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9798293856060

Author

Virta, Johanna Mae

Publication Date

2025-09-19

Peer reviewed|Thesis/dissertation

Exploring Molecular Glues for 14-3-3 Protein-Protein Interactions

by
Johanna Virta

DISSERTATION

Submitted in partial satisfaction of the requirements for degree of
DOCTOR OF PHILOSOPHY

in

Chemistry and Chemical Biology

in the

GRADUATE DIVISION

of the

UNIVERSITY OF CALIFORNIA, SAN FRANCISCO

Approved:

DocuSigned by:

Michelle Arkin

Michelle Arkin

992885B79658434...

Chair

DocuSigned by:

Danica Galonic Fujimori

Danica Galonic Fujimori

82967A5AE18B4D6...

DocuSigned by:

Frank McCormick

Frank McCormick

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Committee Members

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by

Johanna Mae Virta

Acknowledgments

I would like to thank all the members of the Arkin lab, especially team 14-3-3, for being a wonderful thesis lab. Everyone in the lab contributed to my work through insightful and intellectual conversations, feedback at group meetings, trainings on new instruments or teaching me lab techniques. Dr. Michelle R. Arkin, thank you for being a terrific advisor and your support over the last few years, both scientifically and personally. I learned how to be a true scientist in your lab and gained confidence in my work. Michelle crafts the best lab environment to be welcoming and fun. I would also like to thank the rest of the 14-3-3 team, Dr. Markella Konstantinidou, Dr. Dyana Kenanova, and Dr. Holly Vickery. Markella made all my experiments possible by making the compounds. Dyana taught me screening and mentored me during my rotation. Holly was my constant go-to for advice in all things. It was Dyana and Holly who convinced me to come to UCSF and rotate with Michelle; I cannot thank you two enough. From my visit, I knew I would enjoy working with and eventually becoming friends with them. Holly, I would not have agreed to go to Europe for a few months without you. You have been a great friend addition in SF that has and will continue post UCSF. After Dyana and Holly graduated, Markella stuck it out with me on team 14-3-3 through all of the unexpected experimental outcomes and the sea of rotation students.

I would also like to thank Dr. Ziwen Jiang for being my desk/bench neighbor and giving great advice. Dr. Andrew Ambrose, thank you for sharing in our Midwest nostalgia. Thank you, Maryam Pearson, for helping make protein when I didn't have time. Thank you, Dr. Amanda Paulson, for uploading all of my screening data and being quick to answer all of my hits questions. Thank you to the HTS team, Dr. Jezrael Revalde and Zain Alam, for training me on the fancy instruments. Thank you, too, Maryam and Zain for being my lunch buddies the past year or so; it was so much fun to share baked goods and taste test probiotic sodas with you both. To all of my rotation students and mentors at UCSF, thank you for helping me with experiments and learn how to be an effective mentor.

Before I came to UCSF, the Butcher lab at UW-Madison helped shape me into the scientist I am today. Thank you to Dr. Sam Butcher and Dr. Eric Montemayor for mentoring me. Without the experiences I had in the Butcher lab, I would have quit research a long time ago. To my summer internship lab, the Bjorling lab at the UW-Madison vet school, thank you for teaching me a whole new field of research in urology and handling mice. I learned so much from that summer; one of them being that I don't want to work with mice if I can avoid it. These lab experiences propelled me into grad school and were so valuable in helping me decide what I wanted to pursue in a research career.

Moving across the country to SF, I was nervous to start over with new friends, but I lucked out by having my best friend from childhood across the Bay, Dr. Paige Pistono. Thank you for being there always. I can always count on Paige to jump on board for an adventure or plan super fun outings. Whenever Milo isn't available, I know I can count on Paige to join. Thank you, too, for introducing Milo and I to all your Berkeley friends who have become our friends, as well. For being a group of science nerds, we have had so many key adventures that shaped our experience in the Bay Area and led to amazing memories.

I would also like to thank my CCB cohort for going through all the ups and downs of graduate school with me. We started grad school during the worst time, right after covid lockdown in August 2020, having classes all on Zoom, socially distancing, and not being able to participate in the usual events and milestone. It was difficult coming to a new city and not having all the usual festivities going on during our first year, but our regular Tidelands Terrace hangouts made a huge difference. Thank you for all the fun memories throughout grad school.

I would not be where I am today without the love and support of my family. Thank you, Mom, Dad, Katrina, and Stewart for believing me and my big science dreams from the start. I have the best support system. I love you all. Mom and Dad, you two have helped me achieve where I am today with endless support. You have always encouraged me to pursue my dreams in science, helping set up job shadows and editing admission essays. Katrina and Stewart

paved the path for me growing up and have been incredible role models. They also married great additions to the Virta family, Hayley and Matt, who have added to my expansive support system. I love spending time with my family. Thanks to my nephew, who has only been around for a little bit now but has been a constant joy and stress reliver this past year. I love you all! Grandpa Virta didn't get to see me finish college, but he helped curate my intellectual side by being my very first student when I made him play school with me as a young child. I would also like to thank my partner's family, my second family, the Lingard's, for welcoming me in and their support over the years and raising the amazing man I call my partner.

Lastly, I need to thank my partner, Milo. There are too many things to list all of them. Mostly, thank you for moving to SF for me and dealing with living in a city. You have made my SF experience a million times better. You challenge me to try new things and help push me outside of my comfort zone. Our weekend adventures have helped reset me each week. Thank you, too, for dealing with my science ramblings, practice presentations, and helping me refine my science communication skills so that our families didn't look so lost when I told them about my research. Your support has meant the world to me. You are my person, and I love you.

Contributions

Chapter 1 is a manuscript under review:

Stabilization of native protein-protein interactions with molecular glues (2025).

Johanna M. Virta[‡], Markella Konstantinidou[‡], Michelle R. Arkin

[‡]J.M.V. and M.K. contributed equally to this work.

Chapter 2 is a published manuscript:

A Systematic Approach to the Discovery of Protein–Protein Interaction Stabilizers. *ACS Cent. Sci.* **9**, 937–946 (2023).

Dyana N. Kenanova[‡], Emira J. Visser[‡], Johanna M. Virta, Eline Sijbesma, Frederica Centorrino, Holly R. Vickery, Menqi Zhong, R. Jeffrey Neitz, Luc Brunsveld, Christian Ottmann, and Michelle R. Arkin

[‡]D.N.K. and E.J.V. contributed equally to this work.

Chapter 3 is a published manuscript:

Development of a NanoBRET assay for evaluation of 14-3-3 σ molecular glues. *SLAS Discov.* **29**, 100165 (2024).

Holly R. Vickery[‡], Johanna M. Virta[‡], Markella Konstantinidou, Michelle R. Arkin

[‡]H.R.V. and J.M.V. contributed equally to this work.

Chapter 4 is a published manuscript:

Scaffold-hopping for molecular glues targeting the 14-3-3/ER α complex. *Nat Commun.* **16**, 6467 (2025).

Markella Konstantinidou, Marios Zingiridis, Marloes A.M. Pennings, Michael Fragkiadakis, Johanna M. Virta, Jezrael L. Revalde, Emira J. Visser, Christian Ottmann, Luc Brunsveld, Constantinos G. Neochoritis, Michelle R. Arkin

Chapter 5 is a manuscript under review:

Modulation of the 14-3-3 σ /C-RAF autoinhibited complex by molecular glues (2025).

Markella Konstantinidou[‡], Holly R. Vickery[‡], Marloes A.M. Pennings[‡], Johanna M. Virta, Emira J. Visser, Sean Bannier, Mrudula Srikanth, Sabine Z. Cismoski, Lucy C. Young, Maxime C.M. van den Oeterlaar, Frank McCormick, Christian Ottmann, Luc Brunsveld, Michelle R. Arkin

[‡] Authors contributed equally

Chapter 6 is a manuscript in preparation:

Restoring the 14-3-3/CRAF regulatory interaction in Noonan Syndrome using molecular glues (2025).

Johanna M. Virta, Holly R. Vickery, Markella Konstantinidou, Mckenna C. Crawford, Marloes A.M. Pennings, Christian Ottmann, Luc Brunsveld, Michelle R. Arkin

Chapter 7 is unpublished work:

Screening for Noonan mutant-selective 14-3-3/CRAF stabilizing fragments.

Johanna M. Virta

Exploring Molecular Glues for 14-3-3 Protein-Protein Interactions

Johanna Mae Virta

Abstract

14-3-3 regulates the activity of thousands of client proteins in various biological pathways through physical occlusion, structural conformations, and scaffolding protein-protein interactions (PPIs). Utilizing the role of 14-3-3 to regulate client proteins, the Arkin lab has developed molecule glues (MGs) that stabilize native 14-3-3/client interactions, harnessing the negative regulatory interactions to “turn off” client proteins’ functions in disease. By targeting PPIs as opposed to singular proteins, selectivity can be enhanced through selective MGs that bind the unique composite interfaces for a PPI of interest. This also allows for unstructured proteins/regions that were previously difficult to target to gain new induced binding pockets for modulators for the development of novel therapeutics.

The work presented in this dissertation showcases the work on exploring MGs for various 14-3-3/client interactions, with focus on the 14-3-3/ER α and 14-3-3/CRAF complexes. These client proteins represent crucial targets in cancer (ER α and CRAF) and in developmental RASopathies (CRAF) that have distinct functions and binding modes to 14-3-3. Capitalizing on the differences allowed for the development of selective MGs and assays to characterize the effects of stabilizing these 14-3-3/client interactions. Work along each step of the drug discovery and development for 14-3-3/client MGs is presented in this dissertation: screening and validation of stabilizing fragments, optimization of fragment hits into selective MGs, and characterization of cell-active MGs. The lessons learned in each chapter provide insight into 14-3-3 biology in the regulation of transcription factors (ER α) and kinases (CRAF) as well as serving as building blocks to expand stabilization to other native or neomorphic PPIs.

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Chapter 1

Stabilization of native protein-protein interactions with molecular glues

Conspectus

Protein-protein interactions (PPIs) play a key role in homeostasis and are often dysregulated in disease. PPIs were traditionally considered “undruggable” due to their flat surfaces and disordered domains. Recently, the identification of PPI stabilizers or molecular glues (MGs), compounds that bind cooperatively to PPI interfaces, has provided a new direction for the field. MGs offer exciting opportunities for chemical biology and drug discovery, particularly for intrinsically disordered domains. To date, many of the fascinating MGs were discovered serendipitously and their molecular glue mechanism of action was understood retrospectively. Our collaborative contribution has been the development of systematic, rational approaches for the identification, optimization and validation of MGs.

This review focuses on the modulation of the native PPIs between the hub protein 14-3-3 and its client proteins. 14-3-3 recognizes specific phospho-serine/threonine motifs on disordered domains of hundreds of clients and -- depending on the phospho-site -- can activate or inhibit signaling pathways. Until recently, only the natural product fusicoccin-A (FC-A) and its analogs were known to bind at the structured 14-3-3/client interfaces and modulate cellular pathways. The complexity of the natural products significantly hindered chemical biology approaches and did not provide sufficient insight for the systematic, selective targeting of the client of interest.

Inspired by the natural products, our team used fragment-based screens to identify new chemical matter for 14-3-3/client PPIs. Using disulfide-tethering technology, we targeted reversibly either engineered cysteines on 14-3-3 or the native cysteine (C38) on 14-3-3 σ . Five clients (ER α , C-RAF, FOXO1, USP8 and SOS1), representing varying sequences, binding modes and physiological roles, were included in the initial screens. We identified both selective and non-selective fragments, suitable for medicinal chemistry optimization.

Starting from a fragment that stabilized two 14-3-3 clients, estrogen-receptor α (ER α) and C-RAF, we developed cell-active MGs selective for ER α . ER α is a well validated target in

breast cancer and 14-3-3 is a negative regulator that blocks ER α transcriptional activity. We used structure-guided design to optimize ligand-protein interactions at the composite PPI surface. The molecular glues were validated in biophysical assays, including intact mass spectrometry (MS) and fluorescence anisotropy (FA) assays, allowing the quantification of binding, kinetics and cooperativity.

We explored alternative strategies for the identification and optimization of MGs. For the 14-3-3/ER α complex, we demonstrated fragment linking to generate non-covalent stabilizers and a scaffold-hopping approach using multi-component reaction chemistry. For the 14-3-3/C-RAF complex, we used a fragment-merging approach to selectively stabilize the inhibited state of C-RAF. Binding of 14-3-3 to the inhibitory phospho-S259 site prevents C-RAF dimerization and activation, offering an alternative mechanism to block the MAPK pathway. Finally, we validated compounds in cells, using pathway-specific assays and a series of proximity-based NanoBRET assays to measure cellular PPIs.

These approaches have led to first-in-class MGs for the 14-3-3/ER α and 14-3-3/C-RAF targets. Overall, we have developed a systematic platform for the identification of molecular glues for native PPIs applicable to the broad 14-3-3 interactome and beyond.

Introduction

Protein-protein interactions (PPIs) play a fundamental role in maintaining homeostasis and controlling multiple cellular processes under normal conditions.^{5,6} The dysregulation of native PPI networks either through mutations, altered post-translational modifications or disrupted signaling leads to various diseases from malignancies to developmental disorders.^{7,8} From a drug-discovery perspective, the typically flat, large, hydrophobic surfaces of PPIs and the presence of intrinsically disordered domains led to the common characterization of these systems as “undruggable.”^{9,10} In the past 25 years, there have been beautiful examples of PPI inhibition, often taking advantage of transient or “cryptic” pockets in otherwise shallow sites. By contrast, PPIs formed from intrinsically disordered proteins have been harder to address. This situation has begun to change with the discovery of molecular glues (MGs), compounds that bind cooperatively at PPI interfaces that – as individual proteins – lacked well-defined pockets.^{11,12}

MGs include various types of compounds, from natural products to small molecules, with the CELMoDs^{12–15} (CRBN E3 ligase modulatory drugs) being one of the most famous examples. Most of these fascinating compounds were discovered serendipitously, and the understanding of their mechanism of action was retrospective. The challenge of how to rationally discover MGs for a PPI of interest remains a fundamental goal for advancing the field.

To address this issue, we have developed systematic approaches for the discovery and optimization of MGs, particularly for native PPIs. Our main focus is on the hub protein 14-3-3, a scaffolding protein that recognizes specific phospho-S/T motifs on disordered domains of its clients.¹⁶ The interactome of 14-3-3 includes hundreds of clients, involved in many signaling pathways, thus offering exciting opportunities for chemical biology and drug discovery.¹⁷ To date, many 14-3-3 clients are still considered “undruggable” due to their intrinsically disordered domains. However, upon binding to 14-3-3, a structured binary interface is formed that can be rationally targeted by a MG with the appropriate features of shape complementarity and molecular recognition.

In this review we will elaborate on three major aspects of our work on 14-3-3/client MGs:

- 1) screening approaches and techniques we have used for the identification of new chemical matter
- 2) rational, structure-guided medicinal chemistry optimization and the key principles
- 3) development of cell-based assays to validate MGs

The results of these studies are readily applicable to native PPIs and will be useful to experts and non-experts interested in molecular glues.

Screening Approaches

When we started working on these projects, the only cell-active compounds known to stabilize 14-3-3/client interactions were the natural products fusicoccin-A (FC-A), cotylenin-A (CN-A) and their semi-synthetic analogs^{18,19} (Fig 1.1A-B). The co-crystal structures of the natural products bound to the 14-3-3/client interfaces provided valuable proof for the ligandability of the site; however, their complex structures were difficult to translate towards rational, client-selective glues. At the same time, traditional high-throughput screening had met with limited success, and while screening hits provided proof of concept,^{20–22} they were not advanced to cell-active chemistry. To address the need to identify new, advanceable chemical matter, we turned to fragment-based screening approaches, which have been successful for challenging drug targets such as PPI inhibitors.^{23–26} Fragment-based drug discovery is an established philosophy for discovering ligands with low molecular weight and complexity, followed by optimization towards a drug-like molecule.^{27–29} In support, crystallography-based fragment discovery methods have identified diverse chemical structures that bind to 14-3-3/client complexes near the natural-product binding site.^{30–32}

Utilizing a site-directed, disulfide tethering screen³³, we identify cysteine-reactive fragments that bind in the natural-product binding site and stabilize 14-3-3/client interactions.^{1,34}

The principle behind disulfide tethering is that, because the disulfide bond is rapidly reversible, fragment hits are selected based on thermodynamic principles. Our inhouse disulfide library is comprised of ~2000 fragments spanning chemical space. These fragments contain a mixed disulfide, where one side contains a fragment-like molecule attached to a variable linker and the other side contains a solubilizing group such as 2-(dimethylamino)ethane-1-thiol.

While tethering had been used in the discovery of diverse inhibitors,^{35–37} it had not previously been employed for discovery of MGs. Of the 7 highly homologous 14-3-3 isoforms in humans, only the sigma isoform contained a cysteine residue (C38) positioned at one end of the client-binding groove (Fig 1.1A-B). Screening therefore used the 14-3-3 σ protein and client phospho-peptides that mimicked the 14-3-3 binding motif.¹ The phospho-peptides were considered reasonable models for the full-length client protein because the 14-3-3-binding domains containing the phosphorylation site were already intrinsically disordered. The peptide-protein interaction was incubated with a reducing agent (typically BME) and a single dose of fragment (200 μ M) for 3 hours to allow the reaction to reach equilibrium. Fragment binding was measured by the mass shift via intact protein mass spectrometry (MS). To identify fragments that stabilized a PPI cooperatively, screens were run in the presence and absence of the client phospho-peptide; hits were classified as stabilizers (cooperative fragments) if they preferentially bound in the presence versus the absence of the client peptide (or “apo”). Neutral binders showed similar percent tethered in both screens, whereas inhibitory fragments had high percent tethered in the apo screen, but not in the presence of the phospho-peptides. This distinction allowed the classification of fragments based on their potential binding cooperativity (Fig 1.1C). The first examples of tethering for 14-3-3/client interactions discovered cooperative fragments tethered either to the native or engineered cysteine residues near the FC-A binding site (Fig 1.1).³⁴ To evaluate the scope and limitations of the approach, we then screened 14-3-3 σ against the native cysteine (C38) with five diverse client peptides, derived from two transcription factors (ER α and FOXO1), a kinase (C-RAF), a RAS guanine nucleotide exchange factor (SOS1), and

a deubiquitinating enzyme (USP8).¹ The clients encompassed different binding motifs with unique sequences surrounding the phospho-residue to form different composite binding pockets. We identified both selective and non-selective hits for the five clients. Effective hits were validated for four of the clients, with selective fragments discovered for FOXO1 and C-RAF. **Fragment 1** (see below) was a top hit for three clients: ER α , C-RAF, and USP8.

Fragments 5 and 7 were identified as selective stabilizing hits for C-RAF (Fig 1.1D).

We continued to characterize **1**, **5**, and **7** across a series of biophysical and structural assays. In addition to measuring dose responses with MS, we used fluorescence anisotropy (FA) to measure the effect of compound binding on the affinity of the peptide-protein complex. FA assays included fluorescein-labeled phospho-peptide client, at a concentration of peptide and 14-3-3 near the EC₂₀ for the 14-3-3/peptide interaction. Binding of the MG then induced peptide binding to 14-3-3, leading to an increase in anisotropy. Varying the compounds' concentration yielded an EC₅₀ value; titrating 14-3-3 at constant compound concentration yielded a $\Delta AppK_d$ for the 14-3-3/client complex (Fig 1.1E). In these validation assays, **1** acted as a MG for both 14-3-3/ER α and 14-3-3/C-RAF, with a similar EC₅₀ value (1 μ M) for both targets, but higher efficacy of stabilization for C-RAF (81x vs 19x for ER α). **5** and **7** were validated as C-RAF-selective hits with EC₅₀ values of 0.58 μ M (**5**) and 12.2 μ M (**7**) and efficacies of 246-fold (**5**) and 77-fold (**7**).

The validated hits were crystallized with 14-3-3 σ /phospho-peptide complexes to elucidate their key interactions with the composite interface. The structural observations were consistent with the compounds' biophysical activities. For example, comparing multiple fragment hits provided insight into how different scaffolds stabilized C-RAF's interaction with 14-3-3 σ (Fig 1.1D). **Fragment 1** and the C-RAF selective **fragments 5** and **7** represented different chemical scaffolds that uniquely altered the conformation of the C-RAF peptide, particularly around the +4 Val (V263) residue. FOXO1, by contrast, was rigidly oriented in the 14-3-3 σ pocket, but the side chain of the +1 Trp (W25) rotated to interact with two distinct fragment structures. Thus, specific

interactions with residues C-terminal to the phospho-S/T in the client were critical for formation of 14-3-3/client/MG complexes. Furthermore, characteristics of the clients – such as peptide flexibility – facilitated the identification of selective MGs from the disulfide screen. Altogether, disulfide tethering uncovered MG chemotypes for diverse targets with diverse 14-3-3/client structures.

Medicinal Chemistry Strategies

Structure-guided optimization from a disulfide fragment to an irreversible MG

The crystal structures of **1** with the 14-3-3/ER α and 14-3-3/C-RAF complexes served as a starting point for medicinal chemistry optimization (Fig 1.2A). The fragment lacked selectivity and preferentially stabilized C-RAF; however, in the crystal structures, it adopted a similar binding mode with both clients. While the interactions between the fragment and 14-3-3 were similar, distinct interactions were formed between the fragment and the *client phospho-peptides*. On ER α , the phosphorylation occurred on the penultimate residue on the C-terminus (T594) and the ultimate residue (+1) was hydrophobic (V595). This created an open, solvent-exposed interface, where the fragment primarily formed hydrophobic interactions with V595. In stark contrast, the C-RAF phosphorylation occurred as part of an internal sequence (S259), the +1 residue was polar (T260), and the rest of the sequence created a pocket that was shielded from the solvent. The bound fragment was oriented towards T260, and the rest of the peptide was in proximity but did not form favorable interactions. Based on the crystallographic insight, we made structure-guided chemical modifications in five parts of the molecule, aiming to tune the selectivity towards the 14-3-3/ER α complex by optimizing favorable interactions at the PPI interface, and at the same time reducing the binding to the 14-3-3/C-RAF complex via increased steric hindrance (Fig 1.2A).²

The initial fragment had a reversible disulfide linker, which interacted with C38 on 14-3-3 σ . The reversible nature of the bond was advantageous for the tethering screen (see above)

but was unsuitable for cell assays. Therefore, the first two modifications focused on replacing the disulfide with irreversible warheads with varying linker lengths. This change required adaptation of the previously described MS assay, which was now run as a time-course to evaluate binding kinetics. For most compounds, both the MS and the FA assay required overnight incubation to achieve maximum increase in signal. Diverse electrophiles varied significantly in their observed binding affinity and kinetics. The chloroacetamide warhead demonstrated good binding affinity and moderate reactivity and was therefore selected for further SAR optimization.

The next two modifications aimed at optimizing the interactions between the compounds and V595 on ER α . Since the residue was hydrophobic, we replaced the *gem*-dimethyl group with linear or cyclic hydrophobic groups. Increasing the ring size and introducing heteroatoms on the rings (-F, -O, -N) improved both the potency and selectivity towards the 14-3-3/ER α complex. Replacing the ether group with an aniline allowed the compounds to adopt a different conformation, oriented towards the front of the pocket. For both ethers and anilines, the presence of a *p*-chloro-phenyl substituted ring in the K122 pocket was crucial for the stabilization effect. These modifications successfully allowed the formation of specific compound-peptide interactions, both with V595's hydrophobic sidechain and the terminal carboxylic acid.

The last modification, at the rim of the PPI interface, focused on optimizing the warhead linker length and restricting its conformation by introducing spirocycles. The best analog of the series, **compound 181**, showed favorable interactions both with 14-3-3 and ER α , shape complementarity, and a favorable conformation in the composite 14-3-3/ER α interface (Fig 1.2A). The key interactions included a halogen bond with K122 (14-3-3), multiple hydrophobic interactions from the 2,6-dimethyl tetrahydropyran (L218, I219, L222 of 14-3-3 and V595 of ER α) and a water-mediated hydrogen bond between the aniline nitrogen and the terminal carboxylic acid of V595 (ER α). Additionally, it showed fast kinetics in the MS assay and

cooperative binding in the FA assay, leading to a compound EC₅₀ value of 1 μ M for 14-3-3/ER α , with an 18 nM *AppKd* (116-fold stabilization). The compound also showed selectivity when tested against a small panel of eight 14-3-3 clients, including C-RAF. A comparison between **181** and the natural product **FC-A** showed similar cooperativity in the FA and isothermal calorimetry assays, although **181** binding was more strongly driven by enthalpy (DH = -8.6 kcal/mol vs -5.8 kcal/mol for FC-A).

Fragment linking towards non-covalent MGs

Structural information from two series of fragments was also a useful starting point for optimization. We ran a screen with an engineered cysteine on 14-3-3 (C42) in the presence of the ER α phospho-peptide and crystallized the hits, including the one shown in Fig 1.2B (C42-fragment).³⁴ It had the same chlorophenyl binding element as seen in **1**, but tethered to C42 with a shorter linker, putting the chlorophenyl in the same location and leading to a similar degree of 14-3-3/ER α stabilization. A separate crystallographic screen for the 14-3-3/p53 complex had identified a non-covalent amidine fragment, which was bound at the rim of the interface, forming a salt bridge with E14 (14-3-3).³² The amidine fragment was positioned far from the client phospho-peptide and lacked interactions with it. Co-crystallization of the two fragments provided the structural insight for a fragment-linking campaign to yield non-covalent MGs for the 14-3-3/ER α complex.³⁸ In the optimized linked analogs, the original fragment-protein interactions were largely conserved. The best compounds of the series showed 25-fold stabilization of the 14-3-3/ER α complex in the FA protein titration assay. Notably, similarities in the SAR were observed between the covalent and non-covalent series. In both cases, the *p*-chloro-phenyl substituted rings in the K122 pocket and the bulky cyclic rings with heteroatoms (-O or -N) proximal to V595 were necessary for the stabilization of the 14-3-3/ER α complex. To summarize, functionally active disulfides, coupled with structurally adjacent but inactive

fragments, provided a remarkably effective optimization strategy and highlighted a potentially systematic approach to targeted MGs.

A scaffold hopping approach using multi-component reaction chemistry

We recently disclosed a computational scaffold-hopping approach³ using pharmacophore-based docking of virtual libraries based on multi-component reaction chemistry (MCR).^{39,40} MCRs combine at least three starting materials in a single step to form a complex scaffold, thus allowing fast synthesis of analogs and rapid development of SAR. As a starting point, we used **compound 127**, an intermediate analog of our first series of 14-3-3/ER α MGs.² The compound had favorable interactions in the composite surface and was one of the first aniline analogs that showed the optimal ligand conformation (Fig 1.2C).

Our aim in the scaffold-hopping approach was to explore new chemical space by designing a rigid, drug-like scaffold that would maintain the 3D shape and ideal shape complementarity in the 14-3-3/ER α interface. Pharmacophore-based queries using AnchorQueryTM revealed that the most suitable scaffolds derived from the Groebke-Blackburn-Bienaymé reaction (GBB); a three-component reaction using aldehydes, 2-aminopyridines and isocyanides to form to imidazo[1,2-a]pyridines.⁴¹ To target C38, we kept the chloroacetamide warhead constant in most of the analogs since, in our previous experience, it had moderate, tunable reactivity.

Using structure-guided design, we varied one building block at a time, starting with the isocyanide position, which according to our design was expected to occupy the K122 (14-3-3) pocket, in close proximity to the ER α peptide. For this position, we observed different SAR compared to our previous series; the *p*-chloro-phenyl analogs did not result in improved binding/stabilization. Instead, the *o*-position had a more favorable effect, especially in the case of double-*ortho*-methyl substituted rings which correlated with rapid, cooperative binding. We made additional changes in the other two components of the GBB reaction. First, we added

hydrophobic substitutions on the 2-aminopyridines, which led to extensive hydrophobic interactions with 14-3-3 (L218, I219, L222). We also modified the aldehyde building blocks and to a smaller extent the electrophilic warheads -- both positioned at the rim of the interface.

Based on MS data and crystal structures of ternary complexes, we combined the best modifications in **compound 41**, which included the 2,6-dimethylaniline in the isocyanide position, an additional methyl group on the imidazopyridine ring and an *o*-F group on the aryl ring at the rim of the interface (Fig 1.2C). Structurally, the 2,6-dimethylaniline groups in the K122 pocket formed favorable hydrophobic interactions. The first *o*-Me group was oriented in the back of the 14-3-3 pocket, which was primarily hydrophobic, forming hydrophobic interactions with I219 (3.2Å) and facing V595 of ER α ; the second *o*-Me group was oriented in the front of the pocket and formed hydrophobic interactions with F119 of 14-3-3 (3.8 Å). The imidazopyridine ring occupied the hydrophobic pocket of 14-3-3 formed by L218 and I219. The additional methyl group on the imidazopyridine ring formed hydrophobic interactions with L222 of 14-3-3. The *o*-F group on the second aryl ring was oriented “outwards” and made multiple stabilizing interactions both intermolecular (N42 of 14-3-3) and intramolecular with the *o*-Me substituent and aniline nitrogen of the compound itself. These additional interactions favorably restricted the rotational bonds, resulting in fast cooperative binding. Finally, the aniline nitrogen formed a water-mediated hydrogen bond with the terminal carboxylic acid of V595 (ER α).

The optimized covalent analogs from both series, **181** and **41**, had similar potencies in biophysical assays. Both compounds showed fast, cooperative binding in the MS assay and yielded low nanomolar *AppK_d* for 14-3-3/ER α in a TR-FRET assay (5-8 nM). This manuscript also described a surface plasmon resonance (SPR) method to quantify *AppK_d* for 14-3-3/client complexes stabilized by a covalent MG. First, the covalent compounds were pre-incubated with the 14-3-3/ER α complex, followed by immobilization of 14-3-3 on the SPR surface and extensive washing to remove bound peptide. Peptides were then flowed over the SPR surface, and the binding kinetics and *K_d* values were determined. In this format, **181** and **41** yielded

$AppK_d$ values of 3.9 nM and 10.1 nM, respectively, and increased the half-lives from 3.4s to 62.5s and 47.6s, respectively.

Fragment merging for selective MGs stabilizing the 14-3-3/C-RAF complex

In contrast to the C-terminus of ER α , the C-RAF pS259-containing peptide afforded diverse interactions with MGs. Crystal structures of **1** and an analog with a chloroacetamide warhead (**1074202**, Fig 1.2D) revealed unfavorable interactions between the hydrophobic part of the fragment and T260 of C-RAF. We used a fragment-merging approach to combine parts of **1074202** with an aldehyde aryl sulfonamide fragment (**TC-521**) that was reported to bind to 14-3-3/p65 and could position the hydrophilic sulfonamide near T260 (C-RAF).³⁰ The aldehyde of **TC-521** bound reversibly to K122 (14-3-3) via an imine bond. We merged the two fragments and tested a doubly covalent analog (**compound 21**), which included both a cysteine- and lysine-targeting warhead, and a series of only-cysteine-targeting analogs, such as **23**. We thus sought to improve the interactions with T260 and additional C-RAF residues that extended into the MG-binding site. Crystallography showed that **21** was indeed double-covalently bound; however, it lacked specific interactions with the C-RAF peptide.

On the other hand, **23** adopted a surprising, unique, upward conformation, where the C-RAF peptide “wrapped” around the main core of the compound, thus significantly covering the binding groove. The key interactions were formed from the sulfonyl group and included a charged-assisted hydrogen bond with K122 (14-3-3) and a hydrogen bond with T260 (CRAF). The aryl-iodo group was positioned between the hydrophobic pocket formed by L218 and I219 (14-3-3), V263 (C-RAF) and M265 (C-RAF). The chloroacetamide warhead formed two hydrogen bonds with R41 and N42 (14-3-3), in addition to the covalent bond with C38. The binding mode of **23** correlated with fast, cooperative binding in the MS assay and very little binding to 14-3-3 (apo) whereas **21** showed significant binding to 14-3-3 (apo). In FA protein titrations, **23** yielded an $AppK_d$ of 30 nM for the 14-3-3/C-RAF complex – a 280-fold stabilization

– whereas **21** showed only 9-fold stabilization. Additionally, **23** showed remarkable selectivity when tested in a panel consisting of eighty 14-3-3/client complexes in FA format. Only four clients were stabilized by **23**; all showed weaker stabilization than C-RAF259, indicating that the compound's multiple interactions with C-RAF favorably correlated with selectivity.

Cell Assays

In general, properly phosphorylated full-length 14-3-3 client proteins are challenging to isolate. Thus, our biophysical assays have utilized phospho-peptides derived from clients, and it is particularly critical to verify MGs efficacy towards full-length PPIs. We have therefore developed cellular assays to assess PPI stabilization and its downstream effects in target-specific functional assays (Fig 1.3A).

Characterization of full-length PPIs via NanoBRET

To evaluate MG stabilization of the full-length PPIs, we developed a bioluminescence resonance energy transfer (NanoBRET) assay with HaloTag (HT) fused to the C-terminus of 14-3-3 σ and Nanoluciferase (Nluc) fused to the client's N- or C-terminus.⁴ The constructs were transfected into HEK293T cells and fluorescent HT ligand was added. Interaction between the tagged proteins resulted in BRET and HT-ligand fluorescence. The assay was developed to detect a basal level of BRET signal that could be increased upon compound treatment. Assay sensitivity was also enhanced by using HEK293T cells, which did not express endogenous 14-3-3 σ and minimally expressed the clients ER α and C-RAF; thus, the transfected proteins did not compete with their endogenous counterparts.

NanoBRET assays validated the ability of MGs to stabilize 14-3-3/client complexes with overnight MG treatment (Fig. 1.3B, C). Compounds were ranked based on their EC₅₀ values and their maximum fold-increase in normalized BRET signals. Chemically similar, inactive compounds (ER α : **85**, C-RAF: **8**) resulted in no change in signal. The EC₅₀ values of the

compounds in the NanoBRET assays showed a 1-5-fold shift between the values obtained from FA. For example, **181** had an EC₅₀ value of 5.2 μ M in the 14-3-3 σ /ER α NanoBRET (vs 1 μ M in FA). The MCR scaffold **41** had a similar BRET EC₅₀ of 5 μ M (Fig 1.3B); interestingly, **41** resulted in a higher fold- increase in the BRET signal (2.6 vs 1.7 for **181**).³ For C-RAF, **23** was the most potent with the highest maximum BRET signal and an EC₅₀ of 0.18 μ M (Fig 3C). These results indicated that phospho-peptides served as reasonable mimics for the 14-3-3 client proteins and that covalent MGs were cell permeable and selective enough to bind their targets in the presence of other cellular proteins and glutathione.

Biological effects of 14-3-3/client MGs

We then designed orthogonal cell-based assays to evaluate the downstream effects of 14-3-3/client stabilization. For example, 14-3-3 binding to was known to pT594 negatively regulate ER α , blocking binding to chromatin.¹⁸ As expected, in the ER+ breast cancer cell line MCF7, the basal state of ER α was primarily non-phosphorylated at T594, leading to minimal native interaction with 14-3-3 and high ER α -mediated transcription. We expected that, upon binding to 14-3-3, the client phospho-residue would be shielded from dephosphorylation (Fig 3A).⁴² Indeed, upon treatment with **181**, we saw that pT594 was strongly increased, in agreement with increased 14-3-3/ER α complex formation observed by NanoBRET.⁴² Our collaborators then generated a reporter assay in MCF7 cells in which luciferase expression was dependent on ER α binding to the estrogen response element (ERE). In this assay, **181** (1 μ M) strongly inhibited luciferase expression compared to DMSO or the inactive compound. Additional assay formats supported on-target activity for **181**. For instance, we observed a reduction in proliferation in ER+ cells, no change in proliferation in ER- cells, activity in patient-derived organoids, and complex formation using proximity-ligation assays.

We used a similar workflow to characterize MGs that stabilized 14-3-3/CRAF.

Compound 23 and related MGs increased pS259 levels up to 3.2-fold (Fig 1.3D) and increased

the 14-3-3 σ /CRAF interaction seen via co-immunoprecipitation, corroborating the NanoBRET results. We also developed NanoBRET assays to evaluate how 14-3-3/C-RAF stabilization prevented formation of downstream CRAF PPIs. CRAF/NRAS and CRAF/CRAF were known critical steps in the activation mechanism of CRAF;^{43–46} **23** inhibited formation of both these complexes, with IC₅₀ values comparable to the EC₅₀ for 14-3-3/CRAF stabilization. We then monitored dose-dependent reduction in ERK phosphorylation, indicating inhibition of CRAF kinase activity.

Strong upregulation of the RAS/RAF/MAPK pathway leads to cancer, but modest upregulation by activating mutations in the pathway can lead to developmental “RASopathies” such as Noonan Syndrome (NS).^{47,48} We have demonstrated that mutations upstream of 14-3-3/CRAF modestly lower CRAF pS259 and that our MGs protect pS259 in these contexts. More challenging are NS mutations in CRAF near the pS259 site. These mutations, including S257L, impair CRAF’s inhibitory interaction with 14-3-3 and can lead to debilitating infant cardiac hypertrophic myopathy.⁴⁹ **Compound 23** and related MGs restore the 14-3-3/CRAF interaction, protecting phosphorylation of pS259, and reducing levels of pERK.

Conclusions and Perspective

The highlighted papers showcase our progress towards the identification, optimization, and validation of MGs for the 14-3-3 interactome. MS-based fragment screens have enabled the systematic identification of new chemical matter for diverse 14-3-3/client interactions. Disulfide fragments provided multiple starting points for medicinal chemistry by optimizing, merging, scaffold-hopping, or linking fragments; both covalent and noncovalent strategies have been demonstrated. We adapted or developed new biophysical assay formats to validate the unique features of MGs, such as cooperativity. The extraordinary availability of high-resolution, ternary crystal structures from the Ottmann and Brunsveld labs provided the opportunity for rational optimization of client-specific interactions at the 14-3-3/client composite interface, affording high

selectivity for the PPI of interest. These strategies resulted in cell-active MGs for two 14-3-3/client interactions, allowing us to study disordered clients via an unprecedented mechanism.

In addition to the MS tethering screens, 14-3-3-binding fragments have been identified using different assay readouts and screening libraries. Fluorescence polarization (FP) screens have identified fragments covalently bound to cysteine on 14-3-3⁵⁰ or on the phosphopeptide client.⁵¹ Libraries containing aldehydes have targeted imine-formation with K122, a deeply buried residue conserved across 14-3-3 isoforms. Stabilizing fragments were identified for 14-3-3/p65³⁰ and analogs from this screen were further optimized towards 14-3-3/Pin1.³¹ Several non-covalent fragment libraries have also been screened using x-ray crystallography,³² differential scanning fluorimetry,⁵² nuclear magnetic resonance spectroscopy,⁵³ and TR-FRET.⁵⁴

Traditional libraries have also been screened. Two structurally distinct hits were identified for 14-3-3/PMA2 from a 37,000-member library using an ELISA-like assay format.²⁰ The 14-3-3/SLP76 complex was screened using an HTRF assay and a chemically diverse library of 20 K compounds.²¹ Additionally, macrocycles have been reported to restore the impaired activity of the chloride channel CFTR by stabilizing the 14-3-3/CFTR interaction. The macrocycles were identified in an FP screen of 5760 compounds of Cyclenium's proprietary small-molecule macrocycle library and bound to a novel site on the complex.⁵⁵

Unique 14-3-3/client interfaces have enabled unique chemical approaches. A particularly powerful example is ChREBP, one of the few 14-3-3 clients that is not phosphorylated. Our collaborators reported a virtual screening strategy that included phosphate and phosphonate-based compounds that bind in the peptide-phosphate site and selectively stabilize the 14-3-3/ChREBP complex. Structure-guided optimization yielded cell-active MGs that protect beta cells from glucolipotoxicity and therefore show promise for treatment of type 2 diabetes.^{56,57}

Taken altogether, is a wealth of chemical starting points that can be combined and dissected in various ways to develop a suite of MGs and 14-3-3-binding ligands for a variety of

applications. In particular, pan-14-3-3-isoform ligands are needed for tissues that do not express 14-3-3 σ .

We are entering a golden age for chemical biology. Many heretofore undruggable targets can now be addressed using chemistries – including covalent ligands and beyond-rule-of-five molecules – that were once verboten but have demonstrated translation from bench to bedside. Leading the way are bifunctional molecules and molecular glues that induce biology by enhancing proximity between two biomolecules. Our focus is on stabilizing native PPIs because these systems invite systematic discovery and predictable biological outcomes. Native interactions also lay a foundation for learning how to stabilize PPIs more generally. Our 14-3-3 experiences suggest that 1) reversible covalent fragments provide site-directed screens and reduce the three-body problem intrinsic to highly cooperative ternary complex formation, 2) biophysical and structural analysis provides a critical mechanistic understanding of cooperative binding, 3) highly selective MGs are made by optimizing interactions with both sides of the PPI, even if one member of the complex is intrinsically promiscuous, and 4) high selectivity leads to small shifts in EC₅₀ values between biophysical and cell-based measurements of PPI formation. We propose that these strategies and techniques will be broadly applicable to other native PPIs and to the even broader domain of neomorphic interactions.

Figures

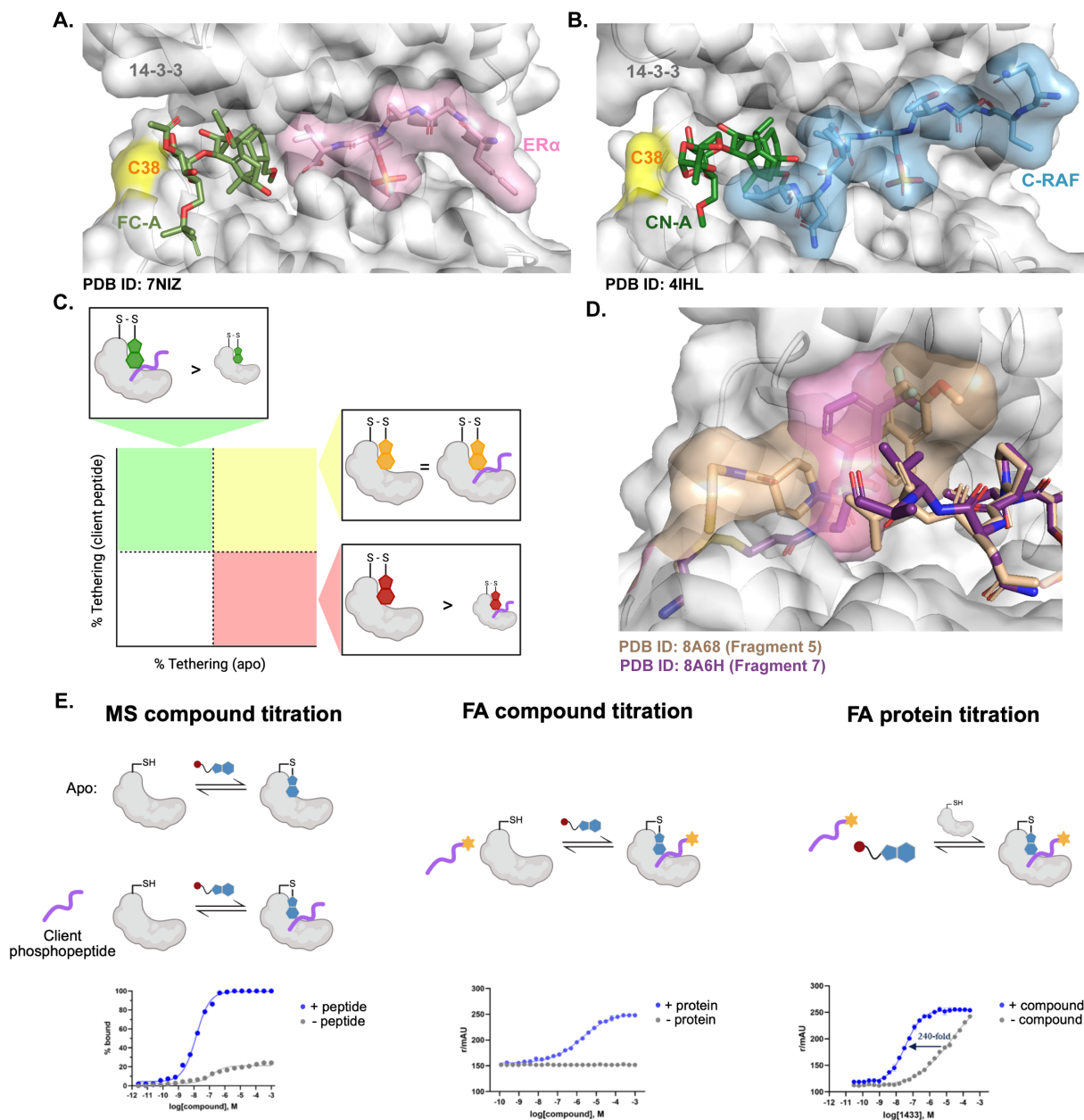
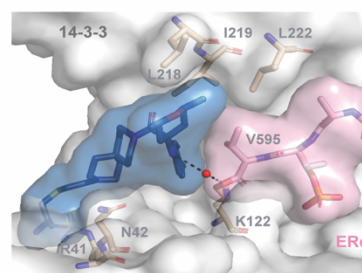
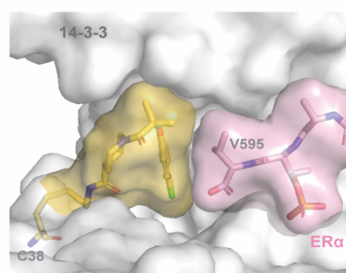
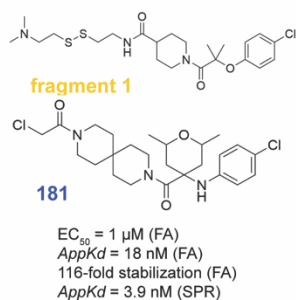


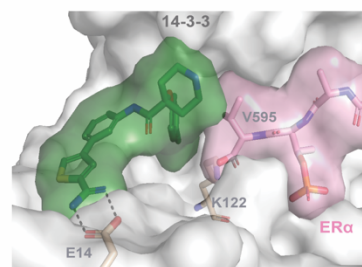
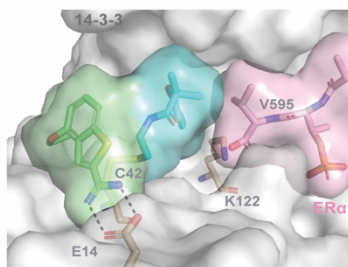
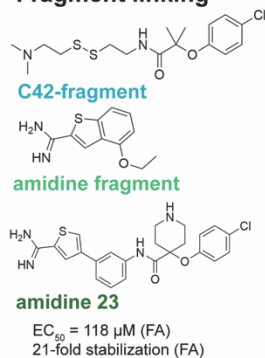
Figure 1.1: Crystal structures of 14-3-3/client interaction and overview of biophysical assays

A-B) Crystal structures of the natural products with 14-3-3, ER α , and C-RAF pS259. Targeted cysteine for MGs highlighted in yellow. **C)** Overview of MS tethering screen. **D)** Crystal structures 14-3-3/C-RAF with selective fragments. **E)** Overview of biophysical assays for validation of hits.

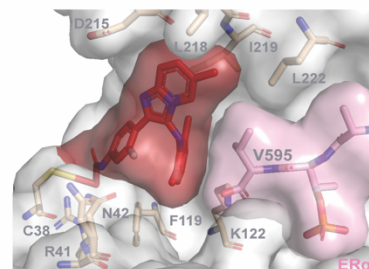
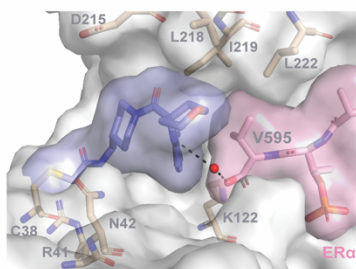
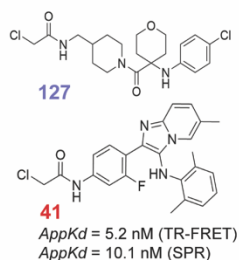
A. Tethering hit to electrophile



B. Fragment linking



C. Scaffold hopping



D. Fragment merging

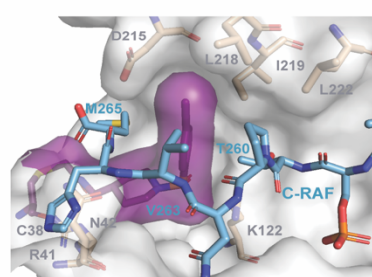
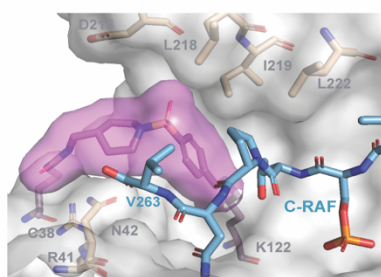
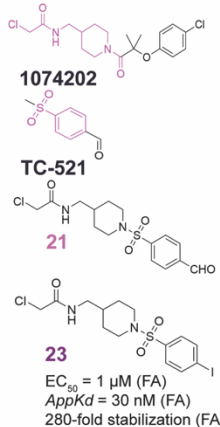


Figure 1.2: Overview of medicinal chemistry strategies for MGs optimization

Chemical structures, structures of MGs/14-3-3/client complexes and biophysical data for the optimized compounds.

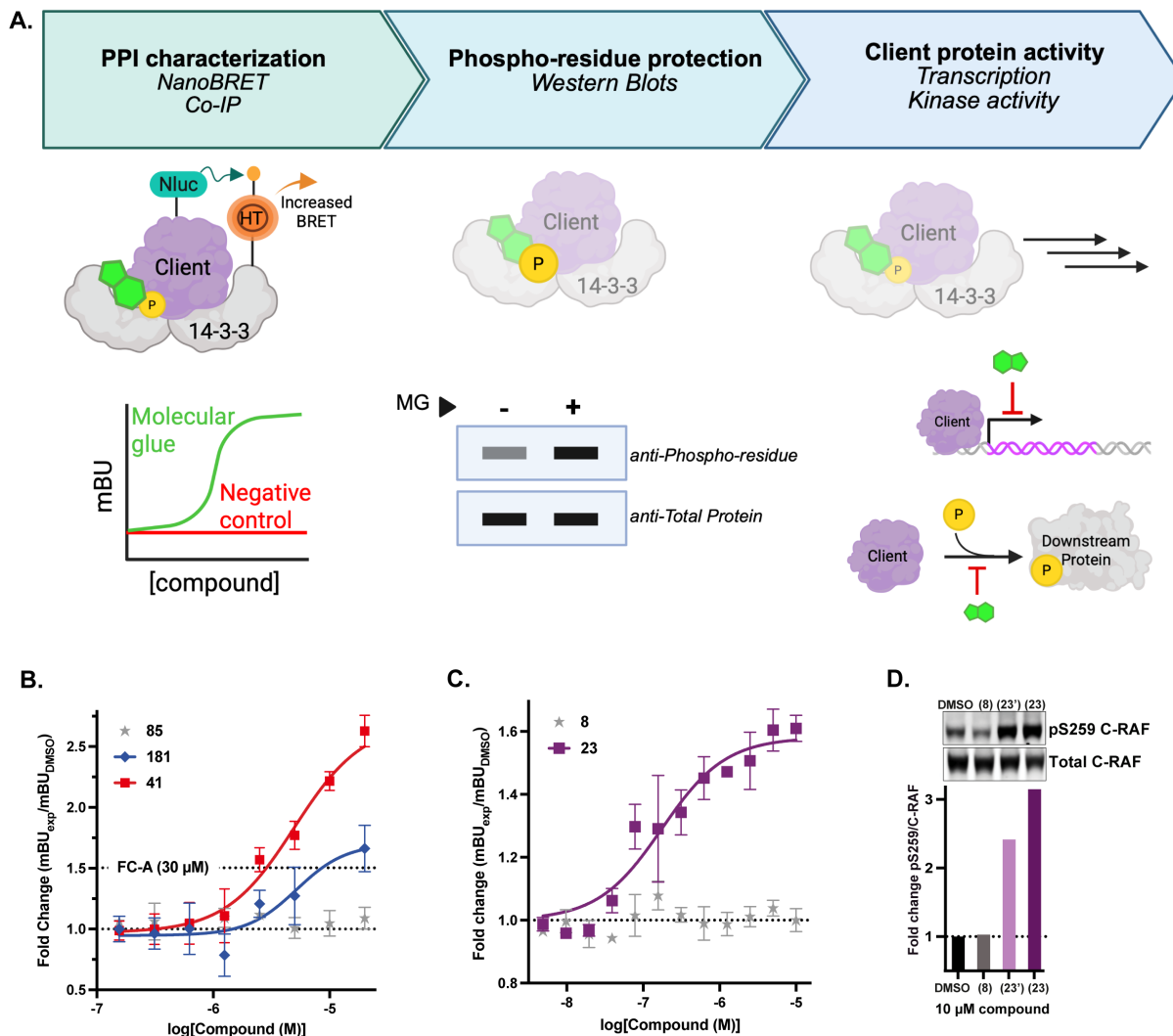


Figure 1.3: Overview of cell assays

A) General workflow for MG validation in cells. **B)** 14-3-3/ER α NanoBRET with MGs and FC-A. **C)** 14-3-3/C-RAF NanoBRET. **D)** Effects on C-RAF phosphorylation upon treatment with MGs (**23** and derivative **23'**) compared to DMSO and **8**.

References

1. Kenanova, D. N. *et al.* A Systematic Approach to the Discovery of Protein–Protein Interaction Stabilizers. *ACS Cent. Sci.* **9**, 937–946 (2023).
2. Konstantinidou, M. *et al.* Structure-Based Optimization of Covalent, Small-Molecule Stabilizers of the 14-3-3 σ /ER α Protein–Protein Interaction from Nonselective Fragments. *J. Am. Chem. Soc.* **145**, 20328–20343 (2023).
3. Arkin, M. *et al.* Rapid scaffold-hopping for molecular glues: from fragments to cell-active probes targeting the 14-3-3/ER α complex. Preprint at <https://doi.org/10.21203/rs.3.rs-6051794/v1> (2025).
4. Vickery, H. R., Virta, J. M., Konstantinidou, M. & Arkin, M. R. Development of a NanoBRET assay for evaluation of 14-3-3 σ molecular glues. *SLAS Discov* **29**, 100165 (2024).
5. Rabbani, G., Baig, M. H., Ahmad, K. & Choi, I. Protein-protein Interactions and their Role in Various Diseases and their Prediction Techniques. *CPPS* **19**, 948–957 (2018).
6. Uversky, V. N. Intrinsic Disorder, Protein–Protein Interactions, and Disease. in *Advances in Protein Chemistry and Structural Biology* vol. 110 85–121 (Elsevier, 2018).
7. Xiong, D., Lee, D., Li, L., Zhao, Q. & Yu, H. Implications of disease-related mutations at protein–protein interfaces. *Current Opinion in Structural Biology* **72**, 219–225 (2022).
8. Zhong, M., Lee, G. M., Sijbesma, E., Ottmann, C. & Arkin, M. R. Modulating protein-protein interaction networks in protein homeostasis. *Curr Opin Chem Biol* **50**, 55–65 (2019).
9. Arkin, M. R. & Wells, J. A. Small-molecule inhibitors of protein–protein interactions: progressing towards the dream. *Nat Rev Drug Discov* **3**, 301–317 (2004).
10. Ruan, H., Sun, Q., Zhang, W., Liu, Y. & Lai, L. Targeting intrinsically disordered proteins at the edge of chaos. *Drug Discovery Today* **24**, 217–227 (2019).
11. Schreiber, S. L. The Rise of Molecular Glues. *Cell* **184**, 3–9 (2021).
12. Konstantinidou, M. & Arkin, M. R. Molecular glues for protein-protein interactions: Progressing toward a new dream. *Cell Chem Biol* **31**, 1064–1088 (2024).

13. Thompson, J. W. *et al.* Profiling CELMoD-Mediated Degradation of Cereblon Neosubstrates. in *Targeted Protein Degradation* (eds. Cacace, A. M., Hickey, C. M. & Békés, M.) vol. 2365 283–300 (Springer US, New York, NY, 2021).
14. Hansen, J. D. *et al.* Discovery of CRBN E3 Ligase Modulator CC-92480 for the Treatment of Relapsed and Refractory Multiple Myeloma. *J. Med. Chem.* **63**, 6648–6676 (2020).
15. Watson, E. R. *et al.* Molecular glue CELMoD compounds are regulators of cereblon conformation. *Science* **378**, 549–553 (2022).
16. Aitken, A. 14-3-3 proteins: a historic overview. *Semin Cancer Biol* **16**, 162–172 (2006).
17. Aghazadeh, Y. & Papadopoulos, V. The role of the 14-3-3 protein family in health, disease, and drug development. *Drug Discovery Today* **21**, 278–287 (2016).
18. De Vries-van Leeuwen, I. J. *et al.* Interaction of 14-3-3 proteins with the estrogen receptor alpha F domain provides a drug target interface. *Proc Natl Acad Sci U S A* **110**, 8894–8899 (2013).
19. Molzan, M. *et al.* Stabilization of physical RAF/14-3-3 interaction by cotylenin A as treatment strategy for RAS mutant cancers. *ACS Chem Biol* **8**, 1869–1875 (2013).
20. Rose, R. *et al.* Identification and Structure of Small-Molecule Stabilizers of 14 – 3 – 3 Protein–Protein Interactions. *Angew Chem Int Ed* **49**, 4129–4132 (2010).
21. Soini, L. *et al.* Identification of molecular glues of the SLP76/14-3-3 protein–protein interaction. *RSC Med. Chem.* **12**, 1555–1564 (2021).
22. Du, Y. *et al.* A Time-Resolved Fluorescence Resonance Energy Transfer Assay for High-Throughput Screening of 14-3-3 Protein–Protein Interaction Inhibitors. *ASSAY and Drug Development Technologies* **11**, 367–381 (2013).
23. Petros, A. M. *et al.* Discovery of a Potent Inhibitor of the Antiapoptotic Protein Bcl-x_L from NMR and Parallel Synthesis. *J. Med. Chem.* **49**, 656–663 (2006).
24. Erlanson, D. A., Davis, B. J. & Jahnke, W. Fragment-Based Drug Discovery: Advancing Fragments in the Absence of Crystal Structures. *Cell Chemical Biology* **26**, 9–15 (2019).

25. Rees, D. C., Congreve, M., Murray, C. W. & Carr, R. Fragment-based lead discovery. *Nat Rev Drug Discov* **3**, 660–672 (2004).
26. Xu, W. & Kang, C. Fragment-Based Drug Design: From Then until Now, and Toward the Future. *J. Med. Chem.* **68**, 5000–5004 (2025).
27. Murray, C. W. & Rees, D. C. The rise of fragment-based drug discovery. *Nat Chem* **1**, 187–192 (2009).
28. Erlanson, D. A., Fesik, S. W., Hubbard, R. E., Jahnke, W. & Jhoti, H. Twenty years on: the impact of fragments on drug discovery. *Nat Rev Drug Discov* **15**, 605–619 (2016).
29. Knight, S., Gianni, D. & Hendricks, A. Fragment-based screening: A new paradigm for ligand and target discovery. *SLAS Discovery* **27**, 3–7 (2022).
30. Wolter, M. *et al.* Fragment-Based Stabilizers of Protein–Protein Interactions through Imine-Based Tethering. *Angew. Chem. Int. Ed.* **59**, 21520–21524 (2020).
31. Cossar, P. J. *et al.* Reversible Covalent Imine-Tethering for Selective Stabilization of 14-3-3 Hub Protein Interactions. *J Am Chem Soc* **143**, 8454–8464 (2021).
32. Guillory, X. *et al.* Fragment-based Differential Targeting of PPI Stabilizer Interfaces. *J Med Chem* **63**, 6694–6707 (2020).
33. Hallenbeck, K. K. *et al.* A Liquid Chromatography/Mass Spectrometry Method for Screening Disulfide Tethering Fragments. *SLAS Discov* 2472555217732072 (2017) doi:10.1177/2472555217732072.
34. Sijbesma, E. *et al.* Site-Directed Fragment-Based Screening for the Discovery of Protein-Protein Interaction Stabilizers. *J Am Chem Soc* **141**, 3524–3531 (2019).
35. Erlanson, D. A., Wells, J. A. & Braisted, A. C. Tethering: fragment-based drug discovery. *Annu Rev Biophys Biomol Struct* **33**, 199–223 (2004).
36. Erlanson, D. A. *et al.* Discovery of a potent and highly selective PDK1 inhibitor via fragment-based drug discovery. *Bioorg. Med. Chem. Lett.* **21**, 3078–3083 (2011).

37. Van Horn, K. S. *et al.* Engaging a Non-catalytic Cysteine Residue Drives Potent and Selective Inhibition of Caspase-6. *J Am Chem Soc* **145**, 10015–10021 (2023).
38. Visser, E. J. *et al.* From Tethered to Freestanding Stabilizers of 14-3-3 Protein-Protein Interactions through Fragment Linking. *Angew Chem Int Ed Engl* **62**, e202308004 (2023).
39. Koes, D. R., Dömling, A. & Camacho, C. J. AnchorQuery: Rapid online virtual screening for small-molecule protein-protein interaction inhibitors. *Protein Sci* **27**, 229–232 (2018).
40. Dömling, A., Wang, W. & Wang, K. Chemistry and biology of multicomponent reactions. *Chem Rev* **112**, 3083–3135 (2012).
41. Boltjes, A. & Dömling, A. The Groebke-Blackburn-Bienaymé Reaction. *Eur J Org Chem* **2019**, 7007–7049 (2019).
42. Visser, E. J. *et al.* Estrogen Receptor α /14-3-3 molecular glues as alternative treatment strategy for endocrine resistant breast cancer. Preprint at <https://doi.org/10.1101/2024.04.25.591105> (2024).
43. Kondo, Y. *et al.* Cryo-EM structure of a dimeric B-Raf:14-3-3 complex reveals asymmetry in the active sites of B-Raf kinases. *Science* **366**, 109–115 (2019).
44. García-Alonso, S. *et al.* Structure of the RAF1-HSP90-CDC37 Complex Reveals the Basis of RAF1 Regulation. <http://biorxiv.org/lookup/doi/10.1101/2022.05.04.490607> (2022) doi:10.1101/2022.05.04.490607.
45. Park, E. *et al.* Cryo-EM Structure of a RAS/RAF Recruitment Complex. <http://biorxiv.org/lookup/doi/10.1101/2022.07.14.500081> (2022) doi:10.1101/2022.07.14.500081.
46. Park, E. *et al.* Architecture of autoinhibited and active BRAF-MEK1-14-3-3 complexes. *Nature* **575**, 545–550 (2019).
47. Riller, Q. & Rieux-Laucat, F. RASopathies: From germline mutations to somatic and multigenic diseases. *Biomedical Journal* **44**, 422–432 (2021).

48. Saint-Laurent, C., Mazeyrie, L., Yart, A. & Edouard, T. Novel therapeutic perspectives in Noonan syndrome and RASopathies. *Eur J Pediatr* **183**, 1011–1019 (2023).
49. Linglart, L. & Gelb, B. D. Congenital heart defects in Noonan syndrome: Diagnosis, management, and treatment. *American J of Med Genetics Pt C* **184**, 73–80 (2020).
50. Shao, Q., Duong, T. N., Park, I., Orr, L. M. & Nomura, D. K. Targeted Protein Localization by Covalent 14–3–3 Recruitment. *J. Am. Chem. Soc.* **146**, 24788–24799 (2024).
51. Sijbesma, E. *et al.* Fluorescence Anisotropy-Based Tethering for Discovery of Protein–Protein Interaction Stabilizers. *ACS Chem Biol* **15**, 3143–3148 (2020).
52. Andlovic, B. *et al.* Fragment-Based Interrogation of the 14–3–3/TAZ Protein–Protein Interaction. *Biochemistry* **63**, 2196–2206 (2024).
53. Sijbesma, E. *et al.* Identification of Two Secondary Ligand Binding Sites in 14-3-3 Proteins Using Fragment Screening. *Biochemistry* **56**, 3972–3982 (2017).
54. Brink, H. J. *et al.* Fragment Screening Yields a Small-Molecule Stabilizer of 14-3-3 Dimers That Modulates Client Protein Interactions. *ChemBioChem* **23**, e202200178 (2022).
55. Stevers, L. M. *et al.* Macrocycle-stabilization of its interaction with 14-3-3 increases plasma membrane localization and activity of CFTR. *Nat Commun* **13**, 3586 (2022).
56. Sijbesma, E. *et al.* Structure-based evolution of a promiscuous inhibitor to a selective stabilizer of protein–protein interactions. *Nat Commun* **11**, 3954 (2020).
57. Katz, L. S. *et al.* Molecular glues of the regulatory ChREBP/14-3-3 complex protect beta cells from glucolipotoxicity. *Nat Commun* **16**, 2110 (2025).

Chapter 2

A systematic approach to the discovery of protein-protein interaction stabilizers

Abstract

Protein-protein interactions (PPIs) are responsible for the proper function of biological processes and, when dysregulated, commonly lead to disease. PPI stabilization has only recently been systematically explored for drug discovery despite being a powerful approach to selectively target intrinsically disordered proteins and hub proteins, like 14-3-3, with multiple interaction partners. Disulfide tethering is a site-directed fragment-based drug discovery (FBDD) methodology for screening small molecules in a quantitative, high-throughput manner. We explore the scope of the disulfide tethering technology for the discovery of selective fragments as starting points for the development of potent small molecule PPI stabilizers and molecular glues using the hub protein 14-3-3 σ . The complexes with 5 biologically and structurally diverse phospho-peptides, derived from the 14-3-3 client proteins ER α , FOXO1, CRAF, USP8, and SOS1, were screened for hit identification. Stabilizing fragments could be found for 4/5 client complexes with a diversified hit-rate and stabilizing efficacy for the different 14-3-3/client phospho-peptides. Extensive structural elucidation revealed the ability and adaptivity of the peptide to make productive interactions with the tethered fragments as key criterion for cooperative complex formation. We validated eight fragment stabilizers, six of which showed selectivity for one phospho-peptide client, and structurally characterized two nonselective hits and four fragments that selectively stabilized CRAF or FOXO1. The most efficacious of these fragments increased 14-3-3 σ /CRAF phospho-peptide affinity by 430-fold. Disulfide tethering to the wildtype C38 in 14-3-3 σ provided diverse structures for future optimization of 14-3-3/client stabilizers and highlighted a systematic method to discover molecular glues.

Introduction

Protein-protein interactions (PPIs) are essential to biology and their dysregulation is central to many diseases including cancer and neurodegeneration.^{1–4} Many of these important PPIs include “hub proteins” that interact with a large number of protein partners, ranging from a few dozen to a few thousand.⁵ Small molecules that inhibit or stabilize individual PPIs within these networks would be powerful tools to understand the effect of a single PPI on cellular function. Although PPIs were historically considered “undruggable”, there has been much progress in developing small molecule PPI inhibitors as biological probes and therapeutics.^{6–10} By contrast, PPI stabilization has remained largely underexplored, despite its potential to be a selective method for the manipulation of a single interaction within a protein network.^{11,12} Stabilization also has the potential to target unstructured, difficult to drug proteins via composite PPI binding pockets.^{13,14} Molecular glue degraders and natural products have demonstrated the therapeutic value of stabilizing native or non-native (neomorphic) PPIs.^{15–17} However, there are few robust, generalizable strategies to discover PPI stabilizers prospectively.^{11,18} Here, we describe a robust and instructive approach, using site-directed fragment based drug discovery (FBDD) to systematically discover molecular glues.

FBDD is a well-established method for the discovery of small molecules towards challenging targets.^{19,20} Fragments are simple chemical building blocks that – owing to their small number of atoms – sample chemical space efficiently. FBDD involves screening for weakly binding fragments that target subsites within a binding site, followed by fragment optimization via linking two fragments or elaborating a fragment-sized core. Disulfide tethering is a method of FBDD that capitalizes on a native or engineered cysteine residue proximal to an envisaged ligand binding site.^{21–24} In the context of orthosteric PPI stabilization, this binding site is composed of both members of the protein complex (the composite PPI interface). Fragments that bind to this site with the correct positioning to form a protein-fragment disulfide bond are detected by intact protein mass spectrometry (MS) in a high-throughput screen.²⁵ We utilize a

library of approximately 1600 disulfide molecules with diverse fragments and linkers between the fragment and the disulfide.²⁶ To test the efficacy of this technology to discover PPI stabilizers, we have selected the hub protein 14-3-3 and a set of its diverse partner proteins. 14-3-3 is ubiquitously expressed in mammals and plays multiple roles within the cell, including phosphorylation protection, conformational changes, subcellular trafficking, and induction or disruption of other PPIs.^{13,27–29} 14-3-3 typically binds to a phosphorylated serine/threonine of intrinsically disordered regions of its clients.³⁰ With several hundred known interacting partners, the 14-3-3-binding proteome provides diverse PPI interfaces with which to test the scope and limitations of our screening technology. Furthermore, 14-3-3/client stabilization could lead to therapeutics in a variety of disease fields including oncology, neurodegeneration, inflammation, and metabolic disease.^{29,31} Previous studies using natural products such as Fusicoccin (FC-A) and Cotylenin-A (CN-A) have shown that stabilizing 14-3-3/client interactions regulates the activity of important cell signaling pathways including estrogen receptor α (ER α) and CRAF, respectively.^{14,32}

We recently demonstrated the utility of disulfide tethering to identify molecular glues of the 14-3-3/ER α PPI. We discovered a series of disulfide fragments that stabilized the complex when bound to an engineered cysteine residue in the binding groove of 14-3-3, enhancing binding of the ER α C-terminal phosphopeptide up to 40-fold.²⁵ We now focus on targeting the native cysteine found in the 14-3-3 sigma isoform (14-3-3 σ), which offers greater translatability for covalent molecules. Of the 7 isoforms found in mammalian cells, 14-3-3 σ is the only one that harbors a cysteine residue proximal to the client binding groove, providing an additional degree of isoform specificity (Figure 2.1A).³⁰ The Protein Data Bank contains dozens of crystallographic structures of 14-3-3 with bound phosphopeptides derived from many of its binding partners, as well as a few examples of cryo-EM structures of full length proteins.^{13,33–35} This wealth of structural information allows for direct visualization of the various 14-3-3/client binding interfaces which could be capitalized on for the discovery of selective fragment stabilizers and the

development of potent lead compounds through structure-guided chemical optimization. For our screens, we utilized the phospho-peptide mimetics of 14-3-3 PPI partners which bind 14-3-3 in a similar fashion to the unstructured regions of the full-length proteins but offer greater synthetic flexibility and simplified crystallography.^{13,34}

Here, we used the disulfide tethering technology to systematically achieve selective PPI stabilization of 14-3-3 client phospho-peptides with diverse sequences and structures. The selected clients are also modulated by 14-3-3 in a way that could be therapeutically useful in cancer, metabolic disease, and/or rare disease.^{14,36–39} For four of the five targets, effective PPI stabilizers were identified. Crystallographic and functional data highlight the molecular recognition of fragments for the distinctive composite PPI interfaces formed by 14-3-3 bound to client phospho-peptides. In particular, the CRAF- and FOXO1-based peptide-protein interactions with 14-3-3 yielded fragments with high selectivity and/or stabilization factors. The diversity of sequences and conformations found in 14-3-3/client complexes make the 14-3-3 interactome particularly promising for small-molecule PPI stabilization; furthermore, the disulfide tethering approach is remarkably effective at selecting chemical starting points for further design of potent and selective PPI stabilizers.

