NEMO interaction with linear and K63 ubiquitin chains contributes to NF-κB activation

Kamyar Hadian1,2+, Richard A. Griesbach1,+, Scarlett Dornauer2, Tim M. Wanger1, Daniel Nagel1, Moritz Metlitzky1, Wolfgang Beisker1, Marc Schmidt-Supprian3 and Daniel Krappmann1, *

Helmholtz Zentrum München – German Research Center for Environmental Health, Institute of Toxicology, Ingolstädter Landstrasse 1, 85764 Neuherberg, Germany, 1 Department Cellular Signal Integration, 2 Assay Development and Screening Platform
3 Max-Planck-Institute for Biochemistry, Institute of Molecular Immunology and Signaltransduction, Am Klopferspitz 18, 82152 Martinsried, Germany

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+ These authors contributed equally to this work
*Address correspondence to: Dr. Daniel Krappmann, Email: daniel.krappmann@helmholtz-muenchen.de, Phone: ++49-(0)89-3187-3461, Fax: ++49-(0)89-3187-3449

The IκB kinase (IKK) complex acts as a gatekeeper of canonical NF-κB signaling in response to upstream stimulation. IKK activation requires sensing of ubiquitin chains by the essential IKK regulatory subunit IKKγ/NEMO. However, it has remained enigmatic whether NEMO binding to K63-linked or linear ubiquitin chains is critical for triggering IKK activation. We show here that the NEMO C-terminus, comprising the ubiquitin binding region and a zinc finger, has a high preference for binding to linear ubiquitin chains. However, immobilization of NEMO, which may be reminiscent of cellular oligomerisation, facilitates the interaction with K63 ubiquitin chains. Moreover, selective mutations in NEMO that abolish association with linear ubiquitin but do not affect binding to K63 ubiquitin are only partially compromising NF-κB signaling in response to TNFα stimulation in fibroblasts and T cells. In line with this, TNFα triggered expression of NF-κB target genes and induction of apoptosis was partially compromised by NEMO mutations that selectively impair the binding to linear ubiquitin chains. Thus, in vivo NEMO interaction with linear and K63 ubiquitin chains is required for optimal IKK activation, suggesting that both type of chains are cooperating in triggering canonical NF-κB signaling.

INTRODUCTION

Induction of gene expression by the transcription factor NF-κB controls many physiological processes, including immunity, differentiation or apoptosis. The IκB kinase (IKK) complex acts as the gatekeeper of canonical NF-κB signaling. In response to extracellular stimuli, IKKs become activated and catalyze the phosphorylation of NF-κB inhibitors (IκB), leading to their ubiquitination and proteasomal degradation (1,2). The IKK complex consists of the two catalytic domains IKKα and IKKβ and the regulatory subunit IKKγ or NEMO (NF-κB essential modulator). NEMO mutations scattered throughout the entire gene are causing severe pathologies like EDA-ID (anhidrotic ectodermal dysplasia with immunodeficiency) and IP (incontinentia pigmenti) (3-6).
NEMO serves as a critical integrating platform coupling upstream receptor signaling to the canonical NF-κB pathway. Biochemical and genetic studies have highlighted a pivotal function of poly-ubiquitination for IKK/NF-κB activation. Upon TNFα or TCR/CD28 stimulation, signaling adaptors RIP1 or MALT1 are modified by covalent attachment of K63 linked ubiquitin (Ubi) chains to recruit NEMO and thereby promote IKK activation (7-9). Recently, the assembly of linear Ubi chains by the LUBAC (linear Ubi chain assembly complex) was shown to promote cytokine triggered IKK activation (10). The Ubi binding surface in NEMO called UBAN (Ubi binding in ABIN and NEMO) or NOA (NEMO-OPTINEURIN-ABIN) is required for signal induced IKK activation. NEMO UBAN has been co-crystallized with linear as well as with K63 Ubi chains (11,12). The crystal structures reveal a bi-partite Ubi binding region from aa 290 to 330, distinguishing between a proximal and a distal moiety of bound diUbi.

In solution, linear diUbi was found to bind with ~100 fold higher affinity to the UBAN than K63 Ubi (13). However, the C-terminal zinc finger (ZF) of NEMO may contribute a second Ubi binding site (14), and it was reported that the UBAN in conjunction with ZF (NEMO UBAN-ZF) displays similar affinities for linear or K63 tetraUbi (15). Thus, the efficiency of linear versus K63 Ubi chains to bind to NEMO in vitro as well as the physiological relevance of these associations for IKK activation has remained unresolved.

Using in vitro association studies we provide evidence that the entire NEMO C-terminus displays a high preference for interacting with linear Ubi chains. However, depending on the assay, also K63 Ubi chains can associate weakly with NEMO through the UBAN domain. By analyzing NEMO UBAN mutations that selectively interfere with binding to linear but not K63 Ubi chains we show that canonical IKK/NF-κB activation is partially compromised upon loss of NEMO binding to linear Ubi chains. Hence, in vivo, different Ubi chains can contribute to optimal NF-κB activation.

**EXPERIMENTAL PROCEDURE**

Prokaryotic expression plasmids- The vector system pASK-IBA3plus (IBA GmbH, Göttingen, Germany) was used for production of bacterial recombinant proteins. This vector is inducible with anhydrotetracycline (ATC, IBA GmbH). Using SacII and NcoI restriction sites, the NEMO sequences were cloned into the multiple cloning site (MCS) leading to in frame fusion of the StrepTagII sequence to the C-terminus. N-terminal StrepTagII was introduced using the 5'-primer. With the 3'-primer stop codons were introduced before the StrepTagII sequence to prevent fusion of the StrepTagII to the C-terminus. HisTag ubiquitin was also cloned into pASK-IBA3plus using the same strategy like N-terminally Strep-tagged NEMO.

