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Supporting Information

Binding Dynamics of a Stapled Peptide Targeting the Transcription Factor NF–Y

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Supporting Information

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Automated peptide synthesis

Peptides **5, 3D^{N}**, **5D^{N}**, **7D^{N}** were synthesized using peptide synthesis robot Syro I (*MultiSynTech*). Ramage amide AM resin was used as a solid support. N-α fluorenylmethoxycarbonyl (Fmoc) protected amino acids were dissolved in 0.33 M ethyl cyano(hydroxyimino)acetate (oxyma), as 0.33 M solutions N,N-dimethylformamide (DMF) and coupling reagents were dissolved in NMP (*c* = 0.33 M). N,N-diisopropylethylamine (DIPEA) was dissolved in N-methylpyrrolidone (NMP) (*c* = 1.33 M). Fmoc protecting group removal was carried out in Piperidine/DMF (2:8, v/v), 2x 5 min followed by wash cycle (6x DMF). Fmoc deprotected amino acid couplings were performed as double coupling of 4 eq. Benzotriazol-1-yl-oxy-trispyrrolidino-phosphonium hexafluorophosphate (PyBOP) (first coupling 40 min) and 4 eq. 1-[bis(dimethylamino)methylene]-1H-1,2,3-triazolo[4,5-b]pyridinium 3-oxid hexafluorophosphate (HATU) (second coupling 40 min) as coupling reagents and were dissolved in DMF. The coupling reaction with 4 eq. Fmoc-protected amino acid was then treated with 4 eq. Oxyma and 8 eq. DIPEA. A capping step was performed after each double coupling cycle by resin treatment with acetic anhydride (Ac_2O) and DIPEA in NMP (1:1:8, v/v/v) for 10 min.

Manual peptide synthesis

Peptides **2**, **3**, **7**, **2D^{N}** were synthesized on H-Rink Amide Chem Matrix resin. Reaction steps were performed in a syringe reactor on an orbital shaker. The workflow for synthesis is in the order of deprotection, coupling and capping of amino acid. The resin was washed with DMF (3x, 1 mL per 50 mg resin), dichloromethane (DCM) (3x, 1 mL per 50 mg resin) and DMF (3x, 1 mL per 50 mg resin) in between reaction steps.

Removal of Fmoc on the N-terminus was done by suspending the resin in Piperidine/DMF (2:8, v/v) solution (1 mL per 50 mg resin) twice for 5 min.

A solution of 4 eq. amino acid, 4 eq. (1-cyano-2-ethoxy-2-oxoethylidenaminooxy)dimethylamino-morpholino-carbenium hexafluorophosphate (COMU) and 4 eq. Oxyma in DMF was activated with 8 eq. of DIPEA. Next, the reaction solution was added to the resin (0.3 mL per 50 mg resin). After 20 min, the solution was discarded and the coupling was repeated. The reaction time was extended to 1 hour for unnatural amino acids and the reaction was performed once.

Ring Closing Metathesis

Ring closing metathesis (RCM) was conducted for macrocyclization of non-natural amino acids. After the completion of the core peptide sequence synthesis, resins were first swollen in dry 1,2-dichloroethane (DCE) for 30 min before performing RCM. A solution of Grubbs first generation catalyst in dry DCE of (4 mg mL^{-1}, 1 mL per 50 mg resin) was added to the resin and a continuous stream of nitrogen was bubbled through the reaction mixture. After 2 hours, the reaction solution was discarded and the resin was washed with dry DCE (3x, 1 mL per 50 mg of resin), DCM (3x) and DMF (3x) respectively. The procedure was repeated an additional three times.

N-terminal modification

For affinity measurements, the final Fmoc deprotected peptides were subsequently fluorescein-5-isothiocyanate(isomer I) (FITC) - 8-amino-3,6-dioxaoctanoic acid (PEG2) labelled or acetylated for crystallization experiments. N-terminal amino group was acetylated using Ac_2O and DIPEA in DMF (1:1:8, v/v/v) for 10 min twice. Afterwards the resin was washed with DCM (3x, 1 mL per 50 mg resin). Before FITC labelling, the flexible spacer PEG2 was N-terminal linked using the amino acid coupling protocol stated above. After removing N-terminal Fmoc, the

resin was suspended in a solution of 5 eq. FITC isomer I and 10 eq. DIPEA in DMF. After overnight reaction, the reaction solution was discarded and the resin was washed with DCM (3x 1 mL per 50 mg resin).

Cleavage, purification and characterization

Prior to final cleavage, the resin was dried under vacuum. A solution of trifluoroacetic acid (TFA) / triisopropylsilane (TIPS) /H_2O (95:2.5:2.5, v/v/v, 1 mL per 50 mg resin) was added to the resin for 3x 1 h. The cleavage solution was then partially evaporated, followed by the addition of cold diethyl ether to precipitate the crude peptide. After centrifugation (15 min, 4°C, 4000 rpm), the supernatant was discarded, the crude product was dissolved in acetonitrile (ACN) /H_2O (1:1, v/v) and lyophilized. Crude lyophilized peptides were re-dissolved in ACN/H_2O and purified by reversed-phase HPLC (Column: Macherey-Nagel Nucleodur C18, 10x125 mm, 110 Å, 5 µm. Solvent A: H_2O + 0.1% TFA Solvent B: ACN + 0.1% TFA. Flow Rate: 6 mL min^{-1}). Pure fractions were subsequently pooled and lyophilized, followed by characterization and quantification. Peptides were characterized using an analytical reversed-phase HPLC (1260 Infinity, Agilent Technology. Column: Agilent Eclipse XDB-C18, 4.6x150 mm, 5 µm. Solvent A: H_2O + 0.1% formic acid (FA) + 0.01% TFA, Solvent B: ACN + 0.1% FA + 0.01% TFA. Flow Rate: 1 mL min^{-1}, 5–95% gradient over 30 min) coupled to an ESI-MS (6120 Quadrupole LC/MS, Agilent Technology). Analytical HPLC chromatograms at 210 nm and MS spectra (masses and m/z ratios) are provided in supporting information (Figure S6). Quantification was performed gravimetrically or via FITC absorption (ε_{495} = 77000 M^{-1} cm^{-1}) in 100 mM sodium phosphate buffer pH 8.5.

