Interaction of an IκBα Peptide with 14-3-3

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ABSTRACT: Inflammatory responses mediated by the transcription factor nuclear factor kappa-light-chain enhancer of activated B cells (NF-κB) play key roles in immunity, autoimmune diseases, and cancer. NF-κB is directly regulated through protein–protein interactions, including those with IκB and 14-3-3 proteins. These two important regulatory proteins have been reported to interact with each other, although little is known about this interaction. We analyzed the inhibitor of nuclear factor kappa B α (IκBα)/14-3-3 interaction via a peptide/protein-based approach. Structural data were acquired via X-ray crystallography, while binding affinities were measured with fluorescence polarization assays and time-resolved tryptophan fluorescence. A high-resolution crystal structure (1.13 Å) of the uncommon 14-3-3 interaction motif of IκBα (IκBαpS63) in a complex with 14-3-3σ was evaluated. This motif harbors a tryptophan that makes this crystal structure the first one with such a residue visible in the electron density at that position. We used this tryptophan to determine the binding affinity of the unlabeled IκBα peptide to 14-3-3 via tryptophan fluorescence decay measurements.

INTRODUCTION

The κB-protein family is well known for their involvement in cell proliferation, cell survival, apoptosis, and regulation of the immune response. The family is divided into transcription factors, summarized as nuclear factor kappa-light-chain enhancer of activated B cells (NF-κB), and their inhibitors (IκB). NF-κB is sequestered by IκB in the cytosol, and upon activation, IκB is phosphorylated and degraded, while NF-κB is translocated into the nucleus. The transcriptional activity of NF-κB regulates over 500 genes, and many of those are involved in a variety of diseases. The most abundant representatives of this protein family are the heterodimer p50/p65 forming NF-κB and its inhibitor of nuclear factor kappa B α (IκBα), which is fundamental for the regulation of NF-κB activity.

The most prominent regulatory pathway of IκBα is its phosphorylation at Ser32 and Ser36, which induces ubiquitination and proteasomal degradation of IκBα. Several studies reported IκBα as an essential component for the inhibition of the transcriptional activity of NF-κB, with the nuclear export of the IκBα/p65 complex depending on a third binding partner, the 14-3-3 proteins. Overexpression of 14-3-3 increases the IκBα amount in the cytosol, while mutation of the possible IκBα motifs for 14-3-3 binding leads to an increased nuclear signal for both IκBα and NF-κB (Figure 1A).

The 14-3-3 proteins are dimeric scaffold proteins that interact with several hundred different proteins. The seven human isoforms (β, γ, ε, ζ, η, σ, and τ) are highly abundant in almost all human cells, and they exist as homo- or heterodimers. The 14-3-3 monomer consists of nine helices that form an amphipathic binding groove. By binding one or two interaction partners within the two binding grooves of the 14-3-3 dimer, the activity or cellular localization of the target proteins is altered. Interaction partners of 14-3-3 bind to 14-3-3 via distinguished phosphorylated motives in most often unstructured regions.

Physical interaction of 14-3-3 and IκBα was demonstrated by co-immunoprecipitation, whereby the truncation analysis of

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IkBα suggested only one active 14-3-3 binding motif. This motif, 59-PRGSEPW-67, follows the 14-3-3 binding motif RXpSXP, although phosphorylation of S63 is not yet reported. Co-immunoprecipitation of the IkBα/14-3-3 complex in the presence and absence of phosphatases revealed no significant differences, indicating that the phosphorylation of this residue might not be required for binding. However, later studies showed that 14-3-3 binding protects its interaction partners from phosphatases, where further investigations are needed to confirm the phosphorylation status of S63 of IkBα. Nevertheless, this region of IkBα is most likely unstructured and therefore compatible with 14-3-3 binding. Fortunately, the binding sequence of IkBα contains a Trp residue at position +3 from the phosphorylation site, which makes this Trp especially interesting. It has been well documented that Trp is an environmentally sensitive fluorophore that is able to reflect even subtle changes in its interaction network by spectral shifts and changes in its quantum yield and fluorescence lifetime. The specific Trp response depends on a number of intramolecular and/or solvent interactions (e.g., Coulombic interaction, local polarity, H-bonding, Trp mobility, etc.), all of which depend on a detailed protein structure. Trp emission can be therefore used for monitoring binding-induced conformational transitions and variations of environmental polarity. Generally, increased hydrophobicity leads to blue spectral shift and prolonged fluorescence lifetime of indole emission. We, therefore, utilized Trp66 fluorescence for the detection of interaction between IkBα peptides and the 14-3-3 protein.