Eucaryotic expression plasmids- In the pPHAGE plasmid, a third generation lentiviral SIN non-replicative vector, the truncated human CD2 (hΔCD2) and human NEMO cDNA were linked by the 18 aa T2A sequence from *Thosea asigna*. The T2A linker co-translationally prevents the formation of its last peptide bond, which allows concomitant expression of two genes under the control of one promoter, in this case the phosphoglycerate kinase (PGK) promoter.

The empty PGK-PHAGE plasmid was supplied with a NotI-SalI-BamHI linker. Subsequently, hΔCD2 (without STOP codon) and the T2A sequence were inserted in frame, each with an EagI/NotI restriction site deletion strategy. Thus, the only remaining NotI site was situated behind PGK-hΔCD2-T2A and could be used to insert NotI-Flag-hNEMO-NotI wt or mutated fragments in frame.

Antibodies and Reagents- Cells were treated with polybrene (Sigma), TNFα (Biomol), and PMA/Ionomycin (Calbiochem).
The following antibodies were used: APC-labeled anti-human CD2 (eBioscience), anti-IκBα (sc-371), anti-NEMO (sc-8330), anti-β-actin (sc-1616; all Santa Cruz), anti-p-IκBα (Cell Signaling), anti-IKKα (05-536; Millipore), anti-IKKβ (05-535; Millipore), anti-FLAG M2 (F3165, Sigma). Anti-HisTag DELFIA Europium-N1 (AD0108; Perkin Elmer)

Protein expression and purification-Recombinant proteins were produced in E. coli strain BL21 codonplus (DE3) RILP (Stratagene) using the pIBA3plus expression system (IBA GmbH, Göttingen, Germany). Bacteria were grown to a density of an OD 600 value of 0.8 - 1.0. Protein expression was induced by addition of 1 mM IPTG and 200 ng/ml anhydrotetracycline (IBA GmbH) and bacteria were cultured o/n at 21°C. Cultures were harvested by centrifugation at 3000 x g and pellets were resuspended in lysis buffer (StrepTagII purification: 100 mM Tris/HCl pH 8.0, 150 mM NaCl, 0.5 mM DTT, 0.5 mg/ml lysozyme and protease inhibitors; His-Tag purification: 75 mM phosphate buffer pH 7.4, 400 mM NaCl, 10 mM imidazol and protease inhibitors) at 4°C. After 20 minutes at RT, the culture suspension was sonicated on ice. 20 minutes centrifugation at 20000 x g (4°C) led to clarification of the lysate from cellular debris. Finally, the supernatant was again centrifuged at 20000 x g (4°C).

Bacterial lysates containing the Streptagged proteins of interest were applied on the StrepTrap columns (GE Healthcare), and bacterial proteins were washed away using washing buffer (100 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.5 mM DTT). Target proteins were eluted with an elution buffer (100 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.5 mM DTT, 2.5 mM D-desthiobiotin). His-tagged proteins were applied on HisTrap columns (GE Healthcare), bacterial proteins were washed away with washing buffer (20 mM phosphate buffer pH 7.4, 500 mM NaCl, 30 mM imidazol) and His-tagged proteins were eluted using a gradient of elution buffer (20 mM phosphate buffer pH 7.4, 500 mM NaCl, 500 mM imidazol). Elution fractions containing the proteins of interest were pooled and concentrated using Amicon Ultra-15 spin columns (Millipore) with an exclusion size of 3 kDa.

K63-diUbi was generated by in vitro ligation of His-Ubi K63R and Ubi-D77 (16). By using these two mutations, directed synthesis of diUb is feasible. To generate the K63-diUbi, the ubiquitin conjugation kit (Boston Biochem, USA) was used in combination with His-Ubi K63R, Ubi-D77, the activating E1 enzyme UBE1 (Boston Biochem, USA) and the K63-specific E2 complex UbcH13/Uev1a (Boston Biochem, USA). Ligation reaction was incubated for 4 hours at 37°C. Excess of non-ligated mono-ubiquitin was removed by subsequent gel filtration (see Suppl. Fig. 1).

Microscale thermophoresis (MST)- The labeling was performed with a reactive dye (NT-647) using N-hydroxy succinimide (NHS)-ester chemistry, which reacts efficiently with the primary amines of proteins to form highly stable dye-protein conjugates. The labeling procedure was performed as previously described (17). MST assay were mainly carried out as described in Wienken et. al. Serial dilutions of unlabeled NEMO proteins were mixed with 200 nM of NT-647-labeled Ubi proteins in MST buffer (25 mM Tris/HCl pH 8.0, 100 mM NaCl, 0.1 % BSA, 0.1 % Tween-20, 0.5 mM DTT) and incubated for 30 minutes. MST assays were measured in a NanoTemper Monolith NT.015T. By plotting NEMO concentration to % changes of normalized fluorescence (ΔFnorm [%]), curve fitting was performed using GraphPad prism software and Kₐ values were determined.

