



# A Synthetic Loop Replacement Peptide That Blocks Canonical NF- $\kappa$ B Signaling

Paul A. Bruno, Alex Morriss-Andrews, Andrew R. Henderson, Charles L. Brooks III, and Anna K. Mapp\*

**Abstract:** Aberrant canonical NF- $\kappa$ B signaling is implicated in diseases from autoimmune disorders to cancer. A major therapeutic challenge is the need for selective inhibition of the canonical pathway without impacting the many non-canonical NF- $\kappa$ B functions. Here we show that a selective peptide-based inhibitor of canonical NF- $\kappa$ B signaling, in which a hydrogen bond in the NBD peptide is synthetically replaced by a non-labile bond, shows an about 10-fold increased potency relative to the original inhibitor. Not only is this molecule, NBD2, a powerful tool for dissection of canonical NF- $\kappa$ B signaling in disease models and healthy tissues, the success of the synthetic loop replacement suggests that the general strategy could be useful for discovering modulators of the many protein–protein interactions mediated by such structures.

Many regulatory protein–protein interactions (PPIs) operate only in certain cellular contexts, and synthetic modulators of such complexes have high intrinsic value due to their inherent context specificity.<sup>[1]</sup> Although numerous cellular signals converge on the master transcriptional activator NF- $\kappa$ B, for example, the PPIs formed in the so-called inhibitor of  $\kappa$ B kinase (IKK) complex only control the canonical activation pathway of the activator, the path often dysregulated in inflammatory diseases and cancer (Figure 1 a).<sup>[2,3]</sup> The only reported inhibitor of this complex is a peptide taken from two of the IKK components, and it selectively attenuates the canonical NF- $\kappa$ B pathway in vitro and in vivo, albeit with modest activity due to its short half life.<sup>[4–6]</sup> A recent elegant dissection of the IKK complex identified the linchpin as a short peptide loop stabilized by an intramolecular hydrogen bond.<sup>[7,8]</sup> Here, we show that this structural feature can be exploited by use of a synthetic loop replacement (SLR) strategy to develop a synthetic inhibitor of the canonical NF- $\kappa$ B signaling pathway that is about 10-fold more effective than the peptide itself. This structure, NBD2, directly engages the IKK component NEMO and blocks NF- $\kappa$ B-mediated transcription of both a reporter construct and endogenous genes.

The IKK complex is a heterotetramer composed of the scaffold protein NEMO and the two kinase components IKK $\alpha$  and IKK $\beta$ .<sup>[9,10]</sup> NEMO is the unique component of this complex, while IKK $\alpha$  and IKK $\beta$  are required in crosstalk for noncanonical NF- $\kappa$ B signaling and other unrelated signaling pathways.<sup>[11,12]</sup>

Structural and biochemical studies have demonstrated that a six amino acid sequence (<sup>737</sup>LDWSWL<sup>742</sup>, also known as the NEMO binding domain or NBD peptide) present in both IKK $\alpha$  and IKK $\beta$  is critical for interaction with NEMO and subsequent activation of canonical NF- $\kappa$ B signaling (Figure 1 b).<sup>[7,13,14]</sup> This short peptide forms a loop stabilized by a hydrogen bond bridge between D738 and S740,<sup>[8]</sup> allowing the hot spot hydrophobic residues W739 and W741 to interact with NEMO over a surface area of about 1800 Å<sup>2</sup> (Figure 1 c,d).<sup>[7]</sup> When conjugated to a cell-penetrating peptide, this six amino acid sequence selectively blocks canonical NF- $\kappa$ B activation (IC<sub>50</sub> ≈ 100 μM).<sup>[13,15–17]</sup> We hypothesized that replacement of the hydrogen bond bridge in the NBD peptide with a non-labile connection would both stabilize this atypical secondary structure and render it less susceptible to degradation, thus producing an effective inhibitor of the NEMO-IKK $\alpha/\beta$  interaction (Figure 1 e).<sup>[18–25]</sup>

