Electronic Supplementary Information

for

Site-selective incorporation of phosphorylated tyrosine into the p50 subunit of NF-κB and activation of its downstream gene CD40

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Table of Contents

Pages

EXPERIMENTAL SECTION

The plasmids containing p50 and p65 subunit genes of NF-κB were purchased from Synbio Technologies, Inc. Ni-NTA agarose was obtained from Qiagen Inc. DNA oligonucleotides were purchased from Integrated DNA Technologies. Ammonium persulfate, acrylamide, *N*, *N′*-methylene-bis-acrylamide, acetic acid, potassium glutamate, ammonium acetate, dithiothreitol, magnesium acetate, phospho(enol)pyruvate, *Escherichia coli* tRNA, isopropyl β-D-thiogalactopyranoside (IPTG), ATP, GTP, CTP, UTP, cAMP, amino acids, rifampicin, formamide, sodium pyruvate, glutamine, phorbol 12-myristate 13-acetate (PMA), calcium ionophore A23187, phenylmethanesulfonyl fluoride (PMSF), Tween-20, Nonidet P40 and Triton-X100 were obtained from Sigma-Aldrich. Tris and SDS were obtained from Bio-Rad Laboratories. [^{35}S]-methionine (1000 Ci/mmol, 10 μCi/μL) and [γ-^{32}P]-ATP (6 Ci/mmol, 10 μCi/μL) were purchased from PerkinElmer Inc. T4 RNA ligase and T4 polynucleotide kinase were purchased from New England Biolabs Inc. SuperSignal West pico Chemiluminescent substrate was obtained from Thermo Fisher Scientific. RPMI 1640 medium, penicillin, streptomycin, and fetal bovine serum were obtained from American Type Culture Collection (ATCC).

The human Jurkat leukemia T cell line was purchased from American Type Culture Collection (ATCC TIB-152). The anti-CD40 goat polyclonal antibody and monkey anti-goat-HRP antibody were obtained from R&D Systems.

Phosphorimager analysis was performed using an Amersham Biosciences Storm 820 equipped with ImageQuant version 5.2 software from Molecular Dynamics. UV spectral measurements were made using a Perkin-Elmer Lambda 20 UV/vis spectrometer. MS/MS analysis was

performed using Thermo Fusion Lumos mass spectrometer. The chemiluminescent immunoassay was scanned using an Azure Imaging System.

Misacylation of Suppressor tRNA-C_{OH} with Phosphotyrosyl-pdCpA. The yeast suppressor $tRNA^{Phe}_{CUA}$-C_{OH} was prepared as reported previously.[1] Activation of the suppressor $tRNA_{CUA}$-C_{OH} was carried out in 200 μL (total volume) of 100 mM Hepes buffer, pH 7.5, containing 2.0 mM ATP, 15 mM $MgCl_2$, 200 μg of suppressor tRNA-C_{OH}, 2.0 A_{260} units of *N*-pentenoyl, bis-*o*-nitrobenzyl protected phosphotyrosyl-pdCpA[2] (5-10 fold molar excess, Scheme 1), 15% DMSO and 400 units of T4 RNA ligase. After incubation at 37 °C for 1 h, the reaction mixture was quenched by the addition of 20 μL of 3 M NaOAc, pH 5.2, followed by 600 μL of ethanol. The reaction mixture was incubated at –20 °C for 30 min, then centrifuged at 15,000 × g at 4 °C for 30 min. The supernatant was carefully decanted and the tRNA pellet was washed with 200 μL of 70% ethanol, and dissolved in 200 μL of RNase free H_2O. The efficiency of ligation was estimated by 8% denaturing PAGE, pH 5.2.[3] The pentenoyl-protected aminoacyl-tRNA was deprotected by treatment with 5 mM aqueous I_2 for 10 min.[4] The reaction mixture was quenched by the addition of 20 μL of 3 M NaOAc, pH 5.2, followed by 600 μL of ethanol. The reaction mixture was incubated at –20 °C for 30 min, then centrifuged at 15,000 × g at 4 °C for 30 min. The supernatant was decanted carefully and the tRNA pellet was washed with 200 μL of 70% ethanol, and dissolved in 60 μL of RNase free H_2O. After treatment with high intensity Hg-Xe light, the aminoacylated suppressor tRNA was used in *in vitro* suppression experiments.

Site-directed Mutagenesis to Obtain the Mutant p50 Gene of NF-κB having TAG Codons at Protein Positions 44, 60, 82 and 90.[5] The wild-type pET16b-Strep-p50 plasmid was purchased from Synbio Technologies. The primers for protein TAG modifications were: 5′- CCA ACA GCA GAT GGC CCA TAG CTT CAA ATT TTA GAG CAA CC-3′ (position 44); 5′-

CGC GGT TTT CGT TTC CGT TAG GTA TGT GAA GGC CCA TCT CAT G-3′ (position 60); 5′- CT AGT GAA AAG AAC AAG AAG TCT TAG CCT CAG GTC AAA ATC TGC AAC-′ (position 82); 5′- CT CAG GTC AAA ATC TGC AAC TAG GTG GGT CCA GCA AAG GTT ATT G-3′ (position 90).

A reaction mixture (25 μL total volume for each primer) contained 200 pmol of primer, 1 mM ATP, 30 units of T4 polynucleotide kinase and 2.5 μL of PNK buffer was incubated at 37 °C for 60 min.

The PCR reaction was carried out in a 50-μL reaction mixture containing 200 ng of template plasmid DNA, 16 pmol of the appropriate primer, 10 nmol of dNTPs, 5 units of *Pfu* DNA polymerase, 2.5 μL of *Pfu* buffer, 40 units of *Taq* DNA ligase and 2.5 μL of *Taq* buffer. An aliquot of 30 μL of mineral oil was added on the top of the reaction mixture. The thermal cycle was programmed as follows: pre-incubated at 65 °C for 5 min, allowing the ligase to repair any nicks in the template DNA; initial denaturation at 95 °C for 2 min; 18 cycles at 95 °C for 1 min, 51 °C for 1 min, 65 °C for 15 min, post-incubation at 75 °C for 7 min. The restriction enzyme *Dpn*I (4 μL) was added to the reaction mixture and incubated at 37 °C for 60 min to eliminate the methylated and hemi-methylated wild-type DNA template. Then the sample was denatured at 95 °C for 30 sec, followed by 2 cycles at 95 °C for 30 sec, 51 °C for 1 min and 70 °C for 7 min. Ten μL of the final sample was transformed into 100 μL of *E. coli* competent cells DH5α. The product was plated on an ampicillin plate, and incubated at 37 °C overnight.

