Comprehensive Experimental and Computational Analysis of Binding Energy Hot Spots at the NF-κB Essential Modulator/ IKKβ Protein-Protein Interface

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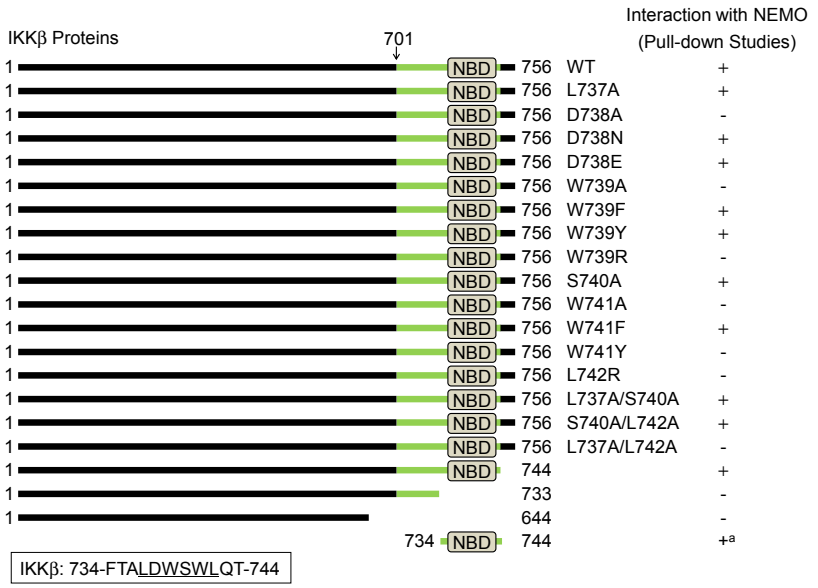
A



B

IKK Proteins	IC_{50} or K_D (μM)
680 NBD 756 β-77 mer	0.0034[a], 1[b]
701 NBD 745 β-45mer	0.047[c], (0.015 - 0.030)[d], 0.0762[e], 0.015[f]
701 NBD 745 β-45mer Mut	>1[d]
701 NBD 745 α/β-45 mer	0.036[c]
718 NBD 745 α/β-28 mer	7[g]
727 NBD 745 α/β-19 mer	20[g]
735 NBD 745 α-11 mer	39[g]
735 NBD 745 β-11 mer	3600[a], 35[b], >10[d], >100[e]
735 NBD 745 β-Peptide	20[h]

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β-45mer:      PAKKSEELVAEAHNLCTLLENAIQDTVREQDQSFTALDWSWLQTE
β-45mer Mut:  PAKKSEELVAEAHNLCTLLENAIQDTVREQDQSFTALDASALQTE
α/β-45mer:    PAKKSEELVAEAHNLCTLLENAIQDTVREQGNSMMNLDWSWLTE-
α/β-28 mer:                            TVREQGNSMMNLDWSWLTE-
α/β-19 mer:                  TLLENAIQDTVREQGNSMMNLDWSWLTE-
α-11 mer:                                    MMNLDWSWLTE-
β-11 mer:                                     TALDWSWLQTE
β-Peptide:                           Propionyl-TALDWSWLQTE-OH
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Figure S1. Literature data on NEMO binding properties of IKKβ point mutants and peptide fragments. (A) Pull-down studies with wild-type (WT) or IKKβ deletion and point mutations were performed with either full-length NEMO-FLAG or GST-NEMO. [1] [a]An 11-mer IKKβ peptide spanning the NBD dose-dependently blocked interaction of both IKKβ and IKKβ with full-length GST-NEMO in pull-down studies, and also disrupted preformed NEMO/IKK interactions.[2] (B) Literature results of measured binding affinities for IKK truncation proteins. [a]Average K_D determined from SPR binding studies (Biacore) in which various concentrations of IKKβ were reacted with surface immobilized biotinylated NEMO (38-196).[4] [b]K_D determined by isothermal titration calorimetry. IKKβ peptides were titrated into solutions containing His-tagged NEMO (38-196).[4] [c]IC_{50} determined when competing for binding of a biotinylated IKKα/β hybrid 45-mer peptide to GST-NEMO (1-196) as determined from concentration dependent FRET studies.[3] [d]IC_{50} determined when competing for binding of a TAMRA-IKKβ 45-mer peptide to NEMO (2-200) as determined from fluorescence polarization studies.[5] [e]K_D determined from SPR binding studies (Biacore) of IKKβ peptides and biotinylated NEMO (2-200).[5] [f]Average K_D determined when competing for binding of a FITC labeled IKKβ peptide to full length NEMO (residues 2-419) in concentration dependent fluorescence anisotropy studies.[6] [g]IC_{50} determined when competing for binding of a biotinylated IKKα/β chimeric 44-mer peptide to GST-NEMO (2-200) as determined from concentration dependent FRET studies.[5] Authors also reported IC_{50} values measured by Alphascreen.[5] [h]IC_{50} determined when competing for binding of a biotinylated IKKβ 11-mer peptide to GST-NEMO (1-196) as determined from concentration dependent FRET studies.[3] [h]IC_{50} determined when competing for binding of a 11-mer IKKβ peptide to His-NEMO-FLAG as determined from concentration dependent pull-down studies.[7] The identified six residue NEMO binding domain (NBD) conserved in IKKα and IKKβ is underlined in the provided sequences in (A) and (B). Regions shown in green in (A) and (B) indicate residues from either a 45-mer IKKβ peptide or IKKα/β hybrid peptide (701-745) which were both successfully crystallized with GST-NEMO (44-111).[3]

