Supporting Information

A Synthetic Loop Replacement Peptide That Blocks Canonical NF-κB Signaling

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Materials and Methods

Peptides used in this study
All peptides were synthesized on CLEAR amide resin (Peptides International) using standard HBTU/HOBT/DIEA solid phase peptide synthesis protocols as previously described.[1] The TFA-cleaved peptides were precipitated with chilled diethyl ether, purified by reverse-phased HPLC using a 0.1% TFA/CH$_3$CN solvent system and verified by electrospray mass spectrometry. Peptides containing allylglycine were incorporated using FMOC-L-allylglycine (AnaSpec) using the methods described above.

Peptide Characterization Data
NBD$_{WT}$ MS (ESI-MS): *m/z*: 1244.8 [M+2H]$^{2+}$, 830.2 [M+3H]$^{3+}$
NBD$_{Mut}$ MS (ESI-MS): *m/z*: 807.7 [M+3H]$^{3+}$
NBD1 MS (ESI-MS): *m/z*: 1241.0 [M+2H]$^{2+}$, 827.7 [M+3H]$^{3+}$
NBD2 MS (ESI-MS): *m/z*: 1227.0 [M+2H]$^{2+}$, 818.3 [M+3H]$^{3+}$
NBD3 MS (ESI-MS): *m/z*: 795.7 [M+3H]$^{3+}$
bNBD$_{WT}$ MS (ESI-MS): *m/z*: 1708.7 [M-H]$^{1-}$, 853.8 [M-2H]$^{2-}$
bNBD2 MS (ESI-MS): *m/z*: 835.8 [M-2H]$^{2-}$

Ring closing metathesis (RCM) reaction
Metathesis reactions were carried out using a Biotage Initiator Microwave Reactor. Metathesis reaction of the dried bis-olefin resin bound FMOC-TALX(W/A)X(W/A)LQTE peptide of **NBD2** and **NBD3** were performed in a nitrogen atmosphere in a glass 10 mL microwave reaction vessel. The microwave vessel containing the dried bis-olefin resin bound peptide was charged with 2 mL of a dry, degassed solution of 0.4 M LiCl in DMF. The resin was allowed to swell with the DMF solution for 1 hour at which point a solution of Hoveyda-Grubbs Generation II catalyst (20 mol%) in dry dichloromethane (0.5 mL) was added to the swollen resin. The vessel containing the resin and catalyst was inserted in the microwave reactor (Biotage Initiator) and radiated (varying power) for 15 minutes at a constant temperature of 100 ^{o}C. After the resin had been subjected to microwave irradiation, the reaction mixture was transferred to a manual peptide synthesizer vessel and washed 3x with dichloromethane and 3x with dimethylformamide to remove any residual catalyst. The octalysine cell-penetrating peptide (8K) and diglycine linker (GG) were added to the resin bound, metathesized peptide using the peptide synthesis methods described above.

Circular Dichroism (CD) studies with SLR peptides
Circular dichroism with the SLR peptides were performed using an Aviv model 202 Circular Dichroism Spectrometer equipped with a 450 watt Xenon arc lamp. Data was collected using wavelength scans with a wavelength range of 190-250 nm and a wavelength step of 1 nm. All samples are represented as the average of 3 scans. The time constant used for the experiment is 100 ms with a monochromator/slit bandwidth of 1 nm. Any wavelength readings that resulted in a dynode voltage reading >600 V were omitted from the data set. All peptide readings were carried out in solution of 10 mM PBS, pH 6.8 at a final concentration of 50 µM. The mean residue molar ellipticity (MRE) was calculated using the following equation:

$$\theta(\deg \cdot cm^2 \cdot dmol^{-1}) = \frac{ellipticity(mdeg) \cdot 10^6}{pathlength(mm) \cdot [protein](\mu M) \cdot n}$$

Where n = # of peptide bonds in protein and ellipticity is the raw data from the instrument. The resulting data was plotted as MRE [θ] (deg cm^2 dmol^{-1}) vs. wavelength (nm).

Proteolytic stability assays
Chymotrypsin type VII (TLCK treated to inactivate residual trypsin activity), salt free, lyophilized powder, $\geq$ 40 units/mg protein was dissolved in 1 mM HCl containing 2 mM $CaCl_2$ at a final concentration of 10 µg/µL in accordance with the manufacturer protocol (Sigma). Peptides **NBD$_{WT}$**, **NBD1**, and **NBD2** were dissolved in proteolysis buffer (100 mM Tris, 10 mM $CaCl_2$, pH 7.8) to a final concentration of 20 µM. Chymotrypsin was added (10 µg) and incubated at room temperature. At the indicated time points (t = 0, 15, 30, 60, 120, 180, 240 min), 25 µL of proteolysis reaction was removed and 175 uL of 1% TFA/H_2O was added to inactivate protease activity. Each peptide/protease reaction sample time point was analyzed by HPLC using a 0.1% TFA/CH_3CN solvent system and overlaid with subsequent time point spectra to indicate any changes in degradation. These spectra were graphically represented using Agilent 1260 workstation software to create 3D overlays of the various time points analyzed.