DELFIA Assay (ELISA-based plate assay)-DELFIA ELISA assays were performed using the manufactory’s protocol (Perkin Elmer). 20 pmol of Streptagged NEMO proteins in
DELFIA assay buffer (Perkin Elmer) were coupled to StrepTactin coated 96-well plates (IBA GmbH). After 2 hours of incubation the unbound fraction was washed away using DELFIA washing buffer (Perkin Elmer) and bound NEMO proteins were incubated with 250 pmol of His-tagged Ubi proteins in DELFIA assay buffer. Again after 2 hours of incubation excess of Ubi proteins were washed away (with DELFIA washing buffer) and 500 ng/ml Europium (Eu)-labeled anti-HisTag antibody in DELFIA assay buffer was added to the reaction for 1 hour. After extensive washing (with DELFIA washing buffer), enhancer solution (Perkin Elmer) was added to activate Eu-fluorescence and this was measured in a BioTek Synergy 2 fluorescence plate reader (Ex: 340 nm – Em: 615 nm).

Cell culture, lentiviral infection, EMSA, Western Blot and Co-IP- MEF and HEK 293T cells were grown in DMEM, Jurkat T cells were cultured in RPMI supplemented with 10% FCS according to standard procedures. Lentiviruses were produced according to (18,19). In short, proviral plasmids (pHAGE-PGK-hΔCD2-T2A-NEMO, pMDL, pVSVG and pREV) were transfected in HEK 293T cells by calcium chloride precipitation. NEMO−/− MEF were incubated with the sterile-filtrated viral supernatant harvested from transfected HEK 293T cells by calcium chloride precipitation. NEMO−/− MEF were incubated with the sterile-filtrated viral supernatant harvested from transfected HEK 293T cells in the presence of 8 µg/ml polybreine for 72 h. Infected cells expressing hΔCD2 were stained with APC-labeled anti-human CD2 and enriched by FACS sorting. A hΔCD2-low population was sorted to yield exogenous NEMO expression at the level of endogenous NEMO from wt MEF. Reconstitution of NEMO deficient Jurkat T cells was performed accordingly. Cells were subsequently expanded and stimulated with 8 ng/ml or 20 ng/ml TNFα (MEF or Jurkat cells, respectively), 400 ng/ml PMA and 600 ng/ml ionomycin (P/I). EMSA and Western blotting was carried out as described previously (20). NEMO-IKKα/β interaction was investigated after Flag IP of Flag-NEMO constructs and subsequent detection of IKKα and IKKβ by Western Blotting. Co-IP and Western Blotting was done as described in (21).

Quantitative Real-Time PCR- Reconstituted MEF were treated with 8 ng/ml TNF alpha for 1 h. mRNA was isolated with QIAGEN RNeasy Mini Kit and subsequently DNase-treated (Promega). cDNA was synthesized using the Invitrogen SuperScript II kit with poly-dT primer. qRT-PCR was basically performed as previously described (21). For data analysis CP values were first normalized to the levels of the murine housekeeping gene porphobilinogen deaminase (PBGD) and afterwards mRNA levels of stimulated samples were related to the values of the corresponding unstimulated control. Finally, levels of wt reconstituted cells were set to 1 and all other data related to this. Following primers were used A20: 5’-GCTCAACTGGTGTCTGAAG-3’ and 5’-ATGAGGCAAGTTCCATCAC-3’; CXCL2: 5’-AGTGAACTGCGCTGAC-3’ and 5’-CTTCAGGGCTAAGGCAA-3’; ICAM1: 5’-GGAGACGCAGAGACCTAAC-3’ and 5’-CGCTCAAGAGGACACCTTCC-3’; PBGD: 5’GGCAGACCTGATGTTGGA-3’ and 5’TGAAGGAAAGGGACATATGG3’

Apoptosis assay- Rate of apoptosis was determined after 22 hours of 8 ng/ml TNFα stimulation in MEF by PE–annexin V and 7AAD staining using the BD Pharmingen apoptosis kit. PE-annexin V stains pre-apoptotic cells and 7AAD dead cells. FACS assays were measured on an LSRII flow cytometer (BD), and data evaluation was carried out using FlowJo software (Treestar).

RESULTS

The NEMO C-terminus is selectively binding to linear Ubi chains in solution- We used microscale thermophoresis (MST), a sensitive type of protein interaction assay, to
investigate the association of the NEMO C-terminus (amino acids 242-419) with fluorescence-labeled diUb or tetraUb in solution. Binding of an interaction partner influences the thermal migration behavior of ubiquitin and the fluorescence depletion in a heated spot of the protein solution was measured in dependency of increasing interactor concentration. K_D values were derived from the depletion curves. All NEMO UBAN-ZF fragments were C- or N-terminally fused to StrepTagII and purified from E. coli (Figure 1A, Supplementary Figure 1A and C). Binding of linear diUb to NEMO UBAN-ZF is evident from the shift in thermal migration of linear diUb induced upon incubation with increasing amounts of NEMO UBAN-ZF (Supplementary Figure 2A). For the binding of linear diUb to UBAN-ZF we determined a K_D of ~3.2 µM (Figure 1B), which is in the range measured by isothermal titration calorimetry for linear diUb and the NEMO UBAN alone (13,22). We were unable to detect an association between K63 diUb and the NEMO UBAN-ZF (Supplementary Figure 2B; Figure 1C). Similar results were obtained with N-terminally StrepTagII tagged NEMO UBAN-ZF and linear or K63 diUb chains (Figure 1D), excluding the possibility that the C-terminal Tag interferes with the function of the ZF.