Heterologous protein expression and purification

The bacterial expression and purification procedure of NF-YB/C was performed similar as previously described using FPLC Ni^{2+}-NTA affinity chromatography followed by tag-cleavage and subsequent Size Exclusion Chromatography (SEC).[1] Chemically competent *Escherichia coli (E. coli)* BL21(*DE3*) cells were transformed with pACYC-duet (Novagen) vector containing the protein-coding gene for N-terminally His_6-tagged NF-YB/C. A main culture (TB) was inoculated at OD_{600} = 0.1 using a LB overnight culture. Cells were grown at 37°C until OD_{600} = 1.0–1.3 was reached. Subsequently, protein expression was induced using 0.5 mM Isopropylthiogalactoside (IPTG), the culture was incubated overnight at 25°C, 150 rpm. Cells were harvested by centrifugation (20°C, 4500 rpm, 10 min, JA 4.5 rotor, BeckmanCoulter) and the cell pellet was dissolved in lysis buffer (25 mM Tris, pH 8.0, 1 M NaCl, 1 mM tris(2-carboxy-ethyl)phosphine (TCEP)). The next steps were performed at 4°C unless otherwise stated. After addition of 100 mM phenylmethylsulfonyl fluoride (PMSF), lysozyme, and DNase I, cells were lysed using a Microfluidizer (LM10, Microfluidics). Lysates were cleared by ultracentrifugation at 4°C (20000 rpm, 45 min, JA 25.5 rotor, BeckmanCoulter). The supernatant was loaded onto a 5 ml HisTrap FF crude column (Cytiva) pre-equilibrated with lysis buffer (flow: 1 ml min^{-1}). The column was washed with 10 column volumes of wash buffer (25 mM Tris, pH 8.0, 1 M NaCl, 1 mM TCEP, 40 mM imidazole). The column bound protein was cleaved off by overnight circulation using PreScission protease in cleavage buffer (25 mM Tris, pH 8.0, 1 M NaCl, 1 mM TCEP). Cleaved NF-YB/C protein solution was concentrated using a 3-kDa cut-off ultra-filtration device (Amicon ultra centrifugal filters, Millipore) up to 5 ml with around 8 mg ml^{-1} protein. Final purification was conducted using size exclusion chromatography (SEC) (Superdex S75 16/600, GE Healthcare Life Sciences) in SEC buffer (25 mM Tris, pH 8.0, 1 M NaCl, 1 mM TCEP). After concentration of pure fractions via ultra-filtration up to 40-70 g L^{-1}, the pure protein was flash-frozen in liquid nitrogen and stored at -80°C.

Supplementary Figures

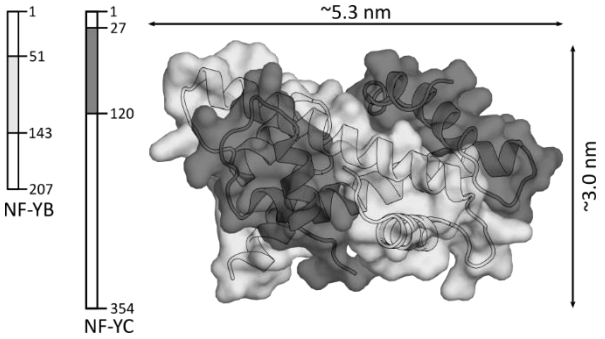


Figure S1 Functional domains of NF-YB (light-grey) and NF-YC (dark-grey) subunits in the NF-YB/C complex. Crystal structure of NF Y B/C (PDB ID 6qms). Shown is the surface representation of subunits B (light-grey) and C (dark-grey). Measurements indicate the outer dimensions of this intertwined heterodimeric complex.

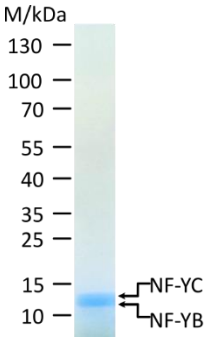


Figure S2 Coomassie stained SDS-PAGE 4–20% gradient gel (GenScript) of the NF-YB/C heterodimer after tag-cleavage and complete purification. Molecular weights of protein standards (M) are indicated in kDa.

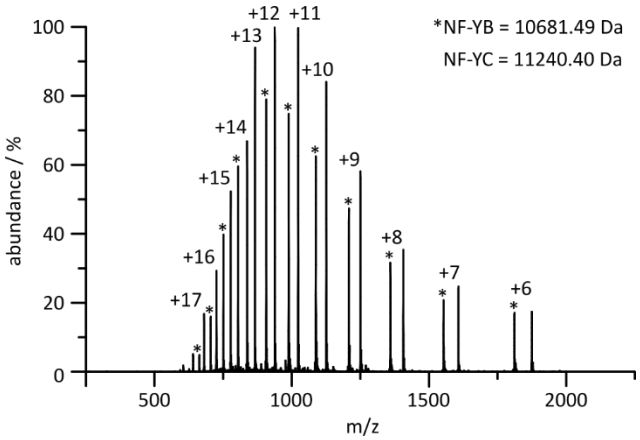


Figure S3 HPLC ESI-MS spectrum of the NF-YB/C heterodimer. Peaks of multiple charged species are shown. Asterisk (*) highlights peaks belonging to NF-YB. Observed masses are indicated. Calculated masses are for NF-YB: 10862.47 Da and NF-YC: 11241.23 Da.

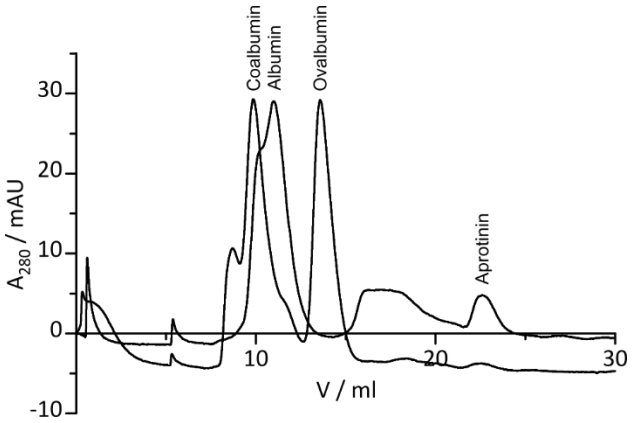


Figure S4 Calibration of analytical SEC with protein standards (Agilent Technologies). Chromatograms from two standard runs on 10/300 S75 Superdex column (Äkta Pure, Cytiva) are shown. Protein standards are labeled on the corresponding peaks. Individual molecular weights and elution volumes: Coalbumin: 75.0 kDa (9.9 ml), Albumin: 66.5 kDa (11.0 ml), Ovalbumin: 43.0 kDa (13.6 ml), Aprotinin: 6.5 kDa (22.5 ml).

