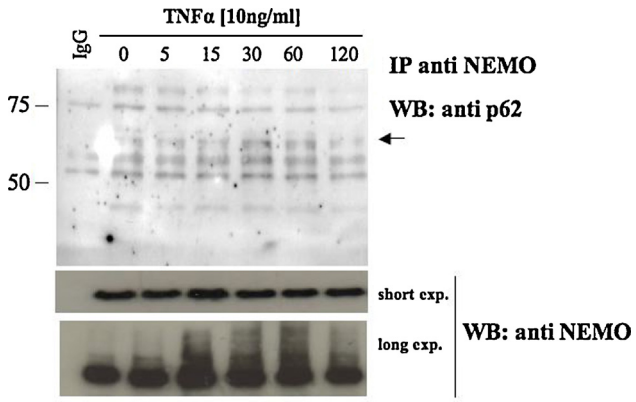

Fig. 1. p62 binds to NEMO. HEK293 cells were treated with TNFα (10 ng/ml) for the indicated time periods. Cell lysates were immunoprecipitated with anti-NEMO antibody or an isotype matched antibody (Ig) and analyzed by immunoblot probed with anti-p62. Membranes were also exposed to anti-NEMO antibody to monitor for efficiency of immunoprecipitation; different exposures for immunoprecipitated NEMO are shown.

2.4. Real-time PCR

Real-time PCR reactions were performed in triplicate as previously described (Vessichelli et al., 2012). The relative transcription level was calculated by using the ΔΔCt method.

3. Results and discussion

While searching for molecules that associating the IKK complex can regulate the NF-κB activity, we noticed that p62 coprecipitates with NEMO in lysates from cells stimulated with TNFα (Fig. 1).

As it is known that NF-κB activation may depend on the ubiquitination state of NEMO (Chen and Chen, 2013; Skaug et al., 2009), and that p62 regulates diverse signal transduction pathways including ubiquitination (Duran et al., 2004, 2011; Sanz et al., 2000; Wooten et al., 2005a), we verified whether p62 can modulate the ubiquitination of NEMO using a short interfering (sh) RNA approach. For this, we first assessed the efficiency of p62-specific shRNA (shp62) in reducing p62 mRNA expressed in HEK293 cells transfected with different sh or control shRNA. One of the p62-targeting sh tested (shp62 #3) results in a 10-fold reduction of p62 mRNA and protein expression (Fig. 2A), and was used for subsequent experiments. Indeed, as shown in Fig. 2B, the ubiquitination state of NEMO following stimulation with TNFα, assessed by immonoblot assay, is greatly reduced in cells that have been depleted of p62. Consistent results were obtained when the ubiquitination state of NEMO was monitored by immunoprecipitation of NEMO followed by immunoblot probed with anti-ubiquitin (Fig. 2C, upper panel) or anti-NEMO (Fig. 2C, lower panel). Also, ubiquitination of NEMO following IL-1β stimulation was impaired in cells that have been depleted of p62 (Fig. 2D). To exclude the possibility that the ubiquitinated material immunoprecipitated by the anti-NEMO antibody represents unrelated protein(s) with coprecipitanting with NEMO, we carried out a "denaturing" immunoprecipitation (see Section 2). The results of these experiments, shown in Fig. 2E, confirm that ubiquitination of NEMO following TNFα stimulation is greatly reduced in the cells depleted of p62. Similar results were obtained when cells were stimulate with IL-1β (data not shown).

We then verified whether in p62 depleted cells activation of NF-κB proceeds normally following stimulation with cytokines, using a luciferase-based NF-κB reporter assay. The results of these experiments, shown in Fig. 3, indicate that while the TNFα signal is normally transduced into cells, that of IL-1β is significantly compromised.