Results and Discussion

Primary Screen for 14-3-3/Client Stabilizers

The disulfide tethering screen targeted C38, a native cysteine on 14-3-3 σ located proximal to the natural product binding pocket within the phospho-peptide recognition groove (Figure 2.1A, Figure S2.1). The cysteine forms a reversible covalent bond with the fragment thiol through disulfide exchange; the amount of bound fragment is measured by MS. A fragment stabilizer is expected to show a higher “% tethering” in the presence of the 14-3-3 σ /client phospho-peptide complex than 14-3-3 σ alone due to cooperativity between the fragment and the peptide (Figure 2.1B). The screening was performed on five different peptide targets

displaying three conceptually distinct 14-3-3 interaction motifs (Figure 2.1C): truncated (ER α),^{14,40} turned (FOXO1),³⁷ and linear (CRAF, USP8, SOS1).^{32,35,38,41}

14-3-3 σ (100 nM) was screened in complex with the 5 client phospho-peptides at a concentration twice their respective K_D values (Figure S2.2). This condition provided a consistent presence of the 14-3-3 σ /phospho-peptide composite interface that the fragments would engage. The 14-3-3 σ /phospho-peptide complex was incubated with a single concentration of fragment (200 μ M) under reducing conditions (250 μ M β -mercaptoethanol) for 3 hours before samples were measured by intact-protein LC/MS. The % tethering threshold for hit selection was three standard deviations (3*SD) above the average % tethering for that condition (Figure 2.2A). In the quadrant of highest interest, potential stabilizing fragments showed % tethering above the tethering threshold in the peptide screen and % tethering below the tethering threshold in the *apo* screen (Figure 2.2B, green quadrant). Neutral compounds showed significant % tethering for both 14-3-3 σ /phospho-peptide and *apo* (Figure 2.2B, yellow quadrant). Potential inhibitory fragments showed significant % tethering above the tethering threshold in the *apo* screen but not in the presence of peptide (Figure 2.2B, red quadrant). Compounds were clustered in a heat map based on % tethering in each of the five peptide screens and *apo* 14-3-3 screen (Figure 2.2C). An overlapping fragment hit cluster was identified for ER α , USP8, and SOS1 (Figure 2.2C, green box), whereas a cluster of unique hit fragments was identified for both CRAF and FOXO1 (Figure 2.2C, yellow boxes), indicating a difference in the abundance of selective stabilizers from the primary screens.

Each 14-3-3 σ /phospho-peptide screen yielded potential stabilizing fragments, but the number and binding efficiency varied (Figure 2.2A, 2.2C, 2.2D, and Tables S2.1-S2.5). The initial screen for ER α yielded 15 hit fragments including 7 unique stabilizers and a 33% tethering threshold. The FOXO1 screen yielded 23 hit fragments including 21 unique stabilizers and a 28% tethering threshold. The CRAF screen yielded 21 fragments including 16 unique stabilizers and a 35% tethering threshold. The USP8 screen yielded 10 hit fragments including 5 unique

stabilizers and a 29% tethering threshold. The SOS1 screen yielded 8 hit fragments including 4 unique stabilizers and a 22% tethering threshold (Figures 2.2A and 2.2D). Figure 2.2E depicts representative chemical structures for each target.

Non-Selective Stabilizing Compound 1

In the initial screen, compound **1** was identified as top hit for ER α , CRAF and USP8 (Figure 2.2). **1** was further characterized by three dose-response experiments. Mass spectrometry (MSDR, analyzing fragment binding to protein, quantified by DR₅₀ values) and fluorescent anisotropy (FADR, analyzing peptide binding to protein in the presence of compound, quantified by EC₅₀ values) defined the binding affinity for the fragment and its effective concentration, respectively (Figure 2.3B). The compound's effect on the 14-3-3/client PPI was then determined by titrating 14-3-3 in a fluorescence anisotropy assay at constant peptide and compound concentrations (quantified by *appK_D*). In all three validation assays, **1** displayed a strong preference for CRAF, followed by ER α and USP8, and had no activity with FOXO1 or SOS1. Compound **1** showed DR₅₀ values of 7 nM for CRAF, 18.1 μ M for ER α , and 24 nM for USP8 (Figure 2.3C) as well as EC₅₀ values of 922 nM for CRAF, 1.31 μ M for ER α , and 3.38 μ M for USP8 (Figure S2.3). In the protein titrations, **1** increased peptide affinity for 14-3-3 σ by 81-fold in the CRAF complex, 19-fold for 14-3-3 σ /ER α , and 4-fold for 14-3-3 σ /USP8 (Figure 2.3D and Table 2.1).

Crystal structures for compound **1** were obtained by co-crystallizing with ER α , CRAF, or USP8 bound to 14-3-3 σ (Figure 2.3E), with clear density for both **1** and the peptides (Figure S2.4). Comparing the three co-crystal structures, the strongest electron density and ligand occupancy for **1** was observed in the co-crystal structure with ER α . For ER α , the phenyl ring of **1** stacked against the +1 Val with a distance of $\sim$ 4 Å (Figure 2.3E). Compound **1** showed an identical binding mode in the presence of CRAF (Figure 2.3F), for which the +1 Thr was 3.5 Å from the phenyl ring, while the remainder of the CRAF peptide wrapped around the fragment.

These additional hydrophobic interactions could explain the higher fold stabilization with the CRAF peptide compared to ER α (Figure 2.3D, 2.3F). Interestingly, **1** shared the binding moiety with N42C-tethered stabilizers that were discovered previously for ER α (Figure 2.3G).²⁵ Whereas compound **1**'s chloro-group was not positioned identically, the longer linker of **1** bridged the larger distance from C38 compared to N42C. In the presence of the USP8 peptide, the phenyl ring of **1** was turned, thereby shifting the fragment up and back into the 14-3-3 σ pocket (Figure 2.3E). This conformational change seemed necessary because the USP8 peptide allowed for less space (Figure 2.3H). While the +1 Ser of UPS8 did not show any specific interaction with **1**, its +4 Ile pushed the fragment towards 14-3-3 σ , which was not an ideal position for this fragment as was reflected by the weak electron density and the minimal stabilization for USP8. By contrast, the +4 Val of CRAF allowed for more space, thereby positioning **1** in a preferred conformation. It is noteworthy that **1** did not stabilize FOXO1 or SOS1 to 14-3-3 σ . A crystallographic overlay of **1** with the FOXO1 peptide showed a steric clash with the +1 Trp of FOXO1, explaining its lack of stabilization (Figure S2.5A). In contrast, the +1 Ala residue of SOS1 would not contact the phenyl ring of **1**, perhaps explaining why no stabilization was observed (Figure S2.5B).

FOXO1 Selective Stabilizers

The FOXO1 peptide showed the highest number of stabilizing hits in our initial screen. For FOXO1, of the 23 initial stabilizers, 21 showed selectivity for the 14-3-3 σ /FOXO1 phospho-peptide complex over *apo* 14-3-3 σ and the other phospho-peptide clients in the initial screen (Figure 2.2D). Interestingly, the unique 21 FOXO-stabilizers had a highly conserved scaffold, with the phenyl ring engaging FOXO1 often decorated with halogens or a triazole moiety (Figure S2.6). Eight of these compounds were validated in the MSDR (Figure S2.7). Of the eight compounds, five compounds had enough material to retest and were active in the FADR assays (Figure 2.4A, Figure S2.8, and Table S2.2). The binding affinity of compound **2** to 14-3-3 σ was

>10,000-fold better in the presence of the FOXO1 phospho-peptide than *apo* 14-3-3 σ and all other phospho-peptide clients (DR_{50} = 360 nM vs. >2 mM; Figure 2.4B and Table 2.2).

Compounds **3** and **4** had DR_{50} values >450-fold and >2,000-fold better, respectively (Table 2.2 and Figure S2.7). Compounds **2**, **3**, and **4** showed the greatest fold-stabilization in the protein titrations decreasing 14-3-3 σ /FOXO1 K_D values 5-fold, 4-fold, and 12-fold (Figure 2.4C, Table 2.2, and Figure S2.9). It should be noted that while a high % tethering was observed for the FOXO1 stabilizers, the protein titrations only showed a modest shift in stabilization. This is likely due to the tight binding of the FOXO1 phospho-peptide, with a K_D value of 50 nM, already close to the limit of detection of this assay. A co-crystal structure for FOXO1/**2**/14-3-3 σ was obtained, with clear density for both **2** and the FOXO1 peptide (Figure S2.10A). The phenyl ring of **2** stacked against the front of the FOXO1 peptide consisting of the +1 Trp and the +2 Pro residues (Figure 2.4D). Strikingly, in the presence of **2**, the Trp of FOXO1 underwent a conformational change to form a hydrogen bond with its NH and the amide carbonyl of **2** (Figure 2.4E). Moreover, the hydroxyl on the phenyl ring of **2** made a hydrogen bond with the S45 of 14-3-3 σ , explaining the benefit of a hydrogen donor or, potentially, acceptor at that position. Compound **3** was also co-crystallized with FOXO1 (Figure S2.10B), showing a highly similar binding mode, but a lack of the hydrogen bonding with S45 of 14-3-3 σ (Figure S2.10C). An overlay of **2** with the other peptides revealed that **2** could not reach the smaller +1 residues in the other client peptides or that the peptides sterically clashed (Figure S2.11), potentially explaining its selectivity for FOXO1 over the other peptides. Previous work discovered imine-based stabilizers for the 14-3-3/Pin-1 complex which, similar to FOXO1, has a +1 Trp.⁴² In that work, the Trp engaged in π - π stacking interactions with an aromatic ring of the stabilizers. By contrast, the +2 Pro of FOXO1 locked the conformation of the +1 Trp and thereby prevented such a π - π stacking interaction with **2**, while the +2 Arg of Pin-1 allowed π - π stacking to take place. Thus, while the compound **2/3** scaffold emphasized the chemical moieties necessary for stabilizing FOXO1, crystal structures also expose a lack of flexibility of the FOXO1 peptide.

CRAF Selective Stabilizers

Following FOXO1, CRAF had the highest number of stabilizers. Of the 21 initial CRAF stabilizers, 16 compounds showed selectivity for the 14-3-3 σ /CRAF phospho-peptide complex over *apo* 14-3-3 σ and the other phospho-peptide clients in the primary screen (Figure 2.2D). Eleven compounds showed a similar scaffold which was remarkably analogous to the conserved scaffold for the FOXO1 stabilizers (Figure S2.12). However, the linker element of these compounds was often longer in the case for CRAF, and the phenyl ring was decorated with large cyclic groups while for FOXO1 only smaller halogen groups were tolerated. This is likely due to the smaller +1 residue of CRAF (Thr for CRAF, Trp for FOXO1), thereby leaving more space for the compound. Furthermore, two CRAF stabilizers were shared with ER α , both of which have a similar size in +1 residue (Val for ER α , Thr for CRAF). Nine of the 16 selective compounds were validated for potency and selectivity in the MSDR (Figure S2.13). Four of the nine compounds (compounds **5-8**; Figure 2.5A) showed activity in FADR (Figure S2.14 and Table S2.3) and stabilization in the protein titrations (Table 2.3 and Figure S2.15).

Compounds **5** and **6** were the most effective stabilizers. Compound **5** had a DR₅₀ value >3,000-fold lower in the presence of the CRAF peptide compared to 14-3-3 σ alone (Figure 2.5B) and showed a 246-fold stabilization of the 14-3-3 σ /CRAF phospho-peptide complex (K_D = 23 μ M to 92 nM; Figure 2.5C). Compound **6** had a DR₅₀ value 230-fold lower in the presence of CRAF compared to *apo* 14-3-3 σ and a 426-fold stabilization of the 14-3-3 σ /CRAF complex (Table 2.3, Figure S2.13 and S2.15).

The crystal structure of **5** with CRAF and 14-3-3 σ revealed a contact between the naphthalene ring of **5** and the +1 Thr residue of CRAF. The methyl group of **5** also seems important for hydrophobic interactions with the +1 Thr residue of CRAF, at a distance of 3.8 Å (Figure 2.5D, Figure S2.16A). An overlay of the CRAF peptide in the presence of **5** with the *apo* CRAF peptide showed no change in conformation of the +1 Thr residue. In contrast, the +4 Val residue of the CRAF peptide changed conformation to make space for **5** (Figure 2.5E).

We also crystallized compound **7** as a representative of the other structural class of the selective CRAF stabilizers (Figure S2.16B). Compound **7** had a DR₅₀ value >228-fold lower in the presence of the CRAF peptide than *apo* 14-3-3 σ (Figure 2.5F) and was less selective for CRAF compared to **5** in the MSDR (Figure S2.13). However, compound **7** showed no stabilization of any of the peptides other than CRAF in the FADR (Figure S2.14C), reflecting the selectivity shown in the primary screen. The weaker 14-3-3 σ binding of **7** (12.2 μ M DR₅₀) was reflected in a somewhat lower stabilization of the 14-3-3 σ /CRAF complex compared to the other chemotype of **5** and **6** (77-fold vs 246- and 426-fold, respectively; Figure 2.5G, Figure S2.15). Co-crystallization of **7** with CRAF and 14-3-3 σ revealed a novel orientation of its phenyl ring towards the roof of 14-3-3 σ , positioning its trifluoromethyl group above the CRAF peptide (Figure 2.5H). While the conformations of **5** and **7** were quite different, an overlay of the two structures shows that the trifluoromethyl group of **7** occupied the same cavity as the naphthalene ring of **5** (Figure S2.16C). Furthermore, an overlay of the CRAF peptide in the presence of **7** with the *apo* CRAF peptide revealed a conformational change of the +4 Val of CRAF, which stacked against the compound, pushing it towards 14-3-3 σ . Additionally, a water-mediated hydrogen bond was formed between **7** and the backbone of CRAF peptide (Figure 2.5I). The lower specificity for CRAF of **7** in the MSDR could be due to its small size, leaving room for alternative +1 residues to have a cooperative effect on 14-3-3 σ engagement. Stabilizer **8** had an almost identical structure to **7**, differing only in a chloro-group in the para-position of the phenyl ring, and showed similar binding modes to **7** in its structure with CRAF (Figure S2.16D and E).

Next to these CRAF selective stabilizers, the non-selective stabilizer compound **1** also showed a large fold-stabilization towards the CRAF peptide (Figure 2.3). A crystallographic overlay of these three scaffolds revealed remarkable differences in conformation of the CRAF/compound interactions (Figure S2.16F). These changes highlight the flexibility of the CRAF peptide, perhaps leading to its facility for stabilization, especially in the case of the

stabilizers' phenyl ring, which can occupy a wide range of positions and conformations in combination with the CRAF client phospho-peptide.

Conclusions

Systematic methods to discover small-molecule stabilizers of PPI would enable chemical biologists to probe challenging biological systems with potency and precision. By trapping proteins in complexes, stabilization can target proteins with intrinsically disordered regions and allow manipulation of a specific PPI from among related hub protein complexes within a network. Disulfide tethering, a powerful FBDD technique, is readily tunable to a specific site on a protein of interest, amenable to HTS, and provides a direct quantitative measurement of fragment binding.

Here, we explored the scope of the disulfide tethering technology using the hub protein 14-3-3 σ and 5 biologically and structurally diverse phospho-peptides derived from the 14-3-3 client proteins ER α , FOXO1, CRAF, USP8, and SOS1. Of the 1600 fragments in the disulfide library, 62 showed activity as stabilizers for one or more phospho-peptides and were assessed by MSDR. 36 of the 62 compounds were taken forward into the FADR assays to determine stabilization of a 14-3-3 client phospho-peptide. Finally, eight compounds showed cooperativity with the 14-3-3 σ /phospho-peptide complex via 14-3-3 σ protein titrations, and six were structurally characterized for their contacts with 14-3-3 σ and the client phospho-peptide via x-ray crystallography (Figure S2.17A). Thus, the disulfide tethering strategy systematically discovered stabilizers for a range of peptide sequences, conformations, and affinities.

Of the 5 peptide targets selected, we discovered stabilizers for four clients, two of which also had selective stabilizers. Fragments increased binding affinity of the 14-3-3 σ /phospho-peptide complex as much as 430-fold in the case of **6** and 250-fold for our best structurally characterized hit, **5**. Selective stabilizers distinguished between phospho-peptide clients due to the unique composite binding surface created by the phospho-peptide/14-3-3 σ interface

(Figures 2.4 and 2.5). The non-selective stabilizers also showed varying degrees of efficacy in stabilizing different clients. Compound **1** facilitated a greater than 80-fold shift in affinity for CRAF, a 19-fold shift for ER α , a more modest 4-fold shift for USP8, but had no effect on SOS1 and FOXO1 (Figure 2.3).

The individual phospho-peptide binding motifs and C-terminal residues following the phosphorylation site create a distinct environment around the 14-3-3 σ C38 fragment binding pocket, dictating what chemical moieties effectively facilitated cooperativity between 14-3-3 σ , the phospho-peptide client, and the fragments. The stabilizers for FOXO1 had a highly conserved scaffold, consistent with the rigidity of this peptide (Figure S2.17B). In contrast, the stabilizers of CRAF were larger and showed more chemical diversity in their scaffold, emphasizing the flexibility of the CRAF peptide. The short ER α peptide resulted in limited selectivity, sharing many stabilizers with CRAF. Lastly, USP8 and SOS1 were the hardest to target, likely due to the proximity of the peptide C-terminus to C38 of 14-3-3 σ , which was also reflected in the small scaffold of the discovered stabilizers from the primary screen (Figure S2.17B). Alternative cysteine tethering mutations could sample different sub-pockets to stabilize peptides which occupy more of the 14-3-3 binding groove. Taken together, the intrinsic diversity of the 14-3-3/phospho-peptide composite binding interface allowed for selectivity and precision when targeting a specific 14-3-3/client PPI.

While the focus of the screen was the discovery of fragment stabilizers, the screen also identified selective inhibitors, non-selective inhibitors, and neutral compounds for each client peptide and 14-3-3 (Figure S2.18). Therefore, disulfide tethering is a versatile tool that can be expanded to meet a wide range of conditions and results in hits that disrupt or stabilize PPIs. 14-3-3 provides an exciting proof of concept due to its large roster of clients, involvement in many biological processes, therapeutic potential, and extensive structural data, but the applicability of FBDD reaches beyond targeting a singular protein. It is due to this ease of access and applicability that disulfide tethering lends itself to the discovery of biological probes

for PPIs and novel therapeutics for previously inaccessible biological challenges and diseases related to intrinsically disordered proteins.

Figures

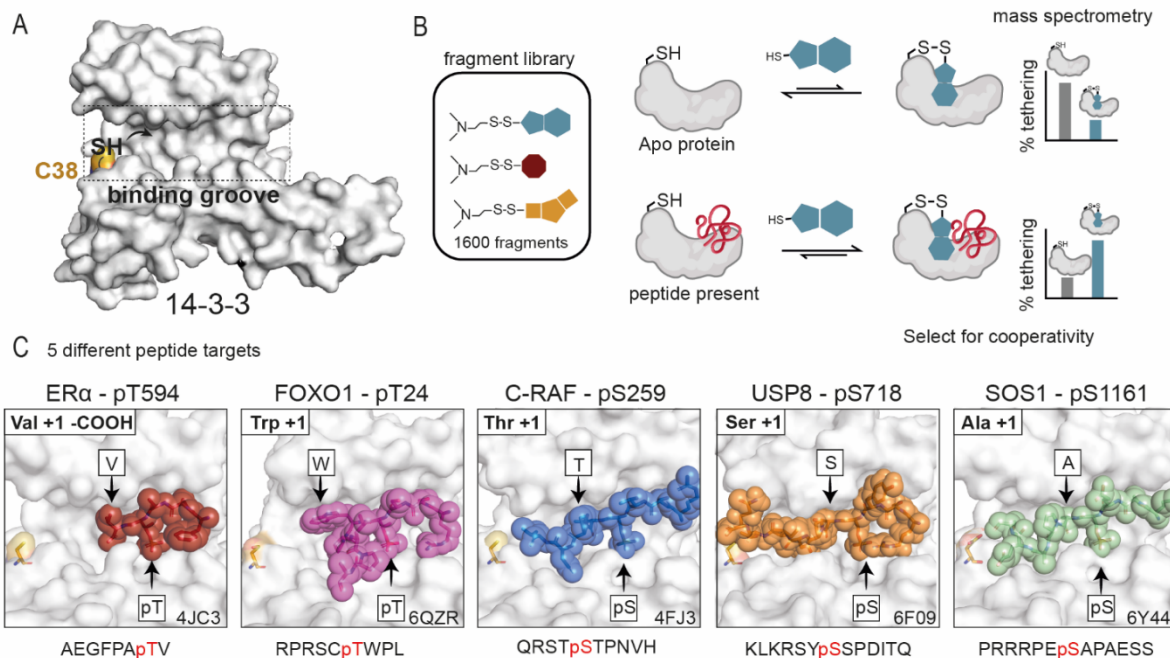


Figure 2.1: 14-3-3/client stabilizer approach

A The client protein binding groove of a 14-3-3 σ monomer (white surface) highlighting the native cysteine (C38; yellow surface) and target thiol. **B** General schematic of the primary disulfide tethering screen. Fragments were incubated with *apo* 14-3-3 σ (white) without any phospho-peptide (top) and 14-3-3 σ with the phospho-peptide client present (bottom). Fragments were assessed for their covalent engagement of C38 via mass spectrometry, termed “% tethering”. Fragments that bind 14-3-3 σ with a higher % tethering in the presence of phospho-peptide than in the *apo* screen are selected for further analysis of cooperativity. **C** Crystallographic structures of the 5 phospho-peptide clients bound in the 14-3-3 σ (white surface) binding pocket showing proximity to C38 (yellow surface). ER α (red sticks) has a C-terminal motif with phospho-threonine (pT) in the penultimate position and C-terminal valine (V) in the +1 position. FOXO1 (pink sticks) has a curved motif with tryptophan (W) in +1 position. CRAF (blue sticks), USP8 (orange sticks), and SOS1 (green sticks) extend to various degrees into the 14-3-3 binding groove, with threonine (T), serine (S), and alanine (A) residues in the +1 position, respectively. PDB left to right: 4JC3, 6QZR, 4FJ3, 6F09, 6Y44.

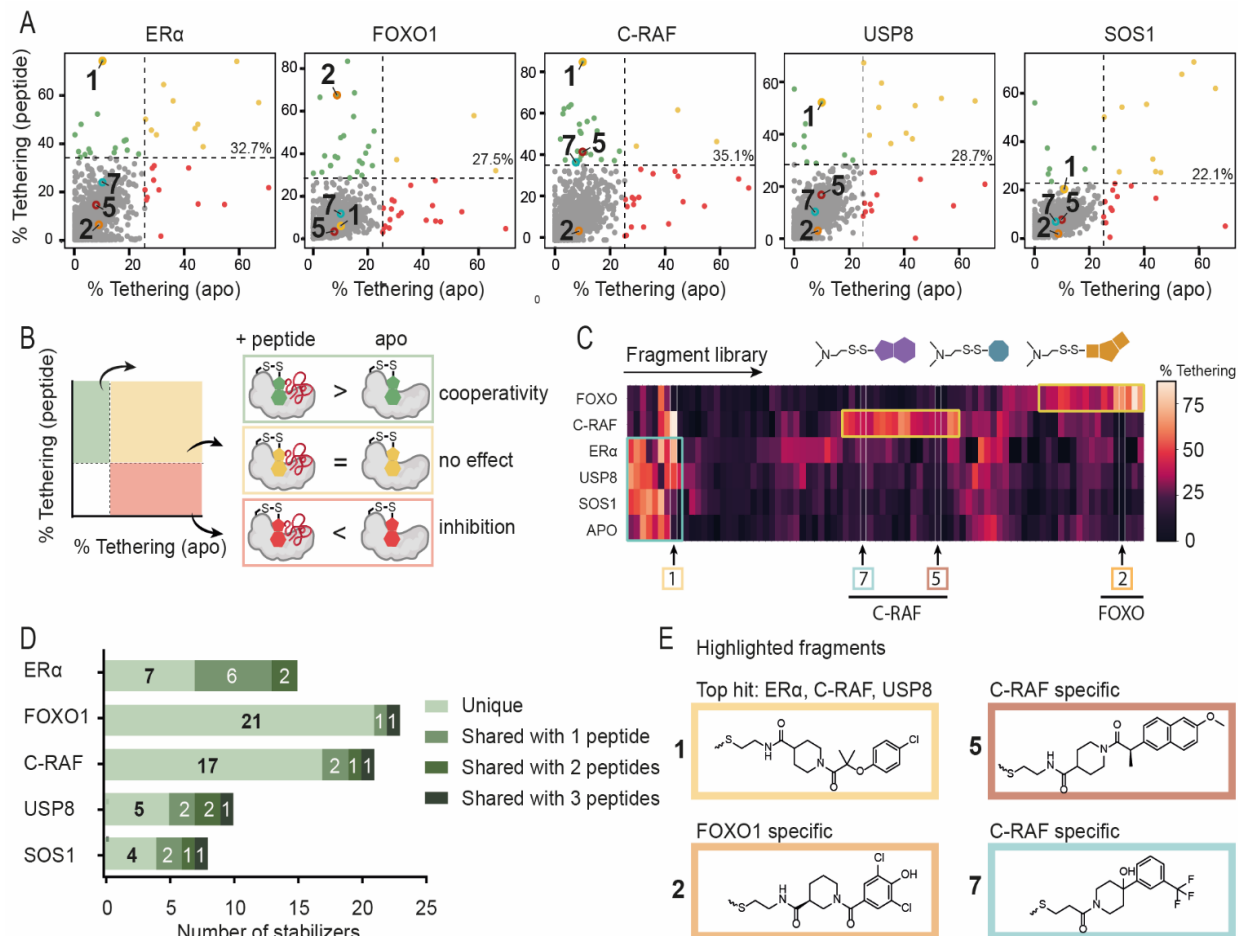


Figure 2.2: Primary tethering screen results

A) Scatterplot data illustrating the correlation of % tethering of fragments to 14-3-3σ in the presence of the phospho-peptide (y-axis) as compared to apo 14-3-3σ (x-axis). Hit selection threshold (mean + 3*SD) in each screen is indicated by a black dashed line. Compounds 1, 2, 5 and 7 are indicated as yellow, orange, red and cyan circles, respectively. **B)** Schematic of compound scatterplots. Quadrants are outlined by dotted lines signifying 3*SD above average % tethering for compounds in the presence of phospho-peptide (horizontal line) and apo 14-3-3σ (vertical line). Compounds in green quadrant showed increased binding to 14-3-3σ in the presence of phospho-peptide, yellow quadrant showed neutral binding to 14-3-3σ, and red quadrant showed a reduced binding in presence of phospho-peptide. **C)** Heat map of hit fragments across all 5 phospho-peptide screens and apo 14-3-3σ screen. Compounds clustered based on % tethering in each screen. Compounds 1, 2, 5 and 7 were of primary interest as non-selective and selective stabilizers. **(D)** Number of stabilizers of each peptide that were: unique, shared with one other peptide, shared with two other peptides, or shared with three other peptides (green bars with the darker color shared with more peptides). **(E)** Chemical structures of highlighted fragment hits 1, 2, 5, and 7.

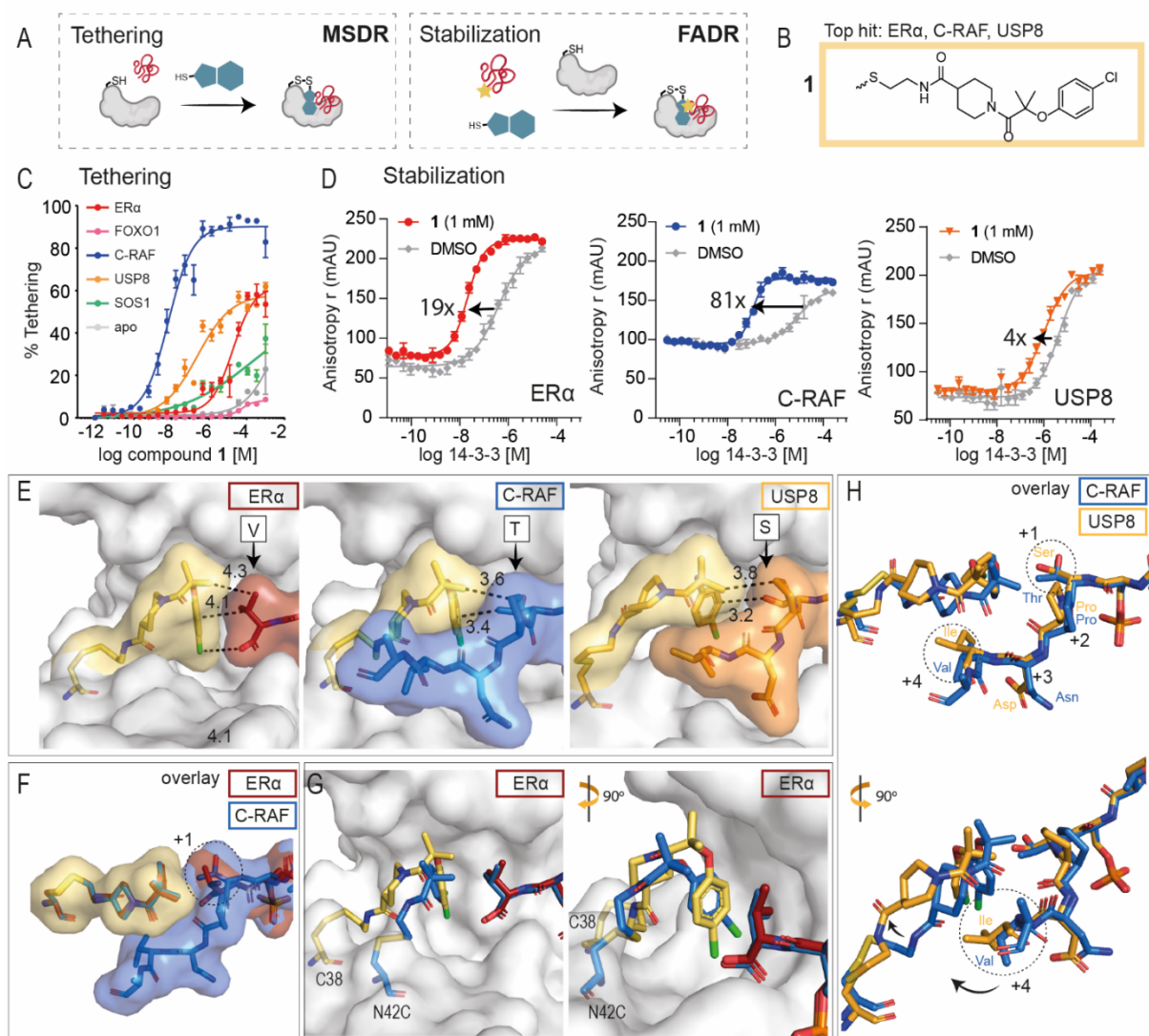


Figure 2.3: Overview of biochemical and structural properties of non-selective stabilizer 1

A) In mass spec dose response (MSDR), the focus was on compound binding to 14-3-3 σ , measured by % tethering; fluorescence anisotropy dose response (FADR) experiments determined degree of stabilization, measured by phospho-peptide binding to 14-3-3 σ in presence of compound. **B**) Chemical structure of stabilizer 1. **C**) MSDR curves for 1 showing percentage of fragment/protein conjugate formation with 14-3-3 σ *apo*, or in the presence of ER α , FOXO1, CRAF, USP8 or SOS1 peptide. **D**) 14-3-3 σ titrations to fluorescein-labeled ER α , CRAF or USP8 in the presence of DMSO or 1 (1 mM), reporting a 19-, 81-, and 4-fold increase of the 14-3-3 σ /peptide binding interface, respectively. **E**) Crystal structure of 1 bound to 14-3-3 σ C38 in complex with (from left to right) ER α peptide, CRAF peptide, and USP8 peptide. Distances are indicated (Å, black dashes). **F**) Overlay of 1's conformations when interacting with ER α and CRAF. **G**) Overlay of 1 (yellow) bound to 14-3-3 σ C38 and previously reported stabilizer (blue) bound to 14-3-3 σ mutant N42C (PDB ID: 6HMT) interacting with ER α phospho-peptides. **H**) Overlay of 1 bound to 14-3-3 σ C38 interacting with CRAF and USP8.

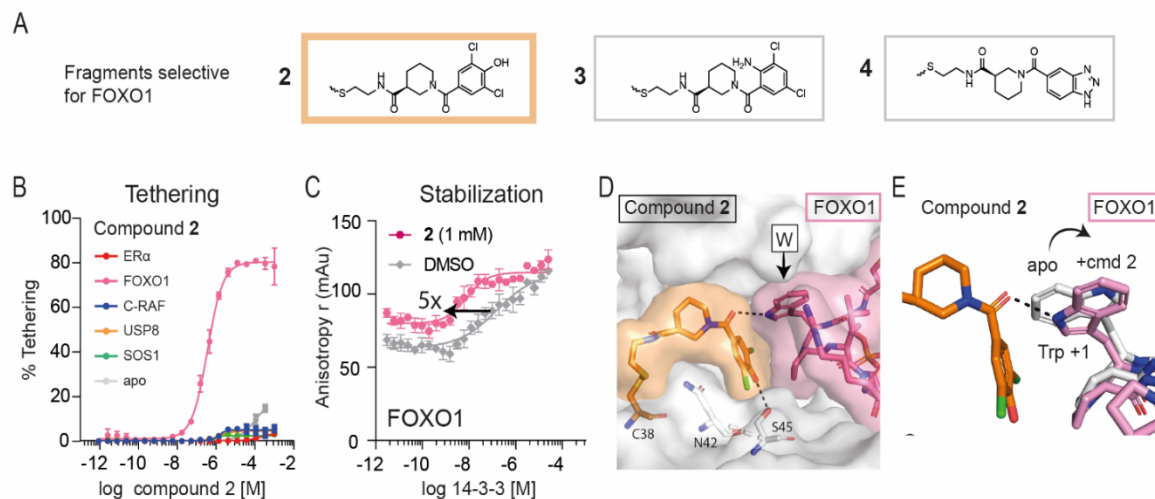


Figure 2.4: Overview of selective stabilizers for FOXO1

A) Chemical structures of highlighted FOXO1 selective stabilizers **2-4**. **B)** MSDR curves for **2** showing percentage of fragment/protein conjugate formation with 14-3-3 σ *apo*, or in the presence of ER α , FOXO1, CRAF, USP8 or SOS1 peptide. **C)** 14-3-3 σ titrations to fluorescein-labeled FOXO1 in the presence of DMSO or **2** (1 mM), reporting a 5-fold increase in 14-3-3 σ /FOXO1 binding. **D)** Crystal structure of **2** (orange) bound to 14-3-3 σ (white) C38 in complex with FOXO1 phospho-peptide (pink). **E)** Overlay of FOXO1 peptide in the *apo*-structure (white) with the FOXO peptide (pink) in presence of **2** (orange).

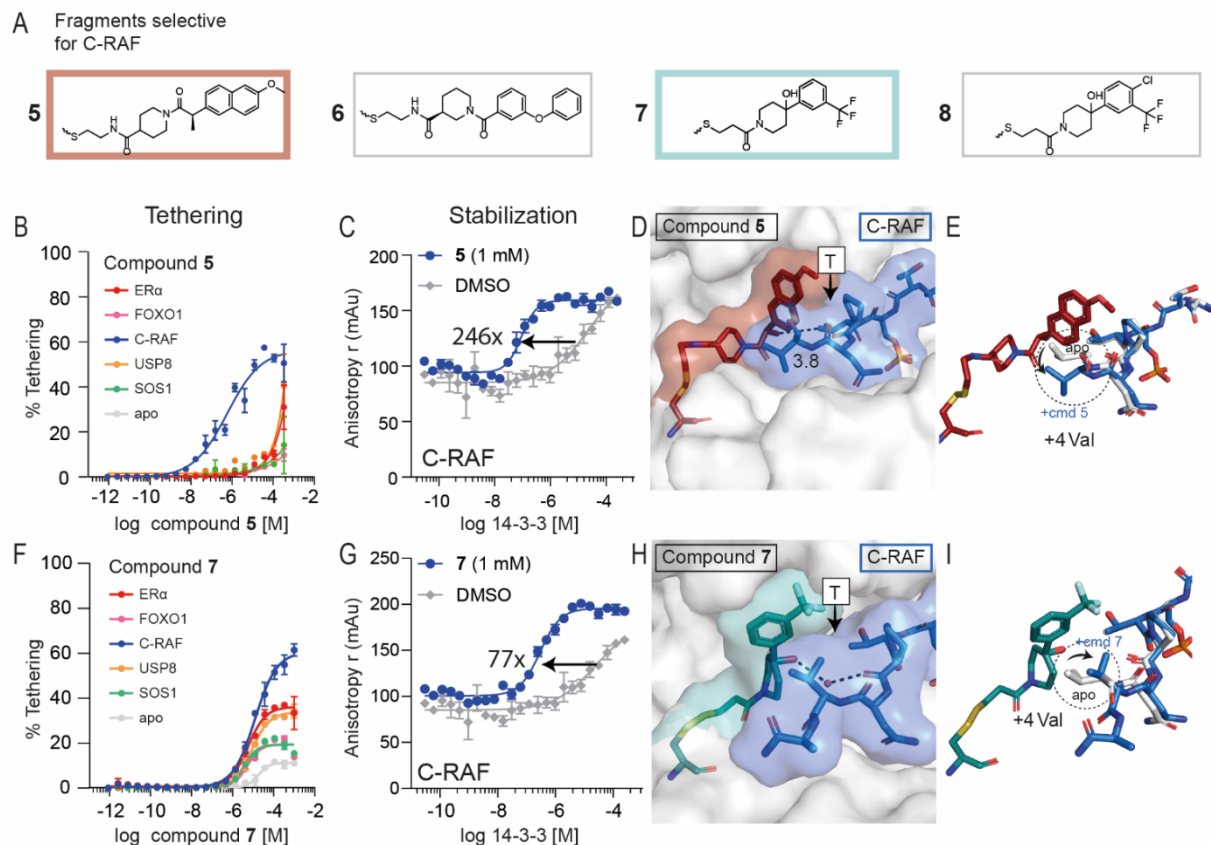


Figure 2.5: Overview of selective stabilizers for CRAF

A) Chemical structures of highlighted CRAF selective stabilizers **5-8**. **B)** MSDR curves for **5** showing percentage of fragment/protein conjugate formation with 14-3-3σ apo, or in the presence of ERα, FOXO1, CRAF, USP8 or SOS1 peptide. **C)** 14-3-3σ titration to fluorescein-labeled CRAF in the presence of DMSO or **5** (1 mM), reporting a 246-fold increase of 14-3-3σ/CRAF binding. **D)** Crystal structure of **5** (red) bound to 14-3-3σ (white) in complex with CRAF phospho-peptide (blue). **E)** Overlay of CRAF peptide in the apo-structure (white) with the CRAF peptide (blue) in presence of **5** (red). **F)** MSDR curves for **7** showing percentage of fragment/protein conjugate formation with 14-3-3σ apo, or in the presence of ERα, FOXO1, CRAF, USP8 or SOS1 peptide. **G)** 14-3-3σ titration to fluorescein-labeled CRAF in the presence of DMSO or **7** (1 mM), reporting a 77-fold increase of 14-3-3σ/CRAF binding. **H)** Crystal structure of **7** (teal) bound to 14-3-3σ (white) in complex with CRAF phospho-peptide (blue). **I)** Overlay of CRAF peptide in the apo-structure (white) with the CRAF peptide (blue) in presence of **7** (teal).

Tables

Table 2.1: Tethering and stabilization of 14-3-3 σ /clients by compound 1

	MSDR (250 μ M BME)	FADR (50 μ M BME)	Protein Titrations (50 μ M BME)		
Peptide	DR₅₀ (μM)	EC₅₀ (μM)	K_{D,app}	K_{D,DMSO}	Fold Stab.
CRAF	0.007	0.922	106 nM	8.5 μ M	81
ER α	18.1	1.31	21 nM	360 nM	19
USP8	0.024	3.38	1.1 μ M	4.5 μ M	4
SOS1	>2 mM	>2 mM	N/A	N/A	N/A
FOXO1	>2 mM	>2 mM	N/A	N/A	N/A
apo	>2 mM	>2 mM	N/A	N/A	N/A

Table 2.2: Properties of selective FOXO1 stabilizers

	MSDR (250 μ M BME)	FADR (50 μ M BME)	Protein Titrations (50 μ M BME)		
Cmpnd	DR₅₀ (μM)	EC₅₀ (μM)	K_{D,app}	K_{D,DMSO}*	Fold Stab.
2	0.36	5.10	7.7 nM	39 nM	5
3	2.2	N/A	10.6 nM	42 nM	4
4	143	N/A	9.7 nM	111 nM	12

*K_D for peptide is accurate within 3-fold range. These values are shown on the same plate as protein titrations with compound.

Table 2.3: Properties of selective CRAF stabilizers

	MSDR (250 μ M BME)	FADR (50 μ M BME)	Protein Titrations (50 μ M BME)		
Cmpnd	DR₅₀ (μM)	EC₅₀ (μM)	K_{D,app}	K_{D,DMSO}*	Fold Stab.
5	0.58	0.22	92 nM	23 μ M	246
6	8.78	1.33	100 nM	42 μ M	426
7	12.2	3.18	294 nM	23 μ M	77
8	3.71	13.5	207 nM	23 μ M	110

*K_D for peptide is accurate within 3-fold range. These values are shown on the same plate as protein titrations with compound.

Supplementary Figures

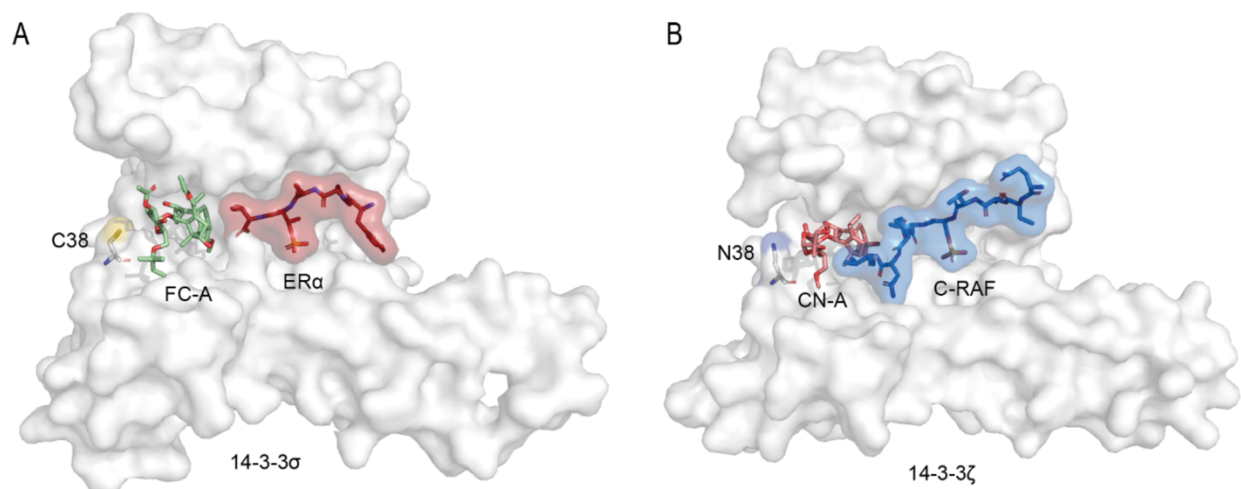


Figure S2.1: Crystal structures of natural product stabilizers

A) Fusicoccin-A (green sticks) and **B)** Cotylenin-A (pink sticks) bound to 14-3-3 (white surface) and clients ER α (red sticks) and CRAF (blue sticks), respectively. Cysteine 38 depicted as white sticks. PDB ID: 4JDD and 4IHL.

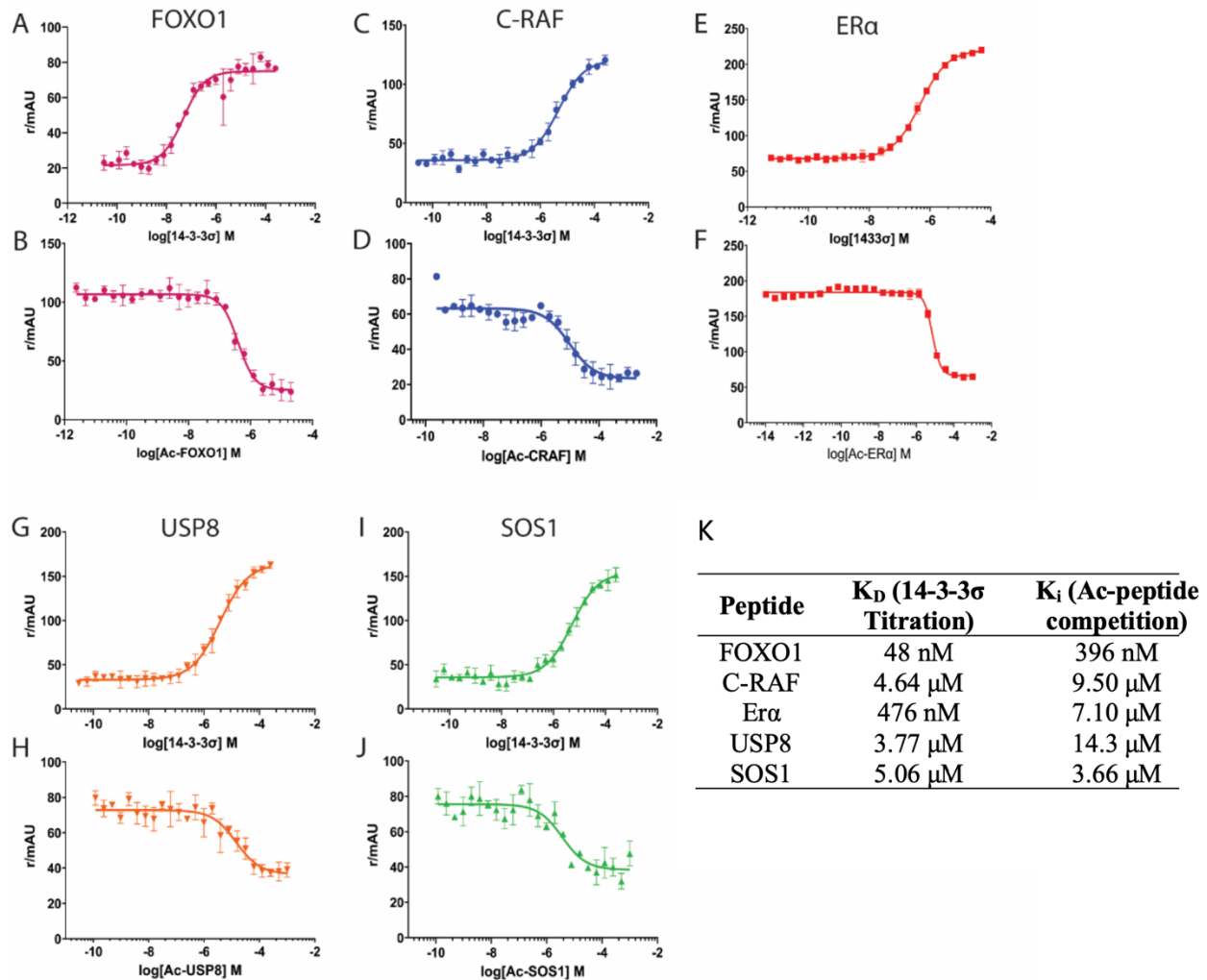


Figure S2.2: 14-3-3 σ /phosphopeptide binding curves

A) Protein titration with 14-3-3 σ in presence of 100 nM FITC-labeled FOXO1 11-mer peptide determined K_D . **B)** Competition assay used to determine K_i of acetylated FOXO1 11-mer peptide (Ac-FOXO1) for mass spectrometry experiments. Ac-FOXO1 was titrated from high to low concentration in presence of 100 nM FITC-FOXO1 peptide and 14-3-3 σ at 3/2 K_D value determined in (A). **C)** Protein titration with 14-3-3 σ in presence of 100 nM FITC-labeled CRAF 36-mer peptide determined K_D . **D)** Competition assay used to determine K_i of acetylated CRAF 11-mer peptide (Ac-CRAF). **E)** Protein titration with 14-3-3 σ in presence of 100 nM FITC-labeled ER α 15-mer peptide determined K_D . **F)** Competition assay used to determine K_i of acetylated ER α 15-mer peptide (Ac-ER α). **G)** Protein titration with 14-3-3 σ in presence of 100 nM FITC-labeled USP8 13-mer peptide determined K_D . **H)** Competition assay used to determine K_i of acetylated USP8 13-mer peptide (Ac-USP8). **I)** Protein titration with 14-3-3 σ in presence of 100 nM FITC-labeled SOS1 13-mer peptide determined K_D . **J)** Competition assay used to determine K_i of acetylated SOS1 13-mer peptide (Ac-SOS1). **K)** Table of peptide affinities to 14-3-3 σ based on data from A-J.

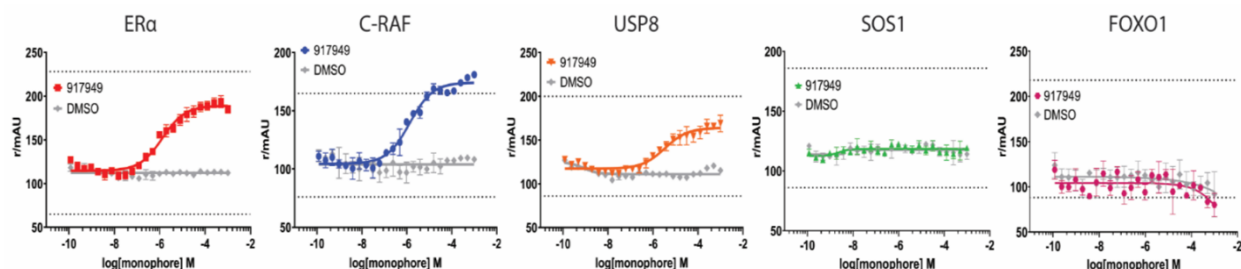


Figure S2.3: FADR curves for non-selective stabilizer 1

Top black dashed line signifies maximum anisotropy control, the value of which was determined by peptide bound to 250 μM 14-3-3 σ (at least 50-fold excess of peptide K_D). Bottom dashed line signifies minimum anisotropy control, value determined by peptide only and no 14-3-3 σ . From left to right: 917949 dose-dependent stabilization of ER α phospho-peptide (red) binding to 14-3-3 σ ; 917949 stabilization of CRAF (blue) binding to 14-3-3 σ ; 917949 stabilization of USP8 (orange) binding to 14-3-3 σ . SOS1 (green) and FOXO1 (pink) showed no stabilization as compared to DMSO control (grey). From left to right EC₅₀ values: 1.31 μM (ER α); 922 nM (CRAF); 3.38 μM (USP8); >2000 μM (SOS1); >2000 μM (FOXO1).

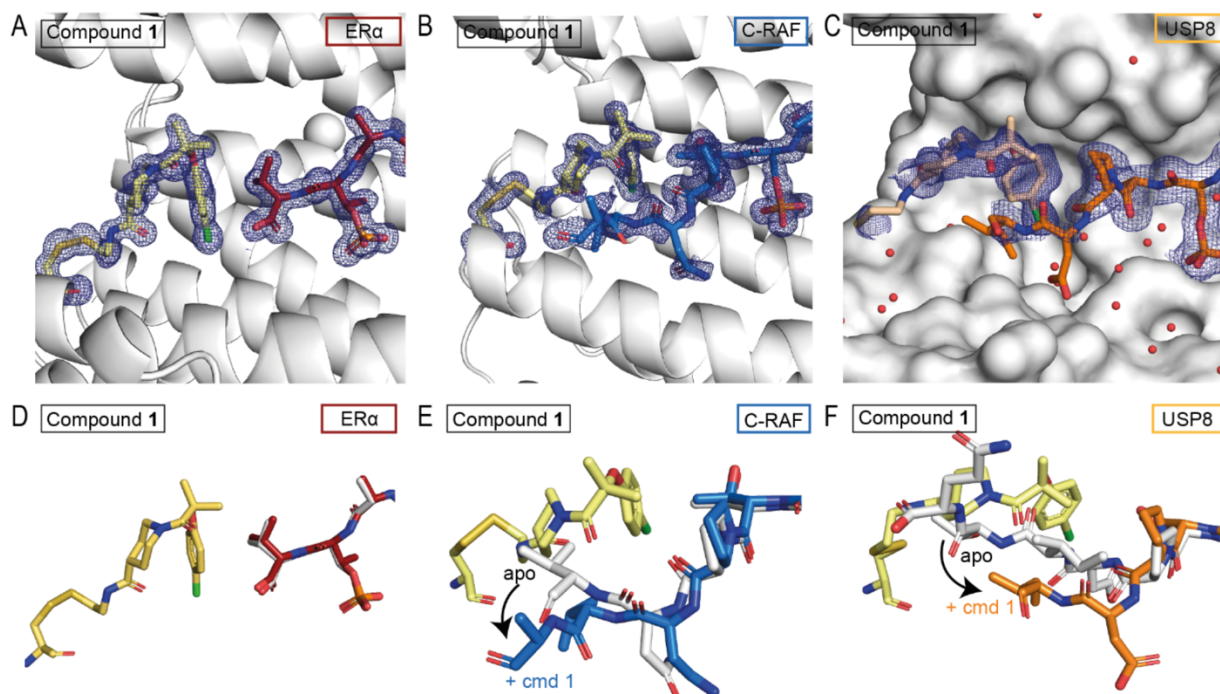


Figure S2.4: Crystal structures of Compound 1 bound to 14-3-3 σ /client complexes

A-C) Crystal structures of Compound 1 (yellow sticks) with **(A)** ER α (red sticks) **(B)** CRAF (blue sticks) and **(C)** USP8 (orange sticks) complexed with 14-3-3 σ (white cartoon and surface). **D-F)** Overlay of crystal structures of compound 1 (yellow sticks) – peptide complex with **(D)** ER α (red sticks when complexed with compound 1, white sticks as apo), **(E)** C-RAF (blue sticks when complexed with compound 1, white sticks as apo), **(F)** USP8 (orange sticks when complexed with compound 1, white sticks as apo). PDB ID apo structures: 4JC3, 4FJ3, 6F09. 2Fo-Fc electron density maps are contoured at 1 σ .

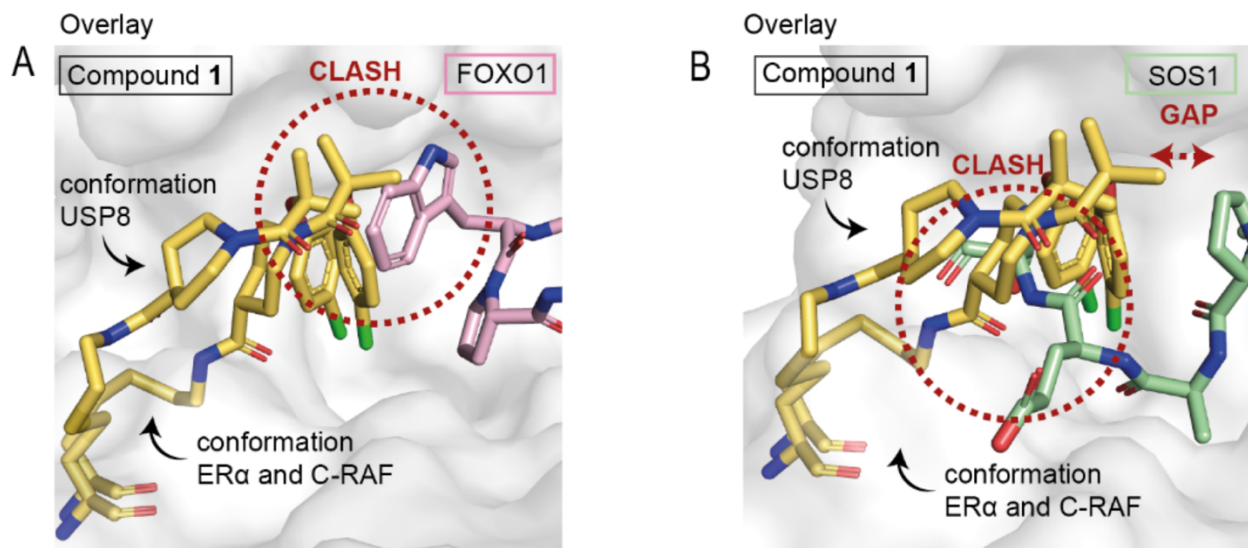


Figure S2.5: Overlays of compound 1 bound with different 14-3-3σ client peptides

Crystallographic overlay of compound **1** (yellow sticks) with **A**) FOXO1 (pink sticks) and **B**) SOS1 (green sticks) in 14-3-3 (white surface), highlighting the clash of compound **1** with these peptides. PDB ID apo structures: 6QZR and 6Y44.

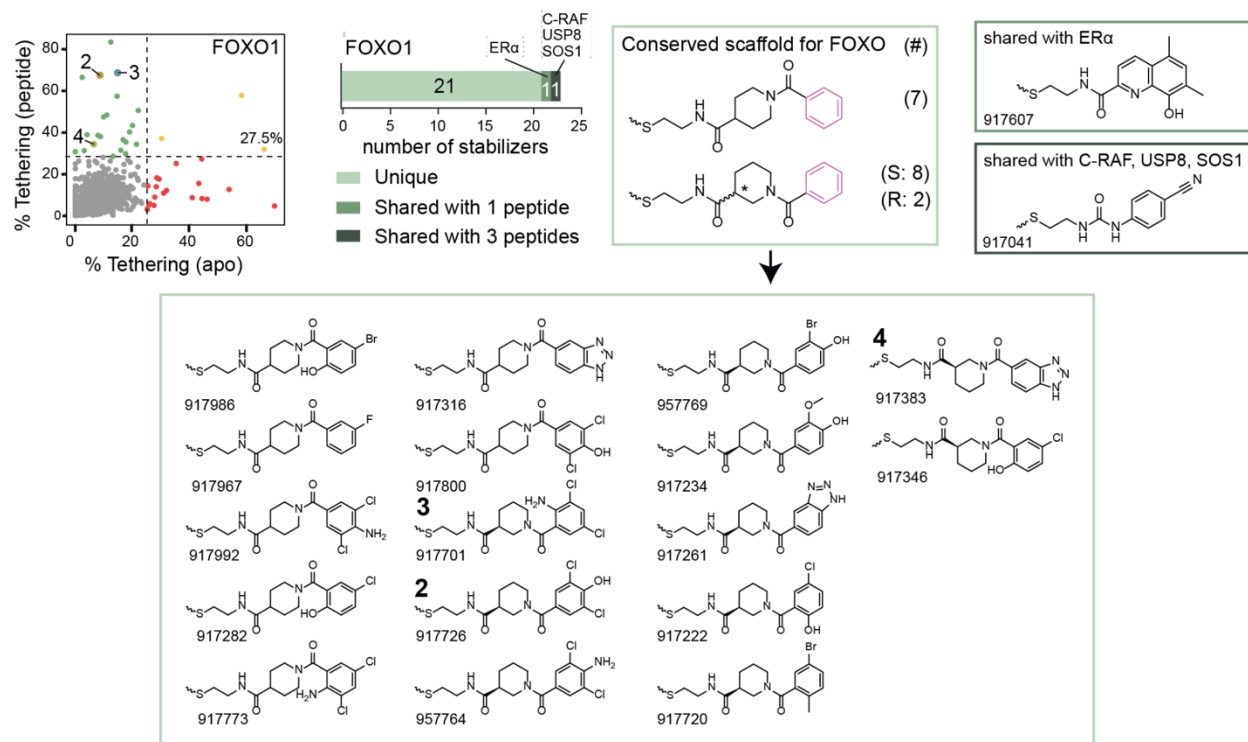


Figure S2.6: FOXO1 stabilizer structures

Scatter plot and chemical structures of the discovered stabilizers for FOXO1 from the primary screen, with in the light green box the unique stabilizers, the darker green box stabilizer shared with ERα and in the darkest green box the stabilizer shared with CRAF, USP8 and SOS1.

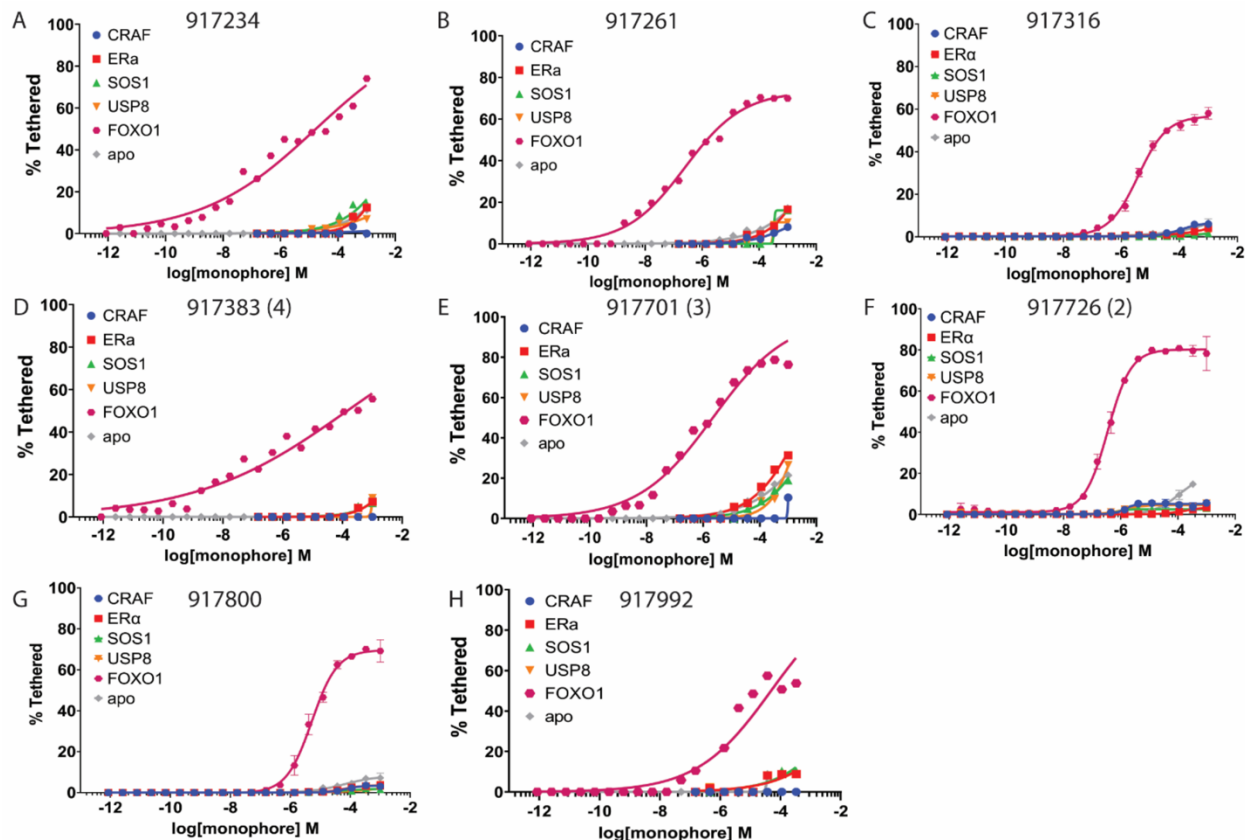


Figure S2.7: MSDR results for top FOXO1 selective stabilizers

A) MSDR for FOXO1 hit compound 917234 showing selectivity for FOXO1 over other four 14-3-3 σ client phospho-peptides and apo. DR₅₀: 16.9 μ M. **B)** MSDR for FOXO1 selective hit compound 917261. DR₅₀: 3.61 μ M. **C)** MSDR for FOXO1 selective hit compound 917316. DR₅₀: 42 nM. **D)** MSDR for FOXO1 selective hit compound 917383 (compound **4** in main text). DR₅₀: 143.4 μ M. **E)** MSDR for FOXO1 selective hit compound 917701 (compound **3** in main text). DR₅₀: 2.2 μ M. **F)** MSDR for FOXO1 selective hit compound 917726 (compound **2** in main text). DR₅₀: 360 nM. **G)** MSDR for FOXO1 selective hit compound 917800. **H)** MSDR for FOXO1 selective compound 917992. DR₅₀: 48.7 μ M.

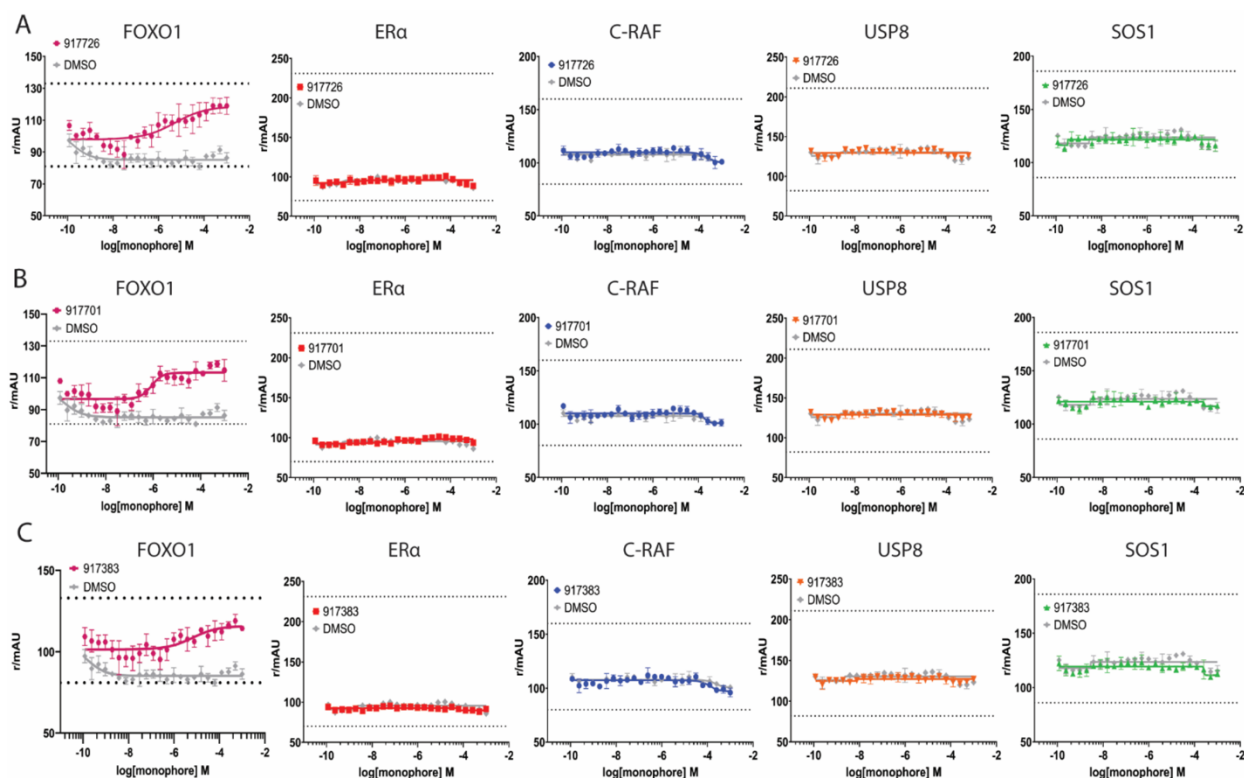


Figure S2.8: FADR for FOXO1 selective stabilizers

Fluorescence anisotropy dose response (FADR) curves for FOXO1 selective stabilizers 917726, 917701, and 917383 (compounds **2-4** in main text, respectively). Top black dashed line signifies maximum anisotropy control, the value of which was determined by peptide bound to 250 μ M 14-3-3 σ (at least 50-fold excess of peptide K_D). Bottom dashed line signifies minimum anisotropy control, value determined by peptide only and no 14-3-3 σ . Controls were measured per plate. **A)** 917726 dose-dependent stabilization of FOXO1 phospho-peptide (pink) binding to 14-3-3 σ . EC₅₀ value with FOXO1: 5.1 μ M. ER α (red), CRAF (blue), USP8 (orange), and SOS1 (green) showed no increase in anisotropy over DMSO control (grey) indicating no stabilization. **B)** 917701 stabilization of FOXO1 (pink) binding to 14-3-3 σ . EC₅₀ value with FOXO1: 850 nM. No increase stabilization over DMSO control (grey) observed for ER α (red), CRAF (blue), USP8 (orange), or SOS1 (green). **C)** 917383 stabilization of FOXO1 (pink) binding to 14-3-3 σ . EC₅₀ value with FOXO1: 6.69 μ M. No increase stabilization over DMSO control (grey) observed for ER α (red), CRAF (blue), USP8 (orange), or SOS1 (green).