NEMO pull-down assays with biotin-labeled, photoreactive SLR peptides
Cell lysates were prepared from a confluent 10 cm^2 plate of HEK293T cells (ATCC). Cells were trypsinized, washed 3x with cold PBS, and pelleted by centrifugation. Cell pellet was resuspended in NP-40 lysis buffer (50 mM Tris, 150 mM NaCl, 1% NP-40, pH 8.0) containing 1x HALT protease inhibitor cocktail (Thermo Fisher) and transferred to an eppendorf tube. The eppendorf tube containing cellular lysate was placed on a rotating carousel at 4 ^{o}C for 15 minutes and then, centrifuged at 14,000 rpm for 15 minutes at 4 ^{o}C. Supernatant was transferred to a new eppendorf tube and protein concentration was determined using Bradford Reagent. Lysate protein concentration was adjusted to a final concentration of 3.5 mg/mL. Peptides **bNBD$_{WT}$** and **bNBD2** were dissolved in 400 µL of cell lysates prepared above to a final concentration of 10 µM. Each peptide/cell lysate sample was split into 200 uL and added to a 30 mm petri dish (Nunc). Each sample was either irradiated with UV light (+UV) or shielded from light (-UV) for 1.5 hours. Performed pull-down with the peptide/cell lysate samples using NeutrAvidin beads according to manufacturer's protocol, performed SDS-PAGE with the indicated samples, and performed western analysis for NEMO/IKKγ (Sigma, I5032).

NF-κB luciferase reporter assay
The NF-κB luciferase reporter plasmid carrying 6 tandem κB-sites (NF-κB-luc), CMV-β-Gal, and pBSSK were generously provided by Dr. Jorge Iñigues-Lluhí (The University of Michigan Pharmacology Department). [2] HeLa cells were grown in Dulbecco's Modified Eagle Medium (DMEM, Invitrogen) supplemented with 10% Fetal Bovine Serum (FBS). Cells were maintained in 5% CO_2 at 37 ^{o}C. For luciferase assays, $4x10^5$ cells were seeded in a 6-well dish and allowed to adhere for 16h. The media was removed and cells were transfected with 400 ng NF-κB-luc, 200 ng CMV- β-Gal, and 1,400 ng pBSSK using Lipofectamine 2000 (Life Technologies) according to manufacturer's instructions. After 4.5h, transfection solution was removed and replaced with DMEM containing 10% FBS. After 24h, cells were trysinized and resuspended in DMEM supplemented with 10% FBS and seeded into a 96-well plate at a density of $8x10^3$ cells per well. After 16h, media was removed and supplemented with Opti-Mem containing vehicle or SLR peptides delivered in DMSO (1% v/v) at the indicated concentrations. After transfected cells incubated with either vehicle or compound for 1h, cells were stimulated with either PBS or IL-1β at a final concentration of 2 ng/mL. After an additional 3h incubation time, media was removed and cells were lysed with 60 µL of passive lysis buffer (Promega). Luciferase and β-Galactosidase activities were determined as previously described.[3] NF-κB luciferase activity and response curve analysis was performed using GraphPad software. The mean and standard deviation were determined using three independent experiments.

<u>Assaying inhibitory effects of SLR peptides against NF-κB endogenous gene expression</u>
For endogenous gene expression analysis, 1×10^5 HeLa cells were seeded into a 24-well plate and allowed to adhere overnight. Media was removed and replaced with Opti-Mem media containing vehicle of SLR peptide delivered in DMSO (1% v/v) at the indicated concentrations. After cells were incubated with either vehicle of peptide for 1h, cells were stimulated with either PBS or IL-1β at a final concentration of 2 ng/mL. After 2h, the media was removed and total RNA was isolated using RNeasy Plus RNA isolation kits (Qiagen) according to manufacturer's protocol. Each RNA sample was used to synthesize cDNA using iScript cDNA synthesis kits (Bio-Rad). Real-Time Quantitative PCR (RT-qPCR) analysis was carried out in an Applied Biosystems StepPlusOne using SYBR green master mix and primers for human genes:

RPL19
Forward, 5'-ATGTATCACAGCCTGTACCTG-3';
Reverse, 5'-TTCTTGGTCTCTCTTCCTCCTTG-3')

MIP3α[4]
Forward, 5'-TACTCCACCTCTGCGGCGAATCAGAA-3';
Reverse, 5'-GTGAAACCTCCAACCCCAGCAAGGTT-3'

IL-8[5]
Forward, 5'-ATGACTTCCAAGCTGGCCGTGGCT-3'
Reverse, 5'- CTCAGCCCTCTTCAAAAACTTCT-3'

Cyclin D1[6]
Forward, 5'- ACAAACAGATCATCCGCAAACAC-3'
Reverse, 5'-TGTTGGGGCTCCTCAGGTTC-3'
.

RT-qPCR analysis was carried out using the comparative C_T Method ($\Delta\Delta C_T$ Method) as previously described[7,8] to estimate MIP3α mRNA levels relative to the reference RPL19 mRNA levels. The reported mean and standard deviation for MIP3α and IL-8 expression were determined using three technical replicates from one representative biological replicate. Three biological replicates were performed. The reported mean and standard deviation for Cyclin D1 were determined using two technical replicates from one representative biological replicate. Two biological replicates were performed. Results from additional replicates are as noted in Supporting Figure 4.

<u>Molecular Dynamics (MD) Simulations</u>
Molecular dynamics simulations were conducted on NVIDIA GTX 680 and 780 GPUs using the CHARMM/OpenMM interface for the peptides LDWSWL, LXWXWL, and metathesized LXWXWL. The CHARMM[9] force field (C36 and CGENFF) was used for all systems, with peptides solvated in TIP3P water. Simulations were run at T=298K for 3 µs per peptide with a time step of 2 fs. Simulations use a van der Waals switching function with cutoffs ctonnb 8Å and ctofnb 9Å, and a cutoff length of 9Å. Particle mesh Ewald was used with a width of κ = 0.56, a radius of kmax = 5 images, cubic order interpolation, and 54 gridpoints in each direction. A cubic box of length 3.98nm was used. A sodium atom was added to the wild-type peptide to neutralize charge; the other systems were neutralized with no added salt. SHAKE was used to

constrain bonds of heavy atoms to hydrogens. As the CHARMM force field does not include explicit fragments for SLR-modified peptides, we obtained these missing parameters and topology files using MATCH,[10] which assigns them by fragment similarity to a training set from the CGENFF CHARMM parameter files. We then patched together the MATCH-generated and CHARMM default parameter and topology files (we include an example script: refer to the README file in patch-example supporting files). To monitor the structure of each peptide we tracked phi/psi angles along the backbone for residue W739 (Figure 2) and also for residues 738-740 in combination (Supp. Figure 3). For the latter we computed an average deviation from the docked configuration for all six phi/psi angles over the three residues as:

$$\Delta = \frac{1}{\sqrt{6}} \sqrt{\sum_{i=738}^{740} \left(\phi_i - \phi_i^{docked}\right)^2 + \left(\psi_i - \psi_i^{docked}\right)^2}$$