As longer K63 Ubi chains were suggested to enhance the affinity to the C-terminus of NEMO (15), we performed MST using linear or K63 tetraUb. Whereas the affinity of linear tetraUb towards C or N-terminally StrepTagII tagged UBAN-ZF was even enhanced compared to linear diUb (K_D ~ 0.34 or 0.94 µM, respectively), a K63 tetraUb did not bind to NEMO independent of the position of the StrepTagII (Figure 1E and F). These data suggest that longer Ubi chains are not per se enhancing the affinity between K63 Ubi chains and NEMO in solution. To verify that K63 diUb and tetraUb are functional, we determined their association to the NZF of TAB2 (TBD-NZF), which was shown to present a selective surface for the interaction with K63 Ubi chains (23). As expected, TBD-NZF bound to K63 diUb and tetraUb with a K_D of ~21.3 µM and ~ 6.9 µM, respectively. However, no association to linear Ubi was observed (Figure 1G and H).

We also determined association of either linear or K63 tetraUb to NEMO UBAN-ZF in StrepTagII pull down experiments and confirmed that the C-terminus of NEMO is exclusively precipitating linear tetraUb after incubation of individual chains or in a mixed chain reaction (Figure 2A and B). Taken together, MST data and pull down experiments provide evidence that in solution the NEMO C-terminus is associating to linear Ubi chains with K_D in the low micromolar range, whereas a putative association to K63 Ubi chains is beyond the limit of detection.

Immobilization of NEMO UBAN-ZF enhances binding to K63 Ubi- Despite the apparent inability of K63 diUb to associate with the NEMO C-terminus in solution, K63 diUb and the NEMO UBAN have been co-crystallized and were found to bind when NEMO UBAN was coated on a biosensor (12,24). We hypothesized that a more static orientation of the NEMO C-terminus might facilitate the interaction of the UBAN with K63 diUb. Therefore, we established DELFIA assays as a very sensitive sandwich-based ELISA method to measure interaction of plate bound NEMO UBAN-ZF with diUb (Figure 3A). For this purpose, StrepTagII tagged NEMO UBAN-ZF was attached to StrepTactin coated plates and incubated with His tagged monoUb, linear diUb or K63
 Binding of NEMO UBAN-ZF to His-Ubi proteins was detected by time-resolved fluorescence (TRF) after extensive washing, using Europium (Eu) labeled anti-His antibody. Interaction of linear His-diUbi with NEMO UBAN-ZF was evident from a strong TRF signal (Figure 3B). Again, the substitution D311N in the NEMO UBAN completely prevented binding to linear His-diUbi. To validate the assay, we tested binding of His-monoUbi to NEMO UBAN-ZF. MonoUbi did not interact with NEMO UBAN-ZF, providing evidence for the reliability of the assay (Figure 3B).

Next, we compared the interaction between linear His-diUbi and K63 His-diUbi with the NEMO UBAN-ZF (Figure 3C). In this setup, we detected binding of the NEMO UBAN-ZF to K63 His-diUbi, even though the TRF signal was ~ 7-8 fold decreased when compared to linear His-diUbi. This reduced affinity of K63 diUbi compared to linear diUbi is in the range that has been observed in a biosensor assay where the NEMO UBAN alone was immobilized (24). The substitution D311N completely prevented association to K63 diUbi, proving that this interaction critically depends on the binding of the distal Ubi moiety to the N-terminal part of the UBAN (12). To determine if longer K63 Ubi chains could enhance the interaction, we used a mixture of K63 Ubi chains ranging from 2-7 Ubi (K63 His-Ubi<sub>2-7</sub>) in length (Figure 3D). Association of K63 His-Ubi<sub>2-7</sub> was slightly enhanced when compared to K63 diUbi. To ascertain that the DELFIA assay did not generally prefer detection of linear versus K63 diUbi, we compared binding of K63 His-Ubi<sub>2-7</sub> and linear His-diUbi to TAB2 NZF fused to StrepTagII. Also in the plate bound assay the NZF domain of TAB2 bound with high preference to K63 Ubi chains when compared to linear diUbi (Figure 3E).

The C-terminal part of UBAN domain is selectively required for association to linear diUbi. The crystal structures of the NEMO UBAN domain bound to linear or K63 diUbi reveal that the distal Ubi moiety is associating with the N-terminal part of the UBAN domain that contains D311 (11,12). Due to the different positioning of the proximal Ubi in linear and K63 diUbi, the C-terminal part of the UBAN domain binds to the proximal Ubi moiety only in the context of linear diUbi (Figure 4A). We purified NEMO UBAN-ZF proteins where residues in the C-terminal part of the UBAN are mutated that are exclusively contacting the distal moiety of linear diUbi (Supplementary Figure 1B). Since Ubi binding requires NEMO dimerization through the surrounding CC2-LZ region (25), we first verified by gel filtration that NEMO UBAN-ZF wt and mutations were predominantly forming solution dimers, ruling out that the mutations are distorting the overall structure of the NEMO UBAN-ZF (Supplementary Figure 3). DELFIA assays revealed that the point mutation of E320A decreased the interaction of NEMO UBAN–ZF with linear diUbi by ~75 % and that the combined mutation of all glutamates (E320A/E324A/E327A) led to 90 % reduced binding (Figure 4B). Moreover, mutations R319A/E320A and R316A/R319A/E320A abolished the association to linear diUbi to the same extent as the D311N mutation in the DELFIA assays. Importantly, all the distal NEMO UBAN-ZF mutations are still binding to the K63 Ubi, confirming that the C-terminal UBAN domain is exclusively responsible for contacting the linear Ubi (Figure 4B). To verify the results, we also determined the affinity of the triple NEMO UBAN-ZF mutations E320A/E324A/E327A and R316A/R319A/E320A to linear diUbi in solution by MST (Figure 4C). Whereas the triple glutamate mutation still bound linear diUbi, even though with a decreased affinity when compared to NEMO UBAN-ZF wt ($K_D \sim 11.4 \, \mu M$ versus $\sim 3.2 \, \mu M$) (compare Figure 1C), mutation of the contact residues
R316A/R319A/E320A completely abolished association of the NEMO C-terminus and linear diUb in solution (Figure 4C).