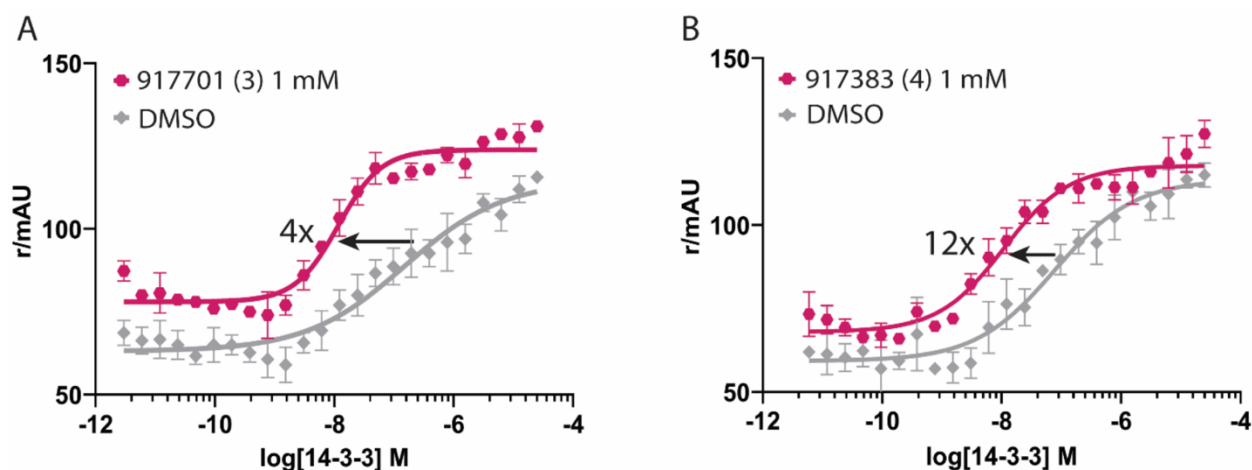


Figure S2.9: Fluorescence anisotropy protein titrations with selective compounds 2 and 3

Fluorescence anisotropy protein titrations for FOXO1 and CRAF selective stabilizers 917701 and 917383 (compounds **2** and **3** in main text, respectively. **A**) Addition of 1 mM 917701 (compound **3**; pink curve) resulted in 4-fold decrease in 14-3-3 σ /FOXO1 K_D (10.6 nM vs. 42 nM) compared to DMSO control (grey curve). **B**) Addition of 1 mM 917383 (compound **4**; pink curve) resulted in 12-fold decrease in 14-3-3 σ /FOXO1 K_D (9.7 nM vs. 111 nM) compared to DMSO control (grey curve).

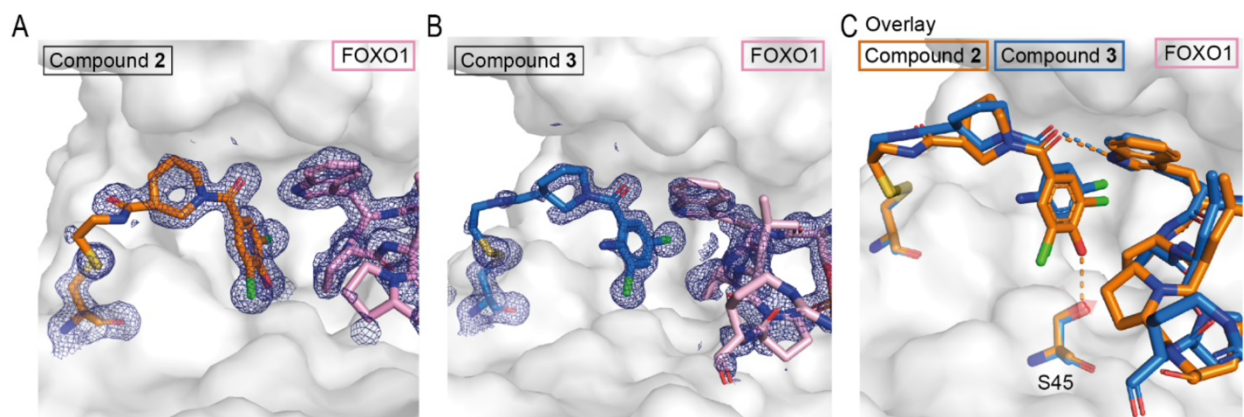


Figure S2.10: Comparison of compounds 1 and 2 binding to the 14-3-3 σ /FOXO1 complex

Crystal structures of **A**) compound **2** (orange sticks) in complex with FOXO1 (pink sticks) and 14-3-3 (white surface) and **B**) compound **3** (blue sticks) with FOXO1 (pink sticks) and 14-3-3 (white surface). **C**) Crystallographic overlay of compound **2** (orange sticks) and compound **3** (blue sticks) with the FOXO1 peptide (peptide color matching compound complexed with) in 14-3-3 (white surface). Hydrogen interactions are depicted as orange dashed lines for compound **2** and blue dashed lines for compound **3**. 2Fo-Fc electron density maps are contoured at 1 σ .

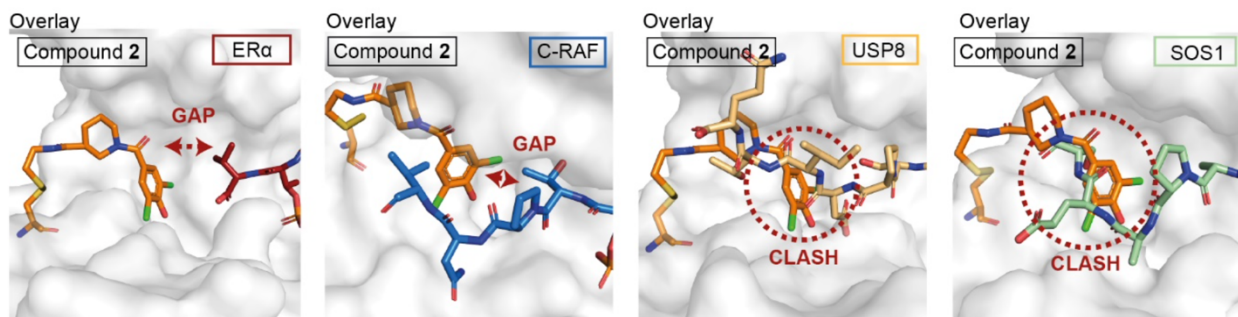


Figure S2.11: Crystallographic overlay of compound 2 with various 14-3-3 σ clients

Crystallographic overlay of compound 2 (orange sticks) with ER α (red sticks), CRAF (blue sticks), USP8 (light orange sticks) and SOS1 (green sticks) in 14-3-3 (white surface). (PDB ID: 4JC3 (ER α), 4FJ3 (CRAF), 6F09 (USP8), 6Y44 (SOS1)).

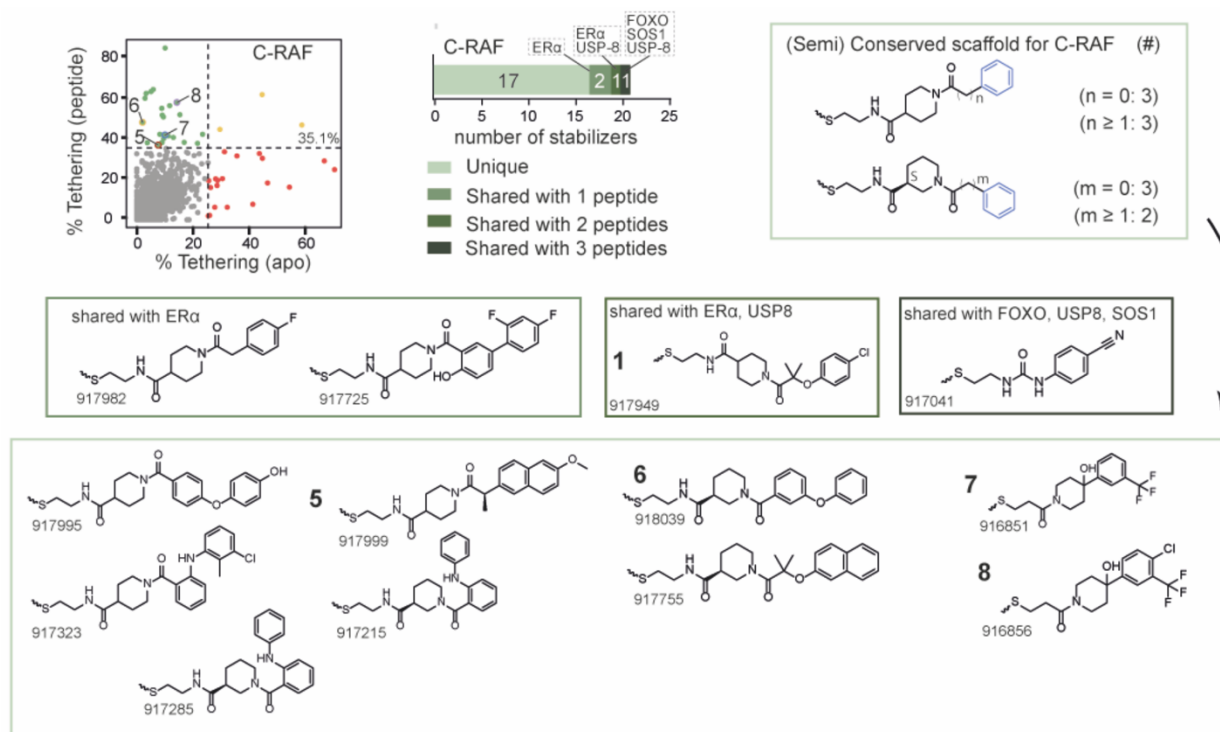


Figure S2.12: CRAF stabilizer structures

Scatter plot and chemical structures of the discovered stabilizers for CRAF from the primary screen, with in the light green box the unique stabilizers, in darker green box the stabilizers shared with ER α , in the darker green box shared with ER α and USP8 (= compound 1) and in the darkest green box shared with FOXO1, USP8 and SOS1.

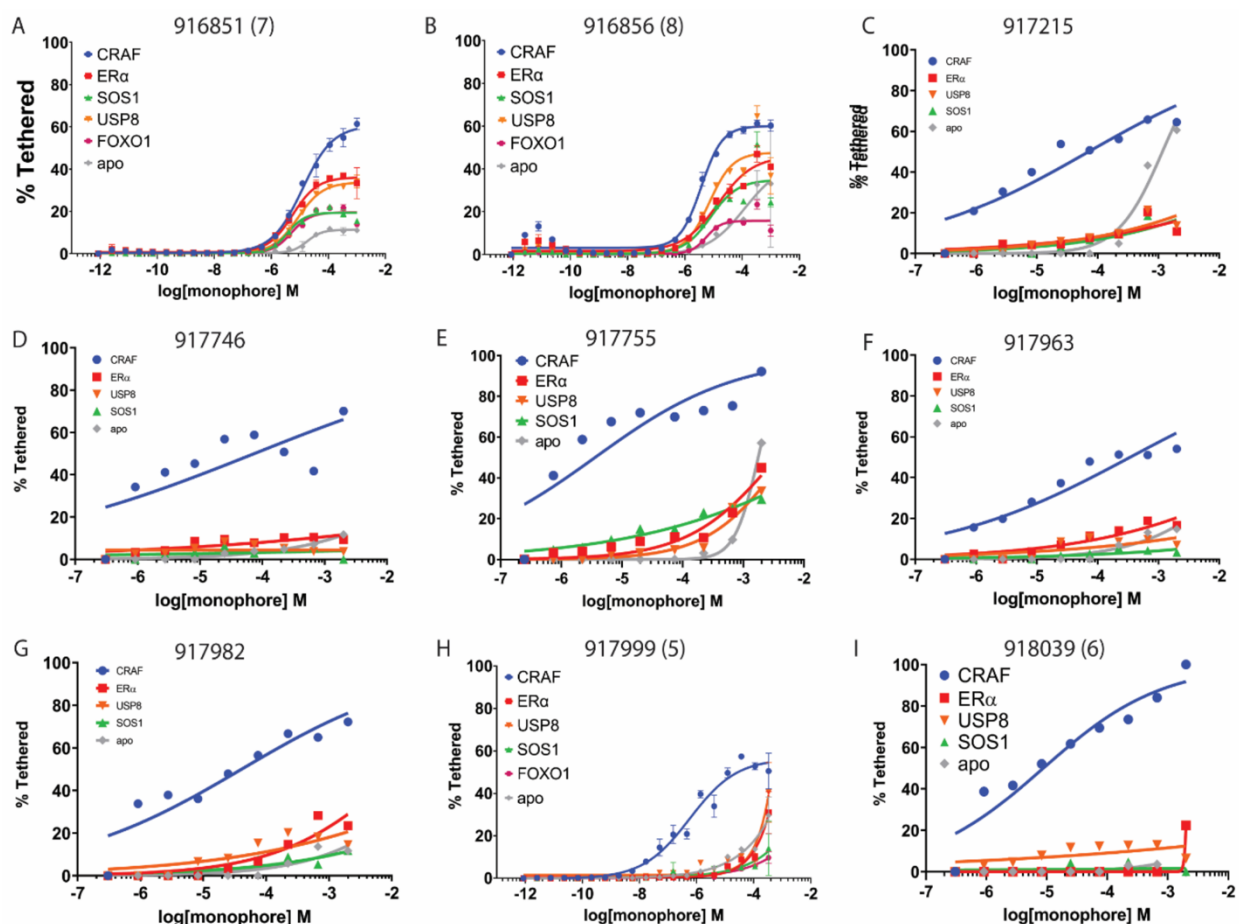


Figure S2.13: MSDR for CRAF selective stabilizers

Mass spectrometry dose response (MSDR) curves (blue) for top CRAF selective stabilizers. **A)** MSDR for CRAF hit compound 916851 (compound **7** in main text) showing preferential binding in presence of CRAF phospho-peptide over other four 14-3-3 client phospho-peptides and apo. DR₅₀ values: 12.2 μ M (CRAF), 5.62 μ M (ER α), 8.54 μ M (USP8), >2000 μ M (SOS1), >2000 μ M (SOS1), >2000 μ M (apo). **B)** MSDR for CRAF hit compound 916856 (compound **8** in main text). 916856 was also a hit stabilizer in ER α primary screen and shows dose dependent engagement of 14-3-3 in MSDR. DR₅₀ values: 4.96 μ M (CRAF), 14.5 μ M (ER α), 7.05 μ M (USP8), >2000 μ M (SOS1), >2000 μ M (FOXO1), >2000 μ M (apo). **C)** MSDR for CRAF selective hit compound 917215. DR₅₀: 5.1 μ M. **D)** MSDR for CRAF selective compound 917746. DR₅₀: 910 nM. **E)** MSDR for CRAF selective compound 917755. DR₅₀: 5.82 μ M. **F)** MSDR for CRAF selective compound 917963. DR₅₀: 5.29 μ M. **G)** MSDR for CRAF selective compound 917982. DR₅₀: 5.45 μ M. **H)** MSDR for CRAF selective hit compound 917999 (compound **5** in main text). DR₅₀: 580 nM. **I)** MSDR for CRAF selective hit compound 918039 (compound **6** in main text). DR₅₀: 8.78 μ M.

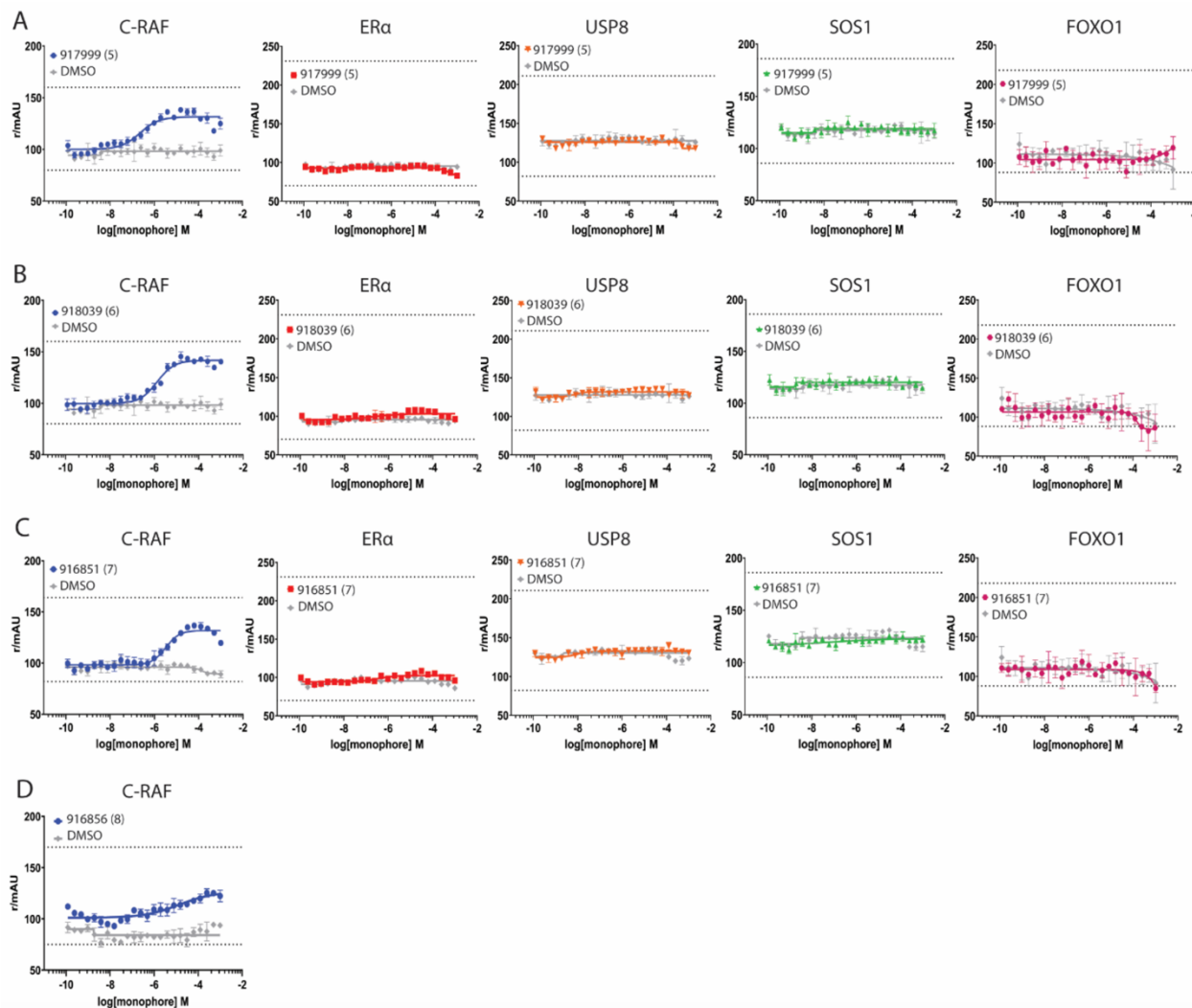


Figure S2.14: FADR for FOXO1 selective stabilizers

Fluorescence anisotropy dose response (FADR) curves for CRAF selective stabilizers 917999, 918039, 916851, and 916856 (compounds **5-8** in main text, respectively). Top black dashed line signifies maximum anisotropy control, the value of which was determined by peptide bound to 250 μ M 14-3-3 σ (at least 50-fold excess of peptide K_D). Bottom dashed line signifies minimum anisotropy control, value determined by peptide only and no 14-3-3 σ . Controls were measured per plate. **A)** 917999 dose-dependent stabilization of CRAF phospho-peptide (blue) binding to 14-3-3 σ . EC_{50} values with CRAF: 220 nM. ER α (red), USP8 (orange), SOS1 (green), and FOXO1 (pink) showed no increase in anisotropy over DMSO control (grey) indicating no stabilization. **B)** 918039 stabilization of CRAF (blue) binding to 14-3-3 σ . EC_{50} value with CRAF: 1.33 μ M. No increased stabilization over DMSO control (grey) observed for ER α (red), USP8 (orange), SOS1 (green), or FOXO1 (pink). **C)** 916851 stabilization of CRAF (blue) binding to 14-3-3 σ . EC_{50} value with CRAF: 3.18 μ M. No increased stabilization over DMSO control (grey) observed for ER α (red), USP8 (orange), SOS1 (green), or FOXO1 (pink). **D)** 916856 stabilization of CRAF (blue) binding to 14-3-3 σ . EC_{50} value: 13.5 μ M. Not sufficient compound for FADR with other peptides.

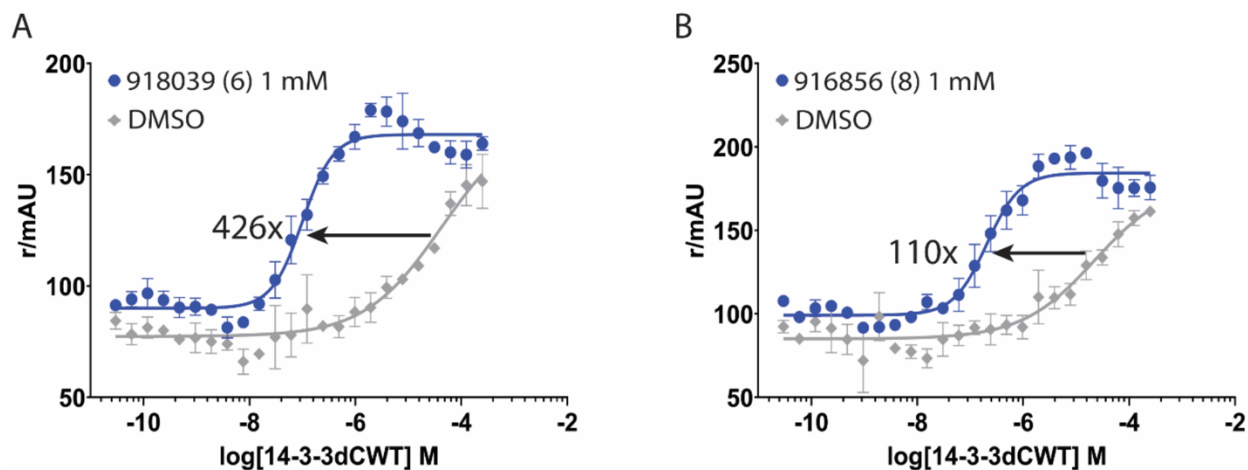


Figure S2.15: FA protein titrations with selective compounds 6 and 8

Fluorescence anisotropy protein titrations for FOXO1 and CRAF selective stabilizers 918039 and 916856 (compounds **6** and **8** in main text, respectively. **A**) Addition of 1 mM 918039 (compound **6**; blue curve) resulted in 426-fold decrease in 14-3-3 σ /CRAF K_D (100 nM vs. 42 μ M) compared to DMSO control (grey curve). **B**) Addition of 1 mM 916856 (compound **8**; blue curve) resulted in 110-fold decrease in 14-3-3 σ /CRAF K_D (207 nM vs. 23 μ M) compared to DMSO control (grey).

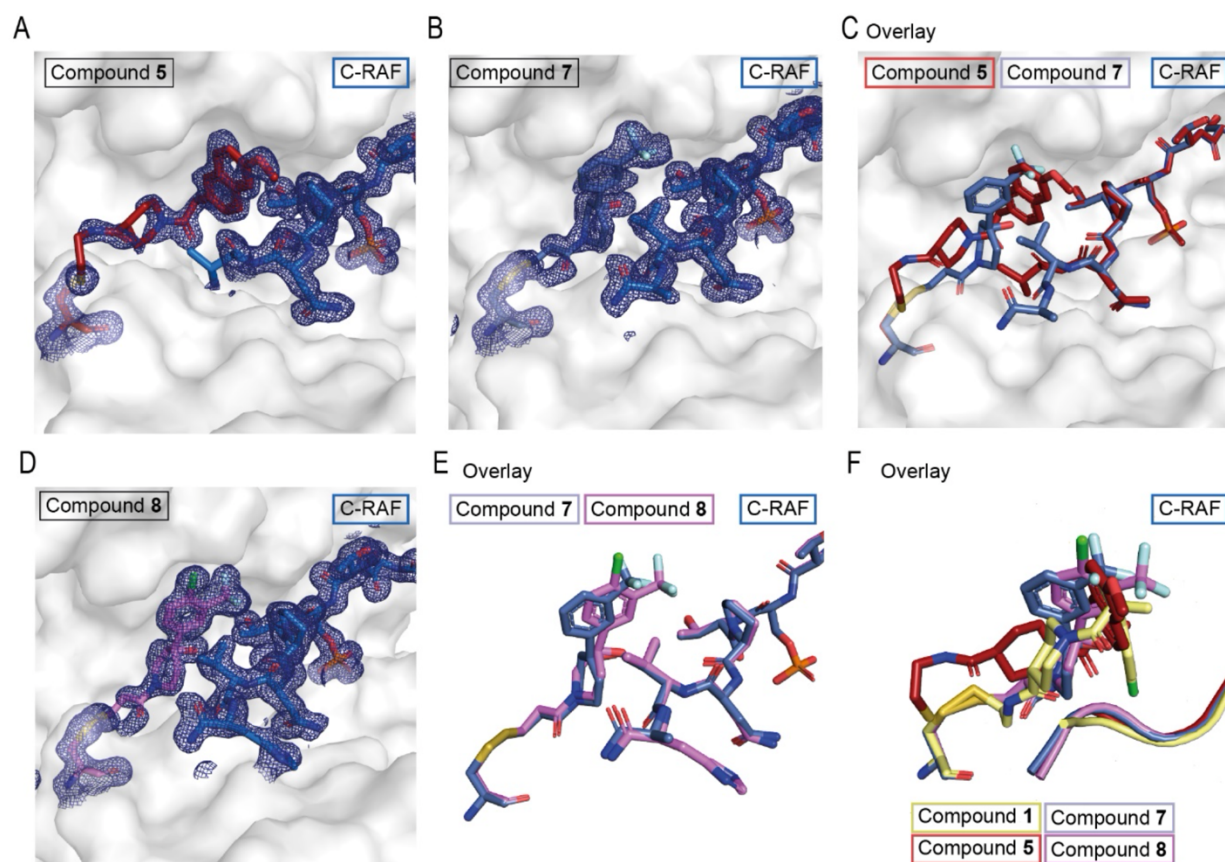
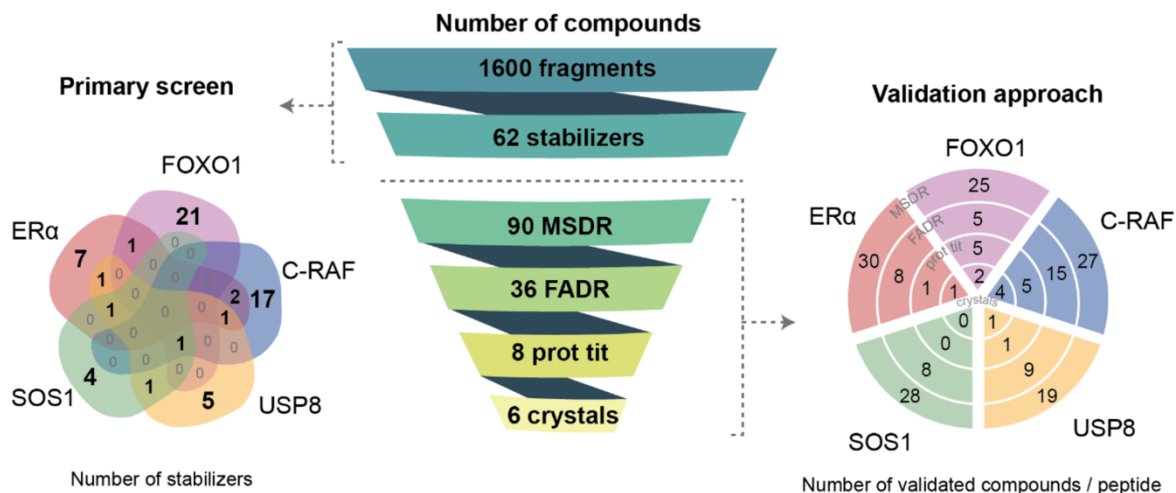


Figure S2.16: Crystal structures of CRAF stabilizers

A) Electron density of compound **5** (red sticks) complexed with C-RAF (blue sticks) in 14-3-3 (white surface). **B)** Electron density of compound **7** (light blue sticks) complexed with CRAF (blue sticks) in 14-3-3 (white surface). **C)** Crystallographic overlay of compound **5** (red sticks) and compound **7** (light blue sticks) with CRAF (color peptide matching the compound complexed with) in 14-3-3 (white surface). **D)** Electron density of compound **8** (violet sticks) with CRAF (blue sticks) in 14-3-3 (white surface). **E)** Crystallographic overlay of compound **7** (light blue sticks) and compound **8** (violet sticks) with CRAF (color peptide matching the compound). **F)** Crystallographic overlay of all crystallized stabilizers with CRAF with compound **1** (yellow sticks), compound **5** (red sticks), compound **7** (light blue sticks) and compound **8** (violet sticks) with CRAF represented as cartoon (color matching the compound complexed with) in 14-3-3 (white surface). 2Fo-Fc electron density maps are contoured at 1σ .

A



B

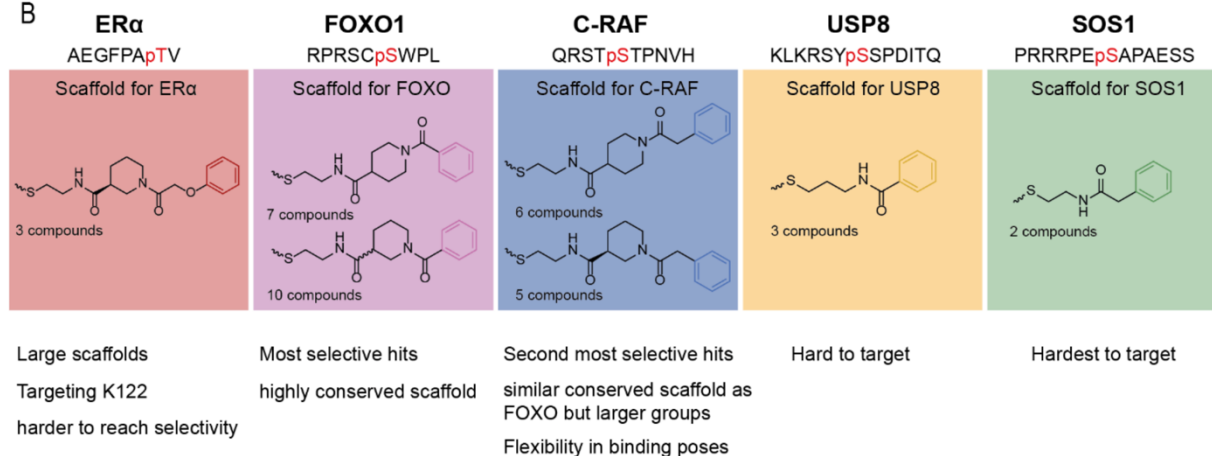
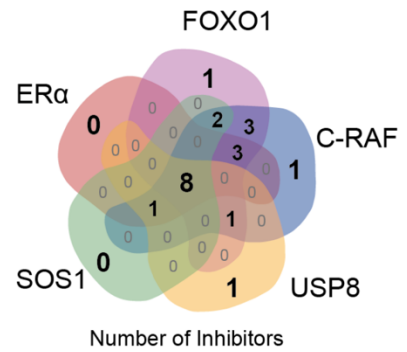


Figure S2.17: Overview of the screens performed

A) The screening funnel starting with the primary screen at the left depicted as a Venn diagram with the number of stabilizers discovered for each peptide and at the right the validation approach taken after the primary screen with the number of validated compounds per peptide per assay. **B)** Conserved scaffold needed for stabilization of each peptide discovered from the primary screen.

A Inhibitors from primary screen



B Neutral binders from primary screen

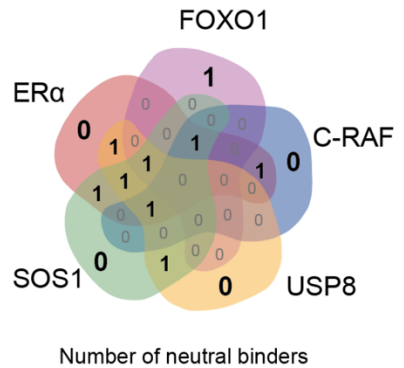
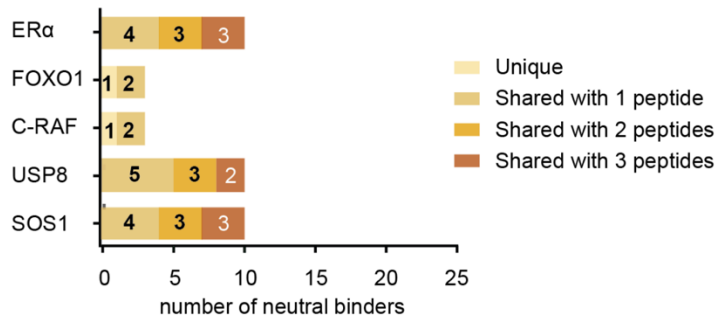


Figure S2.18: Overview of the number of inhibitors and neutral binders discovered

A) Number of discovered inhibitors for each peptide depicted as (left) bar plots and (right) as a Venn diagram. **B)** Number of discovered neutral binders for each peptide depicted as (left) bar plots and (right) as a Venn diagram.

Supplementary Tables

Table S2.1: Tethering and stabilization of 14-3-3 σ /CRAF primary screen hit compounds

SMDC ID	Primary Screen (250 μ M BME)	MSDR (250 μ M BME)	FADR (50 μ M BME)	Protein Titrations (50 μ M BME)		
	% Tethering	DR ₅₀ (μ M)	EC ₅₀ (μ M)	K _{D_app}	K _{D_DMSO}	Fold Stabilization
916851 (7)	42.4	12.2	3.18	294 nM	23 μ M	77
916856 (8)	58.3	4.96	13.5	207 nM	23 μ M	110
916935	44.9	>2000	—	—	—	—
917041	38	>2000	—	—	—	—
917072	62.8	465	>2000	—	—	—
917215	40.4	5.01	>2000	—	—	—
917215	38.3	5.01	>2000	—	—	—
917285	56.7	5.61	—	—	—	—
917285	38.4	5.61	—	—	—	—
917323	42.5	>2000	—	—	—	—
917426	40.9	5.76	—	—	—	—
917692	62.2	>2000	>2000	—	—	—
917725	42.5	—	—	—	—	—
917729	38.5	5.24	—	—	—	—
917746	35.4	<0.91	19.4	—	—	—
917748	15.8+/-17	>2000	—	—	—	—
917755	51.1	5.82	>2000	—	—	—
917845	13.9+/-16	—	—	—	—	—
917866	52.2	>2000	>2000	—	—	—
917908	64.7	>2000	—	—	—	—
917922	47.1	>2000	>2000	—	—	—
917949 (1)	85.3	0.007	0.922	106 nM	8.5 μ M	81
917963	52	5.29	>2000	—	—	—
917982	63.7	5.45	0.58	—	—	—
917988	55.4	>2000	—	—	—	—
917995	41.7	>2000	—	—	—	—
917999 (5)	37.1	0.58	0.22	92	23 μ M	246
918000	60.4	3.95	—	—	—	—
918039 (6)	48.3	8.78	1.33	100 nM	42 μ M	426

Table S2.2: Tethering and stabilization of 14-3-3 σ /FOXO1 primary screen hit compounds

SMDC ID	Primary Screen (250 μ M BME)	MSDR (250 μ M BME)	FADR (50 μ M BME)	Protein Titrations (50 μ M BME)		
	% Tethering	DR ₅₀ (μ M)	EC ₅₀ (μ M)	K _{D_app}	K _{D_DMSO}	Fold Stabilization
917034	29.7	>2000	—	—	—	—
917041	34.3	>2000	—	—	—	—
917222	50.6	—	—	—	—	—
917234	34.3	16.9	—	—	—	—
917261	57.5	3.61	—	—	—	—
917282	48.4	—	—	—	—	—
917316	83.6	0.042	—	117 nM	87 nM	—
917346	36.5	>2000	—	—	—	—
917383 (4)	34.3	143.4	6.69	9.7 nM	111 nM	12
917429	28.5	>2000	—	—	—	—
917607	35.4	—	—	—	—	—
917701 (3)	68.7	2.2	0.85	10.6 nM	42 nM	4
917720	31.4	>2000	—	—	—	—
917726 (2)	67.6	0.36	5.1	7.7 nM	39 nM	5
917748	14.8+/-14	>2000	—	—	—	—
917773	47.5	>2000	—	—	—	—
917800	66.6	4.78	2.41	69 nM	66 nM	—
917845	13.8+/-14	—	—	—	—	—
917848	10.4+/-13	>2000	—	—	—	—
917922	57.9	>2000	—	—	—	—
917967	31.2	>2000	—	—	—	—
917986	38	>2000	—	—	—	—
917992	38.9	48.7	—	—	—	—
957764	30.7	>2000	—	—	—	—
957769	38.6	>2000	—	—	—	—
960037	31.9	—	—	—	—	—
966848	28	>2000	—	—	—	—
966851	37.1	>2000	—	—	—	—
966864	43.4	>2000	—	—	—	—
966943	40.2	>2000	—	—	—	—

Table S2.3: Tethering and stabilization of 14-3-3 σ /ER α primary screen hit compounds

SMDC ID	Primary Screen (250 μ M BME)	MSDR (250 μ M BME)	FADR (50 μ M BME)	Protein Titrations (50 μ M BME)		
	% Tethering	DR ₅₀ (μ M)	EC ₅₀ (μ M)	K _{D_app}	K _{D_DMSO}	Fold Stabilization
916848	40.9	>2000	>2000	—	—	—
916854	35.1	786	—	—	—	—
916856 (8)	35.7	14.5	—	—	—	—
916874	47.3	>2000	—	—	—	—
916933	45.6	>2000	—	—	—	—
916935	43	>2000	—	—	—	—
916936	33.3	>2000	—	—	—	—
916945	56.9	>2000	—	—	—	—
916974	34.9	133	—	—	—	—
917101	38.1	74.1	—	—	—	—
917607	40.5	1900	>2000	—	—	—
917692	47.3	>2000	—	—	—	—
917717	36.7	>2000	>2000	—	—	—
917725	51.5	>2000	—	—	—	—
917730	37.6	>2000	1.51	—	—	—
917748	20.8+/-17	18.5	129	—	—	—
917750	18.3+/-14	>2000	>2000	—	—	—
917770	17.3+/-13	>2000	—	—	—	—
917848	17.2+/-13	>2000	>2000	—	—	—
917878	36.1	>2000	—	—	—	—
917922	73.1	3.67	—	—	—	—
917949 (1)	73.1	18.1	1.31	21 nM	360 nM	19
917952	33.4	>2000	—	—	—	—
917982	37.1	>2000	—	—	—	—
918016	35.7	4.47	—	—	—	—
957766	44.9	459	—	—	—	—
957779	63.6	>2000	—	—	—	—
960009	49.4	—	—	—	—	—
960037	56.2	8.31	—	—	—	—
966784	33.8	>2000	—	—	—	—
966840	43	>2000	—	—	—	—
993941	34.2	—	—	—	—	—
994336	38.2	—	—	—	—	—
994825	39.8	—	—	—	—	—

Table S2.4: Tethering and stabilization of 14-3-3 σ /USP8 primary screen hit compounds

SMDC ID	Primary Screen (250 μ M BME)	MSDR (250 μ M BME)	FADR (50 μ M BME)	Protein Titrations (50 μ M BME)		
	% Tethering	DR ₅₀ (μ M)	EC ₅₀ (μ M)	K _{D_app}	K _{D_DMSO}	Fold Stabilization
916848	0	>2000	4.21	—	—	—
916933	38.3	>2000	—	—	—	—
916945	36.5	>2000	—	—	—	—
916974	39.4	>2000	—	—	—	—
917041	30.1	>2000	>2000	—	—	—
917252	44.1	>2000	—	—	—	—
917340	50.4	648	1140	—	—	—
917644	18.7+/-23	—	—	—	—	—
917659	28.9	>2000	>2000	—	—	—
917692	51.1	240	—	—	—	—
917748	19.5+/-19	—	—	—	—	—
917848	15.5+/-13	>2000	—	—	—	—
917949 (1)	52.3	0.024	3.38	1.1	4.5	4
957766	39.7	1636	>2000	—	—	—
957779	59.8	>2000	>2000	—	—	—
957817	35.1	>2000	—	—	—	—
957821	57.5	—	—	—	—	—
957824	36	>2000	—	—	—	—
957999	29.4	>2000	—	—	—	—
958001	53.9	>2000	>2000	—	—	—
960009	67.6	—	—	—	—	—
960031	12.8+/-13	>2000	—	—	—	—
960037	52.9	1749	>2000	—	—	—
967287	35.8	—	—	—	—	—
967289	30.4	—	—	—	—	—
994439	36.1	—	—	—	—	—
994487	41.1	—	—	—	—	—
994782	31	—	—	—	—	—
994821	48.2	—	—	—	—	—

Table S2.5: Tethering and stabilization of 14-3-3 σ /SOS1 primary screen hit compounds

SMDC ID	Primary Screen (250 μ M BME)	MSDR (250 μ M BME)	FADR (50 μ M BME)	Prot Titrations (50 μ M BME)		
	% Tethering	DR ₅₀ (μ M)	EC ₅₀ (μ M)	K _{D_app}	K _{D_DMSO}	Fold Stabilization
916848	28.3	>2000	1598	—	—	—
916933	32.5	>2000	297	—	—	—
916935	22.3	—	—	—	—	—
916992	28.4	>2000	>2000	—	—	—
917041	23	>2000	—	—	—	—
917101	26.9	>2000	—	—	—	—
917340	27.2	>2000	55.1	—	—	—
917447	37.1	>2000	148	—	—	—
917602	9.33+/-12	>2000	—	—	—	—
917609	11.3+/-14	>2000	—	—	—	—
917611	9.73+/-12	>2000	—	—	—	—
917621	10.7+/-13	>2000	—	—	—	—
917692	27.3	325	>2000	—	—	—
917748	15.1+/-16	4.26	—	—	—	—
917845	9.67+/-12	266	—	—	—	—
917848	14.1+/-15	>2000	—	—	—	—
917879	10.4+/-12	>2000	—	—	—	—
917922	72.9	1912	—	—	—	—
917941	23.2	>2000	>2000	—	—	—
957779	54.1	>2000	—	—	—	—
957795	55.3	>2000	—	—	—	—
957999	37.1	>2000	—	—	—	—
958001	67.8	>2000	—	—	—	—
960009	50	28.2	—	—	—	—
960031	15.2+/-15	>2000	—	—	—	—
960037	61.9	562	241	—	—	—
960039	7.73+/-10	>2000	—	—	—	—
966837	26.3	>2000	—	—	—	—
966840	55.9	>2000	—	—	—	—

Materials and Methods

Protein Expression and Purification

The 14-3-3 σ isoform with a truncated C-terminus after T231 (Δ C; to enhance crystallization) and an N-terminal His6 tag was expressed in RosettaTM 2(DE3)pLysS competent *E. coli* (Novagen) from a pPROEX HTb expression vector. After transformation following manufacturer's instructions, single colonies were picked to inoculate 30 mL precultures (LB), which were added to 1.5 L terrific broth (TB) medium after overnight growth at 37 °C, 250 rpm. Expression was induced upon reaching OD₆₀₀ 1.9–2.1 by adding 400 μ M IPTG. After overnight expression at 30°C, 150 rpm, cells were harvested by centrifugation at 6,500 rpm, resuspended in lysis buffer (50 mM HEPES pH 7.5, 500 mM NaCl, 20 mM imidazole, 10% glycerol, 1 mM TCEP), and lysed by sonication. The His6-tagged protein was first purified by Ni-affinity chromatography (Ni-NTA Agarose, Invitrogen) (Wash buffer 50 mM HEPES pH 7.5, 500 mM NaCl, 20 mM imidazole, 1 mM TCEP; Elution buffer 50 mM HEPES pH 7.5, 500 mM NaCl, 500 mM imidazole, 1 mM TCEP) followed by His-tag cleavage by TEV protease during dialysis (50 mM HEPES pH 7.5, 150 mM NaCl, 1 mM TCEP) overnight at 4 °C. The flowthrough of a second Ni-affinity column was collected and analyzed for purity by SDS-PAGE and Q-ToF LC/MS. The protein was buffer exchanged (Storage buffer 25 mM HEPES pH 7.5, 150 mM NaCl, 1 mM TCEP) and concentrated to ~13 mg/mL and aliquots flash-frozen for storage at –80°C. 14-3-3 σ Δ C used for crystallography was concentrated to ~70 mg/mL and aliquots were flash-frozen for storage at -80 °C.

Peptide Sequences

Peptides for disulfide trapping were ordered from ScenicBio (Houston, Texas) as lyophilized solids. Sequences were as follows: ER α -pp (Ac-KYYITGEAEGF-PA{pT}V-COOH); SOS1-pp (Ac-PRRRPE{pS}APAESS-CONH₂); USP8-pp (Ac- KLKRSY{pS}SPDITQ-CONH₂); CRAF-pp (Ac-RQRST{pS}TPNVH-CONH₂); FOXO1-pp (Ac-RPRSC{pT}WPLPR-CONH₂).

2 mM peptide stocks were suspended in water. Fluorescein-labeled peptides were ordered from ScenicBio and Elim Biopharmaceuticals, Inc. (Hayward, CA) as lyophilized solids. Sequences were as written above with N-terminal 5-FAM-label for ER α -pp, SOS1-pp, USP8-pp, and FOXO1-pp. CRAF-pp sequence was as follows: (5-FAM-QHRYSTPHAFTFNTSSPSSEGLSQRQRST{pS}TPNVH-CONH₂). 20 μ M stocks were suspended in water. Peptides for crystallography were ordered from Genscript Biotech Corp. Sequences were as follows: ER α -pp (Ac-AEGFPA{pT}V-COOH); USP8-pp (Ac-KLKRSY{pS}SPDITQ-CONH₂); CRAF-pp (QRST{pS}TPNVHH-CONH₂); FOXO1-pp (Ac-RPR{pS}C{pT}WPLPR-CONH₂). 5 mM stocks were suspended in Complexation Buffer (25 mM HEPES pH 7.5, 2 mM MgCl₂ and 2 mM β ME).

Disulfide Tethering Screen and Data Processing

The primary disulfide tethering screen was performed by incubating target 14-3-3 σ protein/phospho-peptide complex with small molecule in a 384-well plate format. The UCSF Small Molecule Discovery Center (SMDC) custom 1600 disulfide-containing fragment library was available as 50 mM stock solutions in DMSO. The screen was performed using 100 nM 14-3-3 σ Δ C protein diluted in buffer (10 mM Tris, 250 μ M β -mercaptoethanol (β ME), pH 8.0) and plated in 384-well plates (25 μ L/well). 100 nL of each fragment was pinned from library master plates using non-sterile disposable 384 polypropylene pin tools (V & P Scientific) to give a final concentration of 200 μ M. The peptide screens additionally contained a concentration of peptide equivalent to twice the K_D as established by fluorescence anisotropy and competition experiments: (2 μ M ER α -pp, 10 μ M SOS1-pp, 10 μ M USP8-pp, 18 μ M CRAF-pp, and 750 nM FOXO1-pp). The reactions were incubated at room temperature for 3 hours before being measured by LC/MS (I-class Acquity UPLC/ Xevo G2-XS Quadrupole Time of Flight mass spectrometer, Waters). Data collection and automated processing followed a custom workflow, as previously described. Compound resynthesis was done following the published procedures.

Dose Response LC/MS Experiments

The initial mass spectrometry dose response follow-up experiments were performed under the same conditions as the primary screen with the exception that the compounds were titrated from 50 mM to 23 μ M in a 3-fold dilution series in DMSO. Then 1 μ L of the compound was transferred into 24 μ L of protein-peptide solution for final concentrations of 2 mM – 914 nM and 4% DMSO. A final well of DMSO without compound was used as a control at the end of every dilution series. Compounds which displayed dose dependent activity in the presence of phospho-peptide were taken forward into more extensive dose responses in order to determine DR₅₀ values. Fragments were titrated from 50 mM to 0.129 nM in a 3-fold dilution series in DMSO. Then 1 μ L of the compound was transferred into 24 μ L of protein-peptide solution for final concentrations of 2 mM – 5.16 pM and 4% DMSO. A final well of DMSO without compound was used as a control at the end of every dilution series. Initial validation was done in single replicate. Hits were taken forward to fluorescence anisotropy experiments. DR₅₀ curves were graphed and calculated using log(agonist) vs. response – Variable slope (four parameters) from GraphPad Prism version 9.0 for Mac, GraphPad Software, San Diego, California USA, www.graphpad.com.

Dose Response Fluorescence Anisotropy Experiments

The fluorescence anisotropy compound dose responses were performed using 100 nM fluorescein-labeled peptides (with the exception of FOXO1-pp which was 10 nM) and 14-3-3 σ protein diluted in buffer (10 mM HEPES pH 8.0, 150 mM NaCl, 0.01% TWEEN-20). 14-3-3 σ protein concentration was equivalent to approximately half of the K_D determined by fluorescence anisotropy and competition experiments (CRAF: 1 μ M 14-3-3 σ ; ER α : 250 nM 14-3-3 σ ; FOXO1: 25 nM 14-3-3 σ ; USP8: 2.5 μ M 14-3-3 σ). Fragments were titrated from 50 mM to 11.9 nM in a 2-fold dilution series in DMSO. 1 μ L of compound was transferred in triplicates into 24 μ L of protein-peptide solution for final concentrations of 2 mM – 0.477 nM and 4% DMSO. A final well

of DMSO without compound was used as a control at the end of the dilution series. A row of 5-FAM-labeled peptide alone was used as a lower limit and a row of 5-FAM-labeled peptide with 14-3-3 excess of the K_D was used as an upper limit when calculating the EC_{50} of the fragments. EC_{50} measurements were graphed and calculated using log(agonist) vs. response – Variable slope (four parameters) from GraphPad Prism version 9.0 for Mac, GraphPad Software, San Diego, California USA, www.graphpad.com. 14-3-3 σ protein titrations were performed using 2-fold dilution of 14-3-3 σ in buffer from starting concentration of 250 μ M to 59.6 pM for CRAF and USP8 and 25 μ M starting concentration to 5.96 pM for FOXO1 and ER α . Final well contained only 5-FAM-labeled peptide and compound as a control. 5-FAM-peptides were used at 100 nM except FOXO1 which was at 10 nM. Fragments were added at a single concentration of 1 mM in order to maximize 14-3-3 σ engagement. Apparent K_D values of 14-3-3 σ /phospho-peptide in presence of compounds were compared to K_D values of DMSO control. Fold stabilization (ΔK_D) was determined as the quotient of the apparent K_D of the DMSO control over the apparent K_D with compound. EC_{50} and apparent K_D measurements were graphed and calculated using log(agonist) vs. response – Variable slope (four parameters) from GraphPad Prism version 9.0 for Mac, GraphPad Software, San Diego, California USA, www.graphpad.com.

X-ray Crystallography

The 14-3-3 σ Δ C protein, acetylated ER α , CRAF and FOXO1 peptides and fragments (stock solution of 50mM in DMSO) were dissolved in complexation buffer (25 mM HEPES pH 7.5, 2 mM MgCl₂ and 2 mM β ME) and mixed in a 1:2:2 molar stoichiometry (protein:peptide:fragment) at a final protein concentration of 12 mg/mL. The complex was set up for sitting-drop crystallization after overnight incubation at 4 °C, in a custom crystallization liquor (0.095 M HEPES (pH 7.1, 7.3, 7.5, 7.7), 0.19 M CaCl₂, 24-29 % PEG 400 and 5% (v/v) glycerol). Crystals grew within 10 – 14 days at 4 °C. Crystals were fished and flash-cooled in liquid nitrogen. X-ray diffraction (XRD) data were either collected at the Deutsche Elektronen-Synchrotron (DESY)

PETRA III beamline P11, Hamburg, Germany (all datasets except for 14-3-3 σ /CRAFpS259/Compound 1) or at the Diamond Light Source (DLS) beamline I03, Oxfordshire, United Kingdom (14-3-3 σ /CRAFpS259/Compound 1).

Automatic processing was done by DESY or DLS using XDS9 for data indexing and integration. Initial processed data was then scaled using AIMLESS and Molrep was used for limited molecular replacement using PDB ID's 4JC3 and 3IQU as template. Presence of co-crystallized ligands was verified by visual inspection of the Fo-Fc and 2Fo-Fc electron density maps in Coot. If electron density corresponding to the co-crystallized ligand was present, its structure and restraints were generated using eLBOW before final model rebuilding and refinement was done using phenix.refine and Coot. See Table S2.6 and S2.7 for data collection and refinement statistics. The structures were submitted to the PDB with IDs 8AFN, 8AV0, 8ADM, 8A62, 8A65, 8A68, 8A6H, 8A6F.

References

1. Garlick, J. M.; Mapp, A. K. Selective Modulation of Dynamic Protein Complexes. *Cell Chem. Biol.* 2020, 27 (8), 986–997.
2. Ivanov, A. A.; Revennaugh, B.; Rusnak, L.; Gonzalez-Pecchi, V.; Mo, X.; Johns, M. A.; Du, Y.; Cooper, L. A. D.; Moreno, C. S.; Khuri, F. R.; Fu, H. The OncoPPi Portal: An Integrative Resource to Explore and Prioritize Protein-Protein Interactions for Cancer Target Discovery. *Bioinforma. Oxf. Engl.* 2018, 34 (7), 1183–1191.
3. Kim, M.; Park, J.; Bouhaddou, M.; Kim, K.; Rojc, A.; Modak, M.; Soucheray, M.; McGregor, M. J.; O’Leary, P.; Wolf, D.; Stevenson, E.; Foo, T. K.; Mitchell, D.; Herrington, K. A.; Muñoz, D. P.; Tutuncuoglu, B.; Chen, K. H.; Zheng, F.; Kreisberg, J. F.; Diolaiti, M. E.; Gordan, J. D.; Coppé, J. P.; Swaney, D. L.; Xia, B.; Veer, L. van ’t; Ashworth, A.; Ideker, T.; Krogan, N. J. A Protein Interaction Landscape of Breast Cancer. *Science* 2021, 374 (6563), abf3066.
4. Wright, M. T.; Plate, L. Revealing Functional Insights into ER Proteostasis through Proteomics and Interactomics. *Exp. Cell Res.* 2021, 399 (1), 112417.
5. Hu, G.; Wu, Z.; Uversky, V. N.; Kurgan, L. Functional Analysis of Human Hub Proteins and Their Interactors Involved in the Intrinsic Disorder-Enriched Interactions. *Int. J. Mol. Sci.* 2017, 18 (12), E2761.
6. Arkin, M. R.; Tang, Y.; Wells, J. A. Small-Molecule Inhibitors of Protein-Protein Interactions: Progressing toward the Reality. *Chem. Biol.* 2014, 21 (9), 1102–1114.
7. Celis, S.; Hobor, F.; James, T.; Bartlett, G. J.; Ibarra, A. A.; Shoemark, D. K.; Hegedüs, Z.; Hetherington, K.; Woolfson, D. N.; Sessions, R. B.; Edwards, T. A.; Andrews, D. M.; Nelson, A.; Wilson, A. J. Query-Guided Protein-Protein Interaction Inhibitor Discovery. *Chem. Sci.* 2021, 12 (13), 4753–4762.
8. Linhares, B. M.; Grembecka, J.; Cierpicki, T. Targeting Epigenetic Protein-Protein Interactions with Small Molecule Inhibitors. *Future Med. Chem.* 2020, 12 (14), 1305–1326.

9. Modell, A. E.; Blosser, S. L.; Arora, P. S. Systematic Targeting of Protein-Protein Interactions. *Trends Pharmacol. Sci.* 2016, 37 (8), 702–713.
10. Zhong, M.; Lee, G. M.; Sijbesma, E.; Ottmann, C.; Arkin, M. R. Modulating Protein-Protein Interaction Networks in Protein Homeostasis. *Curr. Opin. Chem. Biol.* 2019, 50, 55–65.
11. Andrei, S. A.; Sijbesma, E.; Hann, M.; Davis, J.; O'Mahony, G.; Perry, M. W. D.; Karawajczyk, A.; Eickhoff, J.; Brunsveld, L.; Doveston, R. G.; Milroy, L.G.; Ottmann, C. Stabilization of Protein-Protein Interactions in Drug Discovery. *Expert Opin. Drug Discov.* 2017, 12 (9), 925–940.
12. Tang, C.; Mo, X.; Niu, Q.; Wahafu, A.; Yang, X.; Qui, M.; Ivanov, A. A.; Du, Y.; Fu, H. Hypomorph Mutation Directed Small Molecule Protein-Protein Interaction Inducers to Restore Mutant SMAD4 Suppressed TGF- β Signaling. *Cell Chem. Biol.* 2021, 28 (5), 636-647.e5.
13. Stevers, L. M.; Sijbesma, E.; Botta, M.; MacKintosh, C.; Obsil, T.; Landrieu, I.; Cau, Y.; Wilson, A. J.; Karawajczyk, A.; Eickhoff, J.; Davis, J.; Hann, M.; O'Mahony, G.; Doveston, R. G.; Brunsveld, L.; Ottmann, C. Modulators of 14-3-3 Protein-Protein Interactions. *J. Med. Chem.* 2018, 61 (9), 3755–3778.
14. De Vries van Leeuwen, I. J.; da Costa Pereira, D.; Flach, K. D.; Piersma, S. R.; Haase, C.; Bier, D.; Yalcin, Z.; Michalides, R.; Feenstra, K. A.; Jiménez, C. R.; de Greef, T. F. A.; Brunsveld, L.; Ottmann, C.; Zwart, W.; de Boer, A. H. Interaction of 14-3-3 Proteins with the Estrogen Receptor Alpha F Domain Provides a Drug Target Inter-face. *Proc. Natl. Acad. Sci. U. S. A.* 2013, 110 (22), 8894–8899.
15. Mayor-Ruiz, C.; Bauer, S.; Brand, M.; Kozicka, Z.; Si-klos, M.; Imrichova, H.; Kaltheuner, I. H.; Hahn, E.; Seiler, K.; Koren, A.; Petzold, G.; Fellner, M.; Bock, C.; Müller, A. C.; Zuber, J.; Geyer, M.; Thomä, N. H.; Kubicek, S.; Winter, G. E. Rational Discovery of Molecular Glue Degradors via Scalable Chemical Profiling. *Nat. Chem. Biol.* 2020, 16 (11), 1199–1207.

16. Chamberlain, P. P.; Cathers, B. E. Cereblon Modulators: Low Molecular Weight Inducers of Protein Degradation. *Drug Discov. Today Technol.* 2019, 31, 29–34.
17. Ślabicki, M.; Kozicka, Z.; Petzold, G.; Li, Y.-D.; Manojkumar, M.; Bunker, R. D.; Donovan, K. A.; Sievers, Q. L.; Koeppe, J.; Suchyta, D.; Sperling, A. S.; Fink, E. C.; Gasser, J. A.; Wang, L. R.; Corsello, S. M.; Sellar, R. S.; Jan, M.; Gillingham, D.; Scholl, C.; Fröhling, S.; Golub, T. R.; Fischer, E. S.; Thomä, N. H.; Ebert, B. L. The CDK Inhibitor CR8 Acts as a Molecular Glue Degradator That Depletes Cyclin K. *Nature* 2020, 585 (7824), 293–297.
18. Hartman, A. M.; Elgaher, W. A. M.; Hertrich, N.; Andrei, S. A.; Ottmann, C.; Hirsch, A. K. H. Discovery of Small Molecule Stabilizers of 14-3-3 Protein-Protein Interactions via Dynamic Combinatorial Chemistry. *ACS Med. Chem. Lett.* 2020, 11 (5), 1041–1046.
19. Pfaff, S. J.; Chimenti, M. S.; Kelly, M. J. S.; Arkin, M. R. Biophysical Methods for Identifying Fragment-Based Inhibitors of Protein-Protein Interactions. In *Protein-Protein Interactions: Methods and Applications*; Meyerkord, C. L., Fu, H., Eds.; *Methods in Molecular Biology*; Springer: New York, NY, 2015; pp 587–613.
20. Erlanson, D. A.; Arndt, J. W.; Cancilla, M. T.; Cao, K.; Elling, R. A.; English, N.; Friedman, J.; Hansen, S. K.; Hession, C.; Joseph, I.; Kumaravel, G.; Lee, W.-C.; Lind, K. E.; McDowell, R. S.; Miatkowski, K.; Nguyen, C.; Nguyen, T. B.; Park, S.; Pathan, N.; Penny, D. M.; Romanowski, M. J.; Scott, D.; Silvian, L.; Simmons, R. L.; Tangonan, B. T.; Yang, W.; Sun, L. Discovery of a Potent and Highly Selective PDK1 Inhibitor via Fragment-Based Drug Discovery. *Bioorg. Med. Chem. Lett.* 2011, 21 (10), 3078–3083.
21. Hallenbeck, K. K.; Davies, J. L.; Merron, C.; Ogden, P.; Sijbesma, E.; Ottmann, C.; Renslo, A. R.; Wilson, C.; Arkin, M. R. A Liquid Chromatography/Mass Spectrometry Method for Screening Disulfide Tethering Fragments. *SLAS Discov. Adv. Life Sci. R D* 2018, 23 (2), 183–192.
22. Raimundo, B. C.; Oslob, J. D.; Braisted, A. C.; Hyde, J.; McDowell, R. S.; Randal, M.; Waal, N. D.; Wilkinson, J.; Yu, C. H.; Arkin, M. R. Integrating Fragment Assembly and Biophysical

- Methods in the Chemical Advancement of Small-Molecule Antagonists of IL-2: An Approach for Inhibiting Protein-Protein Interactions. *J. Med. Chem.* 2004, 47 (12), 3111–3130.
23. Lodge, J. M.; Rettenmaier, T. J.; Wells, J. A.; Pomerantz, W. C.; Mapp, A. K. FP Tethering: A Screening Technique to Rapidly Identify Compounds That Disrupt Protein-Protein Interactions. *MedChemComm* 2014, 5, 370–375.
24. Sadowsky, J. D.; Burlingame, M. A.; Wolan, D. W.; McClendon, C. L.; Jacobson, M. P.; Wells, J. A. Turning a Protein Kinase on or off from a Single Allosteric Site via Disulfide Trapping. *Proc. Natl. Acad. Sci. U. S. A.* 2011, 108 (15), 6056–6061.
25. Sijbesma, E.; Hallenbeck, K. K.; Leysen, S.; de Vink, P. J.; Skóra, L.; Jahnke, W.; Brunsveld, L.; Arkin, M. R.; Ottmann, C. Site-Directed Fragment-Based Screening for the Discovery of Protein–Protein Interaction Stabilizers. *J. Am. Chem. Soc.* 2019, 141 (8), 3524–3531.
26. Burlingame, M. A.; Tom, C. T. M. B.; Renslo, A. R. Simple One-Pot Synthesis of Disulfide Fragments for Use in Disulfide Exchange Screening. *ACS Comb. Sci.* 2011, 13 (3), 205–208.
27. Morrison, D. K. The 14-3-3 Proteins: Integrators of Diverse Signaling Cues That Impact Cell Fate and Cancer Development. *Trends Cell Biol.* 2009, 19 (1), 16–23.
28. Obsilova, V.; Obsil, T. The 14-3-3 Proteins as Important Allosteric Regulators of Protein Kinases. *Int. J. Mol. Sci.* 2020, 21 (22), E8824.
29. Aghazadeh, Y.; Papadopoulos, V. The Role of the 14-3-3 Protein Family in Health, Disease, and Drug Development. *Drug Discov. Today* 2016, 21 (2), 278–287.
30. Gardino, A. K.; Smerdon, S. J.; Yaffe, M. B. Structural Determinants of 14-3-3 Binding Specificities and Regulation of Subcellular Localization of 14-3-3-Ligand Complexes: A Comparison of the X-Ray Crystal Structures of All Human 14-3-3 Isoforms. *Semin. Cancer Bi-ol.* 2006, 16 (3), 173–182.

31. Zhao, J.; Meyerkord, C. L.; Du, Y.; Khuri, F. R.; Fu, H. 14-3-3 Proteins as Potential Therapeutic Targets. *Semin. Cell Dev. Biol.* 2011, 22 (7), 705–712.
32. Molzan, M.; Kasper, S.; Röglin, L.; Skwarczynska, M.; Sassa, T.; Inoue, T.; Breitenbuecher, F.; Ohkanda, J.; Kato, N.; Schuler, M.; Ottmann, C. Stabilization of Physical RAF/14-3-3 Interaction by Cotylenin A as Treatment Strategy for RAS Mutant Cancers. *ACS Chem. Biol.* 2013, 8 (9), 1869–1875.
33. Bank, R. P. D. RCSB PDB: Homepage. <https://www.rcsb.org/> (accessed 2022-06-21).
34. Liau, N. P. D.; Wendorff, T. J.; Quinn, J. G.; Steffek, M.; Phung, W.; Liu, P.; Tang, J.; Irudayanathan, F. J.; Izadi, S.; Shaw, A. S.; Malek, S.; Hymowitz, S. G.; Sudhamsu, J. Negative Regulation of RAF Kinase Activity by ATP Is Overcome by 14-3-3 Induced Dimerization. *Nat. Struct. Mol. Biol.* 2020, 27 (2), 134–141.
35. Ballone, A.; Lau, R. A.; Zweipfenning, F. P. A.; Ottmann, C. A New Soaking Procedure for X-Ray Crystallographic Structural Determination of Protein-Peptide Complexes. *Acta Crystallogr. Sect. F Struct. Biol. Commun.* 2020, 76 (Pt 10), 501–507.
36. Saha, M.; Carriere, A.; Cheerathodi, M.; Zhang, X.; La-voie, G.; Rush, J.; Roux, P. P.; Ballif, B. A. RSK Phosphorylates SOS1 Creating 14-3-3 Docking Sites and Negatively Regulating MAPK Activation. *Biochem. J.* 2012, 447 (1), 159–166.
37. Saline, M.; Badertscher, L.; Wolter, M.; Lau, R.; Gunnarsson, A.; Jacso, T.; Norris, T.; Ottmann, C.; Snijder, A. AMPK and AKT Protein Kinases Hierarchically Phosphorylate the N-Terminus of the FOXO1 Transcription Factor, Modulating Interactions with 14-3-3 Proteins. *J. Biol. Chem.* 2019, 294 (35), 13106–13116.
38. Centorrino, F.; Ballone, A.; Wolter, M.; Ottmann, C. Biophysical and Structural Insight into the USP8/14-3-3 Interaction. *FEBS Lett.* 2018, 592 (7), 1211–1220.
39. Molzan, M.; Schumacher, B.; Ottmann, C.; Baljuls, A.; Polzien, L.; Weyand, M.; Thiel, P.; Rose, R.; Rose, M.; Kuhenne, P.; Kaiser, M.; Rapp, U. R.; Kuhlmann, J.; Ottmann, C.

- Impaired Binding of 14-3-3 to CRAF in Noonan Syndrome Suggests New Approaches in Diseases with Increased Ras Signaling. *Mol. Cell. Biol.* 2010, 30 (19), 4698–4711.
40. Falcicchio, M.; Ward, J. A.; Chothia, S. Y.; Basran, J.; Mohindra, A.; Macip, S.; Roversi, P.; Doveston, R. G. Cooperative Stabilisation of 14-3-3 σ Protein–Protein Interactions via Covalent Protein Modification. *Chem. Sci.* 2021.
41. Molzan, M.; Ottmann, C. Synergistic Binding of the Phosphorylated S233-and S259-Binding Sites of CRAF to One 14-3-3 ζ Dimer. *J. Mol. Biol.* 2012, 423 (4), 486–495.
42. Cossar, P. J.; Wolter, M.; van Dijck, L.; Valenti, D.; Levy, L. M.; Ottmann, C.; Brunsveld, L. Reversible Covalent Imine-Tethering for Selective Stabilization of 14-3-3 Hub Protein Interactions. *J. Am. Chem. Soc.* 2021, 143 (22), 8454–8464.

Chapter 3

Development of a NanoBRET assay for evaluation of 14-3-3 σ molecular glues

Abstract

We report the development of a 384-well formatted NanoBRET assay to characterize molecular glues of 14-3-3/client interactions in living cells. The seven isoforms of 14-3-3 are dimeric hub proteins with diverse roles including transcription factor regulation and signal transduction. 14-3-3 interacts with hundreds of client proteins to regulate their function and is therefore an ideal therapeutic target when client selectivity can be achieved. We have developed the NanoBRET system for three 14-3-3 σ client proteins CRAF, TAZ, and estrogen receptor α (ER α), which represent three specific binding modes. We have measured stabilization of 14-3-3 σ /client complexes by molecular glues with EC₅₀ values between 100 nM and 1 μ M in cells, which align with the EC₅₀ values calculated by fluorescence anisotropy in vitro. Developing this NanoBRET system for the hub protein 14-3-3 σ allows for a streamlined approach, bypassing multiple optimization steps in the assay development process for other 14-3-3 σ clients. The NanoBRET system allows for an assessment of PPI stabilization in a more physiologically relevant, cell-based environment using full-length proteins. The method is applicable to diverse protein-protein interactions (PPIs) and offers a robust platform to explore libraries of compounds for both PPI stabilizers and inhibitors.