***In Vitro* Translation of Mutant NF-κB p50 Subunit Genes (Modified at Protein Positions 44, 60, 82 and 90) and wild-type p50 and p65.**[2] The *in vitro* expression mixture (100 μL total volume) containing 10 μg of plasmid DNA with mutant NF-κB p50 genes (modified at protein positions 44, 60, 82 and 90), 40 μL of premix (35 mM Tris-acetate, pH 7.0, 190 mM potassium

glutamate, 30 mM ammonium acetate, 2.0 mM dithiothreitol, 25 mM magnesium acetate, 20 mM phospho(enol)pyruvate, 0.8 mg/mL of *E. coli* tRNA, 0.8 mM IPTG, 20 mM ATP and GTP, 5 mM CTP and UTP and 4 mM cAMP), 100 μM of each of the 20 amino acids, 30 μCi of [^{35}S]-L-methionine, 10 μg/μL rifampicin, 30 μL of S-30 extract from *E. coli* strain BL-21 (DE-3)-pUCrrnBmut cells and 30-50 μg of phosphotyrosyl-tRNA$_{CUA}$ from which the protecting groups had been removed. The reaction mixture was incubated at 30 ^{o}C for 45 min to maximize the yields. Plasmid DNA containing the wild-type p50 gene was used as a positive control, and an abbreviated tRNA (tRNA-C$_{OH}$) lacking any amino acid was used as the negative control.

A plasmid DNA containing the wild-type p65 gene was used to prepare the p65 protein. An aliquot containing 2 μL of reaction mixture was removed, treated with 2 μL of loading buffer (250 mM Tris-HCl, pH 6.8, containing 10% glycerol, 1% SDS, 0.01% bromophenol blue and 80 mM DTT) and heated at 90 ^{o}C for 2 min. The sample was analyzed by 15% SDS-PAGE at 100 V for 2 h.

Purification of the p50 Protein Subunit of NF-κB with Strep-tactin Sepharose. The crude reaction mixture (100 μL) was loaded on a 30 μL Strep-tactin Sepharose column.[6] The column was washed with 500 μL of 100 mM Tris-HCl, pH 8.3 (washing buffer) and eluted with 100 μL of 100 mM Tris-HCl, pH 8.3, supplemented by 2.5 mM desthiobiotin (elution buffer) three times. An aliquot containing 2 μL of each portion was removed, treated with 2 μL of loading buffer (250 mM Tris-HCl, pH 6.8, containing 10% glycerol, 1% SDS, 0.01% bromophenol blue and 80 mM DTT) and heated at 90 ^{o}C for 2 min. The sample was analyzed by 15% SDS-PAGE at 100 V for 2 h.

For the larger scale crude reaction mixture without ^{35}S-methionine, a one mL reaction mixture was loaded on a 300 μL Strep-tactin Sepharose column. The column was washed with 2500 μL

of 100 mM Tris-HCl, pH 8.3 (washing buffer) and eluted with 300 μL of 100 mM Tris-HCl, pH 8.3, supplemented by 2.5 mM desthiobiotin (elution buffer) three times. The three elution portions were combined together and concentrated using an Amicon® centrifugal filter (cut off 10 kDa). The obtained proteins were quantified with 15% SDS-PAGE using BSA as a control.

Purification of the p65 subunit with Ni-NTA in the native condition.[7-10] The *in vitro* expression mixture for p65 protein (1000 μL) was diluted with 3000 μL of 50 mM Tris-HCl, pH 8.0, and 300 μL of Ni-NTA beads were added. After incubation at 4 ºC for 5 min, the beads were washed with 2000 μL of 50 mM Tris-HCl, pH 8.0, containing 300 mM NaCl and 20 mM imidazole. Finally, the p65 protein was eluted three times with 300 μL of 50 mM Tris-HCl, pH 8.0, containing 300 mM NaCl and 150 mM imidazole. The p65 protein were concentrated using an Amicon® centrifugal filter (cut off 10 kDa). Aliquots of each fraction were analyzed by 15% SDS-PAGE.

"In Gel" Trypsin Digestion.[11] Samples to be digested in the gel were run in 2-4 lanes of a 15% SDS-polyacrylamide gel, stained with Coomassie R-250 and destained until the background was clear. That area of the gel having the p50 was cut from the gel and washed with 0.1 M ammonium bicarbonate (1 h, room temperature). The solution was discarded and 0.1-0.2 mL of 0.1 M ammonium bicarbonate and 10-30 μL of 0.045 mM DTT were added. Gel pieces were incubated at 60 ºC for 30 min, cooled to room temperature and incubated at room temperature for 30 min in dark after the addition of 10-30 μL of 0.1 M iodoacetamide. Gel pieces were washed in 50% acetonitrile/0.1 M ammonium bicarbonate until they became colorless. After discarding the solution, the gel pieces were incubated in 0.1-0.2 mL of acetonitrile (10-20 min at room temperature) and, after removal of solvent, were re-swelled in 50-100 μL of 25 mM ammonium

bicarbonate containing 0.02 μg/μL of trypsin. After incubation at 37 ºC for 4 h, the supernatant was removed to a new tube and the peptides were extracted with 60% acetonitrile-0.1% TFA (20 min at room temperature). The combined fractions were dried and reconstituted in a minimum amount of 60% acetonitrile-0.1% CF_3COOH. The peptides were analyzed using a Thermo Fusion Lumos mass spectrometer with an ESI ion source.

Mass spectra for the full-length p50s. Each phosphorylated p50 protein (300 μL reaction mixture) was purifed with 50 μL Strep-tactin Sepharose beads. The beads were washed with 500 μL of 100 mM Tris-HCl, pH 8.3 (washing buffer) followed by 100 μL pure water. Then the protein was eluted with 100 μL of 0.1% CF_3COOH/H_2O and concentrated using a speed vacuum concentrator. Finally, the proteins were analyzed using Agilent 6530 Quadrupole TOF LC-MS with an ESI ion source.

Formation of p50/p65 complexes. The ^{35}S-methionine labeled wild-type and mutant p50 subunits (100 ng) were mixed with 100 ng ^{35}S-methionine labeled wild-type p65 subunit in 10 μL 50 mM Tris-HCl, pH 8.0, containing 300 mM NaCl. The mixtures were incubated at room temperature for 10 min and analyzed using a 5% native PAGE gel at 100 V for 1 h. The gel was quantified using a phosphorimager.

***In Vivo* Translation in Jurkat Cells.** Jurkat cells (20 mL) stimulated with PMA and calcium ionophore A23187 were harvested by centrifugation at 500 × g at room temperature for 5 min and washed once with 5 mL of cold PBS. The cell pellets were resuspended in 200 μL of 20 mM Hepes, pH 7.9, containing 50 mM KCl, 0.5 mM DTT, 0.2 mM EDTA, 0.5 mM PMSF (phenylmethylsulfonyl fluoride), and 0.17 mM Triton-X100 and incubated at 4 °C for 10 min. The pretreated Jurkat cells were then stored at −80 °C.

The *in vivo* expression mixture (10 μL total volume) contained 30 μCi of [^{35}S]-L-methionine, 5 μL of pretreated Jurkat cells, 8 ng of NF-κB and 3 μL of RPMI-1640 cell culture medium. The reaction mixture was incubated at 37 °C for 1 h. The reaction mixture was treated with 5 μL of loading buffer (250 mM Tris-HCl, pH 6.8, containing 10% glycerol, 1% SDS, 0.01% bromophenol blue and 80 mM DTT) and heated at 90 °C for 2 min. The samples were analyzed by 15% SDS-PAGE at 100 V for 2 h.

