Supporting Information

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SI Materials and Methods

Materials and Cell Culture. The expression vectors for GST-14-3-3 and HexaHis-14-3-3 isoforms and mutants were constructed as previously described (1). Glutathione agarose beads and nickel-charged affinity columns were purchased from GE Healthcare. Anti-Raf-1 and anti-GST antibodies were purchased from Santa Cruz and the anti-PRAS40 antibody was a product of Biosource. The LOPAC library was purchased from Sigma-Aldrich. COS-7 cells were grown in DMEM supplemented with 10% FBS.

Florescence Polarization Assay and Chemical Screening. The 14-3-3 FP assay was carried out in black 384-well microplates in a total volume of 50 μL per well. Assay reaction buffer (1 μM GST-14-3-3γ and 2 nM TMR-pS259-Raf-1 peptide in Hepes buffer; 49 μL) were dispensed into each well. Test compound (1 μL of 2 mM stock in DMSO) was added to the reaction buffer using a Sciclone liquid handler (Caliper LifeSciences) 384-channel low volume heads for a final compound concentration of 40 μM in 2% DMSO. For each assay plate, column 1, 2, 23, and 24 contain controls. The following controls were included for each plate: TMRpS259-Raf-1 peptide alone (blank) and TMR-pS259-Raf-1 peptide with GST-14-3-3γ (negative control). The R18 peptide was added to wells with TMR-pS259-Raf-1 peptide and GST-14-3-3γ and served as a positive control. Plates were incubated at room temperature for 2 h and the FP value [in millipolarization (mP) units] was measured with an Analyst HT reader (Molecular Devices). An excitation filter at 545 nm and an emission filter at 610 to 675 nm were used with a dichroic mirror at 565 nm. Data analysis was conducted using CambridgeSoft software. Compounds with recorded mP values less than three standard deviation (SD) from the negative controls were considered positive hits.

Enzyme-Linked Immunosorbent Assay. The 14-3-3 ELISA assay was developed in 96-well microplates coated with anti-GST antibody (Pierce Biotechnology). Briefly, 50 μL of GST-14-3-3 protein (1 μM) was added to each well of an anti-GST plate and incubated at 4 °C overnight. The plate was washed three times with washing buffer (PBS with 0.05% Tween-20). One microliter of compound was then added to 49 μL of washing buffer and incubated for 2 h before the addition of COS-7 lysate prepared in 1% NP-40 lysis buffer (2). After incubation with COS-7 lysate for 2 h, the plate was washed three times and then incubated with antibodies specific to the 14-3-3 client protein, PRAS40, and peroxidase-labeled antirabbit IgG (50 μL; 1:1,000 dilution) for 1 h at room temperature. Plates were then washed four times and 100 μL of tetramethylbenzidine was added. The reaction was stopped after 10 min with the addition of 100 μL of 0.1 N sulfuric acid. Reactions were read at 450 nm on an Envision™ multilabel plate reader (Perkin Elmer). Results were normalized to the vehicle (DMSO) control wells after subtracting the background signal from the wells without GST-14-3-3. IC_{50} values were calculated using GraphPad software. For experiments shown in Fig. S5, an ELISA assay protocol with the following modifications was used to monitor the 14-3-3ζ/Raf-1 interaction. In this case, 96-well microplates coated with glutathione (Pierce Biotechnology) were used to capture GST-tagged 14-3-3ζ protein. After incubation with cell lysates and extensive washing, anti-Raf-1 antibody was used to detect the presence of Raf-1 in the immobilized GST-14-3-3 protein complex.

GST Pull-Down Assay and Western Blotting. COS-7 cells were lysed in NP-40 buffer (1% NP-40, 10 mM Hepes, pH 7.4, 150 mM NaCl, 5 mM NaF, 2 mM Na_3VO_4, 5 mM $Na_4P_2O_7$, 10 μg/mL aprotinin, 10 μg/mL leupeptin, and 1 mM phenylmethylsulfonyl fluoride). The cell lysates were clarified and used as a source of 14-3-3 client proteins. GST-14-3-3 proteins were purified by glutathione-agarose affinity chromatography according to the manufacturer's instructions. For binding assays, GST-14-3-3 proteins (1 μg) were preincubated with various concentrations of test compounds at room temperature for 10 min. COS-7 cell lysates were added to the 14-3-3/compound complex in a final volume of 20 μL and incubated at 4 °C for 1 h. Glutathione Sepharose, 30 μL, was added to the lysates and incubated at 4 °C for 2 h with rotation. Unbound proteins were removed from the beads by three washes with 1% NP-40 buffer. The fraction that was bound to the beads was analyzed by SDS-PAGE followed by immunoblotting with antibodies specific to Raf-1, PRAS40, and GST antibodies.