Introduction

Protein-Protein Interactions

Protein-protein interactions (PPIs) are crucial for various cellular processes and modulation of these interactions, by inhibition or stabilization, results in profound effects on cellular function.^{1–4} Molecular glues are small molecules that stabilize the interaction between two proteins by binding cooperatively to the PPI interface.^{5–7} Recent work on PPI stabilization has focused on developing molecular glues and bifunctional small molecules, called PROTACs, that link a ubiquitin ligase to a protein of interest to induce the protein's degradation.^{8,9} Generally, PROTACs and molecular-glue degraders induce neomorphic interactions to degrade target proteins selectively.^{6,7,9} However, an intriguing and relatively underexplored approach lies in harnessing the potential of molecular glues as non-degradative stabilizers of PPIs.^{5,10} This novel perspective offers a promising approach to precisely modulate and stabilize PPIs for therapeutic interventions and for a deeper understanding of cellular processes.

Systematic technologies geared towards the identification of native PPI stabilizers^{11–13} tend to be target-directed, compared to cell-based technologies for molecular glue degraders.^{14–16} For instance, we have established biochemical fragment-based assays for the identification, validation, and optimization of PPI stabilizers. Our workflow employs a mass spectrometry (MS)-based site-directed disulfide tethering approach that has identified highly cooperative stabilizers for multiple 14-3-3/client complexes.^{11,12,17} During chemical optimization, PPI stabilizers are evaluated using MS- and fluorescence anisotropy (FA)-based assays. These technologies measure stabilization of 14-3-3 and a peptide derived from the client protein, facilitating screening and x-ray crystallography.^{11,12,18} With effective PPI stabilizers in hand, we sought a systematic approach to measure stabilization of full-length protein complexes. Ideally, such an approach would be suitable to lysate and cellular contexts. Here we describe the validation of NanoBRET assays to evaluate PPI stabilizers for the hub protein 14-3-3 bound to diverse protein partners.

The Hub Protein 14-3-3

14-3-3 modulates the function of hundreds of disease-relevant ‘client’ proteins through binding to linear sequences containing phosphorylated serine and threonine residues. Within the 14-3-3 family there are seven isoforms, each encoded by different genes.^{19,20} Notably, these isoforms exhibit pronounced sequence similarity within the phospho-binding groove, underscoring a shared mechanism of interaction with phosphorylated regions of client proteins.^{21,22} Peptides derived from the client proteins interact with the 14-3-3 binding groove via distinct conformations,^{20,22,23} including: (1) turning out of the groove after a phosphoserine/phosphothreonine (pS/pT) residue, typically guided by a proline in the +2 position, (2) binding straight through the phospho-binding groove, and (3) using a truncated sequence with a pS/pT at the penultimate residue of the protein. The three clients we describe here represent these three binding modes, with CRAF representing mode 1, TAZ reflecting mode 2, and Estrogen Receptor α (ER α) binding as mode 3 (Figure 3.1A; Figure S3.2).^{24–26} Figure 3.1B illustrates the 14-3-3 σ /client pocket we aim to target and example molecular glues.

14-3-3 plays important biological roles in the regulation of CRAF, TAZ, and ER α . 14-3-3 is involved in both the inhibition and activation of CRAF, with CRAF pS259 acting as a regulatory switch that is only phosphorylated in the inhibited state. In the activation of CRAF, pS259 releases from 14-3-3 and can be dephosphorylated.^{27–32} Stabilizing the 14-3-3 σ /CRAF pS259 interaction with molecular glues could therefore represent a novel method of inhibiting the MAPK pathway in various cancers (Figure 3.1C).³² The interaction between 14-3-3 and TAZ is a regulatory mechanism in the Hippo pathway. Phosphorylation of TAZ at S89 promotes association with 14-3-3, preventing TAZ translocation into the nucleus and activation as a transcription factor.^{25,33} Stabilizing the 14-3-3 σ /TAZ pS89 interaction with molecular glues would inhibit the oncogenic role of TAZ in driving cell proliferation and survival (Figure 3.1D). 14-3-3 binds to ER α at the penultimate residue pT594. Binding to 14-3-3 inhibits ER α by preventing its dimerization and DNA binding. This interaction is crucial in the context of ER-positive breast

cancer, where stabilizing the 14-3-3 σ /ER α pT594 interaction may offer a potential therapeutic strategy to disrupt estrogen-driven tumorigenesis (Figure 3.1E).²⁶ Not only do these clients represent different binding modes, but also different subcellular locations and functions.

Distinct classes of molecular-glue stabilizers of 14-3-3/client complexes have been discovered. Fusicoccane natural products and semisynthetic analogs,^{20,26,34–38} non-covalent synthetic compounds,^{39,40} and lysine-reactive fragments bind in the 14-3-3 phospho-binding groove and stabilize clients to all 14-3-3 isoforms.^{41,42} We have also identified disulfide fragments that bind to a cysteine residue at the periphery of the binding groove of the sigma isoform only and stabilize diverse 14-3-3s/client complexes.^{11,12} Structure-guided optimization towards 14-3-3/ER α stabilizers yielded cysteine-reactive molecules with EC₅₀ values in the low μ M range in biochemical assays and the ability to stabilize the complex by 120-fold, eg, $K_{D,app}$ = 2 μ M to 18 nM (Figure S3.1).¹⁸ We and our colleagues have also developed 14-3-3s-specific stabilizers for CRAF and TAZ phosphopeptides. These three chemical series require cell-based validation of their ability to act as molecular glues for 14-3-3s and full-length proteins.

NanoBRET Assay

NanoLuciferase bioluminescence resonance energy transfer (NanoBRET) assays are widely adopted methods for measuring PPI and protein/small-molecule interactions in live cells.^{30,43–46} This innovative approach utilizes fusion proteins to quantify energy transfer, a process contingent upon the proximity between the tagged proteins of interest. NanoBRET assays have been effectively harnessed to measure PPI inhibition in cells and to measure formation of ubiquitin ligase/target complexes for PROTACs, showcasing their utility in probing the effects of modulating PPIs.^{43,47,48} However, to our knowledge, NanoBRET systems have not been used to measure PPI stabilization in the presence of molecular glues. We selected NanoBRET over related technologies such as cell-based fluorescence resonance energy

transfer (FRET) or NanoLuc Binary Technology (NanoBiT) due to its improved sensitivity and geometric flexibility, respectively.^{44,47,49,50}

Here we report a 384-well plate-based cellular NanoBRET method for the evaluation of 14-3-3 σ /client molecular glues. The optimized assay allows for a statistically robust ($Z' = 0.7$ - 0.95) increase in the BRET signal of 14-3-3 σ /client interactions in the presence of small-molecule stabilizers. This 14-3-3 σ /client stabilization NanoBRET methodology can be universally applied to characterize stabilizers for other PPIs, employing a similarly efficient and systematic approach.

Materials & Methods

Construct Design and Cloning

14-3-3 σ and client proteins CRAF, TAZ, and ER α were cloned into the Promega NanoBRET vectors (Promega N1811) using Gibson Assembly (NEB E2611) with a GSSG linker between the protein and the tag. Linker length between the protein of interest and tags can be optimized depending on structural constraints.^{51,52} 17 residues were deleted from the C-terminus of 14-3-3 σ to reduce dynamics of C-terminal tags; therefore, all 14-3-3 σ constructs contain residues 1-231. The sigma isoform was used due to the presence of cysteine 38, which is not present in the other six isoforms, for compound engagement. Additionally, 14-3-3 σ is the only isoform known to preferentially homodimerize.⁵³ 14-3-3 σ Δ C and full-length constructs bind to client proteins with nearly identical affinities and engagement in biochemical and cellular assays (data not shown). NES-TAZ and NES-ER α constructs contain an engineered nuclear export signal (NES) on the N-terminus with a GSSG linker before the NanoLuc tag. Final construct sequences can be found in Table S3.1.

Compounds

CRAF-01-05 were synthesized in house and will be disclosed in due course. TAZ-01-03 were identified in a high-throughput screening campaign; the structures have not been disclosed (Ambagon Therapeutics). ER α -01 and ER α -02 were published as compounds 85 and 181, respectively.¹⁸ Fusicoccin-A was purchased from Enzo Life Sciences (BML-EI334-0001) and XMU-MP-1 was purchased from MedChemExpress (HY-100526).

Cell Lines

HEK293T cells were purchased from ATCC (CRL-3216) and used as recommended. To avoid signal inhibition by endogenous, unlabeled proteins, we selected HEK293T cells because they express minimal to no 14-3-3 σ , TAZ, and ER α . HEK293T cells also have an inactive MAPK pathway, suggesting that transfected CRAF would be phosphorylated at S259. If the endogenous expression level of proteins of interest is a concern, knockdown or knockout can be used to reduce endogenous protein levels. Alternatively, different cell lines can be used with various expression levels of the proteins of interest. MCF7 (HTB-22) and MDA-MB-231 (HTB-26) cells were purchased from ATCC and used as recommended for 14-3-3 σ /ER α studies.

NanoBRET

NanoBRET assays were performed as described by Promega. Four constructs (two for ER α) were generated for each client protein to test in combination with four constructs of 14-3-3 σ . Combination tests were performed at a 1:10 ratio of NanoLuc:HaloTag; ratios of 1:1, 1:10, 1:100, and 1:1000 were then tested with selected combinations. Plasmids were transfected using jetOPTIMUS transfection reagent following the manufacturer's protocol and cells were plated in white, flat bottom TC-treated 384-well microplates (Corning 3570) after transfection in Gibco FluoroBrite DMEM with 4% fetal bovine serum (FBS; Sigma-Aldrich). ER α experiments were conducted with 4% charcoal dextran stripped FBS (Corning 35-072-CF). Each experiment was performed in triplicate and included no-acceptor controls and samples

with the HaloTag NanoBRET 618 Ligand. Plates were read on an EnVision XCite 2105 plate reader at 618 nm (HaloTag) and 460nm (NanoLuc) (Filters: M647 CWL=647nm BW=75nm; M460 CWL=460nm BW=80nm Mirror: LUM/D585).

Fluorescence Anisotropy Measurements

ER α FA measurements were performed as previously described.¹⁸ For CRAF and TAZ, peptides were purchased from Elim Biopharmaceuticals, Inc (CRAF: QRSTpSTPNVH; TAZ: RSHpSSPASLQ).

For CRAF, fluorescein-labeled peptides (5-FAM), full-length 14-3-3 σ protein, the compounds (50 mM stock solution in DMSO) were diluted in buffer (10 mM HEPES, pH 7.5, 150 mM NaCl, 0.1% Tween20, 1 mg/mL Bovine Serum Albumin (BSA; Sigma-Aldrich). Final DMSO in the assay was always 1%. Dilution series of 14-3-3 proteins or compounds were made in black, flat-bottom 384-microwell plates (Greiner Bio-one 784900) in a final sample volume of 10 μ L in triplicate. For compound titrations the initial 50 mM compound stock solutions in DMSO were diluted to 20mM in a 384-well master plate, followed by a serial 2-fold dilution. Then, 500 nl of the dilution series were transferred in the assay plates. A master mix containing 10 nM fluorescein-labeled peptide and 5 μ M full-length 14-3-3 σ (concentration at EC₂₀ value of the protein-peptide complex) in buffer (10 mM HEPES, pH 7.5, 150 mM NaCl, 0.1% Tween20, 1 mg/mL BSA) was dispensed on the assay plates. The final volume per well was 10 μ L, with final top concentration of compounds dose response series at 1mM. Each compound was measured in triplicate, in two independent experiments. Fluorescence anisotropy measurements were performed directly and after overnight incubation at room temperature. Protein titrations were made by titrating 14-3-3 σ in a 2-fold dilution series (starting at 250 μ M) to a mix of fluorescein-labeled peptide (10 nM) and DMSO or compound (100 μ M). Fluorescence anisotropy measurements were performed after overnight incubation at room temperature. Fluorescence anisotropy values were measured using a Molecular Devices ID5 plate reader (filter set λ_{ex} : 485

± 20 nm, λ_{em} : 535 ± 25 nm; integration time: 50 ms; settle time: 0 ms; shake 5sec, medium, read height 3.00 mm, G factor = 1. Data reported are at endpoint. EC_{50} and apparent K_D values were obtained from fitting the data with a four-parameter logistic model (4PL) in GraphPad Prism. Data was obtained and averaged based on two independent experiments.

TAZ FA experiments were done as described for CRAF with the following exceptions: final sample volume was 25 μ L using 384-well plates (Corning 3575), final peptide concentration was 100 nM 5-FAM-TAZ, and final full-length 14-3-3 σ protein concentration was 300 nM in the compound titration. Measurements were taken at 1 hour and 24 hours for compound titration and protein titration, respectively.

Procedure

We have developed a NanoBRET assay to test small molecule molecular glues of 14-3-3 σ /client PPIs (Figure 3.2A). To test new PPIs, eight NanoLuc/HaloTag-fused protein constructs were cloned, and all eight combinations were tested. After selecting the desired combination, four ratios of NanoLuc:HaloTag plasmid were used to determine the optimal transfection ratio before testing compounds to measure their effects on PPI stabilization (Figure 3.2B).

Construct Design

14-3-3 σ and client proteins were cloned into NanoLuc and HaloTag vectors with N- or C-terminal tags. The constructs were named based on the placement of the tag, with “HaloTag-14-3-3 σ ” denoting N-terminal placement of the tag, and “14-3-3 σ -HaloTag” denoting C-terminal placement. Because the C-terminus of ER α is crucial for binding to 14-3-3, ER α was only cloned with N-terminal tags. A nuclear export signal (NES; LPPLERLTL) was inserted at the N-terminus of TAZ and ER α for the “NES-client” constructs.⁵⁴ The DNA binding domain (185-250) was truncated out of ER α to make the “ Δ DBD-ER α ” construct.

Assay Setup

Generally, we followed the manufacturer's protocol.^{43,55} In short, plasmids were transfected in HEK293T cells in bulk for 48 hours. Ratios of NanoLuc:HaloTag plasmids were transfected by keeping the amount of HaloTag plasmid constant and decreasing the amount of NanoLuc plasmid. For example, a 1:10 transfection ratio could include 1 µg of HaloTag plasmid plus 0.1 µg of NanoLuc plasmid. The decrease in the amount of NanoLuc plasmid transfected is reflected in the amount of protein expressed as measured by western blot (Figure S3.3A). Cells were trypsinized and counted before adjusting the cell density to 250,000 cells/mL and adding HaloTag ligand (1 µL ligand/1 mL of media). A no-HaloTag-ligand control was also made wherein DMSO was added instead of ligand at the same % v/v (1 µL/1 mL). 30 µL of the cell/HaloTag ligand or DMSO mixture was added to each well in a 384-well assay plate. Compound (or DMSO) was diluted in media to 4x the final concentration. Then, 10 µL of this 4x compound stock was added to corresponding wells. All samples had the same final percent DMSO of less than 0.5%. After 24 hours, NanoLuc substrate was added, and assay plates were read to measure luminescence in the NanoLuc and HaloTag channels (Figure 3.2C).

An alternative approach can be used to measure NanoBRET in lysate using the detergent digitonin. This approach can be used if the compounds are not cell permeable or are cytotoxic at the desired concentration range. Digitonin permeabilizes the outer membrane to release cytosolic proteins into the media (Figure S3.3B). As exemplified for CRAF, we added digitonin at 200 µg/mL after seeding cells and before dosing with compound.

Data Analysis

The following formulas were used to calculate the raw milliBRET units (mBU), mean corrected mBU, and fold change. Data was then analyzed and visualized in GraphPad Prism to obtain EC₅₀ values by fitting the data with a 4PL model.

$$\frac{\text{acceptor emission value (618nm)}}{\text{donor emission value (460nm)}} * 1,000 = mBU$$

$$\text{mean } mBU_{\text{experimental}} - \text{mean } mBU_{\text{no HaloTag ligand control}} = \text{mean corrected } mBU$$

$$\frac{mBU_{\text{experimental}}}{mBU_{\text{DMSO control}}} = \text{fold change}$$

Results

CRAF/14-3-3σ NanoBRET

To measure cysteine-targeting stabilizers of the 14-3-3σ/CRAF pS259 interaction, eight constructs were cloned for 14-3-3σ/CRAF: HaloTag-14-3-3σ, 14-3-3σ-HaloTag, NanoLuc-14-3-3σ, 14-3-3σ-NanoLuc, HaloTag-CRAF, CRAF-HaloTag, NanoLuc-CRAF, and CRAF-NanoLuc. With these constructs, eight combinations were tested at a 1:10 NanoLuc:HaloTag transfection ratio (Figure 3.3A). Combinations where the NanoLuc was fused to 14-3-3σ and the HaloTag fused to CRAF resulted in low BRET signal. Two combinations, NanoLuc-CRAF/14-3-3σ-HaloTag and CRAF-NanoLuc/14-3-3σ-HaloTag ($Z' = 0.91$ and 0.92 , respectively), were selected for ratio testing. CRAF-NanoLuc/14-3-3σ-HaloTag gave higher signal and was chosen to move forward with. In these ratio tests, 1:10 and 1:100 CRAF-NanoLuc:14-3-3σ-HaloTag gave the highest signals ($Z' = 0.90$, $Z' = 0.95$, respectively; Figure 3.3B). Ratios of 1:1 and 1:1,000 were excluded due to low signal and high background, respectively.

When using NanoBRET to identify molecular glues, careful selection of a combination and ratio is essential to ensure that the protein complex is not completely formed, such that it maintains sensitivity to stabilization by small molecules. Thus, we evaluated both 1:10 and

1:100 in the presence of stabilizers. 1:10 resulted in higher fold-stabilization with molecular glues than 1:100 (Figure S3.4A); thus, we proceeded with this condition.

We tested three compounds, initially identified as molecular glues through MS and FA assays, along with one negative control, in the CRAF/14-3-3 σ NanoBRET system. The negative control was structurally related to the stabilizers but inactive in the FA assay. The molecular glues exhibited up to 1.6-fold increase in NanoBRET signal, and EC₅₀ values in the sub- μ M to low- μ M range (Figure 3.3C). The fold increase in signal and the Z' values were similar between the NanoBRET and FA assays, suggesting similar sensitivity. These compounds were dependent on the presence of C38 in 14-3-3 σ , as mutation of this residue rendered the compounds ineffective in stabilizing the interaction (Figure S3.4B).

In comparing the EC₅₀ values obtained from FA and NanoBRET, it is noteworthy that FA was performed in vitro with a fully phosphorylated CRAF pS259 peptide with the 14-3-3 σ concentration kept at its EC₂₀, while NanoBRET was performed in cells with the full-length CRAF that may not have been fully phosphorylated. Moreover, the degree of phosphorylation of CRAF may increase over the time of compound incubation, since increased 14-3-3 binding protects clients from dephosphorylation. These factors make it difficult to quantitatively compare EC₅₀ values from the biochemical and cell-based binding assays. Nevertheless, the trends in EC₅₀ values track between NanoBRET and FA, with the negative control CRAF-01 exhibiting a non-stabilizing effect. In contrast, CRAF-02 emerged as the most potent stabilizer with an EC₅₀ value of 0.18 μ M and the highest maximum signal. CRAF-03 had a similar EC₅₀ value of 0.20 μ M, but a lower maximum signal. CRAF-04 demonstrated a comparatively diminished stabilizing capacity with an EC₅₀ value of 1.1 μ M, making it the weakest of the three molecular glues (Table 3.1). The difference in signal maximum was reproducible and consistent with the FA data (Figure S3.1A-E). The calculated EC₅₀ values from the NanoBRET assay for CRAF-02 and CRAF-03 were lower than those calculated from FA assays (Table 3.1), but the rank order was maintained.

Using the procedure described above, we introduced the detergent digitonin into the assay to permeabilize the cell membrane, releasing the NanoBRET pair into the media. In the cellular NanoBRET assay, CRAF stabilizers were tested up to 10 μ M based on their cytotoxicity in HEK293T (data not shown). To compare the lysate and cell-based assays, we measured CRAF-05 activity in the CRAF/14-3-3 σ NanoBRET with four doses, from 1 – 250 μ M in lysate and 1 – 10 μ M in cells. The results in both assays were similar, with CRAF-05 showing significant stabilization even at 1 μ M. The lower signal ratio at the maximum concentrations (1.3- vs 1.5-fold) led us to use the cell-based assay going forward (Figure S3.4C). Thus, PPI stabilization can be measured either in cells or in lysates using the same transfection ratio and assay formats.

TAZ/14-3-3 σ NanoBRET

The following four constructs were cloned to test both donor and acceptor tags on the N- or C-terminus of TAZ: HaloTag-TAZ, TAZ-HaloTag, NanoLuc-TAZ, and TAZ-NanoLuc. To aid in developing the 14-3-3 σ /TAZ NanoBRET system, we used an MST1/2 inhibitor, XMU-MP-1 (“XMU”), at 60 μ M to prevent phosphorylation of TAZ, which in turn inhibited the 14-3-3 σ /TAZ interaction (Figure 3.1D). After testing all eight NanoBRET combinations of 14-3-3 σ and TAZ, we chose to move forward with NanoLuc-TAZ/14-3-3 σ -HaloTag because it produced the largest BRET signal (24 mBU) with the most significant difference between untreated and XMU treated samples (fold change = 1.4, Z' = 0.84; Figure 3.4A). NanoLuc:HaloTag ratios were then evaluated, and 1:10 gave the largest fold-change between untreated and XMU treated samples (fold-change = 1.4, Z' = 0.90; Figure 3.4B).

14-3-3 σ controls trafficking of TAZ between the cytoplasm and the nucleus, so we evaluated whether manipulating NanoLuc-TAZ subcellular localization would alter the BRET signal. We engineered a nuclear export signal (NES) into the N-terminus of the TAZ construct before the NanoLuc tag. With this NES-NanoLuc-TAZ (NES-TAZ) construct, the basal

NanoBRET signal increased 3.1-fold (Figure 3.4C). While the raw mBU signal varied between biological replicates, perhaps due to transfection efficiency, the BRET signal was consistently larger with the NES construct.

We compared the 14-3-3 σ /TAZ complex formation with C38-reactive molecular glues, TAZ-02 and TAZ-03, along with an inactive compound, TAZ-01 (Figure S3.5). The NES-TAZ construct led to increased mBU values but decreased the fold-change compared to NanoLuc-TAZ itself. For instance, TAZ-02 increased the BRET signal by 4.2-fold and 2.2-fold with NanoLuc-TAZ and NES-TAZ, respectively (Figure 3.4C). Nevertheless, the compound dose-responses yielded similar EC₅₀ values with both TAZ constructs. The negative control TAZ-01 had no effect on the BRET signal, while TAZ-02 had an EC₅₀ of 730 nM; TAZ-03 did not reach a plateau, with an estimated EC₅₀ of 120 nM (Figure 3.4D). In FA assays, TAZ-02 and TAZ-03 performed similarly to each other with EC₅₀ values of 1.2 μ M and 1.6 μ M, respectively (Figure S3.1F-I; Table 3.1). Thus, the TAZ/14-3-3 σ NanoBRET assays confirmed the stabilizing effects of these molecular glues.

ER α /14-3-3 σ NanoBRET

ER α binds 14-3-3 σ at its penultimate residue, pT594. Therefore, only N-terminal tag constructs were developed for ER α to avoid interference with 14-3-3 σ binding. ER α combination and ratio tests were performed in the presence of a natural product stabilizer, Fusicoccin-A (FC-A) at 30 μ M. Similar to CRAF and TAZ, little to no signal was measured for the combinations in which 14-3-3 σ was tagged with NanoLuc. The NanoLuc-ER α plus 14-3-3 σ -HaloTag combination gave the largest signal and fold stabilization for the positive control FC-A (Z' = 0.79, Figure 3.5A). In the ratio test, 1:10 was the only combination where there was a measurable difference between DMSO and FC-A (Z' = 0.96, Figure 3.5B). Therefore, this ratio was selected to advance to the subsequent testing phase for new 14-3-3 σ specific, cysteine-reactive molecular glues (Figure S3.6A). The most potent molecular glue, ER α -02, resulted in 2.5-fold

stabilization with an EC₅₀ of 2.4 μ M (Figure 3.5C). In FA dose-responses, ER α -02 had a similar EC₅₀ value of 1.0 μ M when tested with the ER α peptide (Figure S3.1J,K).¹⁸ The inactive compound, ER α -01, had no effect in NanoBRET or FA dose response assays (Figure 3.5C, Table 3.1). FC-A resulted in low fold stabilization (Figure 3.5A,B) likely because binding of the NanoBRET pair was diluted by binding to other 14-3-3 isoforms and clients. Because HEK293T cells did not contain endogenous 14-3-3 σ , the sigma-specific stabilizer, ER α -02, was highly effective at binding to the transfected 14-3-3 σ .

Since ER α is also a transcription factor, a significant fraction could be localized in the nucleus, resulting in a low NanoBRET signal. We therefore engineered two cytoplasmic constructs containing an NES at the N-terminus ("NES-ER α ") or removing the DNA-binding domain (aa 185-250; " Δ DBD-ER α "). While both methods increased the BRET signal, from 5-10 to 10-15 mBU, the stabilizer dose response effects were diminished (Figure 3.5D), possibly due to reaching maximal signal of the system. Although these methods did not improve the ER α /14-3-3 σ NanoBRET assay to evaluate molecular glues, they are useful techniques to apply when troubleshooting low NanoBRET signal for other PPI NanoBRET systems, as seen with TAZ.

A benefit of developing the ER α /14-3-3 σ NanoBRET assay in HEK293T cells was the low endogenous expression of both proteins. We extended these experiments to an ER-positive breast cancer cell line, MCF7, where both ER α and 14-3-3 σ were expressed, and MDA-MB-231, an ER-negative breast cancer cell line, where only 14-3-3 σ was endogenously expressed. Comparing the ER α /14-3-3 σ NanoBRET assay in these three cell lines, HEK293T cells had the largest signal window with minimal background (Figure S3.6B-D). Thus, using a cell line that does not endogenously express the proteins of interest ensured that the tagged proteins were not outcompeted by endogenous proteins.

Discussion

14-3-3 σ NanoBRET Summary and Workflow

Traditional NanoBRET assay development involves a four-step workflow: (1) generating eight constructs of 14-3-3 σ and the client tagged with NanoLuc or HaloTag, (2) testing eight combinations, (3) evaluating four ratios of the best combination, and (4) subsequently testing molecular glues (Figure 3.2B). In developing analogous NanoBRET assays for three 14-3-3 σ /client complexes, we learned that the HaloTag acceptor was always preferred on 14-3-3 σ and that varying the subcellular localization of the client protein sometimes improved the BRET signal, depending on the biology of the complex (Table 3.2). Since 14-3-3 σ is the more promiscuous protein compared to these three clients, this could explain the preference for the HaloTag on 14-3-3 σ . The NanoLuciferase donor conjugated to 14-3-3 σ would lead to unquenched BRET when bound to endogenous clients lacking the HaloTag acceptor. Leveraging the insights gained from our results across 14-3-3 σ /client systems, we propose a streamlined workflow that condenses the NanoBRET assay design for 14-3-3 σ /client interactions into three efficient steps: (1) constructing two client constructs (or one depending on client binding restrictions, e.g. ER α), (2) test two combinations, and (3) evaluate molecular glues (Figure 3.6). This simplified workflow promises expedited discovery of 14-3-3 σ /client molecular-glue stabilizers, offering a potentially impactful resource for diverse therapeutic applications.

Assay Throughput

NanoBRET assays to discover and characterize molecular glues can be set up in a low-, medium-, or high-throughput format depending on materials and instruments employed. Generally, we have run approximately 200-2,000 samples at a time. Because optimizing the transfection ratio is necessary to detect PPI stabilizers, the NanoBRET assay requires a transient transfection. This manual step reduces throughput and precision; in particular, raw mBU values can vary between experiments. Nevertheless, with automation for cell seeding and

compound dosing, the NanoBRET assay for PPI stabilizers can be performed in a high-throughput manner.

To increase assay throughput, additional techniques can be implemented. Structural and computational methods can be used to predict optimal tag placements on the proteins of interest. In our system, 14-3-3 binds unstructured domains; thus, computational modeling of NanoLuc and HaloTag conjugated proteins with techniques such as AlphaFold may not accurately depict tag placement and ideal NanoBRET combinations for these multi domain, flexible PPIs.

Conclusions

We describe the development of a NanoBRET assay designed to characterize molecular-glue stabilizers of 14-3-3 σ /client PPIs. Building from the published NanoBRET protocol,⁵⁵ we have identified multiple factors that required careful evaluation. Specific to PPI stabilizers, it is critical to select a combination of protein constructs and transfection ratio that leads to a measurable but incomplete formation of the PPI, such that the assay can detect an increase in BRET signal. Additional general considerations include optimizing the cell permeability and colocalization of the protein partners. For example, digitonin releases cytoplasmic proteins into the media, allowing for higher compound concentrations and effectively transforming the assay into a lysate assay. Furthermore, adding a nuclear export signal increased mBU values for the transcription factor/14-3-3 σ complexes, suggesting that localization motifs should be considered if the BRET signal is low. Lastly, choice of cell line should account for the endogenous expression levels of the proteins of interest. With these considerations, our NanoBRET results closely mirror the sensitivity of FA and the NanoBRET EC₅₀ values were consistent with the FA EC₅₀ values measured for diverse molecular glues. Thus, NanoBRET is a valuable tool to characterize stabilizers targeting 14-3-3 σ /client

interactions. Notably, the distinct advantage of NanoBRET is the physiological relevance of the assay, as it is performed in a cellular environment with full-length proteins.

This comprehensive method can be efficiently adapted and streamlined for the exploration of other 14-3-3 σ client proteins. Moreover, the systematic approach can be readily applied to other hub protein systems and to discover novel molecular glues. As research in this domain progresses, the integration of NanoBRET assays for PPI stabilization could significantly broaden the repertoire of techniques available for studying PPIs and their implications in cellular processes.

Figures

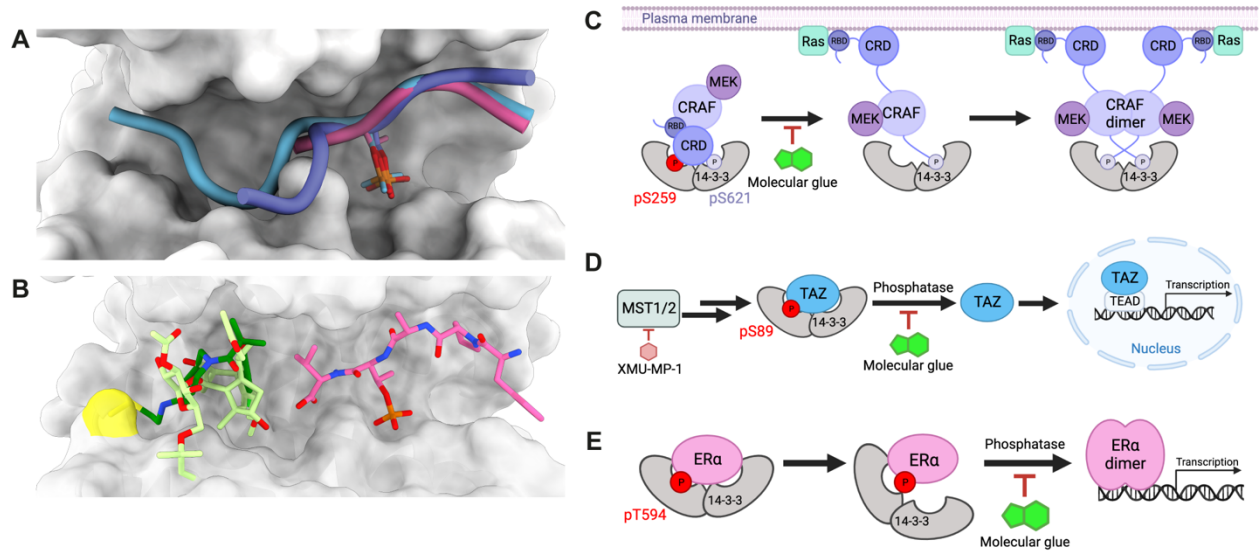


Figure 3.1: 14-3-3 σ /client binding and regulatory mechanisms

A) Crystal structure overlay of 14-3-3 σ (grey) bound to CRAF (purple), ER α (pink), and TAZ (blue) peptides illustrating the three different binding modes. pS/T residues are shown to illustrate the overlap in this position of the peptide. PDBs: CRAF 4IHL, ER α 4JDD, TAZ 3MHR. **B)** Visualization of the molecular glue binding pocket within the 14-3-3 σ phospho-binding groove. Both the fragment stabilizer (dark green) and Fusicoccin-A (light green) bind in the 14-3-3 σ phospho-binding groove proximal to the ER α peptide (pink). 14-3-3 σ C38 is highlighted in yellow. PDB 8AFN with Fusicoccin-A overlaid from PDB 4JDD. **C)** Proposed 14-3-3/CRAF mechanism. 14-3-3 binding to CRAF pS259 (red) maintains the inhibited state of CRAF. 14-3-3 σ /CRAF pS259 molecular glues would prevent the opening and subsequent activation of CRAF. **D)** 14-3-3 regulation of TAZ in the Hippo signaling pathway. MST1/2 activity leads to the phosphorylation of TAZ at pS89 (red). In the nucleus, TAZ binds TEAD transcription factors and activates transcription. 14-3-3 σ /TAZ pS89 molecular glues would localize TAZ in the cytoplasm, impeding its role as a transcription factor. **E)** 14-3-3 regulation of ER α . 14-3-3 binds ER α at pT594 (red) to prevent ER α dimerization and binding to DNA. 14-3-3 σ /ER α pT594 molecular glues would prevent ER α dissociation and activation.

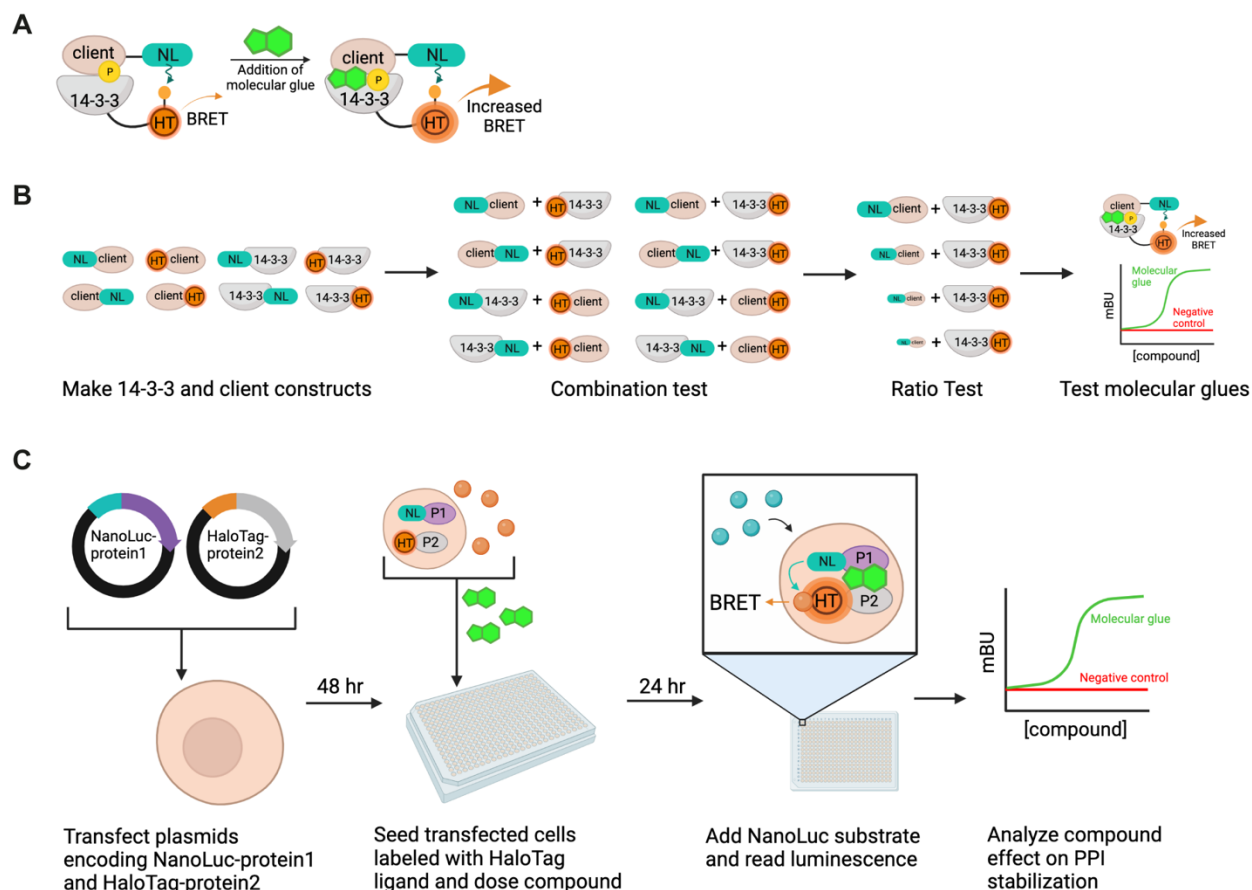


Figure 3.2: NanoBRET assay overview

A) Schematic representation of the 14-3-3/client NanoBRET assay, where one protein is tagged with NanoLuc (NL) and the other with HaloTag (HT). Addition of molecular glues is expected to increase the BRET signal. **B)** Steps to set up a NanoBRET assay for a new PPI. **C)** Procedure to set up NanoBRET assay.

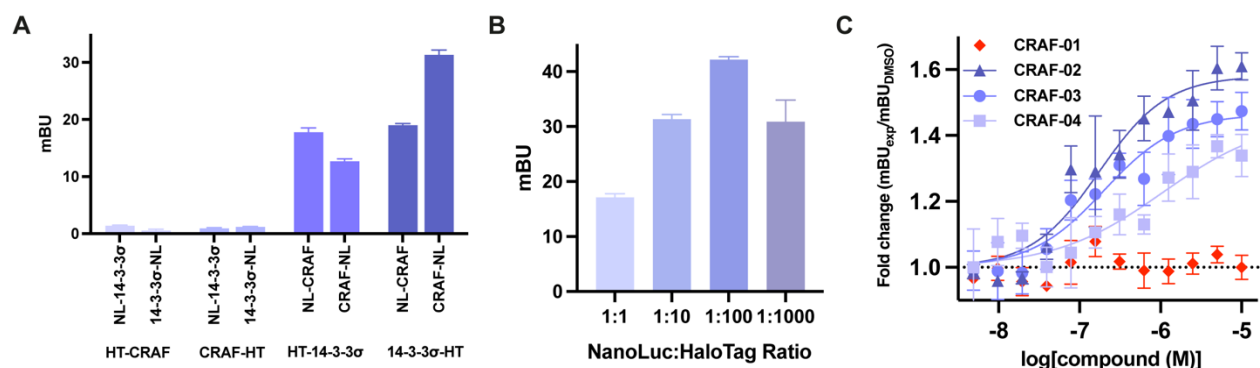


Figure 3.3: NanoBRET results for CRAF/14-3-3σ

A) Combination test ($n = 3$). 14-3-3σ with a C-terminal HaloTag and CRAF with an N- or C-terminal NanoLuc tag resulted in the highest signal. **B)** CRAF-NanoLuc/14-3-3σ-HaloTag ratio test ($n = 3$). **C)** CRAF-NanoLuc/14-3-3σ-HaloTag NanoBRET data with 4 compounds, three stabilizers (CRAF-02-04) and one negative control (CRAF-01) ($n = 3$). Data shown in C are representative of 5 biological replicates.

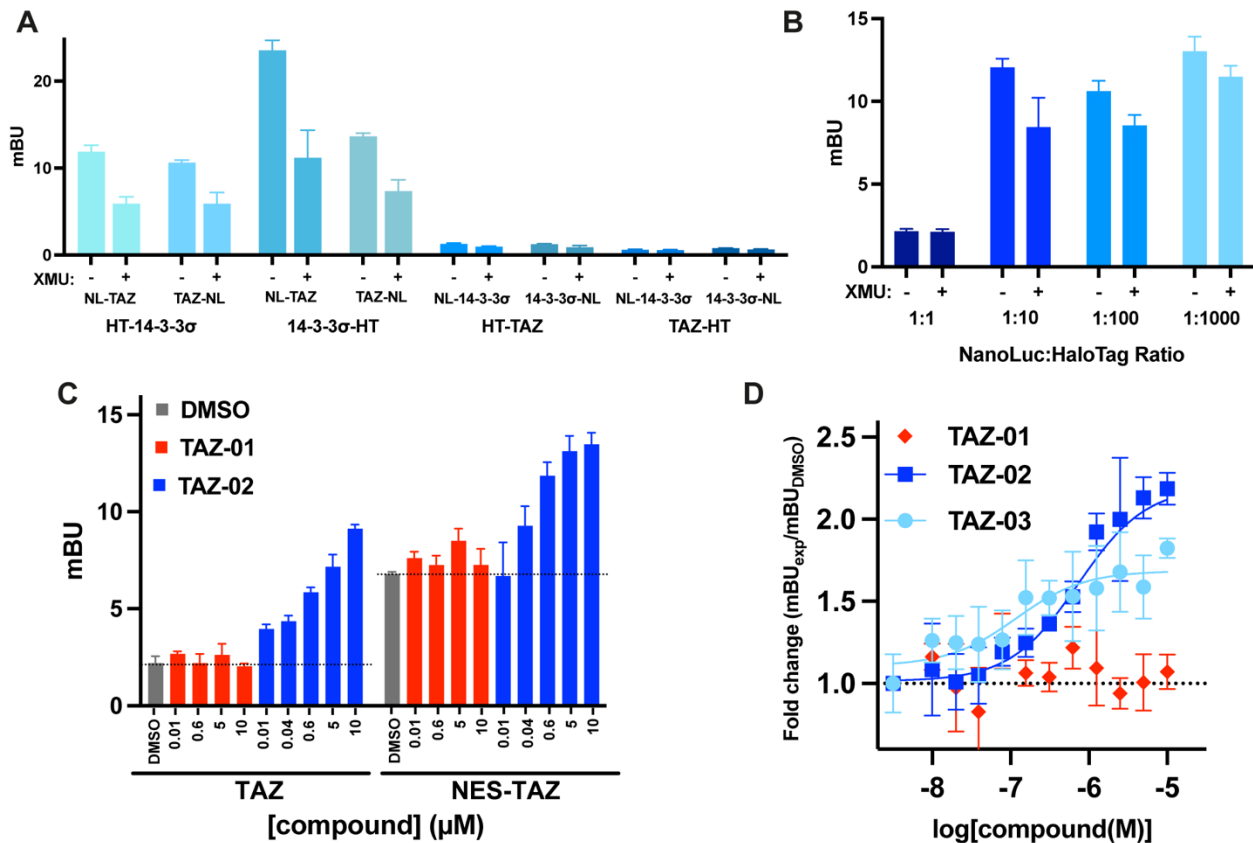


Figure 3.4: NanoBRET results for TAZ/14-3-3σ

A) Combination test comparing untreated and XMU (60 μM) treated samples (n = 3). NanoLuc-TAZ/14-3-3σ-HaloTag gave the highest signal and largest fold change with XMU. **B)** NanoLuc-TAZ/14-3-3σ-HaloTag ratio test (n = 3). **C)** Comparison of TAZ and NES-TAZ constructs with one inactive compound (TAZ-01) and one stabilizer (TAZ-02) (n = 3). A subset of tested compound concentrations is shown. **D)** NES-TAZ/14-3-3σ NanoBRET results with two TAZ/14-3-3σ stabilizers (TAZ-02 and TAZ-03) and TAZ-01 with all data points in the dilution series shown (n = 3). Data shown in C and D are representative of 3 biological replicates.

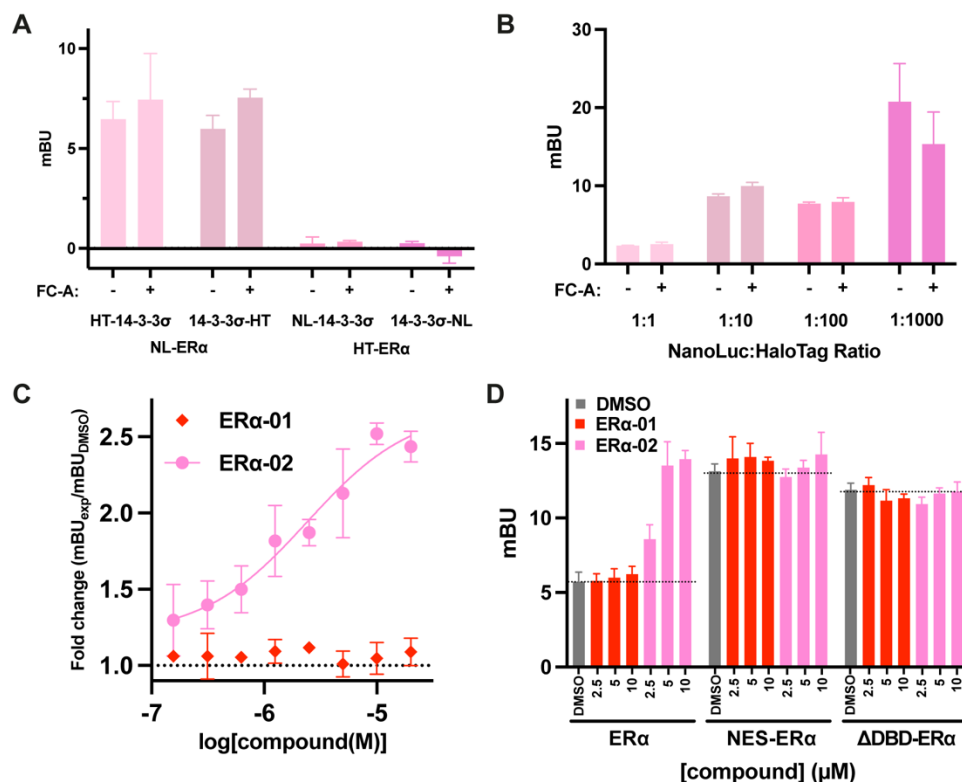


Figure 3.5: NanoBRET results for ERα/14-3-3σ

A) Combination test comparing untreated and FC-A (30 μM) treated samples (n = 3). NanoLuc-ERα/14-3-3σ-HaloTag gave the only significant difference with DMSO vs FC-A treatment. **B)** NanoLuc-ERα/14-3-3σ-HaloTag ratio test (n = 3). **C)** ERα/14-3-3σ NanoBRET results with one molecular-glue stabilizer (ERα-02) and one inactive compound (ERα-01) (n = 3). **D)** Comparison of different ERα constructs with ERα-01 and ERα-02 (n = 3). Data shown in C and D are representative of 3 biological replicates.

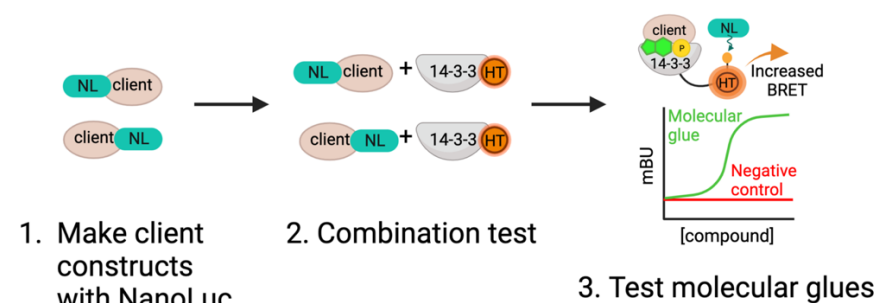


Figure 3.6: Simplified NanoBRET workflow for identification of 14-3-3σ/client molecular glues

Tables

Table 3.1: EC₅₀ values for 14-3-3σ/client NanoBRET and FA (n = 3)

NanoBRET Complex	Compound	NanoBRET EC ₅₀ (μM)	FA EC ₅₀ (μM)
CRAF-NanoLuc/14-3-3σ-HaloTag	CRAF-01	>10	>150
	CRAF-02	0.18	1.0
	CRAF-03	0.20	1.2
	CRAF-04	1.1	1.2
NES-NanoLuc-TAZ/14-3-3σ-HaloTag	TAZ-01	>20	>150
	TAZ-02	0.73	1.2
	TAZ-03	0.12 (est.)	1.6
NanoLuc-ERα/14-3-3σ-HaloTag	ERα-01	>20	>150
	ERα-02	2.4	1.0

Table 3.2: Tag placement for 14-3-3σ/CRAF, TAZ, and ERα NanoBRET assays

14-3-3σ client	NanoLuc	HaloTag
CRAF	CRAF (C-terminus)	14-3-3σ (C-terminus)
TAZ	TAZ (N-terminus)	14-3-3σ (C-terminus)
ERα	ERα (N-terminus)	14-3-3σ (C-terminus)

Supplementary Figures

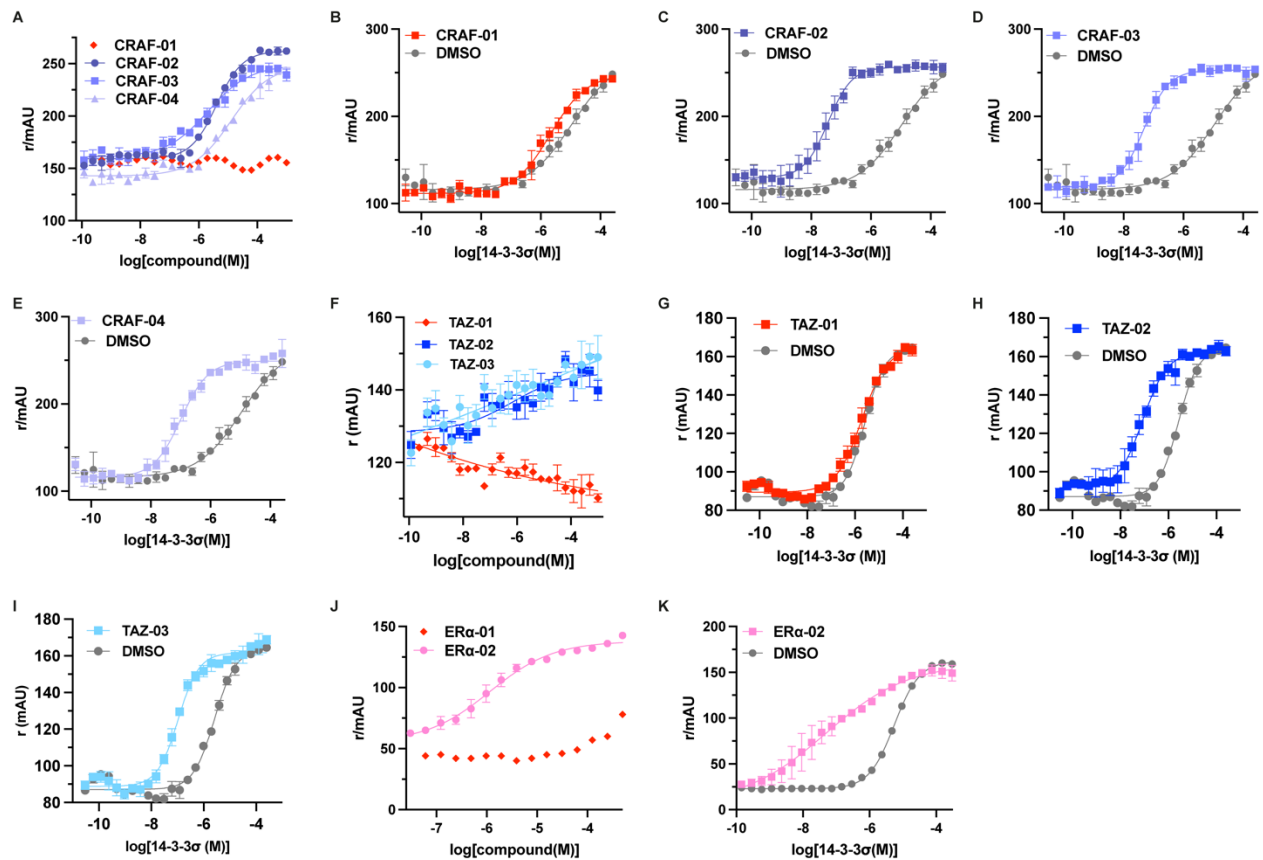


Figure S3.1: CRAF pS259, TAZ pS89, and ERα pT594 peptide FA compound and protein titrations

A) CRAF-01-04 compound titrations. **B)** CRAF-01 protein titration. **C)** CRAF-02 protein titration. **D)** CRAF-03 protein titration. **E)** CRAF-04 protein titration. **F)** TAZ-01-03 compound titrations. **G)** TAZ-01 protein titration. **H)** TAZ-02 protein titration. **I)** TAZ-03 protein titration. **J)** ERα-01 and ERα-02 compound titrations. **K)** ERα-02 protein titration. All experiments n = 3.

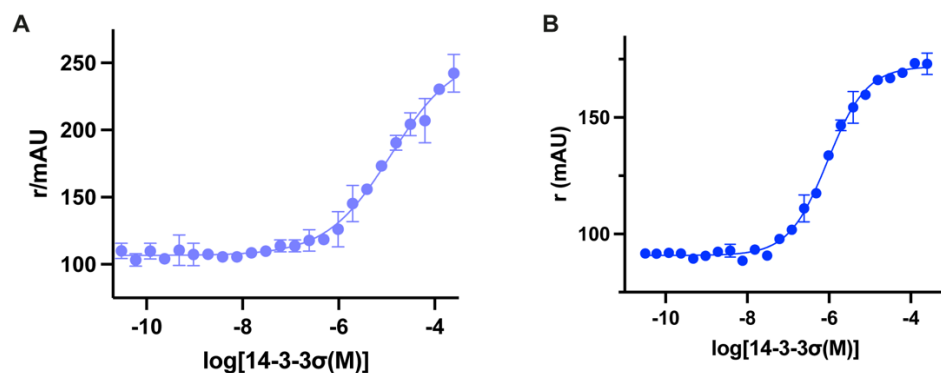


Figure S3.2: 14-3-3/client peptide affinities

A) CRAF pS259 peptide FA KD assay (n = 3). **B)** TAZ pS89 peptide FA KD assay (n = 3).

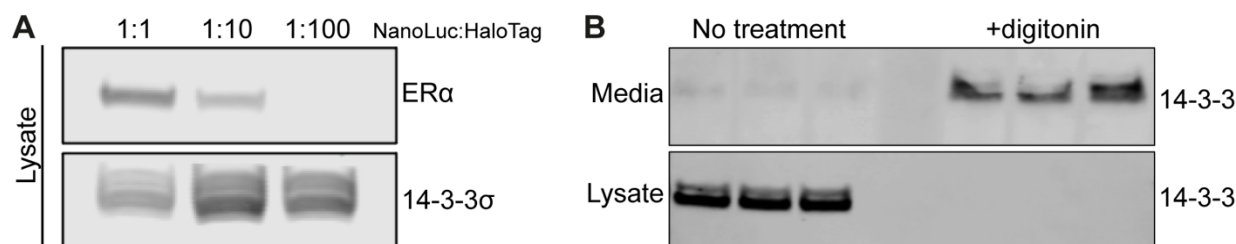


Figure S3.3: Protein expression and effects of detergent

A) NanoLuc:HaloTag plasmid transfection ratios reflected in protein expression in HEK293T cells. **B)** Western blot for 14-3-3 without and with digitonin treatment (n=3). Cytosolic proteins are released into the media when treated when cells are treated with digitonin.

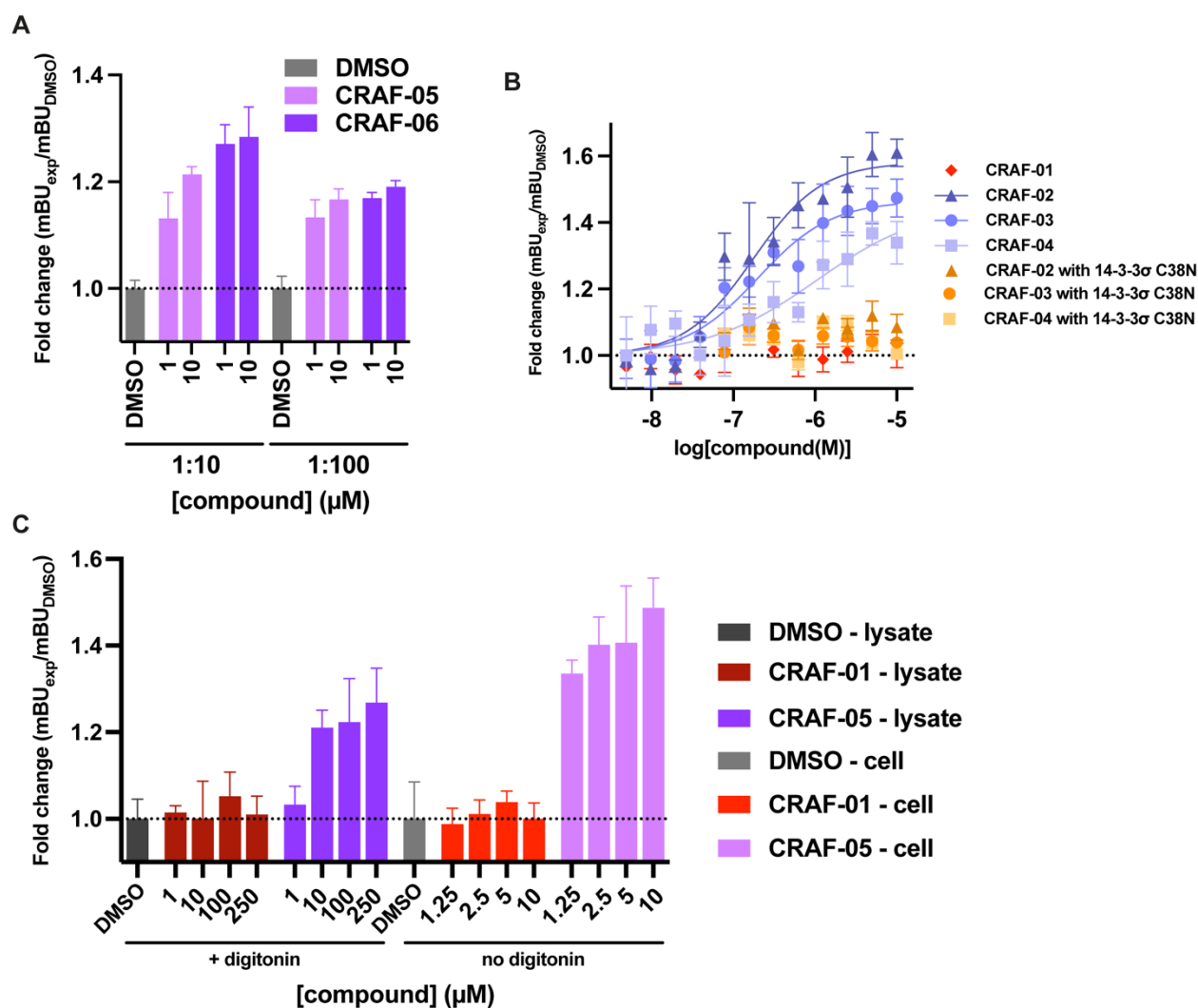


Figure S3.4: CRAF/14-3-3σ NanoBRET optimization

A) CRAF/14-3-3σ 1:10 and 1:100 ratio comparison (n = 3). 1:10 resulted in a larger fold increase. **B)** 14-3-3σ WT vs 14-3-3σ C38N NanoBRET. Stabilizers were dependent on the presence of C38 (n = 3). **C)** Comparison of lysate and in cell NanoBRET assays (n = 3). Both showed stabilization with CRAF-05.

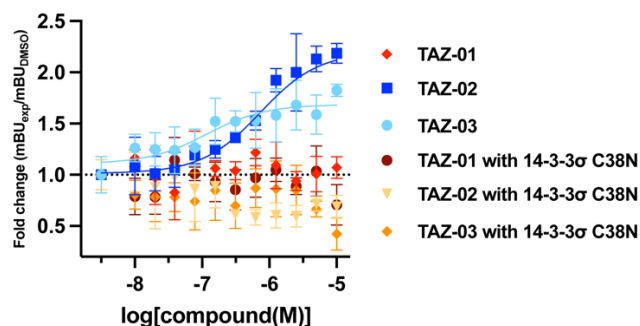


Figure S3.5: TAZ NanoBRET with 14-3-3σ C38N
Compounds bind to C38 (n = 3).

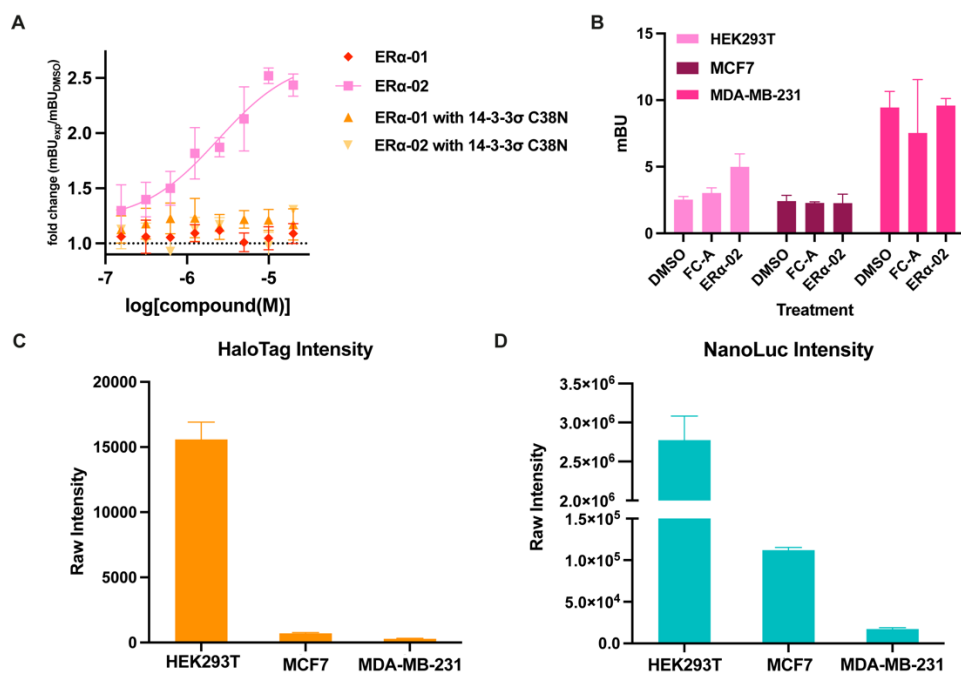


Figure S3.6: ERα/14-3-3σ NanoBRET optimization

A) ERα NanoBRET with 14-3-3σ C38N (n = 3). Compounds are dependent on C38. **B)** ERα/14-3-3σ NanoBRET cell line comparison with FC-A (30 μM) and ERα-02 (10 μM) treatment (n = 3). HEK293T showed a measurable stabilization with ERα-02. **C)** Raw HaloTag luminescence intensity in HEK293T, MCF7, and MDA-MB-231 (n = 3). **D)** Raw NanoLuc luminescence intensity in HEK293T, MCF7, and MDA-MB-231 (n = 3).

