Supplementary Information for

Functional mapping of the 14-3-3 hub protein as a guide to design 14-3-3 molecular glues

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Figure S1. Non-natural peptide screening results. (a) Table of seventeen obtained hit sequences from the non-natural peptide library screen. (b) Positional frequency analysis of identified 14-3-3γ binding hits. Each letter represents a non-natural amino acid and the size of the letter determines its abundance in the hit sequences. Figures taken from Quartararo (2020).

Table S1. Binding characteristics peptide 1. $K_D$ and $\Delta G$ values obtained from fluorescence anisotropy (FA) assays and $K_D$, $\Delta G$, $\Delta H$ and $-T\Delta S$ data obtained from isothermal titration calorimetry (ITC) experiments. Values are given for all measured replicates (3x for FA; 2x for ITC). N.A. = not available.
**Figure S2.** Phosphoserine replacement study. (a) Crystal structure of 14-3-3σ (white surface) bound to the peptide 1 (orange sticks). Enlarged view of the electrostatic interaction between the phosphoserine residue of the 1 and the 14-3-3 phospho-accepting pocket (R129, R56, and Y130). (b) Fluorescence anisotropy assay of 14-3-3σ titration to fixed concentrations fluorescein-labelled peptide (10 nM) and schematic representation of phosphoserine containing peptide (orange) and the tested phosphoserine replacements being serine (blue), glutamic acid (yellow) and aspartic acid (green).
Figure S3. Sequences N-terminal truncated peptides 2a-d. Schematic representation and chemical structure of peptide 1 and the N-terminal truncations. Within these peptides we stepwise remove the N-terminal amino acids leading to peptides 2a-d.

Figure S4. Sequences C-terminal truncated peptides 2e-i. Schematic representation and chemical structure of peptide 1 and the C-terminal truncations. Within these peptides we stepwise remove the C-terminal amino acids leading to peptides 2e-i.
Table S2. Overview $K_d$ and $\Delta\Delta G$ analysis N-terminal truncated peptides 2a-2d. $K_d$ values are determined from the FA-based binding studies of 14-3-3 to each of the peptides. The fold change in binding affinity is determined by dividing the $K_d$ from a peptide with the $K_d$ of the previous (one amino acid longer) peptide. Furthermore the $K_d$ is transformed into $\Delta G$ values which in the end is transformed into a $\Delta\Delta G$ analysis by subtracting the $\Delta G$ from each peptide with the $\Delta G$ from the previous (one amino acid longer) peptide.

| Affinity $K_d$ (M) | 1     | 2a    | 2b    | 2c    | 2d    |
|--------------------|-------|-------|-------|-------|-------|
| Replicate 1 | 1,64E-08 | 2,83E-08 | 1,03E-07 | 5,21E-08 | 2,12E-06 |
| Replicate 2 | 1,81E-08 | 4,63E-08 | 1,08E-07 | 5,68E-08 | 2,60E-06 |
| Replicate 3 | 2,33E-08 | 3,86E-08 | 1,01E-07 | 5,63E-08 | 2,43E-06 |
| Mean | 1,92E-08 | 3,77E-08 | 1,04E-07 | 5,51E-08 | 2,39E-06 |
| Std. deviation | 3,56E-09 | 9,07E-09 | 3,44E-09 | 2,59E-09 | 2,45E-07 |

| Fold change to previous | 1     | 2a    | 2b    | 2c    | 2d    |
|-------------------------|-------|-------|-------|-------|-------|
| Replicate 1 | 1,72 | 3,64 | 0,51 | 40,69 |
| Replicate 2 | 2,56 | 2,32 | 0,53 | 45,81 |
| Replicate 3 | 1,66 | 2,61 | 0,56 | 43,18 |
| Mean | 1,98 | 2,86 | 0,53 | 43,23 |
| Std. deviation | 0,50 | 0,69 | 0,03 | 2,56 |

| $\Delta G$ (kcal/mol) | 1     | 2a    | 2b    | 2c    | 2d    |
|-----------------------|-------|-------|-------|-------|-------|
| Replicate 1 | -10,61 | -10,29 | -9,53 | -9,93 | -7,74 |
| Replicate 2 | -10,56 | -10,00 | -9,50 | -9,88 | -7,61 |
| Replicate 3 | -10,41 | -10,11 | -9,54 | -9,88 | -7,65 |
| Mean | -10,53 | -10,13 | -9,52 | -9,90 | -7,67 |
| Std. deviation | 0,11 | 0,15 | 0,02 | 0,03 | 0,06 |

| $\Delta\Delta G$ (kcal/mol) to previous | 1     | 2a    | 2b    | 2c    | 2d    | N-term |
|----------------------------------------|-------|-------|-------|-------|-------|--------|
| Replicate 1 | 0,32 | 0,76 | -0,40 | 2,19 | 2,88 |
| Replicate 2 | 0,56 | 0,50 | -0,38 | 2,26 | 2,94 |
| Replicate 3 | 0,30 | 0,57 | -0,34 | 2,23 | 2,75 |
| Mean | 0,39 | 0,61 | -0,37 | 2,23 | 2,86 |
| Std. deviation | 0,14 | 0,14 | 0,03 | 0,04 | 0,10 |
Table S3. Overview $K_D$ and $\Delta \Delta G$ analysis C-terminal truncated peptides 2e-2i. $K_D$ values are determined from the FA-based binding studies of 14-3-3 to each of the peptides. The fold change in binding affinity is determined by dividing the $K_D$ from a peptide with the $K_D$ of the previous (one amino acid longer) peptide. Furthermore the $K_D$ is transformed into $\Delta G$ values which in the end is transformed into a $\Delta \Delta G$ analysis by subtracting the $\Delta G$ from each peptide with the $\Delta G$ from the previous (one amino acid longer) peptide.