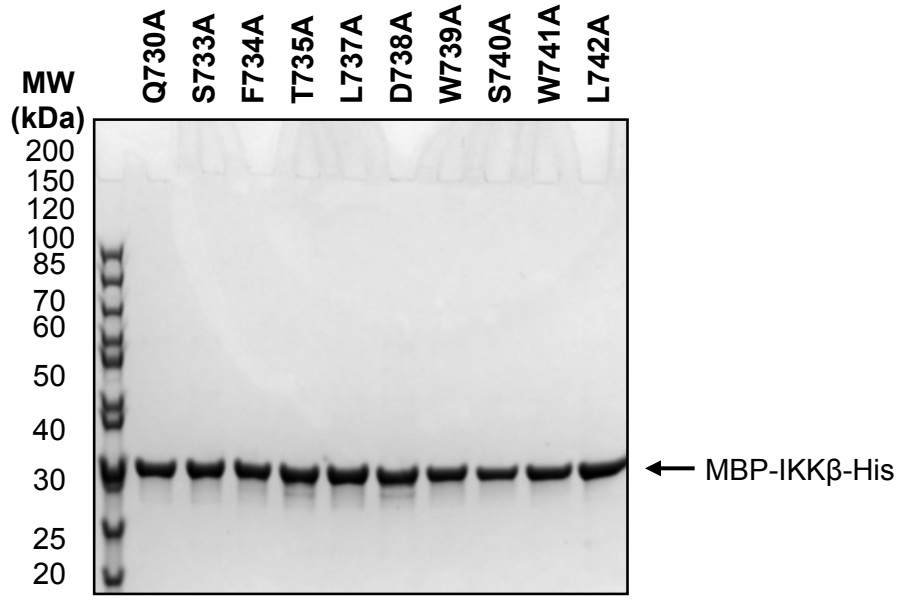
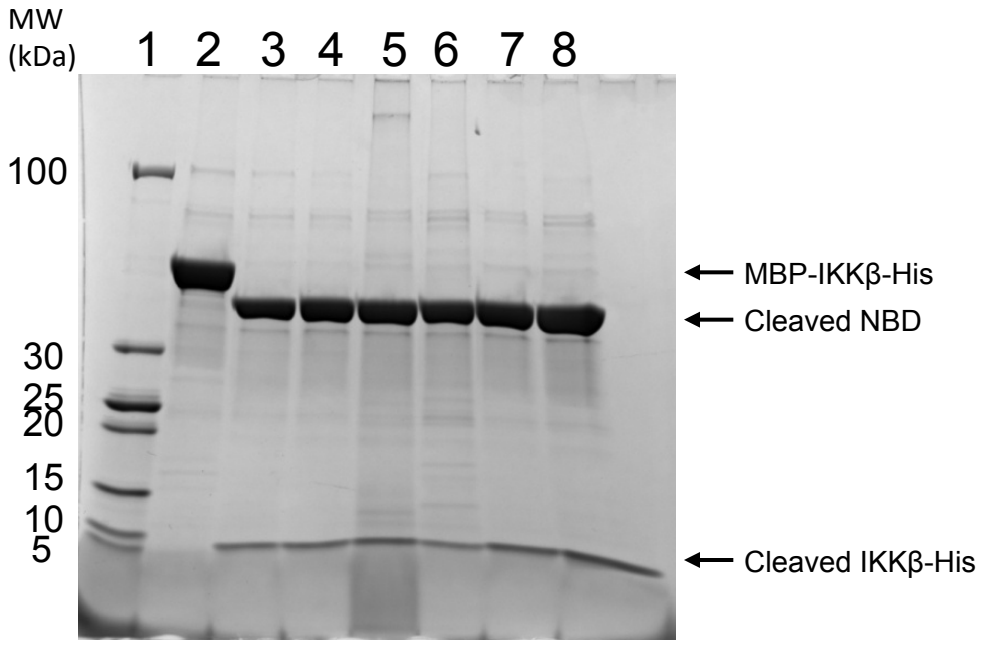


