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Inhibition of Canonical NF- κ B Signaling by a Small Molecule Targeting NEMO-Ubiquitin Interaction

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Michelle Vincendeau^{1,*}, Kamyar Hadian^{1,2,*}, Ana C. Messias^{3,4,*}, Jara K. Brenke², Jenny Halander³, Richard Griesbach^{1,†}, Ute Greczmiel¹, Arianna Bertossi¹, Ralf Stehle^{3,4}, Daniel Nagel¹, Katrin Demski¹, Hana Velvarska³, Dierk Niessing^{3,5}, Arie Geerlof⁶, Michael Sattler^{3,4} & Daniel Krappmann¹

The I κ B kinase (IKK) complex acts as the gatekeeper of canonical NF- κ B signaling, thereby regulating immunity, inflammation and cancer. It consists of the catalytic subunits IKK α and IKK β and the regulatory subunit NEMO/IKK γ . Here, we show that the ubiquitin binding domain (UBAN) in NEMO is essential for IKK/NF- κ B activation in response to TNF α , but not IL-1 β stimulation. By screening a natural compound library we identified an anthraquinone derivative that acts as an inhibitor of NEMO-ubiquitin binding (iNUB). Using biochemical and NMR experiments we demonstrate that iNUB binds to NEMO_{UBAN} and competes for interaction with methionine-1-linked linear ubiquitin chains. iNUB inhibited NF- κ B activation upon UBAN-dependent TNF α and TCR/CD28, but not UBAN-independent IL-1 β stimulation. Moreover, iNUB was selectively killing lymphoma cells that are addicted to chronic B-cell receptor triggered IKK/NF- κ B activation. Thus, iNUB disrupts the NEMO-ubiquitin protein-protein interaction interface and thereby inhibits physiological and pathological NF- κ B signaling.

NF- κ B signaling plays a crucial role in inflammation, immune responses, survival and cell proliferation¹. The canonical NF- κ B signaling can be activated through various pathways including TNF receptor (TNFR), IL-1 receptor (IL-1R) or T-cell receptor (TCR)^{2,3}. Upon ligand binding, proximal signaling events converge at the I κ B kinase (IKK) complex, which acts as the gatekeeper of NF- κ B signaling by phosphorylating and inactivating the inhibitors of NF- κ B (I κ Bs).

The IKK complex consists of two catalytic subunits IKK α /IKK β and the regulatory subunit IKK γ /NEMO (NF- κ B essential modulator)⁴. The IKK regulatory subunit NEMO serves as a critical integrating platform that couples upstream receptor signaling complexes to the catalytic IKKs^{5,6}. Biochemical and genetic studies have highlighted a pivotal function of poly-ubiquitination for IKK/NF- κ B activation. It has been shown that IKK upstream signaling adaptors like RIP1 (TNFR), IRAK1/4 (IL-1R) or MALT1 (TCR) are modified by ubiquitin (Ub) chains to recruit NEMO and thereby promote IKK activation^{2,7,8}. Assembly of Met1-linked (linear) Ub chains by the LUBAC (linear Ub assembly complex) is also essential for IKK/NF- κ B activation in response to various stimuli^{9–12}.

¹Research Unit Cellular Signal Integration, Helmholtz Zentrum München für Gesundheit und Umwelt, Ingolstädter Landstr. 1, 85764 Neuherberg, Germany. ²Assay Development and Screening Platform, Institute of Molecular Toxicology and Pharmacology, Helmholtz Zentrum München für Gesundheit und Umwelt, Ingolstädter Landstr. 1, 85764 Neuherberg, Germany. ³Institute of Structural Biology, Helmholtz Zentrum München für Gesundheit und Umwelt, Ingolstädter Landstr. 1, 85764 Neuherberg, Germany. ⁴Center for Integrated Protein Science Munich at Biomolecular NMR Spectroscopy, Department Chemie, Technische Universität München, Lichtenbergstr. 4, 85747 Garching, Germany. ⁵Biomedical Center of the Ludwig-Maximilians University München, Department of Cell Biology, Großhadener Str. 9, 82152 Planegg-Martinsried, Germany. ⁶Protein Expression and Purification Facility, Institute of Structural Biology, Helmholtz Zentrum München für Gesundheit und Umwelt, Ingolstädter Landstr. 1, 85764 Neuherberg, Germany. *These authors contributed equally to this work. †Present address: Natural and Medical Sciences Institute at the University of Tuebingen, Markwiesenstr. 55, 72770 Reutlingen, Germany. Correspondence and requests for materials should be addressed to D.K. (email: daniel.krappmann@helmholtz-muenchen.de)

The Ub binding surface in NEMO called UBAN (Ub binding in ABIN and NEMO) has been co-crystallized with linear as well as with K63 Ub chains^{13,14}. Despite the fact that the NEMO C-terminus comprising the UBAN and the zinc finger (ZF) domain has a high preference for binding to linear Ub chains, it can also bind K63 or K11 chains^{15–18}. The importance of non-covalent NEMO-Ub interaction for delivering upstream signaling events in response to TNFR, IL-1R or TCR/BCR stimulation to activate the IKK complex has been highlighted by several studies¹⁹. Moreover, chronic B-cell receptor (BCR) signaling in the aggressive activated B-cell (ABC) subgroup of diffuse large B-cell lymphoma (DLBCL) has been shown to require regulatory ubiquitination and the LUBAC^{20,21}. Overexpression of the NEMO_{UBAN} or cell permeable peptides comprising the UBAN or leucine zipper (LZ) regions interfered with ubiquitin association or NEMO oligomerization and impeded NF- κ B activation^{22–24}. Thus, disruption of NEMO-ubiquitin binding may be a promising strategy to inhibit inducible physiological as well as chronic pathological NF- κ B activation.

In this work, we demonstrate by direct comparative analyses that UBAN-dependent NEMO-ubiquitin interaction is required for NF- κ B in response to TNF α , but not IL-1 β stimulation. We identified an inhibitor of NEMO-Ub binding (iNUB), an anthraquinone derivative of the natural product Emodin, as a small molecule that binds to the NEMO_{UBAN}. Using biochemical, biophysical and NMR experiments we show that iNUB acts as a protein-protein interaction (PPI) inhibitor by preventing the recruitment of linear Ub chains. Consistent with the genetic data, iNUB inhibits TNF α , but not IL-1 β -triggered NF- κ B signaling. iNUB interferes with antigen receptor signaling to NF- κ B and acts toxic in human lymphoma cells that are addicted to chronic BCR signaling.

Results

Differential requirement of NEMO_{UBAN} for TNF α and IL-1 β -induced NF- κ B signaling. The critical role for the binding of Ub chains to the NEMO_{UBAN} motif has been demonstrated by multiple NEMO mutations that either directly prevent NEMO-Ub binding or indirectly disturb the structural conformation of the coiled-coil/leucine-zipper (CC2-LZ) region^{13,17,25}. Mutations in NEMO were shown to impair IKK/NF- κ B activation after TNF α and IL-1 β stimulation^{13,26}. However, the requirement of the UBAN and other regions in NEMO for TNF α and IL-1 β -induced NF- κ B signaling has not been directly compared. We used NEMO deficient mouse embryonic fibroblasts (NEMO^{-/-} MEFs) that were reconstituted with NEMO WT or NEMO mutants (Fig. 1a). The co-expressed surface marker human Δ CD2 was used for sorting homogenous populations of infected cells (>90%) that expressed NEMO proteins at equivalent levels (Supplementary Fig. 1a–d)¹⁸. Whereas NEMO WT was able to efficiently rescue defective NF- κ B signaling in response to TNF α or IL-1 β stimulation as reflected by I κ B α phosphorylation and degradation, as well as activation of NF- κ B DNA binding, deletion of the IKK interaction surface (Δ IKK; Δ 44–111) abolished NF- κ B signaling (Supplementary Fig. 1e–g)²⁷. The deletions Δ CC1-1 (Δ 112–150) and Δ CC1-2 (Δ 151–223) in NEMO did not interfere with IKK α/β association and led to a partial or complete inhibition of both TNF α and IL-1 β signaling to NF- κ B, respectively (Supplementary Fig. 1e–g). Similarly, deletion of the linker region between CC1 and CC2 encoded by exon5 (Δ 224–257) reduced NF- κ B activation in response to both inducers (Fig. 1b,c). In contrast to these mutations, removal of the CC2 (Δ 259–304) or the Ub binding region in the UBAN (Δ 296–327) prevented NF- κ B signaling in response to TNF α , but only partially diminished IL-1 β -triggered NF- κ B activation (Fig. 1b,c). Moreover, the NEMO_{UBAN} point mutation D311N or the deletion of the C-terminal zinc finger (E391X: Δ 391–417), two patient derived mutations that cause anhidrotic ectodermal dysplasia with immunodeficiency (EDA-ID), abolished TNF α -induced NF- κ B signaling, but only mildly affected IL-1 β stimulated NF- κ B activation (Fig. 1d,e). Thus, while the NEMO CC2, UBAN and ZF in the C-terminus are essential for TNF α signaling, these domains are largely dispensable for IL-1 β signaling, emphasizing that distinct NEMO surfaces are required for bridging the different receptor pathways to canonical NF- κ B.

Structural characterization of the NEMO_{UBAN} dimer and linear Ub₂ in solution. To explore the possibility of small molecule inhibition to interfere with protein-protein interaction (PPI) of NEMO_{UBAN} and ubiquitin, we first addressed the stoichiometry and conformation of NEMO_{UBAN} alone and in complex with linear Ub₂ (linUb₂) in solution. Whereas a 2:2 stoichiometry had been reported for the NEMO_{UBAN}:linUb₂ complex in co-crystals (PDB 2ZVO)¹³, a 2:1 stoichiometry was described in solution²⁵. Indeed, by size exclusion chromatography (SEC) in combination with static light scattering (SLS) we found that one NEMO_{UBAN} dimer associates with one linear Ub₂, revealing a 2:1 stoichiometry for the complex (Supplementary Fig. 2a,b).

To gain further insight into the structure of the NEMO_{UBAN}:linUb₂ complex, we performed small angle X-ray scattering (SAXS) experiments using NEMO_{UBAN} C347S (258–350). Microscale Thermophoresis (MST) and Isothermal Titration Calorimetry (ITC) measurements confirmed that the C347S mutation in NEMO does not affect the affinity ($K_D \sim 1–2 \mu\text{M}$) and the 2:1 stoichiometry of the NEMO_{UBAN}:linUb₂ complex (Supplementary Fig. 2c,d)^{18,25}. SAXS data also revealed that NEMO_{UBAN} forms a dimer in solution (Supplementary Fig. 2e and 2b). However, the relatively high χ^2 value indicates that the solution conformation of NEMO_{UBAN} is slightly different from the crystal structure (PDB 3FX0)²⁵, suggesting that NEMO_{UBAN} in solution is not strictly linear. Comparison of the SAXS experimental curves for Ub₂ with the theoretical curves back-calculated from the two published crystal structures for linear Ub₂ suggests that the solution conformation of linear Ub₂ is better represented by an ensemble of an open (PDB 3AXC)²⁸ and a compact (PDB 2W9N)²⁹ domain arrangements seen in previous crystal structures with 38%:62% population (Supplementary Fig. 2f). Calculated Porod volumes and MWs from the scattering curves obtained for solutions containing NEMO_{UBAN} C347S and linear Ub₂ at ratios of 2:1 and 2:2 (Fig. 2a,b) are consistent with a complex of two NEMO_{UBAN} molecules (i.e. one NEMO dimer) bound to one linear Ub₂. A DAMMIF model derived from the SAXS data of the 2:1 NEMO_{UBAN}:Ub₂ solution showed an elongated surface with only one hump consistent with an NEMO dimer bound to one Ub₂ in solution (Fig. 2c).