Supplementary Tables

Table S3.1: Amino acid sequences of final NanoBRET constructs

Construct	Amino Acid Sequence
14-3-3σ-HaloTag	MERASLIQKAKLAEQAERYEDMAAFMKGAVEKGEELSCEERNLLSVAYKNVVG QRAAWRVLSSIEQKSNEEGSEEKGPEVREYREKVETELQGVCDTVLGLLD EAGDAESRVFYLMKMGDYRYLAEVATGDDKKRIIDSARSAYQEAMDISK KEMPP TNPIRLGLALNFSVFHYEIANSPEEAI SLAKTTTFDEAMADLHTLSEDSYK DSTLIMQL LRDNLTLWTGSSGEDLYFQSDNDG SEIGTGFPDPHYVEVLGERMHYVDV GPRD GTPVLFLHGNPTSSYVWRNIIPHVAP THRCIAPDLIGMGKSDKPDLYGFFD DHVRF MDAFIEALGLEEVVLVIHDWGSALG FHWAKRNP ERVKGIAFM EFIRPIPTWDEW PE FARETFQAFRTT DVGRKLIIDQNV FIEGTLP MGVVRPLTEV EMDHYREPFL NPVDR EPLWRF PNELPIAGEPA NIVALVEEYMD WLHQSPVPKLL FWGTPGVLIP PAEAARL AKSLPNCKAVD IGPGLNLLQED NPDLIGSEIAR WLSTLEISG
CRAF-NanoLuc	MEHIQGAWKTISNGFGFKDAVFDGSSCISPTIVQQFGYQRRASDDGKLTDP SKTS NTIRVFLPNKQRTVVNVNRNGMSLHDCMKALKVRGLQPECCAVFRLLHEH KGKKA RLDWNTDAASLIGEELQVDFLDHVPLTTHNFARKTFLKLAFCDICQKFL LNGFR CQ TCGYKFHEHCSTKVPTMCVDWSNIRQLLLFPNSTIGDSGVPALPSLT MRRM RESV SRMPVSSQHRYSTPHAFTFNTSSPSSEGSLSQRQRSTSTPNVHMVST LTPV DSR MIEDAIRSHSESASPSALSSSPNNLSPTGWSQPKTPVPAQRERAPVSGT QEKN KI RPRGQRDSSYYWEIEASEVMLSTRIGSGSFGTVYKKGWHGDVAVKIL KVVD PTPE QFQAFRNEVAVLRKTRHVNILLFMGYMTKDNLAIVTQWCEGSSLYKHL HVQET KF QMFQLIDIARQTAQGM DYLHAKNII HRDMKS NNIFLHE GLTVKIG DFGLATV KSRW SGSQQVEQPTGSVLWMAPEVIRMQDNNPFSFQSDVYSYGIVLYELMT GELPY SHI NNRDQIIFMVGRGYASPDLSKLYKNCPKAMKRLVADCVKVKEERPL FPQIL SSIEL LQHS LPKINRSASEPSLHRAAHTEDINACTLTTS PRLPVFG SSGVFT LEDVGD WR QTAGYNLDQVLEQGGVSSLFQNLGVS VTPIQRIV LSGENGL KIDIHVI IPYEGLS GD QMGQIEKIFKVVYPVDDHHFKVILHYGTLVIDGVTPNMIDYFGRPYEGIAVFDGKKIT VTGTLWNGNKIIDERLINPDGSLLFRV TINGVTGWRLCERILA
NanoLuc-TAZ	MVFTLEDFVGDWRQTAGYNLDQVLEQGGVSSLFQNLGVS VTPIQRIVLSGENGLKIDIHVIIPYEGLSGDQMGQIEKIFKVVYPVDDHHFKVILHYGTLVIDGVTPNMIDYFGR PYEGIAVFDGKKITVTGTLWNGNKIIDERLINPDGSLLFRV TINGVTGWRLCERILAGSSGMNPASAPPPPLPPPGQQVIHVTQDLTDLEALFNSVMNPKPSSWRKKILPESF FKEPDSGSHSRQSSTDSSGGHPGPRLAGGAQHVRSHSSPASLQLGTGAGAAGS PAQQHAHLRQQSYDVTDELPLPPGWEMTFTATGQRYFLNHIEKITTWQDPRKAM NQPLNHMNLHPAVSSTPVPQRSMAVSQPNLVMNHQHQQQMAPSTLSQQNHPT QNPPAGLMSMPNALTTQQQQQKLRQLRIQMERERIRMRQEELMRQEALCRQ LPMEAETLAPVQAAVNPPTMTPD MRSITNNSSDPFLNGGPYHSREQSTD SGLGLGCYSVPTTPEDFLSNVDEMDTGENAGQTPMNINPQQTRFPDFLDCLPGTNVDLG TLESEDLIPLFNDVESALNKSEPFLTWL
NES-NanoLuc-TAZ (NES-TAZ)	MLPPLERLTGSSGMVFTLEDFVGDWRQTAGYNLDQVLEQGGVSSLFQNLGVS VTPIQRIVLSGENGLKIDIHVIIPYEGLSGDQMGQIEKIFKVVYPVDDHHFKVILHYGTLVIDGVTPNMIDYFGRPYEGIAVFDGKKITVTGTLWNGNKIIDERLINPDGSLLFRVTI NGVTGWRLCERILAGSSGMNPASAPPPPLPPPGQQVIHVTQDLTDLEALFNSVMN PKPSSWRKKILPESFFKEPDSGSHSRQSSTDSSGGHPGPRLAGGAQHVRSHSSP ASLQLGTGAGAAGSPAQQHAHLRQQSYDVTDELPLPPGWEMTFTATGQRYFLN HIEKITTWQDPRKAMNQPLNHMNLHPAVSSTPVPQRSMAVSQPNLVMNHQHQQ QMAPSTLSQQNHPTQNPPAGLMSMPNALTTQQQQQKLRQLRIQMERERIRMR QEELMRQEALCRQLPMEAETLAPVQAAVNPPTMTPD MRSITNNSSDPFLNGGPYHSREQSTD SGLGLGCYSVPTTPEDFLSNVDEMDTGENAGQTPMNINPQQTRFP DFLDCLPGTNVDLGTLESEDLIPLFNDVESALNKSEPFLTWL

Construct	Amino Acid Sequence
NanoLuc-ERα	MVFTLEDFVGDWRQTAGYNLDQVLEQGGVSSLFQNLGVSVTPIQRIVLSGENGLK IDIHVIIPYEGLSGDQMGQIEKIFKVVPVDDHHFKVILHYGTLVIDGVTPNMIDYFGR PYEGIAVFDGKKITVTGTLWNGNKIIDERLINPDGSLLFRVTINGVTGWRLCERILAG SSGMTMTLHTKASGMALLHQIQGNELEPLNRPQLKIPLERPLGEVYLDSSKPAVYN YPEGAAYEFNAAAAANAQVYGQTGLPYGPGSEAAAFGSNGLGGFPPLNSVSPSP LMLLHPPPQLSPFLQPHGQQVPYYLENEPSGYTVREAGPPAFYRPNSDNRRQGG RERLASTNDKGSMAKESAKETRYCAVCNDYASGYHYGVWSCEGCKAFFKRSIQ GHNDYMCPATNQCTIDKNRRKSCQACRLRKCYEVGMMKGGIRKDRRGGRMLKH KRQRDDGEGRGEVGSAGDMRAANLWPSPLMIKRSKKNLALSALTADQMVSAALLD AEPPILYSEYDPTRPFSEASMMGLLTNLADRELVHMINWAKRVPGFVDLTLDHQQV HLLCAWLEILMIGLVWRSMHEHPGKLLFAPNLLLDNRNQGKCVEGMVEIFDMLLAT SRFRMMNLQGEEFVCLKSIILLNSGVYTFLLSSTLKSLEEKDHIHRVLDKITDTLIHLM AKAGLTLLQQQHQRLAQLLLILSHIRHMSNKGMEHLYSMKCKNVVPLYDLLLLLEMLDA HRLHAPTSRGGASVEETDQSHLATAGSTSSSHSLQKYYITGEAEGFPATV
NES-NanoLuc-ERα (NES-ERα)	MLPPLERLTGSSGMVFTLEDFVGDWRQTAGYNLDQVLEQGGVSSLFQNLGVSV TPIQRIVLSGENGLKIDIHVIIPYEGLSGDQMGQIEKIFKVVPVDDHHFKVILHYGTL VIDGVTPNMIDYFGRPYEGIAVFDGKKITVTGTLWNGNKIIDERLINPDGSLLFRVTI NGVTGWRLCERILAGSSGMTMTLHTKASGMALLHQIQGNELEPLNRPQLKIPLER PLGEVYLDSSKPAVYNYPEGAAYEFNAAAAANAQVYGQTGLPYGPGSEAAAFGS NGLGGFPPLNSVSPSPLMLLHPPPQLSPFLQPHGQQVPYYLENEPSGYTVREAG PPAFYRPNSDNRRQGGRRERLASTNDKGSMAKESAKETRYCAVCNDYASGYHYG VWSCEGCKAFFKRSIQGHNDYMCPATNQCTIDKNRRKSCQACRLRKCYEVGMM KGGIRKDRRGGRMLKHKRQRDDGEGRGEVGSAGDMRAANLWPSPLMIKRSKKN SLALSALTADQMVSAALLDAEPPILYSEYDPTRPFSEASMMGLLTNLADRELVHMINW AKRVPGFVDLTLDHQQVHLLCAWLEILMIGLVWRSMHEHPGKLLFAPNLLLDNRNQG KCVEGMVEIFDMLLATSSRFRMMNLQGEEFVCLKSIILLNSGVYTFLLSSTLKSLEEK DHIHRVLDKITDTLIHLMKAGLTLLQQQHQRLAQLLLILSHIRHMSNKGMEHLYSMK CKNVVPLYDLLLLLEMLDAHRLHAPTSRGGASVEETDQSHLATAGSTSSSHSLQKYYIT GEAEGFPATV
NanoLuc-ΔDBD-ERα (ΔDBD-ERα)	MVFTLEDFVGDWRQTAGYNLDQVLEQGGVSSLFQNLGVSVTPIQRIVLSGENGLK IDIHVIIPYEGLSGDQMGQIEKIFKVVPVDDHHFKVILHYGTLVIDGVTPNMIDYFGR PYEGIAVFDGKKITVTGTLWNGNKIIDERLINPDGSLLFRVTINGVTGWRLCERILAG SSGMKGGIRKDRRGGRMLKHKRQRDDGEGRGEVGSAGDMRAANLWPSPLMIK RSKKNLALSALTADQMVSAALLDAEPPILYSEYDPTRPFSEASMMGLLTNLADRELV HMINWAKRVPGFVDLTLDHQQVHLLCAWLEILMIGLVWRSMHEHPGKLLFAPNLLLD RNQGGKCVEGMVEIFDMLLATSSRFRMMNLQGEEFVCLKSIILLNSGVYTFLLSSTLKS SLEEKDHIHRVLDKITDTLIHLMKAGLTLLQQQHQRLAQLLLILSHIRHMSNKGMEH LYSMKCKNVVPLYDLLLLLEMLDAHRLHAPTSRGGASVEETDQSHLATAGSTSSSHSL QKYYITGEAEGFPATV

References

1. Arkin, M. R., Tang, Y. & Wells, J. A. Small-Molecule Inhibitors of Protein-Protein Interactions: Progressing toward the Reality. *Chem. Biol.* **21**, 1102–1114 (2014).
2. Ran, X. & Gestwicki, J. E. Inhibitors of Protein-Protein Interactions (PPIs): An Analysis of Scaffold Choices and Buried Surface Area. *Curr. Opin. Chem. Biol.* **44**, 75–86 (2018).
3. Andrei, S. A. *et al.* Stabilization of protein-protein interactions in drug discovery. *Expert Opin. Drug Discov.* **12**, 925–940 (2017).
4. Garlick, J. M. & Mapp, A. K. Selective Modulation of Dynamic Protein Complexes. *Cell Chem. Biol.* **27**, 986–997 (2020).
5. Schreiber, S. L. The Rise of Molecular Glues. *Cell* **184**, 3–9 (2021).
6. Dong, G., Ding, Y., He, S. & Sheng, C. Molecular Glues for Targeted Protein Degradation: From Serendipity to Rational Discovery. *J. Med. Chem.* **64**, 10606–10620 (2021).
7. Sasso, J. M. *et al.* Molecular Glues: The Adhesive Connecting Targeted Protein Degradation to the Clinic. *Biochemistry* **62**, 601–623 (2023).
8. Békés, M., Langley, D. R. & Crews, C. M. PROTAC targeted protein degraders: the past is prologue. *Nat. Rev. Drug Discov.* **21**, 181–200 (2022).
9. Chamberlain, P. P. & Hamann, L. G. Development of targeted protein degradation therapeutics. *Nat. Chem. Biol.* **15**, 937–944 (2019).
10. Soini, L., Leysen, S., Davis, J. & Ottmann, C. Molecular glues to stabilise protein-protein interactions. *Curr. Opin. Chem. Biol.* **69**, 102169 (2022).
11. Kenanova, D. N. *et al.* A Systematic Approach to the Discovery of Protein–Protein Interaction Stabilizers. *ACS Cent. Sci.* **9**, 937–946 (2023).
12. Sijbesma, E. *et al.* Site-Directed Fragment-Based Screening for the Discovery of Protein–Protein Interaction Stabilizers. *J. Am. Chem. Soc.* **141**, 3524–3531 (2019).

13. Sijbesma, E. *et al.* Structure-based evolution of a promiscuous inhibitor to a selective stabilizer of protein–protein interactions. *Nat. Commun.* **11**, 3954 (2020).
14. Toriki, E. S. *et al.* Rational Chemical Design of Molecular Glue Degraders. *ACS Cent. Sci.* **9**, 915–926 (2023).
15. King, E. A. *et al.* Chemoproteomics-enabled discovery of a covalent molecular glue degrader targeting NF- κ B. *Cell Chem. Biol.* **30**, 394–402.e9 (2023).
16. Mayor-Ruiz, C. *et al.* Rational discovery of molecular glue degraders via scalable chemical profiling. *Nat. Chem. Biol.* **16**, 1199–1207 (2020).
17. Hallenbeck, K. K. *et al.* A Liquid Chromatography/Mass Spectrometry Method for Screening Disulfide Tethering Fragments. *SLAS Discov. Adv. Sci. Drug Discov.* **23**, 183–192 (2018).
18. Konstantinidou, M. *et al.* Structure-Based Optimization of Covalent, Small-Molecule Stabilizers of the 14-3-3 σ /ER α Protein–Protein Interaction from Nonselective Fragments. *J. Am. Chem. Soc.* **145**, 20328–20343 (2023).
19. Obsilova, V. & Obsil, T. The 14-3-3 Proteins as Important Allosteric Regulators of Protein Kinases. *Int. J. Mol. Sci.* **21**, 8824 (2020).
20. Stevers, L. M. *et al.* Modulators of 14-3-3 Protein–Protein Interactions. *J. Med. Chem.* **61**, 3755–3778 (2018).
21. Obsil, T. & Obsilova, V. Structural basis of 14-3-3 protein functions. *Semin. Cell Dev. Biol.* **22**, 663–672 (2011).
22. Pennington, K. L., Chan, T. Y., Torres, M. P. & Andersen, J. L. 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 (2018).
23. Manschwetus, J. T. *et al.* Binding of the Human 14-3-3 Isoforms to Distinct Sites in the Leucine-Rich Repeat Kinase 2. *Front. Neurosci.* **14**, (2020).

24. Molzan, M. *et al.* Impaired Binding of 14-3-3 to C-RAF in Noonan Syndrome Suggests New Approaches in Diseases with Increased Ras Signaling. *Mol. Cell. Biol.* **30**, 4698–4711 (2010).
25. Sijbesma, E. *et al.* Identification of Two Secondary Ligand Binding Sites in 14-3-3 Proteins Using Fragment Screening. *Biochemistry* **56**, 3972–3982 (2017).
26. De Vries-van Leeuwen, I. J. *et al.* Interaction of 14-3-3 proteins with the estrogen receptor alpha F domain provides a drug target interface. *Proc. Natl. Acad. Sci. U. S. A.* **110**, 8894–8899 (2013).
27. Lavoie, H. & Therrien, M. Regulation of RAF protein kinases in ERK signalling. *Nat. Rev. Mol. Cell Biol.* **16**, 281–298 (2015).
28. Park, E. *et al.* Architecture of autoinhibited and active BRAF–MEK1–14-3-3 complexes. *Nature* **575**, 545–550 (2019).
29. Kondo, Y. *et al.* Cryo-EM structure of a dimeric B-Raf:14-3-3 complex reveals asymmetry in the active sites of B-Raf kinases. *Science* **366**, 109–115 (2019).
30. Martinez Fiesco, J. A., Durrant, D. E., Morrison, D. K. & Zhang, P. Structural insights into the BRAF monomer-to-dimer transition mediated by RAS binding. *Nat. Commun.* **13**, 486 (2022).
31. Sun, Q. & Wang, W. Structures of BRAF–MEK1–14-3-3 sheds light on drug discovery. *Signal Transduct. Target. Ther.* **4**, 1–2 (2019).
32. Park, E. *et al.* Cryo-EM structure of a RAS/RAF recruitment complex. *Nat. Commun.* **14**, 4580 (2023).
33. Freeman, A. K. & Morrison, D. K. 14-3-3 Proteins: Diverse Functions in Cell Proliferation and Cancer Progression. *Semin. Cell Dev. Biol.* **22**, 681–687 (2011).
34. Molzan, M. *et al.* Stabilization of Physical RAF/14-3-3 Interaction by Cotylenin A as Treatment Strategy for RAS Mutant Cancers. *ACS Chem. Biol.* **8**, 1869–1875 (2013).

35. Ballone, A., Centorrino, F. & Ottmann, C. 14-3-3: A Case Study in PPI Modulation. *Molecules* **23**, 1386 (2018).
36. Wolter, M. *et al.* Selectivity via Cooperativity: Preferential Stabilization of the p65/14-3-3 Interaction with Semisynthetic Natural Products. *J. Am. Chem. Soc.* **142**, 11772–11783 (2020).
37. Tanaka, K., Hatano, Y. & Ohkanda, J. Isoform-Selective Fluorescent Labeling of 14-3-3 σ by Acrylamide-Containing Fusicoccins. *Chem. – Eur. J.* **29**, e202301059 (2023).
38. Falcicchio, M. *et al.* Cooperative stabilisation of 14-3-3 σ protein–protein interactions via covalent protein modification. *Chem. Sci.* **12**, 12985–12992 (2021).
39. Richter, A., Rose, R., Hedberg, C., Waldmann, H. & Ottmann, C. An Optimised Small-Molecule Stabiliser of the 14-3-3–PMA2 Protein–Protein Interaction. *Chem. – Eur. J.* **18**, 6520–6527 (2012).
40. Visser, E. J. *et al.* From Tethered to Freestanding Stabilizers of 14-3-3 Protein-Protein Interactions through Fragment Linking. *Angew. Chem. Int. Ed Engl.* **62**, e202308004 (2023).
41. Wolter, M. *et al.* Fragment-Based Stabilizers of Protein–Protein Interactions through Imine-Based Tethering. *Angew. Chem. Int. Ed.* **59**, 21520–21524 (2020).
42. Cossar, P. J. *et al.* Reversible Covalent Imine-Tethering for Selective Stabilization of 14-3-3 Hub Protein Interactions. *J Am Chem Soc* **143**, 8454–8464 (2021).
43. Machleidt, T. *et al.* NanoBRET—A Novel BRET Platform for the Analysis of Protein–Protein Interactions. *ACS Chem. Biol.* **10**, 1797–1804 (2015).
44. Dale, N. C., Johnstone, E. K. M., White, C. W. & Pfleger, K. D. G. NanoBRET: The Bright Future of Proximity-Based Assays. *Front. Bioeng. Biotechnol.* **7**, (2019).
45. Robers, M. B. *et al.* Target engagement and drug residence time can be observed in living cells with BRET. *Nat. Commun.* **6**, 10091 (2015).

46. Cho, E. J. & Dalby, K. N. Luminescence Energy Transfer–Based Screening and Target Engagement Approaches for Chemical Biology and Drug Discovery. *SLAS Discov.* **26**, 984–994 (2021).
47. Wade, M., Méndez, J., Coussens, N. P., Arkin, M. R. & Glicksman, M. A. Inhibition of Protein-Protein Interactions: Cell-Based Assays. in *Assay Guidance Manual* (eds. Markossian, S. et al.) (Eli Lilly & Company and the National Center for Advancing Translational Sciences, Bethesda (MD), 2004).
48. Jiang, Z. *et al.* Adaptor-Specific Antibody Fragment Inhibitors for the Intracellular Modulation of p97 (VCP) Protein–Protein Interactions. *J. Am. Chem. Soc.* **144**, 13218–13225 (2022).
49. Massoud, T. F. & Paulmurugan, R. Chapter 47 - Molecular Imaging of Protein–Protein Interactions and Protein Folding. in *Molecular Imaging (Second Edition)* (eds. Ross, B. D. & Gambhir, S. S.) 897–928 (Academic Press, 2021). doi:10.1016/B978-0-12-816386-3.00071-5.
50. Nadel, C. M., Ran, X. & Gestwicki, J. E. Luminescence complementation assay for measurement of binding to protein C-termini in live cells. *Anal. Biochem.* **611**, 113947 (2020).
51. Dimri, S., Basu, S. & De, A. Use of BRET to Study Protein–Protein Interactions In Vitro and In Vivo. in *The Nuclear Receptor Superfamily: Methods and Protocols* (ed. McEwan, P., Iain J.) 57–78 (Springer, New York, NY, 2016). doi:10.1007/978-1-4939-3724-0_5.
52. Kocan, M. & Pfleger, K. D. G. Study of GPCR–Protein Interactions by BRET. in *Receptor Signal Transduction Protocols: Third Edition* (eds. Willars, G. B. & Challiss, R. A. J.) 357–371 (Humana Press, Totowa, NJ, 2011). doi:10.1007/978-1-61779-126-0_20.
53. Li, Z. *et al.* Determinants of 14-3-3 σ Protein Dimerization and Function in Drug and Radiation Resistance *. *J. Biol. Chem.* **288**, 31447–31457 (2013).

54. Xu, D., Farmer, A., Collett, G., Grishin, N. V. & Chook, Y. M. Sequence and structural analyses of nuclear export signals in the NESdb database. *Mol. Biol. Cell* **23**, 3677–3693 (2012).
55. NanoBRET™ Protein:Protein Interaction System Protocol.
<https://www.promega.com/resources/protocols/technical-manuals/101/nanobret-protein-protein-interaction-system-technical-manual/>.

Chapter 4

Scaffold-hopping for molecular glues for the 14-3-3/ER α complex

Abstract

Molecular glues, small molecules that bind cooperatively at a protein-protein interface, have emerged as powerful modalities for the modulation of protein-protein interactions (PPIs) and “undruggable” targets. The systematic identification of new chemical matter with a molecular glue mechanism of action remains a significant challenge in drug discovery. Here, we present a scaffold hopping approach, using as a starting point our previously developed molecular glues for the native 14-3-3/estrogen receptor alpha (ER α) complex. The novel, computationally designed scaffold is based on the Groebke-Blackburn-Bienaymé multi-component reaction (MCR), leading to drug-like analogs with multiple points of variation, thus enabling the rapid derivatization and optimization of the scaffold. Structure-activity relationships (SAR) are developed using orthogonal biophysical assays, such as intact mass spectrometry, TR-FRET and SPR. Rational structure-guided optimization is facilitated by multiple crystal structures of ternary complexes with the glues, 14-3-3 and phospho-peptides mimicking the highly disordered C-terminus of ER α . Cellular stabilization of 14-3-3/ER α for the most potent analogs is confirmed using a NanoBRET assay with full-length proteins in live cells. Our approach highlights the potential of MCR chemistry, combined with scaffold hopping, to drive the development and optimization of unprecedented molecular glue scaffolds.

Introduction

The stabilization of native protein-protein interactions (PPIs) with small molecules offers an attractive strategy for the activation or inhibition of signaling pathways in a therapeutic context.^{1,2} PPIs were traditionally considered difficult targets due to the lack of well-defined pockets and the presence of large, hydrophobic surfaces.^{3–5} The fundamental understanding of the mechanism of action of molecular glues – small molecules that bind cooperatively at PPI interfaces and strengthen weak, pre-existing interactions – has enabled the stabilization of PPIs by taking into account the elements of cooperativity, molecular recognition and shape complementarity.^{6,7}

A particularly challenging class of PPIs includes proteins that are intrinsically disordered and only become partially structured when bound to a protein partner, such as a chaperone binding to a client protein.^{8,9} 14-3-3 is an abundant scaffolding protein that recognizes specific phospho-serine or phospho-threonine motifs on disordered domains of the client and upon binding creates a structured binding interface.¹⁰ Of note, 14-3-3 proteins lack function – the function is instead encoded on the client protein and in particular on the phospho-site that is being recognized, leading either to activation or inhibition of signaling pathways.¹¹

Among the extensive interactome of 14-3-3, here we focus on its native interaction with the hormone regulated transcription factor estrogen receptor α (ER α). 14-3-3 recognizes the protein sequence surrounding phospho-T594 on the disordered C-terminus on the F-domain of ER α and acts as a negative regulator by inhibiting ER α binding to chromatin and blocking ER α -mediated transcription.^{12,13} To date, ER α small molecule drugs, acting either as inhibitors or degraders, target the adjacent ligand binding domain (LBD).^{14–17} However, mutations in the LBD are often associated with acquired endocrine resistance.¹⁸ Thus, stabilization of the native 14-3-3/ER α PPI could be useful as an alternative strategy to block ER α transcriptional activity in ER α positive breast cancer, especially in cases of acquired endocrine resistance. The feasibility of this approach, targeting the F-domain, is corroborated by studies using the natural product fusicoccin-

A (FC-A) and its semi-synthetic analogs that stabilize the interaction between 14-3-3 and the C-terminus of ER α .¹⁹ Drug-like chemical probes and leads are now required to define the biological impact of targeting the F-domain to inhibit ER α in hormone-positive breast cancer.

We have applied different strategies for the identification of chemical matter to stabilize the 14-3-3/ER α complex. We used a site-directed fragment-based technology, termed “disulfide tethering” with intact mass spectrometry as the readout to identify reversible fragments bound at the native cysteine (C38) of 14-3-3 σ in the presence of a phosphorylated peptide that represented the disordered C-terminus of ER α .²⁰ Rational, structure-guided optimization of the reversible disulfide fragments led to irreversibly covalent, selective molecular glues that bound at the composite surface of 14-3-3 σ /ER α .²¹ For the development of non-covalent molecular glues, we used a fragment-linking approach, derived from the crystal structures of two diverse fragments that were identified in crystallographic and disulfide tethering screens.²² Thus, the 14-3-3/ER α PPI has served as a valuable system to test diverse molecular-glue discovery strategies.

Here, we present a scaffold-hopping approach based on multi-component reaction chemistry (MCR). Multi-component reactions are defined as synthetic approaches where at least three starting materials react in a single step to form a complex scaffold, where basically all or most of the atoms contribute to the newly formed product. This broad definition covers reactions with various synthetic mechanisms.^{23,24} MCR chemistry, due to its highly divergent character, is an enticing strategy for developing new scaffolds and rapid structure-activity relationships (SAR), as it allows the combination of short synthetic routes with high diversity and complexity. MCR has emerged as an attractive alternative to multistep linear convergent synthetic approaches and has been successfully applied to the synthesis of active pharmaceutical ingredients (API).^{25–30} Here, we describe our strategy for the development of a drug-like MCR scaffold stabilizing the 14-3-3 σ /ER α complex. The most potent analogs of the series showed efficacy in orthogonal biophysical assays and cell-based PPI stabilization in the low micromolar range.

Results

Structure activity relationships (SAR)

Structurally, 14-3-3 binds ER α by recognizing phospho-T594, the penultimate residue on the C-terminus of ER α . This creates a large, open, solvent-exposed pocket that can accommodate a small molecule. Although steric factors are not an issue for targeting the composite surface of the 14-3-3/ER α complex, we found it was important to rigidify an initially flexible scaffold to maximize the stabilization effect.²¹ Our aim in this work was to design a scaffold that would be more rigid from the beginning, locking in a favorable three-dimensional shape complementary to the large pocket.

To this end, we used the freely accessible software AnchorQueryTM, which performs pharmacophore-based screening of approximately 31 million compounds that are readily synthesizable through one-step multi-component reaction (MCR) chemistry.^{31,32} Although AnchorQueryTM was originally developed for PPI inhibitors³³, in this case it was successful in proposing MCR scaffolds for PPI stabilizers. AnchorQueryTM requires a ligand-bound crystal structure or docked binding pose as a starting point. We used the crystal structure of the previously disclosed compound **127** (PDB 8ALW) that was bound at the composite surface of the 14-3-3 σ /ER α complex, with a favorable ligand conformation, based on our biophysical data.²¹ The compound formed multiple favorable interactions both with 14-3-3 σ and the phospho-ER α peptide (Fig 4.1a). In the co-crystal structure, the irreversible chloroacetamide warhead of compound **127** formed a covalent bond with C38 of 14-3-3 σ . The *p*-chloro-phenyl ring occupied a small hydrophobic pocket that formed a halogen bond with K122 of 14-3-3. The tetrahydropyran ring adopted a favorable conformation that allowed the formation of hydrophobic interactions with 14-3-3 residues (L218, I219), the terminal Val595 of ER α , and a water-mediated hydrogen bond from the oxygen atom in the ring, which was part of a large water network. The overall ligand conformation also led to a key water-mediated hydrogen bond between the aniline nitrogen and the terminal carboxylic acid of Val595 of ER α , significantly

contributing to molecular recognition. In this compound series, the introduction of large non-aromatic rings, such as the tetrahydropyrane, combined with aniline rings were necessary to limit the multiple ligand conformations and improved the affinity to the complex.

Understanding the binding mode of **127**, we were able to use AnchorQueryTM for a scaffold-hopping approach. The software contains a virtual library; to query the library, the user inputs two feature sets that derive from the binding mode of the initial ligand (compound **127**, in this case). The first feature is an anchor motif on the ligand that is bioisosteric with an amino acid residue. A suitable anchor in our case is the *p*-chloro-phenyl ring that is deeply buried at the PPI interface in the small pocket surrounding K122 of 14-3-3 (Fig. S4.1). This motif serves as the “phenylalanine anchor”, which is kept constant for all pharmacophore-based searches of the database. The second feature set consists of three additional pharmacophore points on different parts of the ligand; the three-point pharmacophore could include all possible ligand-protein interactions, such as hydrogen bond donors or acceptors, aromatic rings, hydrophobic residues or charged ions. Testing different combinations of pharmacophore points allows greater chemical space coverage and the rapid optimization of ligand-protein interactions that might be sub-optimal in the original ligand. We applied an additional filter, limiting the molecular weight of the hits to 400 Da. AnchorQuery then screens a 31M+ member library (2B conformers) from 27 MCR reactions, covering a large chemical space of different MCR scaffolds (from typical Ugi reactions to heterocyclic scaffolds, such as tetrazoles, thiophenes, imidazo[1,2-*a*]pyridines, pyrroles and so on).

Based on the selected pharmacophore points, the software provided a list of potential docking hits from all possible scaffolds ranked according to energy minimization or RMSD fit. Since we were aiming for hits with similar 3D shape to compound **127**, we used the RMSD fit for hit ranking. We screened all 27 MCR reactions, but interestingly all our top hits were based on the Groebke-Blackburn-Bienaymé (GBB) three-component reaction (GBB-3CR).^{34–36} The GBB-3CR utilized aldehydes, 2-aminopyridines and isocyanides, leading to imidazo[1,2-

a]pyridines.^{37,38} This privileged scaffold has been present in several clinical candidates and marketed drugs, such as zolpidem, miroprofen, minodronic acid, and olprinone.³⁹

Comparison of the docking pose of the proposed GBB compounds with the co-crystal structure of compound **127** revealed a considerable shape complementarity and an almost identical 3D shape of the two scaffolds (Fig 4.1b). Additionally, the GBB scaffold was drug-like and more rigid compared to our original ligand, potentially restricting the possible ligand conformations. After selecting the main GBB scaffold we performed more rounds of docking and selection of suitable substituents to achieve favorable ligand – protein interactions with the docking software SeeSAR [version 14.0.0; BioSolveIT GmbH, Sankt Augustin, Germany, 2024, www.biosolveit.de/SeeSAR]. For molecular glues, the goal of structure-guided design was to incorporate the optimal ligand interactions in the composite surface, thus simultaneously improving the interactions with 14-3-3 and ER α . Although MCR chemistry allowed combinatorial synthesis, where all building blocks could be varied for each compound simultaneously, from our previous experience with molecular glues²¹ we were aware that small changes in even one substituent could have a significant effect on cooperative binding and this could be difficult to predict. Thus, we decided to vary one building block at a time and keep the rest of the ligand constant to elucidate the SAR step-by-step.

Once compounds were selected for synthesis, synthetic routes were established using commercially available aldehydes and 2-aminopyridines, whereas isocyanides were synthesized from primary aromatic amines (Fig 4.1c-1d). The selection of building blocks was based on SeeSAR docking, aiming to achieve specific ligand-protein interactions, as explained in detail later. Two general, three-step synthetic routes were developed based on the type of aldehyde building blocks used. In the first synthetic route (route A), Boc-protected aldehydes reacted with 2-aminopyridines and isocyanides in methanol, using scandium triflate as a Lewis acid catalyst to form the GBB intermediate. The Boc-protecting group was removed under acidic conditions and the chloroacetamide warhead was introduced with an amidation reaction, either

with chloroacetyl chloride or an amide coupling. In the second synthetic route (route B), to reduce the cost of certain Boc-protected aldehydes, nitro-substituted aromatic aldehydes were used instead. The GBB-3CR was performed under the same experimental conditions, followed by a nitro-reduction. The nitro-group was reduced either using iron trichloride and zinc or using ammoniotrihydroborate and gold catalysis with Au/TiO₂.⁴⁰ The two reduction methods led to comparable, almost quantitative yields. The last step, as previously, was the introduction of the electrophilic warhead. Thus, the GBB-3CR allowed multiple combinations of the three main building blocks, facilitating the synthesis of analogs. Synthetic details are provided in the Supplementary Information.

Since ER α is phosphorylated on the penultimate residue, the binding interface to 14-3-3, creates a large, solvent-exposed pocket that can accommodate a small molecule. In general, 14-3-3 molecular glues are expected to stabilize 14-3-3/client complexes based on molecular recognition and shape complementarity. For the 14-3-3/ER α complex, the only accessible contact with ER α is the C-terminal V595, which is located close to the small pocket formed around K122 of 14-3-3, as shown for **127** (Fig. 4.1a). Thus, for the MCR scaffold, our first modifications focused on the K122 pocket/V595 composite interface. To test whether this scaffold was suitable for the development of 14-3-3 σ /ER α stabilizers we synthesized a small set of derivatives varying only the isocyanide position, which according to our design was expected to interact with K122 of 14-3-3. We included two types of isocyanides: benzyl and phenyl isocyanides with a diverse substitution pattern on the aryl ring. The size of the pocket allowed the presence of aromatic rings; since surrounding 14-3-3 amino acids were primarily hydrophobic, we chose appropriate hydrophobic substituents for SAR exploration. We maintained the covalent chloroacetamide warhead, based on our extensive investigation of electrophiles in our previous work.²¹

The compounds were tested in an intact mass spectrometry (MS) assay, which monitored the formation of the covalent bond with C38 of 14-3-3 σ . Binding measurements were

made in the presence or absence of the phospho-ER α peptide and as a function of time to distinguish between cooperative stabilizers and neutral binders and to select compounds with fast binding kinetics. The phospho-ER α peptide was used at a concentration 2-fold above the dissociation constant (K_d). The assay was performed as a compound titration (Fig. S4.2-3). We additionally quantified compound binding at 1 μ M (10:1 compound:protein) over several time points (Table S4.1). Throughout this work, we reference the time-course data as bar graphs, using grey bars to represent binding to 14-3-3 σ alone (“apo”) and colored bars to represent binding to 14-3-3 σ /ER α complex.

Testing the first analogs in the MS assay revealed striking differences among benzyl and phenyl analogs (Fig 4.1e, Fig. S4.4, Table S4.1). The benzyl analog **1**, which lacked aryl ring substitutions, was a neutral binder (for example, in the 8hr measurement, at 1 μ M compound we observed 35.7% binding for apo 14-3-3 and 55.7% binding to 14-3-3 in the presence of ER α). Introduction of electron withdrawing groups, such as halogens or nitro-groups in the *o*-, *m*- or *p*-position (compounds **2-9**) reduced binding in the presence of ER α , even in the case of a small F-substitution. The introduction of halogens and their effect in the different positions was based on previous knowledge; during the optimization of our first series of molecular glues targeting the 14-3-3 σ /ER α , the *p*-Cl substituent was beneficial for cooperative binding.²¹ The fact that the SAR did not translate directly between the two series implied potential differences in the binding mode. Interestingly, the non-substituted phenyl analog **10** showed increased binding to 14-3-3 σ , but still lacked cooperativity, since it showed increased binding in the presence or absence of ER α (in the 8hr measurement, at 1 μ M compound we observed 56.3% apo binding and 68.9% binding in the presence of ER α). Introduction of halogens in the *p*-position significantly reduced apo binding, improving compound cooperativity (compounds **11** and **12**). For example, for **11** in the 8hr measurement, at 1 μ M compound binding to 14-3-3 alone was 3.4% and binding in the presence of ER α rose to 24.3%. However, in contrast to the SAR observed in our previous series²¹, less binding was observed for the *p*-Cl analog (**11**), compared to the non-substituted

analog (**10**). Remarkably, a Me-group in the *o*-position (**13**) led to the first molecular glue of the series; compound **13** showed binding in the presence of ER α and very low apo binding (in the 8hr measurement, at 1 μ M compound apo binding was 1.4% and binding in the presence of ER α 42.1%). Bigger substitutions in the *o*-position significantly decreased the observed binding, indicating unfavorable steric effects (**14-16**) (Fig. S4.4).

We solved co-crystal structures of the 14-3-3 σ /ER α complex with compounds **1**, **2** and **10** to elucidate their binding modes (Fig 4.1f-i). The crystal structure of compound **1** revealed a comparable binding mode to the original ligand **127**, supporting the design and docking hypothesis (Fig 4.1f-g). The chloroacetamide moiety of **1** bound covalently to C38 of 14-3-3 σ , and two water-mediated hydrogen bonds were formed between the carbonyl group and R41 and the backbone of E115, and one direct hydrogen bond with N42 of 14-3-3 at 3Å. The imidazopyridine ring was positioned towards the hydrophobic residues (L218, I219, G171 and L222) at the “roof” of the 14-3-3 pocket. In addition, the nitrogen of the five-membered ring formed a hydrogen bond with D215 of 14-3-3, which adopted two conformations upon binding of compound **1**. Importantly, a large water network was formed between the benzylamine of **1** to N42, S45 of 14-3-3, which reached the terminal carboxylic acid of V595 and the pT594 of ER α , and K122 of 14-3-3. The benzyl ring, adjacent to the electrophilic warhead was positioned between the hydrophobic 14-3-3 residues F119 and I168, (Fig 4.1g). The structural overlay of analogs **1** and **2** showed an identical binding mode with the additional *o*-F substituent forming a halogen bond with I219 of 14-3-3 (Fig 4.1h). Replacing the benzyl ring (**1**) with a phenyl ring (**10**) resulted in a slight turn of the compound that placed the aryl rings of both analogs in the same position in the pocket surrounding K122. The water network that connected the amine of compound **1** to residues of 14-3-3 and ER α was conserved upon modification of the benzyl ring (**1**) to a phenyl ring (**10**). The removal of the methylene group positioned the aniline nitrogen of (**10**) closer the terminal carboxylic acid of ER α , in the position previously occupied by the methylene group of (**1**). Additionally, it led to increased axial rotation of the bond between the

nitrogen and the aryl ring, which might have resulted in the increased apo binding in the MS assay, since this axial rotation was not possible for the benzyl analogs (Fig 4.1i).

Since the *o*-Me substitution correlated with improved binding in the presence of ER α in the MS assay and taking into account the rotational nature of the aniline – phenyl ring bond, we designed and synthesized a series of analogs with double *o*-substitutions (Fig 4.2a, Fig. S4.5). The symmetrical 2,6-di-Me analog **17** showed significantly faster binding in the presence of ER α and almost no apo binding, indicating molecular-glue-like binding. Analog **18** with an *o*-Et group in addition to *o*-Me, was weaker, indicating unfavorable steric effects (Fig. S4.5). Analogs **19**, **20**, and **21** with additional *o*-OMe, *o*-F and *o*-Cl groups respectively, showed similar, rapid binding to the symmetric analog **17** and low apo binding. In agreement with our previous observation for the effect of *p*-substitutions (compounds **11** (*p*-Cl) and **12** (*p*-Br)), the introduction of additional substitutions in the *p*-position (-Cl, -Me, -OH) significantly reduced binding (compounds **22-25**), especially for the triple substituted analogs (double *o*- and *p*-positions).

Co-crystal structures of the 14-3-3 σ /ER α complex with compounds **17**, **19**, **20**, **21**, and **25** revealed differences in the positions of the double-*ortho* substituents (Fig 4.2b-e). In the case of the symmetric analog **17** one *o*-Me group was oriented in the back of the pocket, which was primarily hydrophobic, forming hydrophobic interactions with I219 (3.2Å) and facing Val595 of ER α . The second *o*-Me group was oriented in the front of the pocket and formed hydrophobic interactions with F119 (3.8 Å). The formation of these interactions seemed to favorably restrict the axial rotation of the aniline-phenyl bond, locking its position in a highly complementary shape with the composite 14-3-3/ER α surface. In analog **19** the larger *o*-OMe group was positioned in the front of the pocket and formed, together with the aniline nitrogen, a water-mediated hydrogen bond with the terminal carboxylic acid of Val595 of ER α . The orientation of the warhead amide differed in two analogs, but in both cases a direct hydrogen bond with N42 of 14-3-3 was formed (3.0 Å). For the halogen-containing analogs *o*-F (**20**) and *o*-Cl (**21**) the

halogens interacted with I219 and the common *o*-Me group with F119, whereas the aniline nitrogen interacted with the terminal carboxylic acid of Val595 of ER α via a water mediated-hydrogen bond. Our NMR data for these compounds do not imply restricted axial rotation. Our assumption is that most of these analogs could be classified as class-1 atropoisomers,⁴¹ which are commonly treated as achiral. From a chemical perspective, the rotational bond in these cases can sample the full 360 degrees of rotational conformations about the axis. From a biological perspective, they typically bind to a given target in only a subset of these conformations, as observed for analogs **17**, **19**, **20**, and **21**, with analogs **19** and **21** adapting the two extreme rotational positions to form favorable interactions with 14-3-3, as demonstrated by crystallography. The binding mode of the triple substituted analog **25**, although overall comparable with the double substituted analog **17**, showed an additional hydrogen bond between the *p*-OH group and the backbone carbonyl group of I168 (3.2 Å). The presence of this direct interaction seemed to negatively affect the axial rotation of the aniline-phenyl bond, resulting in significantly reduced binding in the MS assay and loss of cooperativity.

Notably, the alignment of the co-crystal structures of compounds **17** and **25** (Fig 4.2e) was almost identical, including the orientation of the common electrophilic warhead. The different interactions based on the compounds' substitution pattern on the *p*-position dramatically affected the observed binding in the mass spec assay (Fig 4.2a). Even though the warhead orientation was the same, compound **17** was a fast, cooperative binder in the MS assay, whereas **25** was barely bound. We therefore concluded that the speed of binding was due to cooperativity and shape complementarity, rather than binding solely driven by the electrophilic warhead.

We synthesized analogs of compound **21**, maintaining the 2-Cl,6-Me substitutions and varied positions X and Y on the scaffold (Fig 4.3a, Fig. S4.6). Position X referred to substitutions on the imidazopyridine ring and was expected to contribute to additional hydrophobic interactions with Val595 of ER α . Position Y included modifications that were expected to

improve interactions with the rim of the 14-3-3 pocket, primarily with variations of the aldehyde building blocks and to a smaller extent with different electrophilic warheads. Although modifications in position Y were quite far from the ER α terminal valine, they could result in altered ligand conformations and additional interactions with 14-3-3. In both cases, even the introduction of small groups led to surprising effects in the binding modes, as revealed by crystal structures.

For position X, aiming for hydrophobic interactions with Val595 of ER α , we introduced groups with a range of radii (-F (**26**), -Cl (**27**), -Me (**28**), -*i*-Pr (**29**), -CF₃ (**30**)). Steric effects seemed to play a bigger role than electronic effects, since the best tolerated substitutions were -Cl (**27**) and -Me (**28**). For position Y, derived from the aldehyde building blocks, the *o*-F analog (**31**) was tolerated, but was weaker compared to the unsubstituted (**21**); a plausible explanation being the rotational nature of the aryl ring–imidazopyridine ring bond, which lost axial symmetry with the presence of the *o*-F group. Introduction of a methylene linker (**32**) or replacement of the aryl ring with a cyclohexyl ring (**33**) that had a similar size made the compounds almost unable to bind in the MS assay. Modifications in the position of the electrophilic warhead were also not tolerated. The less-reactive ester analog (**34**) was inactive, whereas halogenated chloroacetamides (**35-37**) did not lead to the expected mass adducts, showing instability in the MS and unclear chemical reactivity with 14-3-3. A plausible explanation was their increased reactivity, which correlated with reduced stability. The sulfamate warhead (**38**) was tolerated in the MS assay, but was significantly weaker than the chloroacetamide analog, in addition to being less atom efficient.

Crystal structures were solved for **28**, **32**, and **33** and were compared as overlays with analog **21** (Fig 4.3b-e). The -Me group on the imidazopyridine ring of **28** was expected to contribute to hydrophobic interactions with Val595 of ER α . However, it disrupted the previously observed binding mode and instead moved the imidazopyridine ring upwards, in the hydrophobic pocket of 14-3-3 formed by L218, I219 and L222. This upward movement affected

the conformation of L218, which moved upwards but maintained contact with **28**. The movement in the pocket also altered the interactions of the double-*ortho*-substituted aryl ring; while the *o*-Cl still interacted with I219 on the roof of the binding site, the *o*-Me group moved further away from F119 in the bottom of the pocket, and the interaction was lost. The direct hydrogen bond between the amide and N42 and the water-mediated bond between the aniline nitrogen and the carboxylic acid of Val595 were maintained. Overall, the changes in binding mode were associated with slower binding, but a similar apparent K_d in the MS assay (Fig. S4.3). Analog **32**, with an additional methylene group on the electrophilic warhead linker showed a slightly disrupted binding mode. While the biggest difference was the position of the aryl ring next to the longer linker, the imidazopyridine ring also moved further away from ER α ; the latter correlated negatively with the binding observed in the MS assay. In contrast to the previous analogs, the *o*-Cl group on the aryl ring of **32** pointed out of the 14-3-3 pocket and due to increased distance, was unable to interact with F119. Thus, the presence of an additional methylene group on the linker was sufficient to make the compound unable to stabilize the complex. Analog **33** bearing a cyclohexyl ring instead of an aromatic ring maintained the interaction between the *o*-Cl group of the aryl ring and Ile219, but not the interaction of the *o*-Me group with F119. The orientation of the amide bond next to the warhead also differed; however, the hydrogen bond with N42 was still formed. The presence of the cyclohexyl ring, which had the possibility of adopting more conformations compared to the more rigid aryl ring, correlated with reduced binding to the 14-3-3/ER α complex in the MS assay.

Investigation of modifications in positions X and Y provided valuable input in the groups that were tolerated; however, these single-point substitutions did not significantly improve the stabilization effect in the MS assay. Based on the crystallographic input, we synthesized four analogs combining the favorable substitutions from positions X and Y with the symmetric double *o*-Me analog **17**, hypothesizing that a symmetric rotatable bond would correlate with improved potency (Fig 4.4a). This hypothesis was readily confirmed by evaluating the symmetric analogs

39-42 in the MS assay. Analogs **39** and **40** included the -Me group on the imidazopyridine ring (X position) and the -F group on aryl ring (referred to as W position), respectively. Analogs **41** and **42** both had the F group in W position and a -Me or -Cl group in X position, respectively. All analogs showed comparable, fast, cooperative binding to 14-3-3/ER α in the MS assay, indicating that the symmetry of the 2,6-di-Me-aniline ring was more favorable compared to the asymmetric 2-Cl,6-Me. All four analogs showed low apo binding with analog **41** showing the lowest.

Crystal structures were solved for the symmetric analogs **40**, **41** and **42**, highlighting the significance of two rotational bonds in the scaffold: the aniline-aryl ring bond, as previously discussed and the aryl ring-imidazopyridine ring bond (Fig 4.4b-c). The *o*-F substituent in W position, adjacent to the aryl ring-imidazopyridine rotational bond, adopted two different orientations; an inward orientation for analog **40** and an outward orientation for **41** and **42**, which each had an additional substituent on the imidazopyridine ring. With the inward conformation of analog **40**, the *o*-F substituent interacted with P167 of 14-3-3, whereas for **41** and **42** the *o*-F group was oriented to make multiple stabilizing interactions, being approximately 3 Å from N42 of 14-3-3, and the *o*-Me substituent and aniline nitrogen of the compound itself, thus contributing both to ligand-protein interactions and intramolecular interactions, restricting the axial bond rotation. In agreement with previous observations, the presence of a substituent in the X position (**41** and **42**) led to an upward movement of the imidazopyridine ring, orienting the substituent toward the hydrophobic residues I219 and L222. Overall, the presence of the additional substituent on the imidazopyridine ring in the last two analogs, even though in a distant position compared to the *o*-F-substituted ring, correlated favorably with the restriction of the rotational bond on the F-aryl ring. Additionally, since the 14-3-3 σ /ER α composite surface was primarily hydrophobic, the optimized analog **41**, with the additional hydrophobic substituents, achieved favorable shape complementarity (Fig. S4.7). For analogs **41** and **42**, the different substituents of the imidazopyridine ring, -Me (**41**) or -Cl (**42**), did not affect the position

of the stabilizer in the crystal structures; however, in the MS assay the Me group showed lower apo binding to 14-3-3, resulting in higher cooperativity of the ternary complex.

TR-FRET

Up to this point, the SAR was developed using the intact MS assay and supported by crystallography. In our previous work²⁰⁻²², we relied on a fluorescence anisotropy assay (FA) to confirm cooperativity. The FA assay typically used (5-carboxylfluorescein) FAM-labeled phospho-peptides to quantify peptide binding to the compound/14-3-3 complex. For the MCR scaffold, however, this assay proved to be unsuitable, since the extensively conjugated ring system was intrinsically fluorescent in the same wavelengths as the FAM-labeled peptide (480 nm and 520 nm). To circumvent this issue, we turned our attention to far-red fluorescent dyes. The cy5-labeled ER α -peptide with excitation wavelength of 651 nm and emission wavelength of 670 nm, significantly affected the K_d of the 14-3-3/ER α complex. The reported K_d using the acetylated ER α peptide in an isothermal calorimetry (ITC) experiment or the FAM-labeled-ER α in an FA assay is in the range of 1-2 μ M.²¹ The cy5-ER α -peptide, however, resulted in a K_d of 6 nM, indicating significant dye binding (Fig. S4.8).

As a suitable alternative to the FA assay, we developed a time-resolved fluorescence energy transfer (TR-FRET) assay⁴², using HIS-tagged-full-length 14-3-3 σ , biotin-labeled-ER α peptide, an anti-HIS-tag monoclonal antibody conjugated with a Tb(III) cryptate as the donor and streptavidin conjugated with the D2 dye as the acceptor. The observed K_d for this system was 30 nM. To ensure that the observed difference in K_d from ITC or the FAM-labeled-ER α in an FA assay was not related to the biotin-tag on the peptide, we performed a competition assay with the FAM-labeled peptide. The K_d of the biotin-peptide was 2.5 μ M, in good agreement with the FAM-peptide's K_d (Fig. S4.8). This result suggested that the lower K_d in the TR-FRET assay was due to avidity⁴³; since 14-3-3 and the antibody were both dimers and streptavidin was

tetravalent, different multivalent complexes could form, resulting in differences in the observed binding affinity between the labeled and the unlabeled proteins.

We performed assay optimization with 2D-titrations of 14-3-3, biotin-ER α and donor/acceptor ratios. We then tested the synthesized compounds using the optimized experimental conditions: 200 nM 14-3-3 (top concentration, 2-fold dilution), 50 nM biotin-ER α , 0.166 nM MAb anti-6HIS Tb and 6.25 nM SA-D2. The compounds were tested at 100 μ M and DMSO was used as negative control. 14-3-3 was titrated using an Echo acoustic dispenser, followed by the addition of the compounds and the peptide; after 1h incubation at room temperature, the donor and acceptor were added. In contrast to the MS assay, maximum signal was obtained after 2h rather than 16h – a plausible explanation again being avidity. The tight K_d in the TR-FRET assay resulted in a small assay window and a hook effect was observed at higher protein concentrations (ca. 50 – 100 nM 14-3-3).

Nevertheless, the TR-FRET assay was sensitive to the addition of molecular glues. In addition to quantifying fold stabilization for the 14-3-3/ER α complex ($AppKd_{(compound)}/AppKd_{(DMSO)}$), we also quantified the fold increase in the TR-FRET signal (the ratio of observed E_{max} and E_{min}). To validate the TR-FRET assay, we included two positive controls: the natural product **FC-A** and our previously described stabilizer compound **181**²¹. The two compounds gave comparable results. **FC-A** had an $AppKd_{(compound)}$ of 11 nM, fold-stabilization of 3.09, and fold-increase of 5.52. Compound **181** had an $AppKd_{(compound)}$ of 8.6 nM, fold-stabilization of 3.95 and fold-increase of 5.52 (Fig. S4.9, Table S4.2). In good agreement with the MS data, neutral binders **1** and **10** showed only a small signal shift compared to the DMSO control (Fig 4.4d, Fig. S4.9). The 2-Me analog **13** showed 4.19-fold-stabilization and 2.36-fold increase, whereas 2,6-di-Me analog **17** showed 4.78-fold-stabilization and 2.58-fold increase, the same rank order as the MS assay. The more sterically hindered analog **18** (o-Me, o-Et) showed weaker stabilization (3.06-fold-stabilization and 2.14-fold increase). Analogs **19**, **20**, and **21**, all bearing the o-Me group and additional o-OMe, o-F and o-Cl groups respectively,

showed comparable stabilization (4.41 – 5.23-fold stabilization and 2.1 – 2.48-fold increase). The highest stabilization effect was observed for the final symmetric analogs **39** – **42**. Analog **39** with a -Me group in X position had an $AppKd_{(compound)}$ of 7.8 nM, fold-stabilization of 4.35 and fold-increase of 2.61, whereas **40** with the -F group in W position had an $AppKd_{(compound)}$ of 8.4 nM, fold-stabilization of 4.04 and fold-increase of 3.12. Analog **41**, which had both the -Me group in X position and the -F group in the W position had the lowest $AppKd_{(compound)}$ (5.2 nM) and the highest fold-stabilization (6.53) and fold-increase (3.71) of the series. Analog **42**, which differed only in the X position (-Cl instead of -Me group) appeared weaker ($AppKd_{(compound)}$ 8.8 nM, fold-stabilization of 3.87 and fold-increase of 2.97). In summary, while the fold-changes were dampened by avidity, 14-3-3/ER α molecular glues showed the same rank-order in the TR-FRET assay as in the mass spectrometry assay used for initial SAR.

SPR

Surface Plasmon Resonance (SPR) was then used to analyze the kinetic parameters of the ER α peptide binding to 14-3-3 σ in the presence of compounds **181**, **17** and **41**, and to compare the $AppKd_{(compound)}$ and kinetics to the binary ER α /14-3-3 σ interaction (Fig 4.4e, Fig. S4.10 - 11, Table S4.3). Here, 14-3-3 σ tagged with a Twinstrep-tag was captured on a SPR chip coated with Strep-Tactin XT, after which a 2-fold dilution series of acetylated ER α phosphopeptide was injected. For the binary interaction, the fast dissociation rate (k_{off}) reached the limit of detection of the SPR instrument, due to a relative weak interaction with a K_d of 1.1 μ M, which was in line with the ITC and FA experiments. The covalent bond between the chloroacetamide warhead of the compounds and C38 of 14-3-3 σ was formed after overnight incubation in the presence of ER α . Immobilization of this complex on the chip, followed by extensive washing to remove the bound ER α peptide, allowed us to determine the kinetics of ER α binding to the 14-3-3 σ /stabilizer complex. The previously described stabilizer **181** decreased the off-rate to 0.016 s⁻¹ and simultaneously increased the association rate (k_{on}) by a factor of 16, resulting in a low

nanomolar affinity constant (3.9 nM; stabilization = 282-fold). The analogs of the newly designed scaffold, **17** and **41**, both showed an 8-fold increase in association rate compared to the binary interaction. The binding of compound **41** induced a stronger decrease in dissociation rate of ER α compared to **17**, which resulted in K_d values of 10.1 nM and 15.1 nM for **41** and **17**, respectively. The higher stabilizing potency of **41** compared to **17** (stabilization = 110-fold for **41** and 71-fold for **17**) were in rank-order agreement with the TR-FRET data. The decreased dissociation rates in the presence of the stabilizers, especially for **41** and **181**, increased the residence time of ER α binding to 14-3-3 from approximately 3.4 s to 47.6 s and 62.5s, respectively.

NanoBRET

To test the effects of the compounds on the full-length PPI, we used a NanoLuciferase bioluminescence resonance energy transfer (NanoBRET) assay we developed previously⁴⁴. Compounds were tested using a C-terminal fusion 14-3-3 σ -HaloTag and full length, N-terminal fusion NanoLuc-ER α (Fig 4.4f-g; Table S4.4). Briefly, NanoLuc-ER α and 14-3-3 σ -HaloTag plasmids were transfected in 1:10 ratio in hormone deprived HEK293T cells. After 48 hours post transfection, cells were seeded in assay plates with the experimental wells treated with 100 nM HaloTag NanoBRET 618 ligand (Promega) and no-ligand control wells treated with DMSO (v/v). Cells were treated for 24 hours with compounds in 1:2 dilution series starting at 40 μ M. The BRET signal was read and normalized against DMSO treated samples. All active compounds resulted in an increase in BRET signal compared to the negative control, **85**. The previously described compound **181**²¹ stabilized the 14-3-3 σ /ER α complex in cells with an EC₅₀ value of 5.2 μ M and a 1.7-fold increase in the BRET signal, which was higher than the observed BRET signal with the natural product FC-A, tested at 30 μ M. Of the MCR compounds tested, the neutral binder **10** was less effective than **181** (EC₅₀ value > 100 μ M, 1.6-fold increase). Compound **13** showed an improved EC₅₀ and fold-increase compared to **10** (12 μ M and 1.8-fold). Compound **17** had the

lowest EC₅₀ value of 2.7 μ M and showed a 1.8-fold increase in BRET signal, a slight improvement compared to **181**. Compounds **40** and **42** had the same 2-fold-increases in BRET signal, though exhibited slightly different EC₅₀ values (7.2 and 4.6 μ M, respectively). Compound **41** resulted in the highest increase in BRET signal, a 2.6-fold increase; however, it had a similar EC₅₀ value to **181** of 5 μ M. In the NanoBRET assay, compounds **17** and **41** performed most effectively, based on the EC₅₀ value (**17**) and fold-increase in BRET signal (**41**) of the panel of compounds tested. The majority of MCR stabilizers showed similar EC₅₀ values to the previous scaffold represented by **181** but consistently showed improved fold-increase in BRET signal.

Compounds **181**, **17**, and **41**, along with the negative control, **85**, were tested in a NanoBRET assay where the cysteine of interest in 14-3-3 σ C38, was mutated to an asparagine, 14-3-3 σ ^{C38N}-HaloTag (Fig. S4.12a). The BRET signal did not increase for **85**, **181**, or **17** with increasing compound concentration. There was a minimal increase in BRET signal for compound **41**, from 2.5 to 3.5 mBU at 20 μ M **41**. In comparison to the assays done with 14-3-3 σ ^{WT}-HaloTag, the non-normalized BRET signal increased from 6.7 to 16.1 mBU at the same concentration of **41** (data shown as normalized). The natural product FC-A was also tested as a positive control; however, since the compound is non-covalent, the effect was not specific to the 14-3-3 sigma isoform and an increase in BRET signal was observed for both constructs (Fig. S4.12b). In summary, when C38 was not present in 14-3-3 σ , the covalent compounds were unable to bind and stabilize the full-length 14-3-3 σ /ER α complex in cells (Fig. S4.12).

Discussion

Using MCR chemistry, we demonstrated the potential of a scaffold-hopping approach for molecular glues stabilizing a native 14-3-3/client PPI. Our approach combined computational de novo design with multi-component reaction chemistry, which included short, efficient synthetic routes with multiple points of variation, accelerating the synthesis of analogs. Thus, two distinct optimization approaches led to two highly diverse chemical series, converging on similar

efficacies as 14-3-3s/ER α molecular glues. It is noteworthy that the natural product FC-A stabilizes the 14-3-3/ER α complex to a similar extent.²¹ Since ER α terminates at the FC-A pocket, only the C-terminal valine residue is available for binding; it may be that these three distinct series have maximized the binding energy available for gluing this protein-protein interaction.

In the MCR approach, structure-activity relationships were established with an intact mass spectrometry assay, which allowed the distinction between neutral binders and stabilizers – cooperative molecular glues. Overall, the SAR showed that the introduction of even small substituents in the case of molecular glues could profoundly affect their potency and cooperativity. Crystal structures of ternary complexes were crucial in elucidating small changes in the binding modes of otherwise highly similar analogs. Starting from the weak neutral binder **1** and by removing one methylene group, we significantly increased binding to the complex for compound **10**. Introduction of substituents in the *o*-position was sufficient to reduce apo binding and turn the compounds from neutral binders to cooperative molecular glues. Although the number of rotational bonds is generally kept to a minimum, in this case we took advantage of two rotational bonds in the scaffold and with appropriate substituents achieved favorable ligand conformations resulting in increased potency, as shown for analogs **17** and **41**. Notably, most of the synthesized analogs contained the same electrophilic warhead but varied significantly in their binding and stabilization potential. Thus, we were able to demonstrate that binding was not solely driven by the covalency, but rather that specific, favorable interactions of the ligands in the PPI composite surface contributed to molecular recognition and shape complementarity. Additionally, since the 14-3-3/ER α interface was largely hydrophobic, most of the favorable ligand modifications were non-polar, indicating that the overall shape complementarity was a key factor in improving stabilization of the complex.

Several biophysical assays provided complementary strategies for evaluating these novel molecular glues. A TR-FRET assay circumvented the issue of scaffold fluorescence,

which could have been a limiting factor in establishing SAR and allowed the rank-ordering of analogs. A new SPR assay, in which the covalent ligand was pre-associated with 14-3-3s before immobilization, provided an insightful analysis of binding/unbinding kinetics and started to hint at differences between the two series of molecular glues. Taken together, biophysical assays allowed the quantification of cooperativity (MS, TR-FRET) and kinetics (SPR); importantly, the observed SAR was consistent among these three assays. The NanoBRET assay further showed good correlation with the biophysical protein/phosphopeptide assays while demonstrating stabilizing of the full-length proteins in intact cells.

Overall, we showed that the compounds that were binding the fastest and at the lowest concentrations in the presence of the peptide in the mass spectrometry assay also showed the largest stabilization by TR-FRET and NanoBRET. Stabilization was further validated and explained by 100+-fold reductions in K_d for the protein-peptide interaction, driven mostly by reduced off-rates. Thus, the observed ranking and SAR of the compounds translated across different assay formats. Thorough characterization and validation of molecular glue scaffolds in orthogonal assays was beneficial for rational scaffold optimization. Structural studies indicated minor changes in the shapes of the molecules but large changes in stabilization that correlated closely with improved interactions with the protein and shape complementarity. Taken together, the optimized, cell-active MCR scaffold will facilitate chemical biology approaches to study the 14-3-3/ $\text{ER}\alpha$ interaction, which has so far been unexploited for drug discovery. We also propose that the scaffold-hopping strategy will be broadly applicable in the rapidly evolving field of molecular glues, where we are just beginning to explore 'glue-like' chemical space.

Methods

Protein expression and purification

The 14-3-3 σ isoform (full-length for mass spectrometry and TR-FRET assays, ΔC for crystallography) with an N-terminal His6 tag was expressed in RosettaTM 2(DE3)pLysS

competent E.coli (Novagen) from a pPROEX HTb expression vector. After transformation following manufacturer's instructions, single colonies were picked to inoculate 30 mL precultures (LB), which were added to 1.5 L terrific broth (TB) medium after overnight growth at 37°C, 250 rpm. Expression was induced upon reaching OD₆₀₀ 1.9–2.1 by adding 400 µM IPTG. After overnight expression at 30°C, 150 rpm, cells were harvested by centrifugation at 6,500 rpm, resuspended in lysis buffer (50 mM HEPES pH 7.5, 500 mM NaCl, 20 mM imidazole, 10% glycerol, 1 mM TCEP), and lysed by sonication. The His6-tagged protein was purified by Ni-affinity chromatography (Ni-NTA Agarose, Invitrogen) (Wash buffer 50 mM HEPES pH 7.5, 500 mM NaCl, 20 mM imidazole, 1 mM TCEP; Elution buffer 50 mM HEPES pH 7.5, 500 mM NaCl, 500 mM imidazole, 1 mM TCEP) and analyzed for purity by SDS-PAGE and Q-ToF LC/MS. The protein was buffer exchanged (Storage buffer 25 mM HEPES pH 7.5, 150 mM NaCl, 1 mM TCEP) and concentrated to ~16 mg/mL and aliquots flash-frozen for storage at –80°C. The ΔC variant was truncated at the C-terminus after T231 to enhance crystallization and after the first Ni-affinity chromatography column, the construct was treated with TEV protease to cleave off the His6 tag during dialysis (25 mM HEPES, pH 7.5, 200 mM NaCl, 5% glycerol, 10 mM MgCl₂, 250 µM TCEP) overnight at 4 °C. The flow-through of a second Ni-affinity column was subjected to a final purification step by size exclusion chromatography (Superdex 75 pg 16/60 size exclusion column (GE Life Science) (SEC buffer: 25 mM HEPES pH 7.5, 100 mM NaCl, 10 mM MgCl₂, 250 µM TCEP). The protein was concentrated to ~60 mg/mL, analyzed for purity by SDS-PAGE and Q-ToF LC/MS and aliquots flash-frozen for storage at -80 °C.

Peptides

Peptides for mass spectrometry, fluorescence anisotropy and TR-FRET assays were purchased from Elim Biopharmaceuticals, Inc. (Hayward, CA). Peptides for SPR and X-ray crystallography were purchased from GenScript Biotech Corp. The following peptides were used:

Ac-KYYITGEAEGFPA{pT}V-COOH (MS assay, 15-mer),

5-FAM-AEGFPA{pT}V-COOH (FA assay, 8mer ER α -pp),
cy5-KYYITGEAEGFPA{pT}V-COOH (FA assay, 15-mer),
biotin-KYYITGEAEGFPA{pT}V-COOH (TR-FRET assay, 15-mer),
Ac-EGFPA{pT}V-COOH (crystallography and SPR, 7-mer)

Intact mass spectrometry assay

Mass spectrometry dose response assays were performed on a Waters Acquity UPLC/ Xevo G2-XS Q-ToF mass spectrometer. A Waters UPLC Protein BEH-C4 Column (300 Å, 1.7 μ m, 2.1 mm x 50 mm) was used to desalt the samples prior to application on the mass spectrometer. For 19-point MS dose responses, 50 mM compound stocks in DMSO were serially diluted in 3-fold increment in a master plate, then 1000 nl of the compounds were transferred to the assay plates. Master mixes containing 100 nM full-length wild type 14-3-3 σ in the absence or presence of 2 μ M ER α were then dispensed into 384 well plates (Greiner Bio-One, catalog number 784201). Assay buffer was TRIS (10 mM, pH 8.0) and final volume per well was 50 μ l, with final top concentration of compounds dose response series at 1 mM. The reaction mixtures were incubated for 1h at rt before subjected to MS. Four measurements (1h, 8h, 16h, 24h) were performed for time-course experiments. The injection volume for each sample was 6 μ l. 24 μ l of sample were needed for the time-course experiments, so the total volume in the assay plate was adjusted to 50 μ l, to account for the dead volume in the injections. Data collection and automated processing followed a custom workflow, as previously described.⁴⁵ Z Plots were created using GraphPad Prism with the log(agonist) vs. response (variable slope, four parameters) fitting model.

K_D Determination for FAM-, cy5- and Biotin-labeled ER α peptides

For K_D determination, N-terminal fluorescein-labeled ER α peptide (5-FAM) or cy5-labeled ER α peptide and HIS-tag FL 14-3-3 σ were diluted in buffer (10mM HEPES pH 7.5, 150mM NaCl,

0.05% tween 20, 0.05% BGG (bovine gamma globulin)). Two-fold dilution series of 14-3-3 were made in black, round-bottom 384-microwell plates (Greiner Bio-one 784900) in a final sample volume of 10 μ L in triplicates. FAM- or cy5-labeled ER α peptides (final assay concentration 10nM) were dissolved in assay buffer and mixed with the protein dilution series on the plates. Fluorescence anisotropy measurements were performed after 1h incubation at room temperature on an Envision HTS Dual Detector 2105 plate reader (for FAM-labeled ER α peptide: filter set lex: 480, lem: 535, and D505fp/D535 advanced dual mirror). For cy5- labeled ER α peptide filter set lex: 620, lem: 688 nm, and D658fp/D688 advanced dual mirror). Data were reported at endpoint. Prism 10 (GraphPad) was used to generate plots using the [agonist] vs response (variable slope, four parameters) fitting model to determine the K_D values.

For K_D determination of the biotin-labeled ER α peptide a competition assay was performed. 5-FAM- and biotin-labeled ER α peptide were diluted in buffer (10mM HEPES pH 7.5, 150mM NaCl, 0.05% tween 20, 0.05% BGG (bovine gamma globulin)). Two-fold dilution series of biotin-labeled ER α peptide were made in black, round-bottom 384-microwell plates (Greiner Bio-one 784900) in a final sample volume of 10 μ L in triplicates. A mastermix of 14-3-3 σ and 5-FAM-ER α was dispensed on the assay plate (final assay concentrations: 6 μ M 14-3-3 σ (IC₈₀) and 10 nM 5-FAM-ER α). Fluorescence anisotropy measurements were performed after 1h incubation at room temperature using a Molecular Devices ID5 plate reader (filter set lex: 485 $\pm$ 20 nm, lem: 535 $\pm$ 25 nm; integration time: 50 ms; settle time: 0 ms; shake 5 sec, medium, read height 3.00 mm, G-factor = 1). Data were reported at endpoint. Prism 10 (GraphPad) was used to generate plots using the [agonist] vs. response (variable slope, four parameters) fitting model to determine K_D values. The obtained K_D value was corrected using the Cheng-Prusoff equation.

$$K_d = IC_{50} / (1 + [S] / K_m)$$

$$K_d = 6.9 / (1 + 50 \text{ nM} / 30 \text{ nM}) = 2.5 \text{ } \mu\text{M}$$

TR-FRET protein titrations

For assay optimization, 2D titrations of biotin-labeled ER α peptide, HIS-tag FL 14-3-3 σ and streptavidin-D2 were performed in assay buffer (10mM HEPES pH 7.5, 150mM NaCl, 0.05% tween 20, 0.05% BGG (bovine gamma globulin)). The donor (MAb anti-6HIS Tb cryptate gold, (Revvity, 5000 tests #61HI2TLA)) concentration was kept constant at 0.166 nM. For TR-FRET protein titrations, biotin-labeled ER α peptide (50 nM), the compounds or DMSO (100 μ M), MAb anti-6HIS Tb cryptate gold (0.166 nM) and streptavidin-D2 (6.25 nM) were mixed in assay buffer (10mM HEPES pH 7.5, 150mM NaCl, 0.05% tween 20, 0.05% BGG (bovine gamma globulin)). 2-fold serial dilutions of HIS-tag FL 14-3-3 σ were performed (200 nM top assay concentration, 12-point dilution series). The assay was performed in 384-well microplates (Corning 4513, low volume white) at a volume of 10 μ l per well. The following procedure was used: The compounds (50 mM stocks in DMSO) were transferred to echo LDV masterplates. 20 nL were transferred from the masterplate to the assay plate to achieve 100 μ M compound concentration in the assay using Echo acoustic dispensing. The biotin-labeled ER α peptide was dissolved in assay buffer (10mM HEPES pH 7.5, 150mM NaCl, 0.05% tween 20, 0.05% BGG (bovine gamma globulin)) and dispensed in the assay plate using Dragonfly. 14-3-3 dilution series were prepared using Echo acoustic dispensing. Assay plates were incubated for 1hr at room temperature before the addition of a mastermix containing the donor (MAb anti-6HIS Tb cryptate gold) and acceptor (streptavidin-D2) in assay buffer. The mastermix was dispensed with Dragonfly. Assay plates were incubated for 2hr at room temperature prior to TR-FRET measurements using the Envision HTS Dual Detector 2105 plate reader equipped with the TR-FRET filter set (320/615/665 nm) and a D407/D630 advanced dual mirror. A 50 μ s delay was employed to reduce background fluorescence. The TR-FRET signal was obtained through calculating the ratio of 665 nm to 615 nm fluorescence ($\times 1000$), and Prism 10 (GraphPad) was used to generate plots using the [agonist] vs. response (variable slope, four parameters) fitting model. For each compound three independent experiments were performed (n=3).

SPR

The SPR experiments were performed at 25°C using a Biacore X100 (Cytiva) and a 200 nm Strep-Tactin XT derivatized linear polycarboxylate hydrogel chip, medium charge density (XanTec Bioanalytics). All proteins and peptides were dissolved in fresh running buffer prepared with ultrapure water and filtered through a 0.2 µm filter (10 mM HEPES pH 7.4, 200 mM NaCl, 50 µM EDTA, 0.005% P20). First the surface was conditioned with a 1 min injection of 3 M Guanidine HCl. Then, the recombinant 14-3-3σ-Twinstrep protein (250 nM) was captured on flow cell 2 of the sensor chip at a flow rate of 10 µL/min for 2 minutes, which resulted in a capture level of 1000 RU. For the ternary interaction, 14-3-3σ-Twinstrep protein (250 nM) was first incubated overnight with 1 µM Ac-ERα peptide and 20 µM compound prior to immobilization to the chip (similar flow rate and injection time used as for apo 14-3-3). The bound ERα peptide was washed away using running buffer flowed over the chip for 15 min at flow rate of 30 µL/min. Flow cell 1 was left blank as a reference surface. After immobilization of the protein, the Biacore X100 was primed with running buffer. Multi-cycle kinetic measurements were conducted at a flow rate of 30 µL/min. A 2-fold dilution series of analyte (Ac-ERα peptide) in running buffer were injected over the sensor chip for 2 min, followed by dissociation of 3 min (binary interaction), 7 or 13 minutes (ternary interaction) until full dissociation of peptide. For the binary interaction, the highest concentration of ERα was 50 µM, and for the ternary interactions this was 250 nM. Between cycles of one multi-cycle measurement, no regeneration step was performed due to complete dissociation of the analyte. After a measurement, the chip was regenerated by 2 times 30 sec injections at flow rate 10 µL/min of 3 M Guanidine HCl. The data collection rate of the instrument is 1 datapoint/sec. The data was corrected by double subtracting to the reference surface (flow cell 1) and buffer injection (blank) and analyzed using 1:1 interaction fitting model with the BIA evaluation software (2020). All parameters were fitted using global fitting, including the Rmax, because a single immobilization was used for all analyte concentrations. The RI was set constant at 0 RU. Next to kinetic fitting, the affinity fit was made using a 1:1 binding model

by plotting the response (RU) at steady state over the analyte concentration (Fig. S4.11). This was not possible for the data with compound 181 since steady state was not reached for all analyte concentrations.