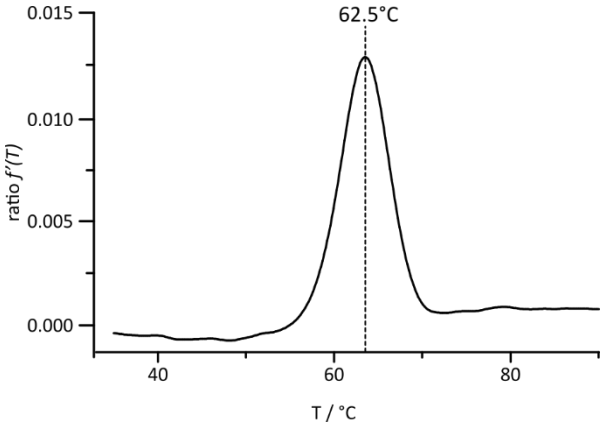


Figure S5 First derivative *f'*(T) curve of thermal unfolding (F_{350}/F_{330}), calculated using Panta Control (v1.6.3) software. The peak indicates the melting point of 62.5°C. (Figure 1D).

2D^{N}

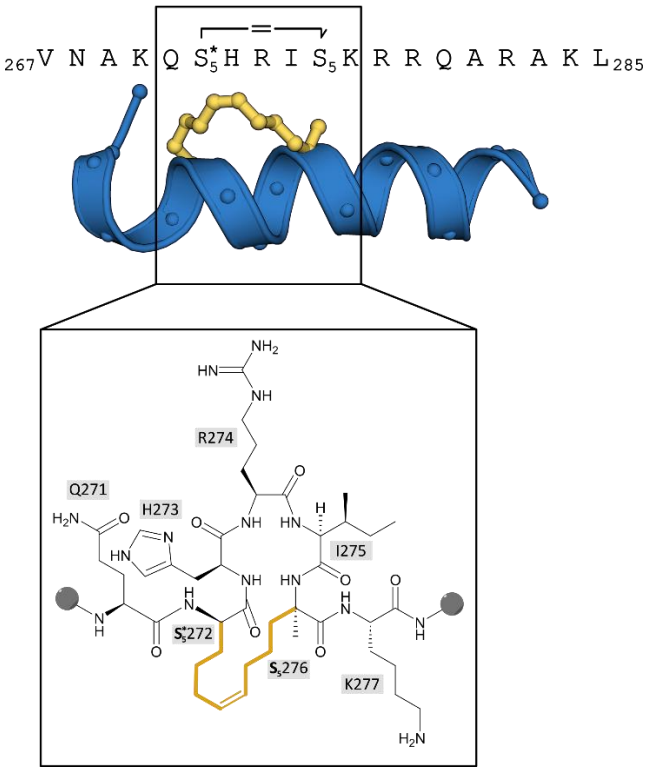
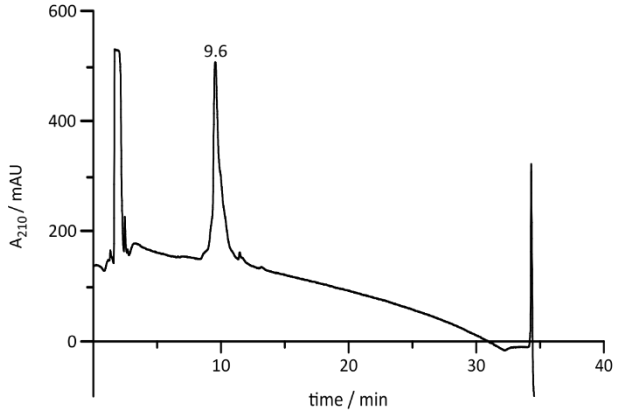


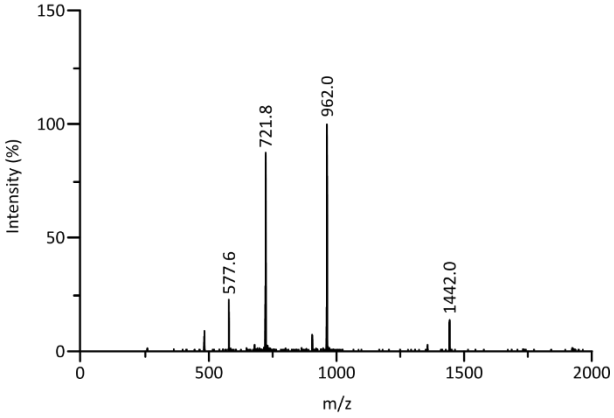
Figure S6 NF-YA derived peptide **2D^{N}** as described earlier.[1] Top panel: the **2D^{N}** peptide sequence. Orange lines indicate hydrocarbon staple, = indicates double bond after ring closing metathesis), **S$_5$***:(S)-2-(4-pentenyl)glycine, **S$_5$**: (S)-2-(4-pentenyl)alanine. Middle panel: **2D^{N}** extracted from the crystal structure (blue cartoon helix, PDB ID: 6qms). Hydrocarbon peptide staple is indicated in orange sticks. Bottom panel: Chemical structure of the **2D^{N}** hydrocarbon peptide staple region (highlighted in orange), including the adjacent amino acids (indicated by black box). Amino acids are labeled.

