

Supplementary Information

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

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Supplementary Methods

Screening of small molecule library

Compound screening was performed using a natural compound library of 320 compounds at a concentration of 10 μ M. This library was derived from Specs (specs.net, Netherlands) containing purified or synthesized natural products with a purity of >80%. The compounds were pre-selected by Specs with focus on diversity and a broad spectrum of natural sources, e.g. plants, fungi, bacteria, sea organisms. DELFIA assay for compound screening was performed as described above.

Compounds

Compounds from Chembridge/Hit2lead.com, USA: Anthraquinone 1 (=iNUB, 5109356), Anthraquinone 2 (5181426), Anthraquinone 3 (5212492), Anthraquinone 4 (5228191), Anthraquinone 5 (5253964), Anthraquinone 6 (5266859), Anthraquinone 7 (5631331), Anthraquinone 8 (5730401), Anthraquinone 9 (7259337), Anthraquinone 10 (7267603); other compounds: Emodin (7451.1, Roth, Germany); Aloe-Emodin derivative (AO-774/41465647, Specs, Netherlands)

Production of NEMO for biophysical and structural studies

The constructs were transformed into *E. coli* strain BL21 (DE3) and cultured at 293 K in ZYM 5052 auto-induction medium¹. Cells were lysed by sonication and His-tagged proteins purified by immobilized metal affinity chromatography (IMAC) using a 5-ml HiTrap Chelating HP column (GE Healthcare). The elution fractions were dialyzed overnight at 277 K in the presence of His-tagged 3C or TEV protease. The cleaved NEMO was further purified by IMAC and subjected to size exclusion chromatography using a HiLoad 16/60 Superdex 75 column

(GE Healthcare). The main elution peak containing NEMO was collected, concentrated to 10-110 mg/ml and stored at 277 K. A protease inhibitor cocktail (complete EDTA-free, Roche) was added to the final preparation according to the manufacturer's instructions in order to prevent degradation. Protein concentrations were determined by measuring the absorbance at 280nm using specific absorbance for NEMO 258-350 and 258-350 C347S of 0.271 mL/mg. Protein production of labeled NEMO and Ub₂ for NMR studies was done by substituting the rich medium by M9 minimal medium with controlled ¹⁵N and ¹³C carbon sources. Production of triple labeled ²H, ¹³C, ¹⁵N-NEMO for assignment was performed using M9 minimal medium, 100% D₂O and uniformly labeled ²H,¹³C-glucose. Purification of the proteins for NMR studies was carried out as described above for the unlabeled proteins.

NMR Spectroscopy

Assignments for linear Ub₂ were obtained by using HNCACB, ¹⁵N-edited NOESY and ¹⁵N-edited TOCSY 3D experiments². Spectra were acquired using a 900 μM ¹³C, ¹⁵N-labeled linear Ub₂ sample at 290 K in 20 mM deuterated Tris-HCl pH 8.0, 50 mM NaCl, 10 % D₂O and at 298 K in 20 mM deuterated Tris-HCl pH 7.5, 50 mM NaCl, 10 % D₂O. Assignments of wild-type NEMO (258-350) were obtained using triple labeled samples in concentrations ranging from 850 μM to 1.2 mM based on data from 3D TROSY-based HNCA, HNCACB, HNCO, HN(CA)CO, HN(CA)CO and HN(CO)CACB with deuterium decoupling. Spectra were recorded at 308 K and sample conditions were 50 mM sodium phosphate pH 7.0, 50 mM NaCl and 10% D₂O. Some experiments were also repeated with NEMO₂₅₈₋₃₅₀ C347S. The software CARA (cara.nmr.ch) was used to assign linear Ub₂ and wild-type NEMO_{UBAN}.

Competition experiments were performed at 298 K using 100 μM of ¹⁵N-labeled linear Ub₂ mixed in a 1:1 ratio with unlabeled NEMO_{UBAN} C347S by adding the compounds (489.2 μM

Emodin, 290.7 μM iNUB, or DMSO-d6 for reference) and monitoring the changes by ^1H , ^{15}N -HSQC experiments (2048 (ω_2) x 256 (ω_1) time domain points and 64 scans). Sample conditions were 20 mM deuterated Tris-HCl pH 8.0, 50 mM NaCl, 0.3% Tween 20 and 10% D₂O. Due to poor compound solubility, some precipitation of the compounds was observed after the experiment. A comparison of peak volumes was made between the spectra of the complex (^{15}N -labeled linear Ub₂ and unlabeled NEMO_{UBAN} C347S) with compound or deuterated DMSO added. Integration was performed using Sparky³ with a Gaussian fit integration mode.

Binding of iNUB and Emodin to NEMO_{UBAN} wild-type was performed at 308 K using 117 μM of ^{15}N -labeled NEMO in 50 mM deuterated Tris-HCl pH 8.0, 50 mM NaCl, 10mM DTT, 0.3% Tween 20 and 10% D₂O by adding the compounds to a final concentration of 870 μM , and monitoring the changes by ^1H , ^{15}N -TROSY experiments (1024k (ω_2) x 128 (ω_1) time domain points and 16 scans). A reference experiment was conducted in identical experimental conditions where the compound was replaced by an addition of the same volume of DMSO-d6.

Binding of iNUB and Emodin to Ub₂ was monitored at 298 K using 50 μM of ^{15}N -labeled Ub₂ in 20 mM deuterated Tris-HCl pH 8.0, 50 mM NaCl, 0.3% Tween 20 and 10% D₂O by adding the compounds to a final concentration of 1 mM and following changes by ^1H , ^{15}N -HSQC experiments (1024 (ω_2) x 128 (ω_1) time domain points and 8 scans). Reference experiments were conducted in identical experimental conditions where the compounds were replaced by addition of the same volume of DMSO-d6.

Titration of Ub₂ to NEMO_{UBAN} was recorded at 308 K using 100 μM ^{15}N -labeled NEMO wild-type in 50 mM deuterated Tris-HCl pH 8, 50 mM NaCl, 10mM DTT, 0.3% Tween 20 and 10% D₂O by adding unlabeled Ub₂ 781 μM solution to final molar ratios Ub₂:NEMO_{UBAN} of 0, 0.06,

0.13, 0.20, 0.39, 0.42, 0.58 and 1.0 and following the changes by ^1H , ^{15}N -TROSY experiments (1024 (ω_2) x 128 (ω_1) time domain points and 32 scans).