X-ray crystallography data collection and refinement

The 14-3-3 $\sigma\Delta$ C protein, acetylated ER α and compounds (50 mM stock in DMSO) were dissolved in complexation buffer (25 mM HEPES pH=7.5, 2 mM MgCl₂ and 100 μ M TCEP) and mixed in a 1:2:3 or 1:2:5 molecular stoichiometry (protein : peptide: compound) with a final protein concentration of 12 mg/mL. The complex was set-up for sitting-drop crystallization after overnight incubation at 4 °C, in a custom crystallization liquor (0.05 M HEPES (pH 7.1, 7.3, 7.5, 7.7), 0.19 M CaCl₂, 24-29% PEG400, and 5% (v/v) glycerol). Crystals grew within 10-14 days at 4 °C. Crystals were fished and flash-cooled in liquid nitrogen. X-ray diffraction (XRD) data were collected at the European Synchrotron Radiation Facility (ESRF Grenoble, France, beamline ID23-1, ID30A-3/MASSIF-3, or ID23-2) or at the Deutsches Elektronen-Synchrotron (DESY Hamburg, Germany, beamline PETRA III). Data was processed using CCP4i2 suite (version 8.0.019). After indexing and integrating the data, scaling was done using AIMLESS. The data was phased with MolRep, using PDB 4JC3 as template. The presence of co-crystallized ligands was verified by visual inspection of the Fo-Fc and 2Fo-Fc electron density maps in COOT (version 0.9.8.93). If electron density corresponding to the co-crystallized ligand was present, its structure, restraints, and covalent bond were generated using AceDRG. After building in the ligand, model rebuilding and refinement was performed using REFMAC5. The PDB REDO server (pdb-redo.edu) was used to complete the model building and refinement. The images were created using the PyMOL Molecular Graphics System (Schrödinger LLC, version 4.6.0).

NanoBRET

NanoBRET assays were performed as previously described.⁴⁴ HEK293T cells were cultured DMEM, high glucose (Gibco) supplemented with 10% charcoal stripped Fetal Bovine Serum (FBS; Gibco) and 1% penicillin/streptomycin. Cells were transfected with a 1:10 ratio of Nanoluc-ERa:14-3-3σ-HaloTag plasmid for 48 hours using jetOPTIMUS transfection reagent (Polyplus). Cells were then seeded at 8,000 cells per well in a 384-well plate (Corning #3570) in FluoroBrite DMEM (phenol red-free; Gibco) with 4% charcoal stripped FBS and treated with 100 nM HaloTag NanoBRET 618 Ligand (Promega) or equivalent volume of DMSO as a no ligand negative control. Following plating, cells were treated for 24 hours with compound in 1:2 dilution series starting at 40 µM (0.35% DMSO final concentration). After 24 hours, the BRET signal was read using an EnVision XCite 2105 plate reader at 618 nm (HaloTag) and 460 nm (Nluc). The final corrected NanoBRET ratio was calculated using the following equation:

$$\text{Corrected BRET ratio} = \left(\frac{618nm}{460nm} \right)_{\text{HaloTag Ligand}} - \left(\frac{618nm}{460nm} \right)_{\text{No ligand control}}$$

The BRET ratios were normalized to samples treated with DMSO.

Docking

Computational design for SAR optimization and docking was performed with SeeSAR version 14.0.0; BioSolveIT GmbH, Sankt Augustin, Germany, 2022, www.biosolveit.de/SeeSAR

Figures

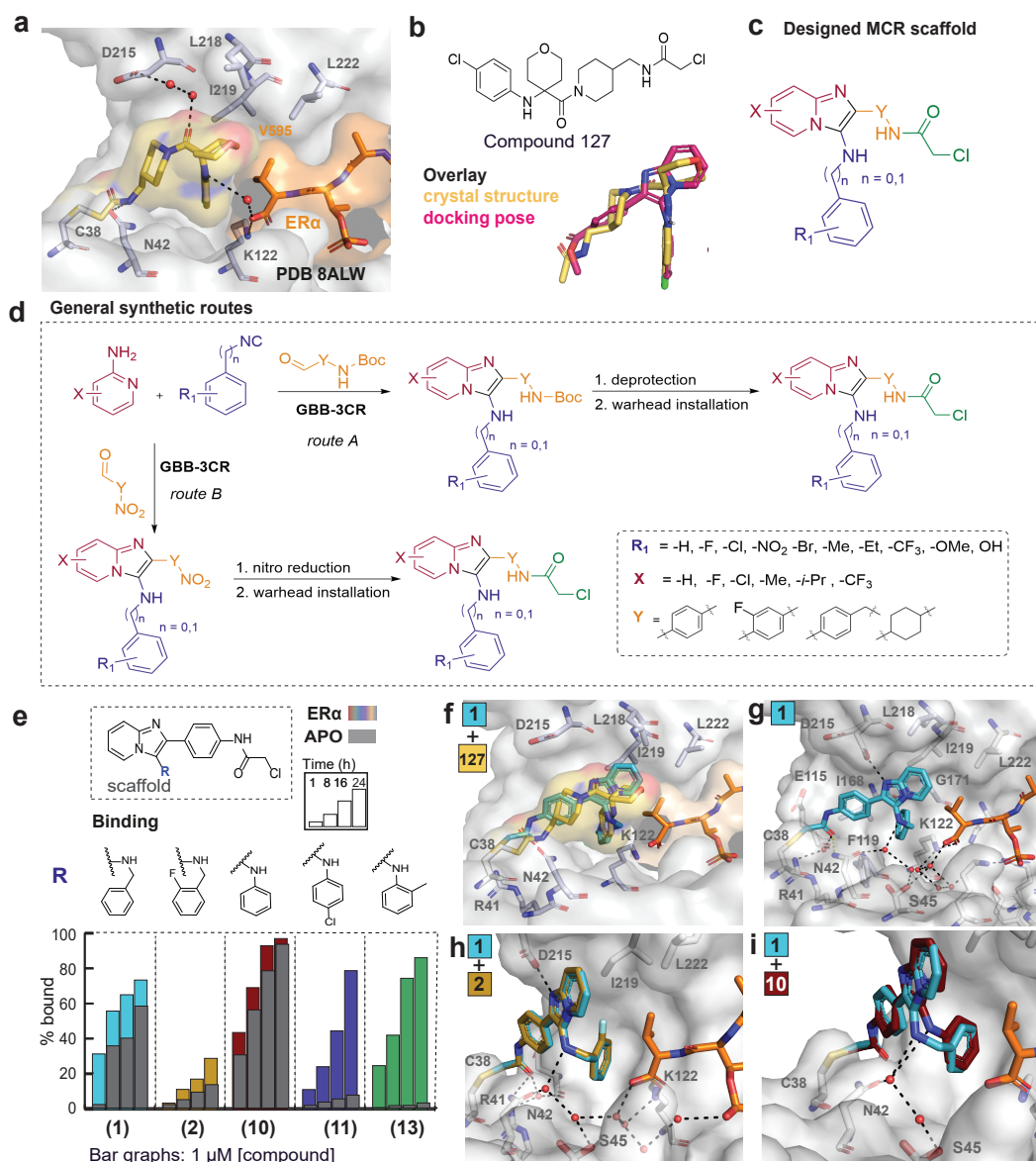


Figure 4.1: Overview of the scaffold hopping approach

SAR and crystal structures of selected benzyl and aniline analogs. **a)** Crystal structure of compound **127** (yellow sticks) with 14-3-3 σ (grey surface) and phospho-ER α peptide (orange sticks). Interacting amino acids are shown as sticks and water molecules as red spheres (PDB 8ALW). **b)** Chemical structure of compound **127** and ligand overlay of compound **127** and docking pose of the new MCR scaffold. **c)** General multi-component reaction scaffold (MCR) based on the Groebke-Blackburn-Bienaymé (GBB) reaction and main points of variation. **d)** Overview of general synthetic routes. Detailed experimental conditions are described in the SI. **e)** Mass Spectrometry (MS) bar graphs at 1 μ M. For each compound, time course experiments were performed with measurements at 1h, 8h, 16h and 24h. ER α data are shown with different colors for each compound, and apo data in grey. **f)** Crystal structure overlay for compounds **1** (cyan sticks) (PDB 9I6Y) and **127** (yellow sticks) (PDB 8ALW) bound to 14-3-3 σ (grey surface) /ER α (orange sticks). **g)** Crystal structure of compound **1** (cyan sticks) (PDB 9I6Y) with 14-3-3 σ /ER α . Interacting amino acid residues are shown as sticks and interacting water molecules as red spheres. **h-i)** Structural overlays of compounds **2** (brown sticks) (PDB 9I6Z), and **10** (dark red sticks) (PDB 9I72) with compound **1** (cyan sticks) (PDB 9I6Y).

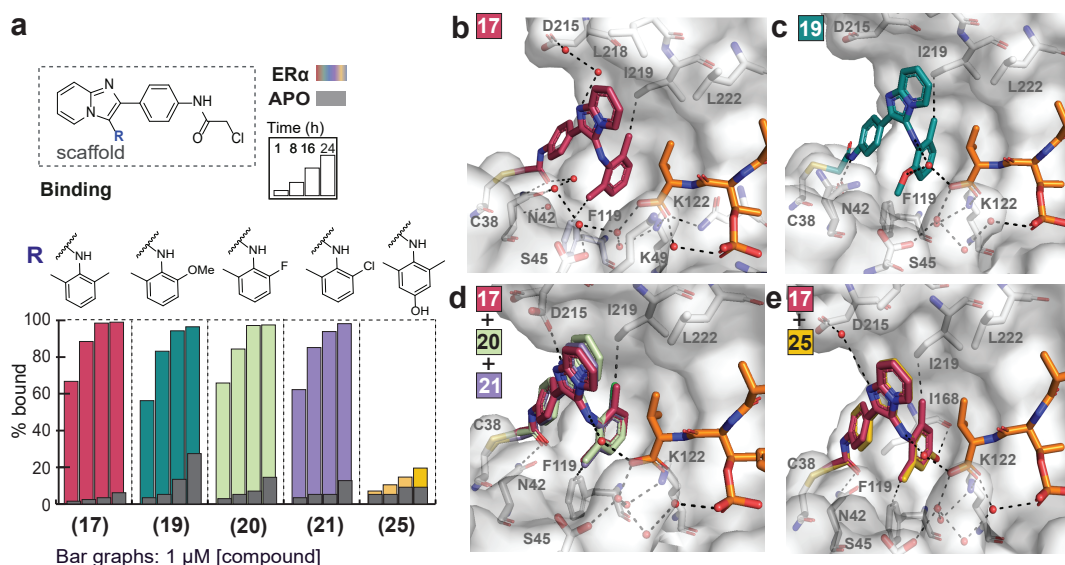


Figure 4.2: SAR and crystal structures of selected double-*ortho* substituted analogs

a) MS bar graphs at 1 μ M. For each compound, time course experiments were performed with measurements at 1h, 8h, 16h and 24h. ER α data are shown with different colors for each compound, and apo data in grey. **b-e)** Crystal structures of 14-3-3 σ /ER α with compounds **17** (dark pink sticks) (PDB 9I70), **19** (teal sticks) (PDB 9I71) and overlays of crystal structures for compounds **17** (dark pink sticks) (PDB 9I70), **20** (pale green sticks) (PDB 9I73), **21** (pale purple sticks) (PDB 9I74) and **25** (bright yellow sticks) (PDB 9I75).

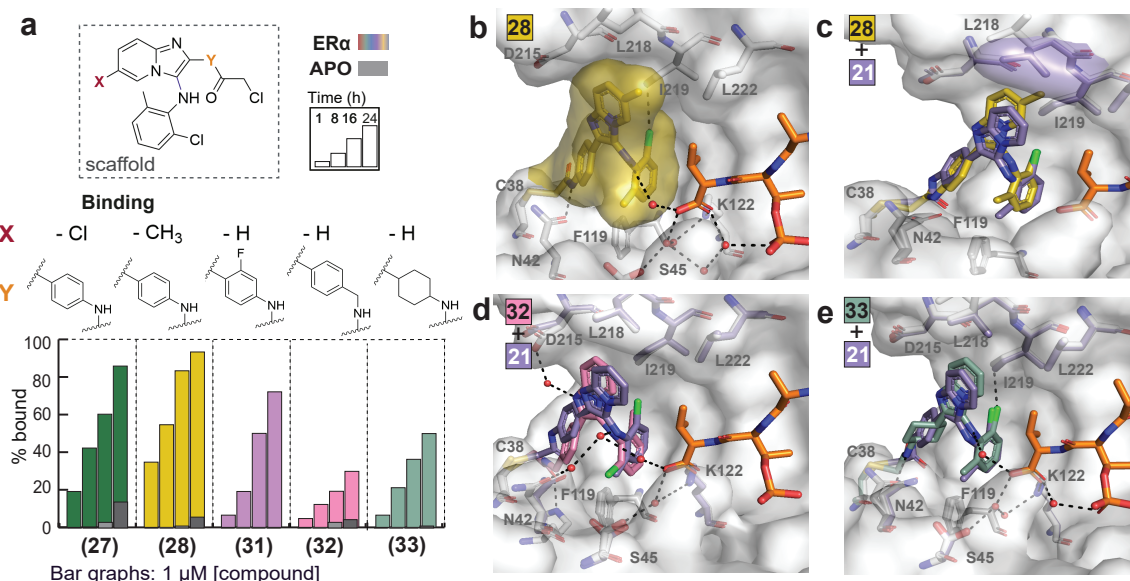


Figure 4.3: SAR and crystal structures of analogs substituted in positions X and Y

a) MS bar graphs at 1 μ M. For each compound, time course experiments were performed with measurements at 1h, 8h, 16h and 24h. ER α data are shown with different colors for each compound, and apo data in grey. **b-e)** Crystal structure of 14-3-3 σ /ER α with compound **28** (dark yellow sticks) (PDB 9I6S), and overlays of crystal structures for compounds **28** (dark yellow sticks) (PDB 9I6S), **32** (pink sticks) (PDB 9I6T) and **33** (emerald green sticks) (PDB 9I6U) with **21** (pale purple sticks) (PDB 9I74).

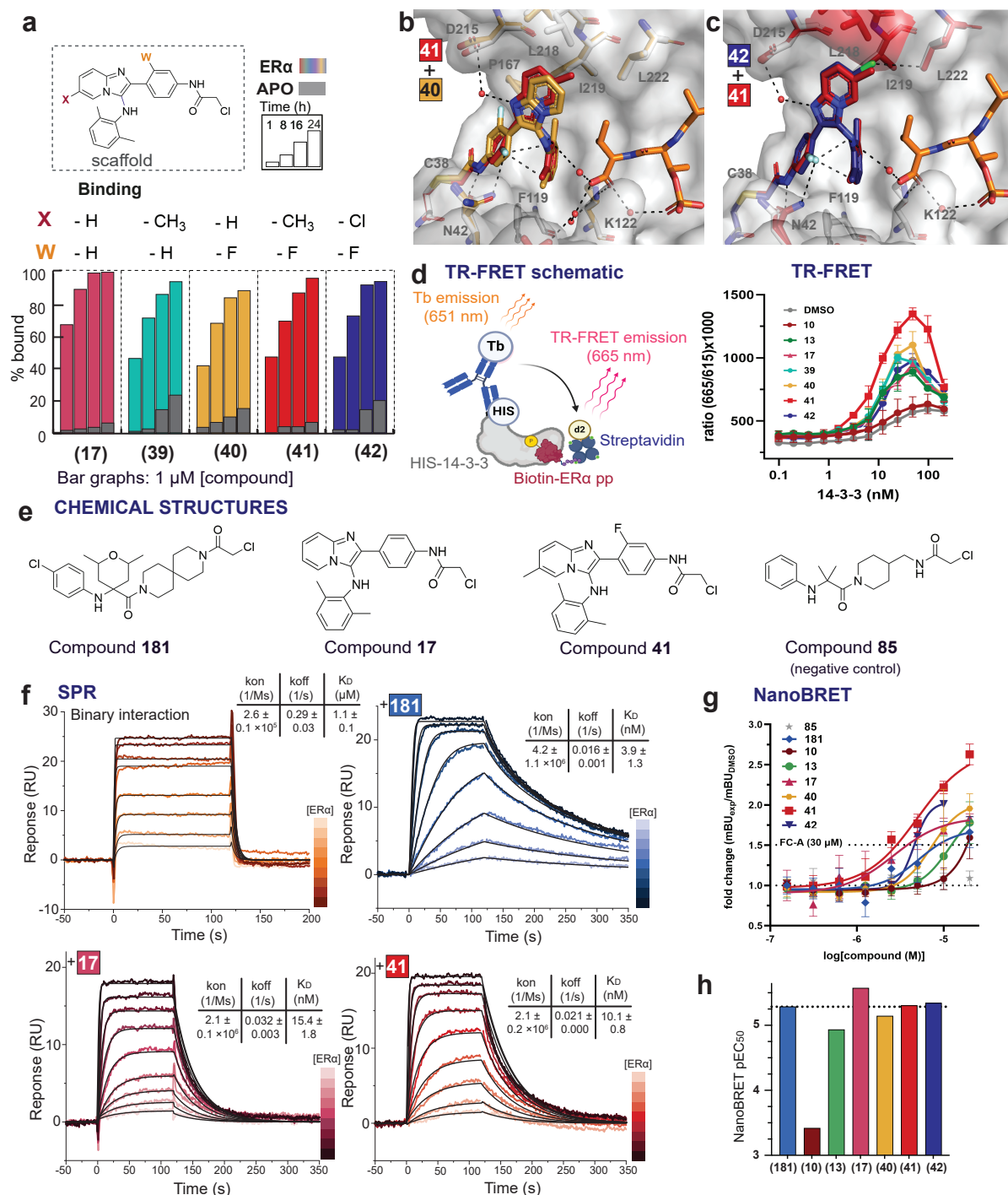


Figure 4.4: SAR and crystal structures of 2,6-di-Me analogs substituted in positions X and W
 Biophysical data (MS, TR-FRET, SPR) and cell data (NanoBRET). **a)** MS bar graphs at 1 μM. For each compound, time course experiments were performed with measurements at 1h, 8h, 16h and 24h. ERα data are shown with different colors for each compound, and apo data in grey. **b-c)** Overlays of crystal structures of 14-3-3σ/ERα ternary complexes with compounds **40** (orange sticks) (PDB 9I6V), **41** (red sticks) (PDB 9I6W), and **42** (blue sticks) (PDB 9I6X). **d)** Time-resolved fluorescence energy transfer (TR-FRET) schematic and protein titration data for representative compounds at 100 μM compound or DMSO. (Figure caption continued on the next page).

(Figure caption continued from previous page). For each compound, three independent experiments were performed (n=3). (TR-FRET schematic was created in BioRender. Konstantinidou, M. (2025) **e**) Chemical structures of compounds **181**, **17** and **41** used in SPR and compound **85**, used in NanoBRET as a negative control. **f**) Surface Plasmon Resonance (SPR) data for the binary 14-3-3 σ /ER α interaction and ternary interactions with **181**, **17** and **41** (mean $\pm$ SD, n=2). **g-h**) 14-3-3 σ -HaloTag/Nluc-ER α NanoLuciferase bioluminescence resonance energy transfer (NanoBRET) assay in HEK293T cells with compound titrations (1:2 dilution, starting at 40 μ M). Data points excluded where compound dosage was toxic to the cells. MCR compounds compared to the previously described stabilizer **181** and **85**, an inactive compound as the negative control. The natural product FC-A was used as a control, dosed at 30 μ M (black dashed line). Bar graphs quantifying pEC₅₀ values. Data points presented are mean values $\pm$ SD (n=3 technical) and representative of 4 biological replicates.

Supplementary Figures

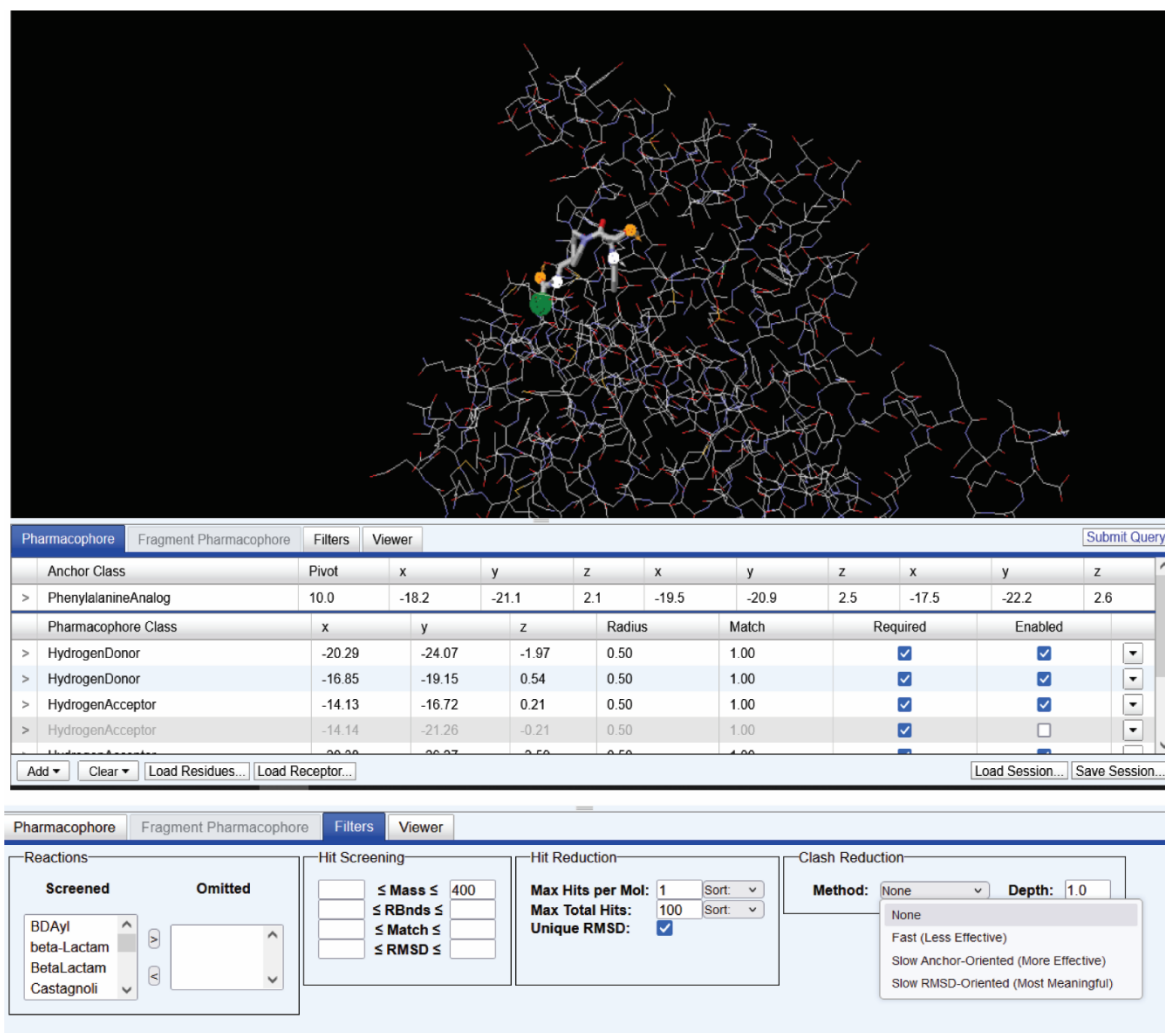


Figure S4.1: Overview of AnchorQuery™

The crystal structure of compound **127** bound to the 14-3-3 σ /ER α complex (PDB 8ALW) was used as a starting point for the design of an MCR scaffold.

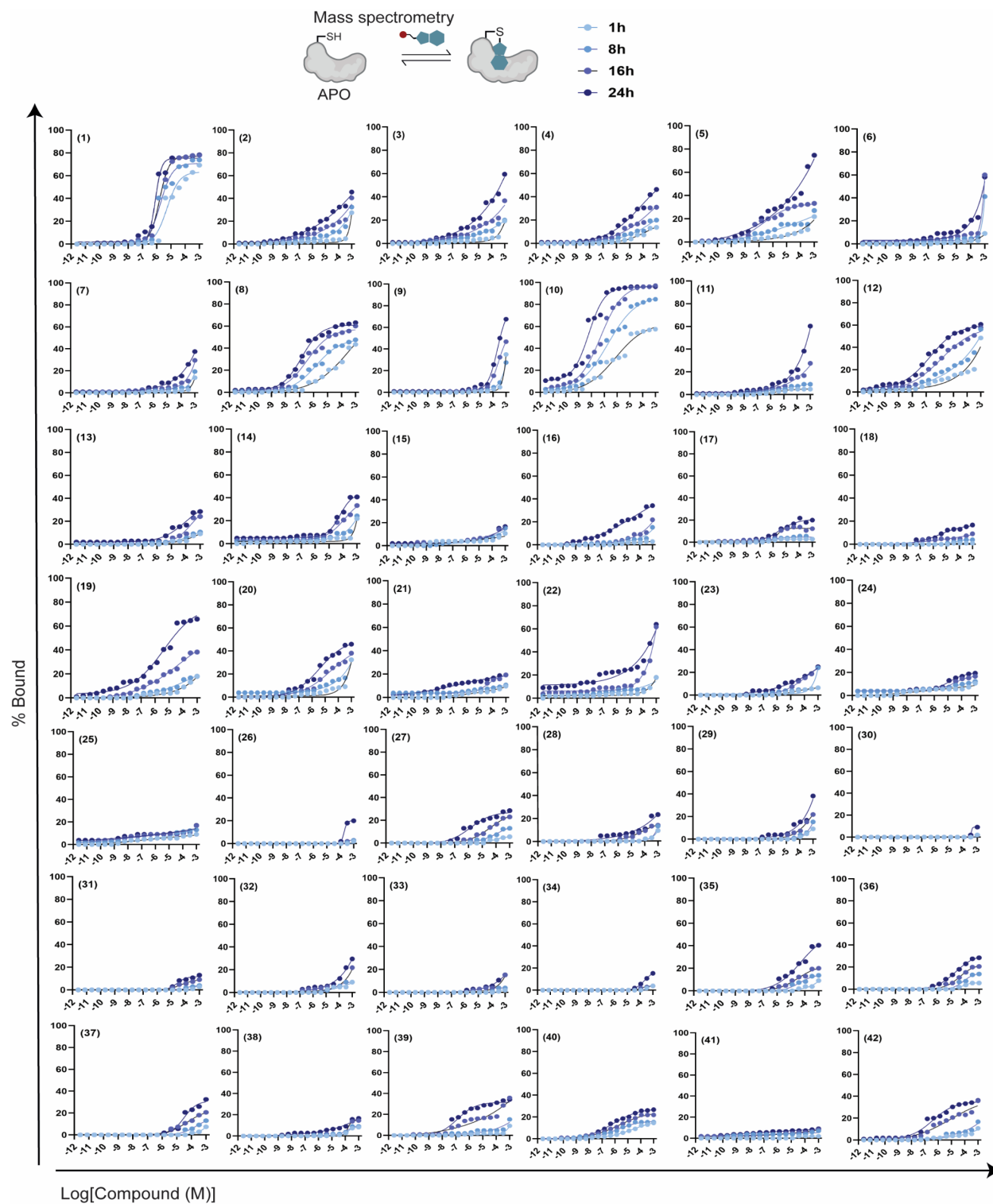


Figure S4.2: MS dose-response curves (apo)

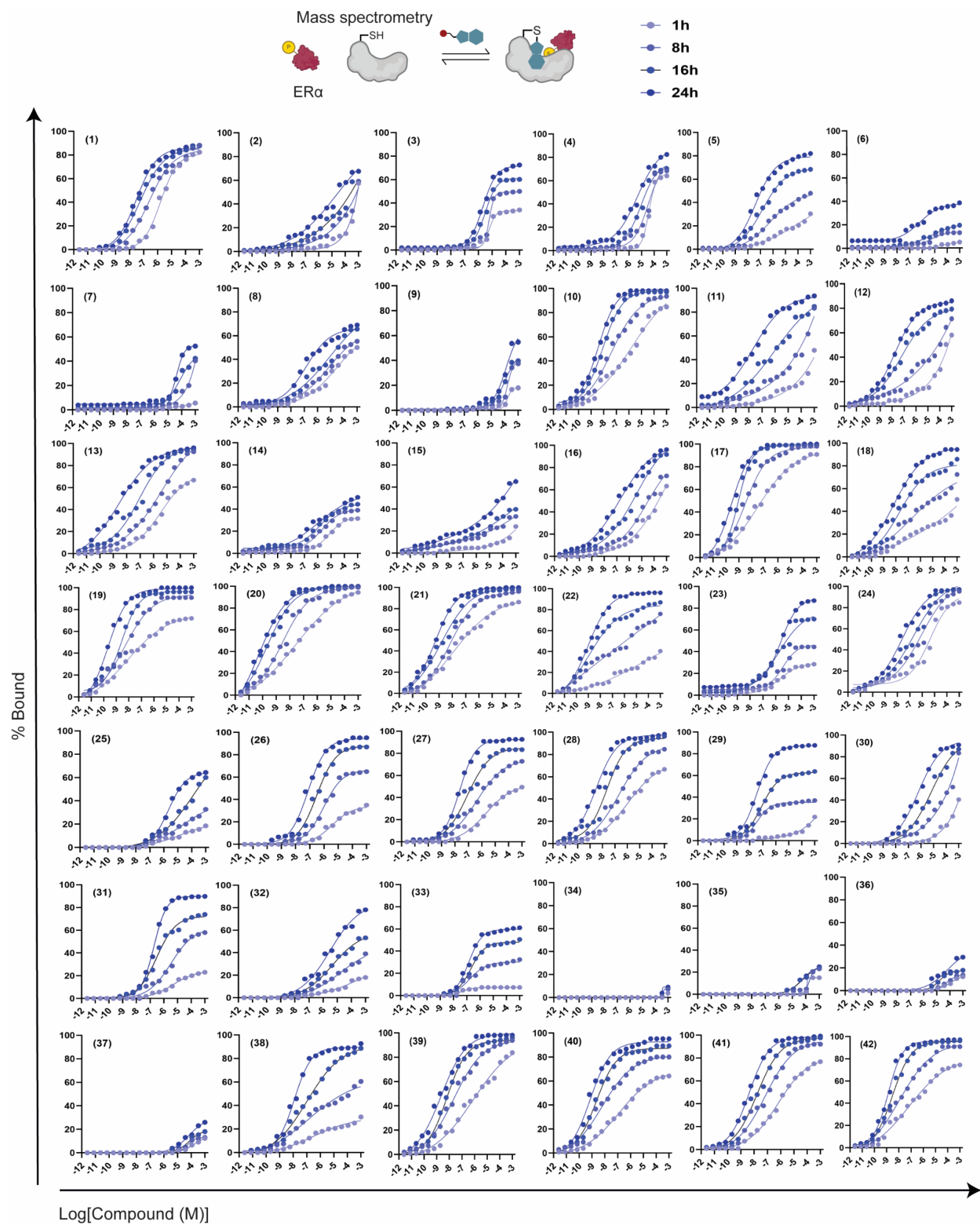


Figure S4.3: MS dose-response curves (ERα)

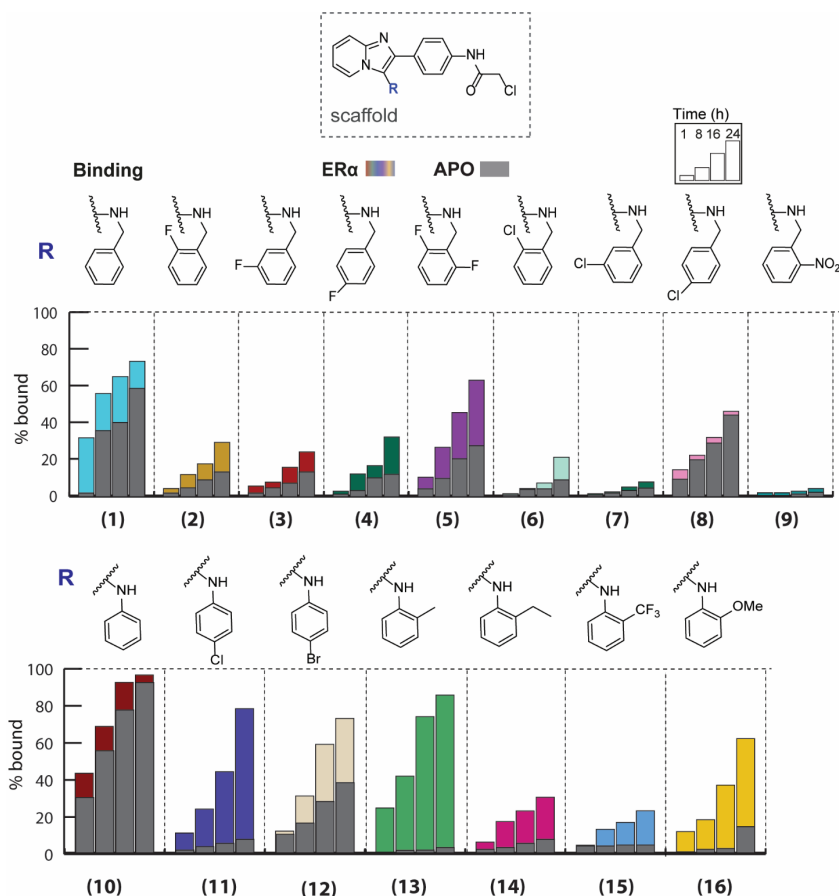


Figure S4.4: SAR of benzyl and phenyl analogs

MS bar graphs at 1 μ M compound. For each compound, time course experiments were performed with measurements at 1h, 8h, 16h and 24h. ER α data are shown with different colors for each compound, and apo data in gray.

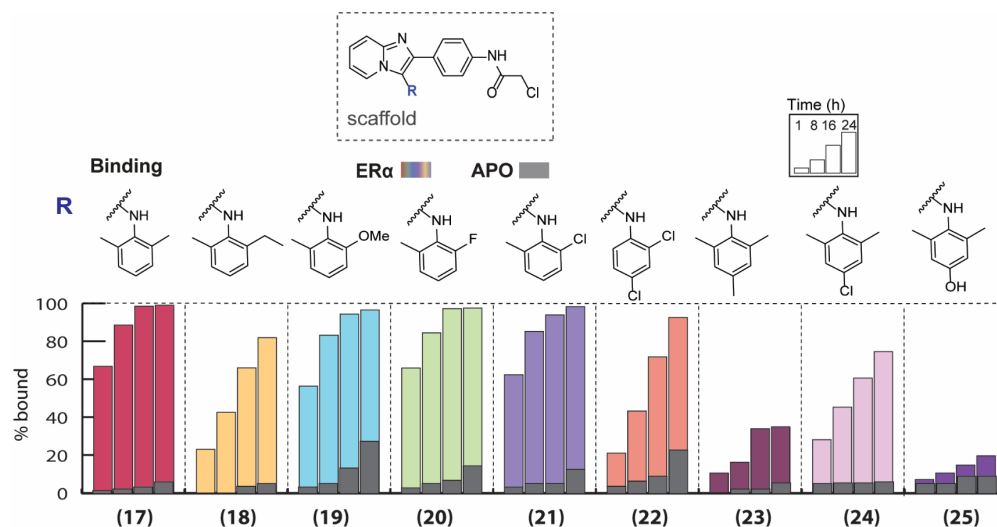


Figure S4.5: SAR of analogs with double o-substitutions

MS bar graphs at 1 μ M compound. For each compound, time course experiments were performed with measurements at 1h, 8h, 16h and 24h. ER α data are shown with different colors for each compound, and apo data in gray.

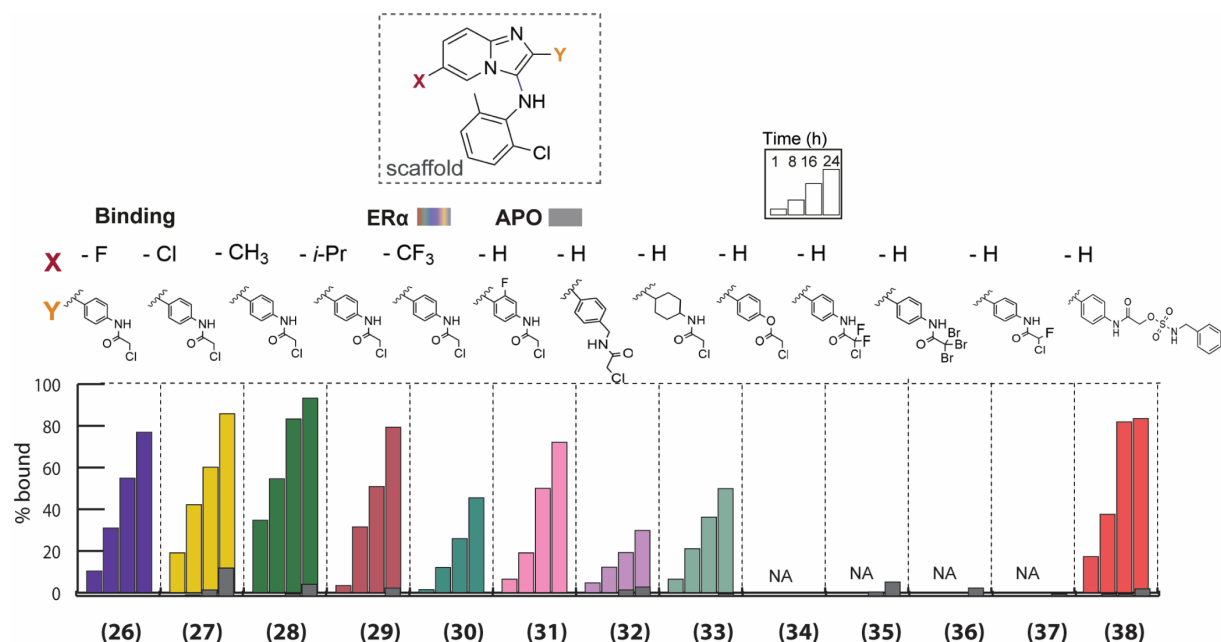


Figure S4.6: SAR of analogs varying positions X and Y on the scaffold

MS bar graphs at 1 μ M compound. For each compound, time course experiments were performed with measurements at 1h, 8h, 16h and 24h. ER α data are shown with different colors for each compound, and apo data in gray.

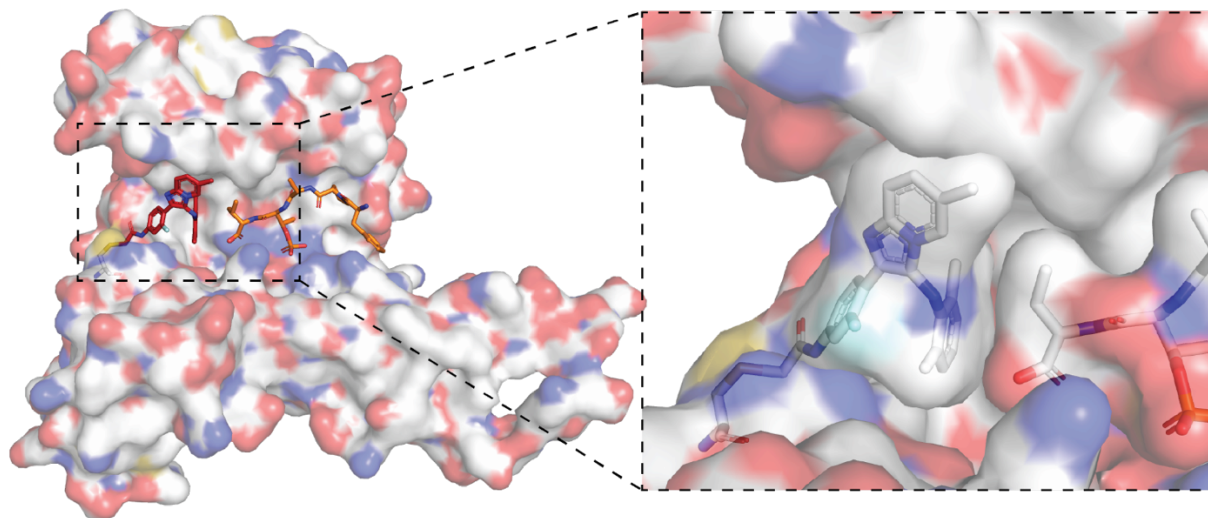


Figure S4.7: Surface representation of 14-3-3 σ bound to the ER α peptide and compound 41

Colored by atom (carbon = white, oxygen = red, nitrogen = blue, sulfur = yellow, fluoro = cyan). The groove occupied by the compound was primarily hydrophobic. Polar amino acids were located at the rim of the interface. The close-up surface representation of the compound indicated favorable shape complementarity with the 14-3-3 σ /ER α complex.

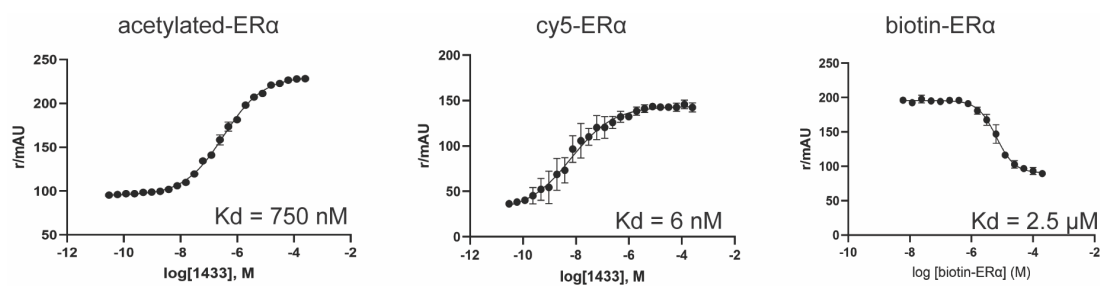


Figure S4.8: K_d determination for FAM-, cy5- and biotin-labeled ER α peptides

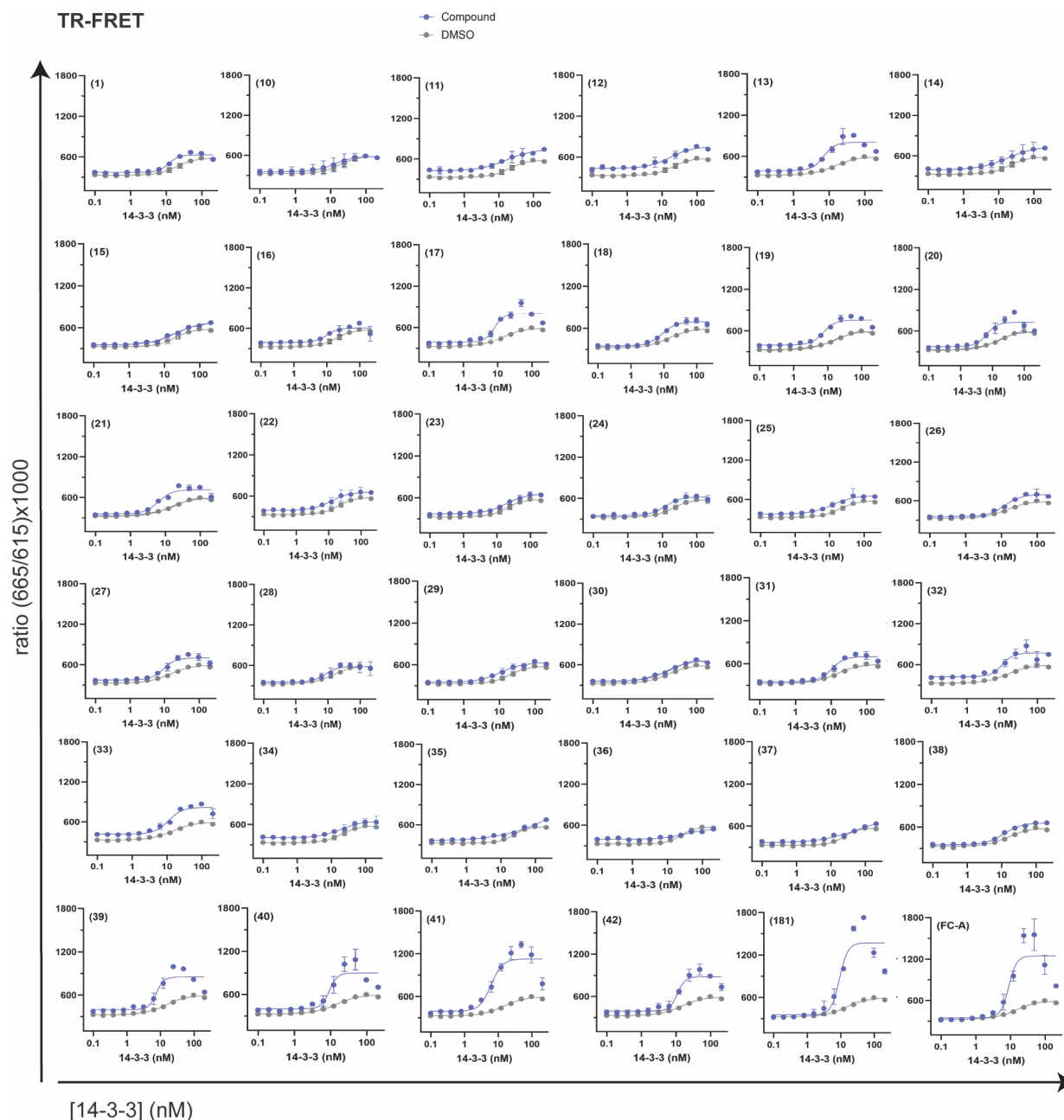


Figure S4.9: TR-FRET protein titrations

For each compound three independent experiments were performed ($n=3$).

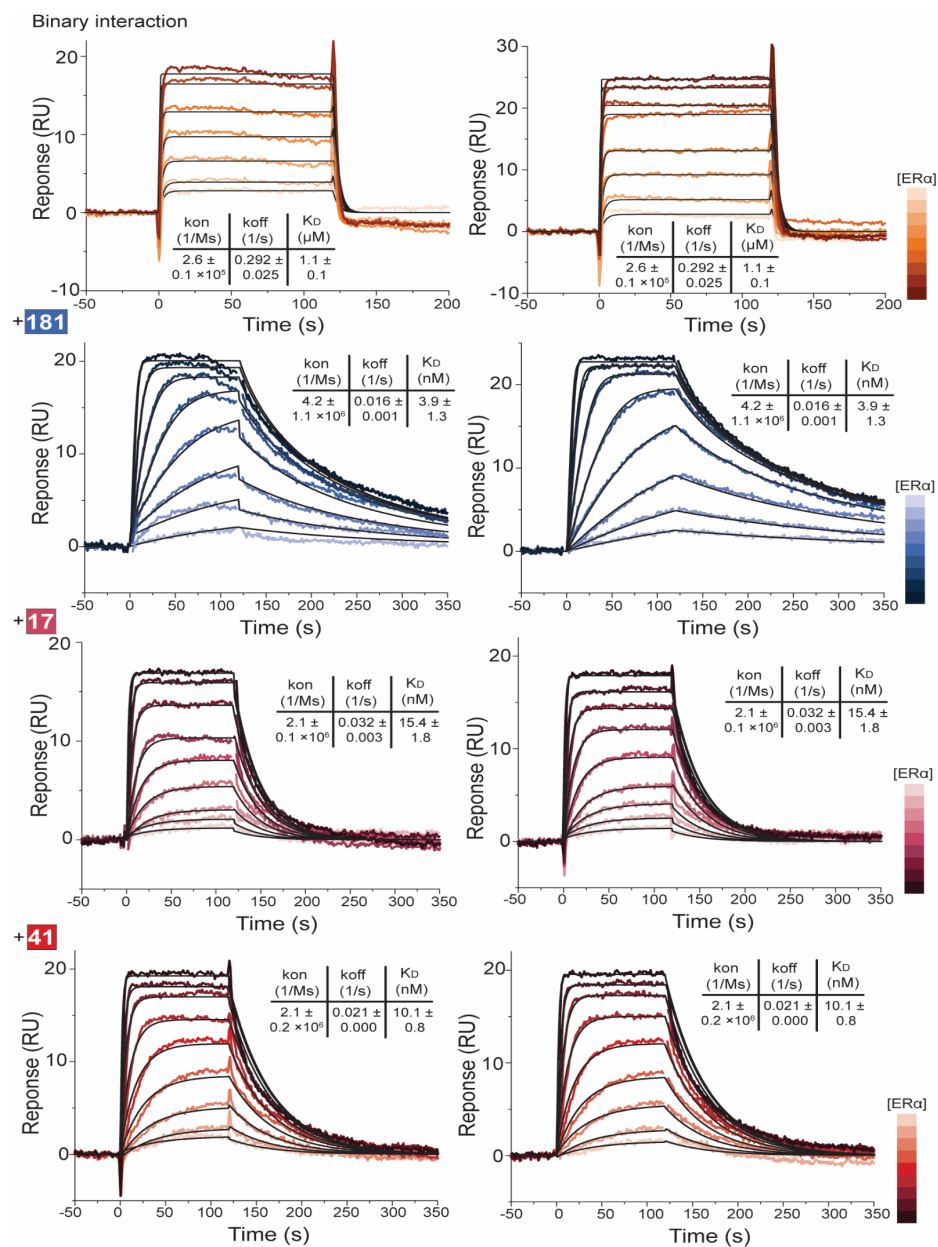


Figure S4.10: SPR replicates

Binary 14-3-3σ/ERα interaction and ternary interactions with **181**, **17** and **41**.

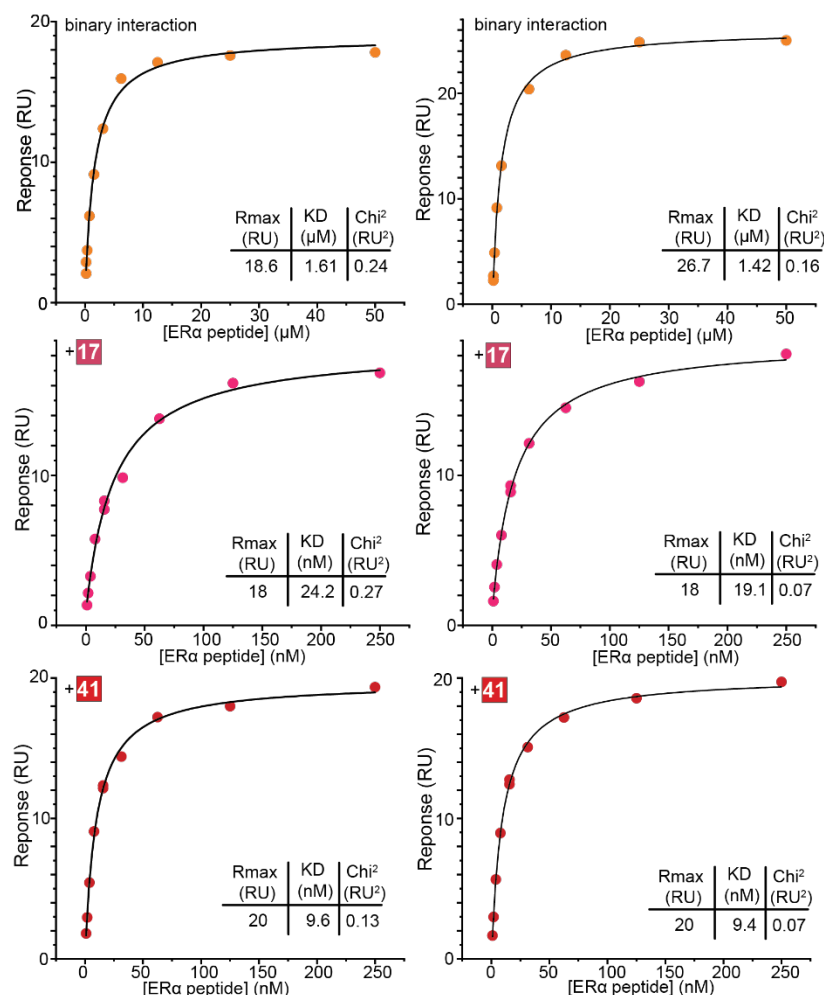


Figure S4.11: Affinity fits of sensorgrams

Affinity fits for SI Figure 10 that reached equilibrium by plotting the response (RU) over the ERα peptide concentration (μM for the binary interaction, nM for compounds **17** and **41**). The Rmax, KD value and Chi² are reported for each replicate.

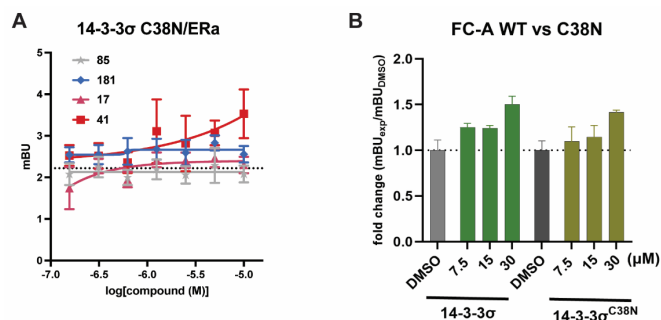


Figure S4.12: 14-3-3/ERα NanoBRET

A) 14-3-3σ^{C38N}/ERα NanoBRET. 1:10 ratio of Nluc- ERα:14-3-3σ^{C38N}-HaloTag plasmids. Cells treated with compound for 24 hours. **B)** 14-3-3σ/ERα NanoBRET and 14-3-3σ^{C38N}/ERα NanoBRET for the natural product FC-A, indicating that the effect is not specific to the sigma isoform. Data points presented are mean values +/- SD (n=3 technical).

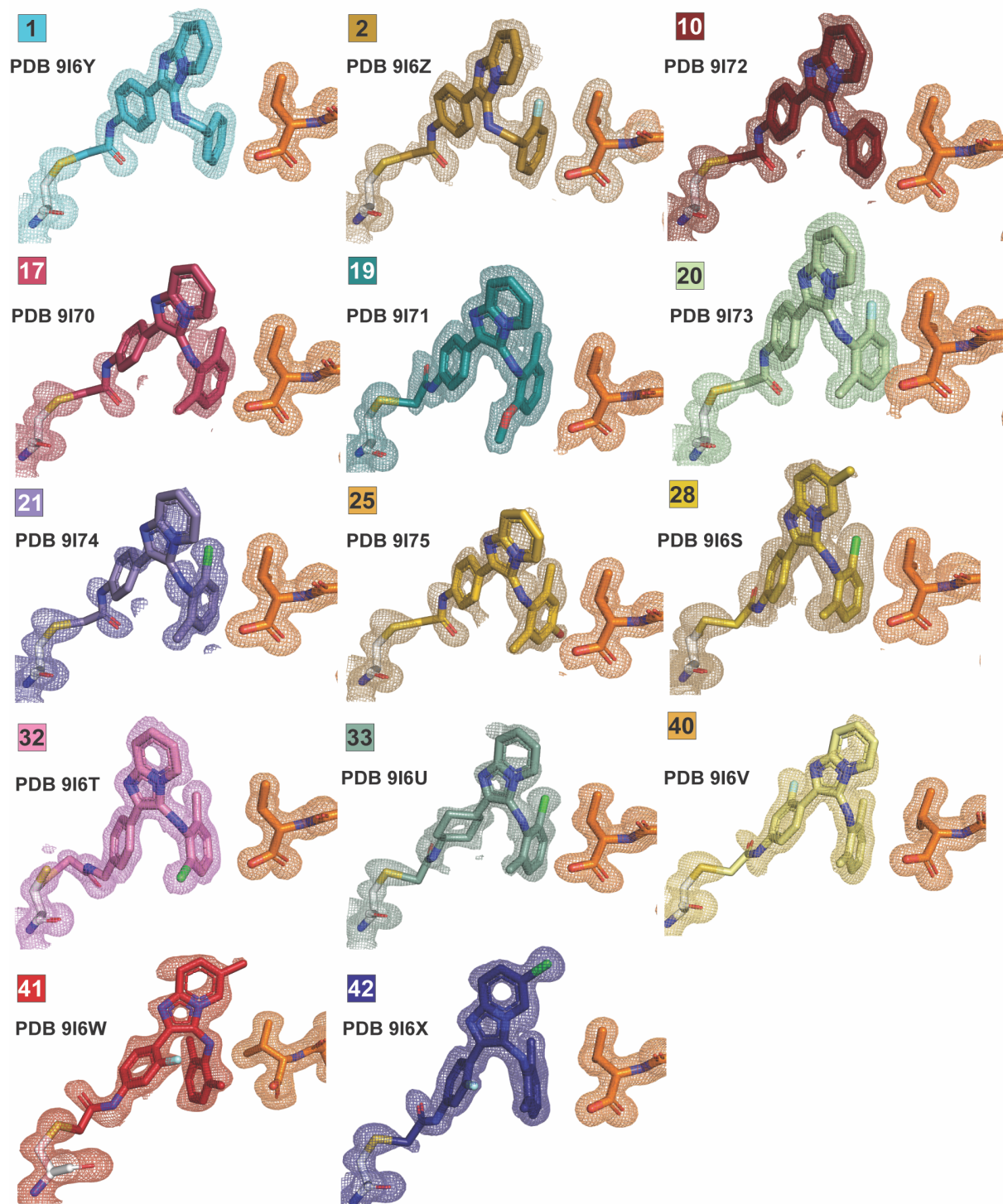


Figure S4.13: Densities and PDB IDs of MCR molecular glues

Represented as sticks, compound number at top left. 2Fo-Fc electron density maps (blue mesh) are contoured at 1σ .

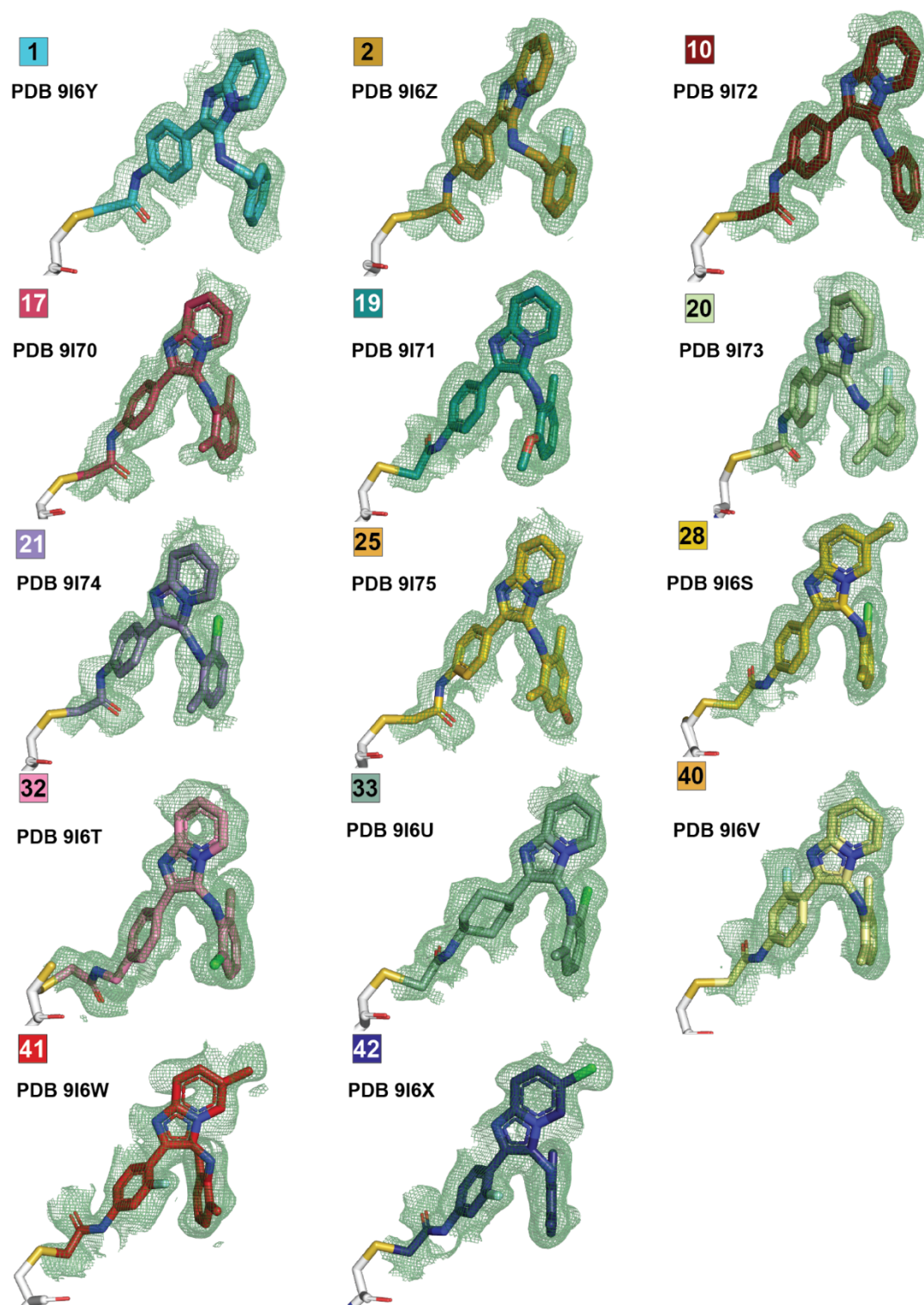


Figure S4.14: Omit density maps (Fo-Fc) of MCR molecular glues
 Represented as sticks, compound number at top left, which are contoured at 1σ .

Supplementary Tables

Table S4.1: Percentage bound

Compound (1 μ M) to 14-3-3 σ (100 nM) measured by mass spectrometry in the absence of peptide (apo) or with ER α peptide (2 μ M) after 1, 8, 16 and 24 hours.

No.	SMDC ID	APO				ER α			
		1h	8h	16h	24h	1h	8h	16h	24h
(1)	1076388	2	35.7	40.1	58.5	31.5	55.7	64.9	73.2
(2)	1083860	2	4.8	9.1	13.4	3.8	11.5	17.3	29
(3)	1083861	2	4.8	7.4	13.4	5.2	7.3	15.4	23.8
(4)	1083857	1.4	3.3	10.2	12.1	2.4	11.8	16.3	31.9
(5)	1084657	4.3	9.9	20.5	27.6	10	26.3	45.3	62.9
(6)	1083858	1	3.9	4.3	9.1	1	3.8	6.9	20.9
(7)	1083859	1	2	3.4	4.7	1	2	4.7	7.4
(8)	1084659	9.5	20	29	44.2	14.1	22	31.7	45.9
(9)	1083866	0	0.5	1.5	2.4	1.5	1.5	2.4	3.8
(10)	1124905	30.5	56.3	78.8	94	43.6	68.9	92.6	96.6
(11)	1083865	1.5	3.4	5.2	7.4	11.4	24.3	44.5	78.5
(12)	1084656	10.2	16.4	28.3	38.7	12.4	31.4	59.2	73.2
(13)	1124899	0	1.4	1.5	2.9	24.9	42.1	74.2	85.8
(14)	1084652	1.9	2.9	5.2	7.4	6.5	17.6	23.3	30.7
(15)	1124900	3.8	3.8	4.3	4.3	4.8	13.4	17.1	23.4
(16)	1124902	0.5	1.9	2.4	14.4	12.2	18.6	37.2	62.3
(17)	1084662	1.5	2.4	3.4	6.1	66.8	88.5	98.5	99
(18)	1124901	0	0	3.8	5.2	23	42.5	66	81.9
(19)	1124904	3.4	5.2	13.4	27.4	56.3	83.2	94.3	96.5
(20)	1124906	2.9	5.2	7	14.5	65.9	84.4	97.2	97.5
(21)	1124907	3.4	5.2	5.2	12.7	62.3	85.2	93.9	98.2
(22)	1084654	3.8	6.5	9.1	22.8	21	43.2	71.7	92.5
(23)	1124903	0.5	2.4	2.4	5.6	10.5	16.2	33.9	34.9
(24)	1124908	5.2	5.6	5.6	6.1	28.1	45.2	60.6	74.5
(25)	1124909	5.2	5.2	9.1	9.1	7.1	10.5	14.7	19.6
(26)	1132336	0	0	0	0	10.4	31	54.9	76.9
(27)	1132335	0	0.5	2.9	13.4	19.1	42.2	60.2	85.8
(28)	1132334	0	0	1	5.6	34.7	54.6	83.3	93.3
(29)	1132332	0	0	0	3.8	3.4	31.5	50.9	79.3
(30)	1132333	0	0	0	0	1.5	12.1	25.9	45.5
(31)	1132337	0	0	0	0	6.5	19.1	50	72.1
(32)	1132338	0	0	2.9	4.3	4.7	12.2	19.2	29.8
(33)	1132339	0	0	0	1	6.5	21.1	36.2	49.9
(34)	1132341	0	0	0	0	0	0	0	0
(35)	1132340	0	0	1.9	6.7	0	0	0	0
(36)	1132342	0	0	0	3.8	0	0	0	0
(37)	1132343	0	0	0	0.9	0	0	0	0
(38)	1133566	0	0.9	0.9	3.4	17.3	37.6	87.9	83.5
(39)	1132364	0.9	2.4	14.5	23.6	46.1	70.9	85.4	93.2
(40)	1132365	3.4	6.5	9.9	15.2	41.6	67.7	83.3	87.7
(41)	1132366	0	3.8	3.8	6.5	47	69	86.3	95.3
(42)	1132367	1.9	1.9	14.5	20.2	47	72.2	91.2	93.3

Table S4.2: TR-FRET protein titrations data

AppKd, *E*_{max} (at observed protein concentration), fold-increase and fold-stabilization values refer to measurements after 2h-incubation at room temperature. Fold increase = *E*_{max}/*E*_{min}. Fold stabilization = *AppKd*_(compound)/*AppKd*_(DMSO). NT = not tested in this experiment, NA = not applicable.

No.	SMDC ID	<i>AppKd</i> compound (nM)	<i>E</i> _{Max}	Fold increase	Fold stabilization
(1)	1076388	NA	-	-	-
(10)	1124905	NA	-	-	-
(11)	1083865	NA	-	-	-
(12)	1084656	NA	-	-	-
(13)	1124899	8.1	887.8	2.36	4.19
(14)	1084652	NA	-	-	-
(15)	1124900	NA	-	-	-
(16)	1124902	NA	-	-	-
(17)	1084662	7.1	991.6	2.58	4.78
(18)	1124901	11.1	742.8	2.14	3.06
(19)	1124904	7.7	818.7	2.1	4.41
(20)	1124906	7.1	894.0	2.47	4.78
(21)	1124907	6.5	772.7	2.28	5.23
(22)	1084654	NA	-	-	-
(23)	1124903	NA	-	-	-
(24)	1124908	16.7	628.6	1.85	2.03
(25)	1124909	NA	-	-	-
(26)	1132336	13.4	757.7	2.1	2.53
(27)	1132335	8.4	761.3	2.1	4.04
(28)	1132334	10	627.7	1.73	3.40
(29)	1132332	NA	-	-	-
(30)	1132333	NA	-	-	-
(31)	1132337	9.7	753.5	2.13	3.50
(32)	1132338	8.6	810	2	3.95
(33)	1132339	12.1	855.8	2.11	2.80
(34)	1132341	NA	-	-	-
(35)	1132340	NA	-	-	-
(36)	1132342	NA	-	-	-
(37)	1132343	NA	-	-	-
(38)	1133566	NA	-	-	-
(39)	1132364	7.8	1017.2	2.61	4.35
(40)	1132365	8.4	1184.8	3.12	4.04
(41)	1132366	5.2	1359.3	3.71	6.53
(42)	1132367	8.8	1036.7	2.97	3.87
(181)	1083744	8.6	1725.3	5.52	3.95
-	FC-A	11	1715.2	5.52	3.09
-	DMSO	34	-	-	-

Table S4.3: SPR data

Fitted parameters of 1:1 binding model, with k_a (association constant), k_d (dissociation constant), K_D (affinity constant), the standard error (SE), and the maximal signal (R_{max}). Other parameters (t_c , χ^2 , U-value) indicate the quality of the fit.

		ER α binary			ER α + 181		
		Replicate 1	Replicate 2	Average $\pm$ SD	Replicate 1	Replicate 2	Average $\pm$ SD
Kinetic fit	k_a (1/Ms)	2.63E+05	2.50E+05	2.6E+05 $\pm$ 9.3E+03	3.41E+06	4.96E+06	4.2E+06 $\pm$ 1.1E+06
	SE (k_a)	2.70E+04	1.40E+04	-	6.40E+04	6.30E+04	-
	k_d (1/s)	0.309	0.274	0.292 $\pm$ 0.025	0.0163	0.0147	0.016 $\pm$ 0.001
	SE (k_d)	0.013	0.005	-	0.0003	0.0002	-
	K_D (M)	1.18E-06	1.10E-06	1.1E-06 $\pm$ 5.6E-08	4.79E-09	2.97E-09	3.9E-09 $\pm$ 1.3E-09
	R_{max} (RU)	20	27	-	21	23	-
	t_c	1.46E+12	6.65E+14	-	1.28E+7	1.14E+7	-
	χ^2 (RU ²)	1.76	1.04	-	0.323	0.176	-
	U-value	15	9	-	4	2	-
Affinity fit	K_D (M)	1.61E-06	1.42E-06	1.51E-06 $\pm$ 1.33E-07	-	-	-
	R_{max} (RU)	18.6	26.7	-	-	-	-
		ER α + 17			ER α + 41		
		Replicate 1	Replicate 2	Average $\pm$ SD	Replicate 1	Replicate 2	Average $\pm$ SD
Kinetic fit	k_a (1/Ms)	2.03E+06	2.12E+06	2.1E+06 $\pm$ 6.5E+04	2.24E+06	1.97E+06	2.1E+06 $\pm$ 1.9E+05
	SE (k_a)	3.40E+04	3.50E+04	-	3.10E+04	2.00E+04	-
	k_d (1/s)	0.0339	0.0301	0.032 $\pm$ 0.003	0.0214	0.0211	0.021 $\pm$ 0.000
	SE (k_d)	0.0003	0.0003	-	0.0002	0.0001	-
	K_D (M)	1.67E-08	1.42E-08	1.5E-08 $\pm$ 1.8E-09	9.55E-09	1.07E-08	1.0E-08 $\pm$ 8.3E-10
	R_{max} (RU)	17	17	-	19	20	-
	t_c	1.58E+13	8.81E+11	-	8.80E+09	3.65E+09	-
	χ^2 (RU ²)	0.228	0.346	-	0.353	0.265	-
	U-value	4	4	-	3	2	-
Affinity fit	K_D (M)	2.42E-08	1.91E-08	2.2E-08 $\pm$ 3.6E-09	9.64E-09	9.37E-09	9.5E-09 $\pm$ 1.9E-10
	R_{max} (RU)	18	18	-	20	20	-

Table S4.4: 14-3-3 σ -HaloTag/Nluc-ER α NanoBRET data

Calculated EC₅₀ values from compound titrations (2-fold dilution series, starting at 40 μ M), pEC₅₀ values, and the maximum fold-increase in BRET signal for each compound. Average EC₅₀ values from 3 biological replicates.