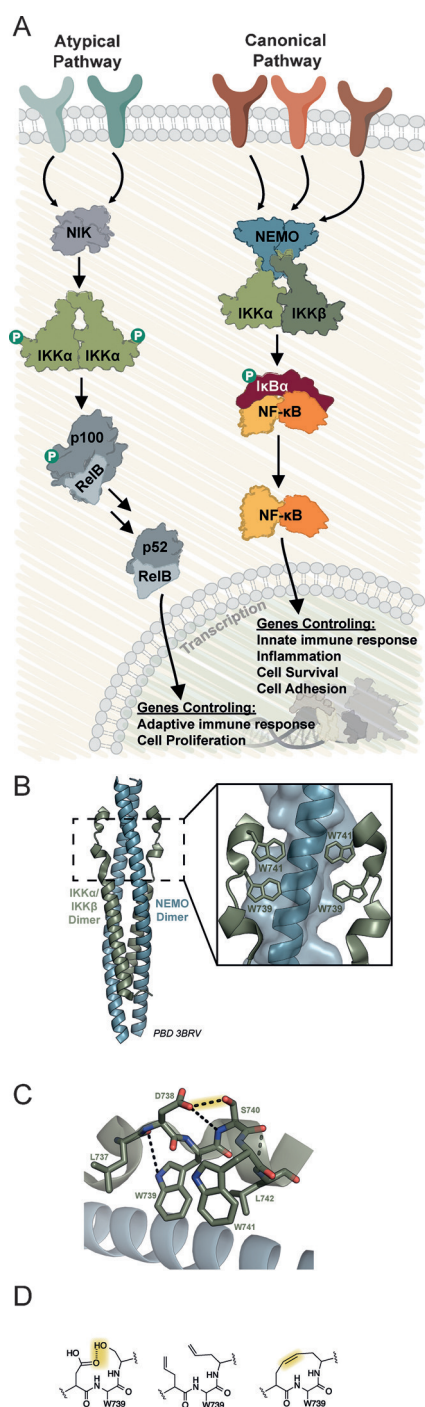
Molecular dynamics (MD) simulations were used to test the initial hypothesis. As illustrated in Figure 2, simulations of the native NBD sequence produced lowest energy conformers that overlap with the reported crystal structure in the docked configuration at the position of W739 (indicated by the blue dot in Figure 2, panel A).<sup>[8]</sup> When D738 and S740 are substituted with allylglycine residues, the lowest free energy conformer is observed to have backbone dihedral angles at W739 that are dissimilar to those in the native sequence, suggesting these modifications increase the local flexibility of the modified NBD peptide (Figure 2, panel B).<sup>[7,14]</sup> However, MD simulations of a peptide in which a C–C bond replaces the loop hydrogen bond reveal that the key dihedral angles are analogous to those in the wild-type NBD peptide (Figure 2, panel C), demonstrating that the constrained version of the NBD sequence can adopt the key dihedral angles needed to interact with NEMO.

A series of NEMO-binding domain (NBD)-derived peptides were designed based upon the above results (Figure 3 a). Each of the structures contains a cell-penetrating peptide (CPP) previously demonstrated to be important for the function of the native peptide.<sup>[13,26]</sup> Synthesis of the peptides was accomplished by standard methods and for peptides NBD2 and NBD3, ring-closing metathesis was performed using Hoveyda–Grubbs generation II catalyst. All of the modified peptides (NBD1, 2 and 3) have circular