Static Light Scattering (SLS)

Static light scattering (SLS) experiments of the different NEMO constructs, Ub₂ and the NEMO_{UBAN}:linUb₂ complex were performed at 303 K using a Viscotek TDA 305 triple array detector (Malvern Instruments) downstream to an Äkta Purifier (GE Healthcare) equipped with an analytical size exclusion column (Superdex 75 or 200 10/300 GL, GE Healthcare) at 277 K. The samples were run in 50mM Tris-HCl pH 8.0, 300mM NaCl, 0.01% (v/v) 1-thioglycerol with concentrations varying from 2.9-7.8 mg/ml at a flow rate of 0.5 ml/min. The molecular masses of the samples were calculated from the refractive index and right-angle light-scattering signals using Omnisec (Malvern Instruments). The SLS detector was calibrated with a 4 mg/ml BSA solution with 66.4 kDa for the BSA monomer and a dn/dc value of 0.185 ml/g for all protein samples.

Isothermal titration calorimetry

ITC measurements were carried out at 298 K using a ITC200 titration microcalorimeter (Microcal, GE Healthcare, NJ).. The calorimetric titration consisted of 32 injections of 0.4 μl of a 1.28 mM NEMO_{UBAN} C347S sample, into the reaction cell containing 200 μl of 90 μM linear Ub₂, at a stirring speed of 1000 rpm. Sample conditions were 50 mM sodium phosphate pH 7.0 and 50 mM NaCl. The heat of dilution was obtained by titrating NEMO_{UBAN} C347S into the sample cell containing only buffer and this was subsequently subtracted from each experimental titration. The ITC data were analyzed using the software Origin provided by Microcal.

Supplementary Table

Table qRT-PCR primers:

Target genes (murine)	FW Primer (5'-3')	Rev Primer (5'-3')
TNFAIP3/A20	GCTCAACTGGTGTCTGAAG	ATGAGGCAGTTTCCATCACC
ICAM-1	GGAGACGCAGAGGACCTTAAC	CGCTCAGAAGAACCACCTTC
VCAM-1	AGTTGGGGATTCTGGTTGTTCT	CCCCTCATTCTTACCACCC
IL-2	GAGTGCCAATTCGATGATGAG	AGGGCTTGTTGAGATGATGC
TNF α	CCACCATCAAGGACTCAAATG	GAGACAGAGGCAACCTGACC
NFKBIA/I κ B α	TTGCTGAGGCACTTCTGAAAG	TCTGCGTCAAGACTGCTACACT
INF γ	GCTCTGAGACAATGAACGCTAC	TCTTCCACATCTATGCCACTTG
β -actin	CCTCTATGCCAACACAGTGC	GTACTCCTGCTTGCTGATCC

Target genes (human)	FW Primer (5'-3')	Rev Primer (5'-3')
CFLAR/c-FLIP	TCAGAATCCTTTCCAGTGGG	TCCAGGCTTTCGGTTTCTTT
BCL2L1/BCL $_{XL}$	AAACTGGGTCGCATTGTGG	TCTCGGCTGCTGCATTGTTC
TNFAIP3/A20	TTTTGTACCCTTGGTGACCCTG	TTAGCTTCATCCAACCTTGC GG
TNF α	CCCAGGGACCTCTCTAATCA	GCTACAGGCTTGCTACTCGG
NFKBIA/I κ B α	CCGCACCTCCACTCCATCC	ACATCAGCACCCAAGGACACC
IL-6	GGTACATCCTCGACGGCATCT	GTGCCTCTTTGCTGCTTTCAC
IL-10	CTGGAGGAGGTGATGCCCAA	ACCTGCTCCACGGCCTTGCT
JunB	GCACTAAAATGGAACAGCCCTT	GGCTCGGTTTCAGGAGTTTG
RPII	GCACCACGTCCAATGACAT	GTGCGGCTGCTTCCATAA

Supplementary References

- 1 Studier, F. W. Protein production by auto-induction in high density shaking cultures. *Protein Expr Purif* **41**, 207-234 (2005).
- 2 Sattler, M., Schleucher, J. & Griesinger, C. Heteronuclear multidimensional NMR experiments for the structure determination of proteins in solution employing pulsed field gradients. *Prog NMR Spectrosc* **34**, 93-158 (1999).
- 3 Goddard, T. D. & Kneller, D. G. SPARKY3. *University of California, San Francisco*.

Supplementary Figure Legends

Supplementary Figure 1. Lentiviral transduction and analyses of NEMO proteins in MEFs.

(a-b) Transduction and sorting of human (h) Δ CD2-T2A-NEMO expressing MEFs. Exemplified sorting of infected MEFs transduced with NEMO Δ 44-111 based on h Δ CD2 surface expression (a). Sorting resulted in homogenous cell populations for all constructs that express h Δ CD2 (>90 %) (a and b). (c and d) Expression levels of NEMO WT and mutant proteins after reconstitution in NEMO^{-/-} MEFs were analyzed by Western Blot and compared to MEFs from wildtype mice. (e) Co-IP of NEMO WT and three N-terminal deletion mutant was performed and analyzed for binding to IKK α and IKK β by Western Blot. (f and g) Effects of NEMO N-terminal 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 (f) or IL-1 β (g). 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.

Supplementary Figure 2. Analysis of NEMO and Ub₂ conformation and stoichiometry of the complex NEMO_{UBAN}:linUb₂.