In this study, the interaction of IkBα and 14-3-3 is characterized by a peptide-based approach. The binding of the IkBα peptide to 14-3-3 was measured by fluorescence polarization (FP) assay and the interface of IkBα and 14-3-3 was structurally characterized by X-ray crystallography. Furthermore, the intrinsic fluorescence of Trp66 of the peptide was used to establish a label-free method for the determination of binding affinities based on fluorescence decay. Our findings expand our knowledge of the IkBα/14-3-3 interaction and further provide a technique for the characterization of binding affinities.

## RESULTS

### 14-3-3 Binding Motif of IkBα

The IkBα protein contains only one 14-3-3 binding site, which follows a typical 14-3-3 binding motif, 57-PRGSEP-66. To investigate the binding affinity of 14-3-3 and IkBα, two peptides were synthesized, representing the unphosphorylated (IkBαpS63) and phosphorylated (IkBαpS63) binding motifs (Figure 1B). In both peptides, the centered Ser63 is flanked by six amino acids on each side of the wild-type sequence of IkBα.

For the initial measurements of the binding affinities, the peptides were N-terminally labeled with a fluorescein isothiocyanate (FITC) dye and fluorescence polarization (FP) assays were performed for both peptides with all seven human 14-3-3 isoforms (Figure 1C,D). The unphosphorylated peptide showed no affinity to either of the seven isoforms, whereas the phosphorylated peptide showed binding. No peptide reached sufficient binding for a reliable calculation of dissociation constant (Kd), although differences between the isoforms were detectable. The Kd values of IkBαpS63 and 14-3-3 are expected to be in the high micromolar to millimolar range, with the best binding to 14-3-3σ and the weakest binding to 14-3-3B (Figure 1D).

This binding behavior was previously reported in the literature for peptides representing 14-3-3 binding motifs. In those cases, a second binding site was essential for sufficient representation of the bivalent binding to the 14-3-3 dimer. Therefore, we elaborated on a hypothetical presence of additional binding sites with the prediction server 14-3-3-Pred. This server analyzes the amino acid sequence of 14-3-3 interaction partners with three different methods, resulting in a

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**Figure 1.** Phosphorylated IkBα peptide bound to 14-3-3. (A) Schematic representation of the key elements of the NF-κB pathway. In short, upon an inflammatory signal (here represented via TNFα and its receptor TNFR), a signaling cascade (thick dashed arrow) leads to proteasomal degradation of IkBα and translocation of NF-κB into the nucleus. Binding of 14-3-3 to IkBα and NF-κB is required for either nuclear export or cytosolic retention of the IkBα/NF-κB complex (red dashed arrow). (B) IkBα protein consists of an unstructured N-terminus, six helical ankyrin repeats, and the proline (P), glutamic acid (E), serine (S), and threonine (T) rich (PEST) domain. The 14-3-3 is supposed to bind to S63 at the unstructured N-terminal region. (C) Fluorescence polarization (FP) with the IkBαpS63 peptide and all human 14-3-3 isoforms. (D) FP with the IkBαpS63 peptide and all human 14-3-3 isoforms.
score for each method plus an overall score. The IK sequence revealed only one site, pT168 (sequence: VLTQSCpT168TPHL; Supporting Information, Table 1), as a likely binding site. This residue is located next to the second ankyrin repeat of the IKα protein (Supporting Information, Figure S1), and previous truncation assays did not reveal any effect on the 14-3-3 binding. Therefore, we continued with the single phosphorylated IKαpS63 peptide only.