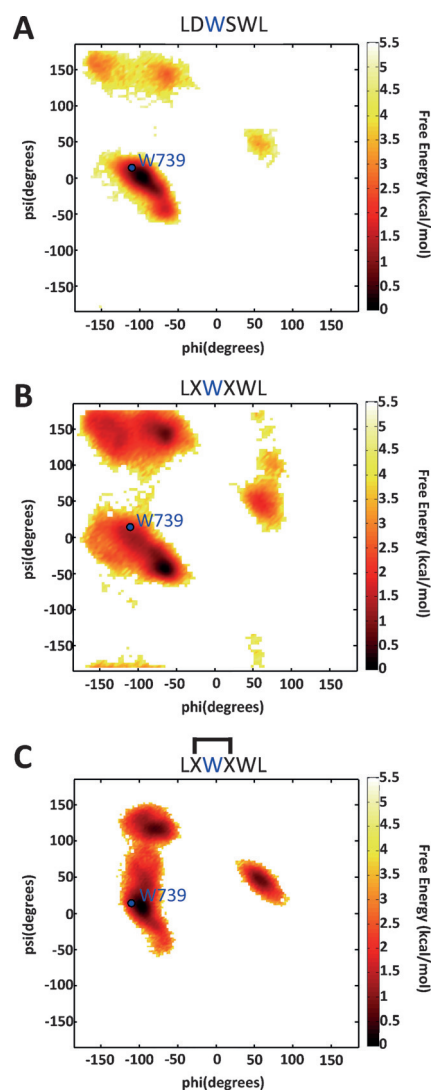
[\*] Dr. P. A. Bruno, Dr. A. Morriss-Andrews, A. R. Henderson, Prof. Dr. C. L. Brooks III, Prof. Dr. A. K. Mapp  
Department of Chemistry, University of Michigan  
Ann Arbor (USA)  
E-mail: amapp@umich.edu

Dr. P. A. Bruno, A. R. Henderson, Prof. Dr. A. K. Mapp  
Life Sciences Institute, Program in Chemical Biology  
University of Michigan, Ann Arbor (USA)

Supporting information for this article can be found under:  
<http://dx.doi.org/10.1002/anie.201607990>.