FITC-**2**

FITC-PEG2-VNAKQYHRILKRRQARAKL-NH_2

MW: 2883.4 Da

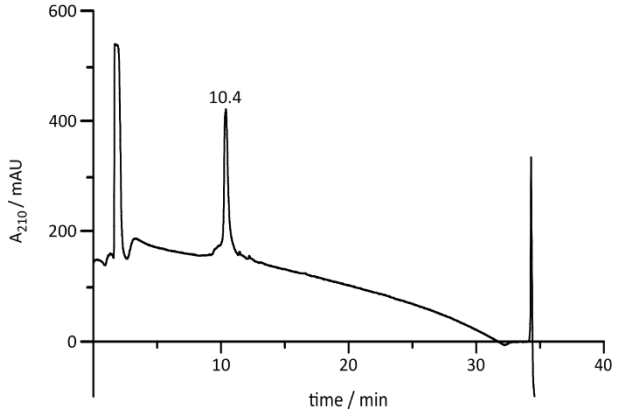


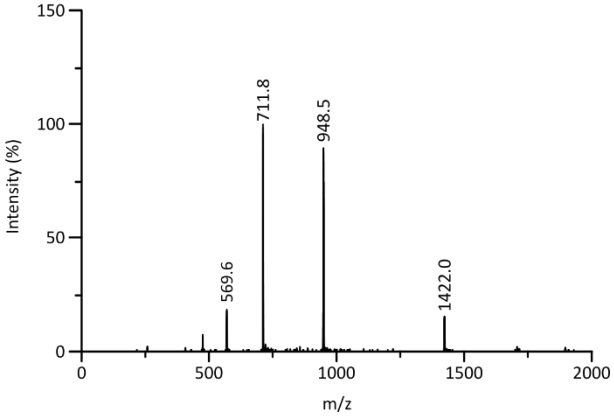


FITC-**2D^{N}**

FITC-PEG3-VNAKQ**S$_5$***HRI**S$_5$**KRRQARAKL-NH_2

MW: 2843.4 Da





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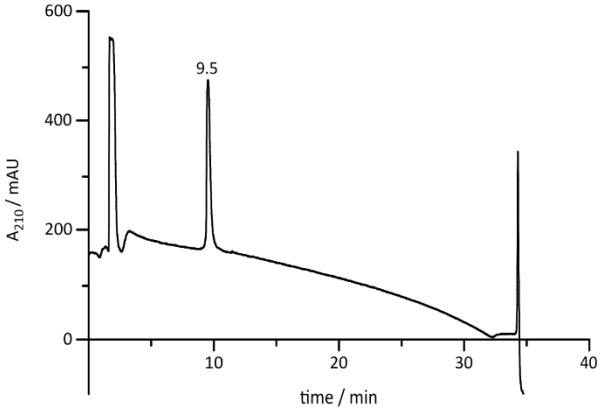

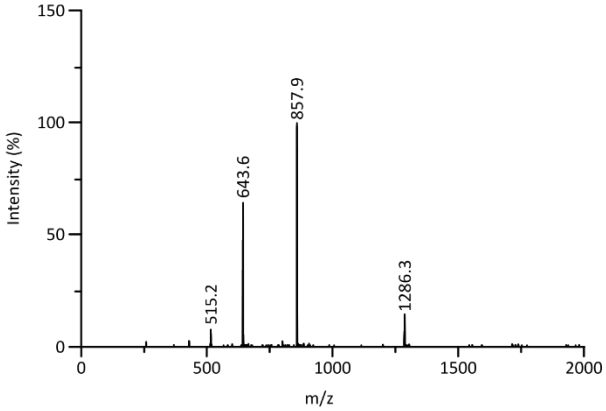

FITC-**3D^{N}**

FITC-PEG2-VNAKQ**S$_5$***HRI**S$_5$**KRRQAR-NH_2

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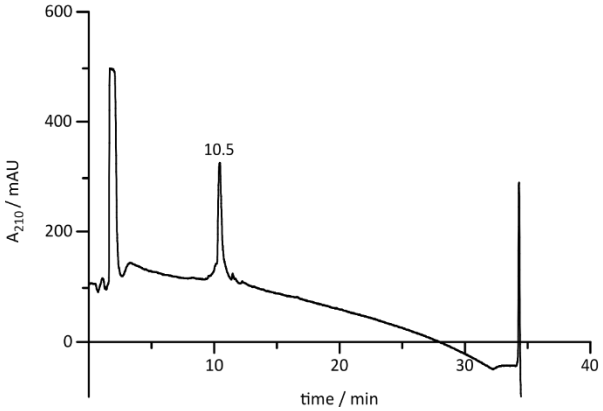

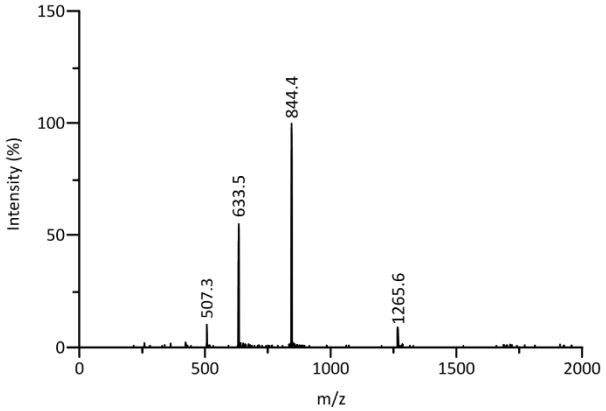

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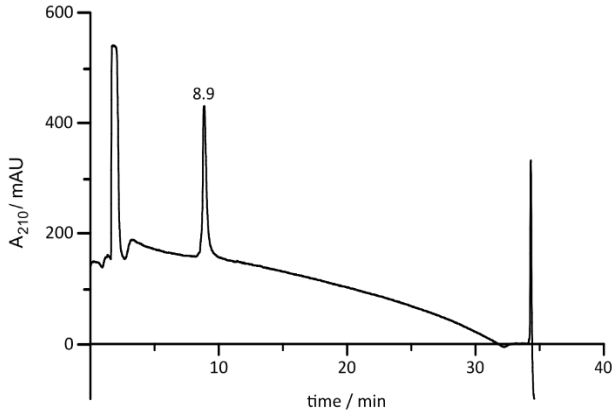
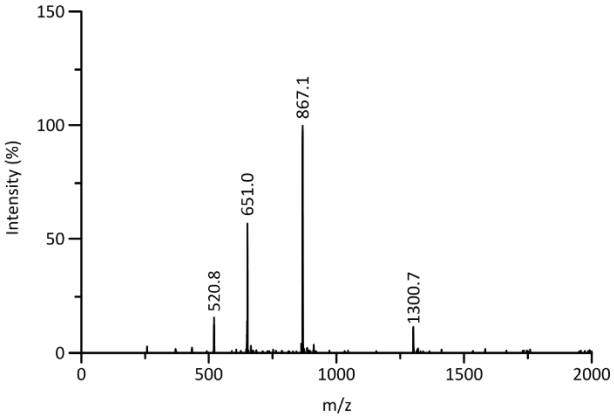
FITC-PEG2-KQYHRILKRRQARAKL-NH_2
MW: 2599.1 Da