| Affinity $K_D$ (M) | 1 | 2e | 2f | 2g | 2h | 2i |
|--------------------|---|----|----|----|----|----|
| Removal            | - | +5 Lys | +4 Nph | +3 bAla | +2 bSer | +1 Nph |
| Replicate 1        | 1,64E-08 | 9,09E-08 | 1,15E-06 | 2,22E-06 | 6,46E-06 | 2,73E-05 |
| Replicate 2        | 1,81E-08 | 1,29E-07 | 1,49E-06 | 2,73E-06 | 7,29E-06 | 2,59E-05 |
| Replicate 3        | 2,33E-08 | 9,25E-08 | 1,20E-06 | 2,14E-06 | 6,83E-06 | 2,96E-05 |
| Mean               | 1,92E-08 | 1,04E-07 | 1,28E-06 | 2,37E-06 | 6,86E-06 | 2,76E-05 |
| Std. deviation     | 2,91E-09 | 1,75E-08 | 1,47E-07 | 2,63E-07 | 3,41E-07 | 1,51E-06 |

| Fold change to previous | 1 | 2e | 2f | 2g | 2h | 2i |
|-------------------------|---|----|----|----|----|----|
| Removal                 | - | +5 Lys | +4 Nph | +3 bAla | +2 bSer | +1 Nph |
| Replicate 1             | 5,54 | 12,69 | 1,92 | 2,91 | 4,23 |
| Replicate 2             | 7,13 | 11,53 | 1,84 | 2,67 | 3,56 |
| Replicate 3             | 3,98 | 12,93 | 1,79 | 3,19 | 4,33 |
| Mean                    | 5,55 | 12,39 | 1,85 | 2,92 | 4,04 |
| Std. deviation          | 1,57 | 0,75 | 0,07 | 0,26 | 0,42 |

| $\Delta G$ (kcal/mol) | 1 | 2e | 2f | 2g | 2h | 2i |
|-----------------------|---|----|----|----|----|----|
| Removal               | - | +5 Lys | +4 Nph | +3 bAla | +2 bSer | +1 Nph |
| Replicate 1           | -10,61 | -9,60 | -8,10 | -7,71 | -7,08 | -6,22 |
| Replicate 2           | -10,56 | -9,39 | -7,95 | -7,59 | -7,00 | -6,25 |
| Replicate 3           | -10,41 | -9,59 | -8,07 | -7,73 | -7,04 | -6,18 |
| Mean                  | -10,53 | -9,53 | -8,04 | -7,67 | -7,04 | -6,22 |
| Std. deviation        | 0,11 | 0,12 | 0,08 | 0,08 | 0,04 | 0,04 |

| $\Delta \Delta G$ (kcal/mol) | 1 | 2e | 2f | 2g | 2h | 2i | C-term |
|------------------------------|---|----|----|----|----|----|--------|
| Removal                      | - | +5 Lys | +4 Nph | +3 bAla | +2 bSer | +1 Nph |        |
| Replicate 1                  | 1,01 | 1,50 | 0,39 | 0,63 | 0,85 | 4,39 |
| Replicate 2                  | 1,16 | 1,45 | 0,36 | 0,58 | 0,75 | 4,30 |
| Replicate 3                  | 0,82 | 1,52 | 0,34 | 0,69 | 0,87 | 4,23 |
| Mean                         | 1,00 | 1,49 | 0,36 | 0,63 | 0,82 | 4,31 |
| Std. deviation               | 0,17 | 0,04 | 0,02 | 0,05 | 0,06 | 0,08 |
Figure S5. Examples of 14-3-3 binders. Crystal structures of 14-3-3σ (white surface) bound to four different phosphorylated peptides (stick representations); Estrogen Receptor alpha (ERα), p53, Estrogen Related Receptor gamma (ERRγ) and NF-kB p65. Crystal structures represent that most peptides bind within their N-terminal side (right from the phosphoserine) into the binding groove, whereas the C-terminal is often lacking or binding outside the binding groove.
Figure S6. Crystal structures of N-terminal truncated peptides 2c-d. (a/b) Structure of 14-3-3 (white surface) bound to either of the N-terminally truncated peptides (blue sticks). A total view and an enlarged view of the peptide binding. The final 2Fo-Fc electron density map is represented as blue mesh contoured at 1σ. PDB: 7ZMU & 7ZMW
Figure S7a. Crystal structures of known PPI stabilizing compounds. Two different 14-3-3 PPIs, namely 14-3-3/ERα (PDB: 6TL3 & 6HHP), 14-3-3/ERRγ (PDB: 6Y3W), in complex with a pyrrolidone compounds or disulfide tethered fragments (yellow sticks) that stabilizes the PPI complex. A total overview of 14-3-3 (white surface) bound to peptide (reg/green spheres) and the stabilizer (yellow sticks) is shown at the left. An enlarged view is given in the middle panel of compound binding at the PPI interface. The right panel envisions the proximity of these compounds to hotspot residues K122 (yellow sticks) and the hydrophobic patch formed by L218, I219 and L222 (yellow sticks and surface).
Figure S7b. Crystal structures of known PPI stabilizing compounds. Two different 14-3-3 PPIs, namely 14-3-3/p65 (PDB: 6YQ2), 14-3-3/PIN1 (PDB: 7BFW), in complex with a pyrrolidone compounds or disulfide tethered fragments (yellow sticks) that stabilizes the PPI complex. A total overview of 14-3-3 (white surface) bound to peptide (blue/pink spheres) and the stabilizer (yellow sticks) is shown at the left. A enlarged view is given in the middle panel of compound binding at the PPI interface. The right panel envisions the proximity of these compounds to hotspot residues K122 (yellow sticks) and the hydrophobic patch formed by L218, I219 and L222 (yellow sticks and surface).
Figure S8. Sequences point mutated peptides 3a, 4a-c, 5a-c. Schematic representation and chemical structure of peptide 1 and the point mutations. Within these peptides point mutations are made at position -1 (Thz) leading to peptide 3a, position +1 (Nph) leading to peptides 4a-c, and position +4 (Nph) leading to peptides 5a-c. Mutations were made to alanine, tyrosine and phenylalanine residues.
Figure S9. FA assay mutant peptides. (a) Fluorescence anisotropy assay of 14-3-3γ titration to a fixed concentration (10 nM) of FITC-labelled 1 (orange) and -1 mutated peptide (blue). (b) Fluorescence anisotropy assay of 14-3-3γ titration to a fixed concentration (10 nM) of FITC-labelled 1 (orange) and +1 mutated peptides (green). (c) Fluorescence anisotropy assay of 14-3-3γ titration to a fixed concentration (10 nM) of FITC-labelled 1 (orange) and +4 mutated peptides (green). (d) Bar plot representation from obtained binding affinities ($K_D$) of mutated peptides and the fold change in affinity between different constructs. All data is recorded in triplicate from three independent experiments. The $K_D$ obtained from each of the independent experiments is shows as single point and the bar represents the mean $K_D$ of these three datapoints.
Table S4. Overview $K_d$ and $\Delta\Delta G$ analysis mutated peptides (FA-based assays). $K_d$ values are determined from the FA-based binding studies of 14-3-3 to each of the peptides. The fold change in binding affinity is determined by dividing the $K_d$ from a peptide with the $K_d$ of 1. Furthermore the $K_d$ is transformed into $\Delta G$ values which in the end is transformed into a $\Delta\Delta G$ analysis by subtracting the $\Delta G$ from each peptide with the $\Delta G$ from 1.