(a-b) Determination of the molecular weight of NEMO_{UBAN} C347S (green), Ub₂ (blue) and NEMO_{UBAN}:linUb₂ complex (red) using size exclusion chromatography in combination with static light scattering. The refractive index and right angle light scattering signals were monitored and used to determine the molecular weight (grey and black). 100 μ l of the protein samples (2.9-7.8 mg/ml) were applied to a Superdex 200 10/300 GL column. NEMO was analyzed at 7.8 mg/ml and eluted as a homodimer of 21.6 kDa (calculated MW is 22.0

kDa), while Ub₂ (2.9 mg/ml) behaved as a monomer of 18.2 kDa (calculated MW is 18.2 kDa). NEMO and Ub₂ were mixed in a 2:1 molar ratio and 100 μl applied to a Superdex 200 10/300 GL column. Two peaks were obtained. The first peak eluted at 14.6 ml and a MW of 33.4 kDa. This peak contained a mixture of the NEMO_{UBAN}:linUb₂ complex and the remaining NEMO dimer. The second peak eluted at 17.3 ml contained the remaining Ub₂. Increasing the Ub₂:NEMO ratio led to an increase in the apparent molecular weight of NEMO (Insert in the chromatogram) to a maximum of 37.3 kDa at a ratio of 12:1. As the *k*_{off} of the NEMO_{UBAN}:linUb₂ complex is relatively low, NEMO dimer and Ub₂ separate in the column, giving rise to a peak of NEMO dimer, which overlaps with the complex, leading to a lower apparent molecular weight (MW) of the complex (33.4 kDa at 2:1 NEMO:Ub₂). However, the apparent MW increases when the Ub₂:NEMO molar ratio is increased. **(c)** Interaction of NEMO_{UBAN} C347S to Ub₂ was measured in MST assays. NEMO_{UBAN} C347S binds Ub₂ with a *K*_D of 1.2 μM. **(d)** Isothermal titration calorimetry (ITC) measurement of the NEMO:Ub₂ interaction. NEMO C347S was titrated into the Ub₂ solution. The numbers show the fitting of the data, with N for stoichiometry, *K*_D for dissociation constant, Δ*H* for enthalpy change and -Δ*S* for entropy change. **(e)** Comparison of the SAXS experimental curve (black) with the theoretical curve back-calculated from the NEMO crystal structure (red; PDB 3FX0; construct 246-337) leads to a quite high χ² value, indicating that the NEMO dimer crystal structure is considerably different than in solution. **(f)** Comparison of the SAXS experimental curves for Ub₂ (black) with the theoretical curves back-calculated from the two published crystal structures for linear Ub₂ PDB 2W9N (red) and PDB 3AXC (green), which do not fit to the experimental scattering well independently (right). An ensemble calculation with a 5 AlaSer flexible linker at the C-terminal part of the first Ub (residues 72-76) leads to a much better fit

(left). The resulting structures have the same domain distances as the published crystal data but different relative orientations of the Ub domains.

Supplementary Figure 3. Assay Development and Screening for NEMO-Ub₂ binding inhibitors.

(a) Schematic overview of the DELFIA assay using Strep-tagged coupled to Streptactin coated plates and His-tagged linear Ub₂. (b) DELFIA signal depends on intact UBAN. Signal by Myc-NEMO_{UBAN}-StrepTagII WT and His-linUb₂ was abolished by the UBAN mutation NEMO D311N. (c) Screening of a natural compound library using the DELFIA assay. Compound (10 μM) treated samples were compared to non-treated positive controls. 4 hits that inhibited the signal by >50% were identified. The overall Z' factor of the screening was 0.78. (d) Chemical structure of the 4 identified hits. (e) Effects of anthraquinone derivatives on NEMO_{UBAN} binding to linear Ub₂. 12 anthraquinone derivatives comprising Aloe Emodin derivative and Emodin were tested in DELFIA binding assays at three concentrations.

Supplementary Figure 4. Effects of the natural compound Emodin on NEMO-Ub₂ binding.

(a) DELFIA assay detection of Myc-NEMO_{UBAN}-StrepTagII (242-350) binding to His tagged linear Ub₂ with or without Emodin. Graph shows the inhibition of NEMO-diUb binding by the addition of increasing Emodin concentrations. (b) NMR of linear Ub₂ in complex with NEMO_{UBAN} in the presence of Emodin. ¹H, ¹⁵N –HSQC spectrum of 100 μM ¹⁵N-labeled Ub₂ with unlabeled NEMO_{UBAN} (1:1 stoichiometry) in the presence of DMSO-d₆ (blue) or 489.2 μM Emodin (red). Ub₂ backbone amides resonances, which changed upon treatment with Emodin are indicated. (c) Mapping of Ub₂ backbone amides perturbed upon Emodin addition in 'b' onto NEMO_{UBAN} structure (PDB 2ZVO). Red spheres indicate peak shifts of

unambiguously assigned amino acids; orange spheres indicate peak shifts of ambiguously assigned or overlapping amino acids, while gray spheres represent the counterpart amino acid on the other Ub moiety. **(d)** Ratio of amide peak volume upon Emodin versus DMSO-d6 addition for the ¹⁵N-labeled linUb₂ complexed with unlabeled NEMO (1:1 stoichiometry).

Supplementary Figure 5. iNUB Toxicity assessment in MEFs.

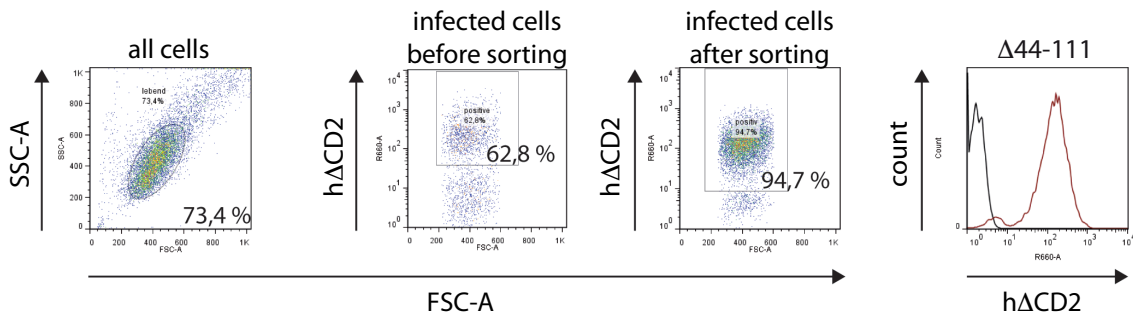
iNUB is not toxic to MEFs up to a concentration of 60 μM. Viability of MEFs was determined by MTT assay.