We used Nuclear Magnetic Resonance (NMR) experiments to analyze the NEMO_{UBAN} binding interface to linear Ub₂ in solution and compared it with the previously reported crystal structure¹³. We could assign 84% of the backbone NMR signals of NEMO_{UBAN} (258–350) and analyzed spectral changes induced upon the addition

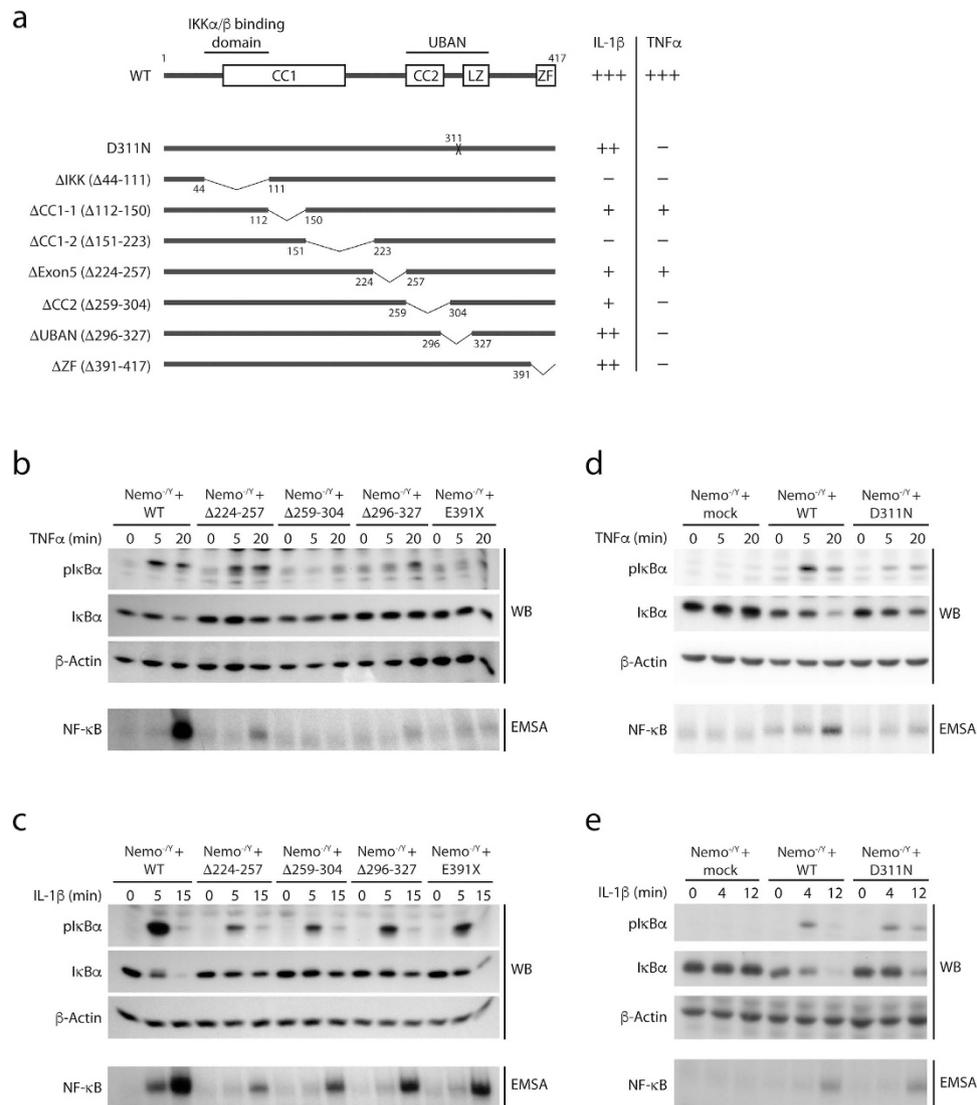


Figure 1. NEMO-Ub binding is essential for TNF α signaling, but not for IL-1 β signaling. (a) Schematic illustration of NEMO deletions or point mutations. (b,c) Effects of NEMO C-terminal deletion mutants on TNF α and IL-1 β signaling. NEMO $^{-/-}$ MEFs were reconstituted with mock, NEMO WT or different C-terminal deletion constructs and stimulated with TNF α (b) or IL-1 β (c). Effects on NF- κ B signaling were investigated by determining I κ B α phosphorylation and degradation in Western Blots as well as NF- κ B-DNA binding by EMSA. (d,e) Effects of NEMO D311N on TNF α and IL-1 β signaling. NEMO $^{-/-}$ MEFs were reconstituted with mock, NEMO WT or NEMO D311N point mutant and stimulated with TNF α (d) or IL-1 β (e). Analysis on NF- κ B signaling were performed as in (b,c).

of unlabeled linear Ub₂ in a 2:1 ratio (NEMO_{UBAN}:Ub₂) (Fig. 2d). Chemical shift perturbations were observed for multiple amino acids on NEMO that are directly contacting linear Ub₂ in the crystal, e.g. V300, A303, Q304, D311, E324, E320. However, additional shifts were obtained for several residues distributed across the entire CC2-LZ region of NEMO that are not directly involved in contacting linear Ub₂ (Fig. 2e). Taken together, the SAXS and NMR data suggest that, in solution, binding of linear Ub₂ to NEMO_{UBAN} induces allosteric effects that modulate the overall structure and dynamics of the NEMO_{CC2-LZ} dimer. Such allosteric effects could also explain that only an asymmetric 2:1 complex is formed as allosteric changes induced by binding of one Ub₂ molecule may prevent binding of a second Ub₂ moiety on the opposite surface.

Identification of NEMO-Ub binding inhibitors from a natural compound library. Due to the relatively low binding affinity of NEMO_{UBAN} to ubiquitin (K_D 1–2 μ M for linear and 12–130 μ M for K63 Ub₂)^{13,15,18,25}, the conformational dynamics of the NEMO_{UBAN} (see Fig. 2) and the selective requirement of the NEMO_{UBAN} for TNF α , but not IL-1 β signaling to NF- κ B (see Fig. 1), we wanted to test whether NEMO-Ub interaction could be susceptible to small molecule inhibition. We used the previously established ‘Dissociation-Enhanced Lanthanide Fluorescent Immunoassay’ (DELFI) technology to detect binding of recombinant Myc-NEMO_{UBAN}-StrepTagII (aa 246–350) and His-linUb₂ in a multi-plate format (Supplementary Fig. 3a)¹⁸. As expected, linear Ub₂ bound

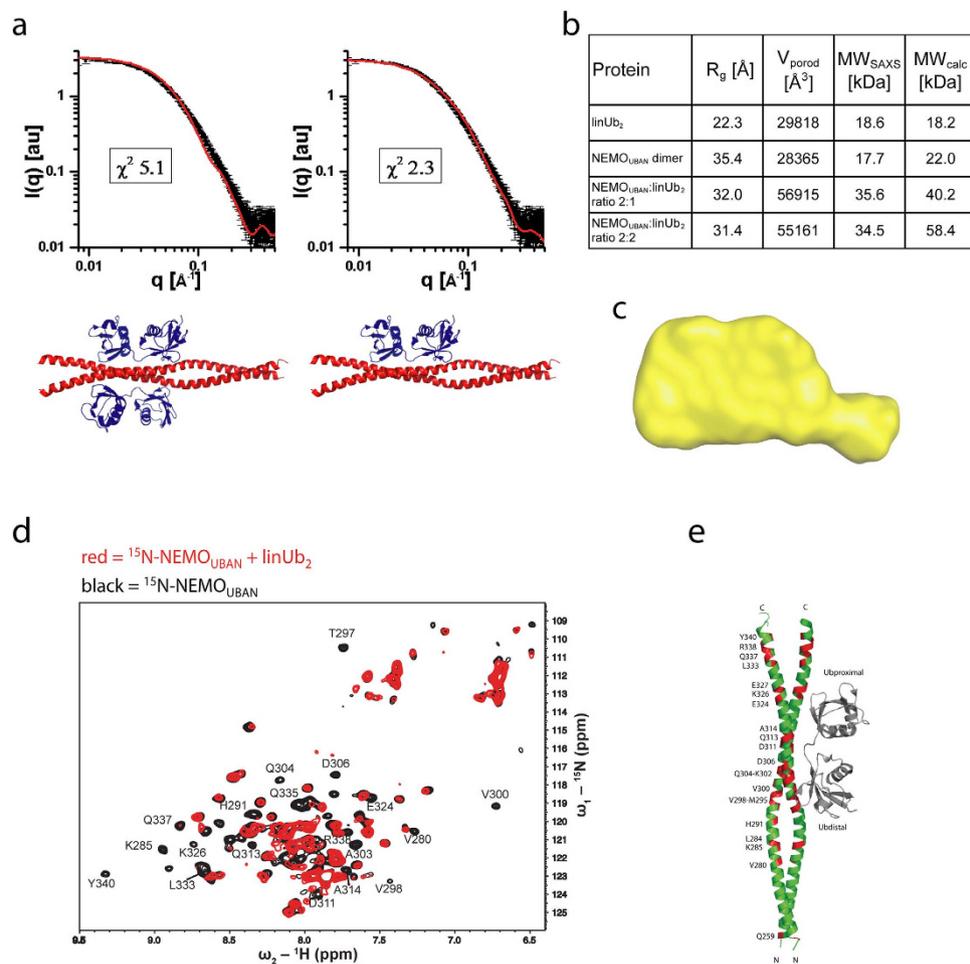


Figure 2. The NEMO-Ub₂ complex has a 2:1 stoichiometry in solution. (a) The 2:1 stoichiometry of the NEMO-Ub₂ complex is supported by comparison of the SAXS experimental curves with theoretical curves back-calculated from either the 2:2 NEMO-Ub₂ co-crystal structure (PDB 2ZVO, 2:2) or a 2:1 complex, where one Ub₂ was removed from the crystal structure. (b) The calculated MWs in the table show that independent of the mixing ratio of NEMO_{UBAN} and Ub₂ a 2:1 complex is formed, and (c) the 2:1 ratio is further supported by the shape of the envelope of *ab initio* modeling with DAMMIF (shown in yellow). (d) NMR analysis of the NEMO_{UBAN}-linUb₂ interaction. ¹H, ¹⁵N TROSY NMR spectrum of 100 μM ¹⁵N-labeled NEMO in the absence (black) or presence of Ub₂ (red) in a 2:1 (NEMO_{UBAN}:Ub₂) ratio. Amides, which disappear upon Ub₂ addition are indicated. (e) Mapping of the backbone amides, which disappear upon linUb₂ addition in panel d onto the NEMO_{UBAN} surface from the crystal structure (PDB 2ZVO).