Identification of transcription of 96 human NF-κB signaling pathway genes in Jurkat cells. Jurkat cells (20 mL) stimulated with 0.3 μM PMA and 1.0 μM calcium ionophore A23187 were harvested by centrifugation at 500 × g at room temperature for 5 min and washed once with 5 mL of cold PBS. The cell pellets were resuspended in 200 μL of 20 mM Hepes, pH 7.9, containing 50 mM KCl, 0.5 mM DTT, 0.2 mM EDTA, 0.5 mM PMSF, and 0.17 mM Triton-X100, and then incubated at 4 °C for 10 min. Then the pretreated Jurkat cells were stored at −80 °C.

The *in vivo* expression mixture (60 μL total volume) contained 25 μL of pretreated Jurkat cells, 100 ng of NF-κB, 5 mM GTP and 25 μL of RPMI-1640 cell culture medium. The reaction mixture was incubated at 30 °C for 1 h. The total RNAs were extracted with the miRNeasy® Mini Kit (Qiagen) following the standard protocol in the handbook. (.https://www.qiagen.com/us/resources/resourcedetail?id=14e7cf6e-521a-4cf7-8cbc-bf9f6fa33e24&lang=en). After a reverse transcription using a RT2 First Strand Synthesis Kit (Qiagen), the RNAs were transduced into corresponding DNAs following the standard protocol (https://www.qiagen.com/us/resources/resourcedetail?id=f4b13eaa-884f-4357-abe6-1a5f9469bc32&lang=en). The generated DNAs were added into a 384-wells plate for real-time

PCR to identify the overexpression of the targets genes. The obtained data were analyzed using Qiagen online tool "RT² Profiler PCR Data Analysis" (https://dataanalysis2.qiagen.com/pcr).

Western Blot Analysis Blot Analysis of Human CD40. The protein samples were loaded on a 15% SDS–PAGE gel. The gel was run at 100 V for 2 h. The proteins were transferred to a nitrocellulose membrane in Novex Tris-glycine transfer buffer with 10% methanol at 60 V for 1 h. The nitrocellulose membrane was blocked with blocking buffer containing 5% BSA at room temperature for 1 h. After being washed three times with TBS buffer (50 mM Tris-HCl, pH 7.5, with 150 mM NaCl) containing 0.05% Tween 20, the nitrocellulose membrane was incubated in 10 mL TBS buffer containing 0.05% Tween 20 and 10 μL of goat anti-human CD40 Ab (R&D Systems) at room temperature for 1 h. After being washed three times with TBS buffer containing 0.05% Tween 20, the nitrocellulose membrane was incubated in 10 mL TBS buffer containing 0.05% Tween 20 and 5 μL of monkey anti-goat-HRP Ab (R&D Systems) at room temperature for 1 h. After being washed three times with TBS buffer containing 0.05% Tween 20, the nitrocellulose membrane was treated with SuperSignal West Pico Chemiluminescent Substrate at room temperature for 2 min, and the signals were scanned using an Azure Imaging System.

5′-^{32}P-Labeling of DNA.[2] A reaction mixture (50 μL total volume) contained 400 pmol of primer (5′-GATCCAAGGGACTTTCCATG-3′, 5′-GCATGGGAATTTCCTACG-3′, 5′-GCATGGGAACTTCCTACG-3′ or 5′-GCATGGGAAACTCCTACG-3′), 6 μL of γ-^{32}P-ATP, 30 units of T4 polynucleotide kinase (PNK) and 5 μL of PNK buffer was incubated at 37 °C for 60 min. The excess γ-^{32}P-ATP was removed using a G-25 column. To each ^{32}P-labeled DNA primer was added a complementary DNA strand (400 pmol). The reaction mixture was heated to 90 °C

for 5 min and cooled to room temperature over a period of 1 h to obtain the double-stranded DNA.

Preparation of Jurkat Cytosolic Fraction.[2] Jurkat cells were cultured at 37 °C in a 5% CO_2 atmosphere and grown in Gibco RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS) containing 50 units/mL of penicillin, and 50 μg/mL of streptomycin. When the cells grew to a density of 5×10^5 cells/mL, they were stimulated with 0.3 μM phorbol 12-myristate 13-acetate (PMA) and 1.0 μM calcium ionophore A23187 at 37 °C for 1 h. The stimulated Jurkat cells (20 mL) were harvested by centrifugation at 500 × g at room temperature for 5 min and washed once with 5 mL of cold PBS. The cell pellets were resuspended in 200 μL of 10 mM Hepes, pH 7.9, containing 10 mM KCl, 1.5 mM $MgCl_2$, 0.5 mM DTT and 0.1% Nonidet P40 and incubated at 4 °C for 10 min. After centrifugation at 1,000 × g at room temperature for 2 min, the supernatant (cytosolic fractions) were collected in new tubes and incubated at 37 °C for 6 h. This Jurkat cytosolic fraction was stored at −80 °C.

NF-κB and DNA Binding Assay. The reaction mixture (10 μL total volume) contained 0 – 8 ng of purified unlabeled (i.e., no ^{35}S-methionine) wild-type or mutant NF-κB containing a phosphorylated tyrosine at position 44, 60 or 82, 8 pmol of double-stranded 5′-^{32}P-labeled DNA probe, 5 mM of GTP and 3 μL of cytoplasmic extract. Each reaction mixture was incubated at 4 °C for 10 min. The reactions were analyzed by 5% native PAGE at 100 V for 80 min.

FIGURES

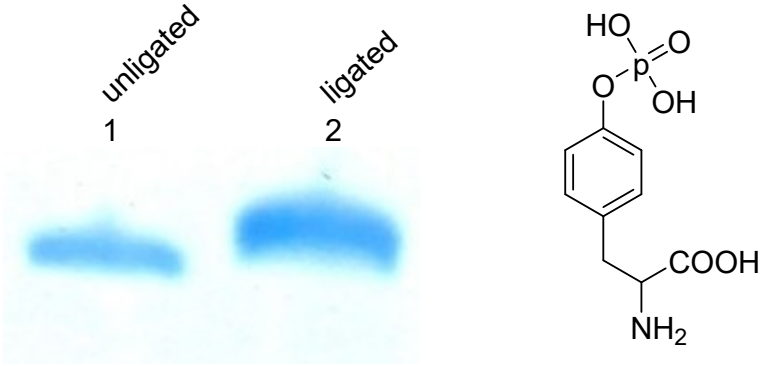

Fig. S1. Bacteriophage T4-mediated ligation of a suppressor tRNA-C_{OH} to phosphotyrosyl-pdCpA (fully protected, cf Scheme 1). The analysis of the extent of ligation was carried out by 8% denaturing PAGE, pH 5.2.