Supporting Figures
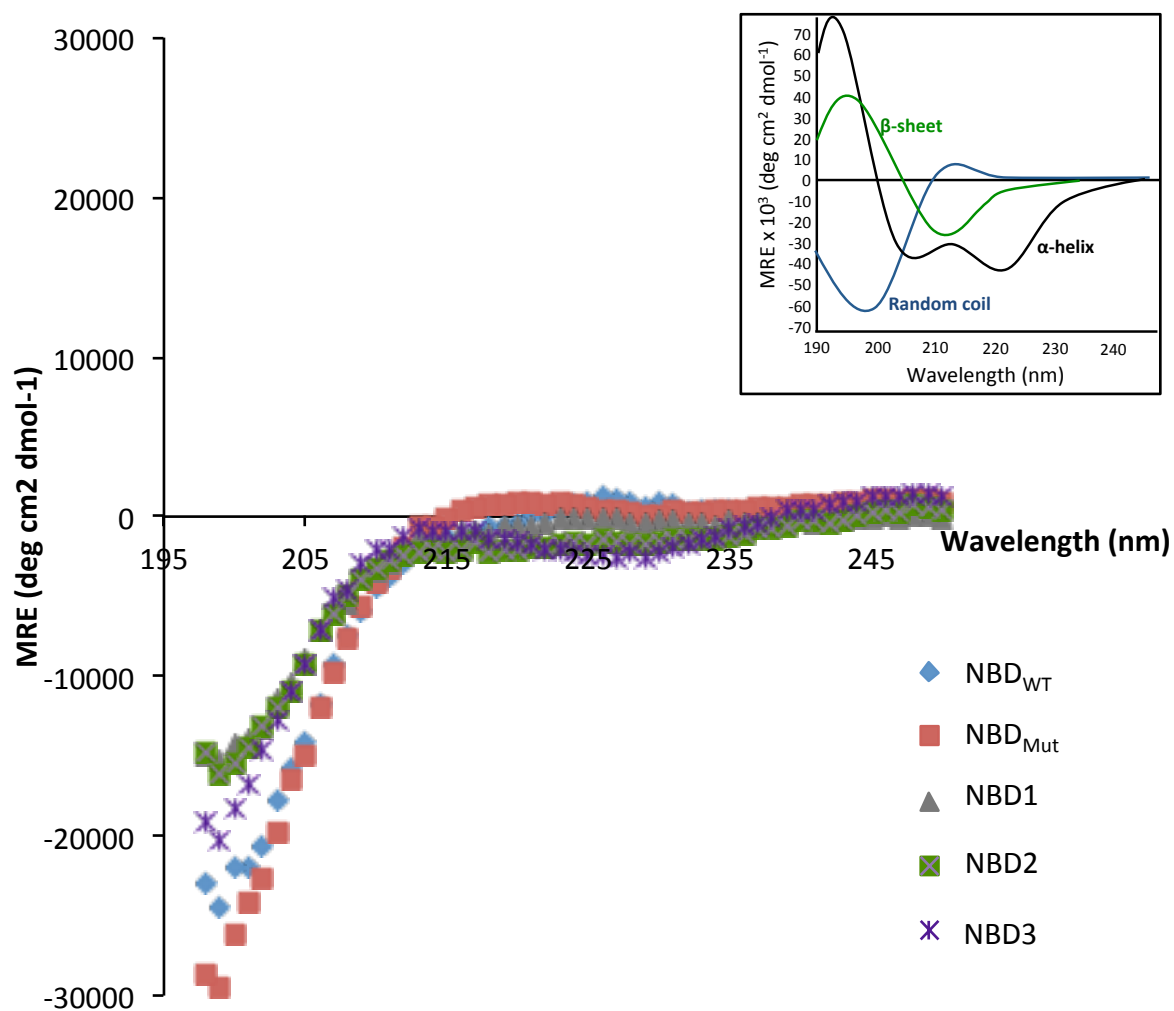
Supporting Figure 1. Circular dichroism as performed with the above indicated peptides. The results above indicate that all of the peptides maintain the flexible random coil structure as indicated by the minima observed at 198 nm and do not display minima consistent with either α-helices or β-sheets. Example spectra for the various secondary structures and their associated minima are included in the inset for comparison.
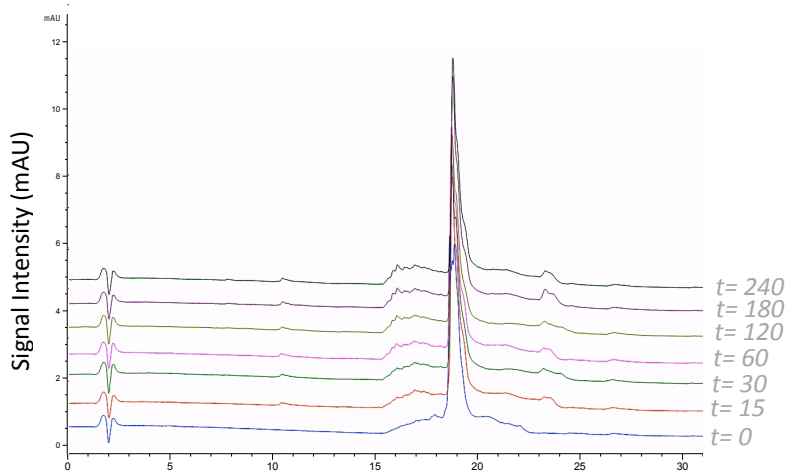
NBD_WT