to NEMO_{UBAN} and association was abrogated by the UBAN mutation D311N (Supplementary Fig. 3b). We screened a small molecule library of 320 purified natural products for their ability to inhibit binding of linUb₂ to NEMO_{UBAN} (Supplementary Fig. 3c). The screening provided robust signal to noise ratio (Z' factor 0.78) and delivered 4 hits, which significantly reduced the signal for NEMO-Ub binding below 50% (Supplementary Fig. 3d). One of the compounds that most severely reduced the signal was a derivative of the anthraquinone Aloe-Emodin. Anthraquinones like Emodin and Aloe-Emodin are natural products, found as active ingredients in Chinese herbs, with demonstrated multiple biological effects including anti-inflammatory and anti-cancer activities³⁰. Since Emodin affects several molecular pathways including NF- κ B signaling³⁰, we wanted to test, if distinct anthraquinones could affect the association of NEMO and ubiquitin. We tested the Aloe-Emodin derivative, Emodin as well as 10 other chemically synthesized anthraquinones for their ability to inhibit the interaction of NEMO and linear Ub₂ in DELFIA (Supplementary Fig. 3e). Interestingly, whereas most anthraquinones including Emodin did not significantly inhibit NEMO-Ub₂ binding, Aloe-Emodin derivative and anthraquinone 1 (8-hydroxy-9,10-dioxo-9,10-dihydro-1-anthracenyl 2-phenylcyclopropanecarboxylate) were able to significantly inhibit this interaction.

iNUB disrupts NEMO-Ub interaction by binding to NEMO_{UBAN}. To investigate the potential impact of distinct anthraquinones on NEMO-Ub binding, we determined the effects of the most effective inhibitor, i.e. anthraquinone 1, which we henceforth call inhibitor of NEMO-Ub binding (iNUB). In DELFIA, iNUB disrupted NEMO_{UBAN} interaction to linUb₂ with an EC₅₀ of 9.3 μM and even though it was not able to completely block the interaction, it reduced the signal more than 70% at 40–80 μM (Fig. 3a).

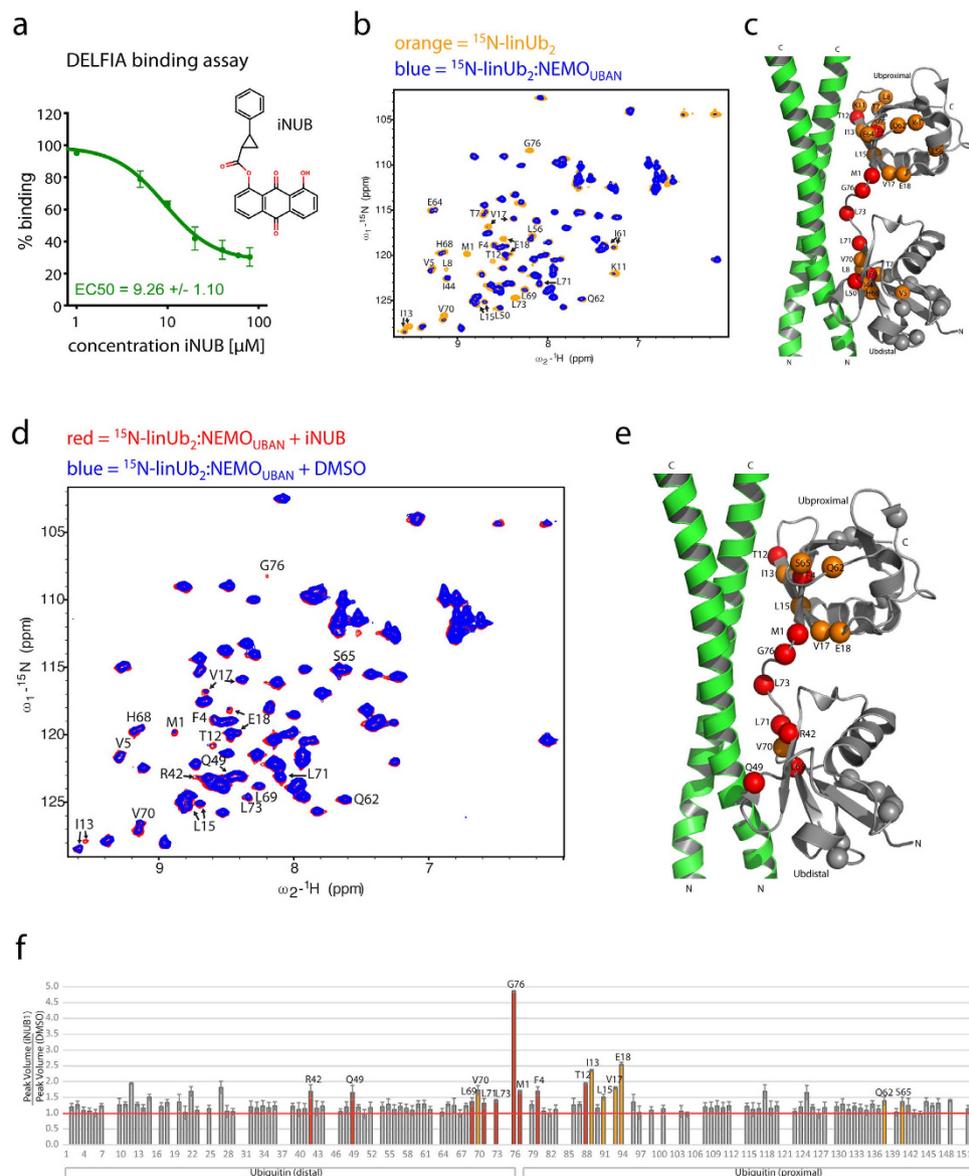


Figure 3. The small molecule iNUB inhibits binding of NEMO_{UBAN} to linUb₂. (a) DELFIA assay detection of StrepTagII-NEMO_{UBAN} (242–350) binding to His-tagged linUb₂ with or without iNUB. Increasing iNUB concentrations disrupted binding of NEMO to linUb₂ binding with an EC₅₀ of 9.26 μM. (b) NMR analysis of the NEMO_{UBAN}-linUb₂ interaction. ¹H, ¹⁵N HSQC spectra of 100 μM ¹⁵N-labeled linUb₂ in absence (orange) and in presence of unlabeled NEMO_{UBAN} C347S (258–350) (blue) at 1:1 stoichiometry. (c) Backbone amide resonances of Ub₂ resonances showing strongest changes upon NEMO_{UBAN} addition are annotated and indicated as spheres on the NEMO crystal structure (PDB 2ZVO). Red spheres indicate residues with unambiguous chemical shift assignments, while orange color shows amides, which could not be unambiguously assigned to the proximal or distal Ub moieties or showing signal overlap. Gray spheres are the amides of the corresponding residues in the other Ub module. (d) ¹H, ¹⁵N HSQC spectra of 100 μM ¹⁵N-labeled Ub₂ bound to the NEMO_{UBAN} (1:1) in the presence of DMSO (blue) or 290 μM iNUB (red). NMR signals of linUb₂ residues that showed reduced signal intensity upon binding to NEMO_{UBAN} are partially restored upon treatment with iNUB. (e) Mapping of Ub residues that show strongest effects upon iNUB addition onto the NEMO_{UBAN} structure as in (d). (f) Ratio of amide peak volume upon iNUB versus DMSO addition for the ¹⁵N-labeled Ub₂ complexed with unlabeled NEMO (1:1 stoichiometry).

We used NMR titrations to confirm that iNUB was able to compete the binding of linUb₂ to NEMO_{UBAN}. NMR experiments recorded with ¹⁵N-labeled linUb₂ in the absence (orange) or presence (blue) of unlabeled NEMO_{UBAN} C347S (Fig. 3b) show large spectral changes upon binding of NEMO (NEMO_{UBAN} : Ub₂ 1:1). Consistent with a previous study (Fig. 3b)²⁵, many Ub₂ backbone amide NMR signals show a significant intensity reduction upon addition of NEMO_{UBAN}, due to severe line broadening. Mapping of the Ub₂ backbone amide resonances with strongest changes onto the crystal structure of NEMO-Ub₂ (PDB 2ZVO) highlights the Ub₂ interface on NEMO

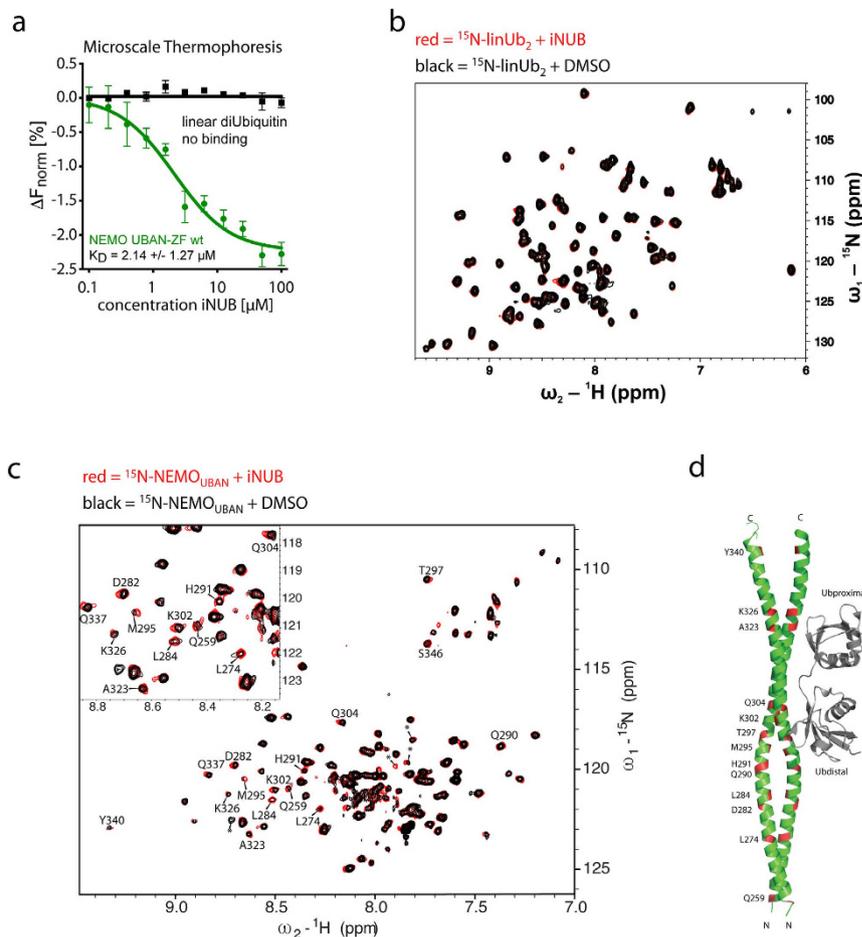


Figure 4. iNUB binds and alters the conformation of the NEMO $_{\text{UBAN}}$. **(a)** Microscale Thermophoresis (MST) assays to determine binding of iNUB to NEMO $_{\text{UBAN}}$ or linUb $_2$. iNUB binds to Myc-NEMO $_{\text{UBAN-ZF}}$ -StrepTagII (242–350) with a K_D of 2.14 μM . **(b)** ^1H , ^{15}N HSQC spectra of 50 μM ^{15}N -labeled linUb $_2$ in the absence (black) and presence of 1 mM iNUB (red). **(c)** NMR analysis of the NEMO $_{\text{UBAN}}$ -iNUB interaction. ^1H , ^{15}N TROSY NMR spectrum of 117 μM ^{15}N -labeled NEMO $_{\text{UBAN}}$ (258–350) with DMSO (black) or 870 μM iNUB (red). Amide signals that are affected upon iNUB addition are indicated. Asterisks indicate unassigned NMR signals that are affected by iNUB. **(d)** Mapping of residues affected upon iNUB addition onto the NEMO $_{\text{UBAN}}$ crystal structure (PDB 2ZVO).

and the essential role of many Ub $_2$ amino acids in contacting NEMO $_{\text{UBAN}}$, which are confirmed by mutagenesis (Fig. 3c)²⁵.