Supplementary Figure 6. Effects of Emodin on NF-κB signaling in MEFs.

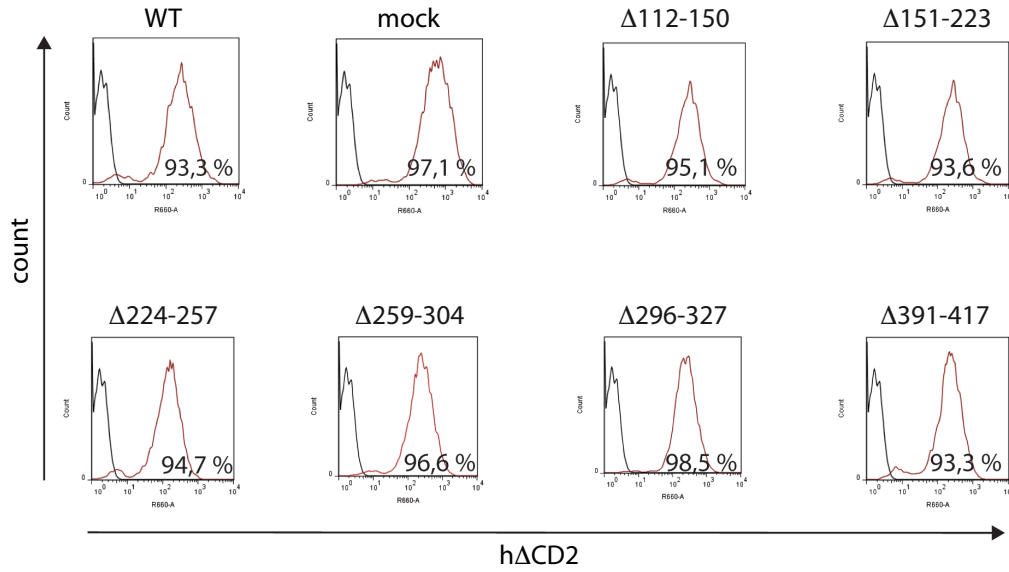
(a) MEFs were treated with DMSO or Emodin (20 and 40 μM) for 6 h and subsequently stimulated with TNFα. NF-κB activation was determined by IκBα phosphorylation and degradation by Western Blot and NF-κB DNA binding by EMSA. **(b)** MEFs were treated with DMSO or Emodin as in a) prior to TNFα or IL-1β stimulation. NF-κB DNA binding activity was analyzed by EMSA and loading was controlled by OCT1 EMSA. **(c)** Effect of Emodin on NF-κB target gene expression after TNFα stimulation. MEFs were treated with 20μM and 40μM Emodin or DMSO and stimulated with TNFα. mRNA was isolated and NF-κB target genes were investigated by qRT-PCR. (n=3; +/- SD). **(d)** Emodin enhances apoptosis after TNFα stimulation. MEFs were stimulated for 24 h in the presence or absence of Emodin (20 and 40 μM) or DMSO. Apoptosis was analyzed by AnnexinV staining and FACS. (n=3; +/- SD).

Supplementary Figure 1

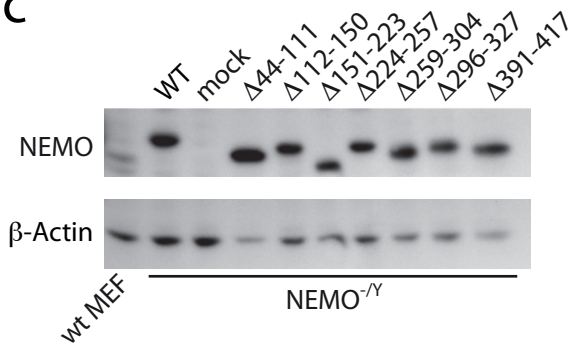
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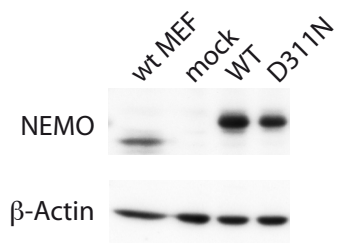
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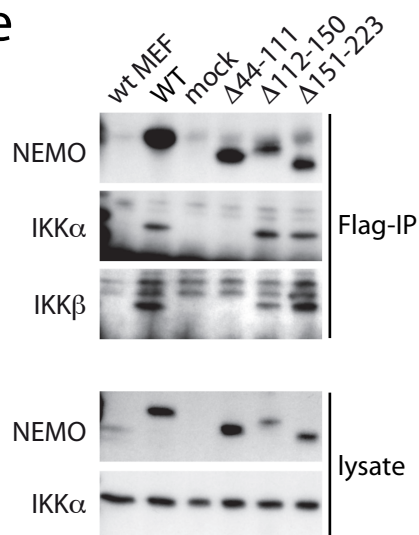
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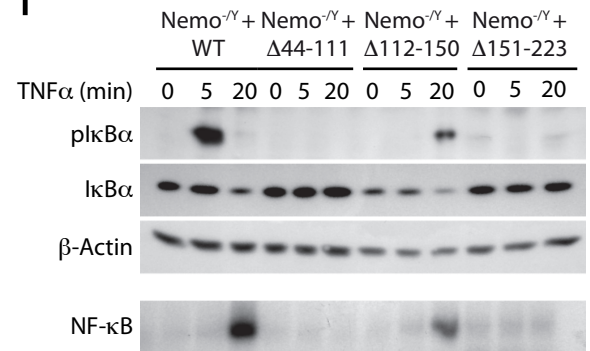
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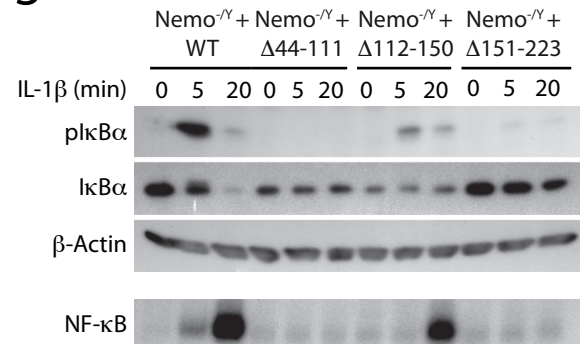
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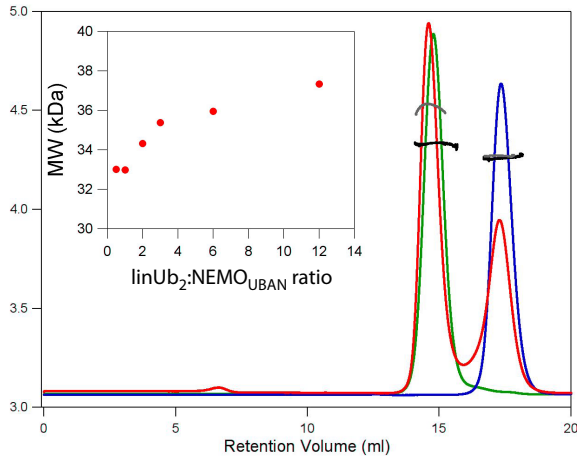