| Affinity Kd (M) | 1 | 3a | 4a | 4b | 4c | 5a | 5b | 5c |
|---------------|--|----|----|----|----|----|----|----|
| Mutant        | - | Nph+4Ala | Nph+1Tyr | Nph+1Phe | Nph+1Ala | Nph +4Tyr | Nph +4Phe | Nph +4Ala |
| Replicate 1   | 5.2E-08 | 1.0E-07 | 4.7E-08 | 4.6E-08 | 1.2E-06 | 1.7E-06 | 1.3E-06 | 3.2E-06 |
| Replicate 2   | 7.1E-08 | 4.2E-08 | 4.1E-08 | 3.3E-08 | 1.2E-06 | 1.2E-06 | 1.2E-06 | 2.2E-06 |
| Replicate 3   | 7.4E-08 | 7.2E-08 | 6.3E-08 | 8.7E-08 | 1.6E-06 | 1.7E-06 | 1.8E-06 | 2.9E-06 |
| Mean          | 6.6E-08 | 7.1E-08 | 5.0E-08 | 5.5E-08 | 1.3E-06 | 1.5E-06 | 1.4E-06 | 2.8E-06 |
| Std. deviation| 1.2E-08 | 3.0E-08 | 1.1E-08 | 2.8E-08 | 2.0E-07 | 2.7E-07 | 3.5E-07 | 5.2E-07 |

| Fold change to 1 | 1 | 3a | 4a | 4b | 4c | 5a | 5b | 5c |
|------------------|--|----|----|----|----|----|----|----|
| Mutant           | - | Nph+4Ala | Nph+1Tyr | Nph+1Phe | Nph+1Ala | Nph +4Tyr | Nph +4Phe | Nph +4Ala |
| Replicate 1      | 1.9 | 0.9 | 0.9 | 24.0 | 33.2 | 24.3 | 62.1 |
| Replicate 2      | 0.6 | 0.6 | 0.5 | 17.0 | 17.1 | 16.9 | 31.0 |
| Replicate 3      | 1.0 | 0.9 | 1.2 | 21.3 | 22.5 | 24.9 | 39.8 |
| Mean             | 1.2 | 0.8 | 0.8 | 20.8 | 24.2 | 22.0 | 44.3 |
| Std. deviation   | 0.7 | 0.2 | 0.4 | 3.5 | 8.2 | 4.4 | 16.0 |

| $\Delta G$ (kcal/mol) | 1 | 3a | 4a | 4b | 4c | 5a | 5b | 5c |
|-----------------------|--|----|----|----|----|----|----|----|
| Mutant                | - | Nph+4Ala | Nph+1Tyr | Nph+1Phe | Nph+1Ala | Nph +4Tyr | Nph +4Phe | Nph +4Ala |
| Replicate 1           | -9.9 | -9.5 | -10.0 | -10.0 | -8.1 | -7.9 | -8.0 | -7.5 |
| Replicate 2           | -9.7 | -10.1 | -10.1 | -10.2 | -8.1 | -8.1 | -8.1 | -7.7 |
| Replicate 3           | -9.7 | -9.7 | -9.8 | -9.6 | -7.9 | -7.9 | -7.8 | -7.5 |
| Mean                  | -9.8 | -9.8 | -10.0 | -9.9 | -8.0 | -7.9 | -8.0 | -7.6 |
| Std. deviation        | 0.1 | 0.3 | 0.1 | 0.3 | 0.1 | 0.1 | 0.1 | 0.1 |

| $\Delta\Delta G$ (kcal/mol) to 1 | 1 | 3a | 4a | 4b | 4c | 5a | 5b | 5c |
|---------------------------------|--|----|----|----|----|----|----|----|
| Mutant                          | - | Nph+4Ala | Nph+1Tyr | Nph+1Phe | Nph+1Ala | Nph +4Tyr | Nph +4Phe | Nph +4Ala |
| Replicate 1                     | 0.4 | -0.1 | -0.1 | 1.9 | 2.1 | 1.9 | 2.4 |
| Replicate 2                     | -0.3 | -0.3 | -0.5 | 1.7 | 1.7 | 1.7 | 2.0 |
| Replicate 3                     | 0.0 | -0.1 | 0.1 | 1.8 | 1.8 | 1.9 | 2.2 |
| Mean                            | 0.0 | -0.2 | -0.1 | 1.8 | 1.9 | 1.8 | 2.2 |
| Std. deviation                  | 0.4 | 0.1 | 0.3 | 0.1 | 0.2 | 0.1 | 0.2 |
Figure S10A. Raw ITC data 1 and 3a. Measured raw thermograms, binding isotherms and derived thermodynamic values for 14-3-3γ binding to 1 and 3a (Thz-1Ala). Used concentrations are state in the graph. A constant blank model is used to correct for heat of injection and independent model is used to model peptide binding (blue line). From this the thermodynamic parameters are determined as noted in each table associated with the thermograms. Each peptide is measured in two independent experiments (replicate 1 and 2) which are both shown here. **For thermodynamic parameters refer to Table S6.
Figure S10B. Raw ITC data 4a and 4b. Measured raw thermograms, binding isotherms and derived thermodynamic values for 14-3-3γ binding to 4a (Nph+1Tyr) and 4b (Nph+1Phe). Used concentrations are state in the graph. A constant blank model is used to correct for heat of injection and independent model is used to model peptide binding (blue line). From this the thermodynamic parameters are determined as noted in each table associated with the thermograms. Each peptide is measured in two independent experiments (replicate 1 and 2) which are both shown here. **For thermodynamic parameters refer to Table S6.
Figure S10C. Raw ITC data 4c and 5a. Measured raw thermograms, binding isotherms and derived thermodynamic values for 14-3-3\(\gamma\) binding to 4c (Nph+1Ala) and 5a (Nph+4Tyr). Used concentrations are state in the graph. A constant blank model is used to correct for heat of injection and independent model is used to model peptide binding (blue line). From this the thermodynamic parameters are determined as noted in each table associated with the thermograms. Each peptide is measured in two independent experiments (replicate 1 and 2) which are both shown here. **For thermodynamic parameters refer to Table S6.
Figure S10D. Raw ITC data 5b and 5c. Measured raw thermograms, binding isotherms and derived thermodynamic values for 14-3-3γ binding to 5b (Nph+4Phe) and 5c (Nph+4Ala). Used concentrations are state in the graph. A constant blank model is used to correct for heat of injection and independent model is used to model peptide binding (blue line). From this the thermodynamic parameters are determined as noted in each table associated with the thermograms. Each peptide is measured in two independent experiments (replicate 1 and 2) which are both shown here. **For thermodynamic parameters refer to Table S6.
Table S5. Overview $K_d$ and $\Delta \Delta G$ analysis mutated peptides (ITC-based assays). $K_d$ values are determined from the ITC-based binding studies of 14-3-3 to each of the peptides. The fold change in binding affinity is determined by dividing the $K_d$ from a peptide with the $K_d$ of peptide 1. Furthermore the $K_d$ is transformed into $\Delta G$ values which in the end is transformed into a $\Delta \Delta G$ analysis by subtracting the $\Delta G$ from each peptide with the $\Delta G$ from peptide 1.