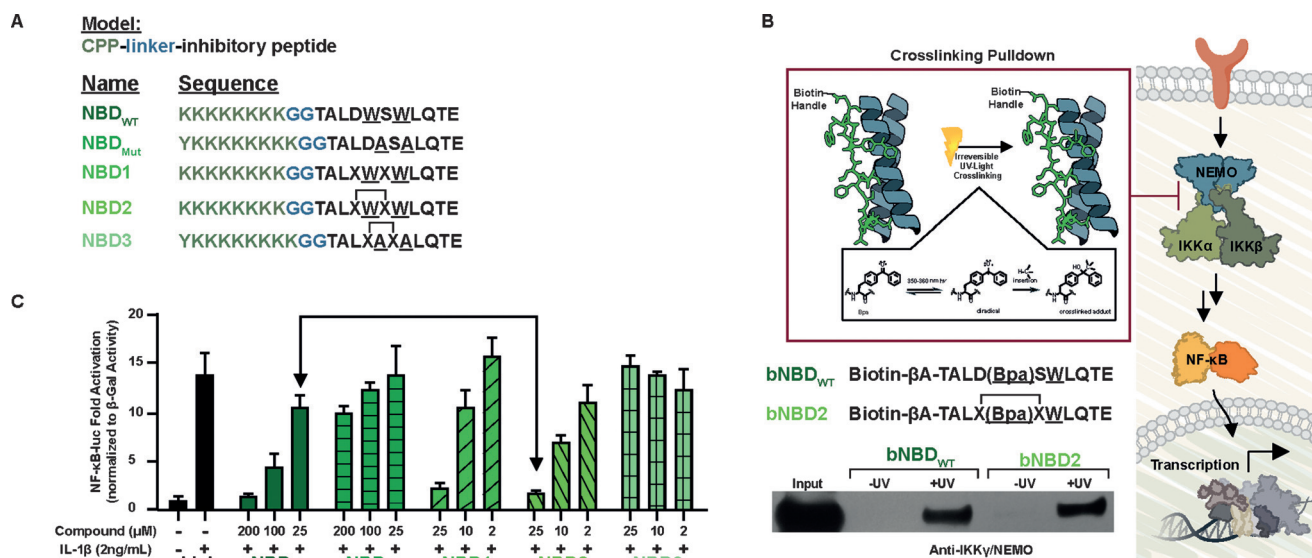


**Figure 1.** A) The signaling cascade of NF- $\kappa$ B can be defined by two pathways, the canonical and non-canonical pathway. The canonical pathway is tightly regulated through the assembly of the IKK complex, a heterotetramer of NEMO-IKK $\alpha$ -IKK $\beta$ . B) The critical assembly point of the IKK complex is mediated by a 4-helix bundle where the hot-spot hydrophobic tryptophan residues of IKK $\alpha$ -IKK $\beta$  make critical interactions with the NEMO dimer. C) The tryptophan residues of IKK $\alpha$ -IKK $\beta$  are able to adopt the unique conformation needed to interact with the NEMO dimer through a hydrogen bond stabilized loop conformation between D738 and S740. D) Replacement of the critical hydrogen bond is possible through incorporation of allylglycine amino acids and subsequent metathesis, allowing us to develop a proteolytically stable inhibitor molecule of this critical PPI. PDB 3BRV.



**Figure 2.** Ramachandran plots generated from molecular dynamics (MD) experiments performed with the indicated peptides. Highlighted in blue, are the observed dihedral angles of hot-spot residue W739 in the NEMO-IKK $\alpha$ / $\beta$  complex crystal structure (PDB: 3BRV). The lowest free energy conformers observed in MD simulations are indicated in dark red for A) wild-type peptide B) the peptide incorporating the allylglycine residues unmetathesized (denoted by X) and C) the peptide with allylglycine residues metathesized (denoted by interconnected X residues).

dichroism spectra analogous to that of the wild-type sequence (see Figure S1 in the Supporting Information).<sup>[27]</sup> However, the modification does confer significant proteolytic stability to the sequence. A time course experiment with NBD<sub>WT</sub>, NBD1, and NBD2 in the presence of chymotrypsin type VII (Sigma), a protease that cleaves the C-terminus of F, Y, L, and W, revealed that NBD<sub>WT</sub> and NBD1 were completely degraded after 15 minutes. Conversely, no proteolytic degradation occurred over 4 h for NBD2 under identical conditions, highlighting the protection afforded to the key hot spot residues (L737, W739, W741, and L742) by the hydrocarbon bridge modification. (Figure S2).<sup>[7]</sup> Significantly, the modification does not alter the ability of the hydrophobic hot spot

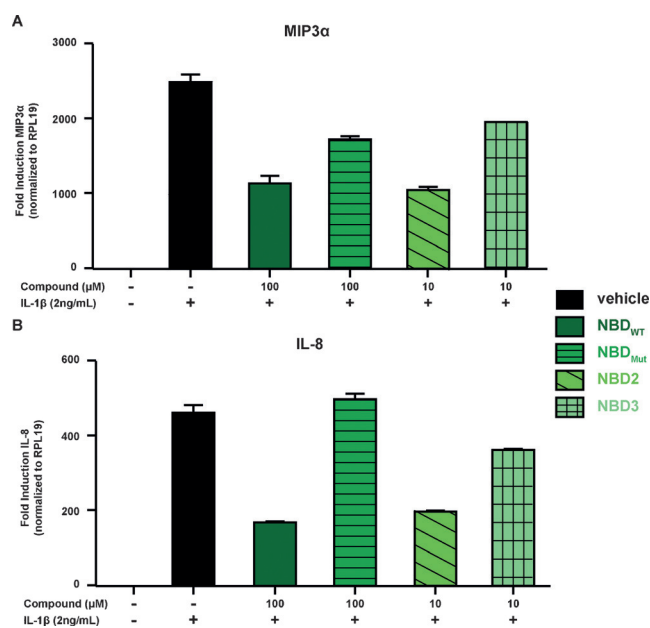


**Figure 3.** A) Peptides synthesized for characterization and cellular experiments. The model used to synthesize the peptides is a Cell Penetrating Peptide (CPP), a diglycine linker (GG), and the inhibitory peptide (NBD peptide). B) NEMO capture experiment performed with a biotinylated, photoreactive version of the NBD<sub>WT</sub> peptide and NBD2 peptide. Pull-down assays were performed with HeLa cellular extracts. C) Effects of the NBD peptides against an NF- $\kappa$ B luciferase reporter. NBD1 displayed significant toxicity and was thus excluded from further study.<sup>[28]</sup> The error is SDOM, determined from three independent experiments.