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Supplementary Figure 2

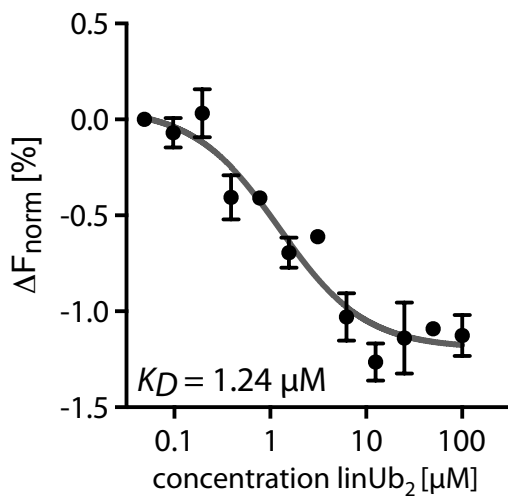
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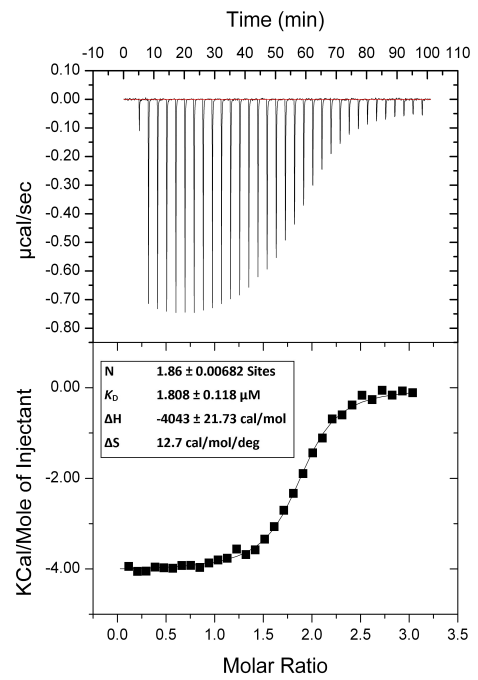
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Protein	MW _{exp} [kDa]	MW _{calc} [kDa]
NEMO _{UBAN} dimer	21.6	22.0
linUb2	18.2	18.2
NEMO _{UBAN} :linUb ₂ ratio 2:1	33.4	40.2 NEMO _{UBAN} :linUb ₂ (2:1)
NEMO _{UBAN} :linUb ₂ ratio 2:12	37.3	40.2 NEMO _{UBAN} :linUb ₂ (2:1)

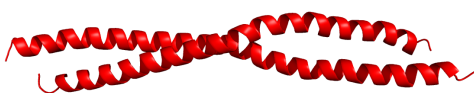
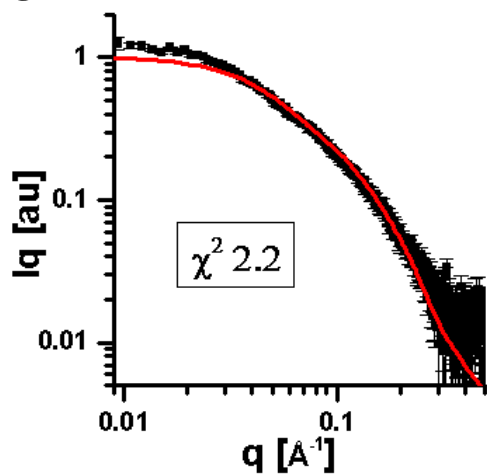
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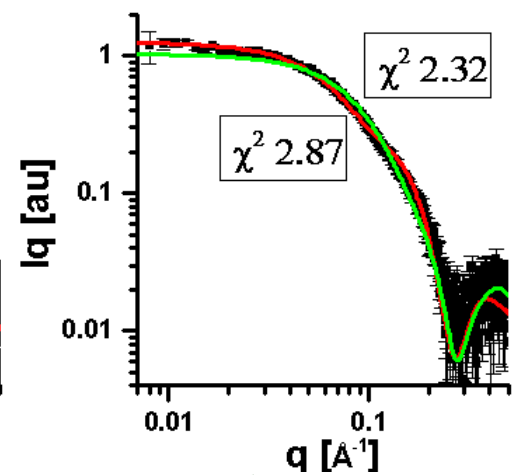
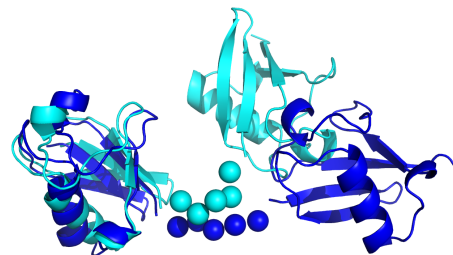
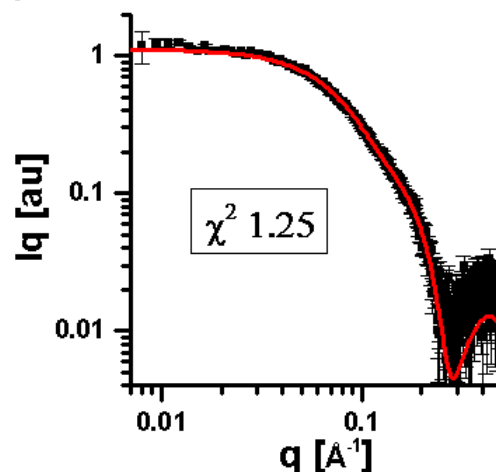
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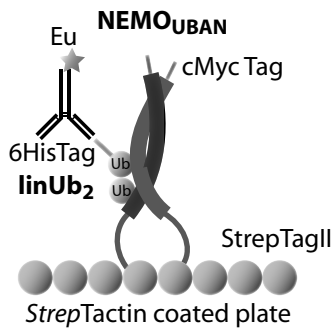