KKKKKKKKGGTALD<u>W</u>S<u>W</u>LQTE

t= 240
t= 180
t= 120
t= 60
t= 30
t= 15
t= 0

NBD1

KKKKKKKKGGTALX<u>W</u>X<u>W</u>LQTE

t= 240
t= 180
t= 120
t= 60
t= 30
t= 15
t= 0

NBD2

KKKKKKKKGGTALX<u>W</u>X<u>W</u>LQTE

t= 240
t= 180
t= 120
t= 60
t= 30
t= 15
t= 0
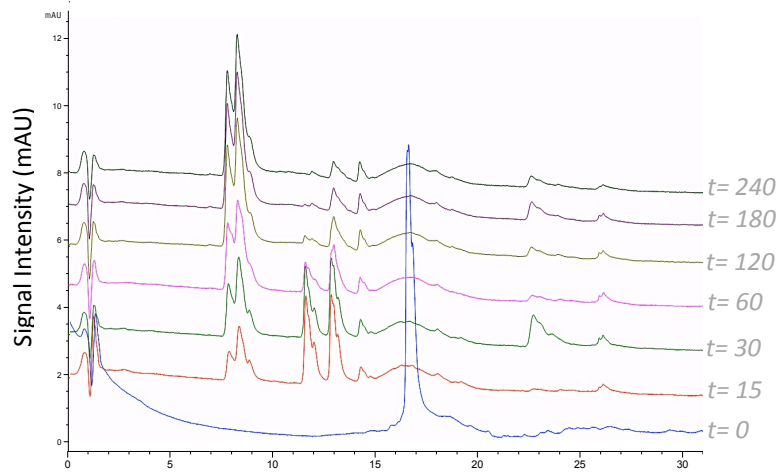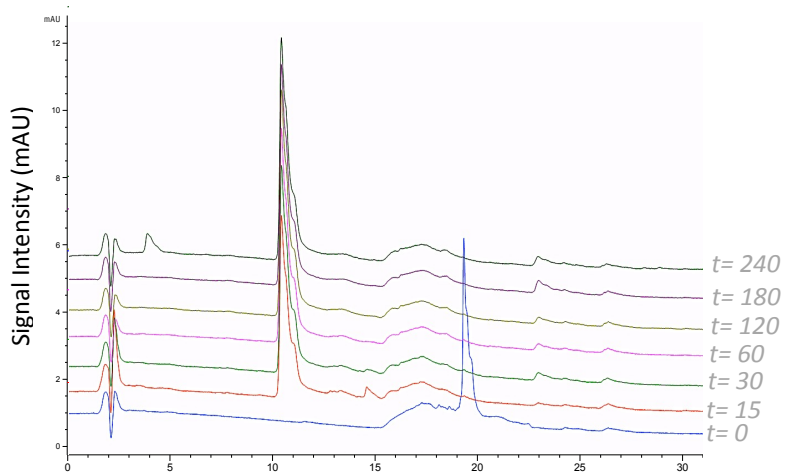
Supporting Figure 2. Purified peptides **NBD_WT**, **NBD1**, and **NBD2** were dissolved in proteolysis buffer at a final concentration of 20 µM. Chymotrypsin was added (0.05 µg/µL final concentration) and reaction samples were removed at the indicated time points above (t = 0, 15, 30, 60, 120, 180, and 240 min) and analyzed by HPLC. The results of time course experiment are displayed as a 3D overlay. Residues indicated by "X" represent incorporated allylglycine residues, with metathesized residues indicated by a bracket between the allylglycine residues