residues to engage NEMO in the context of cellular lysates. As shown in Figure 3b, photoactive variants of NBD<sub>WT</sub> (bNBD<sub>WT</sub>) and NBD2 (bNBD2) capture NEMO in a pull-down experiment in HEK293T cell lysates.

Consistent with the stability and target engagement data obtained *in vitro*, NBD2 is an effective inhibitor of canonical NF- $\kappa$ B signaling in cells. In a luciferase reporter assay, the canonical NF- $\kappa$ B stimulator interleukin-1  $\beta$  (IL-1 $\beta$ )<sup>[2]</sup> potently upregulates the NF- $\kappa$ B-driven reporter (13-fold). Consistent with previous reports, NBD<sub>WT</sub> displays dose-dependent inhibition in this system with approximately 50% inhibition at 100  $\mu$ M (Figure 3c).<sup>[13,26]</sup> In contrast, the negative control peptide (NBD<sub>Mut</sub>) in which critical tryptophan residues W739 and W741 are mutated to alanine has no activity, highlighting the importance of these tryptophan hot spot residues.<sup>[7,8,14]</sup> Notably, NBD2 is approximately an order of magnitude more potent than NBD<sub>WT</sub>. Analogous to the wild-type system, the W739A/W741A double mutant (NBD3) is inactive.<sup>[28]</sup> The activity of NBD2 extends to canonical NF- $\kappa$ B-regulated endogenous genes. MIP-3 $\alpha$  is an NF- $\kappa$ B regulated gene implicated in inflammatory bowel disease,<sup>[29,30]</sup> whereas IL-8 has been identified as a critical factor required for oncogenic metastasis.<sup>[31,32]</sup> In Figure 4, the inhibitory effects of the modified peptides on MIP-3 $\alpha$  and IL-8 gene expression are outlined. Consistent with the reporter gene results, a 10-fold increase in potency for NBD2 compared to the unmodified NBD<sub>WT</sub> peptide was observed. Furthermore, the alanine mutant versions of the peptides (NBD<sub>Mut</sub> and NBD3) displayed significantly attenuated inhibition. Consistent with selectivity for the canonical pathway, no impact on the noncanonical gene target Cyclin D1 was observed (Figure S4).<sup>[33–38]</sup>

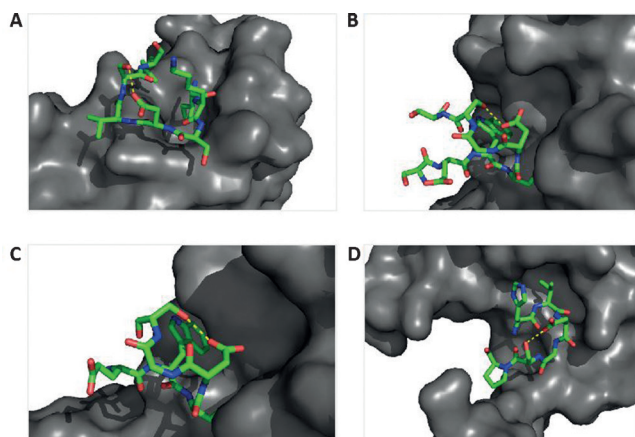
In summary, we implemented a synthetic loop replacement (SLR) strategy to develop an inhibitor against the



**Figure 4.** Effects of NBD peptides against NF- $\kappa$ B regulated gene expression of A) MIP3 $\alpha$  and B) IL-8. The error is SDOM, determined from three technical replicates. Three biological replicates were performed (see SI for additional data).