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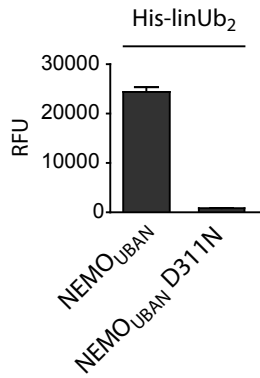


Supplementary Figure 3

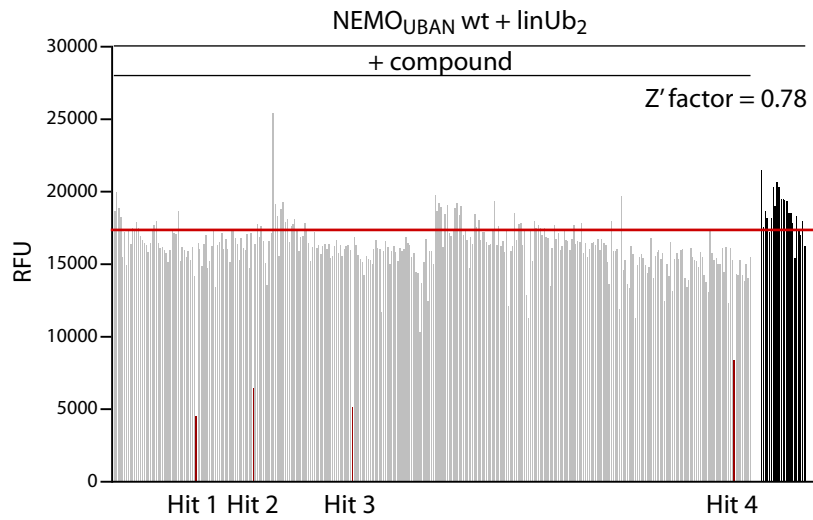
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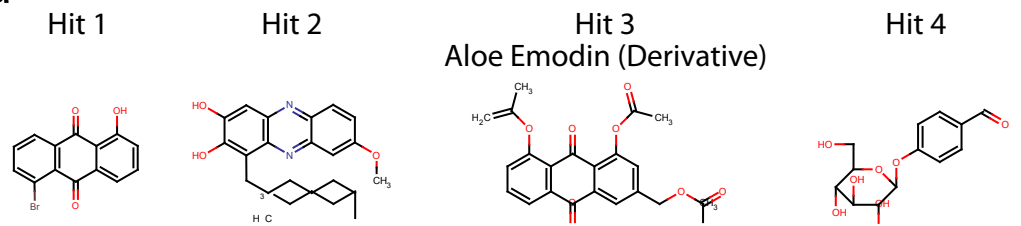
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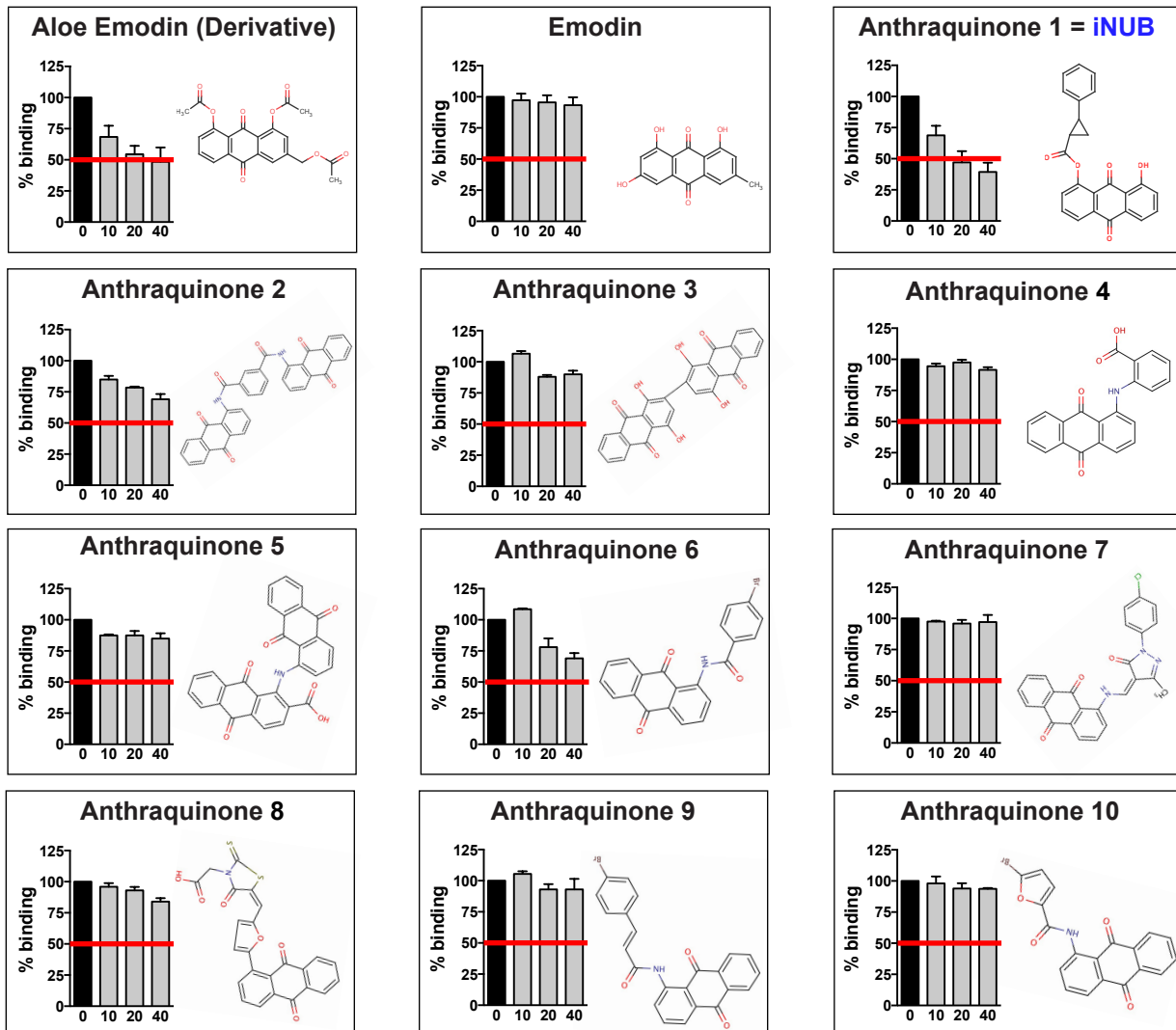
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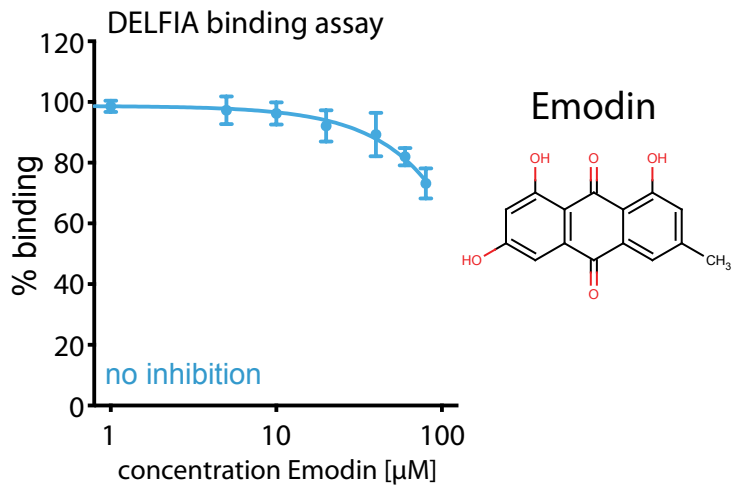