Next, we compared the chemical shifts of ^{15}N -labeled linUb $_2$ when bound to NEMO $_{\text{UBAN}}$ (1:1) in the presence of DMSO or iNUB to test whether the addition of iNUB can compete with the NEMO-Ub $_2$ interaction (Fig. 3d–f). Indeed, iNUB induced the reappearance of many linUb $_2$ signals that were reduced upon NEMO binding. Notably, many of these residues have been previously shown to have a critical role in the NEMO $_{\text{UBAN}}$ and Ub $_2$ interaction based on the crystal structure and/or mutagenesis, e.g. R42, V70 and L73 in the distal Ub moiety and F4, T12 and I13 in the proximal Ub moiety^{13,25}.

In addition, we determined the effects of Emodin, because many studies suggested that Emodin is able to impair NF- κ B signaling³⁰. Confirming our previous results, Emodin did not significantly affect NEMO-Ub $_2$ binding up to 20 μM and slightly reduced binding by approximately 25% at 80 μM (Supplementary Fig. 4a). As expected, incubation of ^{15}N -labeled linUb $_2$ -NEMO $_{\text{UBAN}}$ complex (1:1) with Emodin resulted in fewer changes in chemical shifts of Ub $_2$ amino acids, validating DELFIA results that Emodin exerts only a very mild effect on the NEMO-Ub interaction (Supplementary Fig. 4b–d).

To determine the mechanism of how iNUB disrupts NEMO-linUb $_2$ interaction, we performed MST to elucidate to what component iNUB is binding (Fig. 4a). Titration of increasing iNUB concentrations to fluorescence-labeled NEMO $_{\text{UBAN}}$ or linUb $_2$ revealed that iNUB induced a change in thermophoresis only with NEMO $_{\text{UBAN}}$. Thus, iNUB directly binds to NEMO with a K_D of $\sim 2.14 \mu\text{M}$, which is in the affinity range of NEMO-linUb $_2$ interaction (Supplementary Fig. 2c,d)²⁵. iNUB did not show any binding to linUb $_2$ in MST assays. Consistent with these data, we did not detect significant chemical shifts on ^{15}N -labeled linUb $_2$ upon incubation with iNUB reflecting that iNUB is not binding to Ub $_2$ (Fig. 4b).

We used ^{15}N -labeled NEMO_{UBAN} WT (see Fig. 2) to verify a direct binding of iNUB to NEMO_{UBAN} by NMR (Fig. 4c). iNUB binding induced a number of changes in the NEMO_{UBAN} spectrum. We mapped the residues affected by iNUB incubation onto the crystal structure (Fig. 4d). A number of backbone amide NMR signals exhibit small chemical shifts and an increase in the intensity of NMR signals upon addition of iNUB. Similar to linUb₂ addition (compare Fig. 2d,e), affected residues were not localized to one spot in the UBAN, but scattered throughout the NEMO_{UBAN} dimer. For some of the affected residues, a critical involvement in binding to ubiquitin or maintenance of the NEMO_{UBAN} coiled-coil structure has been demonstrated previously. For example, Q304 establishes hydrophobic interactions with V70 and L8 of the distal Ub₂ moiety¹³. Q304 also forms a hydrogen bond to D306 that stabilizes the dimer and the mutation Q304A weakens NEMO-Ub₂ interaction^{17,25}. Furthermore, A323 is involved in preserving the α -helical structure and, the Incontinentia Pigmenti (IP) associated mutation, A323P completely distorts dimerization and thereby indirectly abrogates Ub binding^{17,25}. Thus, just like binding of linUb₂, iNUB alters the conformational dynamics of the NEMO_{CC2-LZ} and induces changes in amino acids within the NEMO_{UBAN} that are directly involved in binding to Ub and/or preserving the dimer interface. Thereby, iNUB can prevent the formation of the NEMO_{UBAN}:linUb₂ complex.

iNUB inhibits UBAN-dependent NF- κ B activation upon TNF α and TCR/CD28 stimulation. To test if iNUB is affecting NEMO_{UBAN} dependent NF- κ B activation upon cell stimulation, we treated MEFs with 20 or 40 μM of iNUB before stimulation with TNF α or IL-1 β (Fig. 5a,b). In the experimental setup, iNUB did not show cell toxicity up to concentrations of 60 μM as revealed in viability assays (Supplementary Fig. 5). Equivalent to the requirement for NEMO_{UBAN}, iNUB reduced I κ B α phosphorylation/degradation and NF- κ B DNA binding in response to TNF α in a dose dependent manner, while it did not significantly affect IL-1 β induced NF- κ B signaling (Fig. 5a,b). To evaluate inhibitor selectivity, we determined activation of the MAPK JNK in MEFs in response to TNF α and IL-1 β after iNUB treatment (Fig. 5c,d). Here, iNUB did not significantly affect TNF α or IL-1 β stimulated JNK phosphorylation, showing that the compound is selectively acting on the NF- κ B signaling pathway in response to TNF α .

Emodin, which was only mildly competing for NEMO-Ub binding *in vitro* also impaired I κ B α phosphorylation/degradation as well as NF- κ B DNA binding in response to TNF α , but not IL-1 β stimulation (Supplementary Fig. 6a,b). However, cellular effects were considerably weaker when compared to iNUB treatment. Importantly, Emodin was also more selective in targeting TNF α triggered NF- κ B signaling and IL-1 β was not affected (Supplementary Fig. 6b).

We determined induction of NF- κ B target gene activation after iNUB treatment (Fig. 5e,f). Whereas induction of *TNFAIP3/A20*, *ICAM-1* and *VCAM-1* transcripts were decreased by iNUB after TNF α stimulation (Fig. 5e), expression of *TNFAIP3/A20* and *ICAM-1* target genes upon IL-1 β stimulation was not severely affected by iNUB (Fig. 5f). Also, Emodin impaired *TNFAIP3/A20*, *ICAM-1* and *VCAM-1* expression after TNF α stimulation (Supplementary Fig. 6c), but again to a lesser extent than iNUB. Congruent with the anti-apoptotic function of NF- κ B, induction of apoptosis was elevated in TNF α treated cells either in the presence of iNUB or more weakly by incubation of Emodin, revealing that diminished NF- κ B activation renders the cells more susceptible to TNF α -triggered cell death (Fig. 5g and Supplementary Fig. 6d).

Next, we investigated whether iNUB acts upstream of IKK activity in MEFs after TNF α stimulation (Fig. 5h,i). For this, we performed IKK kinase assays, after the cells have been treated with 20 or 40 μM of iNUB. The IKK complex was enriched by NEMO co-immunoprecipitation and IKK activity was measured in an *in vitro* kinase reaction. TNF α -induced IKK activation was markedly reduced in cells pre-treated with iNUB (Fig. 5h). To validate that iNUB is not acting directly on IKK kinases, but rather acts upstream on TNF α stimulation dependent IKK activation, we repeated the experiment in MEFs, but instead of treating the cells, we added iNUB in increasing concentrations to the precipitated IKK complex before the *in vitro* kinase reaction (Fig. 5i). In this case, iNUB was unable to inhibit IKK activity. Moreover, in contrast to a pharmacological IKK β active site inhibitor, iNUB was not able to reduce activity of recombinant IKK β in a pure *in vitro* kinase assay (Fig. 5j). Hence, inactivation of cellular IKK activity by iNUB is not due to a direct inhibition of IKK kinase activity, but rather a disturbed signaling upstream of the IKK complex in response to TNF α signaling.

To verify that iNUB affects NEMO UBAN dependent recruitment of Ub modified proteins in cells, we used NEMO^{-Y} MEFs reconstituted with StrepTagII-NEMO WT or the UBAN mutant D311N (Fig. 5k). In agreement with previous results³¹, polyubiquitinated RIP1 was co-precipitated by StrepTactin pull-down (ST-PD) of NEMO WT after TNF α stimulation and association required an intact UBAN as it was lost in NEMO D311N expressing cells. In the same experimental setup, iNUB treatment (20 and 40 μM) of NEMO WT MEFs also inhibited association of NEMO to ubiquitinated RIP1 (Fig. 5l). Thus, similar to UBAN mutations, iNUB treatment prevented stimulation dependent recruitment of NEMO to ubiquitinated RIP1, suggesting that the inhibitor disconnects the IKK complex from upstream regulators.

The NEMO_{UBAN} domain was also found to be essential for IKK/NF- κ B activation in response to TCR/CD28 co-stimulation¹⁸. To determine if iNUB affects T-cell activation, we tested iNUB on NF- κ B signaling and target gene expression in primary murine CD4⁺ T-cells stimulated with anti-CD3/CD28 antibodies (Fig. 6). Treatment with iNUB inhibited I κ B α phosphorylation upon TCR/CD28 co-engagement of CD4⁺ T-cells (Fig. 6a). iNUB treatment led to a dose-dependent decrease in the induction of the NF- κ B target genes *IL-2*, *TNFAIP3/A20*, *NFKBIA/I κ B α* and *IFN γ* (Fig. 6b,c). Induction of IL-2 secretion was also markedly reduced by iNUB as determined by ELISA (Fig. 6b). Thus, iNUB also impaired NEMO_{UBAN}-dependent TCR/CD28 NF- κ B signaling in primary CD4⁺ T-cells.

iNUB is selectively toxic to IKK/NF- κ B dependent ABC-DLBCL. The survival of ABC-DLBCL tumor cells relies on constitutive NF- κ B activation driven by oncogenic mutations and/or chronic BCR signaling upstream of the IKK complex³². IKK β inhibitors are well-documented to selectively kill ABC-DLBCLs³³ and, thus we wanted to determine whether iNUB also affected pathological NF- κ B signaling that results from