ExoS Activation Assay. To examine the functional effect of **F1** on 14-3-3 proteins, we utilized a 14-3-3-dependent ExoS ADP-ribosyltransferase assay (3). ExoS, secreted from *Pseudomonas aeruginosa*, catalyzes the transfer of ADP-ribose from NAD to various cellular substrates. ExoS enzymatic activity requires 14-3-3 proteins for activation, which has been used for monitoring 14-3-3 function. 14-3-3 proteins are capable of initiating ExoS activity resulting in the transfer of the [^{32}P]ADP-ribose moiety of NAD onto a substrate, soybean trypsin inhibitor (SBTI). This assay is used as a functional readout for 14-3-3 inhibitor screening. Briefly, 6xHis-14-3-3 protein (1 μg/mL) was preincubated with the indicated concentrations of **F1** for 30 min, followed by incubation with ExoS (1 μg/mL) in 10 mM Hepes, pH 7.3, 130 mM NaCl, 100 μg/mL SBTI, 5 μM NAD, 0.35 μCi of [adenylate-^{32}P]NAD^+, and 0.1% bovine serum albumin in a final volume of 20 μL at 25 °C for 10 min. The reaction was terminated by spotting 10 μL of assay mixture onto P81 phosphocellulose paper (Whatman, Maidstone, United Kingdom). The filters were washed three times with 0.75% phosphoric acid and once with acetone. Radioactivity was determined by liquid scintillation counting. Enzyme activities were expressed as picomoles of ADP-ribose incorporated per min per microgram of ExoS. The inhibitory effect of **F1** was expressed as percent inhibition of ExoS activity over vehicle control.

Isothermal Titration Calorimetry (ITC) Analysis. All ITC experiments were carried out with an auto-iTC200 instrument (MicroCal, GE). Test protein (400 μL of 150 μM stock solution), compound (120 μL of 8 mM stock solution), and buffer (400 μL of 10 mM Hepes, pH 7.4, and 100 mM NaCl) were placed in a loading 96 DeepWell PP plate (Nunc, Thermo Fisher Scientific). The test protein solution (200 μL) was automatically transferred by the auto-iTC 200 instrument into the sample cell. Compound solution (2 μL) was titrated stepwise into the protein sample cell using a syringe for a total of 20 injections except that the first injection was 0.4 μL. The equilibrium time between two adjacent injections was 210 s. The binding stoichiometry (n), binding constant (Kd), and thermodynamic parameters (ΔH and ΔS) were determined by fitting the titration curve to a one-site binding mode using the Origin software provided by the manufacturer.

Protein Purification. Bovine 14-3-3ζ was expressed in *E. coli* BL21 (DE3) harboring pET-15b-derived plasmids and purified using Ni^{2+} chelating chromatography essentially as described (3). Hexahistidine tags were removed by thrombin digestion (1 unit/mg of protein). The 14-3-3 protein used for crystallization was further

purified by gel filtration chromatography (Superdex 200 in a Pharmacia FPLC system). ExoS was purified as previously described (4). Similarly, five mutant proteins of 14-3-3ζ (K120E, V176D, R56E, R60E, and K49E) were purified by the two-step purification approach. Protein dimer fractions were collected. For ITC studies, wild-type and mutant 14-3-3ζ proteins were adjusted to 150 μM in a buffer containing 10 mM Hepes, pH 7.4, and 100 mM NaCl. Protein concentrations were determined by UV 280 reading in an Epoch MultiVolume Spectrophotometer System (BioTek).