Crystal Structure of IKα Peptide with 14-3-3σ with Truncated C-Terminus ΔC232-248 (14-3-3σΔC). To obtain structural information on the binding interface, co-crystallization trials were performed. The complex of IKαpS63 with 14-3-3σΔC was grown to the best diverging crystals among all 14-3-3 isoforms, allowing to solve the structure with a high resolution of 1.13 Å (PDB ID: 6Y1J, Supporting Information, Table S2). The crystals belonged to the space group C2221, with one 14-3-3 monomer in the asymmetric unit. One peptide is binding in each of the two binding grooves of the 14-3-3 dimer (Figure 2A, B). The peptide is binding in the proximity of both tryptophan residues of 14-3-3, Trp59, and Trp230 (Figure 2C). In total, seven of the 13 amino acids of the peptide could be assigned to the electron density map (Figure 2D), whereas pSer63 till Trp66 is the most rigid amino acids with the lowest B factors (Supporting Information, Figure S2). Consistently with other 14-3-3/phosphopeptide structures Arg56, Arg129, and Tyr130 of the protein form polar contacts with the phospho group of IKαpS63. Except for phosphoserine, the only additional direct polar contact detected by Pymol software appeared to be between Lys67 of the peptide and Glu14 of the protein (Figure 2E). Nevertheless, more polar contacts are mediated via waters, like Gly62 of the peptide with Asn226 and the backbone of Leu222 of the protein, Glu64 of the peptide and the backbone of Pro167 and Lys122 of the protein, and the backbone of Trp66 of the peptide and Ser45 of the protein. Additionally, there are hydrophobic contacts between Gly62 and Val178, Pro65 and Leu218, Ile219, Leu122, and Trp66, and the aliphatic chain of Lys46 and Gly53, Lys67 and Val46 (Figure 2F).

Measurement of K_D via Mean Tryptophan Fluorescence Lifetime. The crystal structures of IKαpS63 and 14-3-3σΔC revealed extensive contacts between the Trp66 residue and the binding groove of 14-3-3, with the indole moiety being located in a more hydrophobic environment. This binding mode suggested that the mean fluorescence lifetime τ_mean of Trp66 might change upon peptide binding and can be therefore used to detect the binding of the peptide without an artificial fluorescence labeling. To separate the fluorescence of the peptide from the Trp fluorescence of 14-3-3z, we made 14-3-3z fluorescently silent by mutating its two tryptophan residues, Trp59 and Trp228 (equals Trp230 in 14-3-3σ), to phenylalanine (W59F and W228F double mutation of 14-3-3z (14-3-3znoW)). Then, only the fluorescence of the peptide is detected.
Indeed, time-resolved fluorescence decays clearly indicate a progressive formation of IxBtpS63/14-3-3ζnoW complex by a significant increase of the Trp66 lifetime upon 14-3-3ζnoW addition (Figure 3A). This concentration-dependent increase of \( \tau_{\text{mean}} \) ranges from 3.42 ns for the IxBtpS63 alone to 4.30 ns at 763 \( \mu \text{M} \) 14-3-3ζnoW (Figure 3A). In contrast, the unphosphorylated IxBtζS63 indicated a much weaker interaction with 14-3-3ζnoW (Figure 3B). This finding is consistent with the results of the polarization assay shown in Figure 1. The \( \tau_{\text{mean}} \) for the peptide alone was 3.04 ns, while it increased to 3.27 ns at a maximum protein concentration of 640 \( \mu \text{M} \), confirming that the binding sequence needs to be phosphorylated for efficient binding.