Partial impairment of NF-κB signaling by selective interference with NEMO binding to linear Ubi- To analyze the functional impact of NEMO UBAN mutations on TNFα induced NF-κB activation we reconstituted NEMO deficient (NEMO<sup>−/−</sup>) mouse embryonal fibroblasts (MEF). Overexpression of NEMO was shown to inhibit NF-κB activation (26,27). Thus, we chose a lentiviral expression system to co-express Flag-NEMO together with the cell surface marker hΔCD2 to be able to sort for cell expressing different NEMO constructs at equivalent levels in the range of the endogenous protein (Figure 5). Flag-NEMO and hΔCD2 were separated by the co-translational processing sequence T2A ((28); Figure 5A). This allowed us to FACS sort for cells expressing equal NEMO amounts. Re-staining of sorted MEF (Figure 5B) revealed that surface amounts of hΔCD2 directly correlated with intracellular NEMO amounts (Figure 5C and D). Comparison of the TNFα induced NF-κB signaling in reconstituted hΔCD2/NEMO high and low cells showed that increasing amounts of NEMO are indeed strongly impairing NF-κB activation (Figure 5D). Thus, all subsequent reconstitution analyses were carried out with MEF expressing equivalent amounts of wt and mutated NEMO protein at levels slightly above the endogenous NEMO levels in wt MEF (Figure 6A and B left panels). NF-κB signaling was determined by IkBα and phospho-IκBα Western Blotting (WB) and measurement of NF-κB DNA binding by EMSA (Figure 6A and B and Supplementary Figure 4A). Quantification of EMSA results is shown in Figure 6C. As expected, UBAN mutation D311N, which abolished association of linear and K63 Ubi <em>in vitro</em>, is strongly impaired in TNFα dependent NF-κB activation. NEMO carrying the UBAN mutations E320A or E320A/E324A/E327A, which displayed a diminished binding to linear Ubi, efficiently mediated TNFα induced NF-κB activation similar to NEMO wt (Figure 6B and C). Mutations R319A/E320A or R316A/R319A/E320A that completely abrogated association of linear but not K63 Ubi, only partially impaired reconstitution of NF-κB signaling (Figure 6A and C). To exclude that mutations in the NEMO UBAN affect IKK complex assembly, we immunoprecipitated Flag-NEMO from wt, D311N, E320A/E324A/E327A and R316A/R319A/E320A reconstituted MEF and probed for IKKα and IKKβ. As expected, the mutations had no influence on IKK complex composition (Supplementary Figure 4B). Taken together, our results suggest that NEMO binding to linear Ubi chains contributes, but is not exclusively responsible for optimal NF-κB activation.

To verify the results in an independent cellular system, we infected NEMO deficient Jurkat T cells by the same lentiviral approach (Figure 6D). FACS sorted hΔCD2 positive cells expressed NEMO at comparable levels slightly below the endogenous level of Jurkat T cells (left panel). Next, we compared NF-κB activation in response to TNFα (middle) or PMA/Ionomycin (P/I; right) stimulation after reconstitution. Similarly to the results obtained in MEF, the mutation of D311N in NEMO nearly abolished NF-κB activation in response to both stimuli. Whereas NEMO E320A/E324A/E327A was almost fully functional in rescuing TNFα or P/I induced NF-κB activation, the triple R316A/R319A/E320A exchange impaired, but did not completely block NF-κB signaling. Thus, the C-terminal part of the UBAN that contacts the proximal Ubi moiety in linear Ubi is not only contributing to canonical NF-κB in response to TNFα stimulation, but linear chains are also involved in PKC0 dependent IKK/NF-κB activation in T cells.
NEMO binding to linear Ubi partially impairs induction of NF-κB target genes and prevention of apoptosis. To gain more insights into the functional impact of selective disruption of NEMO-linear Ubi binding, we monitored TNFα induced expression of the NF-κB target genes A20 (TNFα induced protein 3, TNFAIP3), CXCL2 (Chemokine (C-X-C-motif) ligand 2) and ICAM1 (Intercellular adhesion molecule 1) by qRT-PCR in MEF reconstituted with NEMO mutations (Figure 7A). Congruent with the result obtained for NF-κB activation the D311N mutation had the most severe effect on the expression of all three genes, while the E320A/E324A/E327A mutation showed almost no influence compared to wildtype NEMO. NF-κB target gene expression was severely reduced in NEMO R316A/R319A/E320A reconstituted cells, even though especially in the case of CXCL2 not quite as strong as with NEMO D311N, which again reflects the partial rescue with the selective liner Ubi binding mutant.

We determined apoptosis induction in fibroblasts as a functional readout for the effectiveness of the NEMO constructs to rescue NF-κB mediated pro-survival signaling after TNFα stimulation (Figure 7B). Whereas expression of NEMO wt clearly counteracts TNFα induced apoptosis when compared to mock reconstituted fibroblasts, NEMO D311N reconstituted cells are showing a significant increase in the level of apoptotic cells. Again, the NEMO E320A/E324A/E327A mutant is acting highly similar to NEMO wt and almost completely preventing TNFα-induced apoptosis. In contrast, NEMO R316A/R319A/E320A reconstitution displays an intermediate phenotype that has less severe effects on the ability of NEMO to prevent apoptosis then the NEMO D311N mutation.