Mass Spectrometry. Covalent adduct formation between 14-3-3ζ-K120 and fragmented **F1** was verified by performing MALDI-TOF-MS analysis of V8-protease (New England BioLab) digested peptide fragments of F1-protein crystals with and without X-ray exposure. Crystals of each type (3–4) were harvested in 10 μL of milliQ water and mixed with an equal volume of SDS sample buffer. The samples were run on a 13% polyacrylamide gel and protein bands were excised into small cubes. The gel pieces were destained using a 50% (v/v) acetonitrile/water mixture containing 10 mM ammonium bicarbonate and dried under a vacuum. Dry gel pieces were rehydrated in 20 to 30 μL of 10 mM ammonium bicarbonate buffer containing 0.2 μg of V8 protease and incubated at 37 °C for 8 h. The digestion reactions were stopped by adding 1 μL of 10% trifluoroacetic acid (TFA). Digested peptides were extracted in 100 μL of a 60% (v/v) acetonitrile/water mixture containing 10 mM ammonium bicarbonate. The peptide extract was dried under vacuum to a final volume of 10 μL and desalted using a C18 zip-tip (Millipore Corp.). Bound peptides were eluted from the zip-tip using 5 μL of a 70% (v/v) acetonitrile/water mixture containing 0.1% TFA. One microliter of the eluted peptide was spotted on a MALDI target plate along with 1 μL of a saturated solution of α-cyano-4-hydroxy cinnamic acid matrix (Sigma). The MALDI-TOF-Mass spectra were recorded in reflection mode and the observed peptide-mass profile was compared against the theoretical mass profile obtained using the Peptide-Mass software on the ExPASy Proteomics Server (http://www.expasy.ch/tools/peptide-mass.html).

1. Subramanian RR, Masters SC, Zhang H, Fu H (2001) Functional conservation of 14-3-3 isoforms in inhibiting bad-induced apoptosis. *Exp Cell Res* 271:142–151.
2. Masters SC, Fu H (2001) 14-3-3 proteins mediate an essential anti-apoptotic signal. *J Biol Chem* 276:45193–45200.
3. Fu H, Coburn J, Collier RJ (1993) The eukaryotic host factor that activates exoenzyme S of Pseudomonas aeruginosa is a member of the 14-3-3 protein family. *Proc Nat'l Acad Sci USA* 90:2320–2324.
4. Knight DA, Finck-Barbancon V, Kulich SM, Babieri JT (1995) Functional domains of Pseudomonas aeruginosa exoenzyme S. *Infect Immun* 63:3182–3186.

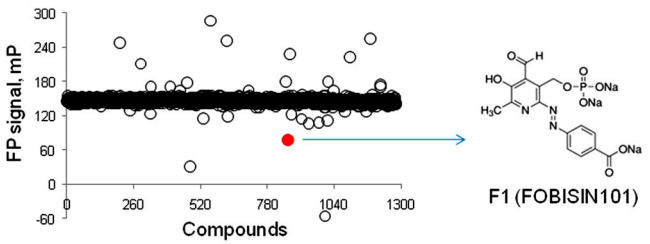

Fig. S1. Histogram of HTS. A LOPAC library (1,280 compounds) was used to screen for molecules that decrease FP signals from a 6xHis-14-3-3γ/pS259-Raf-1-rhodamine complex. Compounds that decreased FP signals by three SDs were cherry picked for dose-response studies. MRS2159 from the library was confirmed as a positive hit (designated FOBISIN101) and further characterized.

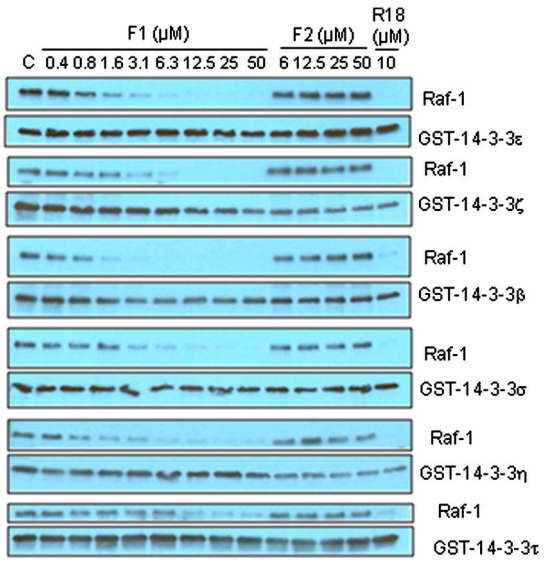

Fig. S2. Inhibition of the interaction of 14-3-3 isoforms with Raf-1 by F1. GST-fusion proteins of each 14-3-3 isoform were incubated with COS-7 lysates in the presence of F1 or F2. GST-14-3-3 protein complexes were isolated by affinity chromatography. The presence of Raf-1 in the GST-14-3-3 complexes was revealed by Western blot analysis with anti-Raf-1 antibodies.