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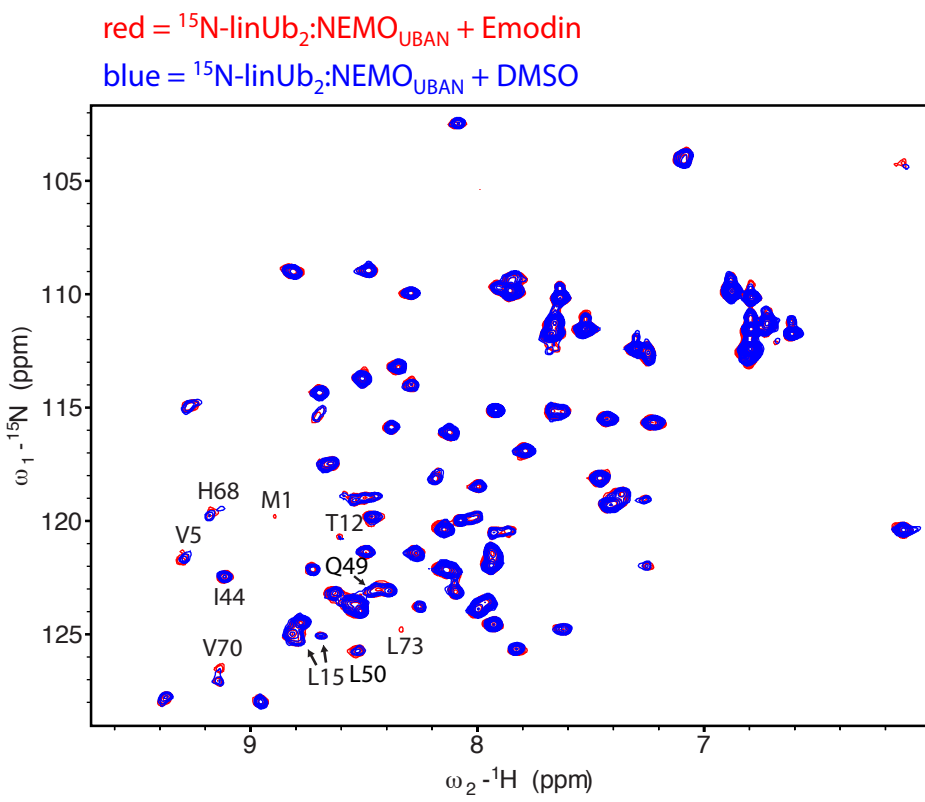