Figure S2. SDS-PAGE analysis of an arbitrary subset of the MBP-IKKβ-His mutant constructs used in these studies. Shown is a representative image showing the sample purity of the MBP-IKKβ-His constructs achieved using the expression and purification conditions discussed in the text. Samples were run under non-reducing conditions.



Lane	Sample	
1.	Molecular weight markers	
2.	MBP-IKKβ-His(C716S) (uncleaved)	
3.	C716S	After treatment with Factor Xa
4.	T735A	
5.	L737A	
6.	D738A	
7.	S740A	
8.	L742A	

Figure S3. SDS-PAGE analysis of a subset of the MBP-IKKβ-His fusion constructs after cleavage with Factor Xa, showing that incubation for 1 h with 1 μg of Factor Xa can completely and cleanly cleave up to 10 mg of MBP-IKKβ-His fusion protein, as described in Materials and Methods. Samples were run under non-reducing conditions.

Competition

```
[task]
  data      = equilibria
  task      = fit
[mechanism]
  L + N <==> N.L     :    K_D1 dissoc  ; L represents *IKKβ, N represents NEMO
  C + N <==> N.C     :    K_D2 dissoc  ; C represents competitor IKKβ mutant (IKKβ_C)
[concentrations]
  L = 15
  N = 15
[constants]
  KD1  =  3.6
  KD2  =  10 ?
[responses]              :    Specific molar response of *IKKβ
  N.L = 0.006 ?
[equilibria]
  variable C
  offset 0.05            :    Value determined from control measurement in which no NEMO
                              (N) was present. Defined in the text as A_F.
  file ./rawdata.txt
[output]
  directory ./outputfit
[end]
```

Figure S4. DYNAFIT 4 code used to analyze inhibition data from the FA competition binding assay to determine the binding affinity (K_{D2}) for NEMO of an unlabeled IKKβ competitor.