| Affinity Kd (M) | 1 | 3a | 4a | 4b | 4c | 5a | 5b | 5c |
|----------------|---|----|----|----|----|----|----|----|
| Mutant         |   | Nph+4Ala | Nph+1Tyr | Nph+1Phe | Nph+1Ala | Nph+4Tyr | Nph+4Phe | Nph+4Ala |
| Replicate 1    |   | 4.5E-08 | 8.9E-08 | 1.3E-07 | 2.6E-08 | 6.7E-07 | 1.6E-06 | 1.1E-06 | 3.5E-06 |
| Replicate 2    |   | 4.5E-08 | 7.0E-08 | 6.1E-08 | 3.8E-08 | 8.6E-07 | 2.6E-06 | 1.8E-06 | 4.3E-06 |
| Mean           |   | 4.5E-08 | 7.9E-08 | 9.4E-08 | 3.2E-08 | 7.6E-07 | 2.1E-06 | 1.5E-06 | 3.9E-06 |
| Std. deviation |   | 4.2E-10 | 1.3E-08 | 4.6E-08 | 8.1E-09 | 1.3E-07 | 7.2E-07 | 5.6E-07 | 6.0E-07 |

| Fold change to 1 | 1 | 3a | 4a | 4b | 4c | 5a | 5b | 5c |
|------------------|---|----|----|----|----|----|----|----|
| Mutant           |   | Nph+4Ala | Nph+1Tyr | Nph+1Phe | Nph+1Ala | Nph+4Tyr | Nph+4Phe | Nph+4Ala |
|                  |   | 2.0   | 2.8   | 0.6   | 15.0  | 15.0  | 34.7  | 23.6  |
|                  |   | 1.5   | 1.3   | 0.8   | 18.9  | 18.9  | 56.7  | 40.8  |
| Mean             |   | 1.8   | 2.1   | 0.7   | 17.0  | 17.0  | 45.7  | 32.2  |
| Std. deviation   |   | 0.3   | 1.0   | 0.2   | 2.7   | 2.7   | 15.6  | 12.2  |

| $\Delta G$ (kcal/mol) | 1 | 3a | 4a | 4b | 4c | 5a | 5b | 5c |
|-----------------------|---|----|----|----|----|----|----|----|
| Mutant                |   | Nph+4Ala | Nph+1Tyr | Nph+1Phe | Nph+1Ala | Nph+4Tyr | Nph+4Phe | Nph+4Ala |
| Replicate 1           |   | -10.0  | -9.6  | -9.4  | -10.3 | -8.4  | -7.9  | -7.9  | -7.4  |
| Replicate 2           |   | -10.0  | -9.8  | -9.8  | -10.1 | -8.3  | -7.6  | -7.8  | -7.3  |
| Mean                  |   | -10.0  | -9.7  | -9.6  | -10.2 | -8.3  | -7.8  | -7.9  | -7.4  |
| Std. deviation        |   | 0.0    | 0.1    | 0.3    | 0.1    | 0.2    | 0.1    | 0.1    |

| $\Delta \Delta G$ (kcal/mol) to 1 | 1 | 3a | 4a | 4b | 4c | 5a | 5b | 5c |
|-----------------------------------|---|----|----|----|----|----|----|----|
| Mutant                            |   | Nph+4Ala | Nph+1Tyr | Nph+1Phe | Nph+1Ala | Nph+4Tyr | Nph+4Phe | Nph+4Ala |
|                                  |   | 0.4   | 0.6   | -0.3  | 1.6   | 2.1   | 2.1   | 2.6   |
|                                  |   | 0.3   | 0.2   | -0.1  | 1.7   | 2.4   | 2.2   | 2.7   |
| Mean                             |   | 0.3   | 0.4   | -0.2  | 1.7   | 2.2   | 2.1   | 2.6   |
| Std. deviation                   |   | 0.1   | 0.3   | 0.1   | 0.1   | 0.2   | 0.1   | 0.1   |
Figure S11. $K_d$ and $\Delta \Delta G$ analysis of mutant peptides. (a) Bar plot representation of $K_d$ values obtained from both ITC and FP experiments for peptide 1 and the Thz-1 (3a), Nph+1 (4a-c), and Nph+4 (5a-c) mutants. (b) Bar plot representation of the DDG contributions of each Thz-1, Nph+1 and Nph+4 Thz-1 (3a), Nph+1 (4a-c), and Nph+4 (5a-c) mutation as obtained in both ITS and FP experiments.
Table S6. Thermodynamic parameters ITC mutated peptides. Obtained $K_d$, $\Delta H$, $-T\Delta S$, and $\Delta G$ values for 1 and point mutated peptides. Two runs were performed for each peptide and for each of the parameters the average and standard deviation has been calculated.

| Run 1 | 1 | 3a Nph+4Ala | 4a Nph+1Tyr | 4b Nph+1Phe | 4c Nph+1Ala | 5a Nph +4Tyr | 5b Nph +4Phe | 5c Nph +4Ala |
|-------|---|---------------|--------------|-------------|-------------|--------------|--------------|--------------|
| Kd    | 4.47E-08 | 8.86E-08 | 1.26E-07 | 2.62E-08 | 6.72E-07 | 1.55E-06 | 1.06E-06 | 3.48E-06 |
| $\Delta H$ | -10.96 | -7.998 | -5.83 | -8.024 | -4.976 | -6.091 | -4.716 | -2.242 |
| $-T\Delta S$ | 0.933 | -1.624 | -3.582 | -2.391 | -3.445 | -1.834 | -3.227 | -5.204 |
| $\Delta G$ | -10.03 | -9.622 | -9.411 | -10.34 | -8.421 | -7.925 | -7.943 | -7.446 |

| Run 2 | 1 | 3a Nph+4Ala | 4a Nph+1Tyr | 4b Nph+1Phe | 4c Nph+1Ala | 5a Nph +4Tyr | 5b Nph +4Phe | 5c Nph +4Ala |
|-------|---|---------------|--------------|-------------|-------------|--------------|--------------|--------------|
| Kd    | 4.53E-08 | 6.97E-08 | 6.09E-08 | 3.76E-08 | 8.56E-07 | 2.57E-06 | 1.85E-06 | 4.33E-06 |
| $\Delta H$ | -10.45 | -6.809 | -7.45 | -6.783 | -4.649 | -4.328 | -4.725 | -3.539 |
| $-T\Delta S$ | 0.426 | -2.955 | -2.393 | -3.345 | -3.629 | -3.299 | -3.096 | -3.779 |
| $\Delta G$ | -10.02 | -9.764 | -9.844 | -10.13 | -8.278 | -7.626 | -7.821 | -7.317 |

