Published in final edited form as:

ACS Chem Biol. 2020 February 21; 15(2): 305–310. doi:10.1021/acscchembio.9b00795.

Analysis of Interactions Stabilized by Fusicoccin A Reveals an Expanded Suite of Potential 14–3–3 Binding Partners

Ananya Sengupta,

Department of Chemistry and Biochemistry, Florida State University, Tallahassee, Florida 32306, United States

Josue Liriano,

Department of Chemistry and Biochemistry, Florida State University, Tallahassee, Florida 32306, United States

Brian G. Miller,

Department of Chemistry and Biochemistry, Florida State University, Tallahassee, Florida 32306, United States

James H. Frederick*

Department of Chemistry and Biochemistry, Florida State University, Tallahassee, Florida 32306, United States

Abstract

Fusicoccin A (FC) is a diterpene glycoside that stabilizes protein–protein interactions (PPIs) between 14–3–3 adapter proteins and their phosphoprotein interaction partners. Recently, FC has gained attention for its pro-apoptotic and neuroprotective properties in cell culture. Although the exact molecular mechanism(s) is (are) unresolved, 14–3–3 PPIs are central to this activity. With the goal of refining the pharmacology of this chemotype, we conducted a systematic analysis of the structural features that govern FC-induced stabilization of 14–3–3 PPIs utilizing a C-terminal phosphorylation recognition motif. This study confirmed that a C-terminal amino acid with a small alkyl group is required for the interaction of FC at canonical C-terminal 14–3–3 PPI interfaces. Using bioinformatics, this structural insight was leveraged to assemble a database of 119 candidate 14–3–3 PPIs that can serve as targets for FC. This group includes a subset of proteins with experimentally determined C-terminal phosphosites that have not been explored as potential targets of FC.

Graphical Abstract

*Corresponding Author: frederich@chem.fsu.edu.

The authors declare no competing financial interest.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at <https://pubs.acs.org/doi/10.1021/acscchembio.9b00795>.

Experimental methods, supplementary fluorescence polarization data, and Table S1 listing a database of 119 candidate C-terminal 14–3–3 PPIs compatible with **1** (PDF)

Protein–protein interactions (PPIs) encompass an important contemporary class of drug targets.^{1,2} Existing small-molecule-based strategies for PPI modulation focus on inhibition. This approach has been effective in instances where a well-defined conformational binding motif (e.g., an α -helix) at the PPI interface supports the rational design of a peptidomimetic.^{3,4} In contrast, the strategy of using a stabilizer molecule to promote PPIs is comparatively underexplored.⁵ In principle, this mode of action provides an avenue to interrogate PPI networks where inhibition is intractable; however, the development of PPI stabilizers is intrinsically challenging because there is no clear starting point for initial ligand identification.

Natural products offer a rich source of molecular scaffolds that can be used to develop PPI stabilizers.⁶ With this idea in mind, we became interested in fusicoccin A (**1**, Figure 1), a complex diterpene glycoside that stabilizes PPIs involving 14–3–3 proteins.^{7,8} These adapter proteins are tightly integrated into phosphorylation-dependent cell signaling pathways, and their aberrant function is linked to the pathobiology of cancer and certain neurological disorders.^{9,10} Nevertheless, 14–3–3 is widely perceived as an intractable target because it forms PPIs with over 500 client proteins.^{11,12} In most cases, these interactions are mediated through a canonical “internal” (RXX_pZXP, X = any residue, _pZ = phosphorylated S or T) phosphorylation recognition motif presented on the surface of client proteins that locates to the amphipathic binding groove of 14–3–3.¹³ Intriguingly, **1** is not compatible with this group of 14–3–3 PPIs. Instead, the selectivity profile of **1** is largely constrained to a distinct part of the 14–3–3 interactome where binding is mediated through an atypical C-terminal phosphorylation recognition motif (X_pZXCOOH).^{14–17}