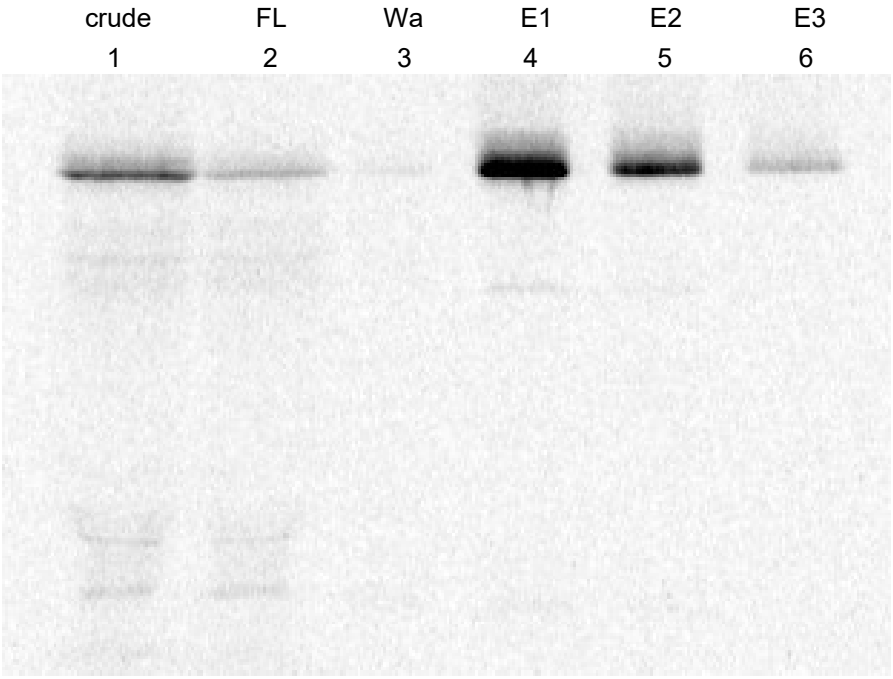

Fig. S2. Purification of the human NF-κB p50 protein having a Strep tag at its C-terminus using a Strep-tactin column. The fractions were analyzed by 15% SDS-PAGE, and visualized using the ^{35}S-methionine incorporated into protein. Lane 1, crude sample; lane 2, flow through fraction; lane 3, washing fraction; lane 4, the first elution; lane 5, the second elution; lane 6, the third elution.

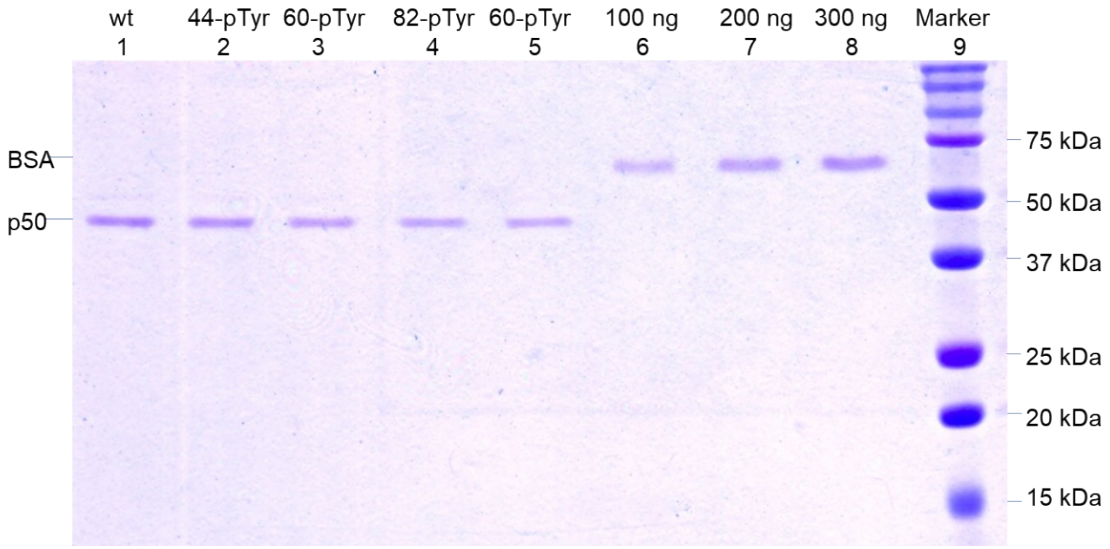

Fig. S3. Quantification of wild-type and phosphorylated p50 proteins. *In vitro* expression of p50 reaction mixtures without ^{35}S-methionine were purified using a Step-tactin column followed by concentration with centrifugal filters. Each purified p50 protein (5 μL) was loaded on a 15% SDS-PAGE gel, and stained with Coomassie blue. The concentration of p50 was determined by comparation to the concentration of BSA.