The change in \( \tau_{\text{mean}} \) due to different protein concentrations encouraged us to use this change for an estimation of the binding affinity of IxBtpS63 to 14-3-3ζnoW. Therefore, \( \tau_{\text{mean}} \) was plotted as a function of the 14-3-3ζnoW concentration, which revealed a binding curve with incomplete saturation (Figure 3C). The data were fitted to a simple 1:1 binding model resulting in a \( K_D \) value of about 370 ± 50 \( \mu \text{M} \) for the IxBtpS63/14-3-3ζnoW complex. It has to be noted that the \( K_D \) values are slightly underestimated since the fitting model neglected the increased quantum yield of the Trp in the complex. To validate the obtained dissociation constant, FP assays with 14-3-3ζ and 14-3-3ζnoW were repeated with the highest possible protein concentrations and both are labeled as IxBtζ/pS63 peptides. The FP assay also showed incomplete saturation, but the fitting converged to the \( K_D \) of about 380 ± 1 and 415 ± 30 \( \mu \text{M} \) for IxBtζ with 14-3-3ζ and 14-3-3ζnoW, respectively (Figure 3D). Although the accuracy of such a fitting might be questionable, the similarity of the fitted dissociation constants is promising. Fortunately, due to the comparable molecular weight of the phosphorylated and unphosphorylated peptide, the plateau of all FP curves in Figure 3D, defined by the overall rotational diffusion of the complex, should be very similar. This makes the comparison of \( K_D \)s more reliable.

Within the uncertainty, the fluorescence decay data agree well with the fluorescence polarization data, whereas both techniques revealed \( K_D \) values close to 400 \( \mu \text{M} \) for the analogous IxBtpS63/14-3-3ζnoW complex. Due to limits of protein solubility, very weak interaction, and thereof inability to reach saturation, the \( K_D \) for the unphosphorylated complex was not reliably quantifiable.

### Discussion

This study focuses on an extension of our knowledge of the IxBtζ interaction with 14-3-3 and establishing label-free measurements of dissociation constants via fluorescence decay on a difficult system. In the first part, we could validate the 14-3-3 binding motif of IxBtζ on the basis of an IxBtζ-peptide−14-3-3 interaction with the traditional FP assay. The biophysical assays showed that phosphorylation of the IxBtζ peptide is necessary for binding to 14-3-3, although the rather weak binding made a quantitative determination of affinity difficult. Regarding the bivalent binding mode due to the two phospho-accepting pockets of the 14-3-3 dimer, this low affinity is consistent with those of other 14-3-3 interactions. Several studies showed similar binding behaviors for single phosphorylated peptides, and double phosphorylated binding partners reached affinities in the low-micromolar to the high-nanomolar range.26,27 The transition from a peptide motif to a protein domain or even full-length protein−protein interaction is expected to considerably enhance the binding affinity. In the case of 14-3-3 PPIs, only a few studies succeeded in the analysis of the complex formation of full-length domains due to the binding of 14-3-3 to unstructured regions of its interaction partners.28,30−36 In the case of calcium/calmodulin-dependent protein kinase kinase 2 (CaMKK2), binding to 14-3-3 was established via unstructured peptide-like motifs, which could be crystallized in a complex with 14-3-3.33 Complementary
small-angle X-ray scattering (SAXS) and binding affinity measurements showed that additional contacts were made with the outside of the 14-3-3 binding groove, while the peptide-like binding conformation in the binding groove was confirmed. Similarly, the crystal structure of the neutral trehalase of *Saccharomyces cerevisiae* (Nth1)/14-3-3 complex revealed that there are extensive interactions between 14-3-3 and Nth1 beyond those involving the 14-3-3 binding grooves. Another example of a biphosphorylated interaction partner of 14-3-3 is procaspase-2. In this case, both binding motifs were tested in fluorescence polarization assays, whereas only one site showed μM binding. However, both phosphorylation sites were necessary to establish a complex formation of the full-length proteins, while no complex formation could be observed for either single phosphorylated procaspase 2. These data suggest that an additional binding site is necessary for efficient binding of *IκB*α to 14-3-3. This can be either via a not yet identified 14-3-3 binding site of *IκB*α or a third binding partner that is simultaneously binding to *IκB*α and 14-3-3. A ternary complex formation has been reported among others for the A20/RAF proto-oncogene serine/threonine-protein kinase (C-Raf)/14-3-3 complexes and glycogen synthase kinase-3β (GSK3β)/Tau/14-3-3 interactions. A high-affinity binding of the second binding site might even mediate binding of the unphosphorylated *IκB*α to 14-3-3, although the isolated motif needs to be phosphorylated to show activity in binding assays. For the *IκBz*/14-3-3 interaction, only one active binding motif could be identified via co-immunoprecipitation, and also the sequence analysis of the *IκBz* protein revealed only one additional possible, but in the structural context unlikely, binding site (pT168; Supporting Information, Table S1 and Figure S1). In this case, it is more likely that the additional binding partner NF-κB is essential to enhance the binding of *IκB*α to 14-3-3. Binding of 14-3-3 to the NF-κB subunit p65 and to *IκB*z is needed for sufficient export of the *IκBα*/NF-κB nuclear export or retention in the cytoplasm.