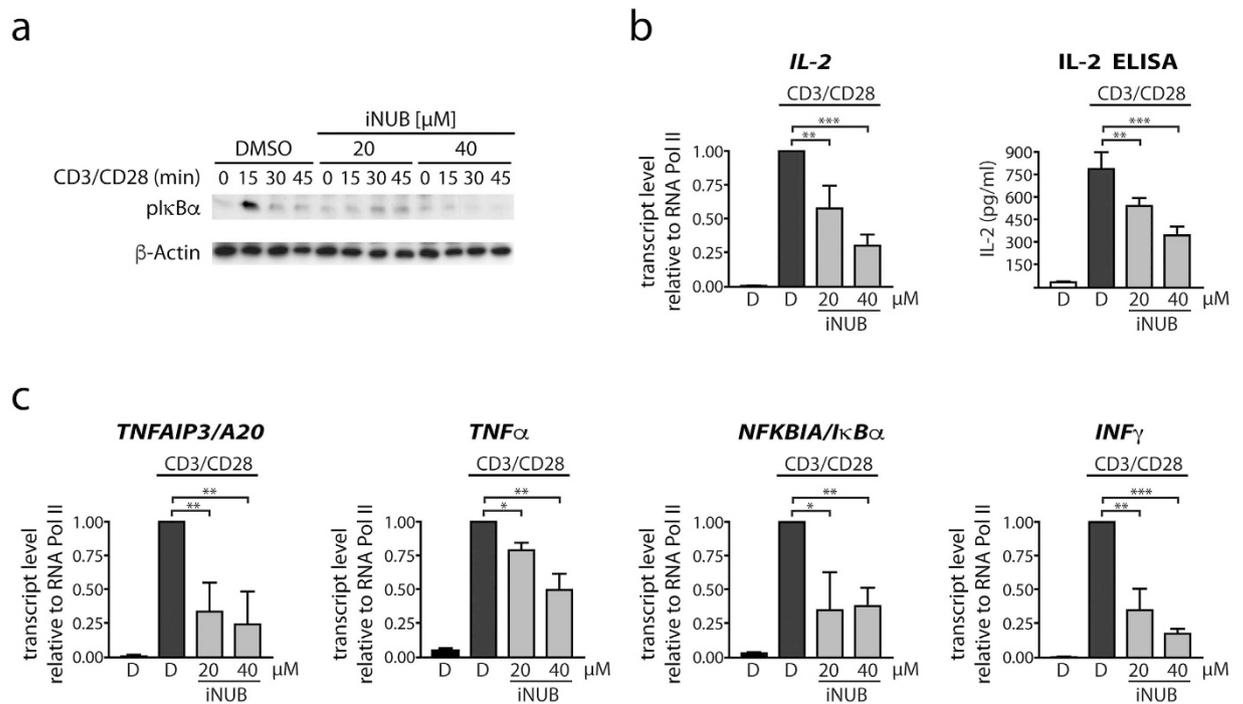


Figure 6. iNUB diminished NF- κ B activation in murine T-cells after TCR/CD28 stimulation. (a) Inhibition of TCR/CD28 induced NF- κ B signaling by iNUB. Primary murine CD4⁺ T-cells were incubated with iNUB (20 μ M, 40 μ M) or DMSO and co-stimulated with anti-CD3/anti-CD28 antibodies. I κ B α phosphorylation was detected by Western Blotting. (b,c) iNUB inhibits NF- κ B gene expression in primary T-cells. CD4⁺ T-cells were treated as in (a,b) IL-2 expression was analyzed on mRNA level by qPCR after 3 h as well as on protein levels by ELISA after 20 h and (c) *TNFAIP3/A20*, *TNF α* , *NFKBIA/I κ B α* and *INF γ* mRNA expression was analyzed after 3 h (n = 3; +/– SD).

uncontrolled antigen signaling in these lymphoma cells. NF- κ B is necessary for maintenance of high expression level of anti-apoptotic and pro-inflammatory factors in ABC-DLBCL, and therefore we evaluated by qPCR the relative mRNA expression levels of a panel of NF- κ B target genes (*c-FLIP*, *Bcl_{XL}*, *TNFAIP3/A20*, *TNF α* , *NFKBIA/I κ B α* , *IL-6*, *IL-10* and *JunB*) in the ABC-DLBCL cell lines HBL-1, OCI-Ly3, TMD8 and RIVA (Fig. 7a). Despite some variations in the extent of inhibition of the different target genes, treatment of iNUB markedly reduced the expression of NF- κ B regulated genes in ABC-DLBCL cells.

Since survival of ABC-DLBCL cells relies on constitutive NF- κ B signaling, we determined the effects of 20 and 40 μ M iNUB treatment on ABC-DLBCL survival by counting of viable cells. To monitor selectivity, we also measured viability in NF- κ B independent germinal center B-cell (GCB) type of DLBCL cells lines (Fig. 7b). Whereas ABC-DLBCL cell lines HBL-1, OCI-Ly3, TMD8 and RIVA were sensitive to iNUB treatment and died in a time and dose-dependent manner, iNUB1 was not toxic to the GCB-DLBCL cells SudHL-4, SudHL-6 and BJAB. We confirmed the results by MTT assays as a second test for cellular viability (Fig. 7c). Again, only ABC-DLBCL cells but not GCB-DLBCL showed a dose-dependent induction of cell toxicity after iNUB treatment. Since NF- κ B inhibition was shown to primarily enhance spontaneous apoptosis in ABC-DLBCL³³, we determined iNUB dependent apoptosis. Indeed, apoptosis was increased by iNUB in ABC but not in GCB-DLBCL cells at 20 and 40 μ M (Fig. 7d) again illustrating the selectivity of treatment. Finally, to verify that iNUB decreased viability of ABC-DLBCL by acting upstream of IKK β , we lentivirally reconstituted HBL-1 cells with a constitutively active IKK β (caIKK β) mutant that drives canonical NF- κ B signaling independent of upstream events³⁴. While in mock infected HBL-1 cells the number of viable cells decreased by 40–50% after 4 days of iNUB treatment, expression of caIKK β promoted a strong resistance to iNUB-induced cell death (Fig. 7e). Thus, iNUB selectively inhibited NF- κ B activation in ABC-DLBCL and thereby counteracted cell survival and enhanced apoptosis.

Discussion

Developing small molecule inhibitors that target PPIs is a challenging task, because the contact surfaces are usually flat and large (~1500–3000 Å²), contain many hydrophobic residues and largely lack distinct pockets for optimal small molecule binding^{35,36}. In this study, we have demonstrated that PPI inhibition can be a novel small molecule strategy to inhibit IKK activation and thus canonical NF- κ B signaling. We found that the anthraquinone derivative iNUB binds to the IKK regulatory subunit NEMO and, thereby alters NEMO dynamics to reduce its interaction with linear Ub₂. In contrast to most PPI surfaces that have been targeted by small molecules, the NEMO-Ub binding interface does not contain distinct pockets, but is composed of extended α -helices forming a dimer. Interaction of the NEMO_{UBAN} and linUb₂ is of relatively low affinity²⁵, which can facilitate the disruption by small molecule compounds. Based on NMR, SAXS and biophysical analyses we propose that iNUB acts through modulating the

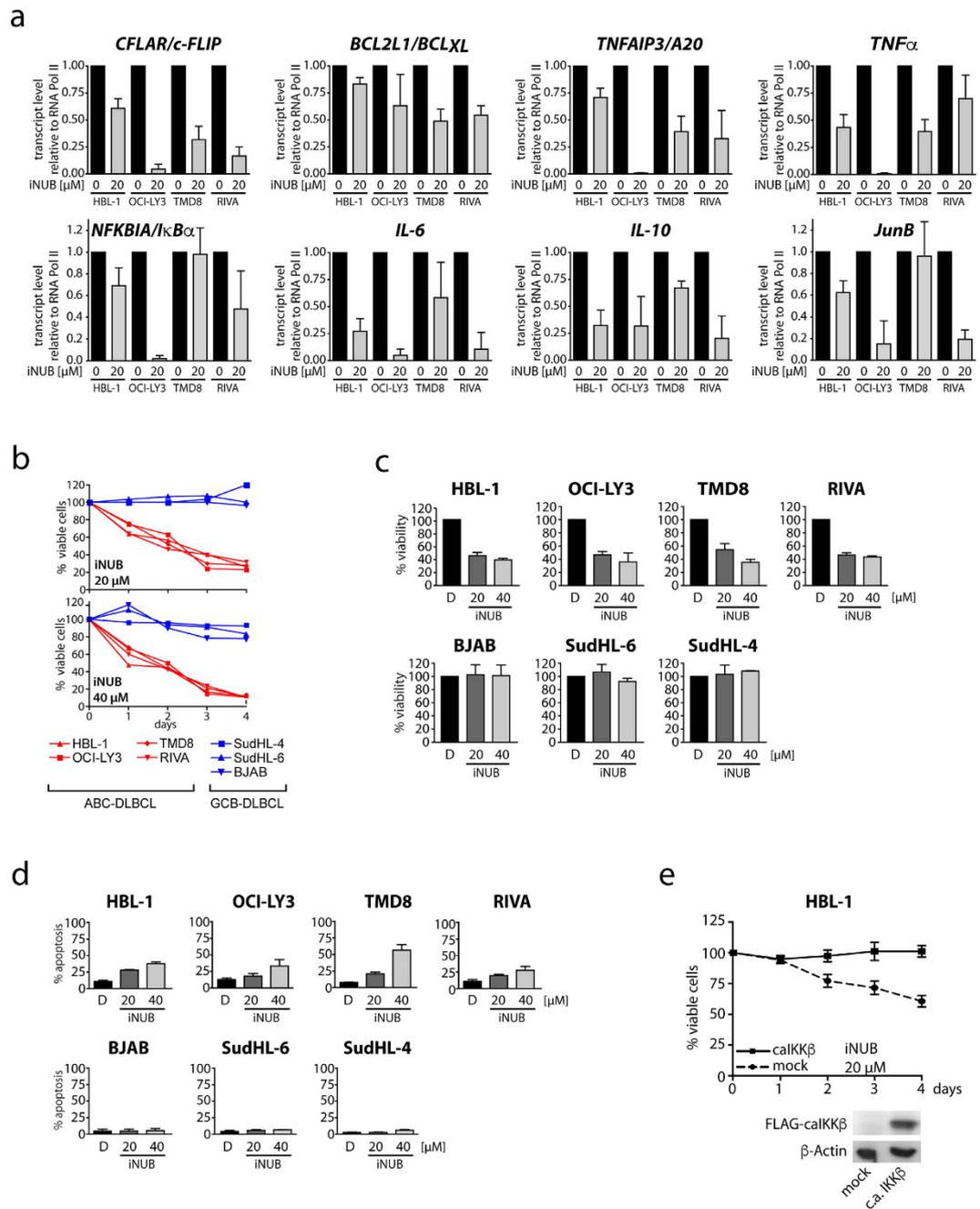


Figure 7. iNUB is toxic to ABC- but not GCB-DLBCL cells. (a) iNUB inhibits expression of NF-κB target genes in ABC-DLBCL cell lines. ABC-DLBCL cells (HBL-1, OCI-Ly3, TMD8, RIVA) were treated with iNUB (20 μM) for 24 h and NF-κB target genes were analyzed by qRT-PCR (n = 3; +/– SD). (b,c) Selective toxicity of iNUB in ABC- and GCB-DLBCL cells. ABC or GCB (SUDHL-4, SUDHL-6, BJAB) DLBCL cells were treated with single dose of 20 μM or 40 μM iNUB or DMSO. (b) Viable cells were counted after trypan blue exclusion over a period of 4 days (n = 4; optional representative experiment) or (c) MTT assay after 3 days of treatment (n = 4; +/– SD). (d) Apoptosis in ABC but not GCB-DLBCLs was induced by iNUB. Apoptotic cells were determined by Annexin-V staining of YO-PRO-3 negative cells and analyzed by FACS (n = 3; +/– SD). (e) Reduced viability of HBL-1 cells after iNUB treatment was rescued by expression of calIKKβ. HBL-1 cells expressing transduced with calIKKβ or mock were treated with iNUB (20 μM) and relative viable cells numbers was determined by trypan blue exclusion over 4 days. Expression of calIKKβ was determined by Western Blotting (n = 3; +/– SD).

structure and/or dynamics of the α-helical arrangement of the CC2-LZ region, as an unexpected and unique mode of action for small molecule inhibitors. We confirmed in cells that iNUB is acting on the level of the IKK complex, but not through direct targeting of catalytic IKKβ, as other conventional ATP-binding pocket inhibitors³⁷. In line

with the *in vitro* data on disrupted UBAN-Ub binding, iNUB inhibits UBAN dependent recruitment of NEMO to poly-ubiquitinated RIP1, a process that was shown to be critical for TNF α induced IKK/NF- κ B activation^{7,8}. iNUB did not inhibit activation of JNK, providing evidence that the compound is selectively impairing NF- κ B signaling in response to TNF α . Further, congruent with the genetic data on the role of the UBAN, iNUB inhibited NF- κ B signaling in response to TNF α and TCR/CD28 stimulation¹⁸, but did not affect UBAN-independent IL-1 β stimulation (see also below). Also in ABC-DLBCL, a well-defined cancer entity whose survival relies on chronic activation of the BCR-IKK-NF- κ B axis, iNUB inhibited NF- κ B activity and selectively killed the tumor cells just like conventional IKK β inhibitors³³. Importantly, various studies have demonstrated the necessity of the LUBAC complex and linear ubiquitination for active BCR signaling^{20,21,38} thereby defining the molecular basis for iNUB inhibition in ABC-DLBCL.