NEMO-IKK $\alpha$ / $\beta$  interaction. Using this strategy, we demonstrated that installation of a hydrocarbon bridge can be used to stabilize the IKK loop structure, a feature necessary for interaction with NEMO. MD simulations demonstrate that the modified NBD2 peptide adopts dihedral angles and conformations similar to the docked configuration observed in the reported crystal structure. Furthermore, we demon-

strated the enhanced proteolytic stability afforded by this synthetic loop replacement modification. Collectively, these characteristics resulted in a synthetic inhibitor that was 10-fold more potent in both an NF- $\kappa$ B driven luciferase reporter assay and against NF- $\kappa$ B endogenous genes. Future structural studies of NBD2 and derivatives alone and in complex with NEMO will be key to dissecting the precise role that the hydrocarbon tether plays in the protein–protein interaction, although this will be challenging given the recalcitrant nature of NEMO.<sup>[7]</sup> A unique aspect of the synthetic loop replacement is that a carbon–carbon bridge is installed in place of a hydrogen bond, rendering it potentially general.<sup>[39–43]</sup> In a recent study by Gavenonis et al. 1407 PPIs mediated by hot-spot-containing loop structures were reported, analogous to the NEMO-IKK interaction.<sup>[44]</sup> A focused analysis of the identified loops presented by Gavenonis et al. demonstrates that there are several loops bearing an *i* to *i*+2 hydrogen-bond-stabilizing feature similar to D738 and S740 in IKK $\alpha$ / $\beta$  bound to NEMO. These identified loops should be ideal candidates for the SLR strategy presented herein (Figure 5) with potential inhibitor applications ranging from autoimmune diseases, anticoagulants, and antivenom to metabolic therapies. A recent study of characterized PPI interfaces found that  $\alpha$ -helices and  $\beta$ -strands make up only 50% of the secondary structure observed at PPI interfaces while the other 50% of characterized PPIs are mediated by non-canonical loop regions, a class of PPIs not readily amenable to previously developed strategies.<sup>[45]</sup> This finding highlights both the opportunity and the necessity of implementing new chemical strategies such as SLR to target PPIs mediated by non-canonical loop regions.<sup>[44]</sup>



**Figure 5.** A subset of highlighted PPIs mediated by loop regions containing reported hot spot residues. This subset of highlighted loop regions are characterized by an *i* to *i*+2 hydrogen bond (yellow dashed line) between aspartic acid and serine residues that would be ideal candidates amenable to the described SLR strategy for inhibitor development. These potential SLR inhibitors could be used to a) inhibit dimerization of outer surface protein C (OspC) from Lyme disease spirochete. b) PDB 1F1M inhibits dimerization of von Willebrand Factor and Botrocetin, a snake venom protein involved in platelet aggregation. c) PDB 1FVU inhibits interaction of blood coagulation factors IX/X-binding protein (IX/X-bp), an anticoagulant isolated from habu snake venom. d) PDB 1IXX inhibits assembly of acetoacetate decarboxylase, a major contributor to diabetic ketoacidosis; PDB 3BGT.

## Acknowledgements

A.K.M. gratefully acknowledges the NIH for support of this work (grant number CA140667). Support from the NIH through grant number GM37554 is acknowledged by C.L.B.