Recently, **1** has gained attention for its pro-apoptotic and neuroregenerative activity in human cell culture.^{18,19} Although the precise molecular mechanism(s) remain(s) undefined, 14–3–3 PPIs contribute to the pharmacology.²⁰ This view is supported by characterized interactions between **1** and various systems that recapitulate C-terminal 14–3–3 PPIs *in vitro*.¹¹ As a result, further development of this chemotype requires a systematic investigation of the structural features that govern stabilization of C-terminal 14–3–3 PPIs

and a comprehensive profile of 14–3–3 client proteins that are compatible with **1**. To this end, Aducci and Camoni reported that the stabilizing effect of **1** is primarily dependent on the identity of the C-terminal residue of the client phospholigand.²¹ Using *in silico* site-directed mutagenesis, this study predicted that C-terminal recognition motifs with hydrophobic, polar, or charged C-terminal residues could be targets for **1**. Ohkanda subsequently showed that only 14–3–3 clients possessing the aliphatic residues I, V, and L at the C-terminus were strongly stabilized by **1**.²² However, this study utilized a semisynthetic variant of **1** harboring a modified diterpene core attached to a non-natural furanose ring system. As a result, we set out to resolve this discrepancy and, in the process, develop a tool to predict C-terminal 14–3–3 PPIs that are compatible with biosynthetic **1**.

Our investigation began by selecting a suitable C-terminal 14–3–3 PPI to benchmark the activity of **1**. As shown in Figure 1, the complex formed between 14–3–3 σ and estrogen receptor alpha (ER α) possessed several attractive features: (i) The 14–3–3 interaction site of ER α is located at the C-terminus of the F-domain, and binding to 14–3–3 is known to be dependent on phosphorylation of the penultimate threonine (T594) *in vivo*. (ii) The dynamics between 14–3–3 σ and ER α have been assayed *in vitro* using a synthetic peptide (15 amino acids) harboring the 14–3–3 recognition sequence (⁵⁹⁰GFPA_pTVCOOH), referred to herein as the ER α C-terminal phospholigand (ER α -ctp, **2**), and (iii) an X-ray structure of the 14–3–3 σ -ER α -ctp-**1** ternary complex (Protein Data Bank [PDB] ID: 4JDD, 2.1 Å resolution) was available to guide our analysis.²³ Notably, among the seven isoforms of 14–3–3 present in humans, 14–3–3 σ is arguably the most important in cancer biology.^{9,24} Therefore, we focused on analyzing interactions between full-length, recombinant 14–3–3 σ (see the SI for details) and the synthetic ER α -ctp mimetic **2** in the presence and absence of **1**.

As shown in Figure 2, we started by establishing a uniform method to evaluate the stabilization induced by biosynthetic **1**. Using isothermal titration calorimetry (ITC), we found that peptide **2** binds to 14–3–3 σ with a K_d of $0.75 \pm 0.14 \mu\text{M}$. In contrast, the K_d of **2** for 14–3–3 σ decreased to $0.02 \pm 0.05 \mu\text{M}$ when $200 \mu\text{M}$ **1** was present. Thus, **1** stabilizes the 14–3–3 σ -ER α -ctp complex by approximately 40-fold. This result was confirmed by fluorescence polarization (FP) experiments. Using this technique, we determined that the apparent affinity of fluorescein-labeled **2** (N-FAM-**2**) and 14–3–3 σ was enhanced by approximately 70-fold, from an apparent K_d of $3.61 \pm 0.41 \mu\text{M}$ to $0.05 \pm 0.02 \mu\text{M}$, when titrated in the presence of $80 \mu\text{M}$ **1**. We also found that **1** stabilized the 14–3–3 σ -N-FAM-**2** complex with an EC_{50} of $3.16 \pm 1.03 \mu\text{M}$. Given that the apparent affinity determined by FP using N-FAM-**2** was comparable to the K_d measured by ITC using **2**, we concluded that the more convenient fluorescence polarization-based assay would be used to analyze 14–3–3/phospholigand interactions moving forward.

Next, we chose to revisit a series of structurally distinct C-terminal 14–3–3 PPIs from the literature. The five targets we selected have been analyzed in the presence and absence of **1** previously;²¹ however, in most cases, existing studies relied on alternative analytical methods, different 14–3–3 isoforms, and/or longer synthetic phosphopeptides. As shown in Table 1, we focused on the interactions of 14–3–3 σ and a series of fluorescein-labeled hexaphosphopeptides replicating the C-terminal recognition sequences of the potassium ion