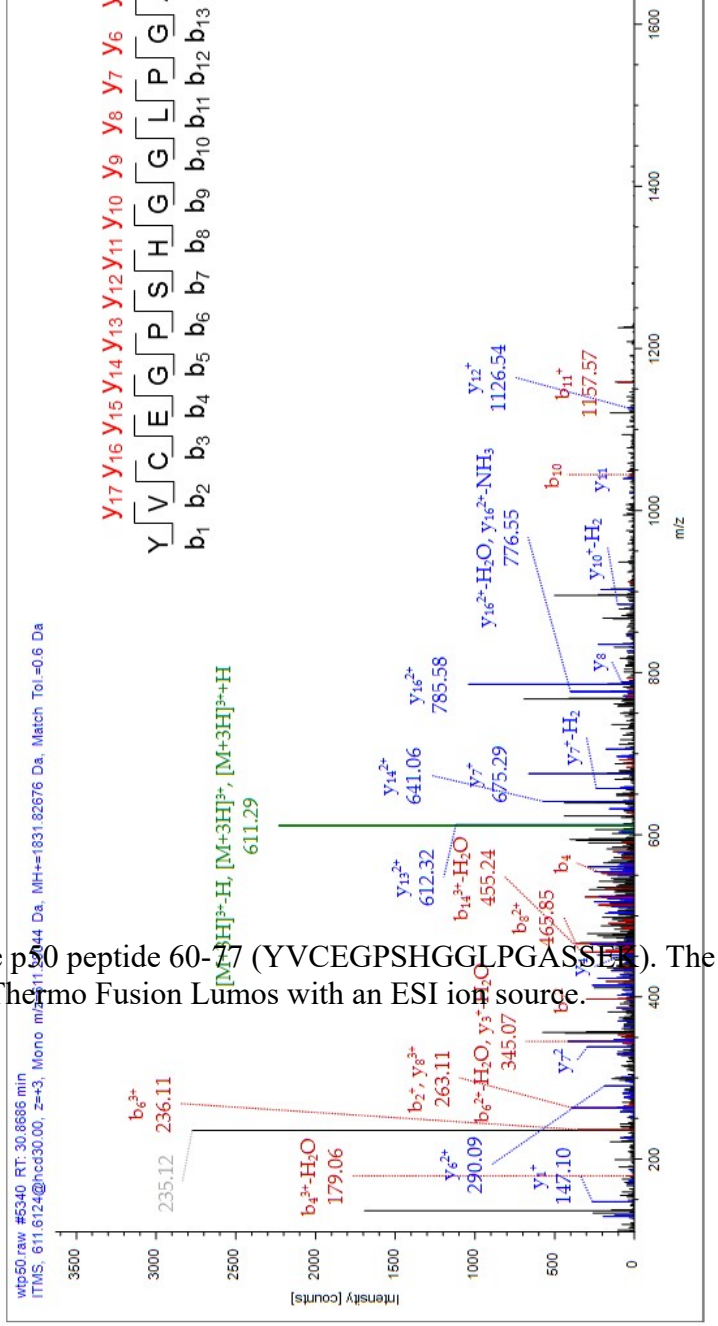

Figure S4. MS/MS of wild-type p53 peptide 60-77 (YVCEGPSHGGLPGASSEK). The peptides were analyzed using a Thermo Fusion Lumos with an ESI ion source.

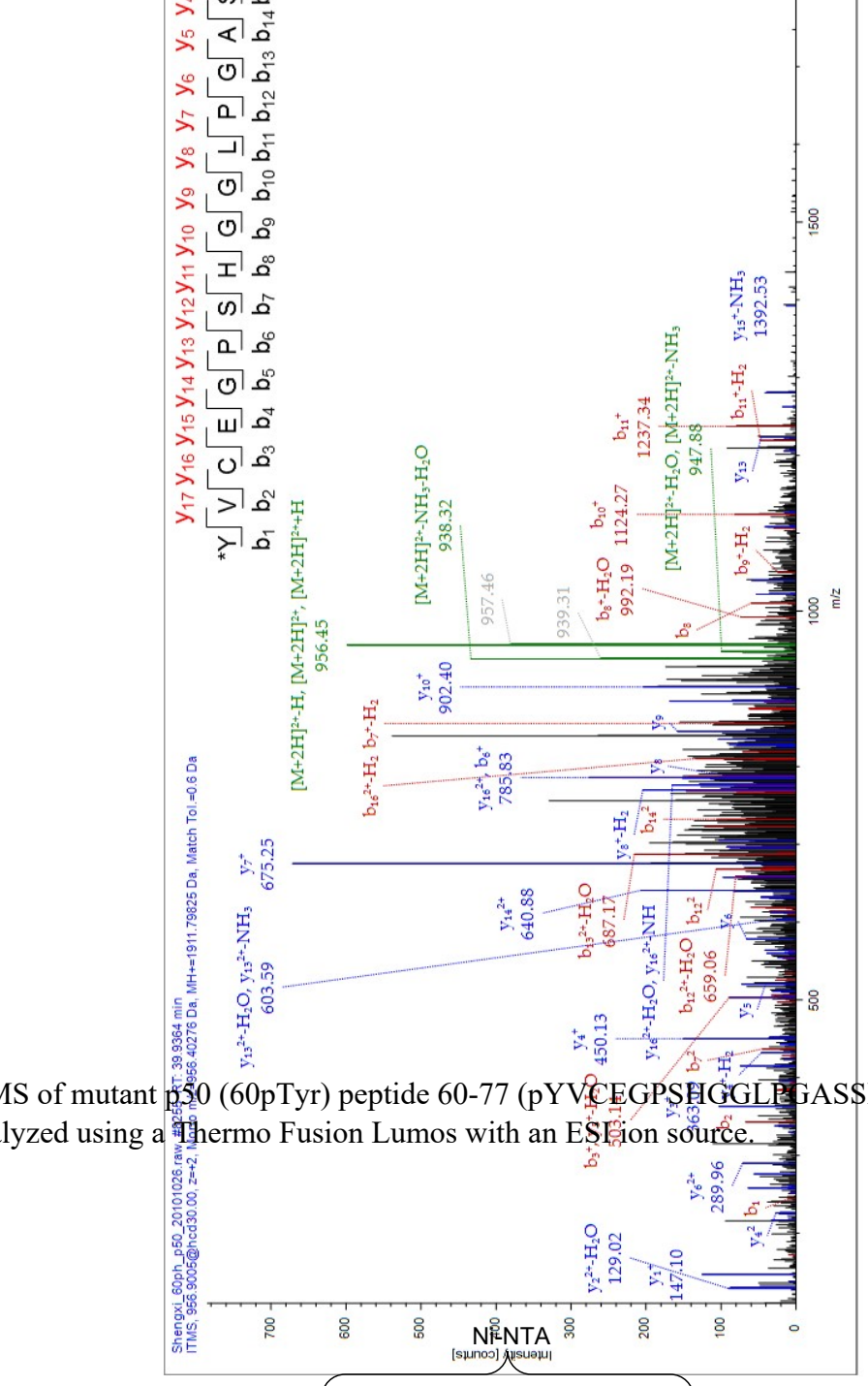

Figure S5. MS/MS of mutant p50 (60pTyr) peptide 60-77 (pYVCEGPSHGGLPGASSEK). The peptides were analyzed using a Thermo Fusion Lumos with an ESI ion source.

Fig. S6. Preparation and purification of the human NF-κB p65 protein prepared by translation *in vitro* with a His-tag at its C-terminus by chromatography on a Ni-NTA column. Elution was carried out with increasing concentrations of imidazole. The fractions were analyzed by 15% SDS-PAGE, and visualized using the ^{35}S-methionine incorporated into protein. Lane 1, wild-type p50 protein as the control; lane 2, *in vitro* expression of p65 protein in the presence of 0.5 μg of plasmid DNA in each reaction (10 μL in total) ; lane 3, *in vitro* expression of p65 protein in the presence of 1.0 μg of plasmid DNA in each reaction (10 μL in total); lane 4, flow through portion from the Ni-NTA column; lanes 5 – 8, elution portion from the Ni-NTA column in the presence of 10 – 150 mM imidazole.