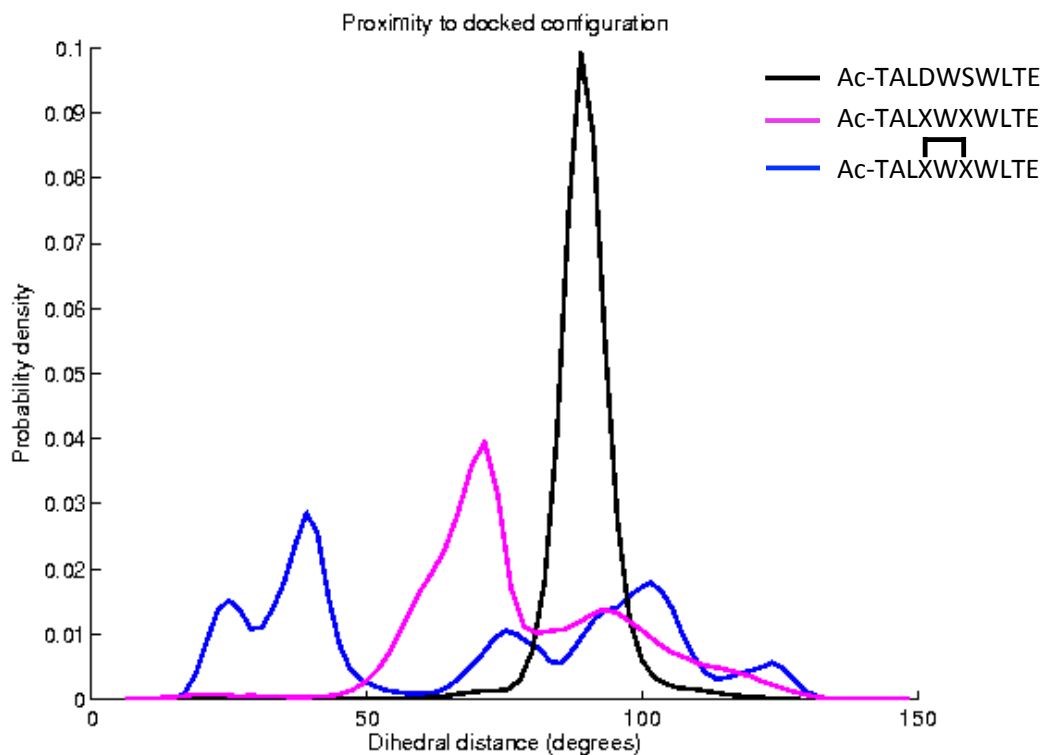
Supporting Figure 3. Molecular dynamics simulations with the above indicated peptides demonstrate that the metathesized version of the peptide adopts a configuration most similar to docked configuration from the reported crystal structure. This was determined using the dihedral distance of IKKβ backbone phi and psi angles from the crystal structure values when in complex with NEMO (PDB 3BRV). Residues indicated by "X" represent incorporated allylglycine residues, with metathesized residues indicated by a bracket between the allylglycine residues. The plot above displays the root mean square deviation of phi and psi angles for residues 738-740 from PDB 3BRV.
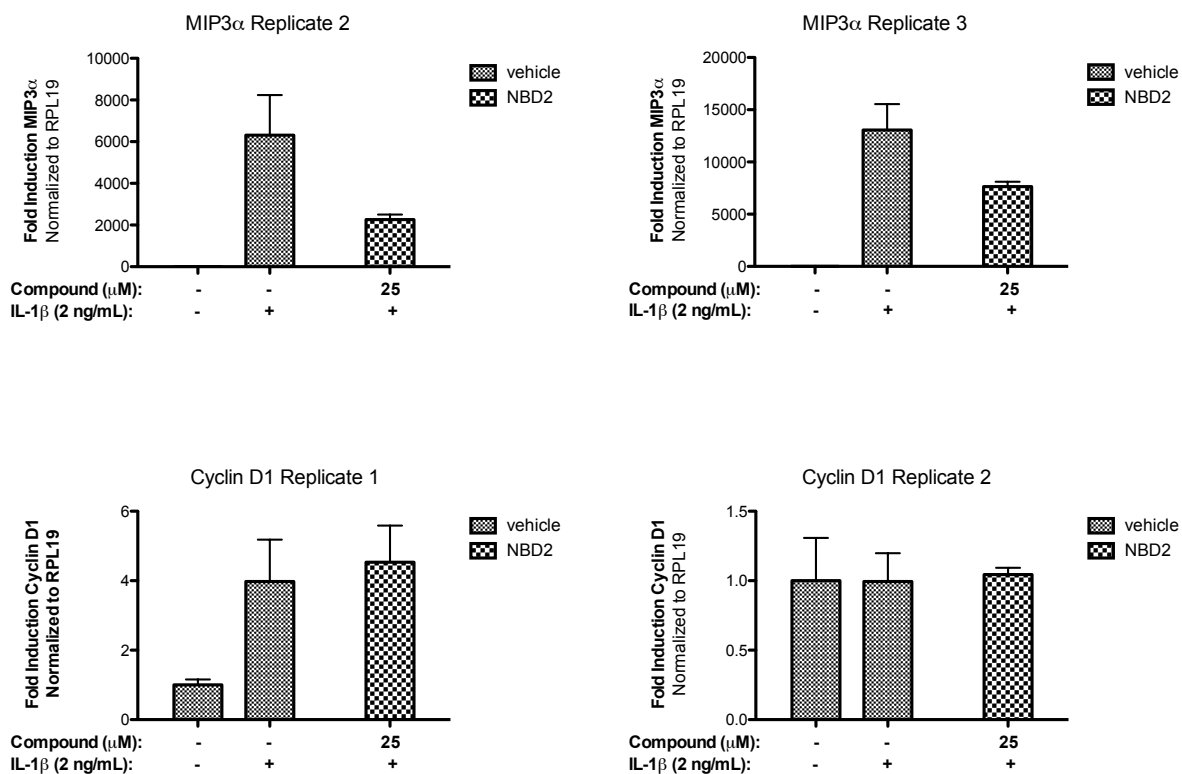