**Keywords:** inhibitors · medical chemistry · peptide loops · peptidomimetics · protein–protein interactions

**How to cite:** *Angew. Chem. Int. Ed.* **2016**, *55*, 14997–15001  
*Angew. Chem.* **2016**, *128*, 15221–15225

- [1] A. L. Garner, K. D. Janda, *Curr. Top. Med. Chem.* **2011**, *11*, 258–280.
- [2] B. Hoesel, J. A. Schmid, *Mol. Cancer* **2013**, *12*, 86.
- [3] J. Albert, S. Baldwin, *J. Clin. Invest.* **2001**, *107*, 3–6.
- [4] B. O. Villoutreix, M. A. Kuenemann, J.-L. Poyet, H. Bruzzoni-Giovanelli, C. Labbé, D. Lagorce, O. Sperandio, M. A. Miteva, *Mol. Inf.* **2014**, *33*, 414–437.
- [5] L. N. Makley, J. E. Gestwicki, *Chem. Biol. Drug Des.* **2013**, *81*, 22–32.
- [6] G. L. Verdine, L. D. Walensky, *Clin. Cancer Res.* **2007**, *13*, 7264–7270.
- [7] M. S. Golden, S. M. Cote, M. Sayeg, B. S. Zerbe, E. A. Villar, D. Beglov, S. L. Sazinsky, R. M. Georgiadis, S. Vajda, D. Kozakov, et al., *J. Am. Chem. Soc.* **2013**, *135*, 6242–6256.
- [8] M. Rushe, L. Silvian, S. Bixler, L. L. Chen, A. Cheung, S. Bowes, H. Cuervo, S. Berkowitz, T. Zheng, K. Guckian, et al., *Structure* **2008**, *16*, 798–808.
- [9] A. V. Gasparian, Y. J. Yao, D. Kowalczyk, L. A. Lyakh, A. Karseladze, T. J. Slaga, I. V. Budunova, *J. Cell Sci.* **2002**, *115*, 141–151.
- [10] E. Zandi, D. M. Rothwarf, M. Delhase, M. Hayakawa, *Cell* **1997**, *91*, 243–252.
- [11] C. Makris, V. L. Godfrey, G. Krähn-Sentfleben, T. Takahashi, J. L. Roberts, T. Schwarz, L. Feng, R. S. Johnson, M. Karin, *Mol. Cell* **2000**, *5*, 969–979.
- [12] A. Oeckinghaus, M. S. Hayden, S. Ghosh, *Nat. Immunol.* **2011**, *12*, 695–708.
- [13] M. J. May, F. D'Acquisto, L. A. Madge, J. Glöckner, J. S. Pober, S. Ghosh, *Science* **2000**, *289*, 1550–1554.
- [14] M. J. May, R. B. Marienfeld, S. Ghosh, *J. Biol. Chem.* **2002**, *277*, 45992–46000.
- [15] I. Strickland, S. Ghosh, *Ann. Rheum. Dis.* **2006**, *65 Suppl 3*, iii75–82.
- [16] D. A. Delfin, Y. Xu, J. M. Peterson, D. C. Guttridge, J. A. Rafael-Fortney, P. M. Janssen, *J. Transl. Med.* **2011**, *9*, 68.
- [17] K. K. Rehman, S. Bertera, R. Bottino, A. N. Balamurugan, J. C. Mai, Z. Mi, M. Trucco, P. D. Robbins, *J. Biol. Chem.* **2003**, *278*, 9862–9868.
- [18] Inspired by success reported by others in the stabilization of canonical secondary structures such as  $\alpha$ -helices and  $\beta$ -strands, references [19–25].
- [19] L. K. Henchey, S. Kushal, R. Dubey, R. N. Chapman, B. Z. Olenyuk, P. S. Arora, *J. Am. Chem. Soc.* **2010**, *132*, 941–943.
- [20] L. D. Walensky, K. Pitter, J. Morash, K. J. Oh, S. Barbuto, J. Fisher, E. Smith, G. L. Verdine, S. J. Korsmeyer, *Mol. Cell* **2006**, *24*, 199–210.
- [21] P.-N. Cheng, C. Liu, M. Zhao, D. Eisenberg, J. S. Nowick, *Nat. Chem.* **2012**, *4*, 927–933.
- [22] R. N. Chapman, P. S. Arora, *Org. Lett.* **2006**, *8*, 5825–5828.
- [23] R. N. Chapman, G. Dimartino, P. S. Arora, *J. Am. Chem. Soc.* **2004**, *126*, 12252–12253.
- [24] P. S. Kutchukian, J. S. Yang, G. L. Verdine, E. I. Shakhnovich, *J. Am. Chem. Soc.* **2009**, *131*, 4622–4627.