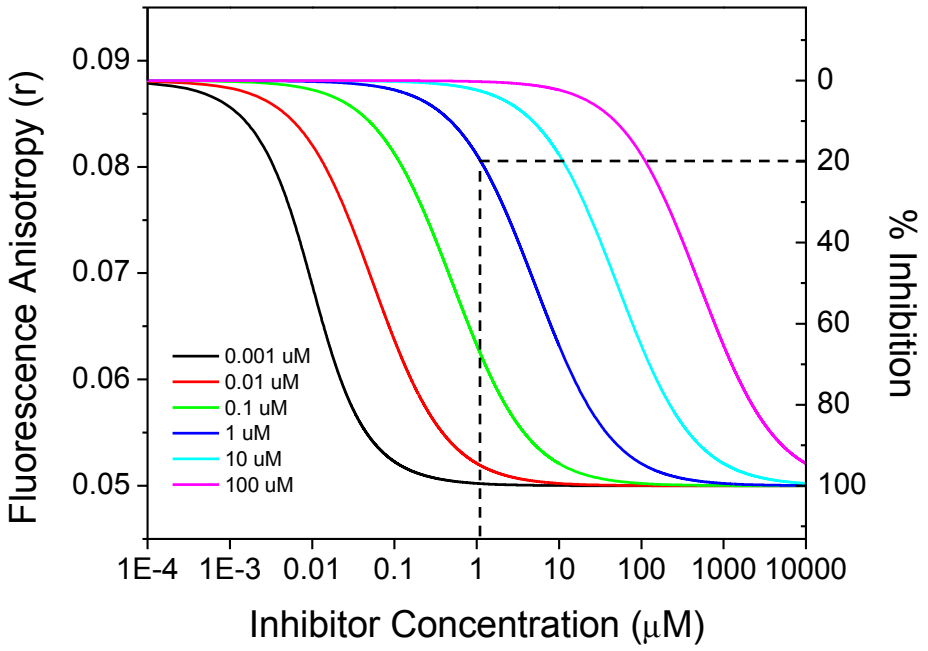
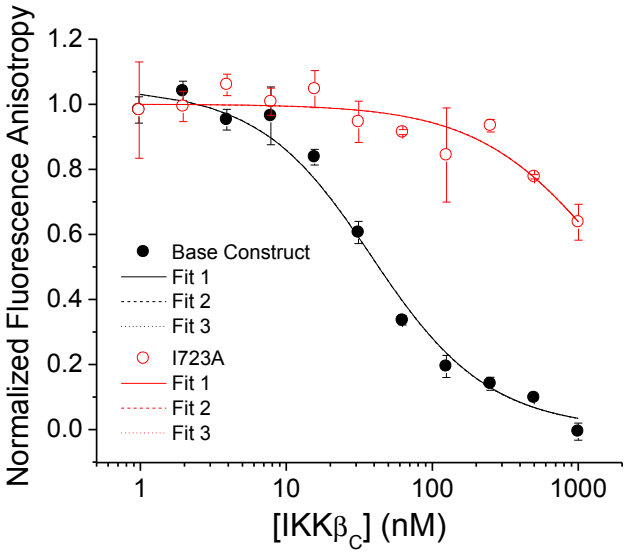


Figure S5. Simulated inhibition curves representing expected response from IKKβ competitors of varying affinities in the FA competitive binding assay. Curves were simulated assuming K_{D1} = 3.6 nM, specific molar response = 0.006 mP per nM, minimum anisotropy A_F = 0.05, [FITC-IKKβ] = 15 nM, and [NEMO] = 9 nM. Simulations are for IKKβ competitors with the following K_{D2} values: 1 nM, black line; 10 nM, red line; 100 nM, green line; 1000 nM, blue line; 10000 nM, cyan line; and 100000 nM, magenta line. Black dashed lines are included to show the relationship between % inhibition at a particular IKKβ competitor concentration and K_{D2}, as described in the text. All curves were simulated using a custom-written DYNAFIT 4 code (Figure S4).



$$A_N = \frac{(A_C - A_F)}{A_B - A_F}$$

	K_{D2} (nM)		ΔΔG (kcal/mol)	
	Base Construct	**I723A**	**Base Construct**	**I723A**
Fit 1 (K_{D1} = 2.4 nM)	5.1	280	1.0	3.3
Fit 2 (K_{D1} = 3.6 nM)	6.7	370	1.1	3.5
Fit 3 (K_{D1} = 5.0 nM)	8.4	450	1.3	3.6

Figure S6. Analysis of the robustness of fitted K_{D2} values obtained by analysis of data from the FA competition binding assay, with respect to error in the assumed value of K_{D1}. Plot shows experimental inhibition data for the base construct (closed circles) and IKKβ mutant I723A (open circles), along with best curve fits obtained using assumed K_{D1} values of 2.4 nM (Fit 1, solid lines), 3.6 nM (Fit 2, dashed lines) or 5.0 nM (Fit 3, dotted lines) for FITC-IKKβ. The solid, dotted, and dashed lines cannot be distinguished because they fall essentially on top of each other. All data were fitted using custom-written DYNAFIT 4 code (Figure S4), as described in the text. The table shows fitted K_{D2} values for the base construct and I723A mutant obtained by fitting with the different fixed values of K_{D1}, showing that the variation in the assumed value of K_{D1} results in a negligible change (<0.3 kcal/mol) in the computed binding free energy ($\Delta G = RT\ln(K_{D2})$ values and, due to cancelation of errors, causes no significant difference in the resulting ΔΔG values.