Noteworthy is also the binding mode of the *IκBα*pS63 peptide as Trp66 at the +3-position of the phosphorylated residue shows a remarkable conformation. Multiple 14-3-3 binding sequences contain aromatic residues; however, even within the constantly growing collection of peptides crystallized in complex with 14-3-3, only a small number contain tryptophan residues. Lately, the first crystal structure was published with a Trp residue, which was actually covered in electron density, located at the +1-position (Figure 4A). Two other examples of peptides binding to 14-3-3 and containing a tryptophan reside are the serine/threonine-protein kinase 26 (MST4) derived peptide (PDB ID: 5XY9) and the serine/threonine-protein kinase STK11 (LKB1) derived peptide (PDB ID: SWXN). The tryptophan residue of MST4 is at the +4-position in relation to the phosphorylated residue, while that of LKB1 is at the −4-position; however, the indol moieties of both tryptophan residues were not visible in the electron density. That makes this study the second one with a Trp residue visible in a 14-3-3 crystal structure.

The amphipathic binding groove of 14-3-3 provides a lot of options for aromatic residues to bind (Figure 4). Multiple structures have been published with aromatic residues at the −1-position or +1-position of the phosphorylated residue, where they make contacts with the hydrophobic residues of the
binding groove of 14-3-3 (Figure 4B,C). An aromatic residue at the +2-position is rare and for the structure shown, the interaction of the yeast Nth1 domain with 14-3-3, the phenylalanine does not make contact with 14-3-3 (Figure 4D). It is only stable in the context of the full domain, which is not shown for clarity. Actually, there is no other structure published with tryptophan at the +3-position in addition to the IxBtSer63. However, there are at least two structures available with a phenylalanine at that position, whereby for one the side chain was not visible in the electron density map (PDB ID: 6GKF; Figure 4E). Nevertheless, the peptide–protein interface of myeloid leukemia factor 1 (Mlf1) and 14-3-3e comprises a phenylalanine at the +3-position with the same orientation as Trp66 of IxBtSer63 (PDB ID: 3UAL; Figure 4E). The conformation of the binding motifs deviates C-terminally from the phospho site more than they do N-terminally, whereas the Mlf1 motif shows the only comparable conformation. Measurements of the distances of the Cγ atoms, Trp66 of IxBt, and phenylalanine of Mlf1 are 1.5 Å apart, while in all other structures, the residues at the +3-position are more than 2.6 Å apart (Supporting Information Table S3). This demonstrates that not only the binding groove in general but also the environment of the phosphorylation site can accommodate bulky hydrophobic residues like aromatic amino acids.