| Average | 1 | 3a Nph+4Ala | 4a Nph+1Tyr | 4b Nph+1Phe | 4c Nph+1Ala | 5a Nph +4Tyr | 5b Nph +4Phe | 5c Nph +4Ala |
|---------|---|---------------|--------------|-------------|-------------|--------------|--------------|--------------|
| Kd      | 4.50E-08 | 7.91E-08 | 9.36E-08 | 3.19E-08 | 7.64E-07 | 2.06E-06 | 1.45E-06 | 3.91E-06 |
| $\Delta H$ | -10.71 | -7.40 | -6.64 | -7.40 | -4.81 | -5.21 | -4.72 | -2.89 |
| $-T\Delta S$ | 0.68 | -2.29 | -2.99 | -2.87 | -3.54 | -2.57 | -3.16 | -4.49 |
| $\Delta G$ | -10.03 | -9.69 | -9.63 | -10.24 | -8.35 | -7.78 | -7.88 | -7.38 |

| Std. deviation | 1 | 3a Nph+4Ala | 4a Nph+1Tyr | 4b Nph+1Phe | 4c Nph+1Ala | 5a Nph +4Tyr | 5b Nph +4Phe | 5c Nph +4Ala |
|----------------|---|---------------|--------------|-------------|-------------|--------------|--------------|--------------|
| Kd            | 4.17E-10 | 1.34E-08 | 4.62E-08 | 8.08E-09 | 1.30E-07 | 7.19E-07 | 5.61E-07 | 5.98E-07 |
| $\Delta H$    | 0.36 | 0.84 | 1.15 | 0.88 | 0.23 | 1.25 | 0.01 | 0.92 |
| $-T\Delta S$ | 0.36 | 0.94 | 0.84 | 0.67 | 0.13 | 1.04 | 0.09 | 1.01 |
| $\Delta G$   | 0.01 | 0.10 | 0.31 | 0.15 | 0.10 | 0.21 | 0.09 | 0.09 |
Figure S12. ITC thermodynamic parameters. Bar plot representation of thermodynamic parameters as obtained from ITC. For each peptide the thermodynamic binding characteristics $\Delta H$, $-T\Delta S$ and $\Delta G$, are shown for its binding to 14-3-3g. (a) 1 and 3a (Thz-1Ala). (b) 1 and 4a-c (Nph+1 mutants). (c) 1 and 5a-c (Nph+4 mutants).
Figure S13. Overlay 14-3-3 binding to AMP/ChREBP and to peptide 2d. (a) Crystal structure of 14-3-3 (white surface) bound to ChREBP (salmon surface) and AMP (yellow sticks) binding as a stabilizer at the interface between 14-3-3 and ChREBP. PDB: 5F74 (b) Hydrogen bonding and electrostatic interactions (black dashes) between AMP and both 14-3-3 and ChREBP highlighting the binding of AMP on the PPI interface. (c) Structural overlay of 14-3-3/ChREBP and the 14-3-3/2d (blue sticks) binary complexes showing complementarity of 2d and ChREBP binding to 14-3-3. (d) Enlarged view of phosphate group of peptide 2d interacting with the phosphoaccepting group of 14-3-3 (black dashes) and its potential interactions with ChREBP (salmon dashes). Showing the potential of 2d to act as a cooperative ligand between 14-3-3 and ChREBP. (e-f) Structural overlay of AMP and 2d binding to 14-3-3 (and ChREBP) showing similarity in binding of both AMP and 2d in the phosphate binding pocket.
Figure S14. Overlay ChREBP and peptide 2c. (a) Crystal structure of 14-3-3 (white surface) bound to 2c (blue sticks), PDB: 7ZMW. (b) Crystal structure of 14-3-3 (white surface) bound to ChREBP peptide (salmon sticks and cartoon), PDB: 5F74. (c) Structural overlay of 2c with thiazolylalanine (Thz) at -1 position (blue sticks) and ChREBP (salmon sticks/cartoon) bound to 14-3-3 (white surface) showing a clear steric clash (red arrow) in peptide binding.
Figure S15. Cooperativity analysis. (a) Cooperativity square representation of the 14-3-3/ChREBP/2d complex formation including binding affinities. The interaction is stabilized by factor alpha (α), reducing the apparent affinity of 14-3-3 to either of the components when pre-bound to the other component. (b) System description given to model to determine our ternary complex formation system and the determined equilibrium equations as generated by the model. (c) Experimental fluorescence anisotropy data of 14-3-3 titration to FITC-labelled 2d in presence of several concentrations ChREBP peptide. All the given $K_D$ and calculated $K_D$ and a factor are given with a 95% confidence interval. (d) Error-landscape plot centered on the determined $K_D$ and α factors. The contours show that there is a valley of parameter combinations that result in a relatively low mean squared error (MSE).
| Protein | Phosphosite | Sequence | PDB | Ref. |
|---------|-------------|----------|-----|------|
| ChREBP | -           | Ac-DKIRLNNAIWRAWYIQYVKRRKSPV-CONH₂ | 5F74 | 1    |
| ERα    | pT594       | Ac-AEGFPA pT V-COOH | 4JC3 | 2,3  |
| SOS1   | pS1161      | Ac-PRRRPE pS APAES-CONH₂ | 6F08 | 4    |
| BRAF   | pS365       | Ac-RDRSS pS APNVH-CONH₂ | - | 5    |
| ERRγ   | pS79        | Ac-KRRRK pS CQA-CONH₂ | 6Y1D | 6    |
| USP8   | pS718       | Ac-KLKRSY pS SPDITQ-CONH₂ | 6F09 | 7    |
| PIN1   | pS72        | Ac-LVKHSQSRPS pS WRQEK-CONH₂ | 7AOG | 8    |
| P65    | pS45        | Ac-EGRSAG pS IPRRRS-CONH₂ | 6YOW | 9    |
### Table S8. Data collection and refinement statistics (molecular replacement) for 14-3-3σΔc in complex with non-natural N-terminally truncated peptides 2c and 2d

|                          | 14-3-3σΔc in complex with N-terminal truncated peptides 2c-d |
|--------------------------|------------------------------------------------------------|
| **PDB**                  | 7ZMU                                                       | 7ZMW |
| **Peptide**              | 2d                                                         | 2c   |
| **Beam**                 | DESY p11                                                  | Homesource |