No.	SMDC ID	EC ₅₀ (μ M)	pEC ₅₀	Fold Increase
(181)	1083744	5.19	5.3	1.66
(10)	1124905	383	3.4	1.59
(13)	1124899	11.7	4.9	1.78
(17)	1084662	2.69	5.6	1.84
(40)	1132365	7.21	5.1	1.96
(41)	1132366	4.97	5.3	2.63
(42)	1132367	4.56	5.3	2.01

References

1. Andrei, S. A. *et al.* Stabilization of protein-protein interactions in drug discovery. *Expert Opin Drug Discov* **12**, 925–940 (2017).
2. Bier, D., Thiel, P., Briels, J. & Ottmann, C. Stabilization of Protein–Protein Interactions in chemical biology and drug discovery. *Progress in Biophysics and Molecular Biology* **119**, 10–19 (2015).
3. Arkin, M. R. & Wells, J. A. Small-molecule inhibitors of protein–protein interactions: progressing towards the dream. *Nat Rev Drug Discov* **3**, 301–317 (2004).
4. Arkin, M. R., Tang, Y. & Wells, J. A. Small-Molecule Inhibitors of Protein-Protein Interactions: Progressing toward the Reality. *Chemistry & Biology* **21**, 1102–1114 (2014).
5. Smith, M. C. & Gestwicki, J. E. Features of protein–protein interactions that translate into potent inhibitors: topology, surface area and affinity. *Expert Rev. Mol. Med.* **14**, e16 (2012).
6. Schreiber, S. L. Molecular glues and bifunctional compounds: Therapeutic modalities based on induced proximity. *Cell Chemical Biology* **31**, 1050–1063 (2024).
7. Konstantinidou, M. & Arkin, M. R. Molecular glues for protein-protein interactions: Progressing toward a new dream. *Cell Chem Biol* **31**, 1064–1088 (2024).
8. Ruan, H., Sun, Q., Zhang, W., Liu, Y. & Lai, L. Targeting intrinsically disordered proteins at the edge of chaos. *Drug Discovery Today* **24**, 217–227 (2019).
9. Santofimia-Castaño, P. *et al.* Targeting intrinsically disordered proteins involved in cancer. *Cell. Mol. Life Sci.* **77**, 1695–1707 (2020).
10. Aitken, A. 14-3-3 proteins: a historic overview. *Semin Cancer Biol* **16**, 162–172 (2006).
11. Pennington, K. L., Chan, T. Y., Torres, M. P. & Andersen, J. L. 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 (2018).

12. De Vries-van Leeuwen, I. J. *et al.* Interaction of 14-3-3 proteins with the estrogen receptor alpha F domain provides a drug target interface. *Proc Natl Acad Sci U S A* **110**, 8894–8899 (2013).
13. Somsen, B. A. *et al.* Molecular basis and dual ligand regulation of tetrameric estrogen receptor α /14-3-3 ζ protein complex. *Journal of Biological Chemistry* **299**, 104855 (2023).
14. Liu, Y., Ma, H. & Yao, J. ER α , A Key Target for Cancer Therapy: A Review. *OTT Volume* **13**, 2183–2191 (2020).
15. Paterni, I., Bertini, S., Granchi, C., Macchia, M. & Minutolo, F. Estrogen receptor ligands: a patent review update. *Expert Opinion on Therapeutic Patents* **23**, 1247–1271 (2013).
16. Scott, J. S. & Barlaam, B. Selective estrogen receptor degraders (SERDs) and covalent antagonists (SERCAs): a patent review (2015-present). *Expert Opinion on Therapeutic Patents* **32**, 131–151 (2022).
17. Scott, J. S. & Klinowska, T. C. M. Selective estrogen receptor degraders (SERDs) and covalent antagonists (SERCAs): a patent review (July 2021–December 2023). *Expert Opinion on Therapeutic Patents* **34**, 333–350 (2024).
18. Jeselsohn, R., Buchwalter, G., De Angelis, C., Brown, M. & Schiff, R. ESR1 mutations—a mechanism for acquired endocrine resistance in breast cancer. *Nat Rev Clin Oncol* **12**, 573–583 (2015).
19. Visser, E. J. *et al.* Estrogen Receptor α /14-3-3 molecular glues as alternative treatment strategy for endocrine resistant breast cancer. Preprint at <https://doi.org/10.1101/2024.04.25.591105> (2024).
20. Kenanova, D. N. *et al.* A Systematic Approach to the Discovery of Protein–Protein Interaction Stabilizers. *ACS Cent. Sci.* **9**, 937–946 (2023).
21. Konstantinidou, M. *et al.* Structure-Based Optimization of Covalent, Small-Molecule Stabilizers of the 14-3-3 σ /ER α Protein–Protein Interaction from Nonselective Fragments. *J. Am. Chem. Soc.* **145**, 20328–20343 (2023).

22. Visser, E. J. *et al.* From Tethered to Freestanding Stabilizers of 14-3-3 Protein-Protein Interactions through Fragment Linking. *Angew Chem Int Ed* **62**, e202308004 (2023).
23. Dömling, A., Wang, W. & Wang, K. Chemistry and Biology Of Multicomponent Reactions. *Chem. Rev.* **112**, 3083–3135 (2012).
24. Zarganes-Tzitzikas, T., Chandgude, A. L. & Dömling, A. Multicomponent Reactions, Union of MCRs and Beyond. *The Chemical Record* **15**, 981–996 (2015).
25. Fotopoulou, E., Anastasiou, P. K., Tomza, C. & Neochoritis, C. G. The Ugi reaction as the green alternative towards active pharmaceutical ingredients. *Tetrahedron Green Chem* **3**, 100044 (2024).
26. Li, X., Zarganes-Tzitzikas, T., Kurpiewska, K. & Dömling, A. Amenamevir by Ugi-4CR. *Green Chem.* **25**, 1322–1325 (2023).
27. Zarganes-Tzitzikas, T., Neochoritis, C. G. & Dömling, A. Atorvastatin (Lipitor) by MCR. *ACS Med. Chem. Lett.* **10**, 389–392 (2019).
28. Znabet, A. *et al.* A highly efficient synthesis of telaprevir by strategic use of biocatalysis and multicomponent reactions. *Chem. Commun.* **46**, 7918 (2010).
29. Wehlan, H., Oehme, J., Schäfer, A. & Rossen, K. Development of Scalable Conditions for the Ugi Reaction—Application to the Synthesis of (*R*)-Lacosamide. *Org. Process Res. Dev.* **19**, 1980–1986 (2015).
30. Váradi, A. *et al.* Synthesis of Carfentanil Amide Opioids Using the Ugi Multicomponent Reaction. *ACS Chem. Neurosci.* **6**, 1570–1577 (2015).
31. Koes, D. *et al.* Enabling Large-Scale Design, Synthesis and Validation of Small Molecule Protein-Protein Antagonists. *PLoS ONE* **7**, e32839 (2012).
32. Koes, D. R., Dömling, A. & Camacho, C. J. AnchorQuery: Rapid online virtual screening for small-molecule protein-protein interaction inhibitors. *Protein Sci* **27**, 229–232 (2018).
33. Neochoritis, C. G. *et al.* Hitting on the move: Targeting intrinsically disordered protein states of the MDM2-p53 interaction. *European Journal of Medicinal Chemistry* **182**, 111588 (2019).

34. Groebke, K., Weber, L. & Mehlin, F. Synthesis of Imidazo[1,2-a] annulated Pyridines, Pyrazines and Pyrimidines by a Novel Three-Component Condensation. *Synlett* **1998**, 661–663 (1998).
35. Blackburn, C., Guan, B., Fleming, P., Shiosaki, K. & Tsai, S. Parallel synthesis of 3-aminoimidazo[1,2-a]pyridines and pyrazines by a new three-component condensation. *Tetrahedron Letters* **39**, 3635–3638 (1998).
36. Bienaymé, H. & Bouzid, K. A New Heterocyclic Multicomponent Reaction For the Combinatorial Synthesis of Fused 3-Aminoimidazoles. *Angewandte Chemie International Edition* **37**, 2234–2237 (1998).
37. Boltjes, A. & Dömling, A. The Groebke-Blackburn-Bienaymé Reaction. *Eur J Org Chem* **2019**, 7007–7049 (2019).
38. Shukla, P., Azad, C. S., Deswal, D. & Narula, A. K. Revisiting the GBB reaction and redefining its relevance in medicinal chemistry: A review. *Drug Discovery Today* **29**, 104237 (2024).
39. Devi, N., Singh, D., K. Rawal, R., Bariwal, J. & Singh, V. Medicinal Attributes of Imidazo[1,2-a]pyridine Derivatives: An Update. *CTMC* **16**, 2963–2994 (2016).
40. Vasilikogiannaki, E., Gryparis, C., Kotzabasaki, V., Lykakis, I. N. & Stratakis, M. Facile Reduction of Nitroarenes into Anilines and Nitroalkanes into Hydroxylamines via the Rapid Activation of Ammonia· Borane Complex by Supported Gold Nanoparticles. *Adv Synth Catal* **355**, 907–911 (2013).
41. Basilaia, M., Chen, M. H., Secka, J. & Gustafson, J. L. Atropisomerism in the Pharmaceutically Relevant Realm. *Acc. Chem. Res.* **55**, 2904–2919 (2022).
42. Degorce, F. HTRF: A Technology Tailored for Drug Discovery - A Review of Theoretical Aspects and Recent Applications. *TOCHGENJ* **3**, 22–32 (2009).

43. Vauquelin, G. & Charlton, S. J. Exploring avidity: understanding the potential gains in functional affinity and target residence time of bivalent and heterobivalent ligands. *British J Pharmacology* **168**, 1771–1785 (2013).
44. Vickery, H. R., Virta, J. M., Konstantinidou, M. & Arkin, M. R. Development of a NanoBRET assay for evaluation of 14-3-3 σ molecular glues. *SLAS Discov* **29**, 100165 (2024).
45. Hallenbeck, K. K. *et al.* A Liquid Chromatography/Mass Spectrometry Method for Screening Disulfide Tethering Fragments. *SLAS Discov* 2472555217732072 (2017)
doi:10.1177/2472555217732072.

Chapter 5

Modulation of the 14-3-3 σ /C-RAF autoinhibited complex by molecular glues

Abstract

Molecular glues, compounds that bind cooperatively at protein-protein interfaces are revolutionizing chemical biology and drug discovery, allowing the modulation of traditional “undruggable” targets. Here, we focus on the native protein-protein interaction (PPI) of C-RAF, a key component of the MAPK signaling pathway, with the scaffolding protein 14-3-3. Although extensive drug discovery efforts have focused on the MAPK pathway, its central role in oncology and developmental disorders (RASopathies), still requires alternative approaches, moving beyond direct kinase inhibition. Indeed, stabilization of native PPIs is a relatively unexplored territory in this pathway. The function of C-RAF is regulated on multiple levels including dimerization, phosphorylation and complex formation with the hub protein 14-3-3. 14-3-3 prevents C-RAF activation by molecular recognition and binding at the phospho-serine 259. We used a fragment-merging approach to design a molecular glue scaffold that would bind to the composite surface of the 14-3-3/C-RAF “auto”inhibited complex. The synthesized molecular glues stabilized the 14-3-3/C-RAF complex up to 300-fold in biophysical assays; their glue-based mechanism of action was confirmed with several crystal structures of ternary complexes. Selectivity among the other RAF isoforms and other RAF phosphorylation sites was evaluated with biophysical assays. The best compounds showed excellent selectivity among a broad panel of 80 14-3-3 clients. Validation in cell assays showed on-target engagement, enhanced phosphorylation levels of the C-RAF pS259 site, reduced RAF dimerization and reduced ERK phosphorylation. Overall, this approach enables chemical biology studies on a C-RAF site that is intrinsically disordered prior to 14-3-3 binding and has not been targeted previously. These molecular glues will be useful as chemical probes and starting points for further drug discovery efforts to elucidate the effect of native PPI stabilization in the MAPK pathway with applications in oncology and RASopathies.

Introduction

RAF kinases are central regulators of the RAS-RAF-MEK-ERK signaling pathway (MAPK), which controls multiple cellular processes, including cell proliferation, differentiation, and survival.¹ Frequent mutations, especially on RAS and the B-RAF isoform, lead to aberrant downstream signaling and occur in different types of cancer.^{2,3} Direct inhibitors for components of the MAPK pathway have been the focus of drug discovery efforts for decades.^{4,5}

Recently, single-molecule cryo-EM has started to elucidate the structural aspects of MAPK protein-protein interactions (PPIs) as well as the underlying dynamics of pathway activation, providing new opportunities for therapeutic interventions.^{6–8} The regulation of RAF kinases is controlled on different levels^{9,10} by phosphorylation, conformation, dimerization, and binding to 14-3-3 proteins^{11,12}, adaptor proteins that recognize specific phospho-serine or phospho-threonine motifs on client proteins.^{13,14} The three RAF isoforms (A-, B-, C-) have highly conserved amino acid sequences and consist of three main domains: the CR1 domain on the N-terminus with the RAS binding domain (RBD) and the cysteine-rich domain (CRD), the CR2 which includes phosphorylation sites, and the C-terminus (CR3), which includes the kinase catalytic domain (CAD).^{15,16} The 14-3-3 binding sites are located in the conserved regions (CRs) on either side of the kinase catalytic domain (Fig 5.1A). The two 14-3-3 binding sites have opposite functions on the regulation of the pathway; the sites located on CR2 (pS214 for A-RAF, pS365 for B-RAF, pS259 for C-RAF) inhibit RAF activation, while sites located on the CR3 domain (pS582 for A-RAF, pS729 for B-RAF, pS621 for C-RAF) are activating.¹⁷

Although cryo-EM structures with full length C-RAF are not yet reported, C-RAF activation is hypothesized to proceed in a similar manner as B-RAF.¹⁸ A recent single-molecule FRET (sm-FRET) study on C-RAF supports this hypothesis.¹⁹ In analogy to the B-RAF cryo-EM structures, in the proposed model C-RAF is maintained in the autoinhibited, closed state via interactions of the N- and C-terminal domains with a 14-3-3 dimer (Fig 5.1B). The pS259 site (corresponding to the B-RAF pS365 site) and the pS621 site (corresponding to the B-RAF

pS729 site) are each bound in an amphipathic, phosphopeptide-binding groove in the 14-3-3 dimer. Upon pathway activation, C-RAF is recruited to the membrane and interacts with RAS via the RBD domain. This is followed by the release of the CRD domain from the autoinhibited complex and its subsequent interaction with RAS and the membrane. This conformational change exposes the inhibitory C-RAF pS259 site to the phosphatase SHOC2-PP1 for dephosphorylation.^{20,21} RAF is then activated through dimerization of the catalytic domains, stabilized through binding to 14-3-3 via the C-terminal phosphorylation sites only (pS621 for C-RAF and pS729 for B-RAF). Homodimers and heterodimers between all three RAF isoforms are reported.^{22,23}

Despite the sequence similarity in RAF isoforms, distinct roles are starting to emerge. Recent reports show that C-RAF depletion inhibits tumor growth but not ERK signaling in K-RAS mutant cells,²³ whereas pan-RAF inhibition affects PI3K-signaling in multiple myeloma.²⁴ The roles of heterodimerization is also an active area of research. The current thinking is that the B-RAF/C-RAF heterodimer is the primary species involved both in native and oncogenic signaling.^{25,26} Notably, the dimerization of C-RAF with A-RAF is also thought to be involved in K-RAS driven tumor growth²³ and RAF heterodimers are an important mechanism linked to RAF inhibitor resistance (including 'paradoxical activation') in cancer therapy.^{27,28}

Here, our aim is to disrupt the transition from the closed C-RAF monomer to the open, active dimer by stabilizing the autoinhibited C-RAF pS259/14-3-3 complex with molecular glues (MGs), as an alternative approach to direct C-RAF kinase inhibition (Fig 5.1C). Although direct, selective inhibition of C-RAF has not yet been achieved clinically, a recent study utilizing bio-orthogonal ligand tethering showed that selective C-RAF inhibition promotes paradoxical activation, thus potentially leading to resistance mechanisms.²⁶ To date, reported stabilizers of the autoinhibited C-RAF pS259/14-3-3 complex are the natural product cotylenin-A²⁹ (CN-A, Fig S5.1) and disulfide-tethered fragments identified by our lab³⁰. These disulfide fragments show two distinct binding conformations in crystal structures, although neither appear to make specific

contacts with the residues C-terminal to C-RAF259³⁰ (Fig S5.1). Here, we used a fragment merging approach to design molecular glues for the unique composite surface of the 14-3-3/C-RAF259 complex, aiming at specific ligand – protein interactions, including the formation of a covalent bond with C38 (on 14-3-3 σ) and polar interactions with T260 (+1 residue on C-RAF259 phosphorylation site). The most potent stabilizer, compound **23** resulted in 280-fold stabilization of the 14-3-3 σ /C-RAF259 complex in fluorescence anisotropy assays, had an EC₅₀ of 0.18 μ M in a cellular 14-3-3/C-RAF NanoBRET assay and partially blocked N-RAS/C-RAF dimer formation in cells. Compound **78**, although less potent than **23**, showed higher selectivity for C-RAF over the other RAF isoforms in cell assays. Thus, a fragment-based development of MGs provided a tunable selectivity that will help elucidate specific roles of RAF complexes in normal and oncogenic signal transduction.

Results

SAR development and biophysical assays

Our scaffold design strategy combined a chloroacetamide piperidine moiety (inspired by analog 1074202, cysteine-targeting 14-3-3/ER α stabilizer³¹) with fragments bearing a sulfonyl group (inspired by TC-521, a lysine-targeting 14-3-3/p65 stabilizer³²) (Fig 5.1D). We hypothesized that the sulfonyl group in the new scaffold would serve to induce a pronounced conformational bend in the MG scaffold and act as a flexible handle for potential polar interactions with T260. Chemical modifications were then performed to optimize the designed scaffold (Fig 5.1E). Modification (I), in close proximity to the peptide, included optimizing the linkage of the two merged fragments and substitutions on the aryl ring. Modification (II) focused on replacing the piperidine ring with spiro-cycles and fused ring systems with varying sizes and orientations. Lastly, combinations of modifications (I) and (II) aimed at exploring synergistic effects.

In analogy to our previous work, two orthogonal assays were used for screening³¹ (Fig S5.2). Briefly, the mass spectrometry (MS) assay monitored the formation of the covalent bond between electrophiles and the native cysteine (C38) on 14-3-3 σ . Dose responses of compounds were performed in the absence and presence of the C-RAF259 phospho-peptide to distinguish between neutral binders and cooperative molecular glues. The experiment was performed as a time-course, with measurements every 8 hours, after an 1h incubation. Additionally, the compounds were tested in a fluorescence anisotropy (FA) assay in the presence of FAM-labeled C-RAF259 peptide. For effective MGs, a significant increase in anisotropy was observed in the overnight measurement. The full dose-response data for all compounds are available in the SI (tables S5.2-S5.5, Fig S5.5-S5.8, Fig S5.15-S5.18, Fig S5.25-S5.32, Fig S5.37-S5.39). The dose-response data from both assays were also visually compared in the form of bar graphs. For the MS data, the bar graphs represented the % bound at 1 μ M compound concentration and 100 nM 14-3-3 σ (10:1 ratio) in the absence or presence of peptide. For FA experiments, the EC₅₀ values from the overnight measurement were calculated and were plotted as bar graphs showing the positive log EC₅₀ value (pEC₅₀). Compounds acting as MGs were expected to show an increase in both types of bar graphs.

The first key modification on the newly designed scaffold focused on the appropriate linkage of the two merged fragments. Chemically, benzyl or aryl sulfonyl chlorides could be reacted with the appropriate piperidine moiety. Initial screening data from both MS and FA assays showed that benzyl analogs were inactive (compounds **6-9**, Fig S5.3, S5.5-S5.7), whereas for phenyl analogs a SAR began to emerge as substituents were added on the phenyl ring (Fig S5.4, S5.5-S5.7). Various substituents in *o*-/*m*-/*p*-positions were added, including electron-withdrawing and electron-donating groups. As a general trend, electron-donating groups were not tolerated, whereas halogens in the *p*-position significantly improved potency, stabilization, and cooperativity (Fig 5.2A,B, Fig S5.4-S5.7, tables S5.2-S5.3). In the MS assay, analogs **11** (*p*-F) and **12** (*p*-Cl) showed binding in the presence of the C-RAF259 peptide and

only very low binding in the apo screen (no peptide), indicating cooperative binding. In FA compound titrations, **11** and **12** showed low micromolar EC₅₀ values (17 ± 3 and 3 ± 1 μ M, respectively) (table S5.1, Fig S5.7). The two compounds were further validated as 14-3-3 σ /C-RAF259 molecular glues in FA protein titrations. 14-3-3 σ was titrated into 10 nM FAM-labeled C-RAF259 peptide in the presence of DMSO or saturating concentration of the compounds (100 μ M). Compounds **11** and **12** decreased the dissociation constant for the 14-3-3s/C-RAF259 peptide complex by 4-fold and 22-fold, respectively (table S5.1, Fig S5.8). Increasing the linker length of the warhead or replacement of the phenyl ring with a pyridine ring led to loss of potency and these modifications were not investigated further (Fig S5.4-S5.7).

To test whether the observed stabilization was unique to halogen substituents or other electron donating groups could be tolerated, the *p*-formyl analog **21** was synthesized. The two covalent warheads of this analog could potentially interact simultaneously with C38 and K122 on 14-3-3 σ . The analog was weak in the MS assay and despite the low EC₅₀ value (5 ± 1.0 μ M) in the FA compound titrations (Fig 25.A,B, Fig S5.4-S5.7), it resulted in only 9-fold stabilization of the 14-3-3s/C-RAF259 complex in the FA protein titrations (table S5.1, Fig S5.8). Based on these observations, the presence of halogens seemed to be crucial, and thus bulkier halogens were introduced (*p*-Br (**22**) and *p*-I (**23**)). Both compounds showed faster binding in the MS assay (Fig 5.2A), and remarkably the *p*-I analog showed almost no binding to 14-3-3a alone; thus, **23** binding was highly cooperative for the 14-3-3 σ /C-RAF259 complex. In the FA assay both compounds showed low micromolar EC_{50s} (1 ± 0.5 μ M). Consistent with high cooperativity, in protein titrations, the *app*K_d of the 14-3-3 σ /C-RAF259 decreased from 8427 nM to 47 nM in the presence of **22** and to 30 nM in the presence of **23**. Thus, **22** and **23** stabilized the 14-3-3 σ /C-RAF 259 complex by 179- and 280-fold, respectively (table S5.1, Fig S5.8).

Crystal structures of the ternary complexes with 14-3-3 σ and a 10-mer C-RAF259 phospho-peptide were solved for **12**, **21**, **22** and **23** (Fig 5.2C, D). Overall, **12**, **22**, and **23** form compact, L-shaped molecules that form multiple interactions with both the C-RAF peptide and

14-3-3. Taking **12** as an example, in addition to the covalent bond with C38 of 14-3-3, two hydrogen bonds were formed between the warhead amide, R41 of 14-3-3a (2.7Å), and the backbone carbonyl of C38 (3.3Å). N42 of 14-3-3 directly interacted with the backbone H264 of C-RAF via two hydrogen bonds (3.0Å). The sulfonyl group of **12** interacted with K122 of 14-3-3 via a charged-assisted hydrogen bond (3.2Å) and with T260 (+1 position) of C-RAF via a hydrogen bond (3.4Å).

Based on our past designs³¹ and the conformation of the aldehyde fragment TC521 (Fig 5.1D), we anticipated that the halogenated/formylated aryl ring would point these substitutions down into a pocket that includes K122 on 14-3-3 σ . Instead, **12** (*p*-Cl) adopted a surprising upward conformation at the 14-3-3/peptide interface (Fig 5.2C, Fig S5.9) with the halogen pointing into a shallow pocket towards the top of the 14-3-3 binding groove, potentially allowing the formation of halogen bonds with D215, L218, I219 of 14-3-3 or V263 of C-RAF to increase the stabilization of the complex. In stark contrast, **21** (*p*-CHO) showed a different binding mode, interacting with C38 and K122 via the two covalent warheads. While this conformation more closely mirrored our prediction, no additional interactions were observed with 14-3-3 or C-RAF (Fig 5.2C). An overlay of the two crystal structures showed a significant conformational change of helix 9 of 14-3-3 for **12** (Fig 5.2C). The helix moved to a downward position, closer to the compound, thus “clamping” the compound in the 14-3-3 binding groove and causing conformational changes to D215, L218 and I219. An overlay with a previously published binary structure further confirmed that the movement of the helix was compound-induced (Fig 5.2C).

Crystal structures of **22** (*p*-Br) and **23** (*p*-I) showed an upward binding mode, consistent with **12** (Fig 5.2D, Fig S5.10). The key ligand-protein and ligand-peptide interactions were maintained. Different water-networks were formed for **22** and **23**. For **22**, N42 formed a water-network that interacted with D215 of 14-3-3. For **23**, a more extensive water-network was observed, which facilitated additional interactions between the compound, N42 (14-3-3) and the C-RAF residues and extended to the phospho-S259 residue. Interestingly, an overlay of the

three structures showed that the C-RAF residues (N262, V263, H264) also adopted different conformations with bigger changes in the case of **23** (Fig 5.2D, Fig S5.10).

Notably, the C-RAF sequence is internal and extends well beyond H264. Although shorter peptides are usually favored for crystallography, we hypothesized that longer peptides might be a better representation of the binding mode for full-length C-RAF. To this end, we solved a binary complex of 14-3-3 σ with a 12-mer C-RAF peptide and an additional ternary complex for **23** (Fig 5.2D, Fig S5.11). The presence of the compound significantly improved the density observed for the C-RAF sequence and allowed the visualization of M265, which “wrapped” around the main core of the compound, thus significantly covering the binding groove. An overlay of the two ternary complexes for **23** with the 10-mer and 12-mer peptides showed similar conformations for N262, V263, and H264; the latter was oriented toward the outer side of the binding groove in both structures. Consistently, helix 9 adopted a downward conformation in both structures with **23** (Fig 5.2D).

Structure-guided optimization led to the synthesis of double- and triple- halogenated derivatives to investigate additional electronic and steric effects at the protein-peptide interface (Fig 5.3A,B, Fig S5.12, Fig S5.5-5.7). While keeping the electron-withdrawing groups in the *p*-position, the introduction of additional *o*-F substituents was less favorable than *m*-F substituents. In *m*-position bulkier substituents than the F-group significantly decreased both the binding in the MS assay and the stabilization in the FA assay, indicating steric hindrance (tables S5.2-S5.3). Analogs **29** (*m*-F, *p*-Cl), **32** (*m*-F, *p*-CF₃) and **37** (*m*-F, *p*-Br) were representative examples of the observed SAR (Fig 5.3A,B, Fig S5.12). Consistent with previous observations, analog **37** bearing a *p*-Br group showed fast and cooperative binding in the MS assay. In the FA compound titrations analogs **29**, **32** and **37** showed similar EC₅₀ values (between 1-5 μ M). In protein titrations, **37** was slightly more potent than **29** and **32** (*app*K_d 92 nM compared to 100 nM and 140 nM, respectively). Triple-substituting analogs were also tested, however in protein titrations, led to lower stabilization compared to **37** (table S5.1, Fig S5.8).

Crystal structures with 14-3-3 σ and 10-mer C-RAF259 were solved for **29**, **32** and **37** (Fig 5.3C, Fig S5.13). In all cases, the compounds showed similar conformations with helix 9 adopting the downward conformation, as previously observed. The additional *m*-F group was positioned inwards and interacted with I219 of 14-3-3 (3.2Å). This orientation supported the observed SAR, where larger substituents in *m*-position were not tolerated. In *p*-position, the bulkier *p*-Br group formed the most favorable interactions, which correlated with faster kinetics and slightly improved stabilization in the biophysical assays. Although other electron-withdrawing groups in *p*-position could potentially interact with the same amino acid residues, differences in the size of the halogens (steric effects), as well as differences in electrostatic effects and σ holes³³ had a direct effect on the biophysical assays.

Thus, the (*m*-F, *p*-Br)-substitution pattern of **37** was kept constant in the next modification, which focused on the piperidine ring (Fig S5.14-S5.18). Substituted piperidines, spiro-cycles and fused ring systems were investigated. Most of these changes had a negative impact on the potency and in certain cases, on the cooperativity, due to increased apo binding in the absence of C-RAF. One notable exception was analog **70**, with a spiro-ring bearing an oxygen atom. Although in the MS assay, it showed less binding than the linear analog **37**, in the FA assay it showed promising stabilization ($EC_{50} = 2 \mu M$, $appK_d$ 58 nM, 95-fold stabilization) (Fig 5.3A,B, Fig S5.14-S5.18). An overlay of the crystal structures of **37** and **70**, showed that the spiro-ring was slightly shifted, compared to the piperidine ring, and formed a hydrogen bond with N42 (14-3-3) at 3.0Å. An extended water-network was observed, similar to analog **23**, reaching the phospho-S259 residue (Fig 5.3C, S5.19).

Regarding the warhead position, two variations of the chloroacetamide electrophile were tested. Both analogs were completely inactive in both assays, indicating potentially steric hindrance and unfavorable geometry that prevented access to C38. Warhead modifications were not pursued further, instead we focused our attention on potential synergistic effects, by combining favorable aryl ring substituents with spiro-cycles (Fig 5.4A,B, Fig S5.20, Fig S5.15-

S5.18). Analogs **78** and **79**, bearing the same oxo-spiro ring as **70** and *p*-Br and *p*-I substituents respectively, showed less binding in the MS assay, which correlated with their poor solubility in the assay conditions. In the FA assay, the two analogs showed potent stabilization with $EC_{50} = 2$ μ M in compound titrations and $appK_d$ 35-40 nM (fold-stabilization 210-240, table S5.1, Fig S5.15-S5.18). To determine whether the aryl ring or the spiro ring were more important for binding and stabilization we synthesized analogs **80** and **83**. Replacing the favorable halogen bonds with a *p*-CF₃ group in analog **80**, while maintaining the spiro-ring dramatically reduced the potency, indicating that the interactions between the aryl ring and V263 (C-RAF) were more significant than the hydrogen bond between the spiro ring and N42 (14-3-3). Similarly, analog **83**, with the same spiro-ring lacking the oxygen, showed slightly reduced potency, compared to **70** ($EC_{50} = 5$ μ M, $appK_d = 100$ nM, 83-fold stabilization). A pair of enantiomers bearing a 5-membered fused system was also tested (**86**, **89**). The *R*-enantiomer showed more binding in the MS assay and potent stabilization in the FA assay ($EC_{50} = 1$ μ M, $appK_d = 28$ nM, 300-fold stabilization), in stark contrast to the *S*-enantiomer, which despite binding in the MS assay, showed no stabilization in the FA assay.

Crystal structures with 14-3-3 σ and 10-mer C-RAF259 were solved for **78**, **79**, **80**, **83** and **86** and revealed unexpected changes (Fig 5.4C, S5.21). Although all ligands showed a similar binding mode as **70**, and the expected hydrogen bond with N42 (14-3-3) when the appropriate oxo-spirocycle was present, significant changes were observed in the interactions with 14-3-3. For **78**, **83** and **86**, helix 9 adopted the downward conformation, “clamping” the compounds and inducing conformational changes in D215, L218 and I219 (14-3-3). However, for **79** and **80**, the helix was in an upward conformation, thus the compounds lacked these interactions with 14-3-3. The C-RAF residues showed smaller dynamic changes, indicating that the formation of the halogen bond with V263 was not affected. This puzzling effect for helix 9 prompted us to further investigate the binding mode and we attempted to solve crystal structures with the longer 12-mer C-RAF peptide, which was more challenging. We obtained

crystals with **78** and **86**, and in contrast to their binding mode with the shorter peptide, in this case 14-3-3 helix 9 was in the upward conformation. Additionally, less density was visible for C-RAF residues with the longer peptide (V263 instead of H264 for the shorter peptide), hinting potentially to unfavorable steric effects from the spiro-cycle. The overall observations imply that the movement of helix 9 was not entirely compound-induced, but it could also be affected by the length of the peptide used for crystallography. Thus, the significance of some of these interactions needs to be interpreted with caution, since the positioning of helix 9 affects the formation of compound-protein interactions.

Selectivity assessment with biophysical assays

After establishing SAR for C-RAF259, we aimed at evaluating potential selectivity; first with the A-RAF and B-RAF inhibiting sites, then with the activating sites and finally in a broader selectivity panel of diverse 14-3-3 clients. We selected 11 compounds for biophysical assays (**22**, **23**, **29**, **32**, **37**, **70**, **78**, **79**, **80**, **83**, **86** and compound **8** as an inactive control, as it lacked an effect on C-RAF259).

The sequences of the RAF isoform inhibiting sites are largely conserved in the regions recognized by 14-3-3; A-RAF214 and C-RAF259 have the same sequence (+1 to +6 amino acids C-terminal to pS259, "TPNVHM"), whereas B-RAF365 differs at the +1 position (A instead of T). The +6/+7 residues for B-RAF also differ compared to A-RAF214 and C-RAF259. More differences occur in the sequences N-terminal to the phosphoserines, which are further away from the compound binding site and were expected to have a minor effect on the selectivity (detailed peptide sequences in the Methods). Based on the binding mode of the compounds, which interacted with the +1 (T), +4 (V) and +6 (M) residues of C-RAF, we thus expected similar binding/stabilization to A-RAF and lower binding/stabilization in the case of B-RAF.

Initially we tried to assess selectivity from a structural perspective, using 12-mer peptides for crystallography. We solved the 14-3-3 binary complexes for A-RAF214, B-RAF365

and ternary complexes with **22**, **23**, **78** and **86** for both peptides (Fig 5.5A, S5.22-S5.24) and compared them to the C-RAF259 structures. For the binary complexes, 14-3-3 helix 9 was in the upward conformation, similar to C-RAF259. The observed density for the RAF peptides extended to the +4 residue (V218 for A-RAF214 and V369 for B-RAF365). In the presence of piperidine-containing compounds **22** and **23** with A-RAF214, the +6 M residue was visible and helix 9 was in the downward conformation. For the spiro-cycles **78** and **86** the visible density did not extend beyond the +4 V residue and helix 9 was in the upward conformation. We noticed similarities between the ternary complexes with A-RAF and C-RAF, especially for **22** and **23** with a water network extending to the phosphate group. The key hydrogen bonds with backbone C38, R41, N42 (14-3-3) and the interactions with V218 and T215 (A-RAF) were consistent. For B-RAF365, for all four compounds the visible density stopped at the +4 V residue and helix 9 was in the downward conformation with the exception of **78**. The hydrogen bonds with backbone C38, R41, N42 (14-3-3) were consistent, however the compounds lacked specific interactions with the +1 A residue, which is typically considered significant for molecular recognition. As mentioned for C-RAF259, the importance of the movement of helix 9 was ambiguous, so instead we tried to correlate the ligand-protein and ligand-peptide interactions with biophysical data.

14-3-3 is considered to recognize between 2 and 20 amino acid residues on the client.³⁴ However, the ideal phospho-peptide length, representative of the recognition motif for various 14-3-3 clients has not been thoroughly investigated. Typically, the +1 residues were expected to be the main driving force for molecular recognition, but in the ternary complexes of our glues with 14-3-3/CRAF259, the +4 and +6 residues were also significantly contributing to binding. Thus, taking into account the differences in C-RAF259 ternary complexes with the 10- and 12-mer peptides, we reconsidered whether this effect might also extend to the biophysical assays and further tested the compounds with peptides with different lengths. Up to this point, to establish the SAR for C-RAF we used an 11-mer acetylated peptide for the MS assay and a 10-mer FAM-

labeled peptide for the FA assay. For a better comparison with crystallography, we tested the compounds again using 15-mer C-RAF peptides, before comparing them to A-RAF and B-RAF peptides. In the MS assay with the 15-mer C-RAF peptide, we noticed similar or increased binding for the piperidine-containing compounds (**22**, **23**, **29**, **32**, **37**), but a significant decrease in binding for the spiro-cycles (**78**, **79**, **80**, **83**, **86**) (table S5.2, Fig S5.25). In FA protein titrations, **22** and **23** appeared more potent with $appK_d$ of 5 nM and 2400-fold stabilization with the 15-mer peptide (30-47 nM, 179-280-fold stabilization with the 10-mer peptide). Compounds **29**, **32** and **37** showed similar $appK_d$ values with both peptides (126 – 154 nM), whereas, in agreement with the MS data, the spiro-cycles (**78**, **79**, **80**, **83**, **86**) showed 2-3-fold weaker stabilization ($appK_d$ 110 – 334 nM) (table S5.3, Fig S5.26). The data with the longer peptide were thus in support of the crystallography data; for the spiro-cycles fewer peptide residues were visible compared to compounds like **22**, where the density extended to the +6 M and the peptide tightly wrapped around the compound. This observation indicates potential steric hindrance of the longer RAF peptides with the spiro-cyclic compounds and testifies to additional opportunities for MG affinity and selectivity regulation.

To determine compound selectivity, we compared the 15-mer C-RAF data to 15-mer A-RAF. In the MS assay, the piperidine-containing compounds (**22**, **23**, **29**, **32**, **37**) showed significant binding, comparable or slightly higher to C-RAF, whereas low binding was observed for the spirocycles (table S5.2, Fig S5.27). In FA protein titrations, there were variations in the $appK_d$ values, however it was difficult to establish a clear trend (table S5.3, Fig S5.28). We also noticed that although the A-RAF and C-RAF 15-mer peptides had similar K_d values (8-10 μ M), the $appK_d$ in the presence of DMSO differed between A-RAF and C-RAF (3 μ M and 12 μ M, respectively). This difference did not affect ranking of the compounds with the individual peptides but resulted in higher fold-stabilization values for C-RAF.

In the case of B-RAF365 we tested two different peptides: a 10-mer and a 15-mer and the results varied considerably, indicating that the 10-mer peptide was probably not a good

representation of this PPI. In both assays, the compounds had a very small effect when the 10-mer peptide was used (table S5.2, S5.5, Fig S5.29-S5.30). The 15-mer B-RAF showed increased binding compared to the 10-mer. When comparing the two B-RAF peptides with A- and C-RAF 15-mer peptides, less binding was observed with the MS assay for the piperidine analogs and consistently low binding for the spiro-compounds (table S5.2, Fig S5.31). A similar effect was observed in the FA assay, where the compounds showed lower stabilization of B-RAF in comparison to the A- and C-RAF 15-mer peptides; **22** and **23** showed $appK_d$ of 5 nM and 448-fold-stabilization; or in other words, they were 3-fold weaker than C-RAF. Consistently, lower fold-stabilization was observed with the spiro-cycles (table S5.3, Fig S5.32). These observations were in good agreement with the crystal structures, where the compounds lacked interactions with the +1 residue and less peptide density was visible compared to A- and C-RAF.

The kinetic fundamentals behind RAF isoform selectivity were evaluated using surface plasmon resonance (SPR) experiments. 14-3-3 σ , tagged with a Twinstrep-tag, was immobilized on a Strep-Tactin XT coated SPR chip. Subsequently, a 2-fold dilution series of acetylated 12-mer C-RAF-, A-RAF, and B-RAF peptides were injected. The analysis of these peptides' binary interactions with 14-3-3 σ revealed K_d values ranging between 2.0 – 2.7 μ M, determined through both kinetic- and affinity-based data fitting (Fig 5.5B, S5.33). The affinities of A-RAF and B-RAF were consistent with results from FA experiments, while for C-RAF, the K_d value measured by SPR was lower compared to FA (2.6 μ M versus 10 μ M, respectively), though this difference might be due to the fast k_{off} , which makes precise measurement difficult. To compare the kinetic parameters of the binary interactions with those of the molecular glue-bound state of 14-3-3 σ , the covalent bond between the chloroacetamide warhead of the compounds and C38 of 14-3-3 σ was formed by overnight incubation in the presence of the C-RAF peptide. This resulting complex was immobilized on the chip and extensively washed to remove the C-RAF peptide. Afterwards, the kinetics of RAF peptides binding to the 14-3-3 σ /molecular glue complex were analyzed. In the presence of **23**, the association rate (k_{on}) increased 100-fold for all three RAF

isoforms compared to their binary interactions, suggesting that the 14-3-3 σ /**23** interface is recognized more rapidly by the RAF peptides than 14-3-3 σ alone. Furthermore, the k_{off} significantly decreased in the presence of **23**, yielding $\text{App}K_{\text{d}(\text{compound})}$ values in the low nanomolar range (Fig 5.5B, S5.34-S5.36). Notably, the presence of the molecular glue resulted in mass-transport limitations, driven by the high association rate and peptide rebinding. These effects were accounted for during the fitting process. The affinity increase was most pronounced for A-RAF and C-RAF (1000-fold and 2000-fold, respectively), whereas a weaker increase (400-fold) was observed for B-RAF, aligning with the selectivity measured in FA experiments. Since the shift in k_{on} was similar for all RAF isoforms, the difference in affinity among isoforms was attributed to a faster k_{off} for B-RAF compared to A-RAF and C-RAF. This indicated that the interactions of +1 and +6 residues (+1A and +6I for B-RAF, +1T and +6M for A-, C-RAF) with **23** were crucial for decreasing the k_{off} , thereby increasing the residence time of RAF binding to 14-3-3 σ . In addition, **22** induced a comparable change in k_{on} for A-RAF and C-RAF binding, relative to **23**. However, the k_{off} was increased, leading to a reduced apparent affinity, in line with FA experiments. While no notable difference was observed between **22** and **23** for B-RAF, the apparent affinity of B-RAF to 14-3-3/**22** complex remained the weakest among the RAF isoforms (Fig. S5.34-S5.36).

Regarding the activating A-RAF582, B-RAF729 and C-RAF621 sites, all isoforms contain the same +1 to +4 residues (EPSL). The +1 glutamic acid in a published binary complex with C-RAF⁶²¹ formed an ionic bond with K122 of 14-3-3 (PDB: 4IEA). We hypothesized that this bond could not be disrupted by the compounds, and it would also prevent the compounds binding, due to repulsive electrostatic interactions with the sulfonyl group of the compounds. This hypothesis was easily confirmed with FA protein titrations, where no stabilization was observed (Fig S5.37-S5.39).

To evaluate selectivity more broadly *in vitro*, we tested **22** and **23** in a selectivity panel that included 80 peptides representing a diverse set of 14-3-3 clients (Fig S5.40). Briefly, the 14-

3-3 σ protein concentration was set at 20% of the maximum effect for each individual peptide. The compounds were incubated overnight at calculated EC₉₀ concentrations (under this assay's conditions: 10.5 μ M for **22** and **23**) in the presence of 14-3-3 and 100 nM client peptides. A client peptide with a high 14-3-3 σ concentration (50 μ M, maximum effect) was used as reference for normalization. As a qualitative output, clients were classified as hits when a positive change in anisotropy of more than 15 units (ca. 15%) was observed. Both compounds showed remarkable selectivity; for **23** only four clients emerged as hits (SOS1, TAZ, KC1A, TSC2), and six for **22** (SOS1, TAZ, ARGH2, H31, KC1A, TSC2). Strikingly, there were no apparent similarities in the peptide sequence for these off-target hits, except for the +2 P, which is a very common residue in 14-3-3 clients (Fig S5.41A). The lack of similarities in the peptide sequences suggested that conformational adaptivity was a key factor in the formation of ternary complexes and molecular recognition among different 14-3-3 clients. FA compound titrations were then performed for the common hits SOS1, TAZ, KC1A, TSC2 (Table S5.4, Fig S5.41B) and FA protein titrations were performed for SOS1, TAZ and TSC2 (Table S5.5, Fig S5.41C). The assay conditions and the C-RAF259 peptide length differed from the ones used to establish the SAR, leading to an EC₅₀ = 5 μ M (vs 1 μ M routinely seen with the standard conditions). Nevertheless, the compounds showed greater fold-stabilization and lower EC₅₀ values for C-RAF, with the second-most prominent hit being TAZ.

Evaluation in cell assays

We further evaluated 10 compounds in cellular assays; the same compounds tested for RAF isoform selectivity, excluding the weak analog **80**. Compound **8**, as previously, served as negative control. A NanoBRET assay was developed to measure binding of full-length 14-3-3 σ to C-RAF in HEK293T cells.³⁵ HEK293T cells only weakly express 14-3-3 σ , express a low level of C-RAF, and have an inactive MAPK pathway; thus, the transfected C-RAF and 14-3-3 σ would be monitored without interference from endogenous expression. C-RAF was tagged with

NanoLuciferase and 14-3-3 σ was tagged with HaloTag/fluorescent HaloTag ligand. Stabilization of the interaction with a molecular glue was expected to lead to an increase in the BRET signal. The tested compounds resulted in a range of increased fold-stabilization (up to 1.6) and cellular EC₅₀ values between 0.1 and 1.2 μ M (Fig S5.42A, table S5.6). Piperidine-containing compounds **22** and **23** showed similar EC₅₀ values (0.20 and 0.18 μ M respectively) and fold-stabilization of 1.5 and 1.6-fold, respectively (Fig 5.6A). For the spiro-cycles **70** and **78**, cellular fold-stabilization was lower (1.3 and 1.4, respectively), in good agreement with the signal window observed in FA data. No increase in the BRET signal was observed for the negative control compound **8**. The stabilization effect was specific to C38 of the σ isoform of 14-3-3 – the molecular glues did not increase the BRET signal when the native C38 was mutated to N, the residue in the other 14-3-3 isoforms (Fig S5.42B).

Binding of full-length 14-3-3 σ /C-RAF was further validated in HEK293T cells through co-immunoprecipitation (co-IP) at 10 μ M compound. NRAS Q61L, an activating mutation of the MAPK pathway, FLAG-C-RAF, and HA-14-3-3 σ were transfected and IP was performed with anti-FLAG beads. We measured a 5.3-fold increase for **23**, 5.1-fold for **22** and 1.6-fold for **78** in a 14-3-3 σ pull-down with FLAG-C-RAF normalized to FLAG band intensity (Fig 5.6B). This effect was not observed without transfecting C-RAF, as shown for **22** (Fig S5.43A).

In addition to binding assays, we quantified the effect of the molecular glues on a 14-3-3 specific function – the protection of phosphorylated residues on the client proteins from phosphatases (Fig 5.6C, table S5.6). By western blots in HEK293T cells, with 10 μ M compound we measured 2.4-fold and 3.2-fold increases in phosphorylation of the C-RAF259 site relatively to total C-RAF for **22** and **23**, respectively. The spirocycles **70** and **78** showed a slightly smaller effect (2.1-fold and 2.2-fold, respectively). In MIA PaCa-2 cells, a KRAS G12C dependent cell line, compounds **22** and **23** showed a 1.4-fold and 1.5-fold increase in endogenous C-RAF pS259 levels relative to total C-RAF (Fig S5.43B, table S5.6).

As mentioned in the introduction, the activation mechanism of C-RAF is assumed to proceed in a similar manner as B-RAF, where the first step is the translocation to the membrane and the interaction with RAS. To study the effect of the molecular glues on this step of the pathway, we developed a NanoBRET assay to measure the interaction between N-RAS Q61L and C-RAF in HEK293T cells (Fig 5.6D). For the quantification of the N-RAS/C-RAF NanoBRET, we set the DMSO-treated cell-signal as 100% and the signal for NRAS/C-RAF R89L, a C-RAF mutation that prevented C-RAF from interacting with RAS, as the 0% control.^{28,36} The stabilization of the interaction between 14-3-3 σ and C-RAF259 by the molecular glues was expected to compete for the interaction of N-RAS/C-RAF. As expected, treatment with **23** resulted in 75% signal reduction of the N-RAS Q61L/C-RAF interaction and 52% with **22**. For **70** and **78** no clear trend was observed. Compound **8** did not affect N-RAS Q61L/C-RAF binding.

The final activation step of the pathway includes the dimerization of RAF kinase domains. Dimer formation is quite complex and different RAF homo- and hetero-dimers can occur, depending on the cell type or cancer type. We aimed to study potential effects of the molecular glues on RAF-dimer formation using a NanoBRET assay. The C-RAF/C-RAF NanoBRET assays were performed with N-RAS Q61L to ensure pathway activation³⁷ (Fig 5.6E). We observed 43% decrease in C-RAF homodimer formation with **22**, 47% decrease with **23**, and ~20% for **70** and **78**.

To test the downstream MAPK effects of stabilizing 14-3-3/C-RAF, we developed a Lumit immunoassay to analyze the levels of pERK. The secondary antibody pair, lysis buffer, and primary antibody concentration were optimized for the ERK/pERK antibodies used (data not shown). 14-3-3 σ and C-RAF were transfected in a 1:1 ratio in HEK293T cells. Cells were treated with **23**, a MEK inhibitor (Trametinib, 100 nM), or a pan-RAF inhibitor (LY3009120, 100 nM) in 0.5% FBS FluoroBrite DMEM for 24 hours, followed by EGF stimulation (100 ng/ μ L, 30 minutes) before performing the Lumit immunoassay protocol. We corrected for cell number

using the fluorogenic live cell GF-AFC substrate. Data was normalized to DMSO-treated samples. Compound **23** dosed at 10 μ M inhibited the phosphorylation of ERK to a similar extent as the MEK inhibitor (Trametinib) and slightly increased the inhibition of pERK compared to the pan-RAF inhibitor (LY3009120); the IC₅₀ value for **23** was 4.4 μ M (Fig 5.6F). The Lumit data suggested stabilization of 14-3-3/C-RAF was an effective target to modulate the MAPK pathway.

We developed additional NanoBRET assays to quantify compound binding to 14-3-3 σ /A-RAF and 14-3-3 σ /B-RAF and determined RAF selectivity in a cellular context (Fig 5.6G, 5.6H, Fig S5.44, table S5.7). Compounds **22** and **23** showed binding in the 14-3-3 σ /A-RAF NanoBRET, giving 1.6-fold and 1.7-fold increase, respectively, at 10 μ M; dose-response data did not plateau. **70** was inactive and **78** showed an effect only in the two highest compound concentrations. Similarly, in the 14-3-3 σ /B-RAF NanoBRET, increased binding was observed in the presence of **22** and **23** (1.40-fold increase) and a minor effect for **70** and **78**. Quantitatively, **22** and **23** showed comparable fold-stabilization for A- and C-RAF, whereas **70** and **78** showed significant selectivity for C-RAF, especially in lower compound concentrations. In all cases, binding was dependent on C38, as the compounds were inactive with the C38N mutant (data not shown). We also tested the formation of C-RAF heterodimers, A-RAF/C-RAF and B-RAF/C-RAF. Compound **23** exhibited inhibition of heterodimers, decreasing the A-RAF/C-RAF interaction by 23% and the B-RAF/C-RAF dimer by 15% (Fig 5.6I).

The compound effect on the phosphorylation protection of the inhibiting A-RAF pS214 site was also quantified and compared to the effect on the C-RAF pS259 site (Fig S5.45, table S5.7). Compound **23** resulted in 1.3-fold increase in the case of A-RAF and 3.2-fold increase in the case of C-RAF. Compound **70** did not increase the A-RAF phosphorylation, whereas it showed 2.1-fold increase for C-RAF. A similar experiment was performed for the B-RAF pS365 site; however, the quantification of the Western blots was inconsistent, potentially due to the antibody used (data not shown).

Taken together, the cell data suggests on-target activity on the C-RAF259/14-3-3 σ complex for the most potent compounds **22** and **23**, a protective effect on C-RAF259 phosphorylation, disruption of C-RAF active kinase dimers, and decrease in phosphorylation of the downstream MAPK target pERK. Regarding SAR, the presence of a piperidine ring was more favorable in cell assays, compared to the spirocycles, supporting our crystal structures, where interactions occur with M265 (+6 residue). Interestingly, while the piperidine containing **22** and **23** favored C-RAF over A- and B-RAFTs, the spirocycles showed an even stronger selectivity for C-RAF.

Discussion

In summary, we describe a fragment-merging approach for the development of cell-active molecular glues targeting the inhibitory 14-3-3 σ /C-RAF259 complex. Validation of the compounds in multiple biophysical assays, from mass spectrometry to fluorescence anisotropy and surface plasmon resonance allowed the quantification of binding, stabilization, and kinetics, elucidating the factors involved in the formation of cooperative ternary complexes. The establishment of SAR for the 14-3-3 σ /C-RAF259 complex was achieved with biophysical assays using phospho-peptides as representations of the full-length client. MS and FA assays were in good agreement in establishing SAR for a given peptide length for C-RAF259. However, we found that the longer C-RAF peptides led to differentiated results, especially in the case of the spirocyclic compounds, where the rigid part of the compound was in close proximity to the flexible region of the peptides. These effects were not exclusively steric, based on space-filling models; rather, we hypothesized that entropy and/or less optimal interactions with the +4 and +6 peptide residues were also involved. The peptide length also had a strong effect on compound evaluation for B-RAF, indicating again that residues involved in molecular recognition can extend well beyond the +1 – here, as far as the +4 and +6 residues. By comparing data using peptides of different lengths, we aimed to emphasize that for 14-3-3 clients where the

phosphorylation site lies on a highly dynamic loop, conformational adaptivity can have a significant effect both on the biophysical assays and crystallography. Thus, in cases where the sequence is internal the length should be chosen carefully.

From crystal structures with different RAF peptide lengths, we observed differences not only in the peptide conformation, which was expected, but also in the positioning of helix 9 of 14-3-3. The movement was not always compound- or peptide-specific, but it could have a significant effect on the formed ligand-protein interactions. It is noteworthy that the downward positioning of this helix is consistent with a cryo-EM structure of 14-3-3/B-RAF (PDB 6NYB), indicating that it is unlikely to be a crystallographic observation (Fig S5.46). Full length, inhibitory 14-3-3/C-RAF cryo-EM structures are still missing, thus a direct comparison with our crystal structures is not feasible yet.

We further evaluated the 10 most potent compounds from the biophysical assays in cell assays with the full-length RAF clients. We noticed good potency correlation in most of the cases. Compounds **22** and **23** were the most potent in cell assays and stabilized both the 14-3-3/C-RAF and 14-3-3/A-RAF complexes, whereas spirocycles **70** and **78** showed greater selectivity for C-RAF in the NanoBRET assay and the protection of phosphorylation. Taken together, our cell data indicate that the molecular glues showed on target activity, stabilizing the 14-3-3 σ /C-RAF interaction and protecting the pS259 site from dephosphorylation. Mechanistically, we see the expected inhibition of the N-RAS/C-RAF interaction, as well as C-RAF dimerization in an activated pathway for the most potent analogs. These data indicate that the molecular glues targeted the “auto”inhibited 14-3-3/C-RAF monomer, thus shifting the equilibrium away from the active form of C-RAF. The effect on the structurally similar A-RAF isoform was chemotype-dependent, with piperidine compounds **22** and **23** being less selective compared to spirocycles **70** and **78**. The observed differences in A-RAF stabilization are intriguing, considering the sequence similarity with C-RAF on the 14-3-3 binding groove. Possible explanations could be differences in the N-terminus, dynamics, or regulation on a cellular level.

The similarity of the A-RAF isoform could explain the observed inhibition of A-RAF/C-RAF complexes with **23** whereas the B-RAF/C-RAF dimer was minimally affected.

Quantifying the downstream effects of stabilizing 14-3-3/C-RAF, **23** prevented the phosphorylation of ERK to similar levels as other modes of MAPK modulation. However, **23**, was slower to modulate the pathway than Trametinib or LY3009120, taking 18 to 24 hours compared to 3 hours. We hypothesize that the time delay is due to the slow reaction of the covalent warhead, rather than the intrinsic biology of 14-3-3/C-RAF stabilization. The kinetics do complicate the biological analysis, since the MAPK pathway includes multiple feedback loops and varied dimer formation. These might explain why we do not see full inhibition of NRAS/C-RAF, nor do we observe on-target cell death in cancer cells like MIA PaCa-2. Additionally, recent literature suggests that C-RAF has roles independent from the MAPK signaling pathway, which we have not explored. For instance, Venkatanarayan *et al.* showed that C-RAF depletion inhibits tumor growth but not ERK signaling in KRAS mutant cells, and upon C-RAF loss, A-RAF dimers promote ERK signaling, leading to cell-cycle arrest.²³ Sanclemente *et al.* reported that in KRASG12V/Trp p53 mutant lung tumors, systematic abrogation of C-RAF expression did not inhibit canonical MAPK signaling, which in contrast to MEK inhibitors, resulted in limited toxicities.³⁸ For these types of tumors, significant levels of phospho-MEK and phospho-ERK expression were retained, despite the elimination of C-RAF expression in most of their cells.³⁸ Alternatively, the C-RAF preference of our glues may limit phenotypic changes. Müller *et al.* showed that selective knockdown of a single RAF isoform had at best partial effects on the phosphorylation levels of MEK1/2 and ERK1/2 indicating functional redundancy and/or compensatory mechanisms between the RAF isoforms, while pan-RAF activity affected PI3K-dependent signaling.²⁴ Deciphering the precise effect of these molecular glues on the C-RAF biology and broader pathway changes is beyond the scope of the current study. We believe, however, that our molecular glues will be useful chemical biology tools that extend beyond direct inhibition and focusing on regulation of protein-protein interactions. Additionally, our cell-active

compounds could be useful starting points for medicinal chemistry campaigns aiming either at pan-RAF/14-3-3 glues or RAF-isoform specific glues to further elucidate pathway biology.

Methods

Protein expression and purification

The 14-3-3 σ isoform (full-length for mass spectrometry and fluorescence anisotropy assays, ΔC for crystallography) with an N-terminal His6 tag was expressed in Rosetta™ 2(DE3)pLysS competent *E. coli* (Novagen) from a pPROEX HTb expression vector. After transformation following manufacturer's instructions, single colonies were picked to inoculate 30 mL precultures (LB), which were added to 1.5 L terrific broth (TB) medium after overnight growth at 37°C, 250 rpm. Expression was induced upon reaching OD₆₀₀ 1.9–2.1 by adding 400 μ M IPTG. After overnight expression at 30 °C, 150 rpm, cells were harvested by centrifugation at 6,500 rpm, resuspended in lysis buffer (50 mM HEPES pH 7.5, 500 mM NaCl, 20 mM imidazole, 10% glycerol, 1 mM TCEP), and lysed by sonication. The His6-tagged protein was purified by Ni-affinity chromatography (Ni-NTA Agarose, Invitrogen) (Wash buffer 50 mM HEPES pH 7.5, 500 mM NaCl, 20 mM imidazole, 1 mM TCEP; Elution buffer 50 mM HEPES pH 7.5, 500 mM NaCl, 500 mM imidazole, 1 mM TCEP) and analyzed for purity by SDS-PAGE and Q-ToF LC/MS. The protein was buffer exchanged (Storage buffer 25 mM HEPES pH 7.5, 150 mM NaCl, 1 mM TCEP) and concentrated to ~16 mg/mL and aliquots flash-frozen for storage at –80 °C. The ΔC variant was truncated at the C-terminus after T231 to enhance crystallization and after the first Ni-affinity chromatography column, the construct was treated with TEV protease to cleave off the His6 tag during dialysis (25 mM HEPES, pH 7.5, 200 mM NaCl, 5% glycerol, 10 mM MgCl₂, 250 μ M TCEP) overnight at 4 °C. The flow-through of a second Ni-affinity column was subjected to a final purification step by size exclusion chromatography (Superdex 75 pg 16/60 size exclusion column (GE Life Science) (SEC buffer: 25 mM HEPES pH 7.5, 100 mM NaCl, 10 mM

MgCl₂, 250 μM TCEP). The protein was concentrated to ~60 mg/mL, analyzed for purity by SDS-PAGE and Q-ToF LC/MS and aliquots flash-frozen for storage at -80 °C.

For SPR, the 14-3-3 σ isoform (FL) with a N-terminal His6-tag and C-terminal Twinstrep-tag was expressed in BL21(DE3) competent E.coli (Novagen) from a pET28a expression vector. After transformation, following manufacturer's instructions, single colonies were picked to inoculate 30mL precultures (LB), which were added to 1.5 L terrific broth (TB) medium after overnight growth at 37 °C, 250 rpm. Protein expression was induced at OD₆₀₀ ~ 0.8 by adding isopropyl β-D-1-thiogalactopyranoside (IPTG; 0.4 mM) and cells were harvested by centrifugation (10 min, 4 °C, 16,000 × g) after overnight expression (18 °C, 140 rpm). Pellets were resuspended in wash buffer (50 mM Tris pH 8.0, 300 mM NaCl, 12.5 mM imidazole and 2 mM β-mercaptoethanol (βME)). After homogenizing the cells (40 bar, Emulsiflex-C3 homogenizer), the soluble fraction was collected by centrifugation (30 min, 4 °C, 40,000 × g) and loaded onto a Ni²⁺-affinity column pre-equilibrated with wash buffer. After a washing step (wash buffer + 20 mM imidazole), the bound protein was eluted with 200 mM imidazole. After Ni-affinity chromatography the elution fraction was loaded on a StrepTactin XT column (Iba lifesciences) (wash buffer 100 mM Tris pH 8, 150 mM NaCl, 1 mM EDTA; elution buffer 100 mM Tris pH 8, 150 mM NaCl, 1 mM EDTA, 50 mM biotin) and analyzed for purity by SDS-PAGE and Q-ToF LC/MS. The protein was buffer exchanged (storage buffer 25 mM HEPES pH 8, 100 mM NaCl, 10 mM MgCl₂, 0.5 mM TCEP) and concentrated to 34.8 mg/mL and aliquots flash-frozen for storage at -80 °C.

Peptides

Peptides for mass spectrometry assays were purchased from Elim Biopharmaceuticals, Inc. (Hayward,CA). Peptides for X-ray crystallography were purchased from GenScript Biotech Corp. Fluorescein-labeled (5-FAM) peptides for fluorescence anisotropy assays were purchased from Elim Biopharmaceuticals, Inc. (Hayward,CA) or GenScript Biotech Corp.

The following acetylated peptides were used in the MS assay:

C-RAF pS259 (11-mer): *Ac*-RQRST{pS}TPNVH-CONH₂,

C-RAF pS259 (15-mer): *Ac*-SQRQRST{pS}TPNVHMHV-CONH₂,

A-RAF pS214 (15-mer) *Ac*-LQRIRST{pS}TPNVHMHV-CONH₂,

B-RAF pS365 (10-mer): *Ac*-DRSS{pS}APNVH-CONH₂,

B-RAF pS365 (15-mer): *Ac*-GQRDRSS{pS}APNVHIN-CONH₂.

The following 5-FAM-labeled peptides were used in the FA assay:

C-RAF pS259 (10-mer): 5-FAM-QRST{pS}TPNVH-CONH₂,

C-RAF pS259 (15-mer): 5-FAM-SQRQRST{pS}TPNVHMHV-CONH₂,

A-RAF pS214 (15-mer) 5-FAM-LQRIRST{pS}TPNVHMHV-CONH₂,

B-RAF pS365 (10-mer): 5-FAM-DRSS{pS}APNVH-CONH₂,

B-RAF pS365 (15-mer): 5-FAM-GQRDRSS{pS}APNVHIN-CONH₂,

A-RAF pS582 (15-mer): 5-FAM-PKIERSA{pS}EPSLHRT-CONH₂,

B-RAF pS729 (15-mer): 5-FAM-PKIHRSR{pS}EPSLNRA-CONH₂,

C-RAF pS621 (15-mer): 5-FAM-PKINRSA{pS}EPSLHRA-CONH₂.

The following peptides were used for crystallography:

C-RAF pS259 (10-mer): QRST{pS}TPNVH,

C-RAF pS259 (12-mer): RQRST{pS}TPNVHM (used also for SPR),

A-RAF pS214 (12-mer): RIRST{pS}TPNVHM (used also for SPR),

B-RAF pS365 (12-mer): RDRSS{pS}APNVHI (used also for SPR).

The following peptides were used in the FA selectivity panel:

C-RAF pS259: FAM[Ahx]RQRST{pS}TPNVHMHVS-CONH₂,

SOS1 pS1161: FITC-Ahx-PRRRPE{pS}APAESS-CONH₂,

TAZ pS89: FITC-Ahx-QHVRSH{pS}SPASLQLGTGA-CONH₂,

KC1A pS218: FAM-Ahx-LMYFNRT{pS}LPWQGLK-CONH₂,

TSC2 pS1254: FAM-Ahx-LYKSL{pS}VPAASTAK-CONH₂,

ARHG2 pS886: FAM-Ahx-PVDPRRR{pS}LPAGDAL-CONH₂.

Intact mass spectrometry assay

Mass spectrometry dose response assays were performed on a Waters Acquity UPLC/ Xevo G2-XS Q-ToF mass spectrometer. A Waters UPLC Protein BEH-C4 Column (300 Å, 1.7 µm, 2.1 mm x 50 mm) was used to desalt the samples prior to application on the mass spectrometer. For 19-point MS dose responses, 50 mM compound stocks in DMSO were serially diluted in 3-fold increment in a master plate, then 1000 nL of the compound solutions were transferred in the assay plates. Master mixes containing 100 nM full-length wild type 14-3-3σ in the absence or presence of either 18 µM C-RAF259 or 16 µM A-RAF214 or 5 µM B-RAF365 were then dispensed into 384 well plates (Greiner Bio-One, catalog number 784201). Assay buffer was TRIS (10 mM, pH 8.0) and final volume per well was 50 µL, with final top concentration of compounds dose response series at 1 mM. The reaction mixtures were incubated for 1h at rt before subjected to MS. Four measurements (1h, 8h, 16h, 24h) were performed for time-course experiments. The injection volume for each sample was 6 µL. 24 µL of sample were needed for the time-course experiments, so the total volume in the assay plate was adjusted to 50 µL, to account for the dead volume in the injections. Data collection and automated processing followed a custom workflow, as previously described.³⁹ z-Plots were created using GraphPad Prism with the log(agonist) vs. response (variable slope, four parameters) fitting model.

Fluorescence anisotropy measurements

N-terminal fluorescein-labeled peptides (5-FAM), 14-3-3σ FL protein, the compounds (50 mM stock solution in DMSO) were diluted in buffer (10 mM HEPES, pH 7.5, 150 mM NaCl, 0.1% Tween20, 1 mg/mL Bovine Serum Albumin (BSA; Sigma-Aldrich). Final DMSO in the assay was

less than 1%. Dilution series of 14-3-3 proteins or compounds were made in black, round-bottom 384-microwell plates (Greiner Bio-one 784900) in a final sample volume of 10 μ L in triplicates.

For *compound titrations* echo acoustic dispensing was used to prepare 2-fold serial dilution series. A master mix containing 10 nM fluorescein-labeled C-RAF-pS259 peptide and 5 μ M full length 14-3-3 σ (concentration at EC₂₀ value of the protein-peptide complex) in buffer (10 mM HEPES, pH 7.5, 150 mM NaCl, 0.1% Tween20, 1 mg/mL BSA) was dispensed in the assay plates. The final volume per well was 10 μ L, with final top concentration of compounds dose response series at 1mM. Each compound was measured in triplicates, in two independent experiments. Fluorescence anisotropy measurements were performed directly and after overnight incubation at room temperature. The high protein concentration used in this assay limits the sensitivity for potent compounds (EC₅₀ < 1 μ M). *Protein titrations* were made by titrating 14-3-3 σ in a 2-fold dilution series (starting at 250 μ M) to a mix of fluorescein-labeled peptide (10 nM) and DMSO or compound (100 μ M). Fluorescence anisotropy measurements were performed after overnight incubation at room temperature. Protein titrations were more sensitive than compound titrations for highly potent compounds and the *appK_D* values obtained were not limited by the protein concentration used. Fluorescence anisotropy values were measured using a Molecular Devices ID5 plate reader (filter set lex: 485 $\pm$ 20 nm, lem: 535 $\pm$ 25 nm; integration time: 50 ms; settle time: 0 ms; shake 5 sec, medium, read height 3.00 mm, G-factor = 1. Data reported are at endpoint. EC₅₀ and apparent K_d values were obtained from fitting the data with a four-parameter logistic model (4PL) in GraphPad Prism 10 for Windows. Data was obtained and averaged based on two independent experiments.