Supplementary Figure 4

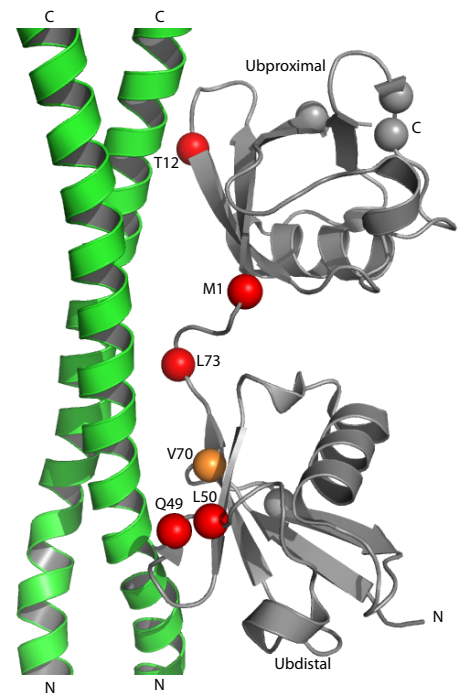
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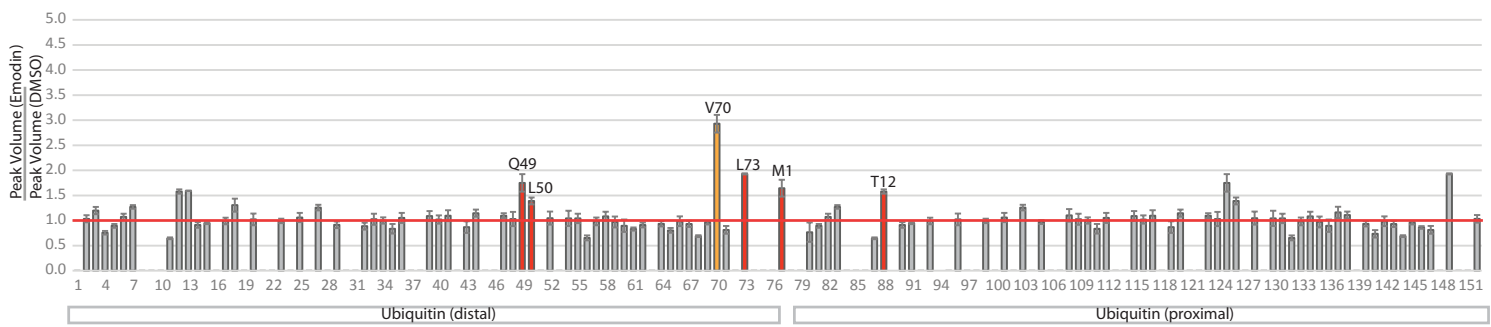
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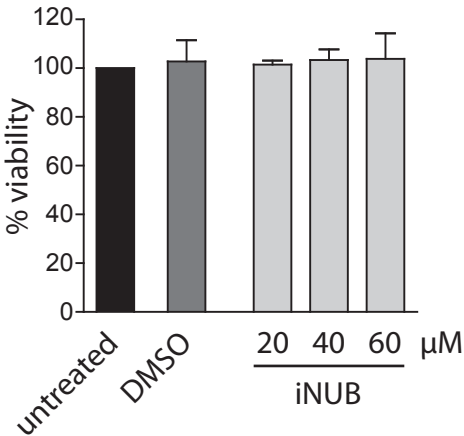
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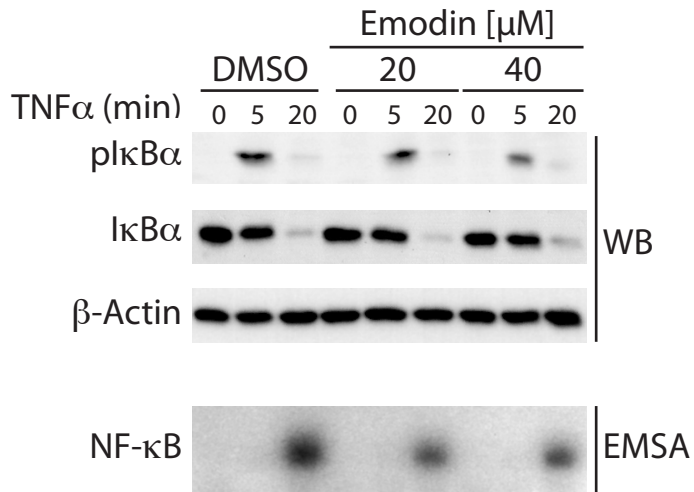


Supplementary Figure 5

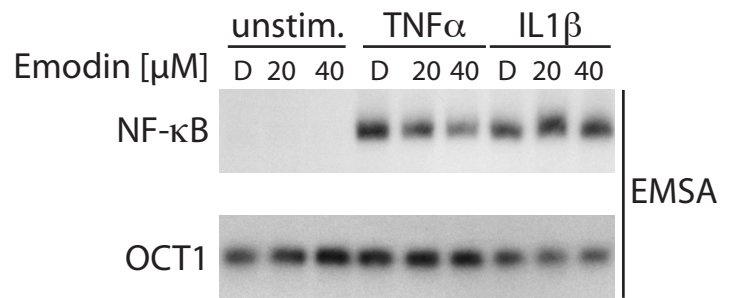


Supplementary Figure 6

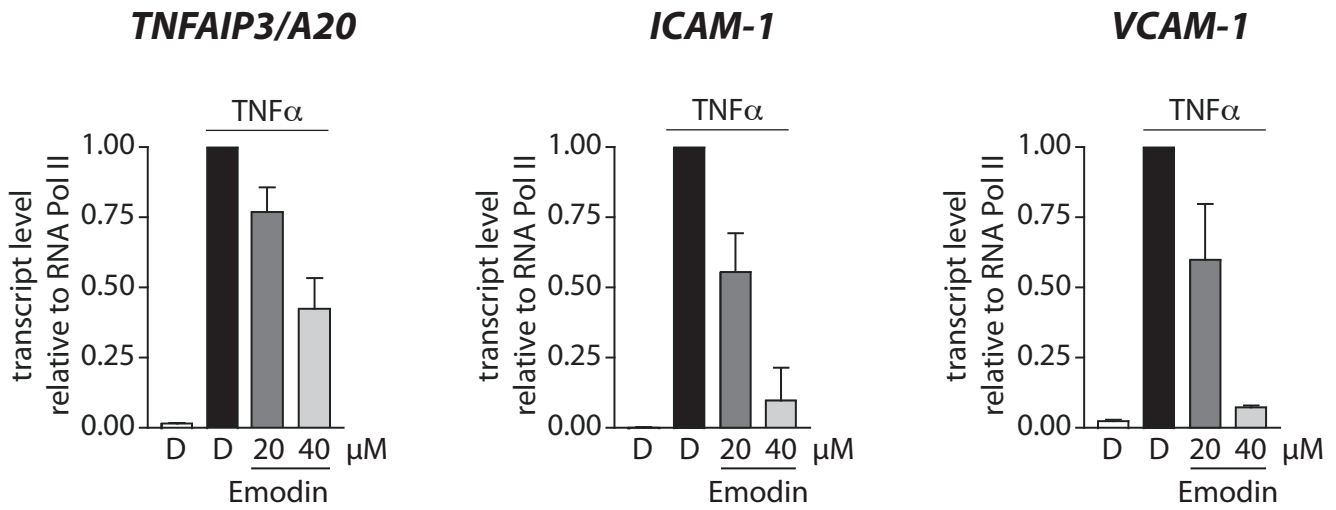
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