- [25] C. E. Schafmeister, J. Po, G. L. Verdine, *J. Am. Chem. Soc.* **2000**, *122*, 5891–5892.
- [26] K. Khaja, P. Robbins, *Pharmaceuticals* **2010**, *3*, 110–124.
- [27] N. J. Greenfield, *Nat. Protoc.* **2006**, *1*, 2876–2890.
- [28] The construct NBD1, which contains the unmetathesized allylglycine residues, also exhibited dose-dependent inhibition of NF- $\kappa$ B driven luciferase activity. However, the NBD1 construct also exhibited dose-dependent inhibition of the constitutively expressed  $\beta$ -gal reporter signal consistent with off-target activities and thus, was excluded from further study.
- [29] J. H. Kwon, S. Keates, S. Simeonidis, F. Grall, T. A. Libermann, A. C. Keates, *J. Biol. Chem.* **2003**, *278*, 875–884.
- [30] 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.
- [31] C. Kunsch, C. A. Rosen, *Mol. Cell. Biol.* **1993**, *13*, 6137–6146.
- [32] S. Lee, Y. J. Kim, S. Kwon, Y. Lee, S. Y. Choi, J. Park, *BMB Rep.* **2009**, *42*, 265–270.
- [33] N. D. Perkins, *Nat. Rev. Mol. Cell Biol.* **2007**, *8*, 49–62.
- [34] F. Wang, Y. Shi, S. Yadav, H. Wang, *Toxicology* **2010**, *273*, 8–12.
- [35] C. Albanese, K. Wu, M. D'Amico, C. Jarrett, D. Joyce, J. Hughes, J. Hulit, T. Sakamaki, M. Fu, A. Ben-Ze'ev, et al., *Mol. Biol. Cell* **2003**, *14*, 585–599.
- [36] S. D. Westerheide, M. W. Mayo, V. Anest, J. L. Hanson, A. S. Baldwin, *Mol. Cell. Biol.* **2001**, *21*, 8428–8436.
- [37] K.-J. Park, V. Krishnan, B. W. O'Malley, Y. Yamamoto, R. B. Gaynor, *Mol. Cell* **2005**, *18*, 71–82.
- [38] J. Zhang, M. A. Warren, S. F. Shoemaker, M. M. Ip, *Endocrinology* **2007**, *148*, 268–278.
- [39] For previous work replacing nonpolar interactions with hydrocarbon tethers see references [40–42].
- [40] A. Glas, D. Bier, G. Hahne, C. Rademacher, C. Ottmann, T. N. Grossmann, *Angew. Chem. Int. Ed.* **2014**, *53*, 2489–2493; *Angew. Chem.* **2014**, *126*, 2522–2526.
- [41] Y. Shin, K. A. Winans, B. J. Backes, S. B. H. Kent, A. J. A. Ellman, C. R. Bertozzi, *J. Am. Chem. Soc.* **1999**, *121*, 11684–11689.
- [42] J. F. Reichwein, C. Versluis, R. M. Liskamp, *J. Org. Chem.* **2000**, *65*, 6187–6195.
- [43] S. M. Miles, R. J. Leatherbarrow, S. P. Marsden, W. J. Coates, *Org. Biomol. Chem.* **2004**, *2*, 281–283.
- [44] J. Gavenonis, B. A. Sheneman, T. R. Siegert, M. R. Eshelman, J. A. Kritzer, *Nat. Chem. Biol.* **2014**, *10*, 716–722.
- [45] M. Guharoy, P. Chakrabarti, *Bioinformatics* **2007**, *23*, 1909–1918.

Received: August 16, 2016

Published online: October 28, 2016