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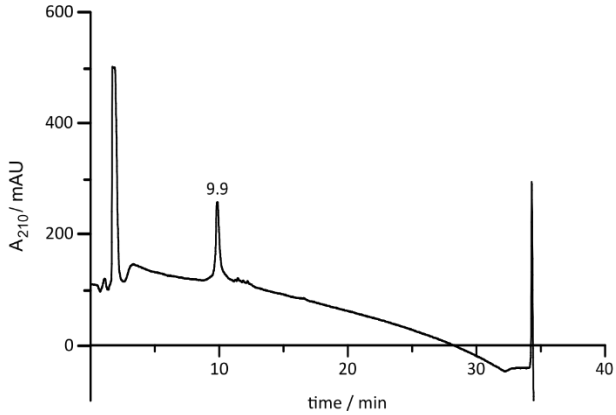

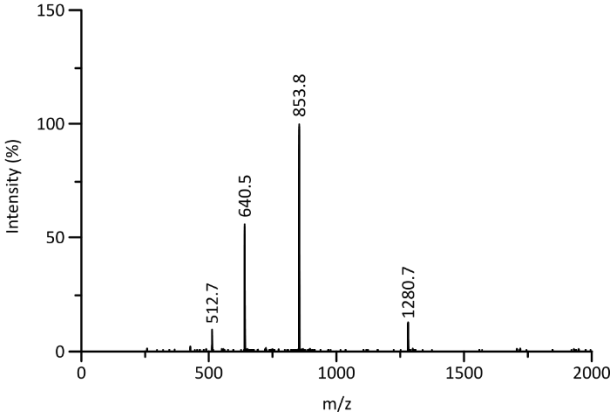

FITC-**7**

FITC-PEG2-KQYHRILKRRQAR-NH_2

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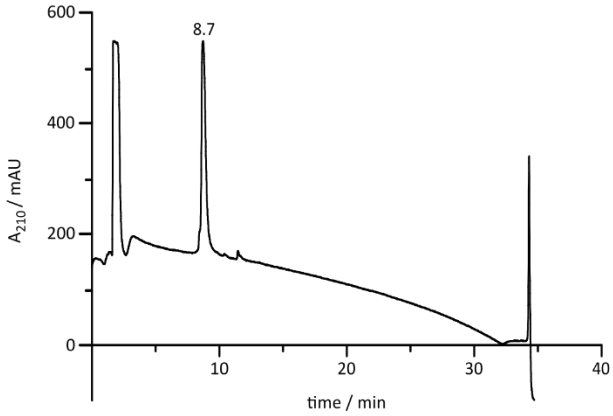

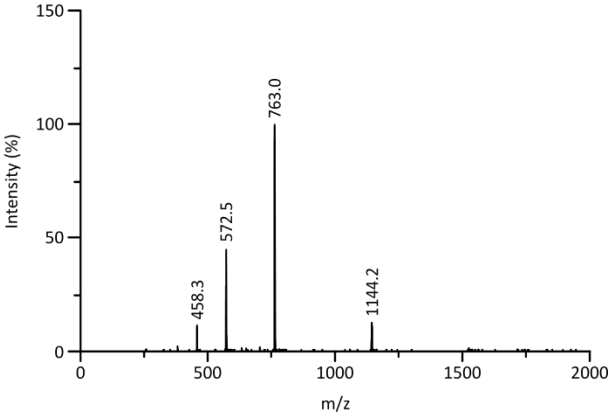

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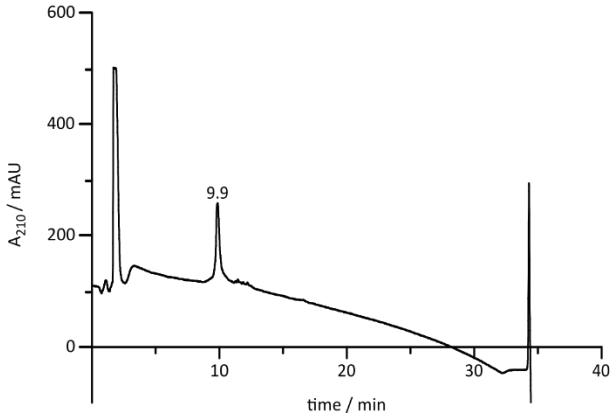
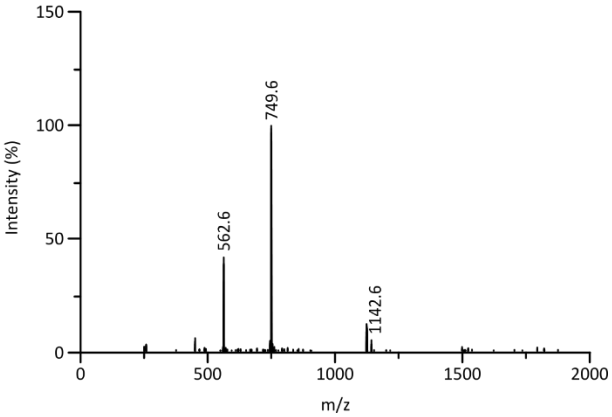
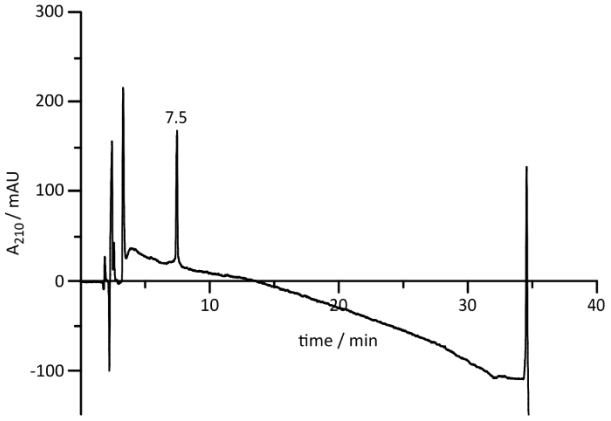
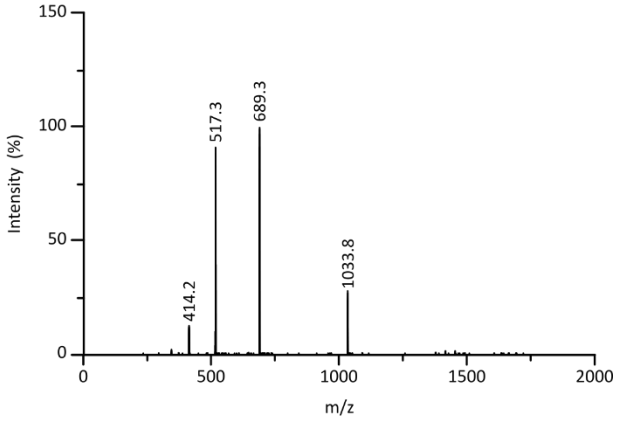