Supporting Figure 4. Effects of NBD2 peptide against NF-κB regulated gene expression of the canonical gene target MIP3α and the noncanonical gene target Cyclin D1 (see manuscript for references). The error is SDOM, determined from two technical replicates. Three biological replicates were performed for MIP3α and two biological replicates were performed for Cyclin D1.
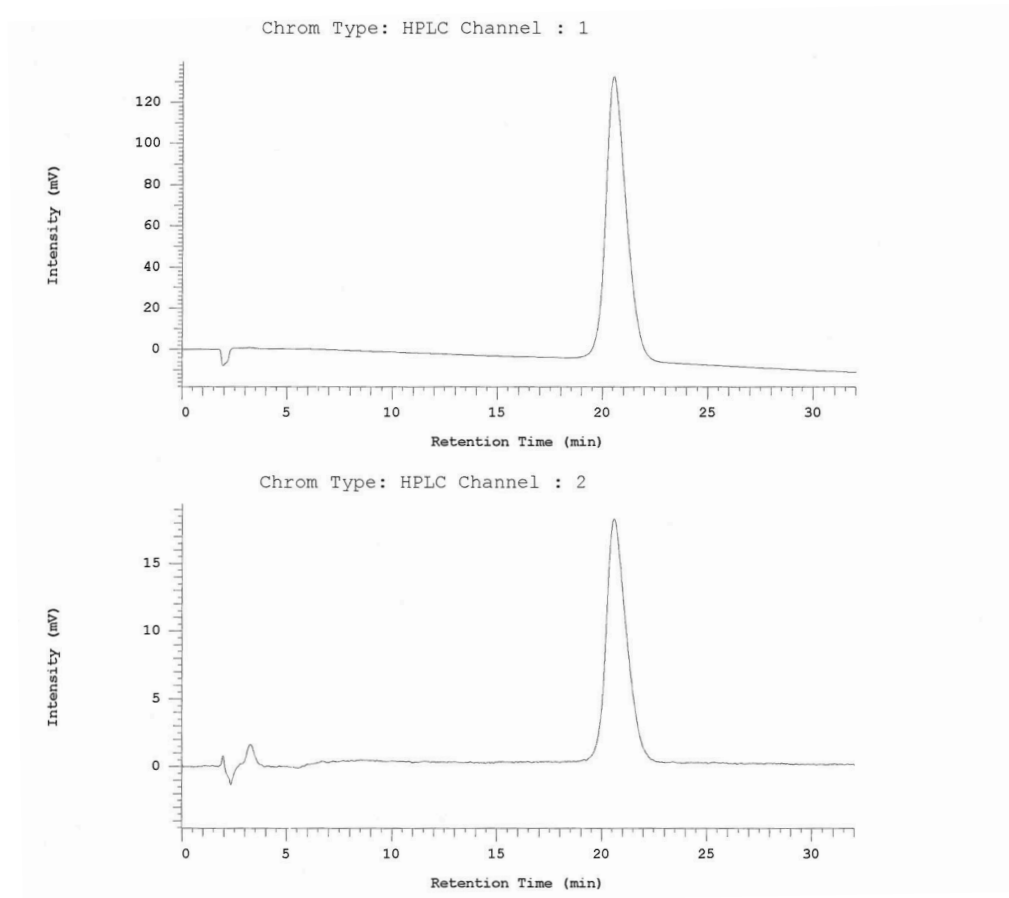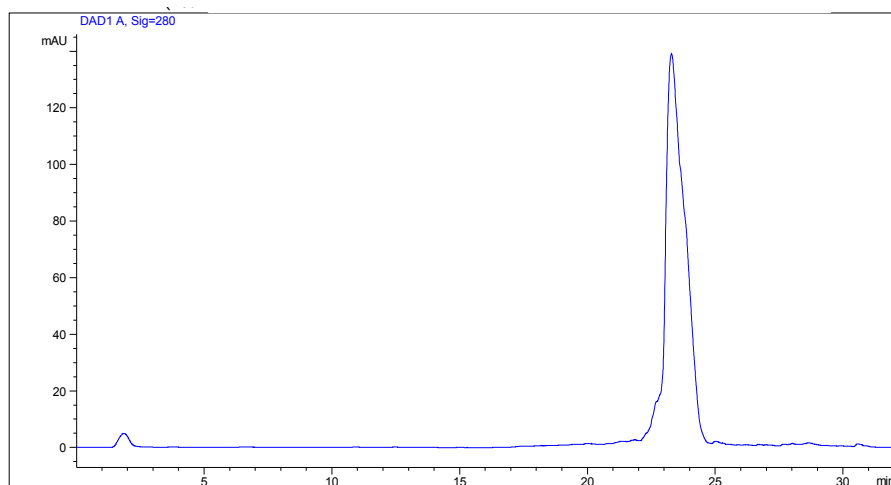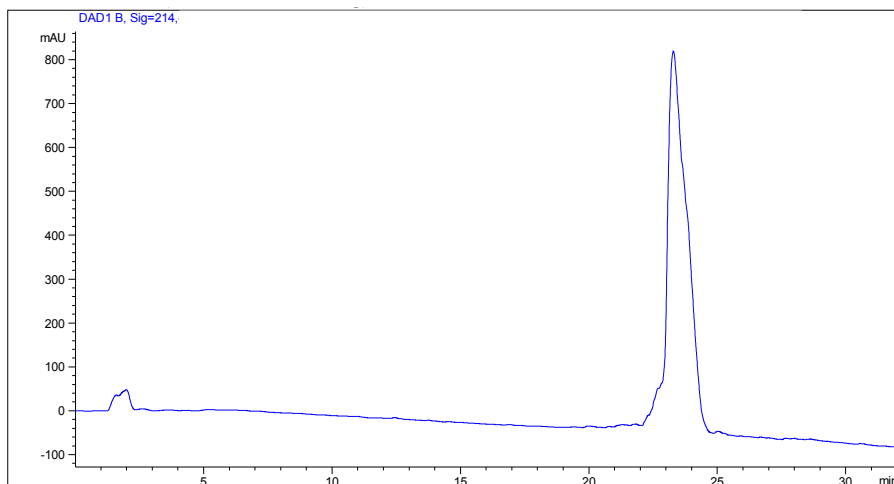
HPLC Analytical Traces
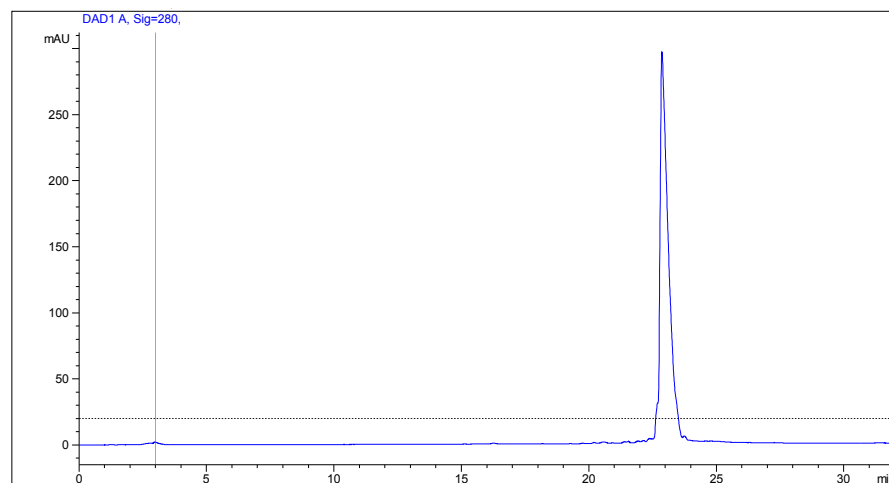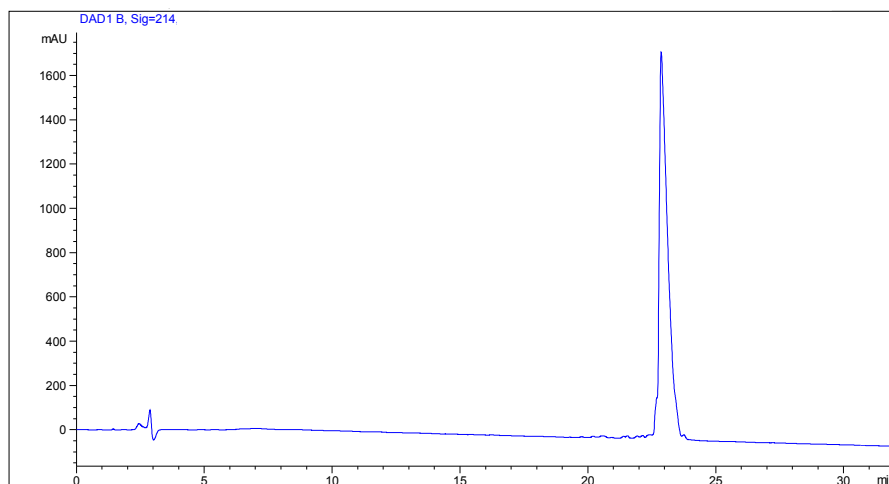
Analytical HPLC UV/Vis trace of **NBD$_{WT}$**, monitored at 214 nm (top) and 280 nm (bottom). Analytical sample was run in a 0.1% TFA/ACN mobile phase. The sample was injected with an isocratic flow of 90% H$_2$O (w/ 0.1% TFA) and 10% ACN. After 2 mins, a gradient flow was performed from 10-40% ACN over 30 mins.