The tryptophan, clearly visible in the electron density, offers a nice opportunity to use its environmentally sensitive emission for direct biophysical affinity measurements without a need for a bulky artificial dye that could eventually bias interactions between IxBt peptides and 14-3-3. For KD quantification, we used the FP assay with externally labeled protein, which relies on changed hydrodynamic properties of the complex. In parallel, we applied intrinsic Trp fluorescence directly reflecting interaction-induced variations of the Trp microenvironment. For our system, both methods gave comparable results, proving the importance of the IxBt phosphorylation and yielding KD of the IxBtSer63/14-3-3ζnoW complex formation in the 10⁻⁴ M range.

Future research will further evaluate the binding of IxBt/ p65 to 14-3-3 and analyze the contribution of this complex regarding the nuclear export mechanism of IxBt and p65.

**MATERIALS AND METHODS**

**Peptide Synthesis.** Peptides were synthesized via Fmoc-solid-phase peptide synthesis with an automated Intavis MultiPep RSi peptide synthesizer with a Rink amide AM resin (Novabiochem; 0.59 mmol/g loading). After the synthesis was completed, the resin was split, with half of the peptide N-terminally acetylated (1:1:3 acetic anhydride (Ac2O)/pyridine/N-methyl-2-pyrrolidone (NMP)) and the other one isothiocyanate (FITC)-labeled via a β-alanine linker as described above. The peptides were cleaved with a 2.5:2.5:2.5:0.9:2.5 EDT/TIS/MQ/TF Mixture and purified with a preparative reversed-phase high-performance liquid chromatography (HPLC) system with mass spectrometry (MS) detection (Supporting Information, Figure S3). The purified peptide was lyophilized and stored at −30 °C.

**14-3-3 Expression.** The 14-3-3 isoforms were transiently expressed in *Escherichia coli* Nico21(DE3) or BL21(DE3) competent cells via a pPROEX HTb plasmid. The cells were grown to a density of OD600 = 0.8–1, and protein expression was induced with 0.4 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) for 18 h by 18 °C. The proteins were purified following standard procedures for Ni-affinity chromatography. For crystallography, the His6-Tag was removed by TEV cleavage and an additional Ni-NTA column, followed by size-exclusion chromatography ensuring the highest purity. All proteins were dialyzed against 25 mM N-(2-hydroxyethyl)-piperazine-N’-ethanesulfonic acid (Hepes) pH 7.5, 100 mM NaCl, 10 mM MgCl₂, 0.5 mM tris(2-carboxyethyl)phosphine, and stored at −80 °C. The exact mass of the proteins was confirmed via hybrid quadrupole time-of-flight (QTOF) measurements, whereby the number of peaks in the chromatogram indicated the purity of the protein (Supporting Information, Figure S4).

**Crystallography.** 14-3-3ζΔC232-248 and IxBtSer63/pS63 complexes were mixed in a ratio of 1:2 in a final protein concentration of 12 mg/mL in crystallography buffer (20 mM Hepes pH 7.5, 2 mM MgCl₂, 2 mM β-mercaptoethanol) and incubated overnight at 4 °C. In a hanging drop approach, the peptide/protein complex was mixed in a 1:1 ratio with a precipitation buffer (0.95 mM Hepes pH 7.1, 28% PEG400, 5% glycerol, 0.19 M CaCl₂), crystals grew within one week. The crystals could be directly fished and flash-cooled in liquid nitrogen. The data set was measured at the Synchrotron DESY beamline P11, Hamburg, Germany.

For the indexing, integration, and molecular replacement, the CCP4 suite was used (iMoRF, AIMless, and MolRep). As a search model for molecular replacement, PDB code 4FR3 was used. The refinement was done with phenix and the model building in Coot.

**Fluorescence Polarization (FP) Assay.** The FP was measured with FITC-labeled peptides at a final concentration of 100 nM in fluorescence polarization buffer (10 mM Hepes, pH 7.4, 150 mM NaCl, 0.1% Tween 20, 1 mg/mL bovine serum albumin (BSA)). The 14-3-3 isoforms were titrated as indicated. All data sets were measured in at least three independent experiments in Corning black round-bottom 384-well plates with a Tecan Infinite F500 plate reader (excitation 485 nm, emission 535 nm).