channel Task-3,²⁵ the platelet adhesion receptor GpIB α ,²⁶ the G-protein coupled receptor GPR15,²⁷ the interleukin 9 receptor α chain (IRL9),²⁸ and the cyclin-dependent kinase inhibitor p27Kip1.²⁹ Importantly, our analysis of the 14–3–3 σ -Task3-ctp complex in the absence ($K_d = 3.15 \pm 1.12 \mu\text{M}$) and presence of 80 μM **1** ($K_d = 0.10 \pm 0.05 \mu\text{M}$) were in agreement with data reported by Ottmann using a similar FP assay and identical interaction partners.²⁵ We also observed a stabilizing effect of **1** in combination with 14–3–3 σ and either GpIB α -ctp ($S_F = 14 \pm 2$) or GPR15-ctp ($S_F = 7 \pm 2$). Notably, both sequences possess a C-terminal leucine, and the magnitude of increased affinity induced by **1** is consistent with literature reports.²¹ In contrast, the IRL9-ctp, which terminates in phenylalanine, was not strongly stabilized by **1** ($S_F = 3 \pm 1$). Similarly, **1** did not markedly enhance the affinity of 14–3–3 σ for the p27Kip1-ctp, which possesses a C-terminal phosphothreonine ($S_F = 3 \pm 2$), and intriguingly, the innate affinity of the 14–3–3 σ -p27Kip1-ctp complex itself was diminished (apparent $K_d = 82.2 \pm 31.0 \mu\text{M}$). Taken together, these results reinforce the idea that the identity of the final residue within the C-terminal recognition motif is a key determinant of the activity of **1** at 14–3–3 PPI interfaces. These data also suggest that the C-terminal residue plays a direct role in innate binding to 14–3–3.

Analysis of the 14–3–3 σ -ER α -ctp-**1** ternary structure shown in Figure 1 shows that interactions between **1** and client phospholigand **2** in the 14–3–3 σ binding groove are restricted to a hydrophobic contact between the 5–8–5 tricyclic core of **1** and the isopropyl side chain of the C-terminal valine within **2**. On the basis of this observation, we hypothesized that amino acids with small alkyl side chains could make similar contacts with **1** and therefore support formation of a ternary complex. However, it was more challenging to predict which of these C-terminal residues might support binding to 14–3–3 σ in the absence of **1**, particularly given the intrinsic plasticity of the 14–3–3 binding groove that can accommodate hundreds of client phosphoproteins.¹² Therefore, to interrogate these features systematically, we analyzed a focused library of *N*-FAM-**2** variants with single amino acid changes at the C-terminus.

As summarized in Table 2, we observed a significant difference in the affinity of ER α -ctp variants for 14–3–3 σ depending on the identity of the C-terminal residue. The results were divided into three groups: The first group contains hydrophobic residues I and V, which had micromolar affinities for 14–3–3 σ (apparent $K_d \sim 3.0$ – $3.5 \mu\text{M}$). A second group includes residues F, L, and A, which exhibited moderate affinities for 14–3–3 σ (apparent $K_d \sim 10.5$ – $20.0 \mu\text{M}$). The last group contained T, N, S, G, and D, which showed comparably weak affinity for 14–3–3 σ (apparent K_d 50.0–200 μM). These titrations highlight a clear preference for docking of a hydrophobic C-terminal residue in the 14–3–3 binding groove.

Next, we compared the affinities of our phospholigand library with 14–3–3 σ in the presence of 80 μM **1**. Consistent with our hypothesis, variants of *N*-FAM-**2** bearing a C-terminal residue with an alkyl side chain were stabilized by **1**. We found that residues I, V, L, A, T, and S supported a substantial decrease in apparent K_d in the presence of **1** (>20-fold, Table 2). The effect of **1** on the alanine variant was particularly striking. In this case, the apparent affinity to 14–3–3 σ was enhanced by approximately 30-fold, from a K_d of $19.9 \pm 6.99 \mu\text{M}$ to $700 \pm 50 \text{ nM}$, in the presence of 80 μM **1**. When interpreted in terms of ΔG of stabilization ($\text{kcal}\cdot\text{mol}^{-1}$), the relative ranking of C-terminal residues was found to be V or L (2.5) > I

(2.3) > T (2.2) > A (2.0) > S (1.8). In contrast, variants of *N*-FAM-2 harboring F, N, G, and D at the C-terminus were not stabilized to a large extent by **1** ($\Delta G < 0.9$ kcal/mol). These results point to an upper (N) and lower (G) constraint on the size of amino acid side chains that support interactions with **1** in the 14–3–3 binding groove. In this regard, hydrophobic residues L, V, and I with branched side chains were optimal, and the stabilizing effect of **1** decreased as the size of the C-terminal residue was decreased (i.e., V $\rightarrow$ A $\rightarrow$ G). A direct comparison of the V and T variants of *N*-FAM-2 indicated that polar functional groups were tolerated but diminished the stabilizing effect of **1**. Consistent with this model, we found that the S variant of *N*-FAM-2 was stabilized by 22-fold in the presence of **1**, whereas the corresponding A variant was stabilized by 29-fold under identical conditions. In contrast, slightly larger polar C-terminal residues (i.e., N) and C-terminal residues with small, charged side chains (i.e., D) disrupt hydrophobic contacts with **1**.