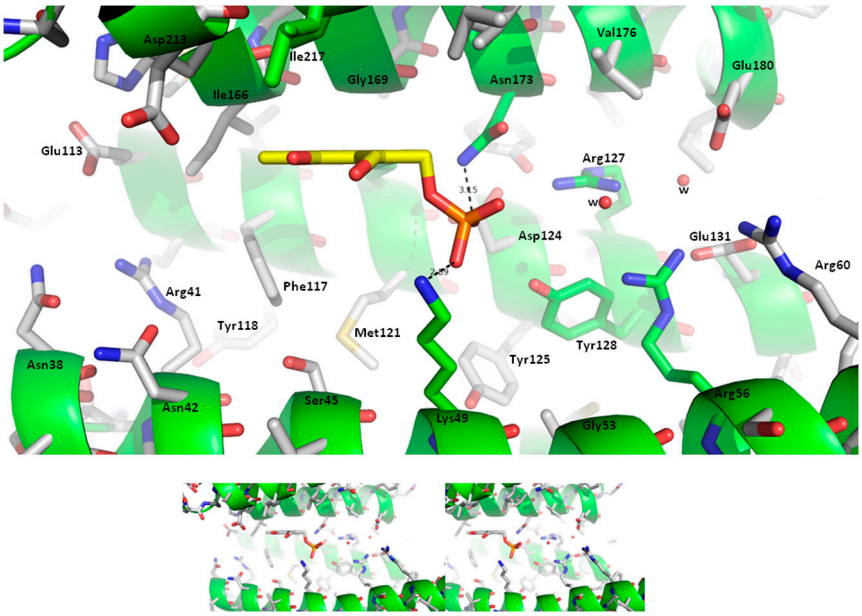

Fig. S3. Structural details of 14-3-3ζ and F1 fragment interactions with labeled residues (upper box) and in stereo images (lower box).

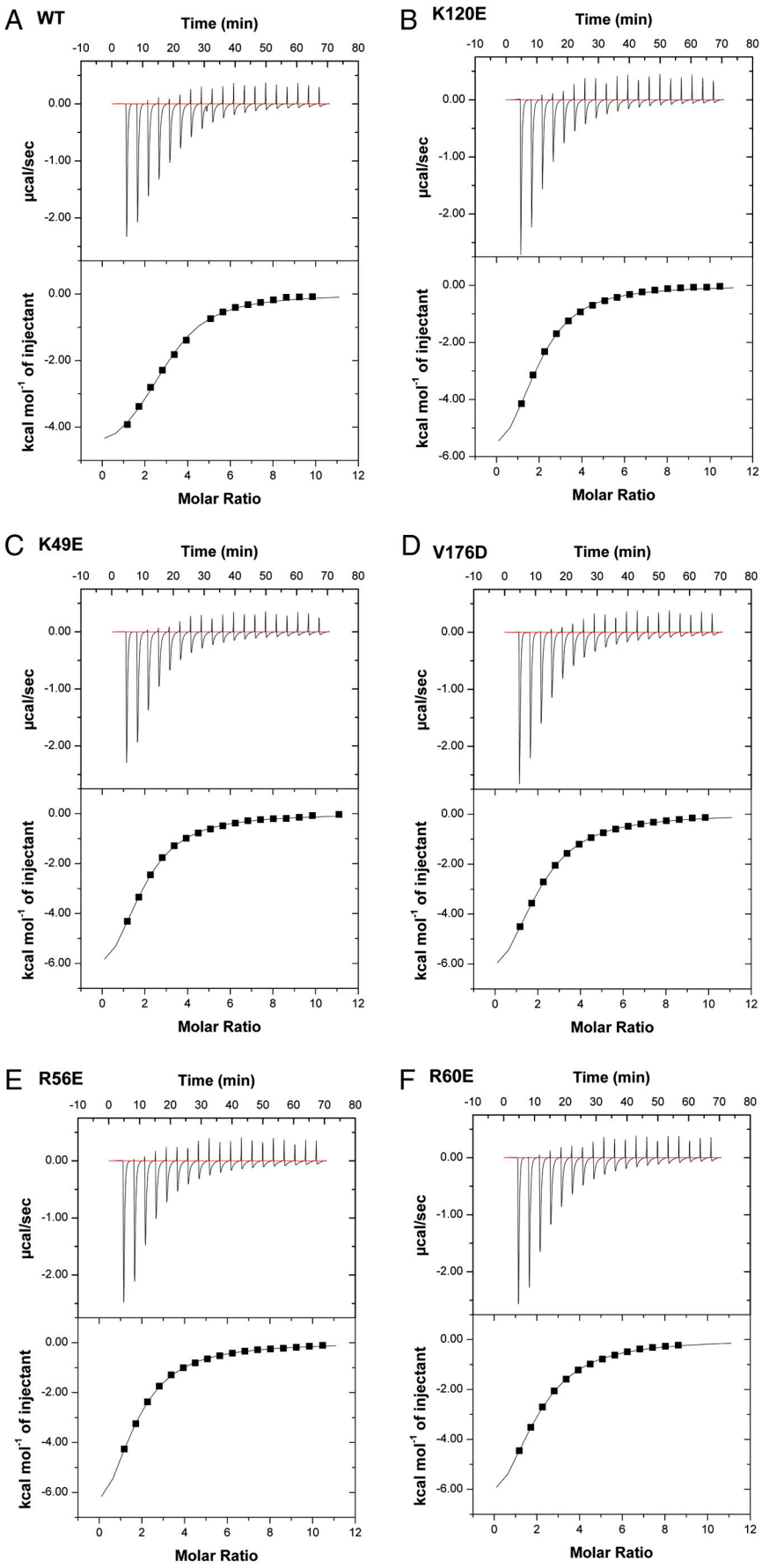
A WT
B K120E
C K49E
D V176D
E R56E
F R60E
Time (min)
µcal/sec
kcal mol-1 of injectant
Molar Ratio