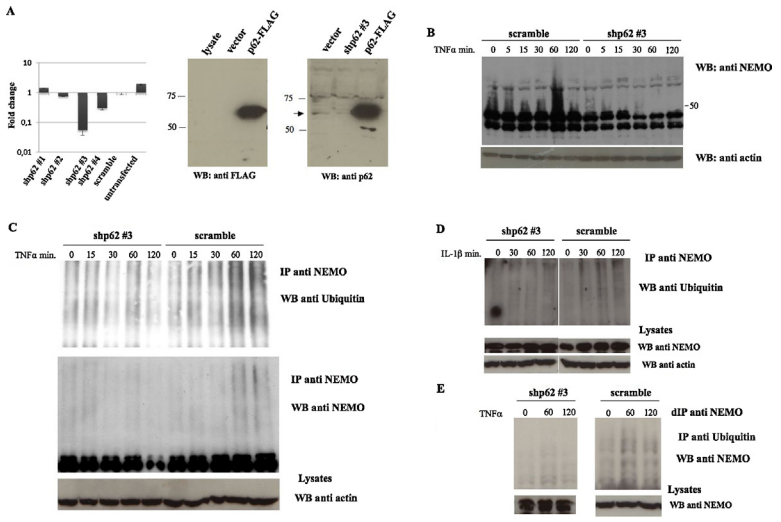

Fig. 2. p62 depletion impairs non-degradative NEMO ubiquitination. (A) *Left panel*: HEK293 cells were trasfected with lentiviral vectors encoding for 4 different shRNAs targeting human p62 or a control shRNA (scramble). After selection, p62 mRNA levels normalized to actin were quantified by real-time PCR. *Right panels*: HEK293 cells were transfected with empty vector or with vectors expressing a FLAG-tagged version of p62 or an shRNA targeting human p62, as indicated. 24 h later, cell lysates were separated by SDS-PAGE and blotted onto membranes probed with anti FLAG or anti p62 antibodies, as indicated. (B) Control and p62-depleted HEK293 cells were treated with TNFα (10 ng/ml) for the indicated time periods and the ubiquitination state of NEMO was monitored by immunoblot assay. (C) Control and p62-depleted HEK293 cells were stimulated with TNFα for the indicated time periods and cell lysates were immunoprecipitated with anti-NEMO antibody. The ubiquitination state of NEMO was monitored by immunoblot assay probed with anti-ubiquitin (upper panel) or anti-NEMO (lower panel). (D) Control and p62-depleted HEK293 cells were treated with IL-1β (20 ng/ml) and the ubiquitination state of NEMO was monitored as in C). (E) Control and p62-depleted HEK293 cells were stimulated with TNFα for the indicated time periods and cell lysates were immunoprecipitated with anti-NEMO antibody in denaturing condition (see Section 2). The ubiquitination state of NEMO was monitored by immunoblot assay probed with anti-ubiquitin.

Since it has been shown that p62 channels the signal that connects IL-1β receptor and TRAF6 to activation of NF-κB (Sanz et al., 2000), and knowing that this pathway may require ubiquitination of NEMO (Skaug et al., 2009; Tokunaga et al., 2009), we wanted to verify at which level p62 is involved in this pathway by monitoring the ubiquitination state of NEMO following TRAF6 expression. Indeed, in p62 depleted cells TRAF6 is unable to promote ubiquitination of NEMO (Fig. 4A), although still able to associate this protein (Fig. 4B).

In this manuscript, we have established an important point about the biology of p62. This multifunctional protein, involved in a diverse range of biological processes ranging from autophagy to cell transformation and to bone morphogenesis and cancer (Duran et al., 2004, 2011; Sanz et al., 2000; Wooten et al., 2005a; Moscat, 2012), has already been involved in the signal transduction pathway that starting from IL-1β receptor leads to activation of the transcription factor NF-κB (Sanz et al., 2000). Interestingly, p62 has also been shown to facilitate K63-polyubiquitination of TRAF6, thereby mediating nerve growth factor-induced activation of the NF-κB pathway (Wooten et al., 2005b). Furthermore, the ubiquitin-associated domain of p62 displays a preference for binding K63-polyubiquitinated substrates, supporting the hypothesis that p62 may act as a critical ubiquitination modulating factor (Seibenhener et al., 2004). In this context, the finding here reported that p62 also regulates the ubiquitination state of NEMO adds another important piece to our understanding of the ubiquitination events of that occur in the cell and to our comprehension of the of the molecular mechanisms that regulate activation of NF-κB.