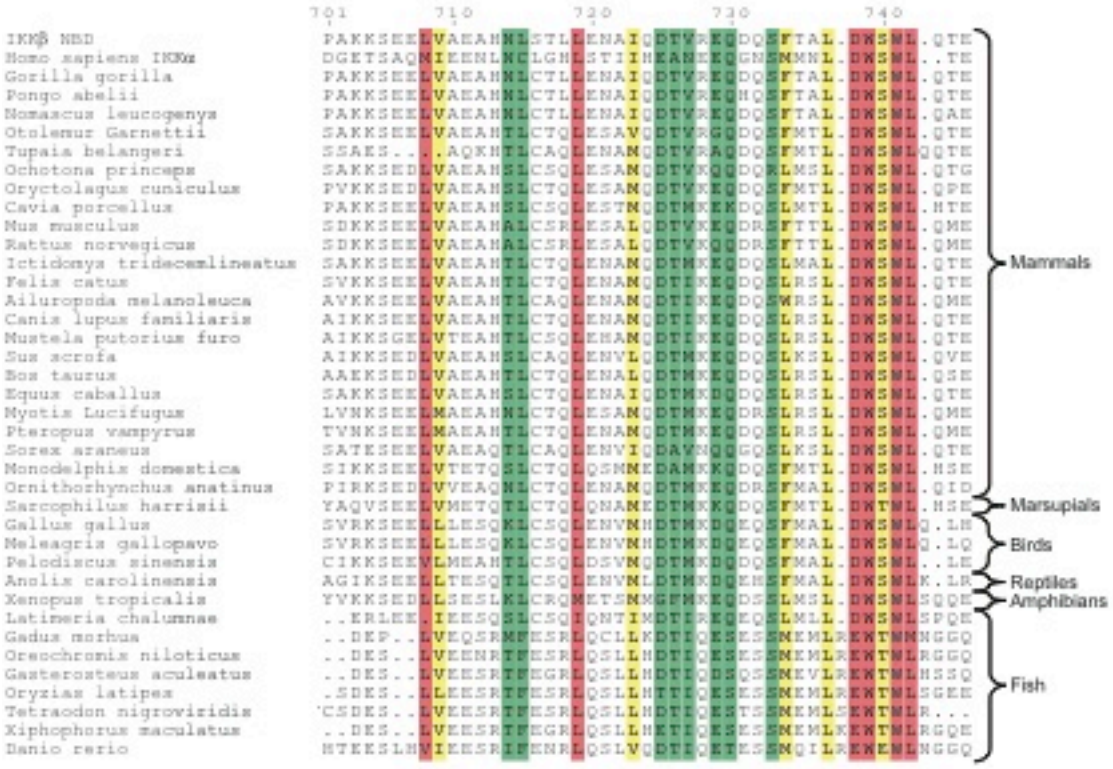


Figure S7. Sequence alignment of human IKKβ(701-745) with the corresponding region of human IKKα and with IKKβ ortholog sequences from the Ensembl Genome Browser (http://useast.ensembl.org/index.html). Sequences were aligned using the ClustalW2 tool with the default settings for each parameter, except for the multiple sequence alignment gap open parameter which was set to 5. All available ortholog sequences are included that (a) contain significant homology (<50% identity) with human IKKβ(701-745) and (b) contain the WxWL motif near the C-terminus.