Fig. S4. Thermodynamic measurements of the binding of FOBISIN 101 to wild-type 14-3-3ζ (*A*) and five mutants (*B*. K120E, *C*. K49E, *D*. V176D, *E*. R56E, *F*. R60E) from isothermal titration calorimetry. Residues that were mutated are centered around the binding site for the phosphate group of its peptide ligands and the proposed site for uncleaved FOBISIN 101 (Fig. 2). For each protein sample, the upper box is the raw heating power over time and the lower box is a fit of the integrated energy values normalized for each injection. The ITC experiments were performed at least four times. Thermodynamic values obtained are listed in Table S2.

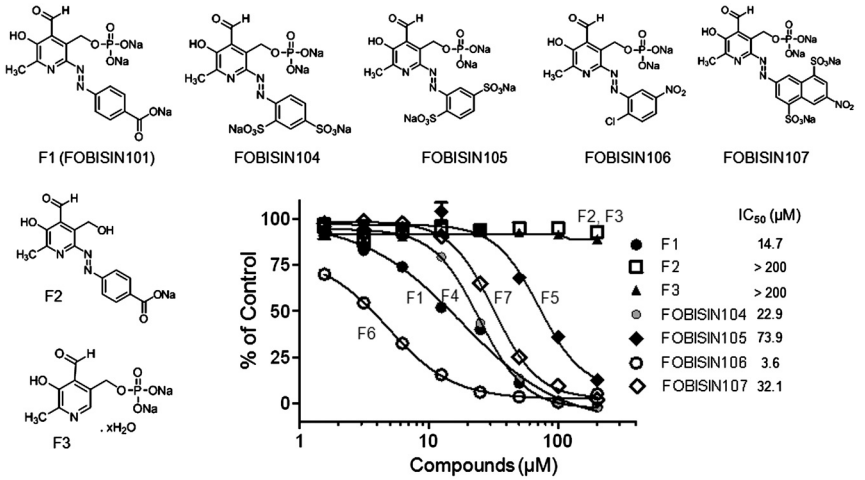

Fig. S5. Inhibition of the interaction between 14-3-3ζ and Raf-1by **F1** and its analogues in an ELISA assay. Interaction of Raf-1 with GST-14-3-3ζ immobilized on a glutathione-coated plate gave rise to robust ELISA signals as detected by anti-Raf-1 antibody. The data are normalized to the vehicle (DMSO) control wells after subtracting the background signal from the wells without GST-14-3-3ζ protein. IC_{50}s were estimated using GraphPad software. Tested compounds include FOBISIN 104 (pyridoxalphosphate-6-azophenyl-2′,4′-disulfonic acid tetrasodium salt), 105 (Pyridoxalphosphate-6-azophenyl-2′,5′-disulfonic acid tetrasodium salt), 106 (2-[(2-Chloro-5-nitrophenyl)azo]-5-hydroxy-6-methyl-3-[(phosphonooxy)methyl]-4-pyridinecarboxaldehyde disodium salt), and 107 (pyridoxalphosphate-6-(2′-naphthylazo-6′-nitro-4′,8′-disulfonate) tetrasodium salt).