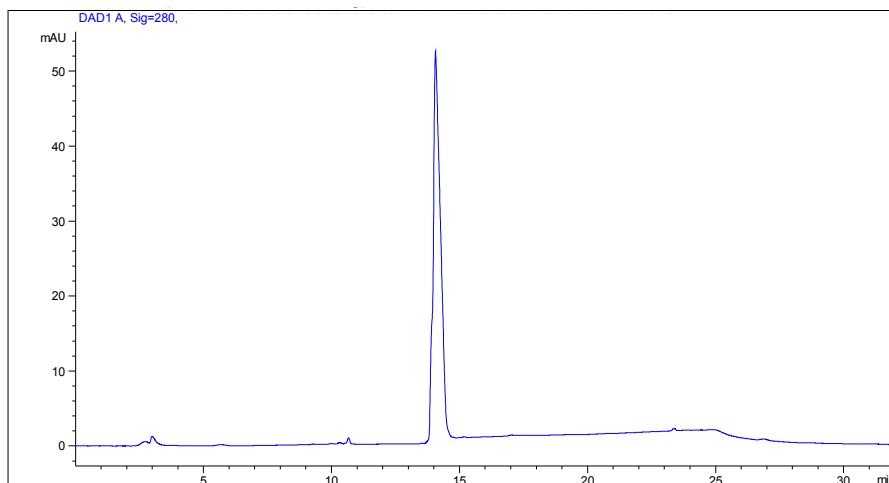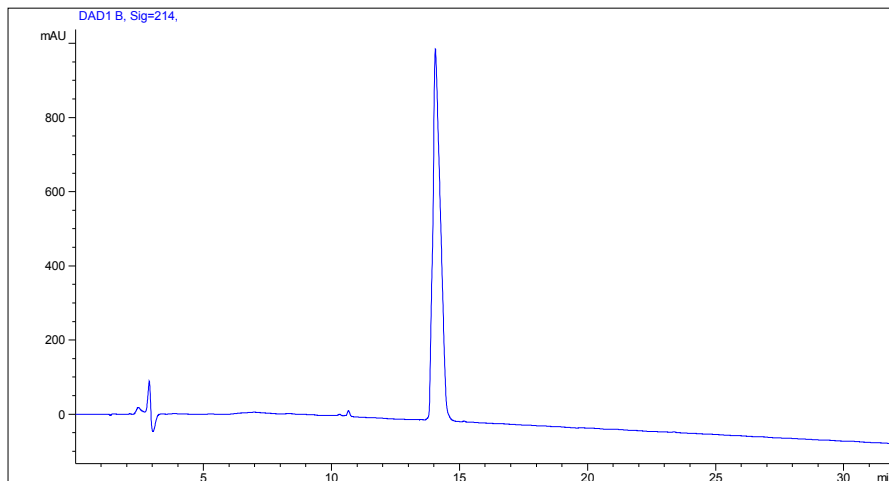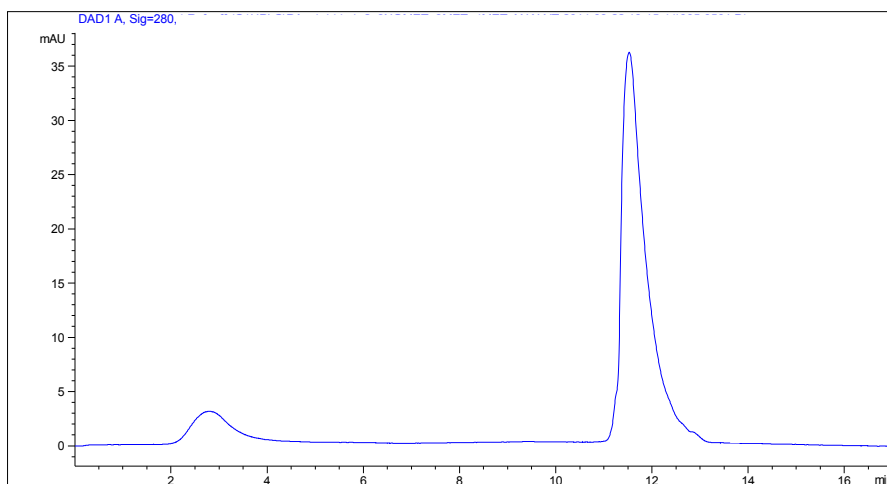
Analytical HPLC UV/Vis trace of **NBD$_{Mut}$**, monitored at 214 nm (top) and 280 nm (bottom). Analytical sample was run in a 0.1% TFA/ACN mobile phase. The sample was injected with an isocratic flow of 90% H$_2$O (w/ 0.1% TFA) and 10% ACN. After 2 mins, a gradient flow was performed from 10-40% ACN over 30 mins.
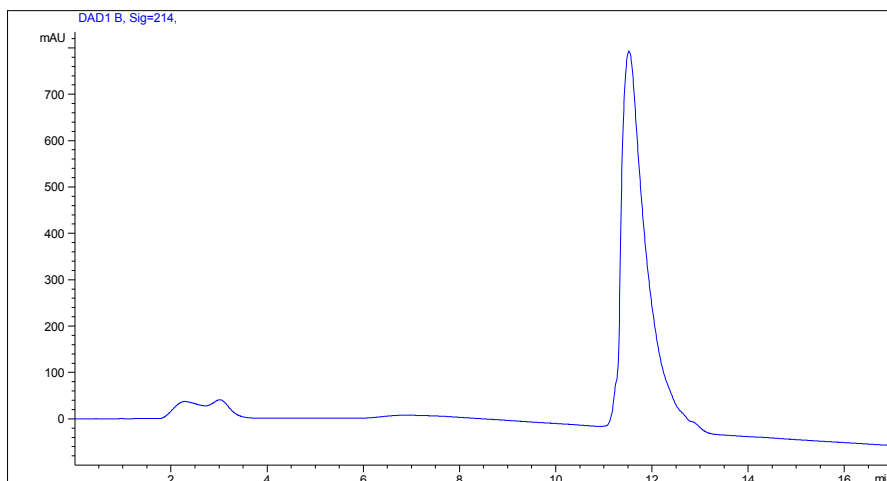
Analytical HPLC UV/Vis trace of **NBD1**, monitored at 214 nm (top) and 280 nm (bottom). Analytical sample was run in a 0.1% TFA/ACN mobile phase. The sample was injected with an isocratic flow of 90% H_2O (w/ 0.1% TFA) and 10% ACN. After 2 mins, a gradient flow was performed from 10-40% ACN over 30 mins.