Altogether, our data suggest that selective interference with NEMO-linear Ubi binding is impairing, but does not completely abrogate NF-κB functional responses, which is in line with the differential capacity of the NEMO mutations mediate NF-κB activation after TNFα stimulation.

DISCUSSION

We find that the entire NEMO C-terminus displays in vitro a very high preference for binding to linear over K63 Ubi chains. This is consistent with previous data showing that the NEMO UBAN alone represents a selective surface for interaction with linear diUbi (11,13,29). Even though the NEMO ZF was suggested to bind monoUbi and to enhance interaction with longer K63 Ubi chains (14,15), K_D measurements by MST are not confirming that the ZF augments interaction of the UBAN with K63 Ubi chains. Nevertheless, in plate bound assays K63 Ubi can also associate with NEMO and this interaction critically depends on residue D311 which is contacting the distal Ubi moiety in either linear or K63 diUbi (11,12). We conclude that the binding of NEMO to distinct Ubi chains is highly dependent on the type of assay. As we used a truncated NEMO mutant that comprises the UBAN-linker-ZF domains (amino acid 242-419) for all in vitro assays, it is well conceivable that the N-terminus may also contribute to the association of distinct Ubi chains in the full-length NEMO.

At a first glance, the enhanced binding of K63 Ubi to plate bound NEMO seems to represent an artificial scenario. However, cellular oligomerization of NEMO is critical for NF-κB signaling (30) and thus higher order oligomers of NEMO may promote a more static orientation that could facilitate K63 Ubi binding. Interestingly, the crystal structure also revealed that one K63 diUbi couples to the UBAN of two NEMO dimers that are aligned in parallel (12). Also our plate bound analyses suggest that a dual binding of one K63 diUbi to the distal UBAN regions of two NEMO dimers may enhance the affinity. This may become relevant, as our
reconstitutions of MEF and Jurkat T cells clearly indicate that selective destruction of linear Ubi binding to the NEMO UBAN is not sufficient to abrogate NF-κB signaling. Thus, \textit{in vivo} only the N-terminal part of the NEMO UBAN surrounding D311 that contacts the distal moiety of linear and K63 Ubi is absolutely essential for triggering activation of canonical NF-κB signaling. Interestingly, three recent reports highlight SHARPIN as a new member of the LUBAC complex including HOIL-1 and HOIP, which catalyzes assembly of linear chains to upstream components of the TNF receptor pathway (31-33). These studies show that TNF signaling is highly dependent on linear ubiquitin chain, but in accordance with our data, the signaling is not completely abolished in SHARPIN knockout cells.

Overexpression of NEMO has been suggested to inhibit optimal activation of NF-κB (26,27). By utilizing a tightly controlled lentiviral expression system, we confirmed that overexpression of NEMO is in fact strongly interfering with NF-κB activation and thus a critical issue for analyzing the physiological impact of NEMO mutations. By sorting cell populations that express homogenous NEMO amounts, we can show quantitative differences in the capacity of distinct UBAN mutations to reconstitute NF-κB signaling. By this approach we were able to demonstrate in two different cell types (fibroblasts and Jurkat T cells) that only the combined interruption of linear and K63 Ubi binding to the UBAN abrogates TNFα mediated NF-κB activation. These differential levels of NF-κB activation have biological consequences as NF-κB dependent target gene expression and NF-κB induced anti-apoptotic pathways in fibroblasts are also affected. Moreover, we show for the first time that besides K63 Ubi the binding of linear Ubi to NEMO UBAN also controls NF-κB signaling in response to T cell activation. Thus, the combined requirement for linear and K63 Ubi binding is apparently a common mechanism to integrate specific upstream signaling pathways to the IKK complex.

Our comprehensive analysis suggests that the NEMO UBAN domain is bi-functional, because it binds with low affinity to K63 and with high affinity to linear Ubi chains and both types of chains can contribute to NF-κB activation in response to TNFα or P/I stimulation. Thus, in cells the NEMO oligomerization and local concentrations of distinct Ubi chains will determine the occupancy of the UBAN. It is conceivable that consecutive binding of K63 and linear Ubi chains to the UBAN could cooperate in optimal IKK activation. Possibly, low affinity binding to K63 Ubi chains mediates NEMO recruitment to the TNFR, where the linear ubiquitin assembly complex (LUBAC) increases the local concentrations of linear Ubi (10,34). Within the TNFR complex, an exchange of K63 to linear Ubi chain binding to UBAN could be required to promote full IKK activation. Such a cooperative model is in agreement with our observation that selective destruction of linear Ubi binding to the NEMO UBAN is not sufficient to completely prevent NF-κB signaling. Recent findings demonstrate that cIAP1/UbcH5 mediate the attachment of K11 Ubi chains to RIP1 thereby facilitating the recruitment of NEMO (24). In fact, this is in line with the observation that neither the lack of K63 Ubi chains nor linear chains were sufficient to completely abrogate NF-κB signaling (35). Hence, a detailed analysis of the NEMO-K11 Ubi binding mode will be necessary to fully understand the role of K11 modified RIP1 in the TNFα receptor pathway. In light of the recent reports about the significant role of LUBAC and linear Ubiquitin chains in the TNF pathway (31-33) future studies have to further reveal how different types of Ubi chains cooperate to activate NF-κB.
REFERENCES