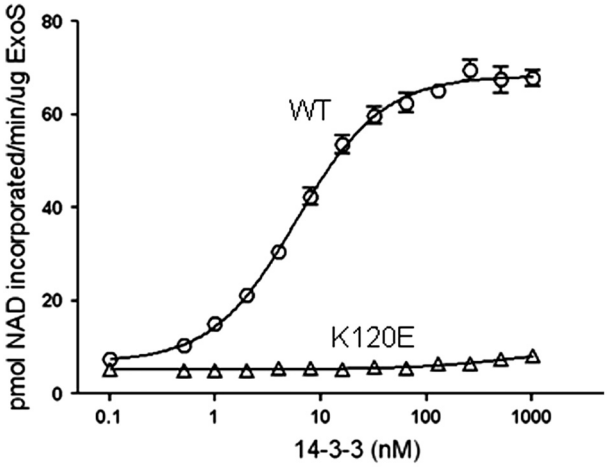

Fig. S6. Activation of ExoS ADP-ribosyltransferase activity by 14-3-3ζ in comparison to 14-3-3ζ/K120E. ExoS was incubated with ^{32}P-NAD and STBI as substrates. While addition of wild-type 14-3-3ζ resulted in an increase in ExoS activity, as measured by pmol ADP-ribose incorporated into STBI per min, addition of the charge-reversal mutant, K120E, failed to activate ExoS.

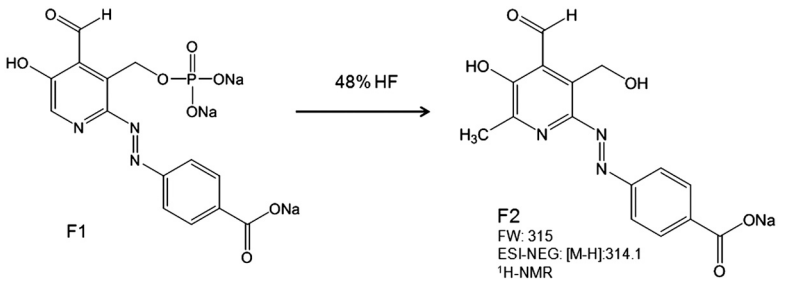

Fig. S7. Chemical synthesis of compound F2 from F1.

Table S1. Data collection and refinement statistics (molecular replacement)

Data collection	14-3-3ζ (FOBISIN 101)
Space group	$P6_5$
Cell dimensions	($\alpha = \beta = 90°$; $\gamma = 120°$)
a, b, c (Å)	94.75, 94.75, 237.86
Beamline (SERCAT)	APS 22-ID
Wavelength (Å)	0.97926
Resolution (Å)	33.77-2.39 (2.48-2.39)*
R_{merge}	0.114 (0.722)
$I/\sigma I$	12.5 (1.8)
Completeness (%)	99.2 (100.0)
Redundancy	5.0 (4.0)
Observed reflections	240,793
Unique reflections	47,706 (4,785)
Refinement	
Resolution (Å)	2.39
No. Reflections	44,678
R_{work}/R_{free}	0.235/0.286
No. atoms	
Protein	7,284
FOBISIN 101	108
Water	86
B-factor (Å^2)	
Protein (A/B/C/D)	54.3/60.9/58.5/62.6
FOBISIN 101 (A/B/C/D)	57.4/62.2/63.3/66.1
Water	48.7
rms deviations	
Bond lengths (Å)	0.007
Bond angles (°)	1.1

*Values in parentheses are for highest-resolution shell.

Table S2. Thermodynamic constants obtained by ITC for the binding of FOBISIN 101 to wild-type 14-3-3ζ protein and the mutants

	*K*d (μM)	ΔH (kcal/mol)	$-T\Delta S$ (kcal/mol)
WT	75.8	−5.2	−0.5
K120E	97.1	−7.5	2.0
K49E	109.5	−8.4	3.0
V176D	132.5	−8.9	3.6
R56E	140.9	−10.2	5.0
R60E	142.9	−9.1	3.9

Decreased binding affinity of FOBISIN 101 to each mutated protein was observed, with R56E and R60E exhibiting the most significant effects. This decrease is likely due to the unfavorable entropy change, as $-T\Delta S$ increases from −0.5 to 5.0 Kcal/mol. Overall, it appears that the binding affinity of the protein to FOBISIN 101 is only slightly affected by mutating a single residue at the binding site, which contains a cluster of positively charged residues.