With an improved picture of the structural criteria required for stabilization by **1**, we employed bioinformatics to establish the scope of potential 14–3–3 client phosphoproteins that might serve as biological targets for **1** *in vivo*. Thus, using the C-terminal recognition motif as a search criterion, we performed a BLAST search to identify human proteins terminating in the motif -XZB_{COOH} (Z = T or S and B = V, L, I, T, A, or S).³⁰ This inquiry returned over 800 unique FASTA sequences, which were further subjected to filtering algorithms. Using NetPhos 3.1,³¹ we compiled a list of proteins with predicted phosphorylation at the penultimate S or T residue. These putative client phosphoproteins were then filtered using the 14–3–3 Prediction Tool.³² This analysis returned a list of 119 C-terminal 14–3–3 PPIs that are candidate targets for **1** (the full list is reported in the SI). Next, utilizing the PhosphoSitePlus database, we evaluated each candidate phosphoprotein for experimentally validated phosphorylation at the predicted phosphosite.³³ This search uncovered a subset of proteins within our initial list that have a reported C-terminal 14–3–3 phosphorylation recognition motif (Figure 3). These proteins are the most likely to be new targets for **1** *in vivo*. Importantly, our bioinformatics analysis also captured the C-terminal 14–3–3 PPIs previously reported with **1**, including the sequences shown in Table 1.

As shown in Figure 3, this study identified several disease relevant proteins that have not been explored as interaction partners for 14–3–3 or as biological targets of **1**. Thus, in an initial effort to validate the results of our search, we probed interactions between 14–3–3 σ and phosphopeptides replicating the putative C-terminal 14–3–3 recognition domains of two structurally distinct candidate proteins from our list. The first was nucleoporin 160 (Nup160-ctp, **3**),³⁴ which binds to 14–3–3 σ with an apparent K_d of 5.34 ± 0.31 μ M in the absence of **1** and with an apparent K_d of 0.25 ± 0.02 μ M when titrated in the presence of 80 μ M **1** (Figure 3A). The affinity of the second candidate protein, TNF receptor 19 (TNFRSF19-ctp, **4**),³⁵ was also improved from an apparent K_d of 538 ± 141 μ M to 38.0 ± 2.91 μ M, when titrated in the presence of 80 μ M **1** (Figure 3B). Importantly, the 80 μ M concentration of **1** used in these experiments exceeds the concentrations of **1** reported to illicit phenotypic changes in cell culture (~ 2 – 20 μ M) and is far beyond the EC_{50} recorded for the 14–3–3 σ -ER α -cpt PPI *in vitro* (~ 3 μ M).^{18,23} It is also noteworthy that the sequences of these phospholigands are different at every position except for the C-termini, and although **3** was a superior ligand for 14–3–3 σ , the magnitude of stabilization afforded by **1** was comparable for **3** and **4** (20-fold

vs 14-fold, Figure 3C). As such, these results demonstrate that the presence of a small alkyl residue at the C-terminus is the main requirement for stabilization of canonical C-terminal 14–3–3 PPIs by **1**.

In conclusion, we have shown that for C-terminal 14–3–3 PPIs, **1** is best accommodated in the 14–3–3 binding groove when the client phosphoprotein terminates with V, L, I, T, A, or S. Using bioinformatics, we have leveraged this observation to assemble a database of 119 candidate 14–3–3 PPIs that can be targets for **1** *in vivo*. This group is notably enriched in proteins terminating with L, V, or I (68 out of 119), the residues found to be most compatible with **1** in Table 2. To further validate this analysis, we have demonstrated that interactions between 14–3–3 σ and potential phospholigand targets identified from our search are enhanced by **1** *in vitro*. More broadly, this study points to an expanded family of potential molecular targets for **1**. As a result, we contend that chemical synthesis will play a critical role unlocking the utility of **1** as a chemical probe of 14–3–3 functions.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

ACKNOWLEDGMENTS

This work was supported by NIH grants R01-GM125926 to J.H.F. and R01-GM115388 to B.G.M. B.G.M. acknowledges support from the Pfeiffer Endowed Professorship for Cancer Research. We thank E. Bienkiewicz (FSU) for assistance in developing fluorescence polarization assays. We are also grateful to B. Stefanovic (FSU) for access to his microplate reader.