#### Data collection

|                          | 14-3-3σΔc | N-terminal truncated peptides 2c-d |
|--------------------------|-----------|-----------------------------------|
| **Space group**          | C 2 2 21  | C 2 2 21                          |
| **Cell dimensions**      |           |                                   |
| a, b, c (Å)              | 82.3 112.0 62.7 | 82.0 111.6 62.5 |
| α, β, γ ()               | 90, 90, 90 | 90, 90, 90                        |
| **Resolution (Å)**       | 45.55 – 1.60 (1.63 – 1.60) | 33.02 – 1.80 (1.84-1.80) |
| I / σ(I)                 | 8.2 (3.5) | 11.2 (3.0)                       |
| **Completeness (%)**     | 100.0 (99.9) | 97.4 (90.1)                   |
| **Redundancy**           | 13.4 (12.3) | 6.0 (5.0)                       |
| **CC1/2**                | 0.988 (0.917) | 0.996 (0.893)                  |

#### Refinement

|                          | 14-3-3σΔc | N-terminal truncated peptides 2c-d |
|--------------------------|-----------|-----------------------------------|
| **No. reflections**      | 38533     | 26185                             |
| **Rwork/Rfree**          | 0.253/0.294 | 0.179/0.221                |
| **No. atoms**            |           |                                   |
| Protein                  | 1975      | 1871                             |
| Ligand/ion               | 112       | 127                              |
| Water                    | 253       | 267                              |
| **B-factors**            |           |                                   |
| Protein                  | 16.22     | 12.48                            |
| Ligand/ion               | 17.61     | 26.05                            |
| Water                    | 25.98     | 22.17                            |
| **R.m.s. deviations**    |           |                                   |
| Bond lengths (Å)         | 0.012     | 0.011                            |
| Bond angles ()           | 1.24      | 1.26                             |
| **Ramachandran**         |           |                                   |
| favored (%)              | 98.29     | 97.86                            |
| outliers (%)             | 0.00      | 0.00                             |
**Experimental procedures**

**14-3-3y protein expression (FP/ITC experiments)**

A pPROEX HTb expression vector encoding the human 14-3-3 protein gamma (14-3-3γ) with an N-terminal his6-tag was transformed by heat shock into NiCo21 (DE3) competent cells. Single colonies were cultured in 50 mL LB medium (100 μg/mL ampicillin). After overnight incubation at 37 °C, cultures were transferred to 2 L TB media (100 μg/mL ampicillin, 1 mM MgCl2) and incubated at 37 °C until an OD600 nm of 0.8-1.2 was reached. Protein expression was then induced with 0.4 mM isopropyl-β-d-thiogalactoside (IPTG), and cultures were incubated overnight at 18°C. Cells were harvested by centrifugation (8600 rpm, 20 minutes, 4°C) and resuspended in lysis buffer (50 mM Hepes, pH 8.0, 300 mM NaCl, 12.5 mM imidazole, 5 mM MgCl2, 2 mM βME) containing cOmplete™ EDTA-free Protease Inhibitor Cocktail tablets (1 tablet/100 ml lysate) and benzonase (1 μl/100 ml). After lysis using a C3 Emulsiflex-C3 homogenizer (Avestin), the cell lysate was cleared by centrifugation (20000 rpm, 30 minutes, 4°C) and purified using Ni2+-affinity chromatography (Ni-NTA superfloow cartridges, Qiagen). Typically two 5 mL columns (flow 5 mL/min) were used for a 2 L culture in which the lysate was loaded on the column washed with 10 CV wash buffer (50 mM Hepes, pH 8.0, 300 mM NaCl, 25 mM imidazole, 2 mM βME) and eluted in several fractions (2-4 CV) of elution buffer (50 mM Hepes, pH 8.0, 300 mM NaCl, 250 mM imidazole, 2 mM βME). Fractions containing the 14-3-3 protein were combined and dialyzed into 25 mM HEPES pH 8.0, 100 mM NaCl, 10 mM MgCl2, 500 μM TCEP. Finally, the protein was concentrated to ~60 mg/mL, analyzed Q-Tof LC/MS, and aliquots were flash-frozen for storage at ~80 °C.

**14-3-3σΔc protein expression (Crystallography)**

A pPROEX HTb expression vector encoding the human 14-3-3 protein sigma truncated after T231 (14-3-3σΔc) and with an N-terminal his6-tag was transformed by heat shock into NiCo21 (DE3) competent cells. Single colonies were cultured in 50 mL LB medium (100 μg/mL ampicillin). After overnight incubation at 37 °C, cultures were transferred to 2 L TB media (100 μg/mL ampicillin, 1 mM MgCl2) and incubated at 37 °C until an OD600 nm of 0.8-1.2 was reached. Protein expression was then induced with 0.4 mM isopropyl-β-d-thiogalactoside (IPTG), and cultures were incubated overnight at 18°C. Cells were harvested by centrifugation (8600 rpm, 20 minutes, 4°C) and resuspended in lysis buffer (50 mM Hepes, pH 8.0, 300 mM NaCl, 12.5 mM imidazole, 5 mM MgCl2, 2 mM βME) containing cOmplete™ EDTA-free Protease Inhibitor Cocktail tablets (1 tablet/100 ml lysate) and benzonase (1 μl/100 ml). After lysis using a C3 Emulsiflex-C3 homogenizer (Avestin), the cell lysate was cleared by centrifugation (20000 rpm, 30 minutes, 4°C) and purified using Ni2+-affinity chromatography (Ni-NTA superfloow cartridges, Qiagen). Typically two 5 mL columns were used for a 2 L culture in which the lysate was loaded on the column washed with 10 CV wash buffer (50 mM Hepes, pH 8.0, 300 mM NaCl, 25 mM imidazole, 2 mM βME) and eluted with several fractions (2-4 CV) of elution buffer (50 mM Hepes, pH 8.0, 300 mM NaCl, 250 mM imidazole, 2 mM βME). Fractions containing the 14-3-3 protein were combined and dialyzed into 25 mM HEPES pH 8.0, 100 mM NaCl, 10 mM MgCl2, 500 μM TCEP. Finally, the protein was concentrated to ~60 mg/mL, analyzed Q-Tof LC/MS, and aliquots were flash-frozen for storage at ~80 °C.