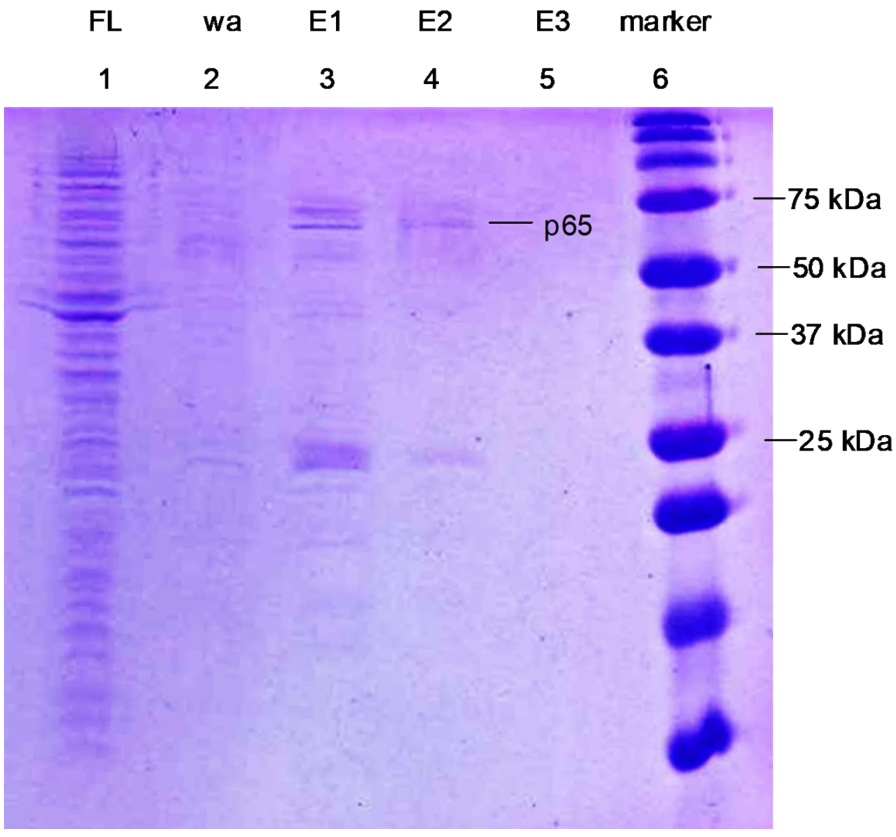

Figure S7. *In vitro* expression of p65 subunits of NF-κB in a 1000 μL reaction mixture without ^{35}S-methionine. The p65 protein was purified with Ni-NTA column. The fractions were analyzed by 15% SDS-PAGE, and visualized using Coomassie blue staining. Lane 1, flow through portion from the Ni-NTA column; lane 2, washing portion from the Ni-NTA column in the presence of 10 mM imidazole; lanes 3 – 5, elution portion from the Ni-NTA column in the presence of 150 mM imidazole; lane 6, protein marker.

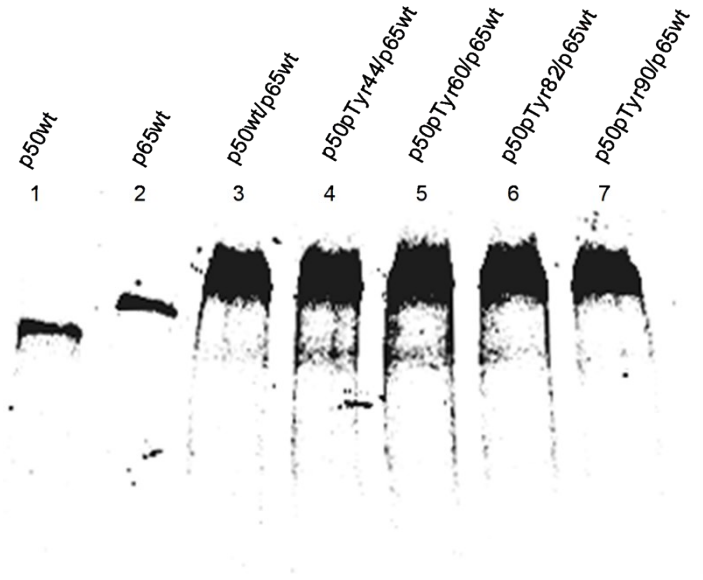

Fig. S8. Analysis of the formation of NF-κB (p50/p65) heterodimer following admixture of radiolabeled p50 and p65 subunits. ^{35}S-methionine labeled wild-type and modified p50 subunits, and ^{35}S-methionine labeled wild-type p65 subunit were prepared by *in vitro* translation. Admixture of p65 with individual p50 subunits was carried out in 50 mM Tris-HCl, pH 8.0, containing 300 mM NaCl. The mixtures were incubated at room temperature for 10 min and analyzed on a 5% native polyacrylamide gel. Lane 1, 50 ng of wild-type p50; Lane 2, 50 ng of wild-type p65; the remaining lanes contained 100 ng of wild-type p65 in addition to 100 ng of wild-type p50 (lane 3); 100 ng of p50 phosphorylated on Tyr44 (lane 4); 100 ng of p50 phosphorylated on Tyr60 (lane 5); 100 ng of p50 phosphorylated on Tyr82 (lane 6); 100 ng of p50 phosphorylated on Tyr90 (lane 7).

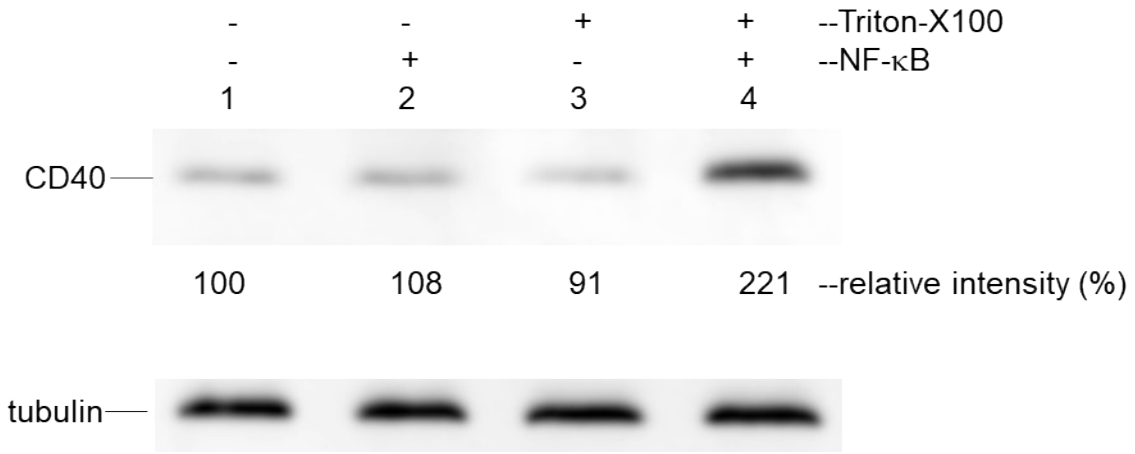

Figure S9. Western blot analysis of *in vivo* expression of CD40 in Jurkat cells in the presence of Triton-X100. The NF-κB containing phosphorylated tyrosine at position 60 of p50 subunit was added to the reaction with and without pre-treatment by Triton-X100. Lane 1, Jurkat cells without added NF-κB and Triton-X100; lane 2, Jurkat cells with added NF-κB without pre-treatment by Triton-X100; lane 3, Jurkat cells pre-treated by Triton-X100 without adding NF-κB; lane 4, Jurkat cells pre-treated by Triton-X100 followed by adding NF-κB.