REFERENCES

- (1). Wells JA, and McClendon CL (2007) Reaching for the highhanging fruit in drug discovery at protein–protein interfaces. *Nature* 450, 1001–1009. [PubMed: 18075579]
- (2). Scott DE, Bayly AR, Abell C, and Skidmore J (2016) Small molecules, big targets: drug discovery faces the protein–protein interaction challenge. *Nat. Rev. Drug Discovery* 15, 533–550. [PubMed: 27050677]
- (3). Arkin MR, Tang Y, and Wells JA (2014) Small-molecule inhibitors of protein–protein interactions: progressing toward the reality. *Chem. Biol* 21, 1102–1114. [PubMed: 25237857]
- (4). Azzarito V, Long K, Murphy NS, and Wilson AJ (2013) Inhibition of α -helix-mediated protein–protein interactions using designed molecules. *Nat. Chem* 5, 161–173. [PubMed: 23422557]
- (5). Thiel P, Kaiser M, and Ottmann C (2012) Small-molecule stabilization of protein–protein interactions: an underestimated concept in drug discovery. *Angew. Chem., Int. Ed* 51, 2012–2018.
- (6). Milroy L-G, Grossmann TN, Hennig S, Brunsfeld L, and Ottmann C (2014) Modulators of protein–protein interactions. *Chem. Rev* 114, 4695–4748. [PubMed: 24735440]
- (7). Fullone MR, Visconti S, Marra M, Fogliano V, and Aducci P (1998) Fusicoccin effect on the *in vitro* interaction between plant 14–3–3 and plasma membrane H⁺ATPase. *J. Biol. Chem* 273, 7698–7702. [PubMed: 9516476]
- (8). Wurtele M, Jelic-Ottmann C, Wittinghofer A, and Oecking C (2003) Structural view of a fungal phytotoxin acting on a 14–3–3 regulatory complex. *EMBO J.* 22, 987–994. [PubMed: 12606564]
- (9). Hermeking H (2003) The 14–3–3 cancer connection. *Nat. Rev. Cancer* 3, 931–943. [PubMed: 14737123]
- (10). Foote M, and Zhou Y (2012) 14–3–3 proteins in neurological disorders. *Int. J. Biochem. Mol. Biol* 3, 152–164. [PubMed: 22773956]