**Peptide synthesis**

The peptides 1 and 2a-d were synthesized on 50 μmol scale on a Rink amide MBHA resin (Novabiochem, 0.52 mmol/g loading) using an automated Intavis MultiPep RSi peptide synthesizer. Deprotection was performed twice per cycle with 20% v/v piperidine in DMF for 8 minutes. Fmoc-protected amino acids were mixed in 5 equivalents of HBTU and 9 equivalents of DIPEA in DMF, and coupled to the resin. With the exception of phosphoserine, which was coupled once for 60 minutes, all amino acids were coupled to the resin twice for 30 minutes. Capping of unreacted amino acids was performed with a mixture of acetic anhydride/pyridine/DMF (1:1:3) for 5 minutes. βAla was incorporated as a spacer between the sequence and Lys(Alloc), to which a FITC fluorophore will be coupled for fluorescence anisotropy studies. The peptides 1 and 2e-i were synthesized manually on a 50 μmol scale using Rink amide MBHA resin (Novabiochem, 0.52 mmol/g loading) via Fmoc based solid phase peptide synthesis. Deprotection was
performed twice per cycle with 20% v/v piperidine in N,N-dimethylformamide (DMF) for five minutes. Fmoc-protected amino acids were mixed with 9 equivalents HBTU (0.38 M in DMF) and 15 equivalents of DIPEA, and coupled to the resin for 30 minutes (except pS, which is coupled for 60 minutes).

Peptides 1 and 2e-i were labeled with fluorescein via a 5-((fluorenylmethylene)oxycarbonyl-amino)-3-oxapentanoic acid (O1-Pen) linker using 5 equivalents of FITC and 7.5 equivalents of DIPEA reacting overnight with continuous agitation. Removal of protecting groups and cleavage of the resin was performed by incubation in a mixture of trifluoroacetic acid (TFA), H₂O, and triisopropylsilane (TIS) (96.5:2.5:1) for 2 hours with continuous agitation, followed by precipitation in an excess of ice-cold diethyl ether (Et₂O).

Peptides 1 and 2a-d were FITC labeled via a three-step approach. The free amine on the N-terminus was protected as follows: to a solution of di-tert-butyl decarbonate (10 eq, 400 mM) in DCM was added DIEA (10 eq) and solution was added to the resin. Coupling was allowed to proceed for 1 h. At this time, resin was washed 3x with DCM and coupling was repeated as described. Resin was washed 5x with DCM. Alloc removal was achieved as follows: resin was treated with a solution of tetrakis(triphenylphosphine)palladium(0) (0.5 eq, 20 mM) and phenylsilane (20 eq) in DCM, 2x 45 min. Resins were then washed 3x with DCM, then 3x with DMF. FITC was installed on the free amine on each C-terminal lysine by treating resin with fluorescein isothiocyanate isomer I (10 eq, 400 mM in 4:1 DMF:DCM) and DIEA (15 eq) for at least 1.5 h. Reactions were kept under aluminum foil for the duration of the coupling. Reaction mixtures were then drained and resins were washed 3x with DMF, 3x with DCM, and dried under reduced pressure. Removal of protecting groups and cleavage of the resin was performed by incubation in a mixture of trifluoroacetic acid (TFA), H₂O, and triisopropylsilane (TIS) (98.5:2.5:1) for 2 hours with continuous agitation, followed by precipitation in excess of ice-cold diethyl ether (Et₂O).

All peptides were purified using preparative HP-LC. This was performed using a Gemini S4 110A 150 x 21.20 mm column using ultrapure water with 0.1% formic acid (FA) and acetonitrile with 0.1% FA with various gradients. Correct mass and purity of peptides was identified using analytical liquid-chromatography coupled with mass-spectrometry (LC-MS) was performed on a C4 Jupiter SuC4300A 150 x 2.0 mm column using ultrapure water with 0.1% formic acid (FA) and acetonitrile with 0.1% FA, in general with a gradient of 5% to 100% acetonitrile over 10 minutes, connected to a Thermo Fisher LCQ Fleet Ion Trap Mass Spectrometer. The purity of the samples was assessed using a UV detector at 254 nm.

**Fluorescence anisotropy (FA) - binary binding studies:** 14-3-3γ was titrated in a 2-fold dilution series (starting at 100 or 400 μM 14-3-3γ) to 10 nM of fluorescein-labeled peptide in FA buffer (10 mM HEPES pH 7.4, 150 mM NaCl, 0.1% (v/v) Tween20, 0.1% (w/v) BSA). Dilution series were made in a polystyrene (non-binding) low-volume Corning Black Round Bottom 384-well plates (Corning 4514 or 4511). Measurements were performed directly after plate preparation, using a Tecan Infinite F500 plate reader at room temperature (λex: 485 ± 20 nm; λem: 535 ± 25 nm; mirror: Dichroic 510; flashes: 20; integration time: 50 ms; settle time: 0 ms; gain: optimal; and Z-position: calculated from well). Wells containing only FITC-peptide were used to set as G-factor at 35 mP. All data were analyzed using GraphPad Prism (7.00) for Windows and fitted using a four-parameter logistic model (4PL). Each measurement was performed in three independent experiments, average and standard deviations were calculated in Excel (see SI tables S1-S4).

**ΔΔG analysis:** Based on the obtained binding affinities from the FA binding studies the fold change in binding affinity is determined by dividing the Kᵣ of one peptide with the Kᵣ from another peptide (fold change = Kᵣ_peptide1/Kᵣ_peptide2). Furthermore the Kᵣ can be change into a ΔG (in kcal/mol) value using ΔG = (ln(Kᵣ))² * 8.314*298/4184. Final ΔΔG value is obtained by subtracting ΔG values from one peptide with the ΔΔG value of another peptide (see SI tables S2-S4).

**Fluorescence anisotropy (FA) - ChREBP titrations:** ChREBP peptide was titrated in a 2-fold dilution series (starting at 100 μM) to 10 nM of fluorescein-labeled peptide 2c and 2d in presence and absence of, respectively, 2 μM and 40 nM 14-3-3γ in FA buffer (10 mM HEPES pH 7.4, 150 mM NaCl, 0.1% (v/v) Tween20, 0.1% (w/v) BSA). Dilution series were made in a polystyrene (non-binding) low-volume Corning Black Round Bottom 384-well plates (Corning 4514 or 4511). Measurements were performed directly after plate preparation, using a Tecan Infinite F500 plate reader at room temperature (λex: 485 ± 20 nm; λem: 535 ± 25 nm; mirror: Dichroic 510; flashes: 20; integration time: 50 ms; settle time: 0 ms; gain: optimal; and Z-position: calculated from well). Wells containing only FITC-peptide were used to set as G-factor at 35 mP. All data were analyzed using GraphPad Prism (7.00) for Windows and fitted using
a four-parameter logistic model (4PL). Each measurement was performed in two independent experiments.