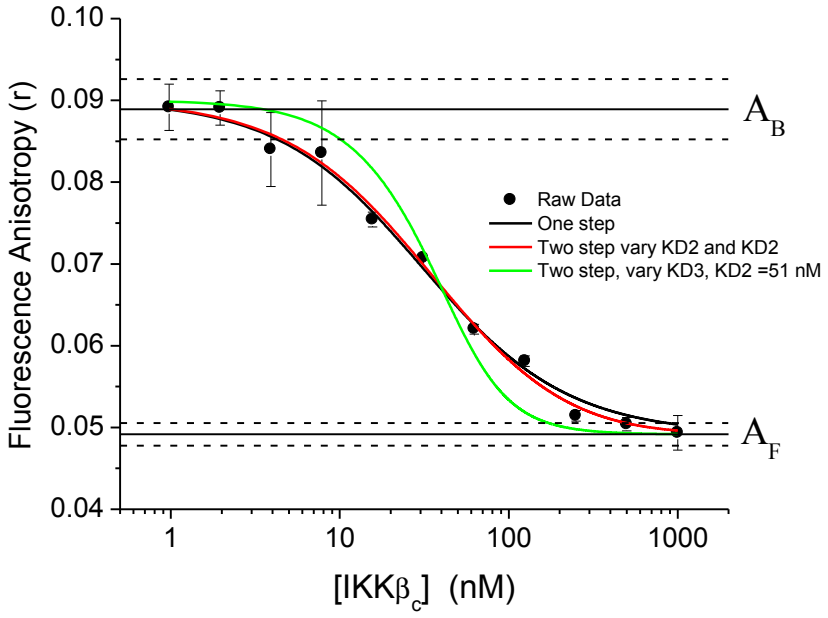


Figure S8. Comparison of single site and cooperative binding models as explanations for IKKβ inhibition binding data. The data points are from Figure 3C of the manuscript. The black curve shows the original fit to an independent site binding mechanism (Scheme 1 in the manuscript). The red curve shows the best fit to a cooperative binding model in which the first and second K_D values were allowed to vary unconstrained. This fit gave a best-fit value for K_{D2} of 505 nM, which in the form of the model we used indicates that this second step does not contribute significantly to the variation in signal observed over the competitor concentration range used. The green curve shows the best fit obtained if K_{D1} is set to a value 10-fold higher than the single-site value of 5.1 nM but K_{D2} is allowed to vary freely, showing that there is no value K_{D2} can adopt that gives a good fit to the data.

Table S1. Density Overlap calculated from focused FTMap analysis of NEMO(44-111)

Mutant	FTMap Density Overlap (# Probe Atoms)
L708A	363
V709A	206
N714A	0
L715A	446
L719A	361
I723A	0
D725A	-10
T726A	-28
V727A	0
E729A	0
Q730A	0
S733A	0
F734A	270
T735A	-1
L737A	167
D738A	0
W739A	511
S740A	0
W741A	386
L742A	269

Table S2. Complementary Oligonucleotide Primers used to Create the Base Construct by Site-Directed Mutagenesis of the MBP-IKKβ-His Construct[a]

Primer	DNA Sequence
C716S-s	GTGGCGGAAGCCCATAACCTGAGCACCCTGCTGGAAAATGCAATTC
C716S-a	GAATTGCATTTTCCAGCAGGGTGCTCAGGTTATGGGCTTCCGCCAC

[a]Sequences are given in the 5' → 3' direction. The codon position of the serine mutation (C716S) is underlined for the sense (s) primer.

Table S3. Complementary Oligonucleotide Primers used for Site-Directed Mutagenesis of the Base Construct[a]