- (11). Stevers LM, Sijbesma E, Botta M, MacKintosh C, Obsil T, Landrieu I, Cau Y, Wilson AJ, Karawajczyk A, Eickhoff J, Davis J, Hann M, O'Mahony G, Doveston RG, Brunsveld L, and Ottmann C (2018) Modulators of 14–3–3 protein–protein interactions. *J. Med. Chem* 61, 3755–3778. [PubMed: 28968506]
- (12). Pennington KL, Chan TY, Torres MP, and Andersen JL (2018) The dynamic and stress-adaptive signaling hub of 14–3–3: emerging mechanisms of regulation and context-dependent protein–protein interactions. *Oncogene* 37, 5587–5604. [PubMed: 29915393]
- (13). Yaffe MB, Rittinger K, Volinia S, Caron PR, Aitken A, Leffers H, Gambin SJ, Smerdon SJ, and Cantley LC (1997) The structural basis for 14–3–3:phosphopeptide binding specificity. *Cell* 91, 961–971. [PubMed: 9428519]
- (14). Coblitz B, Wu M, Shikano S, and Li M (2006) C-terminal binding: An expanded repertoire and function of 14–3–3 proteins. *FEBS Lett.* 580, 1531–1535. [PubMed: 16494877]
- (15). Johnson C, Crowther S, Stafford MJ, Campbell DG, Toth R, and MacKintosh C (2010) Bioinformatic and experimental survey of 14–3–3 binding sites. *Biochem. J* 427, 69–78. [PubMed: 20141511]
- (16). Ottmann C, Weyand M, Sassa T, Inoue I, Kato N, Wittinghofer A, and Oecking C (2009) A structural rationale for selective stabilization of antitumor interactions of 14–3–3 proteins by cotylenin. *J. Mol. Biol* 386, 913–919. [PubMed: 19244612]
- (17). Fusicoccin A also stabilizes 14–3–3 PPIs involving noncanonical recognition motifs: Stevers LM, Lam CV, Leysen SFR, Meijer F, vanScheppingen DS, de Vries RMJM, Carlile GW, Milroy LG, Thomas DY, Brunsveld L, and Ottmann C (2016) Characterization and small-molecule stabilization of the multisite tandem binding between 14–3–3 and the R domain of CFTR. *Proc. Natl. Acad. Sci. U. S. A* 113, 1152–1161.
- (18). deVries-van Leeuwen IJ, Kortekaas-Thijssen C, NzigouMandouckou JA, Kas S, Evidente A, and de Boer AH (2010) Fusicoccin A selectively induces apoptosis in tumor cells after interferon-alpha priming. *Cancer Lett.* 293, 198–206. [PubMed: 20153922]
- (19). Kaplan A, Morquette B, Kroner A, Leong S, Madwar C, Sanz R, Banerjee SL, Antel J, Bisson N, David S, and Fournier AE (2017) Small-molecule stabilization of 14–3–3 protein–protein interactions stimulates axon regeneration. *Neuron* 93, 1082–1093. [PubMed: 28279353]
- (20). Variants of 1 with enhanced affinity for C-terminal 14–3–3 PPIs in vitro exhibit improved efficacy in cancer cell culture: Andrei SA, de Vink P, Sijbesma E, Han L, Brunsveld L, Kato N, Ottmann C, and Higuchi Y (2018) Rationally designed semisynthetic natural product analogues for stabilization of 14–3–3 protein–protein interactions. *Angew. Chem., Int. Ed* 57, 13470–13474.
- (21). Paiardini A, Aducci P, Cervoni L, Cutruzzola F, Di Lucente C, Janson G, Pascarella S, Rinaldo S, Visconti S, and Camoni L (2014) The phytotoxin fusicoccin differentially regulates 14–3–3 proteins association to mode III targets. *IUBMB Life* 66, 52–62. [PubMed: 24408864]
- (22). Ohkanda J, Kusumoto A, Punzalan I., Masuda R, Wang C, Parvatkar P, Akase D, Aida M, Uesugi M, Higuchi Y, and Kato N (2018) Structural effects of fusicoccin upon upregulation of 14–3–3 phospholigand interaction and cytotoxic activity. *Chem. - Eur. J* 24, 16066–16071. [PubMed: 30238524]
- (23). De Vries-van Leeuwen IJ, da Costa Pereira D, Flach KD, Piersma SR, Haase C, Bier D, Yalcin Z, Michalides R, Feenstra KA, Jimenez CR, de Greef TFA, Brunsveld L, Ottmann C, Zwart W, and de Boer AH (2013) Interaction of 14–3–3 proteins with estrogen receptor alpha F domain provides a drug interface. *Proc. Natl. Acad. Sci. U. S. A* 110, 8894–8899. [PubMed: 23676274]
- (24). Li Z, Liu JY, and Zhang JT (2009) 14–3–3-sigma, the double-edged sword of human cancers. *Am. J. Transl. Res* 1, 326–340. [PubMed: 19956445]
- (25). Anders C, Higuchi Y, Koschinsky K, Bartel M, Schumacher B, Thiel P, Nitta H, Preisig-Muller R, Schlichthorl G, Renigunta V, Ohkanda J, Daut J, Kato N, and Ottmann C (2013) A semisynthetic fusicoccane stabilizes a protein–protein interaction and enhances the expression of K⁺ channels at the cell surface. *Chem. Biol* 20, 583–593. [PubMed: 23601647]
- (26). Camoni L, Di Lucente C, Visconti S, and Aducci P (2011) The phytotoxin fusicoccin promotes platelet aggregation via 14–3–3glycoprotein Ib-IX-V interaction. *Biochem. J* 436, 429–436. [PubMed: 21395556]