Fluorescence anisotropy (FA) - 2D-14-3-3/ChREBP titrations: 14-3-3γ was titrated in a 2-fold dilution series (starting at 300 μM 14-3-3γ) to 20 nM of fluorescein-labeled peptide 2d in FA buffer (10 mM HEPES pH 7.4, 150 mM NaCl, 0.1% (v/v) Tween20, 0.1% (w/v) BSA) in presence of 2-fold dilution series of unlabeled ChREBP peptide (0.01-10 μM). Dilution series were made in a polystyrene (non-binding) low-volume Corning Black Round Bottom 384-well plates (Corning 4514 or 4511). Measurements were performed directly after plate preparation, using a Tecan Infinite F500 plate reader at room temperature (λex: 485 ± 20 nm; λem: 535 ± 25 nm; mirror: Dicroic 510; flashes: 20; integration time: 50 ms; settle time: 0 ms; gain: optimal; and Z-position: calculated from well). Wells containing only FITC-peptide were used to set as G-factor at 35 mP. All data were analyzed using GraphPad Prism (7.00) for Windows and fitted using a four-parameter logistic model (4PL). Data was obtained and averaged based on two independent experiments.

Cooperativity analysis:
To determine the cooperativity parameters from the 2D-titration of 14-3-3/ChREBP/2d (as described above) we have used the general framework for straightforward model construction of multi-component thermodynamic equilibrium systems as described by Geertjens et al. (2021). This general platform generates a model to describe multi-component equilibrium systems when given a system description. In our case we gave the following system description:

R + P = RP; K0

RP + C = RPC; K0h / α

R + C = RC; K0h

RC + P = RPC; K0h / α

with R = 14-3-3, P = non-natural peptide (labelled), and C = ChREBP. The framework determined based on this system description the equilibrium equations needed to determine K0h and α. The data from 2D-titrations was provided to the model including the K0h at 1.96 mM, P_tot = 10 nM, and the variable concentrations of 14-3-3 and ChREBP at each datapoint. From this the model determined the K0h and α (with 95% confidence interval) and the error-landscape of the determine parameters.

Fluorescence anisotropy (FA) – Selectivity study: Peptides representing 14-3-3 binding partners (including ChREBP) were titrated in a 2-fold dilution series (starting at 100 μM) to 20 nM of fluorescein-labeled peptide 2d in presence and absence of, respectively, 2 μM 14-3-3γ in FA buffer (10 mM HEPES pH 7.4, 150 mM NaCl, 0.1% (v/v) Tween20, 0.1% (w/v) BSA). Dilution series were made in a polystyrene (non-binding) low-volume Corning Black Round Bottom 384-well plates (Corning 4514 or 4511). Measurements were performed directly after plate preparation, using a Tecan Infinite F500 plate reader at room temperature (λex: 485 ± 20 nm; λem: 535 ± 25 nm; mirror: Dicroic 510; flashes: 20; integration time: 50 ms; settle time: 0 ms; gain: optimal; and Z-position: calculated from well). Wells containing only FITC-peptide were used to set as G-factor at 35 mP. All data were analyzed using GraphPad Prism (7.00) for Windows and fitted using a four-parameter logistic model (4PL). Data was obtained and averaged based on two independent experiments.

Isothermal titration calorimetry (ITC)
Final dialysis fluid from protein expression was frozen as 2 mL aliquots to serve as ITC buffer. Protein and peptide were dissolved and diluted in this buffer to reported concentration. DMSO was added if reported and matched in cell and syringe. Samples were degassed for 10 min prior to measurement at 450 mmHg. The reference cell was filled with 300 μL degassed MilliQ water and sample cell with 300 μL of peptide or protein mixture. Syringe was loaded with at least 200 μL protein or peptide sample. Measurements were performed on an Affinity ITC LV (TA instruments), with injection size set to 2 μL, stirring speed of 150 rpm and temperature at 25 °C. The data was processed and analyzed in NanoAnalyze v3.11. The baseline was manually inspected and corrected, after which a blank constant model was fitted to correct for the heat of injection. Subsequently an independent model was fitted, which the Nanoanalyse uses to report the thermodynamic binding properties reported in this paper.

Crystallography
14-3-3αΔC/2c: Peptide 2c was soaked in preformed crystals of 14-3-3αΔC (truncated after T231 to reduce flexibility) with cJun peptide11, which grew in 28% (v/v) PEG400, 5% glycerol, 0.2 M CaCl2, 0.1 M HEPES pH 7.5 within two weeks. The soaked crystal was fished after 14 days of incubation and flash-frozen in liquid nitrogen. Diffraction data was in-house collected at 100 K. X-ray diffraction data were
collected on an in-house Rigaku Micromax-003 (Rigaku, Europe, Kemsing Sevenoaks, UK) equipped with a Dectris Pilatus 200K detector, with the following settings: 360 image, 0.5°/image, 20 s exposure time.

14-3-3αΔC/2d: Peptide 2d and 14-3-3αΔC protein were mixed in complexation buffer (25 mM HEPES pH 7.5, 100 mM MgCl₂, and 2 mM βME) using a final 14-3-3 concentration of 12 mg/mL and a 1:4 protein-peptide molar ratio. After 1h complexation at room temperature, sitting drop crystallization wells were set-up using 250 nL complex mixture and 250 nL precipitation buffer (95 mM HEPES pH 7.1, 0.19 M CaCl₂, 5% glycerol and 27% PEG400). Crystals grew within 14 days at 4 °C. Suitable crystals were fished and flash-cooled in liquid nitrogen. X-ray diffraction data were collected at the p11 beamline of PETRA III facility at DESY (Hamburg, Germany) with the following settings: 1440 image, 0.25°/image, 100% transmission and 0.1 s exposure time. Initial data processing was performed at DESY using XDS after which pre-processed data was taken towards further scaling steps, molecular replacement and refinement.

Data was processed using the CCP4i2 suite (version 7.1.18). DIALS was used to index and integrate the data after which scaling was done using AIMLESS. The data was phased with MolRep, using protein data bank (PDB) entry 4JC3 as a template. A three dimensional structure of peptide was generated using AceDRG, which was thereafter build in based on visual inspection Fo-Fc and 2Fo-Fc electron density map. Sequential model building (based on visual inspection Fo-Fc and 2Fo-Fc electron density map) and refinement were performed with COOT and REFMAC, respectively. Finally, alternating cycles of model improvement (based on isotropic b-factors and standard set of stereochemical restraints: covalent bonds, angels, dihedrals, planarities, chiralities, non-bonded) and refinements were performed using coot and phenix.refine from the Phenix software suite (version 1.20.1-4487). Pymol (version 2.2.3) was used to make the figures and the structures were deposited in the protein data bank (PDB) with IDs: 7ZMU and 7ZMW.

See SI table S8 for crystal statistics.
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