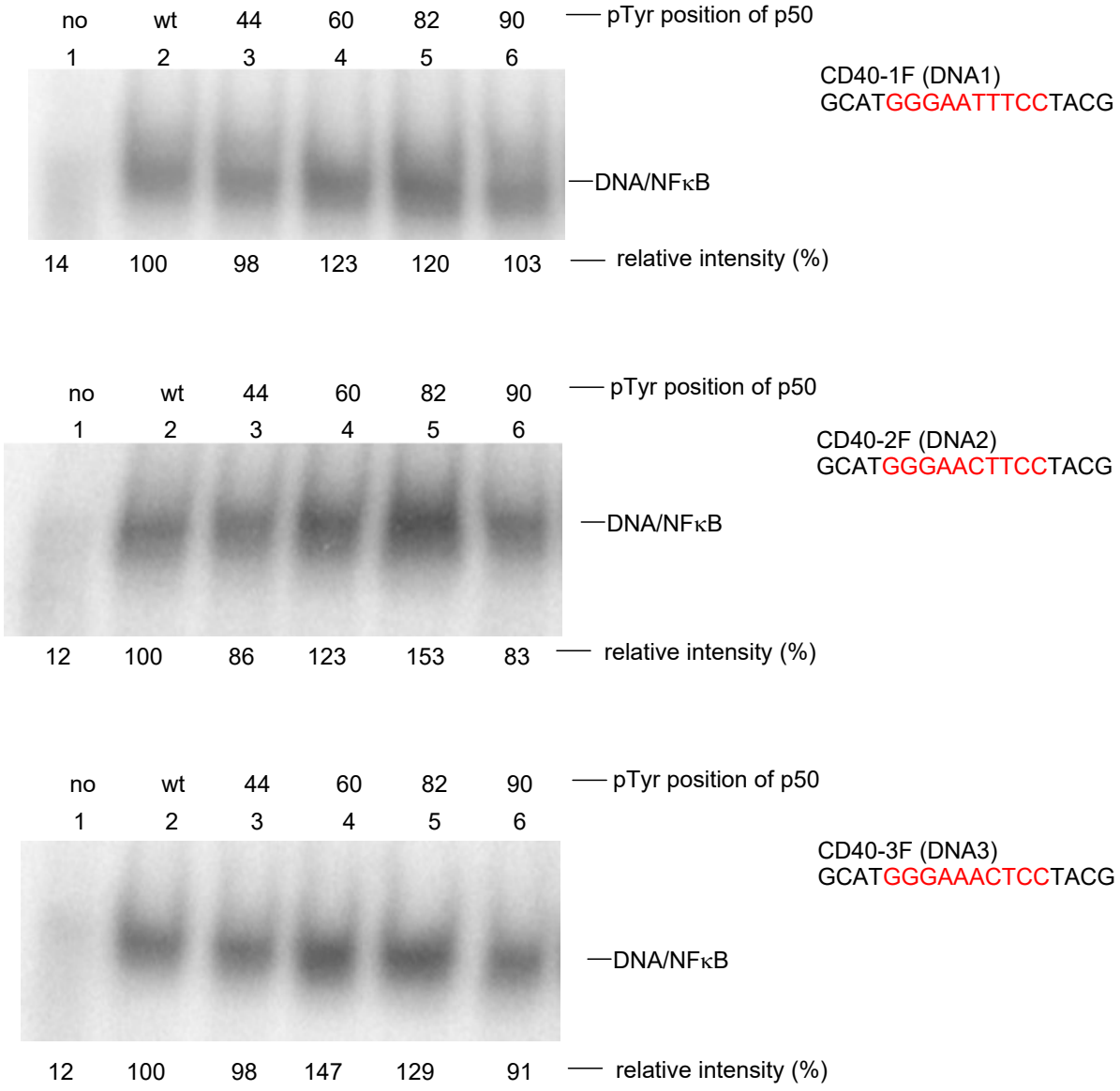

Fig. S10. CD40 promoter DNA binding by (modified) NF-κBs using the three reported sequences (DNA1, DNA2 and DNA3). All lanes contained 5′-^{32}P-end labeled CD40 promoter DNA, 5 mM GTP and 3 mL of cytoplasmic cell extract from activated Jurkat cells. Lanes 1 and 2 employed no or wild-type NF-κB; lanes 3 – 6 contained NF-κB (p50/p65) prepared *in vitro* as separate subunits in which p50 was phosphorylated stoichiometrically at positions 44, 60, 82 or 90, respectively. The relative intensities were calculated with ImageQuant version 5.2 software from Molecular Dynamics based on the ^{32}P signal. The value of the band for wild-type NF-κB was defined as 100%. The statistical significance was calculated using the Student's t-test. In upper panel: wild-type vs pTyr60-p50, $p < 0.05$; wild-type vs pTyr82-p50, p

< 0.05. In middle panel: wild-type vs pTyr60-p50, $p < 0.05$; wild-type vs pTyr82-p50, $p < 0.05$. In lower panel: wild-type vs pTyr60-p50, $p < 0.05$; wild-type vs pTyr82-p50, $p < 0.05$.