The critical role of Ub binding to the NEMO_{UBAN} for IKK activation has been demonstrated by structural studies^{13,14,17,25}. Moreover, inactivating point mutations within the UBAN and adjacent regions are causing IP in females and EDA-ID in males³⁹, revealing that small alterations within this region exert strong effects on activation of NF- κ B signaling. Indeed, we demonstrated by NMR that iNUB directly binds to the CC2-LZ region of NEMO and induces a number of chemical shift perturbations and resonance intensity changes. Some of these changes include residues that have been shown to be directly involved in Ub binding or NEMO dimerization^{13,17}. However, other perturbations throughout the entire CC2-LZ region include residues in NEMO that are not expected to be involved in direct contacts with Ub₂ and go far beyond the direct interactions mapped in the co-crystals¹³. Importantly, similar results were obtained when the interaction of linUb₂ with NEMO_{CC2-LZ} was analyzed using NMR. These data suggest that many chemical shift perturbations observed are not caused by the direct binding of either iNUB or linUb₂ to specific amino acids, but rather reflect that iNUB or linUb₂ disturbs the α -helical structure and dynamics of the NEMO dimer to provoke these extensive changes. Moreover, the conformational alterations may also be important for activation of IKK β ⁴⁰. Importantly, the structural and biophysical data suggest that iNUB is inducing similar, but less severe changes to the NEMO_{CC2-LZ} dimer as linUb₂. Thus, iNUB seems to allosterically alter the structural conformation and dynamics of the NEMO_{UBAN} in a way that prevents its binding to linear Ub₂.

Also, the widespread changes may explain the obvious discrepancy in the NEMO_{UBAN}:Ub₂ stoichiometry between the co-crystals and in solution assays. We confirmed the previously observed 2:1 stoichiometry of NEMO_{UBAN}:Ub₂ in solution²⁵. Using SAXS data we could build a DAMMIF model showing a structure that best fits to the 2:1 NEMO stoichiometry of the NEMO_{UBAN}:Ub₂ complex. This complex is not symmetric as described for the crystal, but linUb₂ is forming a hump and covering exclusively one side of the NEMO dimer. Together with the extensive spectral changes throughout the CC2-LZ seen in NMR, these data indicate that, in solution, binding of linUb₂ to one side of the NEMO dimers precludes the interaction of a second Ub₂ to the opposing surface. Thus, the 2:2 stoichiometry in the co-crystal may be induced by the crystal packing¹³.

In cells, iNUB is inhibiting TNF α , but not IL-1 β driven NF- κ B signaling. By direct comparison in a well-defined reconstitution system, we provide evidence that the NEMO_{UBAN} mutant D311N is required for NF- κ B activation in response to TNF α , but largely dispensable for IL-1 β stimulation. Moreover, even the removal of the UBAN or the C-terminal ZF domain of NEMO only partially impaired IL-1 β signaling to NF- κ B, despite the fact that it abrogated TNF α stimulation. In contrast, several N-terminal deletions resulted in equivalent decreases in NF- κ B activation after TNF α or IL-1 β treatment, ruling out a general variation within the experimental system. Given the pivotal role of Ub conjugation for IKK activation in all NF- κ B signaling pathways, we were surprised to see such differences, and previous studies have reported decreased NF- κ B activation in cells with NEMO_{UBAN} or NEMO_{ZF} mutations after IL-1 β stimulation^{13,26,41–43}. Zhang *et al.* reported severely reduced IKK activation after IL-1 α stimulation in MEFs from NEMO^{D311N/Y} knock-in mice²⁶. However, no direct comparison for the requirement of the NEMO_{UBAN} for NF- κ B activation between TNF and IL-1 stimulation was performed. Interestingly, NF- κ B signaling was reduced in fibroblasts from human EDA-ID patients carrying NEMO D311G mutations in response to TNF α and IL-1 β , but in both cases there was still considerable NF- κ B activation observed⁴¹. Thus, cell-specific and maybe even signal-specific (e.g. IL-1 α versus IL-1 β) variations may contribute to differential requirements of NEMO domains to trigger IKK/NF- κ B in response to different stimuli. In fact, also the amount of NEMO is highly critical for signal propagation¹⁸. To minimize cell variability, we used reconstituted NEMO^{-Y} MEFs and worked with FACS sorted cell pools that express equivalent NEMO amounts. Also other studies found fundamental differences in NEMO-dependent processes to NF- κ B after TNF α and IL-1 β stimulation. NEMO_{UBAN} displays a > 100 fold higher affinity for M1 versus K63 Ub₂^{13,17,25} and an Ub replacement strategy provided evidence for a requirement of K63-linked ubiquitination in IL-1 β , but not for TNF α signaling⁴⁴. In line with this, IL-1 β -triggered recruitment of NEMO to supramolecular clusters required K63-linked Ub chains, whereas it did not for TNF α stimulation⁴⁵. Also, cysteine-to-serine substitutions in the C-terminal ZF of NEMO have been shown to affect IKK activation in response to TNF α , but not IL-1 β ⁴⁶. Even though we cannot fully resolve the discrepancies to some of the previous publications, our NEMO reconstitution provide clear evidence for differences in the requirement of C-terminal NEMO UBAN and ZF domains in the TNF α and IL-1 β response. Congruent with our genetic complementation analyses, iNUB inhibited IKK/NF- κ B activation and target gene expression in response to TNF α stimulation, but it did not affect IL-1 β signaling in cells. Thus, small molecules that interfere with the binding of NEMO to Ub chains can be a strategy to selectively impair certain signaling pathways without affecting NF- κ B response to other stimuli.

The potential of modulating Ub binding properties of the NEMO UBAN was previously shown by generating cell permeable peptides comprising the N-terminal part of the UBAN (UBI peptide)²². The UBI peptide contains the D311R, which impairs binding to Ub chains. However, mechanistically it was shown to bind to NEMO, thereby competing for Ub association and impairing NF- κ B signaling after LPS or TNF α stimulation. Interestingly, in contrast to iNUB the UBI peptide only prevents association of K63-linked chains, while not affecting the binding of linear poly-Ub. Further, the steroidal lactone Withaferin A (WA) was shown to inhibit NF- κ B signaling and to modulate NEMO-Ub binding properties of NEMO in cells and *in vitro*^{47,48}. In contrast to iNUB, WA is covalently attached to the NEMO_{ZF} causing a gain-of-function that selectively enhances binding of NEMO to K48-linked

Ub chains. However, how this impacts NF- κ B signaling is currently unclear and it was also shown that WA can inactivate IKK β directly by targeting Cys179 in the kinase domain⁴⁹.

iNUB belongs to the class of anthraquinones that comprise natural products and the pharmacologically active ingredients of Chinese herbs. Most studied is the anthraquinone Emodin, whose anti-inflammatory and anti-cancer activity is well-established³⁰. Emodin affects multiple pathways including NF- κ B signaling, but the exact molecular targets are largely unresolved. Compared to iNUB, Emodin is a much weaker inhibitor of NEMO-Ub interaction and it is also less potent in inhibiting TNF α -triggered NF- κ B activation and target gene expression. However, despite the mild effects on NF- κ B signaling, Emodin still severely impacted on NF- κ B target gene expression and apoptosis induction. Thus, our data indicate that Emodin may not primarily act on upstream signaling, but also seems to inhibit NF- κ B activity further downstream, e.g. at the transcriptional level. Most likely also the pharmacological effects of iNUB will not only be restricted to prevention of NEMO-Ub binding, but we provide evidence that this mode of action contributes to NF- κ B inhibition and downstream effects. Thus, our data provide a proof of concept that it is possible to dissect the cellular targets of these naturally-derived products and to optimize their activity, which may be of interest for future therapeutic approaches.

Methods

Reagents. TNF α (50435; Biomol Germany); IL-1 β (211-11B; PeproTech); antibodies: anti-I κ B α (sc-371, Santa Cruz, USA), anti-p-I κ B α (9246; Cell Signaling), anti-NEMO (FL-419, Santa Cruz), anti- β Actin (sc-1616, Santa Cruz), anti-IKK β (05-535; Millipore), anti-IKK α (14A231; Imgenex), anti-JNK1/2 (9252; Cell Signaling), anti-p-JNK1/2 (9251; Cell Signaling), anti-Rip1 (3493; Cell Signaling) anti-FLAG M2 (F3165, Sigma); anti-mCD3 (553057, BD Biosciences); anti-hCD28 (553294, BD Biosciences); anti-IgG (307-005-003, Dianova). Tris-d11 (CD4035P1, Cortecnet); DL-dithiothreitol-d10 (CD570P1, Cortecnet); D₂O (151882, Aldrich); DMSO-d6 (156914, Aldrich); ¹⁵NH₄Cl (CN80P100, Cortecnet); D-glucose ¹³C6 (CC860P20, Cortecnet); D-glucose ¹³C6-d7 (CCD860P20, Cortecnet).

Cell Culture. DLBCL cell lines were cultured as previously described⁵⁰. Mouse embryonic fibroblasts (MEF), NEMO^{-Y} MEFs, reconstituted NEMO^{-Y} MEFs and HeLa cells were cultured in DMEM supplemented with 10% FCS and 1% Pen/Strep. All cells were cultured in an H₂O-saturated atmosphere with 5% CO₂ at 37 °C.

Stimulation and Western Blot. MEF cells were treated with the indicated amounts of iNUB or DMSO for 6 hours. Subsequently, MEF or HeLa cells were stimulated with 8 ng/ml TNF α or 1 ng/ml IL-1 β . Murine primary T-cells were stimulated with mCD3/mCD28 (BD Biosciences) and hamster IgG antibodies (Dianova) on plates for 3 hours (qRT-PCR analysis) and 20 hours (ELISA analysis), respectively. DLBCL cell lines were treated with iNUB for 24h before cells were lysed for Western Blot analysis. Western blotting was carried out as described earlier⁵¹.