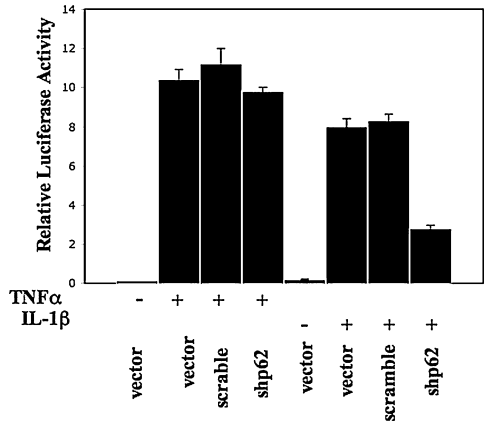

Fig. 3. p62 depletion impairs NF-κB activation following IL-1β stimulation. HEK293 cells expressing shRNAs targeting human p62 or a control shRNA (*scramble*) were cotransfected with pNF-κB – luciferase plasmid and a β-galactosidase reporter vector. 24 h later, cells were treated with the indicated stimuli (TNFα, 20 ng/ml; IL-1β, 20 ng/ml) for 4 h and luciferase activity was determined. Data shown represents relative luciferase activity normalized against β-galactosidase activity and is representative of six independent experiments performed in triplicate.

Non-degradative ubiquitination has gained attention in recent years as a mechanism of protein scaffold building for complex formation during signal-induced NF-κB activation pathway (Chiu et al., 2009; Skaug et al., 2009). This concept is further strengthened by the fact that deubiquitinating enzymes, which disassemble polyubiquitin chains or remove ubiquitin from a substrate, are clearly involved in downregulating NF-κB (Harhaj and Dixit, 2011; Sun, 2010; Hymowitz and Wertz, 2010; Stilo et al., 2008).

Our results also indicate that the requirement for p62 in ubiquitination of NEMO and NF-κB activation appear to be different for TNFα and IL-1β stimulation. In fact, while p62 is required for ubiquitination of NEMO following stimulation with both cytokines, only NF-κB activation in response to IL-1β treatment is compromised in cells depleted of p62. This data is in agreement with a recent work by Xu et al., in which, using ubiquitin-replacement technology, K63 chains were shown to be indispensable for IL-1β-induced, but not TNFα-induced, NF-κB activation (Xu et al., 2009). Thus, although ubiquitin chains play crucial roles in signaling and possibly in IL-1β-induced NF-κB activation, they may not be essential for canonical NF-κB activation, at least in some occasions, such as TNFα stimulation.