Analytical HPLC UV/Vis trace of **NBD2**, 214 nm (top) and 280 nm (bottom). Analytical sample was run in a 0.1% TFA/ACN system. The sample was injected with an isocratic flow of 90% H_2O (w/ 0.1% TFA) and 10% ACN. After 2 mins, a gradient flow was performed from 10-40% ACN over 30 mins.

Analytical HPLC UV/Vis trace of **NBD3**, 214 nm (top) and 280 nm (bottom). Analytical sample was run in a 0.1% TFA/ACN system. The sample was injected with an isocratic flow of 90% H_2O (w/ 0.1% TFA) and 10% ACN. After 2 mins, a gradient flow was performed from 10-40% ACN over 15 mins.

References

[1] Z. Wu, G. Belanger, B. B. Brennan, J. K. Lum, A. R. Minter, S. P. Rowe, A. Plachetka, C. Y. Majmudar, A. K. Mapp, *J. Am. Chem. Soc.* **2003**, *125*, 12390–12391.

[2] J. W. Højfeldt, O. Cruz-Rodríguez, Y. Imaeda, A. R. Van Dyke, J. P. Carolan, A. K. Mapp, J. A. Iñiguez-Lluhí, *Molecular Endocrinology* **2014**, *28*, 249–259.

[3] J. A. Iñiguez-Lluhí, D. Pearce, *Molecular and Cellular Biology* **2000**, *20*, 6040–6050.

[4] T. Nakayama, R. Fujisawa, H. Yamada, T. Horikawa, H. Kawasaki, K. Hieshima, D. Izawa, S. Fujiie, T. Tezuka, O. Yoshie, *Int. Immunol.* **2001**, *13*, 95–103.

[5] S. Lee, Y. J. Kim, S. Kwon, Y. Lee, S. Y. Choi, J. Park, *BMB rep* **2009**.

[6] R. Suzuki, K. Takemura, M. Tsutsumi, S. Nakamura, N. Hamajima, M. Seto, *Am. J. Pathol.* **2001**, *159*, 425–429.

[7] T. D. Schmittgen, K. J. Livak, *Nature Protocols* **2008**, *3*, 1101–1108.

[8] K. J. Livak, T. D. Schmittgen, *Analysis of Relative Gene Expression Data Using Real-Time Quantitative PCR and the 2− ΔΔC T Method Methods 25 (4): 402–408*, Find This Article Online, **2001**.

[9] B. R. Brooks, C. L. Brooks, A. D. Mackerell, L. Nilsson, R. J. Petrella, B. Roux, Y. Won, G. Archontis, C. Bartels, S. Boresch, et al., *J Comput Chem* **2009**, *30*, 1545–1614.

[10] J. D. Yesselman, D. J. Price, J. L. Knight, C. L. Brooks III, *J Comput Chem* **2012**, *33*, 189–202.