Electrophoretic Mobility Shift Assay (EMSA). For EMSA analysis, MEF cells were pre-treated with iNUB or DMSO for 6 hours and stimulated with 8 ng/ml TNF α or 1 ng/ml IL-1 β for several time points. EMSA was performed as described previously⁵². Briefly, cells were lysed and 2 μ g of protein extract were incubated with a ³²P-dATP-labeled, double-stranded NF- κ B oligonucleotide probe (5'-CAGGGCTGGGGATTCCCCATCTCCACAGG-3') or OCT1 oligonucleotide probe (5'-TGTCGAATGCAAATCACTAGAA-3') and separated on native polyacrylamide gel electrophoresis before autoradiography.

Production of NEMO for biophysical and structural studies. For screening, the NEMO₂₅₈₋₃₅₀ construct was cloned, expressed and purified from *E. coli* strain BL21¹⁸. For other biochemical and biophysical measurements NEMO₂₅₈₋₃₅₀ or NEMO₂₅₈₋₃₅₀ C347S were cloned into pET vectors containing a 3C or TEV protease cleavable N-terminal His₆-tags. Details on purification and removal of epitope tags by proteolytic cleavage are given in the Supporting Information.

Microscale Thermophoresis (MST). Recombinant proteins were produced as described earlier using *E. coli* strain BL21-CodonPlus (DE3) RILP (Stratagene) and the pIBA3plus expression system (IBA GmbH, Göttingen, Germany). MST assays were mainly carried out as previously described¹⁸. Serial dilutions of iNUB or Emodin were incubated with NT647-labeled NEMO_{UBAN-ZF} proteins for 30 minutes. MST assays were measured in a NanoTemper Monolith NT.115. Compound concentrations were plotted against percent changes of normalized fluorescence (ΔF_{norm} [%]), curve fitting was done with GraphPad Prism software and K_D values were determined.

Small angle X-ray scattering (SAXS) analysis. SAXS measurements were performed on a Rigaku BIOSAXS1000 instrument with a HF007 microfocus generator equipped with a Cu-target at 40 kV and 30 mA. Transmissions were measured with a photodiode beamstop, q-calibration was made by an Ag-behenate measurement. Measurements were done in four 900 second frames, which were averaged. In these conditions no radiation damage was detected. Circular averaging and background subtraction was done with the Rigaku SAXSLab software v 3.0.1r1. Fits were made with crysol, ensembles with EOM, and distance distribution functions with GNOM, as provided with the ATSAS package v 2.5.0-2⁵³. Molecular weights were calculated from the Porod Volumes. Three-dimensional *ab initio* models were generated using the DAMMIF software⁵³.

SAXS measurements were made at 293 K with the following concentrations, NEMO_{UBAN} C347S four concentrations between 2.8 and 9.9 mg/ml, linear Ub₂ five concentrations in the range of 0.9 to 9.9 mg/ml and three concentrations each for the 2:1 and the 2:2 complex in the range of 3.5 to 12.9 and 4.9 to 12.1 mg/ml respectively. The buffer contained 300 mM NaCl and 50 mM Tris-HCl at a pH of 8.0. No concentration dependent effects were

detected. Agreement of SAXS theoretical curves back-calculated from published crystal structures to experimental curves was evaluated by χ^2 values.

NMR Spectroscopy. All NMR spectra were recorded on a Bruker AvanceIII 800 MHz spectrometer equipped with a TCI cryogenic or TXI room temperature probe head equipped with field gradient coils. All data-sets were processed using NMRPipe⁵⁴. Details for NMR spectroscopy are given in the Supplement.

DELFI Assay. Recombinant proteins were produced as described for MST assays. 20 pmol of StrepII-tagged NEMO proteins (UBAN domain) in DELFIA assay buffer (PerkinElmer) were bound to StrepTactin coated plates (IBA GmbH) and unbound proteins were removed after 2 h incubation by washing. Subsequently, bound NEMO proteins were incubated with several concentrations of iNUP or Emodin for 30 minutes before adding 125 pmol His-linUb₂ in DELFIA assay buffer for further 2 h incubation. Again, unbound proteins were removed after the incubation time by washing. Finally, 500 ng/ml europium-labeled anti-His-tag antibody was added and incubated for 1 h. After extensive washing enhancer solution (PerkinElmer Life Sciences) was added and the signal was measured in a PerkinElmer Envision plate reader (excitation, 340 nm; emission, 615 nm).

Viability, MTT and Apoptosis Assays. Viability, MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) and Apoptosis Assays were carried out as described earlier⁵⁰. Briefly, DLBCL cell lines were incubated with iNUP or DMSO in the indicated final concentrations for 24 hours. MEF cells were pretreated for 6 hours with iNUP or DMSO, respectively, before cells were stimulated with 8 ng/ml TNF α . Cell viability was analyzed by counting cells after trypan blue staining or by MTT cytotoxicity testing always in comparison to DMSO-treated control cells. Apoptosis rates were determined with Annexin-V-FITC staining of YO-PRO-3 negative cells (BD Pharmingen) after 24 hours of compound treatment in DLBCL and MEF cells. For FACS analysis an LSRII flow cytometer (BD) was used, and data were analyzed with FlowJo software (Treestar). Viability of mock (GFP) and $\text{caIKK}\beta$ -GFP expressing cells in response to iNUP treatment was determined by counting trypan blue stained cells.

Lentiviral transduction. Cloning of h Δ CD2-T2A-NEMO constructs into lentiviral vector, viral production, transduction of MEFs and sorting of h Δ CD2 positive cells was performed as described previously¹⁸. Expression of constitutively active Flag-IKK β ($\text{caIKK}\beta$) mutant (SS176, 180EE) was conducted as described⁵⁰. pLVTHM (Addgene plasmid # 12247), psPAX2 (Addgene plasmid # 12260) and pMD2.G (Addgene plasmid # 12259) were a gift from Didier Trono⁵⁵.

Quantitative qRT-PCR. cDNA generated from DNA-free RNA samples by reverse transcription was analyzed using LC-480 SybrGreen PCR mix (Roche) on a LC480 II Lightcycler system (Roche). 1 μ g RNA was transcribed with Superscript II (Invitrogen) according to the manufacturer's protocol, using random hexamers, with a final RNase H digestion step. The qRT-PCR experiments after TNF α stimulation were performed as described¹⁸, while qRT-PCR experiments in murine primary cells were carried out as described previously⁵⁰. Quantification of NF- κ B target genes were done with RNA PolII as control. All primer sequences are in the supplement.

In vitro Kinase Assays. MEF cells were either pre-treated with iNUP before stimulation with TNF α or pre-stimulated with TNF α following a post-treatment with iNUP of the precipitated active IKK complex. In both cases IKK β activity was investigated after immunoprecipitation (IP) of the IKK complex using an anti-NEMO antibody. Subsequently, recombinant GST-I κ B α (aa 1–72) was added to the precipitated IKK complex and the activity was analyzed by detection of pI κ B α in Western blot assays. Co-IP and Western blotting was done as described previously⁵². Recombinant active IKK β was incubated with iNUP or an IKK β inhibitor (sc-514, Santa Cruz) for 30 minutes. Subsequently, GST-I κ B α was added and pI κ B α was analyzed by Western Blotting.

Detection of cellular interaction of NEMO and ubiquitinated RIP1. NEMO^{-/-} MEF cells were transduced with StrepTagII-tagged constructs (mock, NEMO wildtype and NEMO D311N). Cells were grown in 150 mm cell culture plates until 90% confluence. Cells were either left untreated or treated with DMSO/iNUP for 6 hours and subsequently stimulated with 10ng/mL TNF α . Afterwards cells were lysed in 500 μ l lysis buffer (25 mM HEPES pH 7.5, 150 mM NaCl, 0.2% NP-40, 10% glycerol, 1 mM DTT, 10 mM sodium fluoride, 8 mM β -glycerophosphate, 300 μ M sodium vanadate and protease inhibitor cocktail) and StrepTactin pull-down (ST-PD) was performed by adding StrepTactin sepharose (IBA lifesciences) and incubating overnight. Co-precipitated RIP1 ubiquitination was detected in Western Blot analysis using RIP1 D94C12 antibody from cell signaling.

References

- Hayden, M. S. & Ghosh, S. Shared principles in NF-kappaB signaling. *Cell* **132**, 344–362, doi: 10.1016/j.cell.2008.01.020 (2008).
- Oeckinghaus, A., Hayden, M. S. & Ghosh, S. Crosstalk in NF-kappaB signaling pathways. *Nat Immunol* **12**, 695–708, doi: 10.1038/ni.2065 (2011).
- Thome, M., Charton, J. E., Pelzer, C. & Hailfinger, S. Antigen receptor signaling to NF-kappaB via CARMA1, BCL10, and MALT1. *Cold Spring Harb Perspect Biol* **2**, a003004, doi: 10.1101/cshperspect.a003004 (2010).
- Scheidereit, C. IkappaB kinase complexes: gateways to NF-kappaB activation and transcription. *Oncogene* **25**, 6685–6705, doi: 10.1038/sj.onc.1209934 (2006).
- Clark, K., Nanda, S. & Cohen, P. Molecular control of the NEMO family of ubiquitin-binding proteins. *Nat Rev Mol Cell Biol* **14**, 673–685, doi: 10.1038/nrm3644 (2013).
- Napetschnig, J. & Wu, H. Molecular basis of NF-kappaB signaling. *Annu Rev Biophys* **42**, 443–468, doi: 10.1146/annurev-biophys-083012-130338 (2013).