- (27). Okamoto Y, and Shikano S (2011) Phosphorylation-dependent C-terminal binding of 14–3–3 proteins promotes cell surface expression of HIV co-receptor GPR15. *J. Biol. Chem* 286, 7171–7181. [PubMed: 21189250]
- (28). Sliva D, Gu M, Zhu XY, Chen J, Tsai S, Du X, and Yang YC (2000) 14–3–3 zeta interacts with the alpha-chain of human interleukin 9 receptor. *Biochem. J* 345, 741–747. [PubMed: 10642536]
- (29). Fujita N, Sato S, and Tsuruo T (2003) Phosphorylation of p27Kip1 at threonine 198 by p90 ribosomal protein S6 kinases promotes its binding to 14–3–3 and cytoplasmic localization. *J. Biol. Chem* 278, 49254–49260. [PubMed: 14504289]
- (30). Altschul SF, Gish W, Miller W, Myers EW, and Lipman DJ (1990) Basic local alignment search tool. *J. Mol. Biol* 215, 403–410. [PubMed: 2231712]
- (31). Blom N, Gammeltoft S, and Brunak S (1999) Sequence and structure-based prediction of eukaryotic protein phosphorylation sites. *J. Mol. Biol* 294, 1351–1362. [PubMed: 10600390]
- (32). Madeira F, Tinti M, Murugesan G, Berrett E, Stafford M, Toth R, Cole C, MacKintosh C, and Barton GJ (2015) 14–3–3-Pred: Improved methods to predict 14–3–3 binding phosphopeptides. *Bioinformatics* 31, 2276–2283. [PubMed: 25735772]
- (33). Hornbeck PV, Kornhauser JM, Tkachev S, Zhang B, Skrzypek E, Murray B, Latham V, and Sullivan M (2012) PhosphoSitePlus: a comprehensive resource for investigating the structure and function of experimentally determined post-translational modifications in man and mouse. *Nucleic Acids Res.* 40, D261–D270. [PubMed: 22135298]
- (34). Vasu S, Shah S, Orjalo A, Park M, Fischer WH, and Forbes DJ (2001) Novel vertebrate nucleoporins Nup133 and Nup160 play a role in mRNA export. *J. Cell Biol* 155, 339–354. [PubMed: 11684705]
- (35). Loftus JC, Dhruv H, Tuncali S, Kloss J, Yang Z, Schumacher CA, Cao B, Williams BO, Eschbacher JM, Ross JTD, and Tran NL (2013) TROY (TNFRSF19) promotes glioblastoma survival signaling and therapeutic resistance. *Mol. Cancer Res.* 11, 865–874. [PubMed: 23699535]

Figure 1.

A key contact between **1** (khaki) and the C-terminal valine of **2** (purple) in the 14–3–3 binding groove (gray) revealed by analysis of the 14–3–3 σ ER α -ctp·**1** ternary complex (PDB 4JDD).

Figure 2.

Stabilization of the 14–3–3σ-ERα-ctp complex by fusicoccin A (1): (a) Isothermal titration calorimetry (ITC) of ERα-ctp (2) binding to 14–3–3σ in the absence (innate) or presence (stabilized) of 200 μM 1. (b) Fluorescence polarization (FP) measurements of fluorescein-labeled N-FAM-ERα-ctp (N-FAM-2) in the absence (innate) or presence (stabilized) of 80 μM 1 titrated with 14–3–3σ to obtain the apparent K_d of the 14–3–3σ-ERα-ctp complex. (c) Fluorescence polarization (FP) measurements of N-FAM-2 and 14–3–3σ titrated with 1 to obtain an EC_{50} value for its stabilizing activity toward the 14–3–3σ-ERα-ctp complex. (d) A comparison of ITC and FP methods for measuring the fold stabilization (S_F) induced by 1. Error bars indicate the mean ± SD of at least two experiments.

candidate 14-3-3 client phosphoprotein	gene	sequence	predicted phosphosite	reported phosphorylation at predicted site	Uniprot I.D.
Nucleoporin 160	NUP160	¹⁴³¹ LYRRTL _{COOH}	T1435	Yes	Q12769
Tumor necrosis factor receptor superfamily member 19	TNFRSF19	⁴¹⁸ QLGSL _{COOH}	S422	No	Q9NS68
SH3 Domain-binding protein 5-like	SH3BPSL	³⁸⁸ QRSVSL _{COOH}	S392	Yes	Q7L8J4
Calcium-binding and coiled-coil domain protein 2	CALCOCO2	⁴⁴¹ VFCHSL _{COOH}	S445	Yes	Q13137
Coiled-coil domain containing protein 87	CCDC87	⁸⁴⁴ SSAASL _{COOH}	S848	Yes	Q9NVE4
Cell division cycle 7-related protein kinase	CDC7	⁵⁶⁹ FKDMSL _{COOH}	S573	Yes	O00311
Tumor-associated calcium signal transducer 2	TACSTD2	³¹⁸ RKEPSL _{COOH}	S322	Yes	P09758
Nucleophosmin	NPM1	²⁸⁹ QWRKSL _{COOH}	S293	Yes	P06748
Ribonuclease H2 subunit A	RNASEH2A	²⁹⁴ ESATSL _{COOH}	S298	Yes	O60447
5-Hydroxytryptamine receptor 2C	HTR2C	⁴⁵³ ERISSV _{COOH}	S457	Yes	P28335
E3 ubiquitin-protein ligase MARCH9	MARCH9	³⁴¹ MRVTTV _{COOH}	T345	No	Q86YJ5
Protein FAM118B	F118B	³⁴⁶ IRGCST _{COOH}	S350	Yes	Q9BPY3
Proapoptotic nucleolar protein 1	PANO1	²¹⁰ PRSAST _{COOH}	S214	No	I0J062
Protein Smaug homolog 1	SAMD4A	⁷¹³ DRTSTI _{COOH}	T717	Yes	Q9UPU9
Potassium voltage-gated channel subfamily B member 1	KCNB1	⁸⁵³ TRDQSI _{COOH}	S857	No	Q14721
Mis18-binding protein 1	MIS18BP1	¹¹²⁷ SNSDSA _{COOH}	S1131	Yes	Q6P0N0
Pleckstrin homology-like domain family A member 1	PHLDA1	³⁹⁶ STSNSA _{COOH}	S400	Yes	Q8NA92