**FOOTNOTES**

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FIGURES LEGENDS

**Fig. 1.** In solution, recombinant NEMO UBAN-ZF wt selectively binds linear Ubi.
(A) Scheme of the NEMO construct. NEMO UBAN-ZF with the UBAN domain and the mutation site D311 is indicated. CC1/CC2: coiled coil domains 1/2. LZ: leucin zipper. ZF: zinc finger. UBAN: ubiquitin binding domain. S: StrepTagII. (B) C-term Strep NEMO UBAN-ZF wt bound linear diUb with a $K_D$ of 3.24 $\mu$M, but not K63-linked diUb. (C) Microscale thermophoresis (MST) assays of NEMO UBAN-ZF D311N mutation, which is not able to bind linear Ubi. (D) MST experiments were carried out with N-terminally Strep-tagged NEMO UBAN-ZF wt as described in B. It bound linear diUb with a $K_D$ of 2.71 $\mu$M, but not K63-linked diUb. (E) Linear tetraUb chains bound to C-term Strep NEMO UBAN-ZF wt with a $K_D$ of 0.34 $\mu$M, while no binding was detectable with K63-linked tetra Ub. (F) Linear tetraUb chains also bound to N-terminally Strep-tagged NEMO UBAN-ZF wt with a $K_D$ of 0.94 $\mu$M, whereas no binding was detectable with K63-linked tetra Ub. (G) The TBD-NFZ domain of the K63-linked ubiquitin recognizing protein TAB2 served as an independent control for the validity of the assay. TAB2 did not recognize linear diUb, whereas K63-linked diUb was bound with a $K_D$ of 21.33 $\mu$M. (H) Linear tetraUb also did not bind TAB2 TBD-NFZ, while K63-linked tetraUb was bound by TAB2 TBD-NZF with a $K_D$ of 6.88 $\mu$M. All data represent the mean from 3 independent experiments and error bars indicate SD. $\Delta F_{norm}$ = change normalized fluorescence

**Fig. 2.** Pull down experiments of N-terminally or C-terminally Strep tagged NEMO-UBAN-ZF with linear and K63-linked tetraUb.
(A) Western blot of StrepTagII pull down experiment with N-term Strep NEMO-UBAN-ZF. Left panel: 10 % input of linear and K63-linked tetraUb chains. Right panel: N-term Strep NEMO-UBAN-ZF wt was only capable of recognizing linear tetraUb but not K63-linked tetraUb. D311N mutation was unable to bind any of the investigated tetraUb chain. (B) In an approach where a mixture of both chain types (linear and K63) was provided for recognition, C-term Strep NEMO-UBAN-ZF wt only pulled down linear tetraUb.

**Fig. 3.** Immobilized NEMO UBAN-ZF binds to linear and more weakly to K63-linked Ubi chains.
(A) Experimental set-up of the DELFIA plate-based binding assay: Recombinant cMyc-NEMO-UBAN-ZF-StrepTagII dimer is bound to a StrepTactin coated plate. His-labeled diUb binds to NEMO and can be detected by an Europium(Eu)-labeled anti-His-antibody. (B) NEMO-UBAN-ZF wt showed a strong and robust signal when incubated with linear His-diUb compared to background level (no NEMO) or the NEMO UBAN-ZF D311N mutation. Also no interaction of NEMO-UBAN-ZF and monoUbi was detected. (C) NEMO UBAN-ZF wt showed a weak interaction with K63 His-diUb compared to binding to linear His-diUb. (D) The binding of NEMO-UBAN-ZF to K63 Ubi was enhanced by use of a mixture of His-Ubi$_{2-7}$ chains. (E) The functionality of K63 Ubi chains for DELFIA plate assays was validated by the use of TAB2 NZF domain, which specifically recognized K63 His-Ubi$_{2-7}$. All data represent the mean from 3 independent experiments and error bars indicate SD. RFU = Relative Fluorescence Unit

**Fig. 4.** Directed mutagenesis of NEMO selectively impairs binding to linear diUb.
(A) Crystal structures of NEMO UBAN dimers co-crystallized with either K63 diUb (left panel) or linear diUb (right panel). D311 contacting the distal Ubi of K63 and linear diUb as well as
contact residues solely involved in the recognition of the proximal Ubi in linear diUbi are indicated. (B) DELFIA plate assay of NEMO-UBAN-ZF wt and mutations targeting the linear ubiquitin specific recognition site. Mutation of the C-terminal UBAN domain selectively impedes binding of linear Ubi. Association of wt and mutations D311N, R319A/E320A, R316A/R319A/E320A, E320A or E320A/E324A/E327A to linear His-diUbi (left) and K63 HisUbi$_{2-7}$ (right) was measured by plate coupled DELFIA assays. For comparison binding of wt was set to 100 %. (C) In MST, the NEMO UBAN-ZF R316A/R319A/E320A was not binding to linear diUbi, whereas NEMO UBAN-ZF E320A/E324A/E327A interacted with linear diUbi with a lower affinity of 11.39 µM compared to NEMO UBAN-ZF wt (compare Fig. 1A). All data represent the mean from 3 independent experiments and error bars indicate SD. $\Delta F_{\text{norm}} = \text{change normalized fluorescence}$

Fig. 5. Expression levels of reconstituted NEMO in MEF.
(A) Scheme of the lentiviral construct used to reconstitute MEF or Jurkat T-cells. Promoter: Phosphoglycerate kinase promoter (PGK). Surface infection marker: human truncated CD2 (hΔCD2). The T2A sequence mediates interruption of its own translation, thus creating two polypeptides under the control of one promoter. Triple-Flag-tagged full-length wild type human NEMO was used for reconstitution. (B) FACS analysis of infection marker (hΔCD2) levels in the course of the NEMO reconstitution experiment. Staining with anti-hCD2-APC. Left panel: uninfected NEMO deficient MEF. Middle panel: Infected cells before sorting. Right panel: CD2-positive cell populations after sorting. Two populations (NEMO high and low) are indicated: (C) Re-staining of “high” and “low” cells from (B) right panel (D) Western blot and EMSA analysis of reconstituted NF-κB signaling in wt, low and high MEF. Quantification of NEMO levels yielded wt:low:high = 1:8:20. In the case of the NEMO high cells, the NF-κB signaling is severely impaired. The NEMO levels of the cells actually used for experimentation matched the endogenous NEMO levels of wt MEF much closer. See Fig 6(A) and (B) left panels.