**Time-Resolved Tryptophan Fluorescence Measurements.** Measurements of time-resolved Trp emission were performed on an apparatus comprised of a femtosecond Ti:sapphire laser (Chameleon Ultra II; Coherent) with the repetition rate reduced to 4 MHz by a pulse picker (APE). The fluorescence signal was collected using time-correlated single-photon counting detection SPC150 (Becker&Hickl) with a cooled MCP-PMT (R3809U-50; Hamamatsu). Trp fluorescence was excited at 298 nm by a tripled output of the laser and the emission was isolated under the “magic angle” conditions at 355 nm using a combination of a monochromator and a stack of UG1 and BG40 glass filters (Thorlabs) placed in front of the input slit. Fluorescence decays were typically accumulated in 1024 channels with a time resolution of 50 ps/channel until 105 counts in the decay maximum were reached. All experiments were performed at 23 °C in a buffer containing 20 mM Tris–HCl (pH 7.5), 150 mM NaCl, 1 mM ethylenediaminetetraacetic acid (EDTA), and 5 mM dithiothreitol (DTT). The IxBtSer63/pS63 peptide and 14-3-3ζnoW concentrations were 1 and 0–763 μM, respectively. Fluorescence decays were analyzed by the model-independent maximum entropy method allowing accurate determination of the mean fluorescence lifetime τmean calculated as τmean = ∑αiτi/∑αiτi where τi and αi are the fluorescence lifetime components and the corresponding amplitudes revealed by the analysis, respectively.
Supporting Information
The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.9b04413.

Results of the 14-3-3pred webserver for IκBα (uniprot ID: P25963) (Table S1); crystal structure of IκBα (Figure S1); B-factor coloring of 14-3-3ζΔC and the IκBαpS63 peptide (Figure S2); crystallographic statistics of the X-ray structure of the IκBαpS63/14-3-3ζΔC232-248 complex (PDB ID: 6Y1J) (Table S2); quantitative comparison of 14-3-3 binding motifs (Table S3); LC–MS analysis of the in-house synthesized and purified peptides (Figure S3); and QTOF measurements of the purified 14-3-3 isoforms (Figure S4) (PDF)

Accession Codes
Given are the accession codes of UniProt. 14-3-3β: P31946 (1433B_HUMAN) 14-3-3γ: P61981 (1433G_HUMAN) 14-3-3ζ: P62258 (1433E_HUMAN) 14-3-3δ: P63104 (1433Z_HUMAN) 14-3-3ζ: Q04917 (1433F_HUMAN) 14-3-3ζ: P31947 (1433S_HUMAN) 14-3-3: P27348 (1433T_HUMAN)

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Abbreviations
14-3-3ζ/ιW, W59F W228F double mutation of 14-3-3ζ, 14-3-3ζ, 14-3-3ζ with truncated C-terminus ΔC232-248; A20, TNF α induced protein 3, also known as TNFAIP3; CalMK2, calcium/calmodulin-dependent protein kinase kinases 2; C-Raf, RAF proto-oncogene serine/threonine-protein kinase; DTT, dithiothreitol; EDT, 1,2-ethanedithiol; EDTA, ethylenediaminetetraacetic acid; FITC, fluorescein isothiocyanate; FP, fluorescence polarization; GSK3β, glycogen synthase kinase-3 β; IκBα, inhibitor of nuclear factor kappa B α; Kd, dissociation constant; LKB1, serine/threonine-protein kinase STK11; Mbl1, myeloid leukemia factor 1; MST4, serine/threonine-protein kinase 26; NF-κB, nuclear factor kappa-light-chain-enhancer of activated B cells; Ntb1, neutral trehalase of Saccharomyces cerevisiae; PEST, proline (P), glutamic acid (E), serine (S), and threonine (T) rich sequence; SAXS, small-angle X-ray scattering; τ mean, mean fluorescence life

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