Figure 3.

Select proteins that are putative targets for fusicoccin A (1): (a) FP measurements of fluorescein-labeled Nup160-ctp (3) in the absence (innate) or presence (stabilized) of 80 μM **1** titrated with 14-3-3 σ to obtain the apparent K_d of the 14-3-3 σ -Nup160-ctp complex. (b) The same FP experiment repeated with fluorescein-labeled TNFRSF19-ctp (4). (c) A comparison of the fold-stabilization (S_F) induced by **1** using phospholigands 3 and 4. Error bars indicate the mean $\pm$ SD of at least two experiments.

Table 1.

Apparent K_d and Fold Stabilization (S_F) of Known C-Terminal 14–3–3 PPIs in the Absence and Presence of 80 μM **1**^a

client protein	phospholigand sequence ^b	innate K_d (μM)	K_d (μM) in presence of 1 ^c	S_F
Task3	RKKR _p SV _{COOH}	3.15 ± 1.12	0.10 ± 0.05	14 ± 2
GpIB α	YSGH _p SL _{COOH}	22.5 ± 9.71	1.69 ± 0.30	14 ± 2
GPR15	KRSV _p SL _{COOH}	7.36 ± 0.36	1.10 ± 0.09	7 ± 1
IRL9	ARSW _p TF _{COOH}	3.16 ± 0.32	1.54 ± 0.26	3 ± 1
p27Kip1	ALRRQ _p T _{COOH}	82.2 ± 31.0	39.9 ± 8.09	3 ± 2

^aReported apparent K_d is the mean $\pm$ standard deviation of at least two experiments.

^bSynthetic hexapeptides were labeled at the *N*-termini with the fluorophore fluorescein.

^cTitration carried out in the presence of 80 μM **1**. See the Supporting Information for details.

Table 2.

Impact of the Modulation of the C-Terminal Residue within ER α -ctp on the Apparent Affinity of the 14–3–3 σ -ER α -ctp Complex in Both the Absence and Presence of **1**^a

C-terminal residue (X)	innate K_d (μ M)	K_d (μ M) in the presence of 1 ^b	G stabilization ($\text{kcal}\cdot\text{mol}^{-1}$) ^c
I	3.03 $\pm$ 0.31	0.06 $\pm$ 0.01	2.3
V	3.61 $\pm$ 0.41	0.05 $\pm$ 0.02	2.5
F	10.5 $\pm$ 3.36	4.03 $\pm$ 0.26	0.6
L	11.0 $\pm$ 2.72	0.17 $\pm$ 0.02	2.5
A	19.9 $\pm$ 6.99	0.70 $\pm$ 0.05	2.0
T	50.0 $\pm$ 13.3	1.32 $\pm$ 0.14	2.2
S	79.8 $\pm$ 9.62	3.54 $\pm$ 0.44	1.8
N	59.5 $\pm$ 13.5	32.4 $\pm$ 4.75	0.3
G	144 $\pm$ 60.0	36.5 $\pm$ 17.3	0.8
D	173 $\pm$ 82.3	48.2 $\pm$ 0.26	0.7

^a Apparent K_d reported as the mean $\pm$ standard deviation of two experiments.

^b Titrations carried out in the presence of 80 μ M **1**. See the SI for details.

^c G stabilization was calculated using mean K_d values in the absence and presence of **1**.