Primer	DNA Sequence
L708A-s	CGGCGAAAAAATCTGAAGAAGCGGTGGCGGAAGCCCATAACCTG
L708A-a	CAGGTTATGGGCTTCCGCCACCGCTTCTTCAGATTTTTTCGCCG
V709A-s	GCGAAAAAATCTGAAGAACTGGCGGCGGAAGCCCATAACCTGAGC
V709A-a	GCTCAGGTTATGGGCTTCCGCCGCCAGTTCTTCAGATTTTTTCGC
N714A-s	CTGAAGAACTGGTGGCGGAAGCCCATGCGCTGAGCACCCTGCTGGAAAATGC
N714A-a	GCATTTTCCAGCAGGGTGCTCAGCGCATGGGCTTCCGCCACCAGTTCTTCAG
L715A-s	CTGGTGGCGGAAGCCCATAACGCGAGCACCCTGCTGGAAAATGC
L715A-a	GCATTTTCCAGCAGGGTGCTCGCGTTATGGGCTTCCGCCACCAG
L719A-s	GCCCATAACCTGAGCACCCTGGCGGAAAATGCAATTCAGGATACG
L719A-a	CGTATCCTGAATTGCATTTTCCGCCAGGGTGCTCAGGTTATGGGC
I723A-s	GCACCCTGCTGGAAAATGCAGCGCAGGATACGGTTCGTGAACAG
I723A-a	CTGTTCACGAACCGTATCCTGCGCTGCATTTTCCAGCAGGGTGC
D725A-s	CTGCTGGAAAATGCAATTCAGGCGACGGTTCGTGAACAGGATCAG
D725A-a	CTGATCCTGTTCACGAACCGTCGCCTGAATTGCATTTTCCAGCAG
T726A-s	GCTGGAAAATGCAATTCAGGATGCGGTTCGTGAACAGGATCAGAGC
T726A-a	GCTCTGATCCTGTTCACGAACCGCATCCTGAATTGCATTTTCCAGC
V727A-s	GGAAAATGCAATTCAGGATACGGCGCGTGAACAGGATCAGAGCTTTACCG
V727A-a	CGGTAAAGCTCTGATCCTGTTCACGCGCCGTATCCTGAATTGCATTTTCC
E729A-s	GCAATTCAGGATACGGTTCGTGCGCAGGATCAGAGCTTTACCGC
E729A-a	GCGGTAAAGCTCTGATCCTGCGCACGAACCGTATCCTGAATTGC
Q730A-s	GCAATTCAGGATACGGTTCGTGAAGCGGATCAGAGCTTTACCGCGCTGG
Q730A-a	CCAGCGCGGTAAAGCTCTGATCCGCTTCACGAACCGTATCCTGAATTGC
S733A-s	CGGTTCGTGAACAGGATCAGGCGTTTACCGCGCTGGATTGGTCTTGG
S733A-a	CCAAGACCAATCCAGCGCGGTAAACGCCTGATCCTGTTCACGAACCG
F734A-s	GGTTCGTGAACAGGATCAGAGCGCGACCGCGCTGGATTGGTCTTGG
F734A-a	CCAAGACCAATCCAGCGCGGTCGCGCTCTGATCCTGTTCACGAACC
T735A-s	GTGAACAGGATCAGAGCTTTGCGGCGCTGGATTGGTCTTGGCTG
T735A-a	CAGCCAAGACCAATCCAGCGCCGCAAAGCTCTGATCCTGTTCAC
L737A-s	GAACAGGATCAGAGCTTTACCGCGGCGGATTGGTCTTGGCTGCAGACG
L737A-a	CGTCTGCAGCCAAGACCAATCCGCCGCGGTAAAGCTCTGATCCTGTTC
D738A-s	GATCAGAGCTTTACCGCGCTGGCGTGGTCTTGGCTGCAGACGGAAG
D738A-a	CTTCCGTCTGCAGCCAAGACCACGCCAGCGCGGTAAAGCTCTGATC
W739A-s	GATCAGAGCTTTACCGCGCTGGATGCGTCTTGGCTGCAGACGGAAGG
W739A-a	CCTTCCGTCTGCAGCCAAGACGCATCCAGCGCGGTAAAGCTCTGATC
S740A-s	GCTTTACCGCGCTGGATTGGGCGTGGCTGCAGACGGAAGGAGG
S740A-a	CCTCCTTCCGTCTGCAGCCACGCCCAATCCAGCGCGGTAAAGC
W741A-s	GCTTTACCGCGCTGGATTGGTCTGCGCTGCAGACGGAAGGAGGTGG
W741A-a	CCACCTCCTTCCGTCTGCAGCGCAGACCAATCCAGCGCGGTAAAGC
L742A-s	CCGCGCTGGATTGGTCTTGGGCGCAGACGGAAGGAGGTGGAGG
L742A-a	CCTCCACCTCCTTCCGTCTGCGCCCAAGACCAATCCAGCGCGG

[a]Sequences are given in the 5'→3' direction. The codon position of each mutation is underlined for the sense (s) primer.

Table S4. IKKβ Peptides Used in Fluorescence Anisotropy Studies	
Peptide	Sequence
IKKβ(701-745)	PAKKSEELVAEAHNLCTLLENAIQDTVREQDQSFTALDWSWLQTE
FITC-IKKβ	FITC-Ahx-PAKKSEELVAEAHNLCTLLENAIQDTVREQDQSFTALD WSWLQTE[a]

[a]Ahx = 6-aminohexanoic acid

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