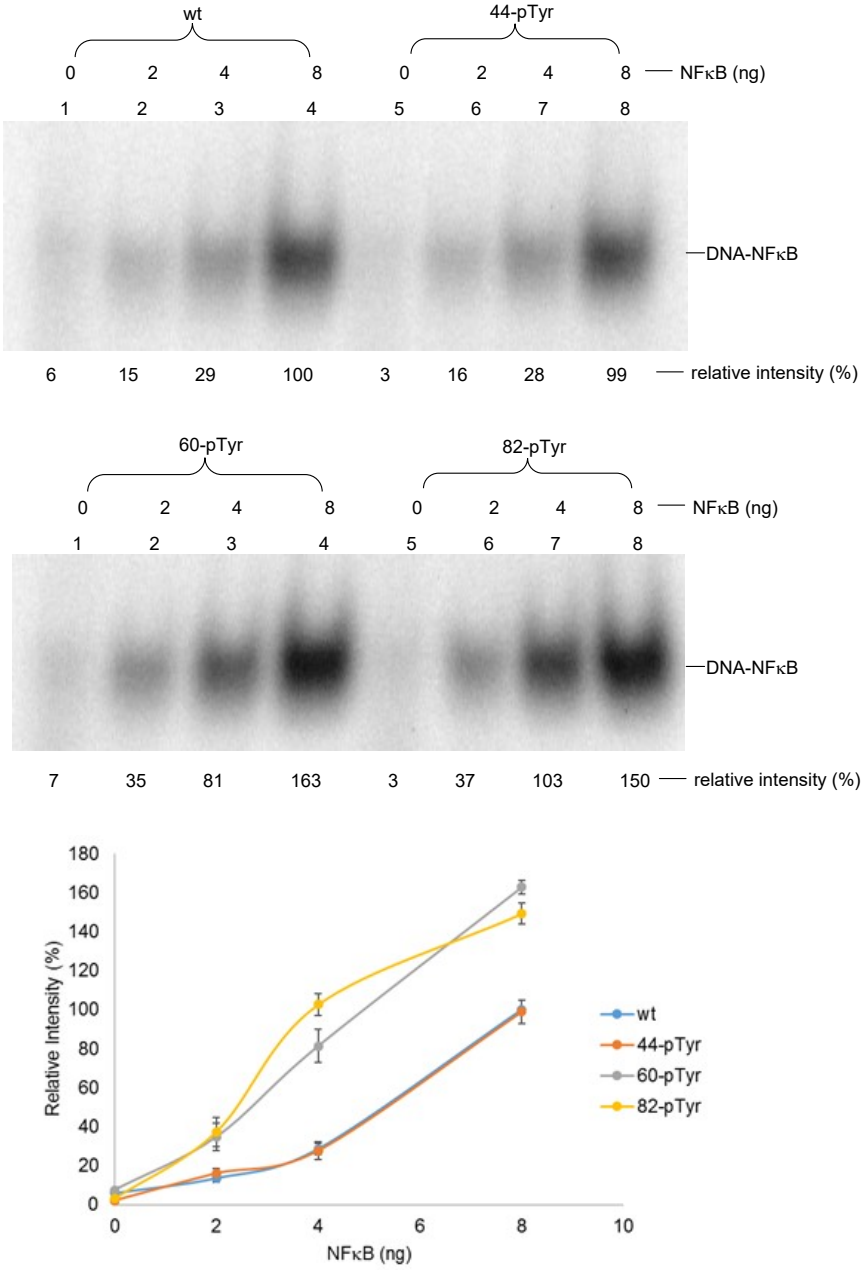

Figure S11. Concentration-dependent binding of wild-type NF-κB and three modified NF-κBs containing a single phosphorylated Tyr moiety to a CD40 promoter (DNA2) flanked by two tetranucleotides (CD40-2F, GCATGGGAACTTCCTACG). The underlined nucleotides are from the promoter sequence. The reaction was carried out as in the legend to Figure 3. The reaction mixture (10 μL total volume) contained 0 – 8 ng of purified NF-κB, 8 pmol of double-stranded 5′-^{32}P-labeled DNA probe, 5 mM of GTP and 3

μL of cytoplasmic extract. The incubation was carried out at 4 °C for 10 min. The samples were analyzed by 5% native PAGE at 100 V for 1 h. The relative intensities were calculated with ImageQuant version 5.2 software from Molecular Dynamics based on the ^{32}P signal. The band in the sample adding wild-type NF-κB was defined as 100%.

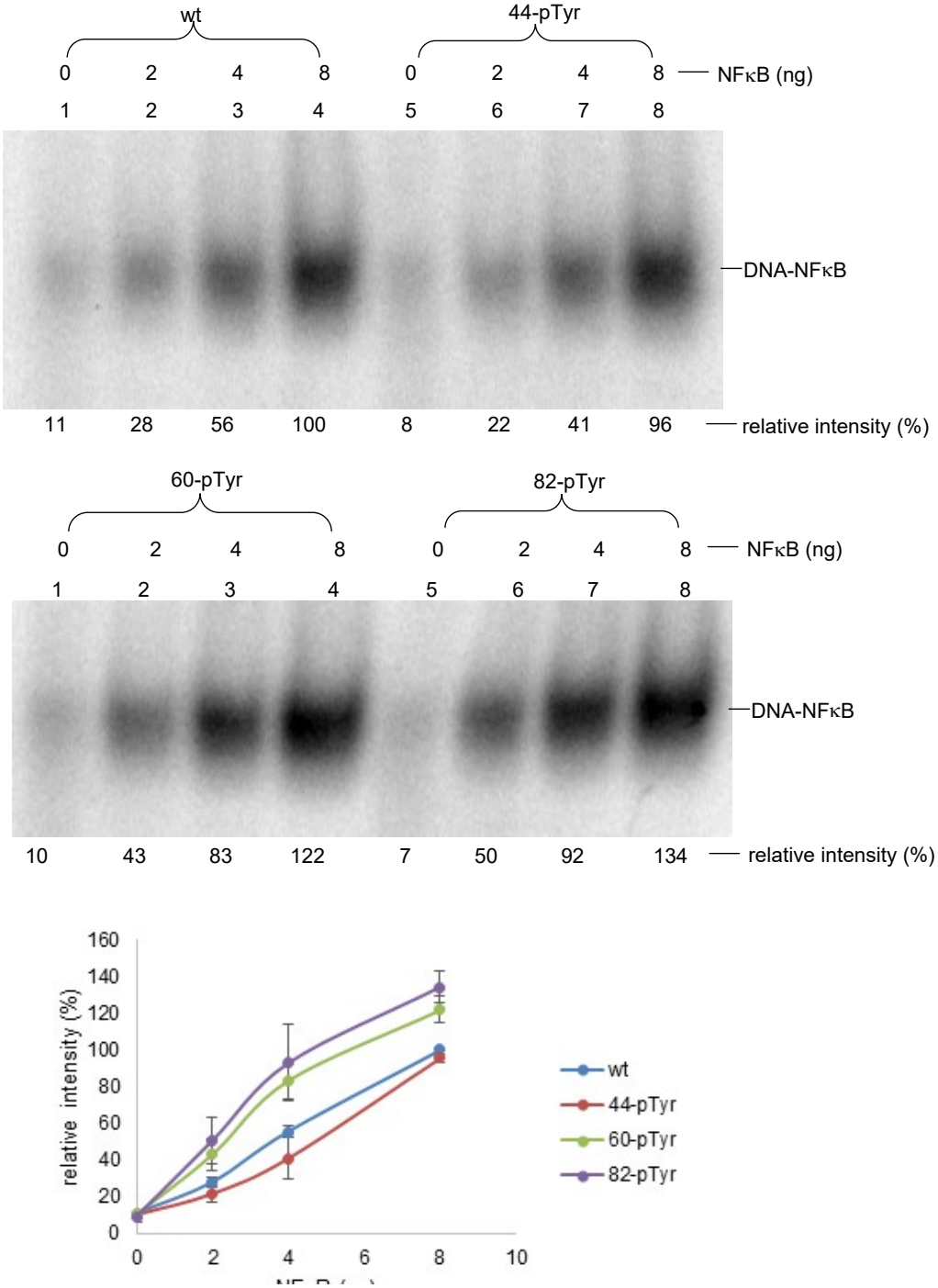

Figure S12. Concentration-dependent binding of wild-type NF-κB and three modified NF-κBs containing a single phosphorylated Tyr moiety to a CD40 promoter (DNA3) flanked by two tetranucleotides (CD40-3F, GCATGGGAAACTCCTACG). The underlined nucleotides are from the promoter sequence. The reaction was carried out as in the legend to Figure 3. The reaction mixture (10 μL total volume) contained

0 – 8 ng of purified NF-κB, 8 pmol of double-stranded 5′-^{32}P-labeled DNA probe, 5 mM of GTP and 3 μL of cytoplasmic extract. The incubation was carried out at 4 ^{o}C for 10 min. The samples were analyzed by 5% native PAGE at 100 V for 1 h. The relative intensities were calculated with ImageQuant version 5.2 software from Molecular Dynamics based on the ^{32}P signal. The band in the sample adding wild-type NF-κB was defined as 100%.

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