Fig. 6. Reconstitution of NEMO deficient MEF and Jurkat T cells.
NEMO deficient MEF or Jurkat T cells were lentivirally reconstituted with NEMO wt or mutation constructs. (A and B) Expression of NEMO wt and NEMO in NEMO$^{-/-}$ MEF was analyzed by WB (upper panels) and the extent of NF-κB signaling was evaluated by determining p-IκBα and IκBα amounts by WB and NF-κB DNA binding by EMSA (lower panels). Whereas mutation D311N almost completely prevented NF-κB signaling, mutation R319A/E320A and R316A/R319A/E320A led to reduced NF-κB signaling and mutations E320A and E320A/E324A/E327A had almost no discernible effect on NF-κB activation. (C) Quantification of NF-κB DNA binding after TNFα stimulation from three independent EMSA experiments with SD. Actin was used as internal control. (D) Expression of NEMO wt and mutations in NEMO deficient Jurkat T cells was analyzed by WB (left panels). NF-κB signaling was determined after TNFα (middle panel) or PMA/Ionomycin (P/I) (right panel) stimulation according to A and B. P/I reenacts T-cell receptor stimulation and serves as a TNF independent control for downstream signaling involving NEMO. In T cells mutations R316A/R319A/E320A showed impaired NF-κB activation after TNFα and P/I stimulation, whereas mutations E320A/E324A/E327A are only slightly reduced.
Fig. 7. TNFα induced NF-κB target gene transcription and susceptibility to apoptosis in NEMO-reconstituted MEF

(A-C) NEMO-reconstituted MEF were stimulated with TNFα for 1 h and NF-κB target gene mRNA levels (A20, CXCL2 and ICAM1) were determined by quantitative PCR. Bars indicate mRNA levels of TNFα stimulated cells versus unstimulated control cells. For comparison, all calculated values were related to levels of wt reconstituted cells, which were set to 1. In accordance with all previous results, mutations D311N and R316A/R319A/E320A led to reduced NF-κB target gene transcript levels whereas mutation E320A/E324A/E327A had only mild effects. (D) NEMO-reconstituted MEF were assayed for early apoptosis after 22 h TNFα treatment. Late apoptotic cells were excluded in the FACS analysis and levels of annexin V positive cells are depicted in the graph. NEMO wt reconstitution is able to completely rescue TNFα induced apoptosis of knock out cells. NEMO D311N shows elevated annexin V levels and thus is only partially able to prevent apoptosis. Mutation R316A/R319A/E320A shows decreased apoptosis levels in comparison to D311N, and E320A/E324A/E327A is even more capable to rescue.
Figure 1

A

NEMO

D311N

UBAN 290 — 330

UBAN-ZF-Strep

Strep-UBAN-ZF

1 — CC1 — CC2 — LZ — ZF 419

B

NEMO UBAN-ZF wt (C-term Strep)

dLub (K63)

K_D = 3.24 μM

+/- 1.54 μM

dLub (linear)

no binding

C

NEMO UBAN-ZF D311N (C-term Strep)

dLub (K63)

no binding

dLub (linear)

n/a

D

NEMO UBAN-ZF wt (N-term Strep)

dLub (K63)

K_D = 3.71 μM

+/- 1.15 μM

dLub (linear)

no binding

E

NEMO UBAN-ZF wt (C-term Strep)

tetraUbi (K63)

no binding

tetraUbi (linear)

K_D = 0.34 μM

+/- 0.23 μM

F

NEMO UBAN-ZF wt (N-term Strep)

tetraUbi (K63)

no binding

tetraUbi (linear)

K_D = 0.94 μM

+/- 0.98 μM

G

TAB2 (TBD-NZF)

dLub (linear)

no binding

dLub (K63)

K_D = 21.33 μM

+/- 14.66 μM

H

TAB2 (TBD-NZF)

tetraUbi (linear)

no binding

tetraUbi (K63)

K_D = 6.88 μM

+/- 6.63 μM
Figure 2

A

NEMO UBAN-ZF (242-419)
N-terminal StrepTagII

4Ubi (linear) 4Ubi (K63)

α-Ubi

α-NEMO

4Ubi (linear) Input (10%) 4Ubi (K63) Input (10%)

NEMO UBAN-ZF wt
NEMO UBAN-ZF D311N

B

NEMO UBAN-ZF (242-419)
C-terminal StrepTagII

4Ubi (linear) 4Ubi (K63)

α-Ubi

α-NEMO

4Ubi (linear) Input (10%) 4Ubi (K63) Input (10%)

NEMO UBAN-ZF wt
NEMO UBAN-ZF D311N
Figure 6
NEMO interaction with linear and K63 ubiquitin chains contributes to NF-κB activation
Kamyar Hadian, Richard A. Griesbach, Scarlett Dornauer, Tim M. Wanger, Daniel Nagel, Moritz Metlitzky, Wolfgang Beisker, Marc Schmidt-Supprian and Daniel Krappmann

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