Ac-5**D**^{N}
Ac-KQ**S**$_5$*HRI**S**$_5$KRRQARAKL-NH$_2$
MW: 2066.6 Da





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Ac-KQ**S**$_5$*HRI**S**$_5$KRRQAR-NH$_2$
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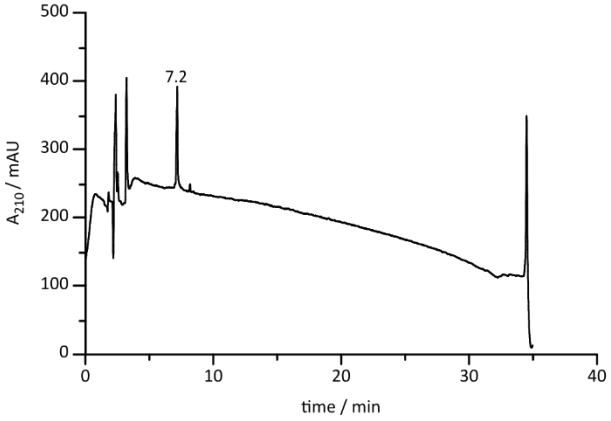

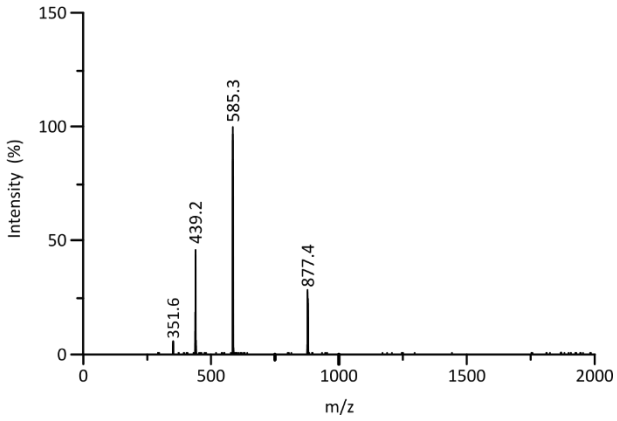

Figure S7 HPLC/ESI-MS analysis. Analysis of peptides FITC-**2**, FITC-**2D**^{N}, FITC-**3**, FITC-**3D**^{N}, FITC-**5**, FITC-**5D**^{N}, FITC-**7**, FITC-**7D**^{N}, Ac-**5D**^{N} and Ac-**7D**^{N} respectively, performed on Agilent HPLC system using ZORBAX Eclipse XDB-C18 column. Left chromatogram shows Absorption (λ = 210 nm) over time (t in min) and right diagram shows relative intensity of MS-ions (m/z).

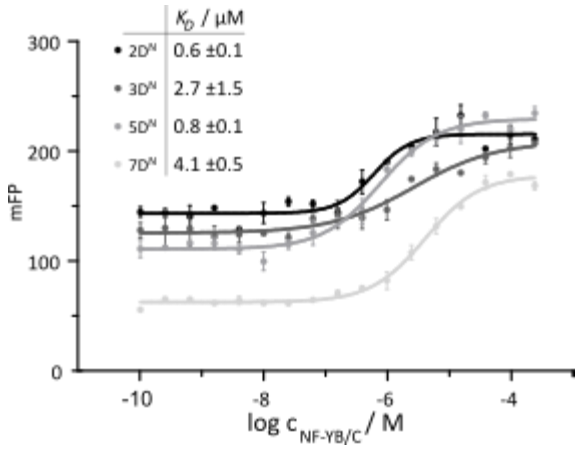

Figure S8 Fluorescence polarization measurements of FITC-labelled stapled peptides **2D^N, 3D^N, 5D^N, 7D^N** binding to NF-YB/C. Measurements were performed as triplicates ($c_{peptide}$ = 40 nM, $c_{NF\text{-}YB/C}$ = 1.6 10^{-9} – 2.4 10^{-4} M).

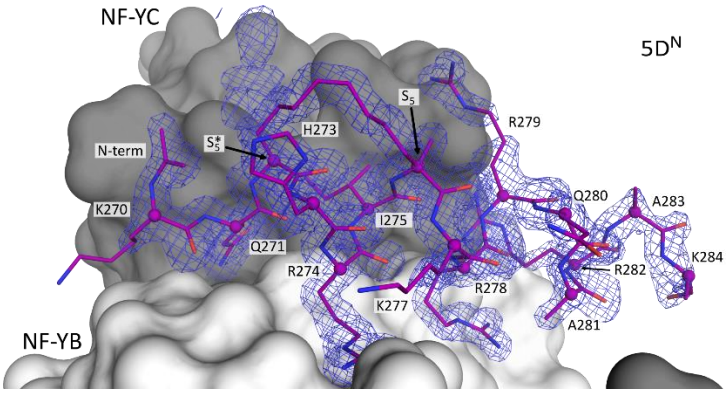

Figure S9 2*m*Fo-*D*Fc electron density (contoured at 1 rmsd) of the **5D^N** bound NF-YB/C complex structure with **5D^N** build into the density (pink sticks, PDB ID 8qu2). NF-YB/C protein surfaces are shown in dark and light grey. Peptide hydrocarbon staple is shown and labeled by its according building blocks **S$_5$*** and **S$_5$**.