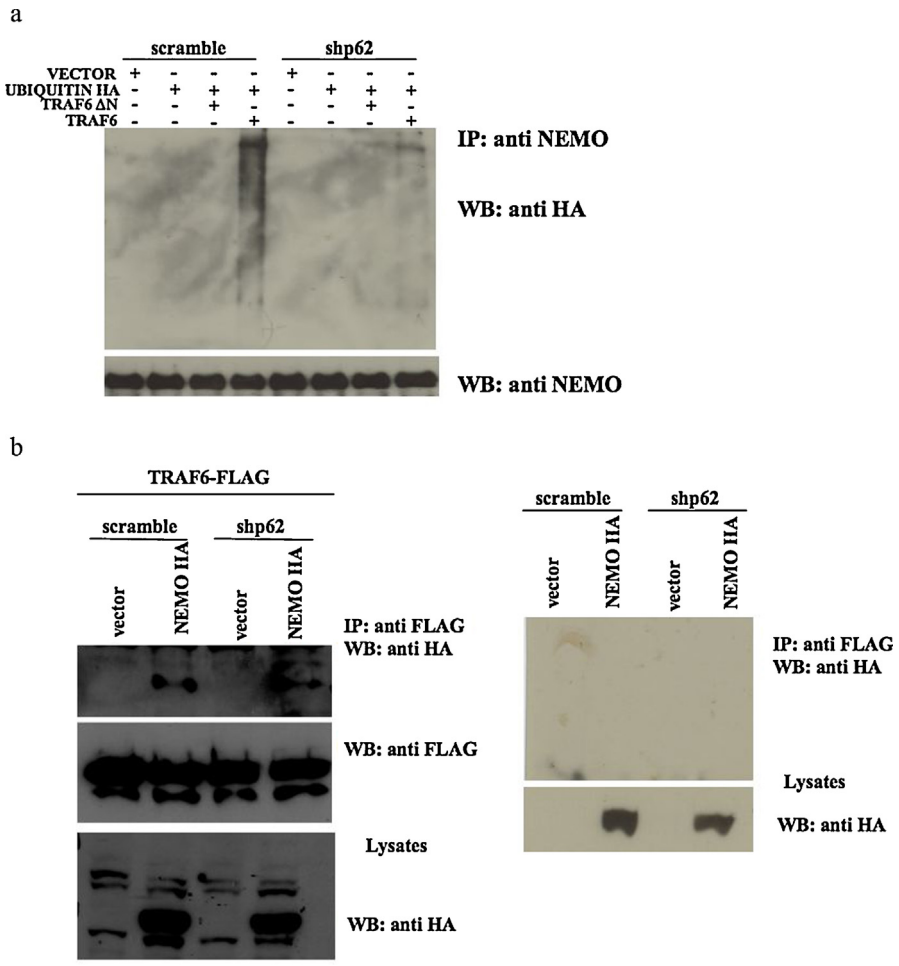

Fig. 4. p62 depletion impairs TRAF6-mediated ubiquitination of NEMO. (A) HEK293 cells were infected with lentiviral vectors encoding for shRNAs targeting p62 or a control sequence (scramble). After selection, cells were transiently cotransfected with an expression vector encoding for full-length TRAF6 or a deleted version of TRAF6 lacking the E3 ubiquitin ligase domain (TRAF6ΔN), together with a vector expressing HA-tagged ubiquitin, as indicated. 24 h after transfection, cell lysates were immunoprecipitated with anti-NEMO antibody and analyzed by immunoblot probed with anti-HA. (B) *Left panel*: HEK293 cells were cotrasfected with the indicated constructs; cell lysates were immunoprecipitated with anti-FLAG antibody and analyzed by immunoblot probed with anti-HA. *Right panel*: control for specific binding of NEMO with TRAF6.

In any case, much more work is required to clarify the molecular details of the signal transduction pathways involving p62 and ubiquitination events in general.

References

Abbott, D.W., Yang, Y., Hutti, J.E., Madhavarapu, S., Kelliher, M.A., et al., 2007. Coordinated regulation of Toll-like receptor and NOD2 signaling by K63-linked polyubiquitin chains. Mol. Cell. Biol. 27, 6012–6025.

Baltimore, D., 2009. Discovering NF-κB. Cold Spring Harb. Perspect. Biol. 1, a000026.

Bonizzi, G., Karin, M., 2004. The two NF-κB activation pathways and their role in innate and adaptive immunity. Trends Immunol. 25, 280–288.

Chen, J., Chen, Z.J., 2013. Regulation of NF-κB by ubiquitination. Curr. Opin. Immunol. 25 (1), 4–12.

Chiu, Y.H., et al., 2009. Ubiquitin in NF-κB signaling. Chem. Rev. 109, 1549–1560.

Deng, L., Wang, C., Spencer, E., Yang, L., Braun, A., et al., 2000. Activation of the IkappaB kinase complex by TRAF6 requires a dimeric ubiquitin-conjugating enzyme complex and a unique polyubiquitin chain. Cell 103, 351–361.

Duran, A., et al., 2004. The atypical PKC-interacting protein p62 is an important mediator of RANK-activated osteoclastogenesis. Dev. Cell 6, 303–309.

Duran, A., et al., 2011. p62 is a key regulator of nutrient sensing in the mTORC1 pathway. Mol. Cell 44, 134–146.

Geetha, T., Jiang, J., Wooten, M.W., 2005. Lysine 63 polyubiquitination of the nerve growth factor receptor TrkA directs internalization and signaling. Mol. Cell 20, 301–312.

Ha, H., Han, D., Choi, Y., 2009. TRAF-mediated TNFR-family signaling. Curr. Protoc. Immunol. 11 (Charter 11: Unit 11.9D).