7. Ea, C. K., Deng, L., Xia, Z. P., Pineda, G. & Chen, Z. J. Activation of IKK by TNF α requires site-specific ubiquitination of RIP1 and polyubiquitin binding by NEMO. *Mol Cell* **22**, 245–257, doi: 10.1016/j.molcel.2006.03.026 (2006).
8. Conze, D. B., Wu, C. J., Thomas, J. A., Landstrom, A. & Ashwell, J. D. Lys63-linked polyubiquitination of IRAK-1 is required for interleukin-1 receptor- and toll-like receptor-mediated NF-kappaB activation. *Mol Cell Biol* **28**, 3538–3547, doi: 10.1128/MCB.02098-07 (2008).
9. Tokunaga, F. *et al.* Involvement of linear polyubiquitylation of NEMO in NF-kappaB activation. *Nat Cell Biol* **11**, 123–132, doi: 10.1038/ncb1821 (2009).
10. Haas, T. L. *et al.* Recruitment of the linear ubiquitin chain assembly complex stabilizes the TNF-R1 signaling complex and is required for TNF-mediated gene induction. *Mol Cell* **36**, 831–844, doi: 10.1016/j.molcel.2009.10.013 (2009).
11. Boisson, B. *et al.* Human HOIP and LUBAC deficiency underlies autoinflammation, immunodeficiency, amylopectinosis, and lymphangiectasia. *J Exp Med* **212**, 939–951, doi: 10.1084/jem.20141130 (2015).
12. Boisson, B. *et al.* Immunodeficiency, autoinflammation and amylopectinosis in humans with inherited HOIL-1 and LUBAC deficiency. *Nat Immunol* **13**, 1178–1186, doi: 10.1038/ni.2457 (2012).
13. Rahighi, S. *et al.* Specific recognition of linear ubiquitin chains by NEMO is important for NF-kappaB activation. *Cell* **136**, 1098–1109, doi: 10.1016/j.cell.2009.03.007 (2009).
14. Yoshikawa, A. *et al.* Crystal structure of the NEMO ubiquitin-binding domain in complex with Lys 63-linked di-ubiquitin. *FEBS Lett* **583**, 3317–3322, doi: 10.1016/j.febslet.2009.09.028 (2009).
15. Dyrnek, J. N. *et al.* c-IAP1 and UbcH5 promote K11-linked polyubiquitination of RIP1 in TNF signalling. *EMBO J* **29**, 4198–4209, doi: 10.1038/emboj.2010.300 (2010).
16. Laplantine, E. *et al.* NEMO specifically recognizes K63-linked poly-ubiquitin chains through a new bipartite ubiquitin-binding domain. *EMBO J* **28**, 2885–2895, doi: 10.1038/emboj.2009.241 (2009).
17. Grubisha, O. *et al.* DARPIn-assisted crystallography of the CC2-LZ domain of NEMO reveals a coupling between dimerization and ubiquitin binding. *J Mol Biol* **395**, 89–104, doi: 10.1016/j.jmb.2009.10.018 (2010).
18. Hadian, K. *et al.* NF-kappaB essential modulator (NEMO) interaction with linear and lys-63 ubiquitin chains contributes to NF-kappaB activation. *J Biol Chem* **286**, 26107–26117, doi: 10.1074/jbc.M111.233163 (2011).
19. Hinz, M. & Scheidereit, C. The IkappaB kinase complex in NF-kappaB regulation and beyond. *EMBO Rep* **15**, 46–61, doi: 10.1002/embr.201337983 (2014).
20. Yang, Y. *et al.* Essential role of the linear ubiquitin chain assembly complex in lymphoma revealed by rare germline polymorphisms. *Cancer Discov* **4**, 480–493, doi: 10.1158/2159-8290.CD-13-0915 (2014).
21. Dubois, S. M. *et al.* A catalytic-independent role for the LUBAC in NF-kappaB activation upon antigen receptor engagement and in lymphoma cells. *Blood* **123**, 2199–2203, doi: 10.1182/blood-2013-05-504019 (2014).
22. Chiaravalli, J. *et al.* Direct inhibition of NF-kappaB activation by peptide targeting the NOA ubiquitin binding domain of NEMO. *Biochem Pharmacol* **82**, 1163–1174, doi: 10.1016/j.bcp.2011.07.083 (2011).
23. Agou, F. *et al.* Inhibition of NF-kappa B activation by peptides targeting NF-kappa B essential modulator (nemo) oligomerization. *J Biol Chem* **279**, 54248–54257, doi: 10.1074/jbc.M406423200 (2004).
24. van Wijk, S. J. *et al.* Fluorescence-based sensors to monitor localization and functions of linear and K63-linked ubiquitin chains in cells. *Mol Cell* **47**, 797–809, doi: 10.1016/j.molcel.2012.06.017 (2012).
25. Lo, Y. C. *et al.* Structural basis for recognition of diubiquitins by NEMO. *Mol Cell* **33**, 602–615, doi: 10.1016/j.molcel.2009.01.012 (2009).
26. Zhang, J., Clark, K., Lawrence, T., Pegg, M. W. & Cohen, P. An unexpected twist to the activation of IKK β : TAK1 primes IKK β for activation by autophosphorylation. *Biochem J* **461**, 531–537, doi: 10.1042/BJ20140444 (2014).
27. Rushe, M. *et al.* Structure of a NEMO/IKK-associating domain reveals architecture of the interaction site. *Structure* **16**, 798–808, doi: 10.1016/j.str.2008.02.012 (2008).
28. Komander, D. *et al.* Molecular discrimination of structurally equivalent Lys 63-linked and linear polyubiquitin chains. *EMBO Rep* **10**, 466–473, doi: 10.1038/emboj.2009.55 (2009).
29. Rohaim, A., Kawasaki, M., Kato, R., Dikic, I. & Wakatsuki, S. Structure of a compact conformation of linear diubiquitin. *Acta Crystallogr D Biol Crystallogr* **68**, 102–108, doi: 10.1107/S0907444911051195 (2012).
30. Shrimali, D. *et al.* Targeted abrogation of diverse signal transduction cascades by emodin for the treatment of inflammatory disorders and cancer. *Cancer Lett* **341**, 139–149, doi: 10.1016/j.canlet.2013.08.023 (2013).
31. Wu, C. J., Conze, D. B., Li, T., Srinivasula, S. M. & Ashwell, J. D. Sensing of Lys 63-linked polyubiquitination by NEMO is a key event in NF-kappaB activation [corrected]. *Nat Cell Biol* **8**, 398–406, doi: 10.1038/ncb1384 (2006).
32. Young, R. M., Shaffer, A. L., 3rd, Phelan, J. D. & Staudt, L. M. B-cell receptor signaling in diffuse large B-cell lymphoma. *Semin Hematol* **52**, 77–85, doi: 10.1053/j.seminhematol.2015.01.008 (2015).
33. Lam, L. T. *et al.* Small molecule inhibitors of IkappaB kinase are selectively toxic for subgroups of diffuse large B-cell lymphoma defined by gene expression profiling. *Clin Cancer Res* **11**, 28–40 (2005).
34. Calado, D. P. *et al.* Constitutive canonical NF-kappaB activation cooperates with disruption of BLIMP1 in the pathogenesis of activated B cell-like diffuse large cell lymphoma. *Cancer Cell* **18**, 580–589, doi: 10.1016/j.ccr.2010.11.024 (2010).
35. Wells, J. A. & McClendon, C. L. Reaching for high-hanging fruit in drug discovery at protein-protein interfaces. *Nature* **450**, 1001–1009, doi: 10.1038/nature06526 (2007).
36. Gul, S. & Hadian, K. Protein-protein interaction modulator drug discovery: past efforts and future opportunities using a rich source of low- and high-throughput screening assays. *Expert Opin Drug Discov* **9**, 1393–1404, doi: 10.1517/17460441.2014.954544 (2014).
37. Hideshima, T. *et al.* MLN120B, a novel IkappaB kinase beta inhibitor, blocks multiple myeloma cell growth *in vitro* and *in vivo*. *Clin Cancer Res* **12**, 5887–5894, doi: 10.1158/1078-0432.CCR-05-2501 (2006).
38. Satpathy, S. *et al.* Systems-wide analysis of BCR signalosomes and downstream phosphorylation and ubiquitylation. *Mol Syst Biol* **11**, 810, doi: 10.15252/msb.20145880 (2015).
39. Fusco, F. *et al.* Alterations of the IKBKG locus and diseases: an update and a report of 13 novel mutations. *Hum Mutat* **29**, 595–604, doi: 10.1002/humu.20739 (2008).
40. Xu, G. *et al.* Crystal structure of inhibitor of kappaB kinase beta. *Nature* **472**, 325–330, doi: 10.1038/nature09853 (2011).
41. Hubeau, M. *et al.* New mechanism of X-linked anhidrotic ectodermal dysplasia with immunodeficiency: impairment of ubiquitin binding despite normal folding of NEMO protein. *Blood* **118**, 926–935, doi: 10.1182/blood-2010-10-315234 (2011).
42. Schrofelbauer, B., Polley, S., Behar, M., Ghosh, G. & Hoffmann, A. NEMO ensures signaling specificity of the pleiotropic IKK β by directing its kinase activity toward IkappaB α . *Mol Cell* **47**, 111–121, doi: 10.1016/j.molcel.2012.04.020 (2012).
43. Windheim, M., Stafford, M., Pegg, M. & Cohen, P. Interleukin-1 (IL-1) induces the Lys63-linked polyubiquitination of IL-1 receptor-associated kinase 1 to facilitate NEMO binding and the activation of IkappaB α kinase. *Mol Cell Biol* **28**, 1783–1791, doi: 10.1128/MCB.02380-06 (2008).
44. Xu, M., Skaug, B., Zeng, W. & Chen, Z. J. A ubiquitin replacement strategy in human cells reveals distinct mechanisms of IKK activation by TNF α and IL-1 β . *Mol Cell* **36**, 302–314, doi: 10.1016/j.molcel.2009.10.002 (2009).
45. Tarantino, N. *et al.* TNF and IL-1 exhibit distinct ubiquitin requirements for inducing NEMO-IKK supramolecular structures. *J Cell Biol* **204**, 231–245, doi: 10.1083/jcb.201307172 (2014).
46. Makris, C., Roberts, J. L. & Karin, M. The carboxyl-terminal region of IkappaB kinase gamma (IKK γ) is required for full IKK activation. *Mol Cell Biol* **22**, 6573–6581 (2002).

47. Jackson, S. S. *et al.* Withaferin A disrupts ubiquitin-based NEMO reorganization induced by canonical NF- κ B signaling. *Exp Cell Res* **331**, 58–72, doi: 10.1016/j.yexcr.2014.09.034 (2015).
48. Hooper, C., Jackson, S. S., Coughlin, E. E., Coon, J. J. & Miyamoto, S. Covalent modification of the NF- κ B essential modulator (NEMO) by a chemical compound can regulate its ubiquitin binding properties *in vitro*. *J Biol Chem* **289**, 33161–33174, doi: 10.1074/jbc.M114.582478 (2014).
49. Heyninck, K., Lahtela-Kakkonen, M., Van der Veken, P., Haegeman, G. & Vanden Berghe, W. Withaferin A inhibits NF- κ B activation by targeting cysteine 179 in IKK β . *Biochem Pharmacol* **91**, 501–509, doi: 10.1016/j.bcp.2014.08.004 (2014).
50. Nagel, D. *et al.* Pharmacologic inhibition of MALT1 protease by phenothiazines as a therapeutic approach for the treatment of aggressive ABC-DLBCL. *Cancer Cell* **22**, 825–837, doi: 10.1016/j.ccr.2012.11.002 (2012).
51. Kloo, B. *et al.* Critical role of PI3K signaling for NF- κ B-dependent survival in a subset of activated B-cell-like diffuse large B-cell lymphoma cells. *Proc Natl Acad Sci USA* **108**, 272–277, doi: 10.1073/pnas.1008969108 (2011).
52. Eitelhuber, A. C. *et al.* Dephosphorylation of Carma1 by PP2A negatively regulates T-cell activation. *EMBO J* **30**, 594–605, doi: 10.1038/emboj.2010.331 (2011).
53. Petoukhov, M. V. *et al.* New developments in the program package for small-angle scattering data analysis. *J Appl Crystallogr* **45**, 342–350, doi: 10.1107/S0021889812007662 (2012).
54. Delaglio, F. *et al.* NMRPipe: a multidimensional spectral processing system based on UNIX pipes. *J Biomol NMR* **6**, 277–293 (1995).
55. Wiznerowicz, M. & Trono, D. Conditional suppression of cellular genes: lentivirus vector-mediated drug-inducible RNA interference. *J Virol* **77**, 8957–8961 (2003).

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Author Contributions

M.V., K.H., A.C.M., R.S., A.G., M.S. and D.K. have conceived and designed the experiments. M.V., K.H., A.C.M., J.H., J.K.B., R.G., U.G., A.B., R.S., D.N., K.D., H.V. and A.G. performed the experiments. M.V., K.H., A.C.M., R.S., D.N., A.G., M.S. and D.K. analyzed and interpreted the data. M.V., K.H., A.C.M., A.G., M.S. and D.K. conceived, drafted and finalized the manuscript.

Additional Information

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