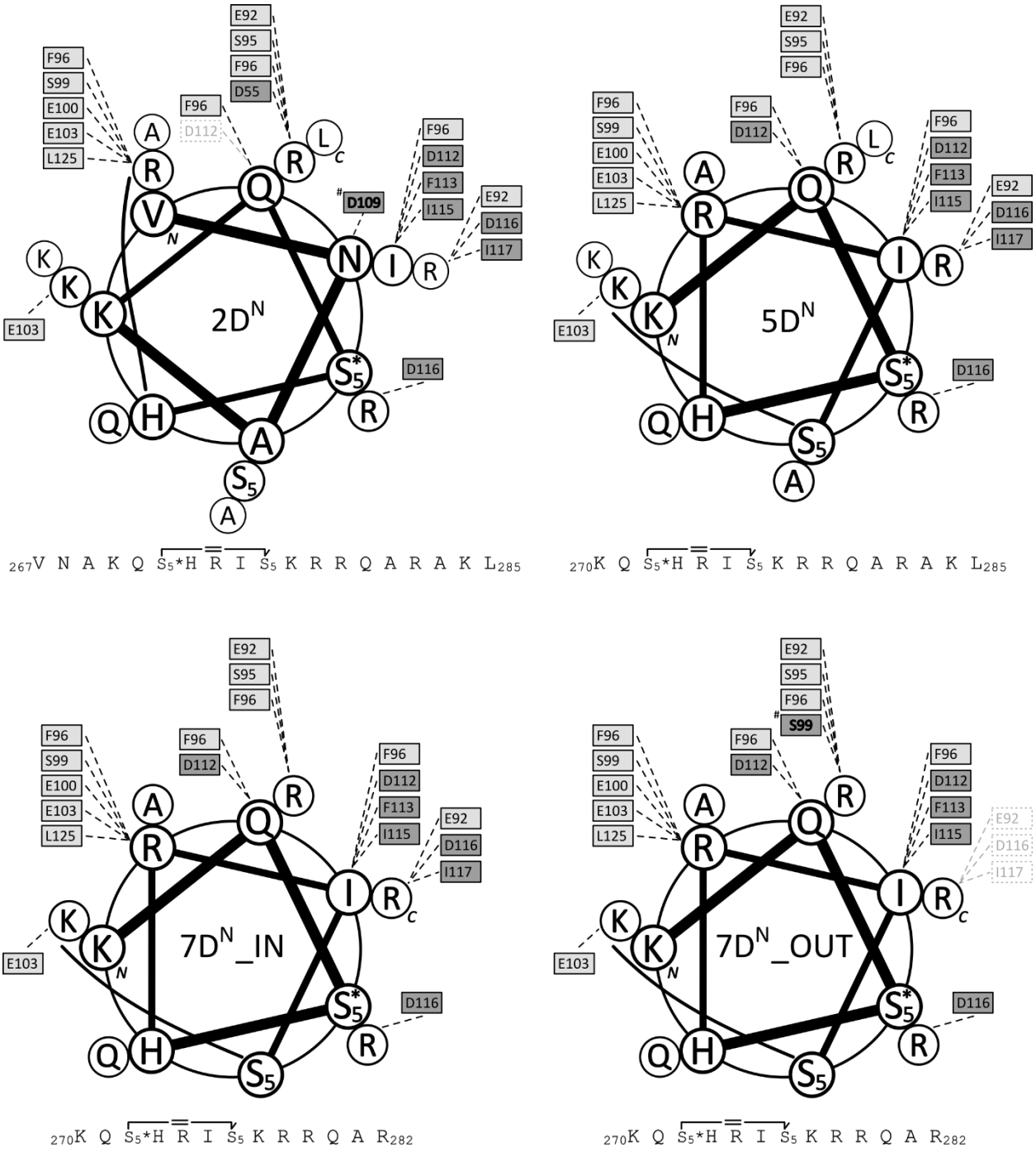

Figure S10 Helix wheel and primary structure representation of **2D^N**, **5D^N**, **7D^N**_IN and **7D^N**_OUT. Amino acid side chain contacts from crystal structures (PDB ID 6qmq, 8qu2, 8qu3, 8qu4) are indicated. #: interaction is unique for this peptide. Fainted entry indicates that interaction is missing in the current peptide, but present in all others. Light grey boxes indicate interaction with side chains of the NF-YB sub-unit, dark grey boxes indicated interaction with side chains of NF-YC sub-unit. *N* and *C*-termini of the peptides are indicated.

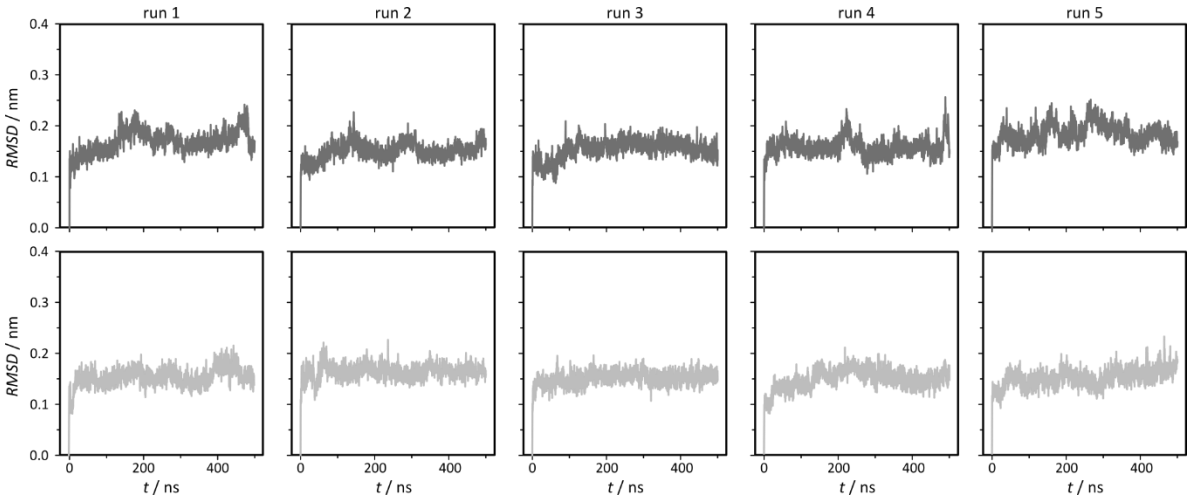

Figure S11 RMSD time traces for 500 ns of the root mean square deviation (RMSDs) of the Cα-atoms of the NF-YB/C heterodimer with respect to their starting structure. MD simulations were performed using five different starting velocities (run 1-5) from the 7DN_IN crystal structure (PDB ID 8qu3, top panel, dark grey) and five from the 7DN_OUT crystal structure (PDB ID 8qu4, bottom panel, light grey). The trajectories are aligned on chains B and C of the heterodimer.