Harhaj, E.W., Dixit, V.M., 2011. Deubiquitinases in the regulation of NF-κB signaling. Cell Res. 21, 22–39.

Hayden, M.S., Ghosh, S., 2008. Shared principles in NF-κB signaling. Cell 132, 344–362.

Hymowitz, S.G., Wertz, I.E., 2010. A20: from ubiquitin editing to tumour suppression. Nat. Rev. Cancer 10, 332–341.

Kanayama, A., Seth, R.B., Sun, L., Ea, C.K., Hong, M., et al., 2004. TAB2 and TAB3 activate the NF-kappaB pathway through binding to polyubiquitin chains. Mol. Cell 15, 535–548.

Moscat, J., 2012. Diaz-Meco MT. p62: a versatile multitasker takes on cancer. Trends Biochem. Sci. 37 (June (6)), 230–236.

Sanz, L., et al., 2000. The atypical PKC-interacting protein p62 channels NF-kappaB activation by the IL-1–TRAF6 pathway. EMBO J. 19, 1576–1586.

Seibenhener, M.L., Babu, J.R., Geetha, T., Wong, H.C., Krishna, N.R., Wooten, M.W., 2004. Sequestosome 1/p62 is a polyubiquitin chain binding protein involved in ubiquitin proteasome degradation. Mol. Cell. Biol. 24 (18), 8055–8068.

Skaug, B., et al., 2009. The role of ubiquitin in NF-κB regulatory pathways. Annu. Rev. Biochem. 78, 769–796.

Stilo, R., Varricchio, E., Liguoro, D., Leonardi, A., Vito, P., 2008. A20 is a negative regulator of BCL10- and CARMA3-mediated activation of NF-kappaB. J. Cell Sci. 121 (Pt 8), 1165–1171.

Sun, S.C., 2010. CYLD: a tumor suppressor deubiquitinase regulating NF-κB activation and diverse biological processes. Cell Death Differ. 17, 25–34.

Sun, S.C., 2011. Non-canonical NF-κB signaling pathway. Cell Res. 21, 71–85.

Tokunaga, F., et al., 2009. Involvement of linear polyubiquitylation of NEMO in NF-κB activation. Nat. Cell Biol. 11, 123–132.

Vessichelli, M., et al., 2012. Neuroepithelial transforming gene 1 (Net1) binds to caspase activation and recruitment domain (CARD)- and membrane-associated guanylate kinase-like domain-containing (CARMA) proteins and regulates nuclear factor κB activation. J. Biol. Chem. 287 (17), 13722–13730.

Walsh, M.C., Kim, G.K., Maurizio, P.L., Molnar, E.E., Choi, Y., 2008. TRAF6 autoubiquitination-independent activation of the NFkappaB and MAPK pathways in response to IL-1 and RANKL. PLoS ONE 3 (12), e4064.

Wooten, M.W., et al., 2005a. The p62 scaffold regulates nerve growth factor-induced NF-kappaB activation by influencing TRAF6 polyubiquitination. J. Biol. Chem. 280, 35625–35629.

Wooten, M.W., Geetha, T., Seibenhener, M.L., Babu, J.R., Diaz-Meco, M.T., Moscat, J., 2005b. The p62 scaffold regulates nerve growth factor-induced NF-kappaB activation by influencing TRAF6 polyubiquitination. J. Biol. Chem. 280 (42), 35625–35629.

Xu, M., et al., 2009. A Ubiquitin replacement strategy in human cells reveals distinct mechanisms of IKK activation by TNFa and IL-1b. Mol. Cell 36, 302–314.

Zotti, T., et al., 2011. TRAF7 protein promotes Lys-29-linked polyubiquitination of IkappaB kinase (IKKgamma)/NF-kappaB essential modulator (NEMO) and p65/RelA protein and represses NF-kappaB activation. J. Biol. Chem. 286 (26), 22924–22933.

Zotti, T., Vito, P., Stilo, R., 2012. The seventh ring: exploring TRAF7 functions. J. Cell. Physiol. 227 (3), 1280–1284.