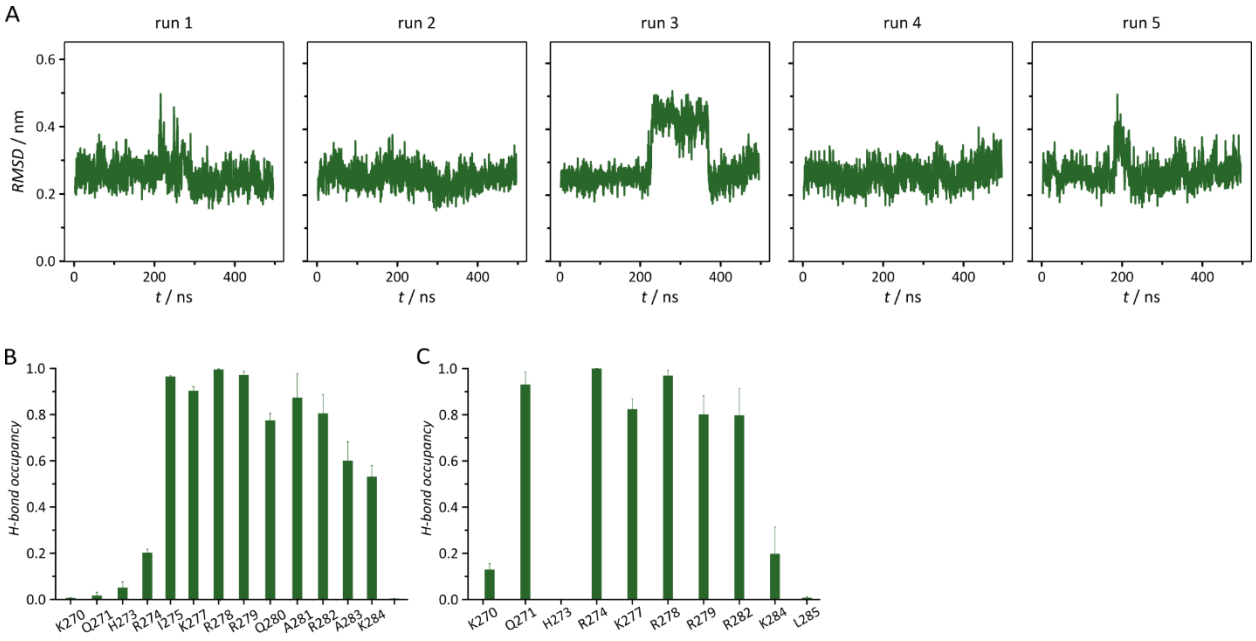

Figure S12 A) Time traces of the root mean square deviation (RMSD) of the Cα-atoms of the **5D^{N}** peptide with respect to the 5DN starting template over 500 ns. Five MD simulations were performed (run 1-5) started from the **5D^{N}** (PDB ID 8qu2). The RMSDs of the peptide are calculated after aligning the complex on chains B and C of the 7D^{N}_OUT crystal structure to capture the dynamics of the peptide. B) H-bond occupancy within the **5D^{N}** peptide. The occupancy of a specific hydrogen bond is averaged per system. A hydrogen bond is considered if the donor-H acceptor distance <0.25 nm and for the donor-H acceptor angle >120°. The error bars represent the standard error of the mean, calculated as the standard deviation of the average values over the independent runs. The calculation included all the atoms in the system using MDTraj.[2] C) H-bond occupancy between **5D^{N}** and the NF-YB/C heterodimer. Experimental conditions analog to B).

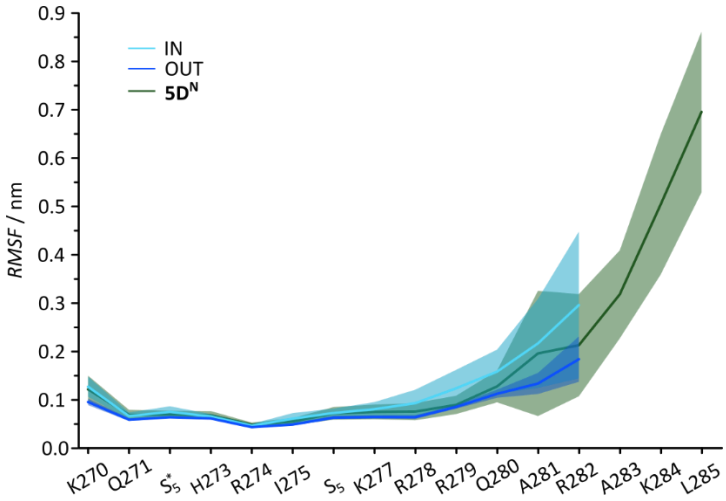

Figure S13 C_α root mean square fluctuations (RMSF) of **5D^N** in the 5DN set of simulations (dark green) as well as **7D^N** in the 7DN_IN (light blue) and 7DN_OUT (dark blue) sets of simulations. The RMSF was calculated for the whole complex, after removing overall translation and rotation in the complex, and shown for 5DN. The shaded area indicates the standard deviation of the values calculated over each independent simulation.

Supplementary Tables

m/z_{obs}	m/z_{calc}	ion
639.9	639.9	$[M+17H]^{17+}$
*662.3	662.2	$[M+17H]^{17+}$
679.8	679.9	$[M+16H]^{16+}$
*703.4	703.6	$[M+16H]^{16+}$
725.1	725.1	$[M+15H]^{15+}$
*750.4	750.4	$[M+15H]^{15+}$
776.8	776.9	$[M+14H]^{14+}$
*803.9	803.9	$[M+14H]^{14+}$
836.5	836.5	$[M+13H]^{13+}$
*865.7	865.7	$[M+13H]^{13+}$
906.1	906.2	$[M+12H]^{12+}$
*937.7	937.8	$[M+12H]^{12+}$
988.4	988.5	$[M+11H]^{11+}$
*1022.9	1022.9	$[M+11H]^{11+}$
1087.2	1087.2	$[M+10H]^{10+}$
*1125.1	1125.1	$[M+10H]^{10+}$
1207.9	1207.9	$[M+9H]^{9+}$
*1249.9	1250.0	$[M+9H]^{9+}$
1358.7	1358.8	$[M+8H]^{8+}$
*1406.0	1406.1	$[M+8H]^{8+}$
1552.7	1552.7	$[M+7H]^{7+}$
*1606.7	1606.9	$[M+7H]^{7+}$
1811.3	1811.3	$[M+6H]^{6+}$
*1874.4	1874.5	$[M+6H]^{6+}$

Table S1. Observed and expected masses of NF-YB and NF-YC (*) of the ESI-MS envelope (Figure S2).

References

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[2] R. T. McGibbon, K. A. Beauchamp, M. P. Harrigan, C. Klein, J. M. Swails, C. X. Hernández, C. R. Schwantes, L. P. Wang, T. J. Lane, V. S. Pande, Biophysical Journal 2015, 109(8), 1528-1532.