Selectivity panel

For single point measurements: N-terminal fluorescein-labeled peptides (5-FAM) and 14-3-3 σ FL protein were diluted in buffer (10 mM HEPES, pH 7.4, 150 mM NaCl, 2 mM MgCl₂, 0.1% Tween20, 1 mg/mL Bovine Serum Albumin (BSA), 100 μ M β -mercaptoethanol (β -ME)) transferred to black, round-bottom 384-microwell plates (Greiner Bio-one 784900). The concentration of the FAM-peptide panel was 100 nM, and the 14-3-3 σ protein concentration was set at 20% of the maximum effect for each individual peptide. Compounds 22 and 23 were dispensed to the 384 well plate at 10.5 μ M using the Tecan D300e digital dispenser. As reference a TAZ peptide with a high 14-3-3 σ concentration (50 μ M, maximum effect) was used. Plates were incubated overnight at room temperature, after which anisotropy values were measured using a BioTek Synergy H1 microplate reader (excitation at 485 nm, emission at 528 nm). The signal plotted is the anisotropy value with compound minus the anisotropy value without compound, giving the anisotropy change (Δ AU). *Compound titrations* were performed to follow-up the single point selectivity panel. A mastermix containing 10 nM of FAM-labeled peptides (C-RAF, SOS1, TAZ, KC1A, TSC2) and the corresponding 14-3-3 σ concentration at EC₂₀ of the protein-peptide complex in buffer (10 mM HEPES, pH 7.4, 150 mM NaCl, 2 mM MgCl₂, 0.1% Tween20, 1 mg/mL BSA, 100 μ M β -ME) was dispensed in assay plates. Compounds 22 and 23 were dispensed with the Tecan D300e digital dispenser in a 3-fold dilution series (starting at 250 μ M). Plates were incubated overnight at room temperature, after which anisotropy values were measured using a BioTek Synergy H1 microplate reader (excitation at 485 nm, emission at 528 nm). *Protein titrations* were performed by titrating 14-3-3 σ FL protein in a 2-fold dilution series (starting at 300 μ M) to a mix of 10 nM FAM-labeled peptides (CRAF, SOS1, TAZ, TSC-2) and 100 μ M of compounds 22 and 23, or DMSO in buffer (10 mM HEPES, pH 7.4, 150 mM NaCl, 0.1% Tween20, 1 mg/mL BSA). After overnight incubation, anisotropy values were measured using a Tecan Infinite F500 plate reader (filter set

excitation: 485±20 nm, emission: 535±25 nm, mirror: Dichroic 510, flashes: 20, integration time: 50 ms, settle time: 0 ms, gain: optimal).

Surface plasmon resonance

The SPR experiments were performed at 25°C using a Biacore X100 and a 200 nm Strep-Tactin XT derivatized linear polycarboxylate hydrogel chip, medium charge density (XanTec Bioanalytics). All proteins and peptides were dissolved in fresh running buffer prepared with ultrapure water and filtered through a 0.2 µm filter (10 mM HEPES pH 7.4, 200 mM NaCl, 50 µM EDTA, 0.005% P20). First the surface was conditioned with a 1 min injection of 3 M Guanidine HCl. Then, the recombinant 14-3-3σ-Twinstrep protein (250 nM) was captured on flow cell 2 of the sensor chip at a flow rate of 10 µL/min for 2 minutes, which resulted in a capture level of 1000 RU. For the ternary interaction, 14-3-3σ-Twinstrep protein (250 nM) was first incubated overnight with 1 µM Ac-C-RAF peptide and 20 µM compound prior to immobilization to the chip. The bound C-RAF peptide was washed away using running buffer flowed over the chip for 15 min. Flow cell 1 was left blank as a reference surface. After immobilization of the protein, the Biacore X100 was primed with running buffer. Multi-cycle kinetic measurements were conducted at a flow rate of 30 µL/min. A 2-fold dilution series of analyte (Ac-A-, B-, C-RAF peptides) in running buffer were injected over the sensor chip for 2 min, followed by dissociation of 3 min (binary interaction), 15 minutes (ternary interaction). For the binary interaction, the highest concentration of the RAF peptides was 50 µM, and for the ternary interactions this was 250 nM. Between cycles of one multi-cycle measurement, no regeneration step was performed due to complete dissociation of the analyte. After a measurement, the chip was regenerated by 2 times 30 sec injections of 3 M Guanidine HCl. The data was corrected by double subtracting to the reference surface (flow cell 1) and buffer injection and analyzed using 1:1 interaction fitting model with the BIA evaluation software (2020).

X-ray crystallography data collection and refinement

The 14-3-3 σ Δ C protein, Ac-C-RAF/B-RAF/A-RAF and compounds (50 mM stock in DMSO) were dissolved in complexation buffer (25 mM HEPES pH=7.5, 2 mM MgCl₂ and 100 μ M TCEP) and mixed in a 1:2:3 or 1:3:5 molecular stoichiometry (protein : peptide : compound) with a final protein concentration of 11 or 12 mg/mL. The complex was set-up for sitting-drop crystallization after overnight incubation at 4 °C, in a custom crystallization liquor (0.05 M HEPES (pH 7.1, 7.3, 7.5, 7.7), 0.19 M CaCl₂ 24-29% PEG400, and 5% (v/v) glycerol). Crystals grew within 10-14 days at 4 °C. Crystals were fished and flash-cooled in liquid nitrogen. X-ray diffraction (XRD) data were collected at the European Synchrotron Radiation Facility (ESRF Grenoble, France, beamline ID23-1, ID30A-3/MASSIF-3, or ID23-2). Initial data processing was performed at ESRF using autoPROC after which pre-processed data was taken towards further scaling steps, molecular replacement, and refinement.

Data was processed using CCP4i2 suite (version 8.0.003). After indexing and integrating the data, scaling was done using AIMLESS. The data was phased with MolRep, using PDB 3IQU as template. The presence of co-crystallized ligands was verified by visual inspection of the Fo-Fc and 2Fo-Fc electron density maps in COOT (version 0.9.6). If electron density corresponding to the co-crystallized ligand was present, its structure and restraints were generated using AceDRG. After building in the ligand, model rebuilding and refinement was performed using REFMAC5. The PDB REDO server (pdb-redo.edu) or phenix.refine from Phenix software suite (version 1.19.2-4158) was used to complete the model building and refinement. The images were created using the PyMOL Molecular Graphics System (Schrödinger LLC, version 4.6.0).

NanoBRET assays

NanoBRET assays were performed as previously described.³⁵ Briefly, eight constructs were generated, and combination and ratio tests were performed in HEK293T cells for development

of the initial 14-3-3 σ /C-RAF NanoBRET. All NanoBRET assays were performed in HEK293T cells at a 1:10 transfection ratio of NanoLuc:HaloTag plasmids. Compounds were tested in a 2-fold dilution series starting at 10 μ M with C-RAF-NanoLuc (C-terminal tag)/14-3-3-HaloTag (C-terminal tag). 14-3-3 σ /A-RAF and 14-3-3 σ /B-RAF NanoBRET were performed with the same conditions and tag placements. N-RAS/C-RAF NanoBRET was performed with C-RAF-NanoLuc (C-terminal tag)/HaloTag-NRAS Q61L (N-terminal tag) with HA-14-3-3 σ transfected in addition at a 1:1 ratio with C-RAF-NanoLuc. C-RAF/C-RAF NanoBRET used C-RAF-NanoLuc/HaloTag-C-RAF with HA-14-3-3 σ and FLAG-N-RAS Q61L at a 1:1 ratio with C-RAF-NanoLuc. Data was analyzed as previously described.³⁵ Briefly, milliBRET units (mBU) were calculated as a ratio of acceptor/donor and mean mBU values were corrected by subtracting the mean mBU of controls with no HaloTag ligand. Fold change was calculated by dividing mBU_{compound} by mBU_{DMSO control}. For N-RAS/C-RAF NanoBRET, 100% was considered the DMSO mBU and 0% the mBU value of NRAS/C-RAF R89L.

Cell culture and western blots

HEK293T (CRL-3216) and MIA PaCa-2 (CRL-1420) cells were purchased from ATCC. Both cell lines were grown in DMEM, high glucose (Gibco) with 10% Fetal Bovine Serum (FBS). All compound treatments were 24 hours. Anti-phospho-C-RAF pS259 (Invitrogen 44-502), anti-C-RAF (BD Biosciences 610152), anti-phospho-A-RAF pS214 (Thermo-Fischer BS-3005R), anti-A-RAF pS214 (OriGene Technologies TA803662), anti-phospho-B-RAF pS365 (Thermo-Fischer BS-3013R), anti-phospho-ERK (Cell Signaling Technology 4370S), anti-ERK (Santa Cruz Biotechnology sc-514302), anti-Vinculin (Cell Signaling Technology 13901S), anti-FLAG (Cell Signaling Technology 8146S), and anti-14-3-3 σ (Invitrogen SD2070) antibodies were used for protein detection. Antibodies were used at a 1:1,000 dilution except anti-Vinculin, which was used at 1:2,000. Western blots were imaged on a LI-COR imaging system and analyzed using Image Studio.

Co-immunoprecipitation

HEK293T cells were dosed with 10 μ M compound for 24 hours before lysis (Triton X-100).

Clarified lysate was incubated with Pierce anti-FLAG magnetic beads (Thermo Fisher A36797) following the manufacturers protocol. Lysate and IP samples were analyzed using antibodies described above.

Lumit Immunoassay

Lumit immunoassays were performed according to the manufacturer (Promega W1331). Briefly, HEK293T cells were transfected in a 1:1 ratio of HA-14-3-3 σ :FLAG-C-RAF in 10% FBS DMEM (Gibco). 24 hours after transfection, cells were re-seeded in 384-well plates (10,000 cells per well) in 0.5% FBS FluoroBrite DMEM media (Gibco). Cells were treated with compound **23** in a 2-fold dilution series, starting at 10 μ M, or LY3009120 (100 nM; Selleck Chemicals S7842) or Trametinib (100 nM; Selleck chemicals S2673) for 24 hours before performing the Lumit protocol. After compound treatment, media was replaced with 10 μ L media supplemented with fluorogenic live cell GF-AFC substrate (50 μ M) for normalization purposes and 100 ng/mL EGF to stimulate the MAPK pathway. Cells were incubated for 30 minutes before proceeding. The protocol was optimized for the antibodies used: digitonin (0.1%) lysis buffer, 100 ng/ μ L pERK antibody (Cell Signaling Technology 4376), 100 ng/ μ L ERK antibody (Santa Cruz Biotechnology 514302), and 0.1 μ L/well of secondary antibodies (SmBiT mouse, LgBiT rabbit). The ratio of cell lysate:antibody:substrate was 2:2:1. Cells were lysed for 40 minutes at 800 rpm before incubation with the antibody mix for 90 minutes (2 minutes at 400 rpm prior to incubation). After antibody incubation, substrate was added and incubated for 2 minutes at 400 rpm. Luminescence was then read on an EnVision Xcite 2105 plate reader. Luminescence was normalized against the total fluorescence from the GF-AFC (Luminescence/Total fluorescence). Values were then normalized to DMSO treated cells (experimental/DMSO). Data was analyzed

using GraphPad Prism and fit using a 4PL model. Data represents the average of 3 technical replicates, confirmed over 3 biological replicates.

Docking

Computational design for SAR optimization and docking was performed with SeeSAR version 11.2.0; BioSolveIT GmbH, Sankt Augustin, Germany, 2022, www.biosolveit.de/SeeSAR

Figures

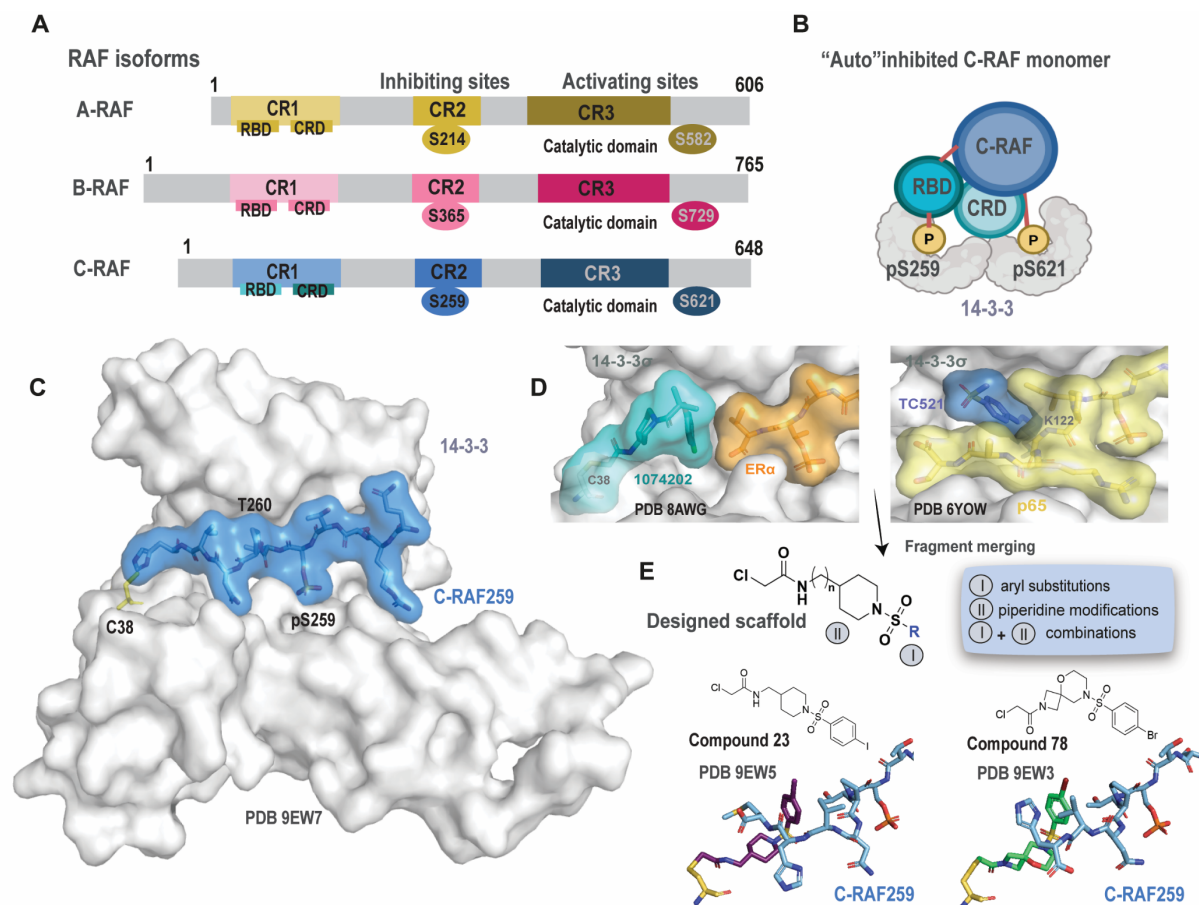


Figure 5.1: RAF isoforms, 14-3-3/C-RAF pS259 complex and fragment merging approach

A) Inhibiting and activating sites on A-, B- and C-RAF. **B)** Proposed model for the auto-inhibited C-RAF monomer complex bound to a 14-3-3 dimer. **C)** Crystal structure of 14-3-3 σ (gray surface) bound to a phospho-C-RAF259 peptide (cyan surface). C38 (14-3-3) is shown as yellow sticks. PDB: 9EW7 **D)** Left: crystal structure of 1074202/14-3-3 σ /ER α (PDB 8AWG). Right: crystal structure of TC521/14-3-3/p65 (PDB 6YOW). **E)** Top: Fragment merging approach towards a designed scaffold for the 14-3-3 σ /C-RAF pS259 complex. Bottom: Representative examples and their binding mode (14-3-3 is omitted for clarity, C38 is shown as yellow sticks, and C-RAF as cyan sticks).

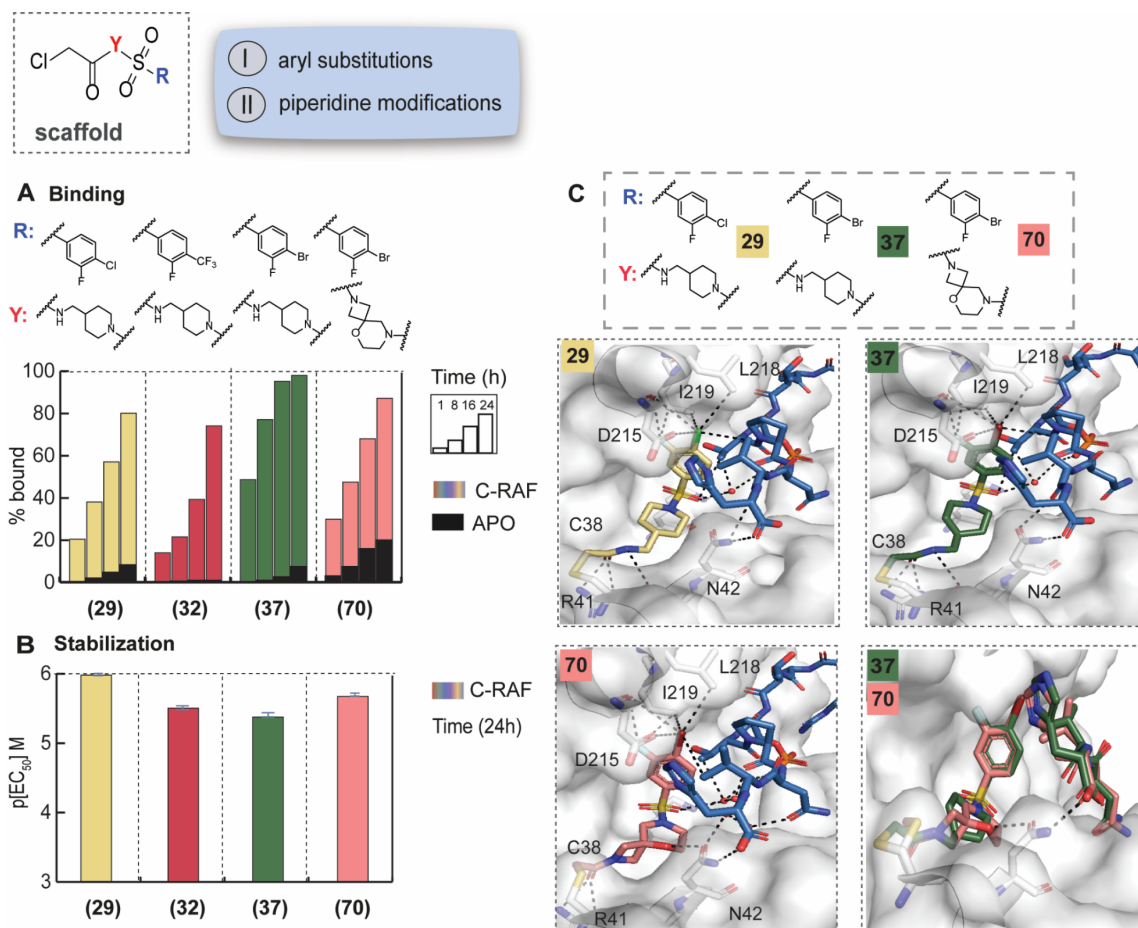


Figure 5.3: SAR and crystal structures of selected phenyl and spiro analogs

A) MS bar graphs at 1 μ M. For each compound, time course experiments were performed with measurements at 1h, 8h, 16h and 24h. C-RAF259 data are shown with different colors for each compound, and apo data in black. **B)** Bar graphs of FA compound titration pEC₅₀ values after overnight incubation. C-RAF259 data are shown with different colors for each compound. Fig S5.22 – S5.27. **C)** Top: Crystal structures of **29** (yellow sticks) and **37** (green sticks) with 14-3-3 σ (white surface) and C-RAF pS259 10-mer peptide (blue sticks). Bottom: crystal structure of **70** (salmon sticks) with 14-3-3 σ /C-RAF259 10-mer and overlay with **37**. Interacting water molecules are shown as red spheres.

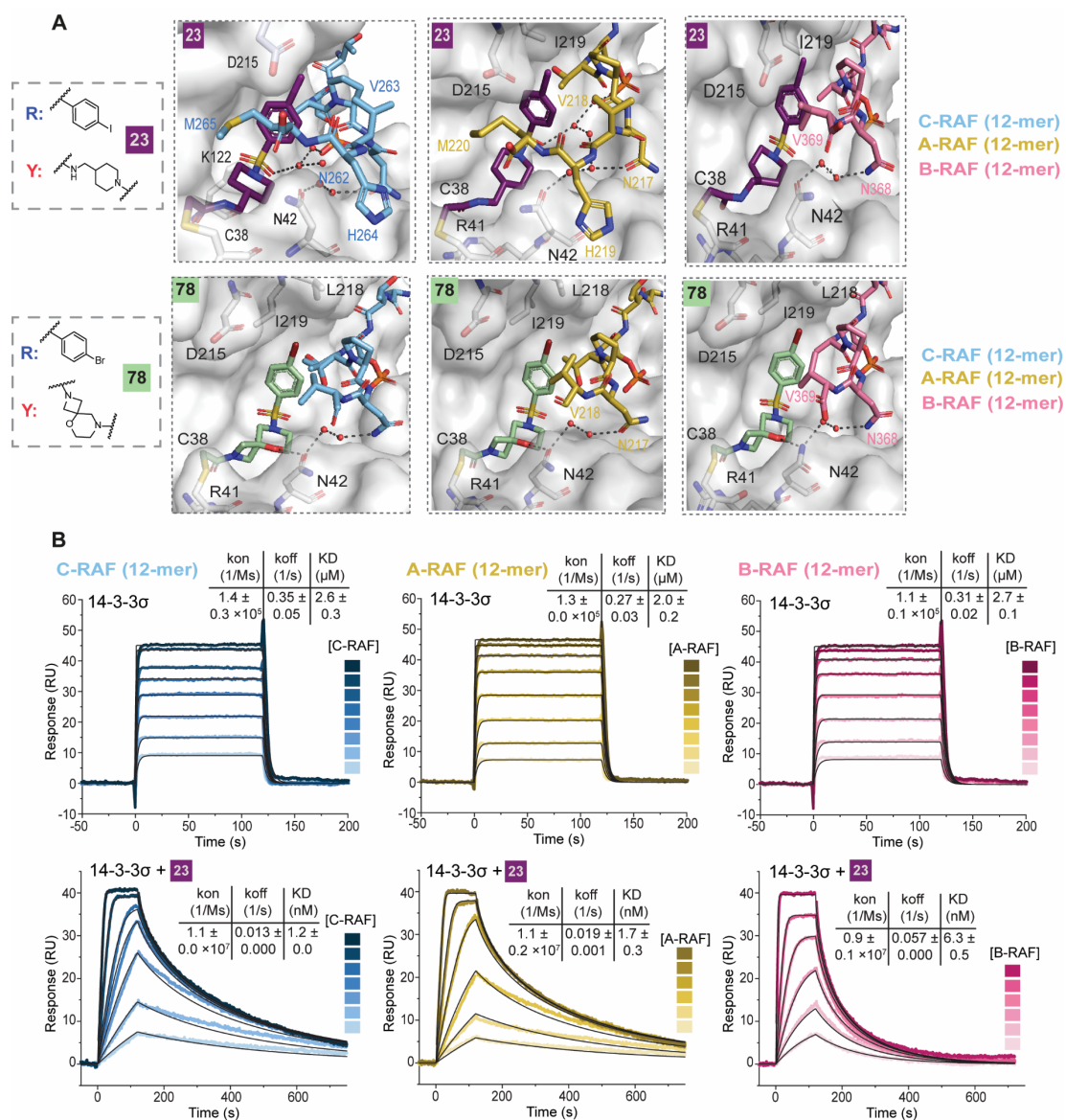


Figure 5.5: Crystallography and SPR with RAF isoforms

A) Crystal structures of **23** (top) and **78** (bottom) with 14-3-3σ/C-RAF-259 (left), 14-3-3σ/A-RAF214 (middle) and 14-3-3σ/B-RAF365 (right) 12-mer peptides. Interacting water molecules are shown as red spheres. **B)** SPR data of A-RAF (yellow), B-RAF (pink), C-RAF (blue) 12-mer peptides binding to 14-3-3σ (top panel), and 14-3-3σ covalently bound to molecular glue **23** (parameters shown mean ± SD, n=2).

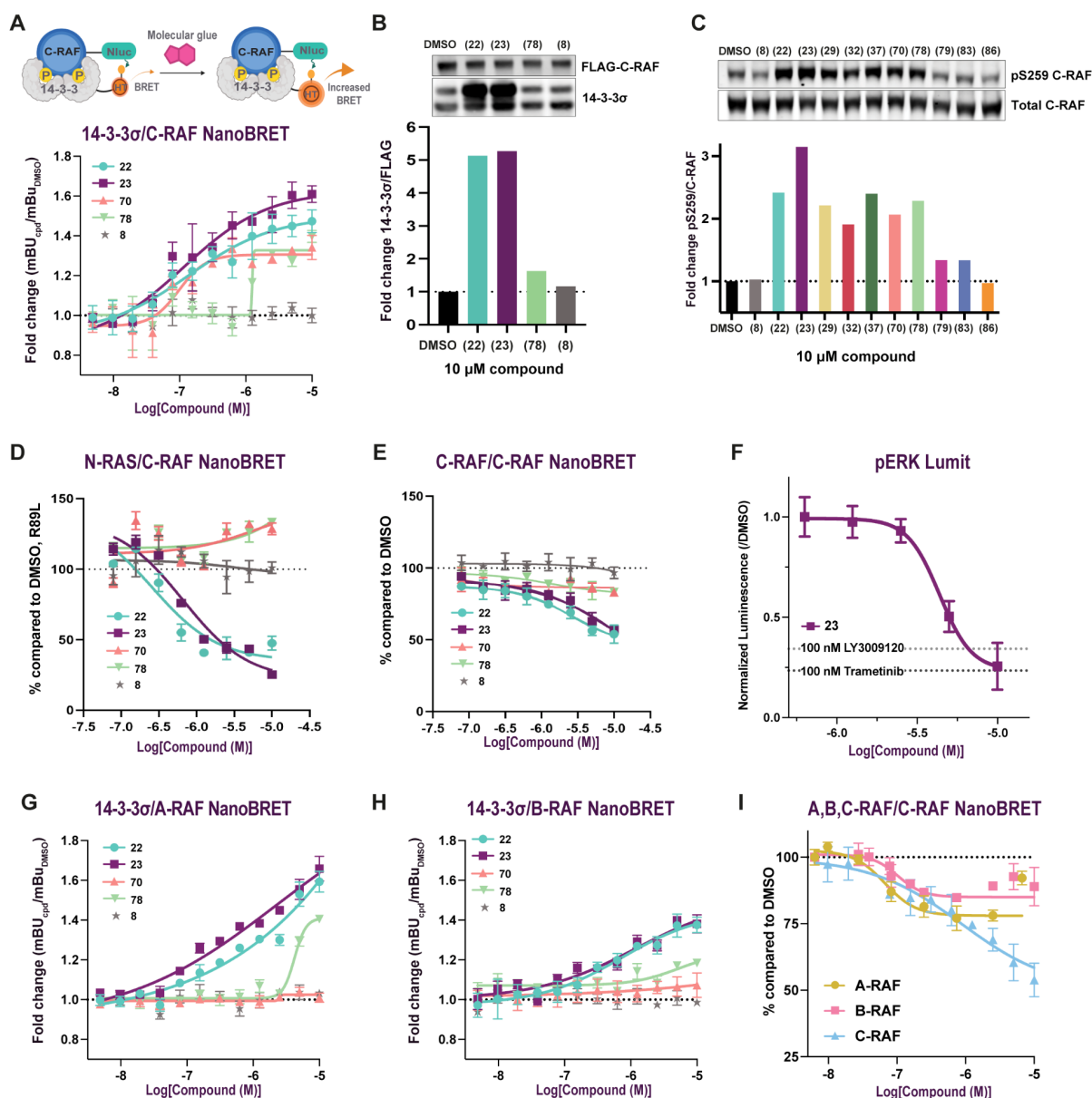


Figure 5.6: Evaluation of MGs in cell assays

A) 14-3-3σ/C-RAF NanoBRET schematic (left) and dose-response curves in HEK293T cells. **B)** Co-IP western blots and quantification. **C)** Protection of phosphorylation of the C-RAF pS259 site in HEK293T cells. **D)** N-RAS/C-RAF NanoBRET; 100% was considered the DMSO mBU and 0% the mBU value of NRAS/C-RAF R89L, which does not bind to C-RAF. **E)** C-RAF/C-RAF homodimers NanoBRET dose-response curves. **F)** Lumit immunoassay for pERK levels in dose-responses with **23**. **G)** 14-3-3σ/A-RAF NanoBRET. **H)** 14-3-3σ/B-RAF NanoBRET. **I)** NanoBRET for heterodimer formation for A-RAF/C-RAF and B-RAF/C-RAF, compared to C-RAF homodimers NanoBRET for **23**.

Supplementary Figures

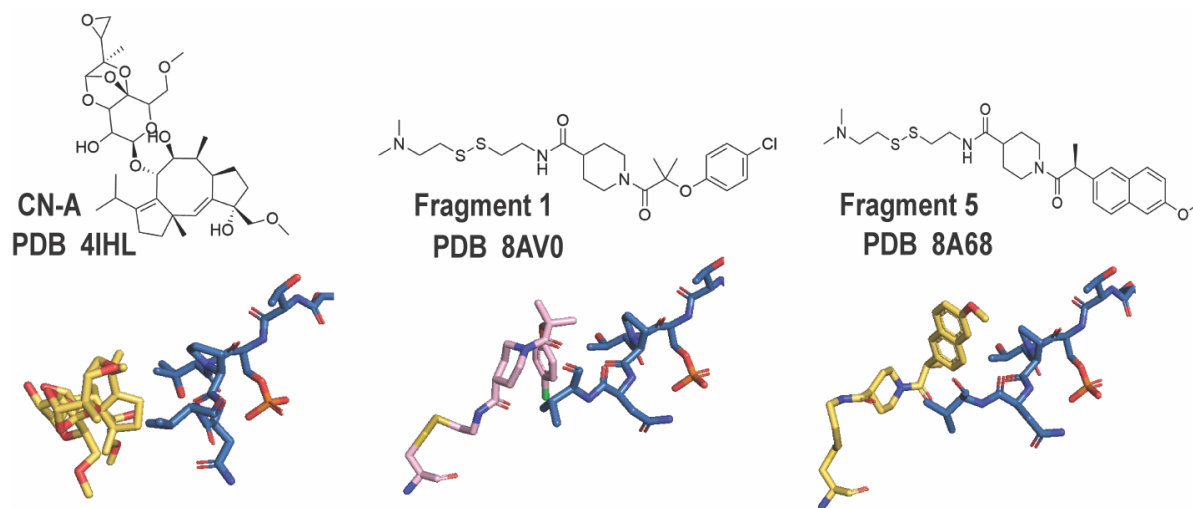


Figure S5.1: Crystal structures of known molecular glues bound to 14-3-3/C-RAF259
(14-3-3 is omitted for clarity, C38 is shown as yellow sticks, and CRAF as blue sticks).

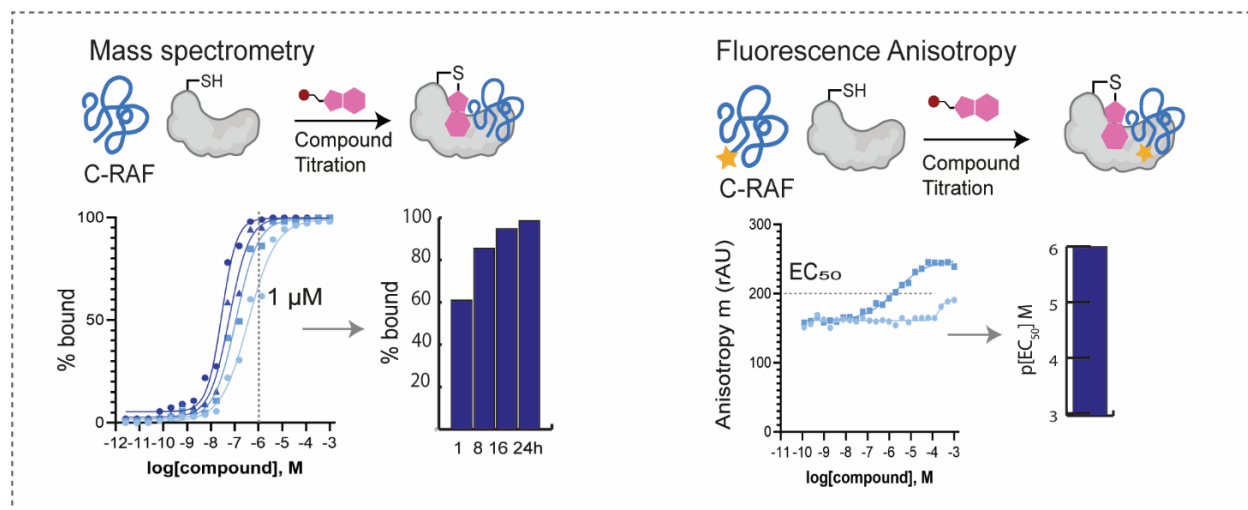
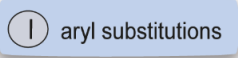
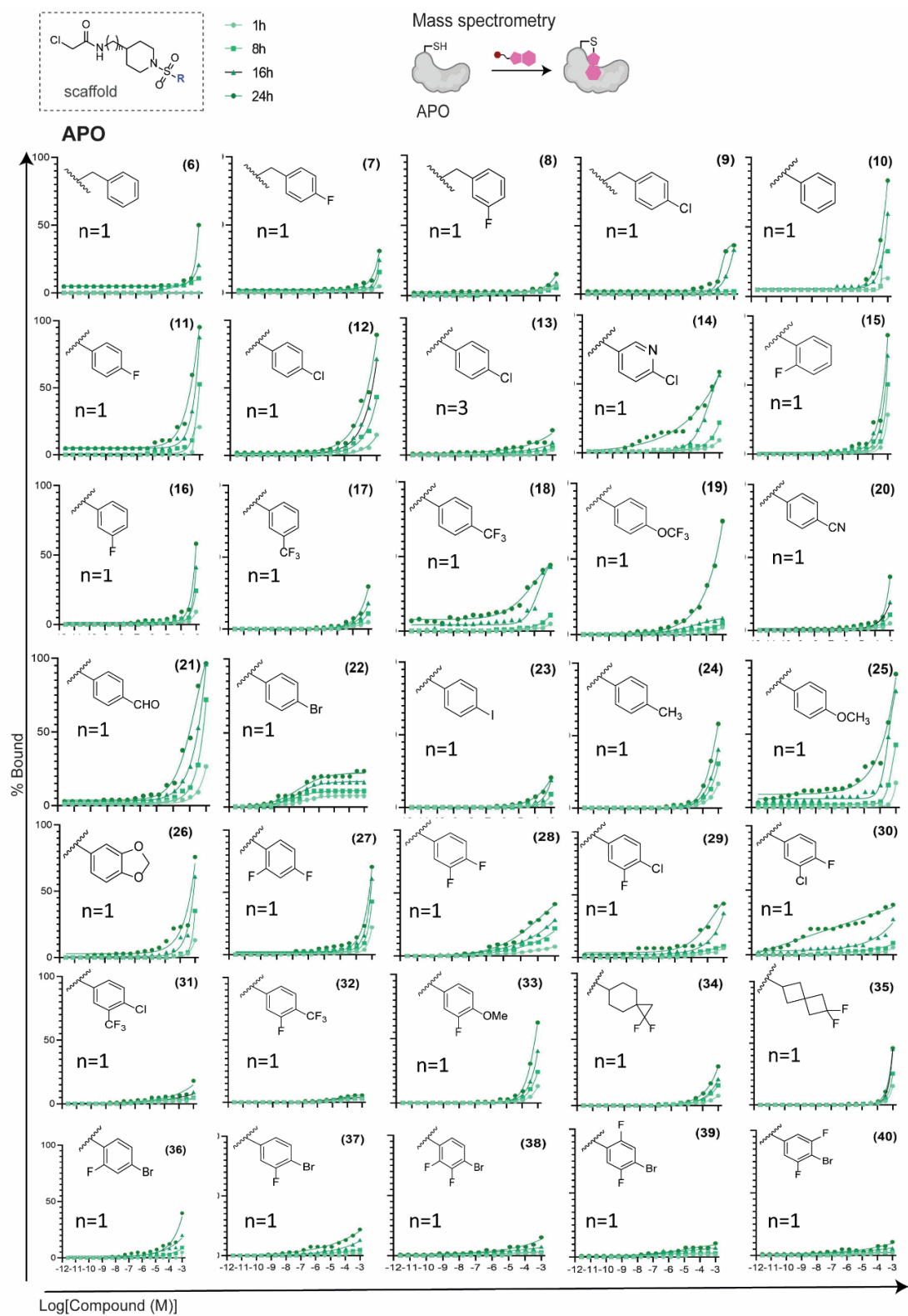


Figure S5.2: Overview of assays

Left: mass spectrometry time-course experiments were performed at 1h, 8h, 16h and 24h in the presence of C-RAF259 phosphopeptide. Bar graphs were used to represent % bound at 1 μ M compound concentration (1:10 [protein]:[compound] ratio). Right: fluorescence anisotropy compound titrations were performed in the presence of FAM-labeled C-RAF259 phosphopeptide. EC_{50} values were calculated from the overnight measurement. pEC_{50} bar graphs represent the positive log EC_{50} value.



A) MS bar graphs sat 1 μ M. For each compound, time course experiments were performed with measurements at 1h, 8h, 16h and 24h. C-RAF259 data are shown with different colors for each compound, and apo data in black. **B)** Bar graphs of FA compound titration pEC₅₀ values after overnight incubation. C-RAF259 data are shown with different colors for each compound. Inactive compounds are described as non-applicable (NA).



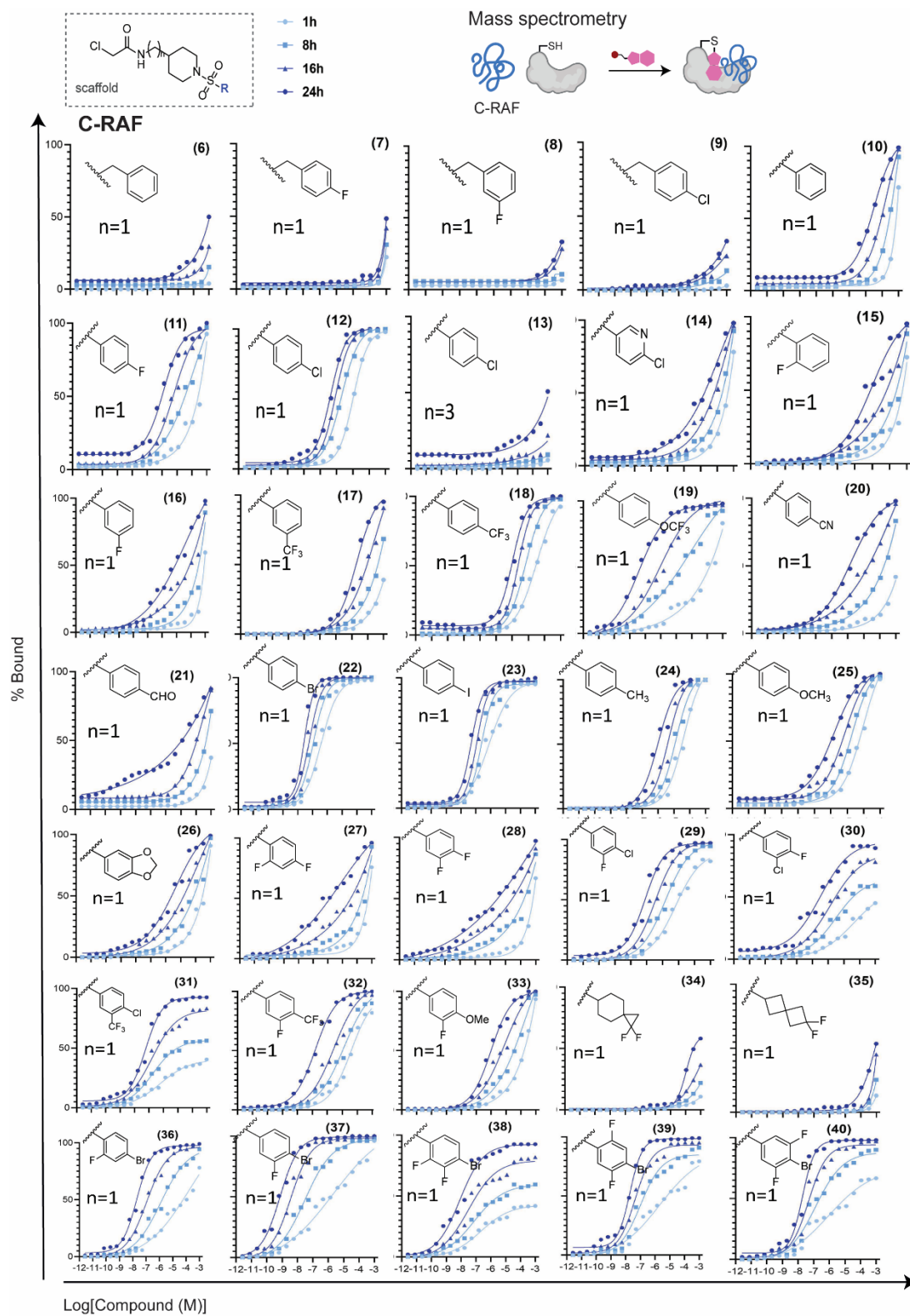


Figure S5.6: MS dose-response curves (C-RAF259) for aryl-ring modifications

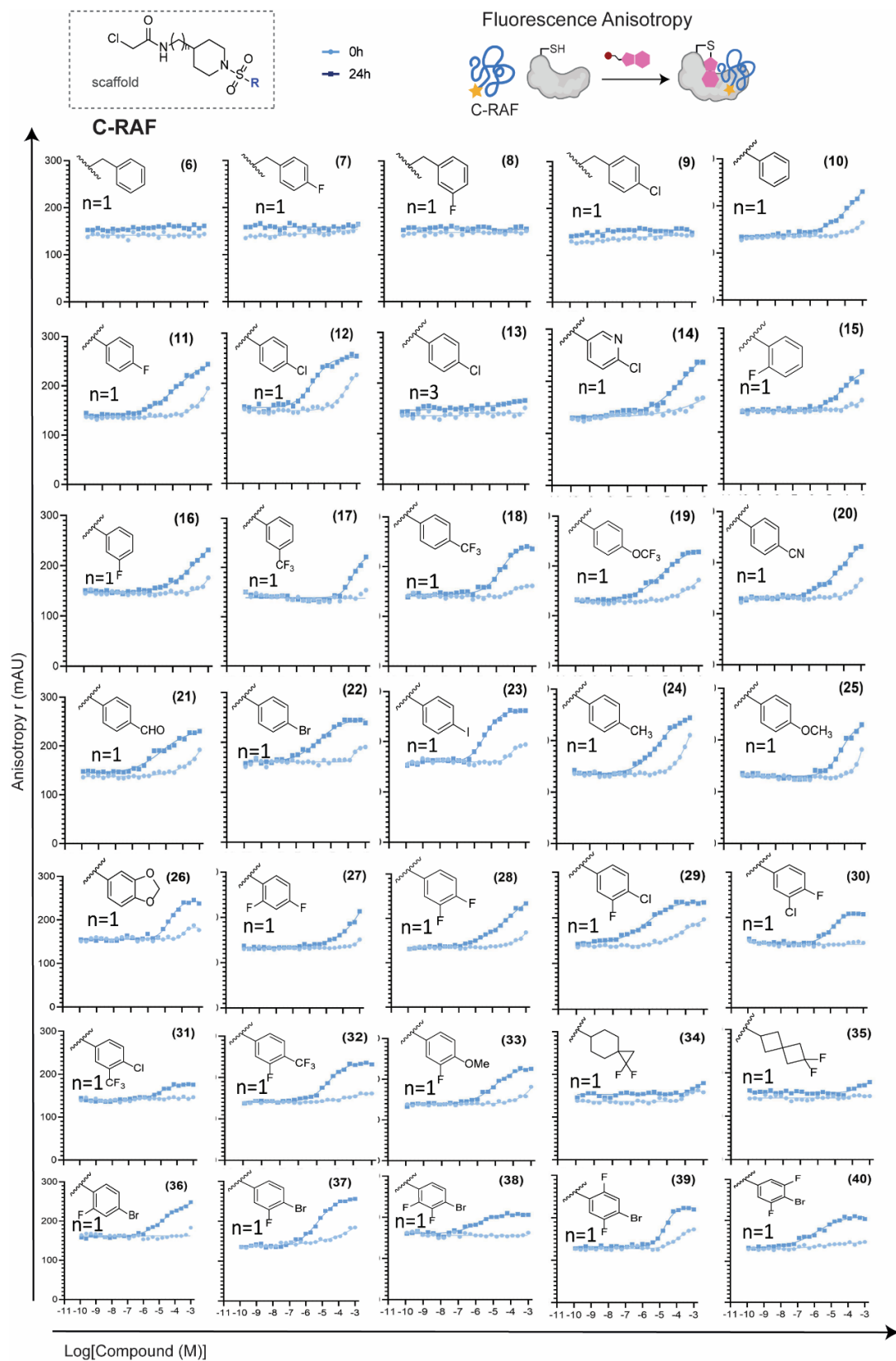


Figure S5.7: FA compound titrations (C-RAF259) for aryl-ring modifications

C-RAF 259: Protein titrations

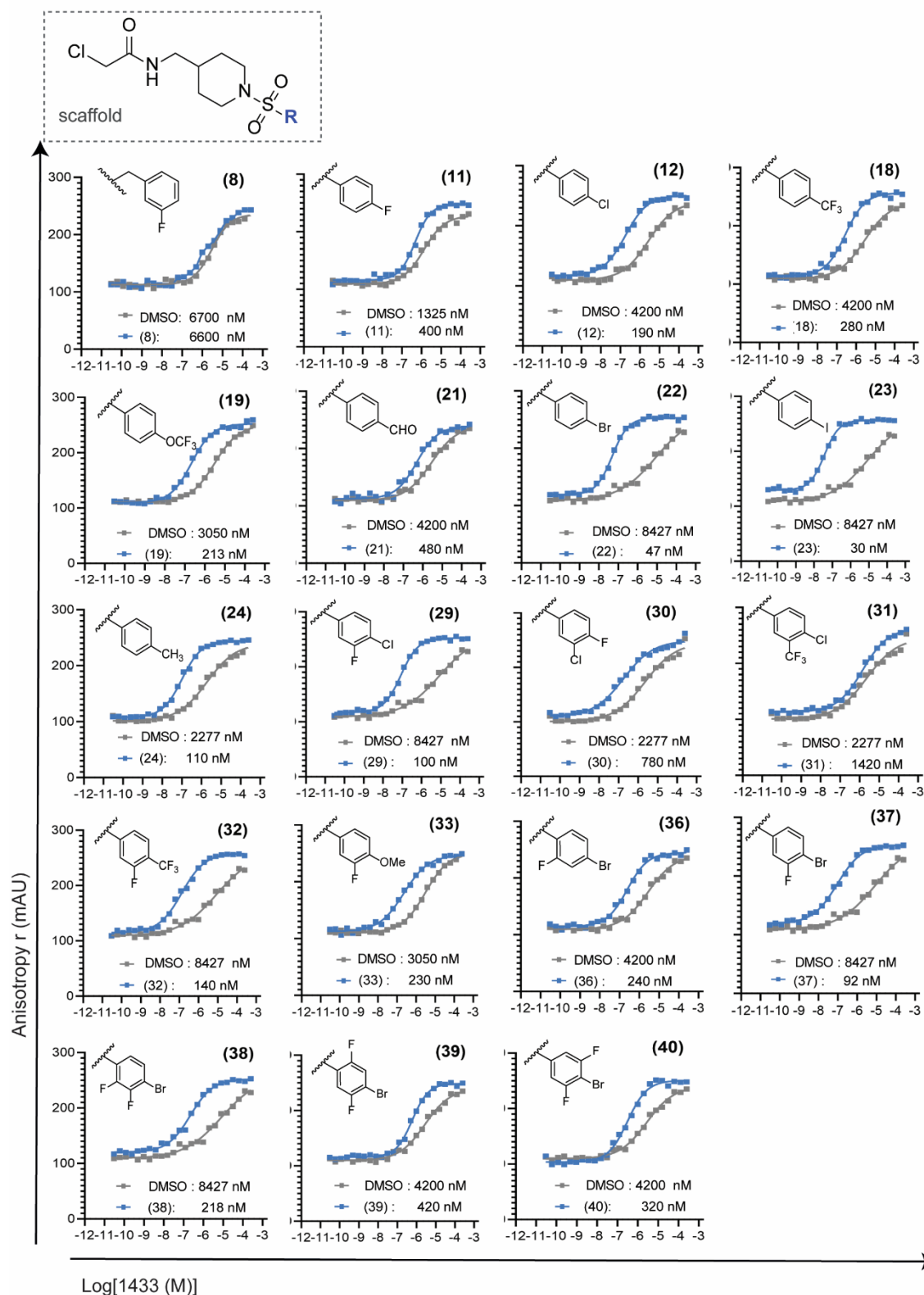
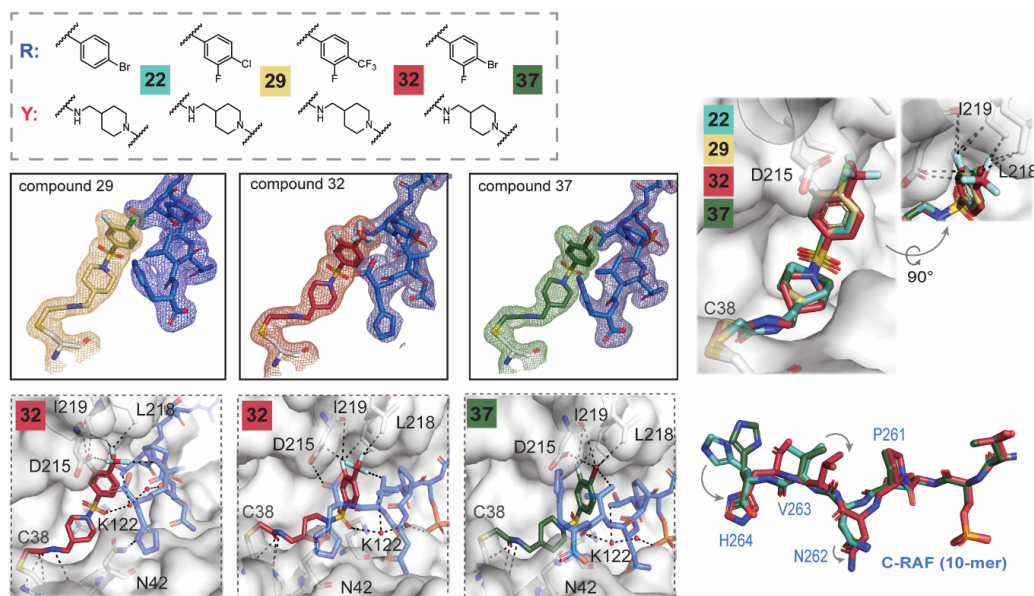
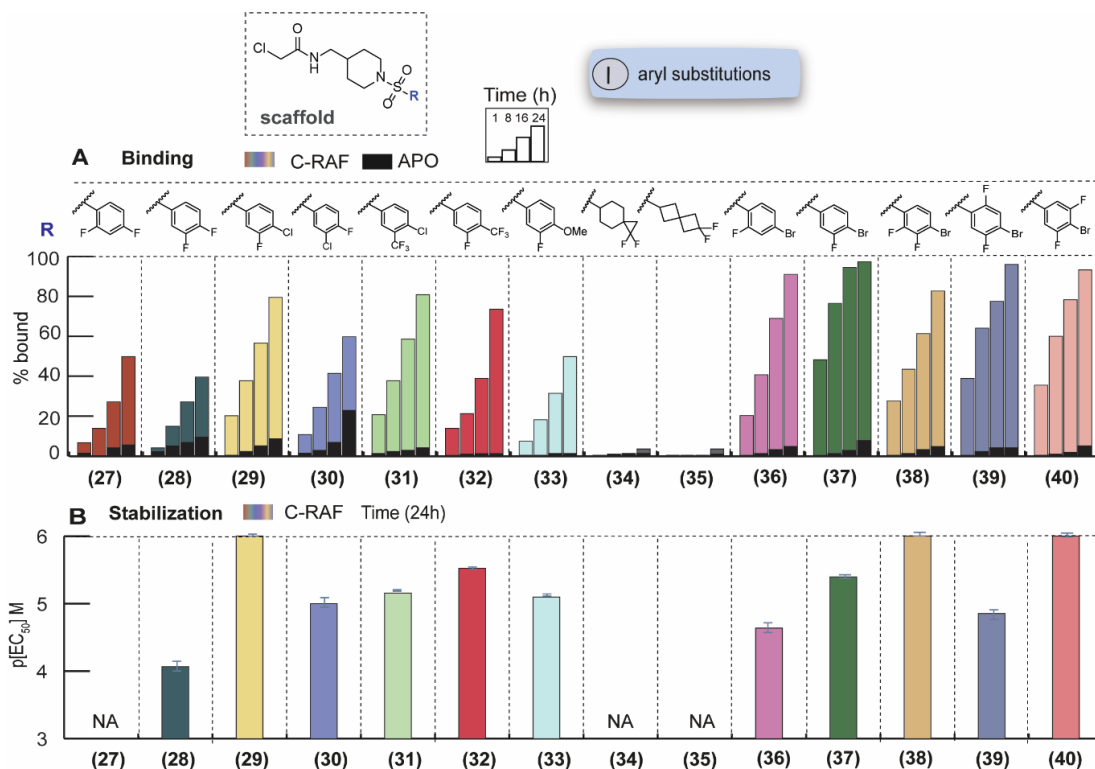


Figure S5.8: FA protein titrations (C-RAF259) for aryl-ring modifications



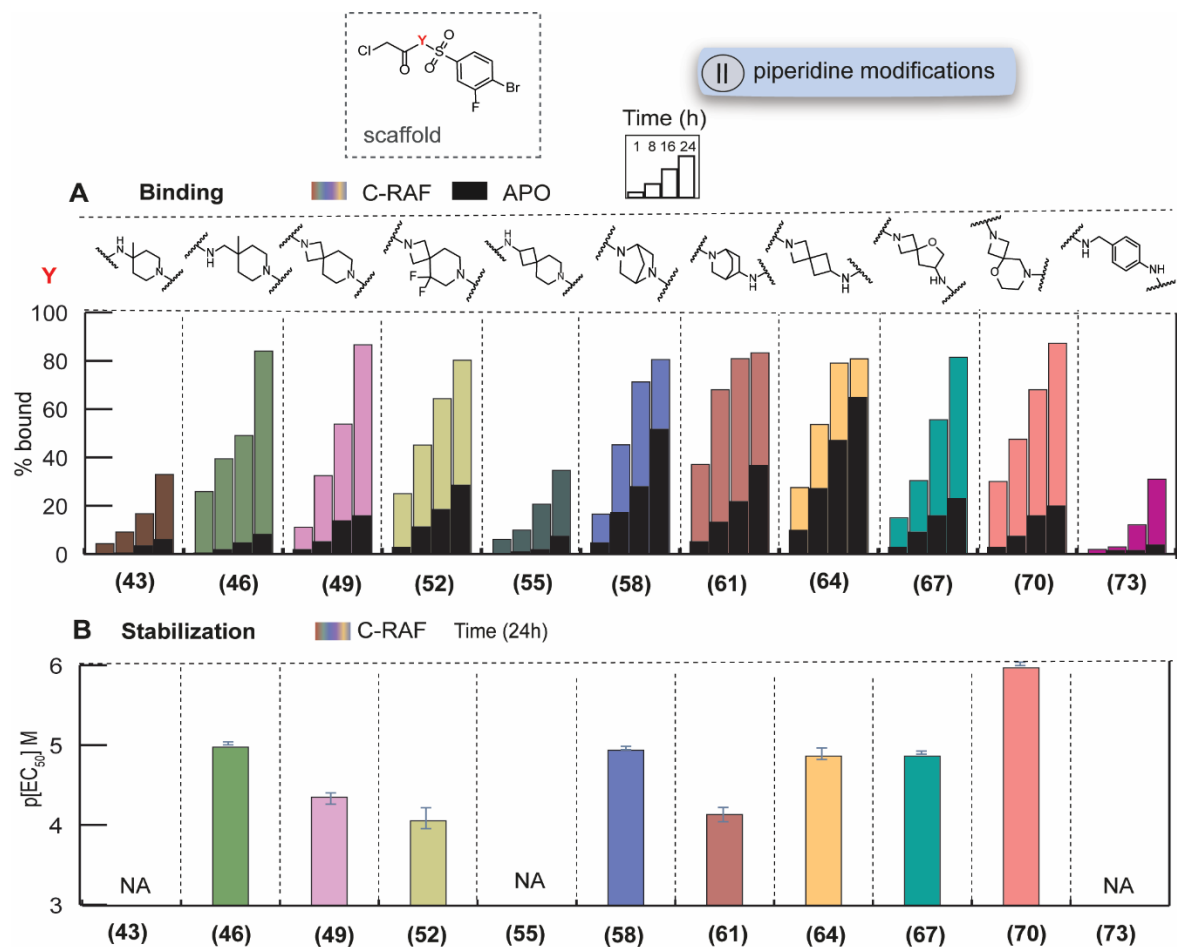


Figure S5.14: SAR of modifications on the piperidine position

A) MS bar graphs sat 1 μ M. For each compound, time course experiments were performed with measurements at 1h, 8h, 16h and 24h. C-RAF259 data are shown with different colors for each compound, and apo data in black. **B)** Bar graphs of FA compound titration pEC_{50} values after overnight incubation. C-RAF259 data are shown with different colors for each compound. Inactive compounds are described as non-applicable (NA).

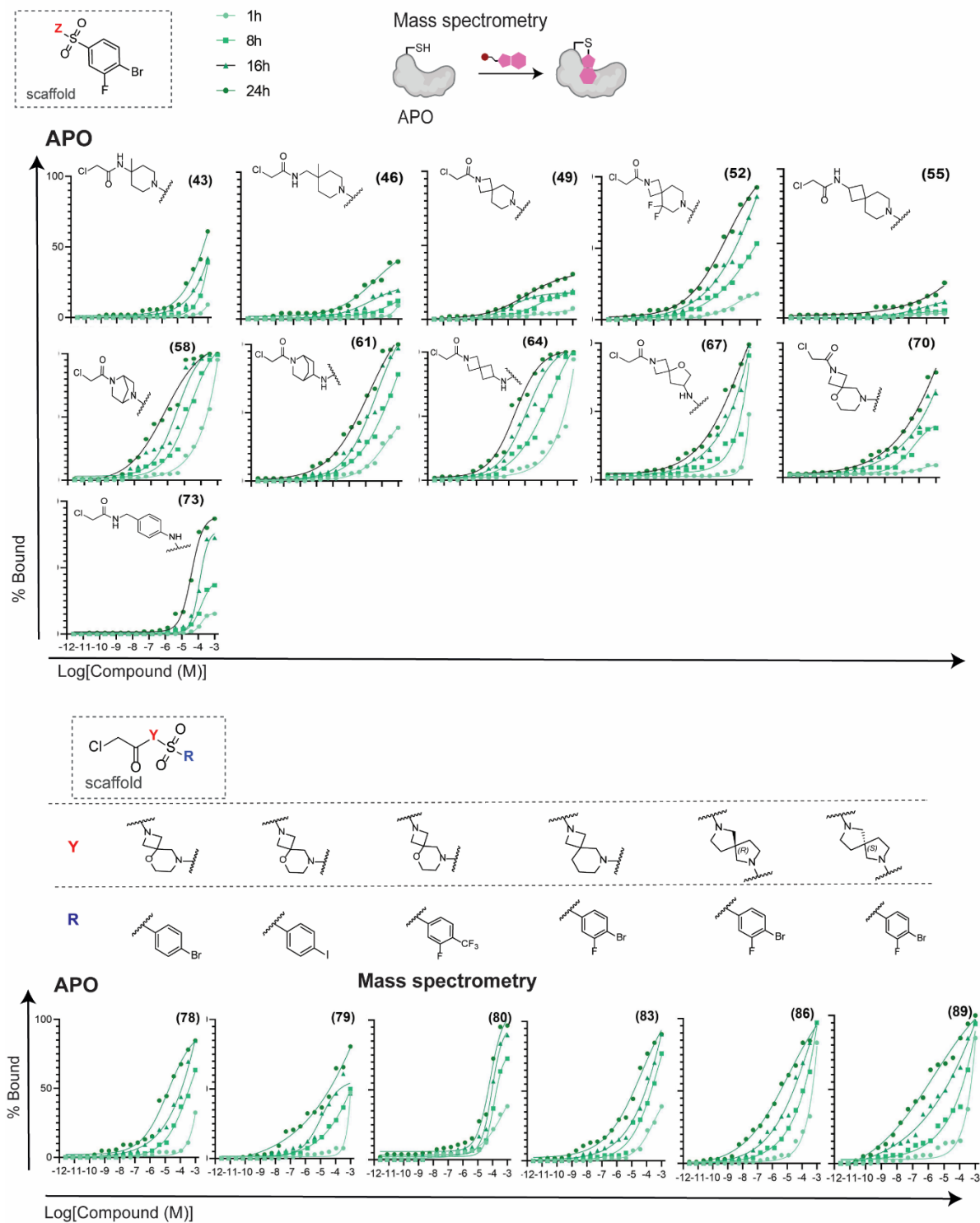


Figure S5.15: MS dose-response curves (apo) for piperidine modifications and combinations

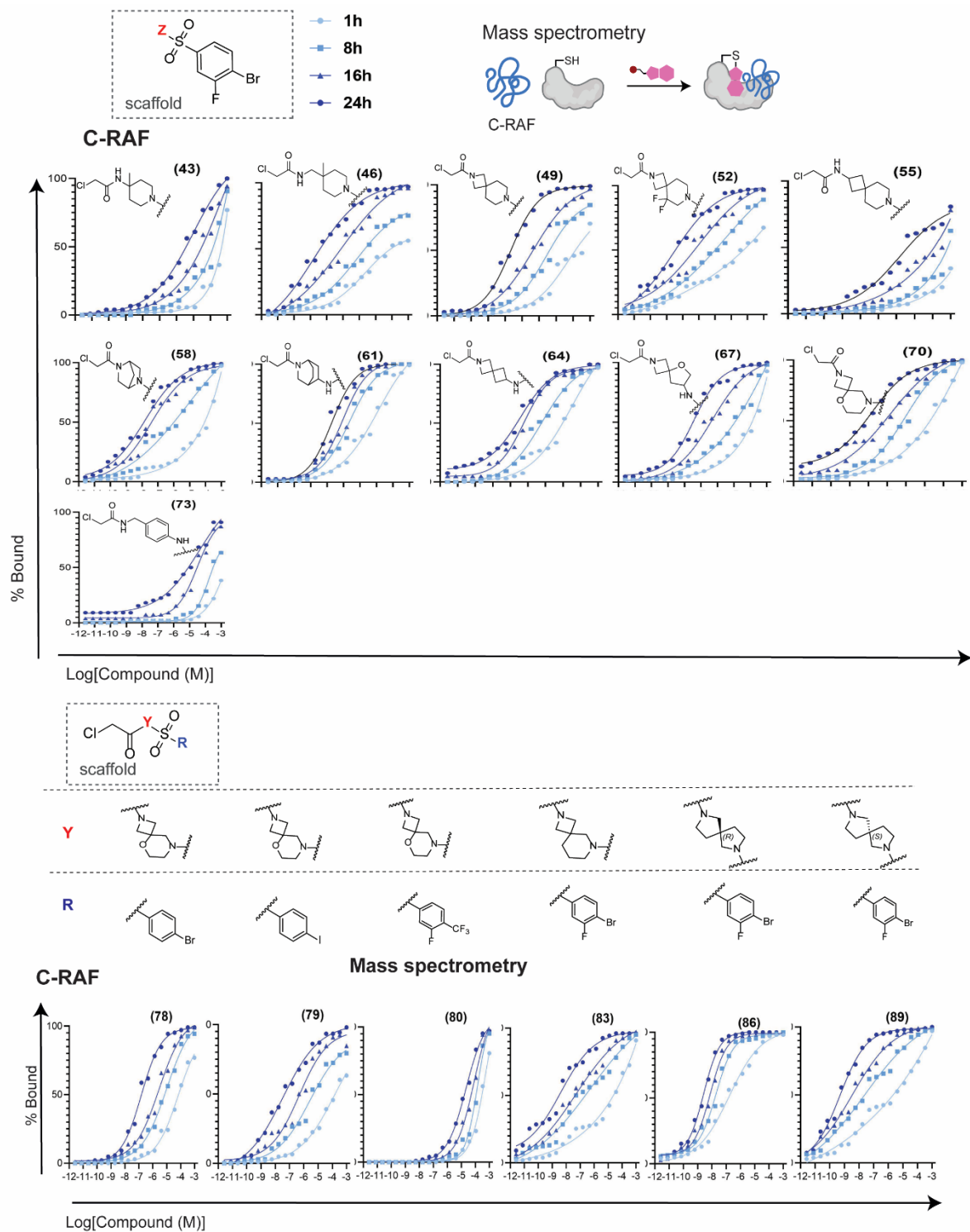


Figure S5.16: MS dose-response curves (C-RAF259) for piperidine modifications and combinations

C-RAF 259: Protein titrations

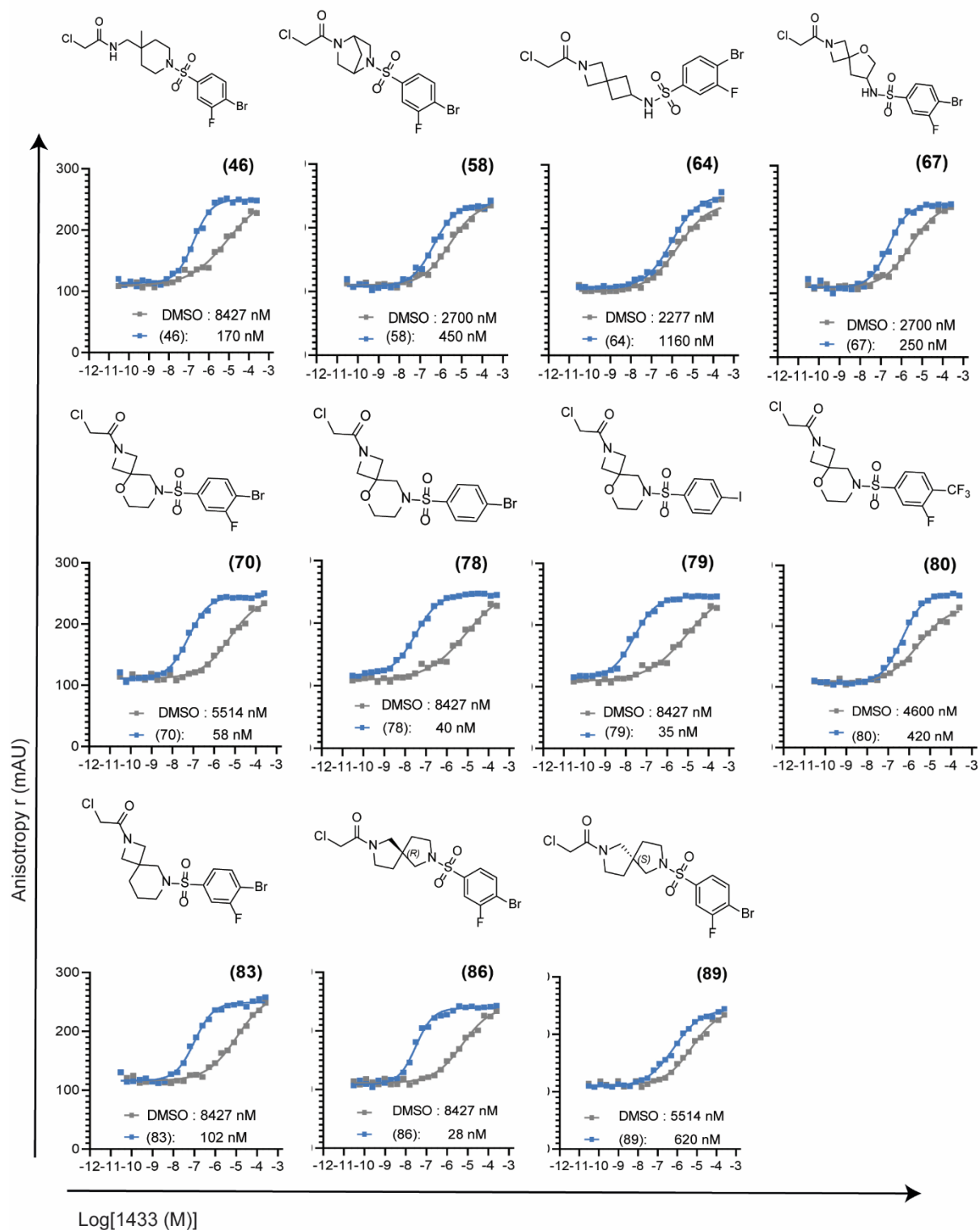


Figure S5.18: FA protein titrations (C-RAF259) for piperidine modifications and combinations

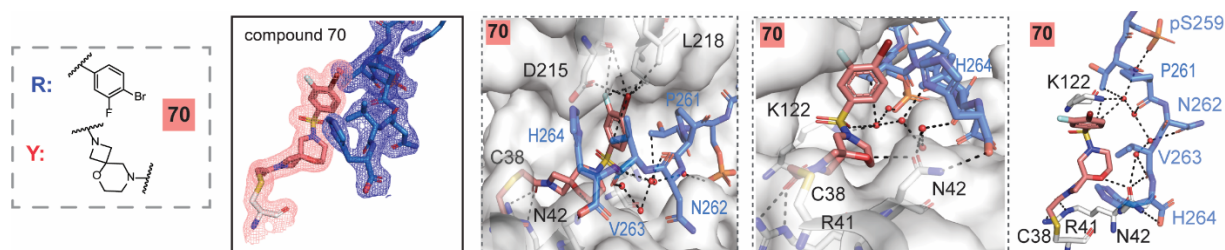


Figure S5.19: Crystal structure of 70 in complex with C-RAF pS259 10-mer peptide and 14-3-3 σ
Close-up views on interacting amino acids interacting water molecules are shown as red spheres (70: salmon). 2Fo-Fc electron density maps (mesh) were contoured at 1 σ .

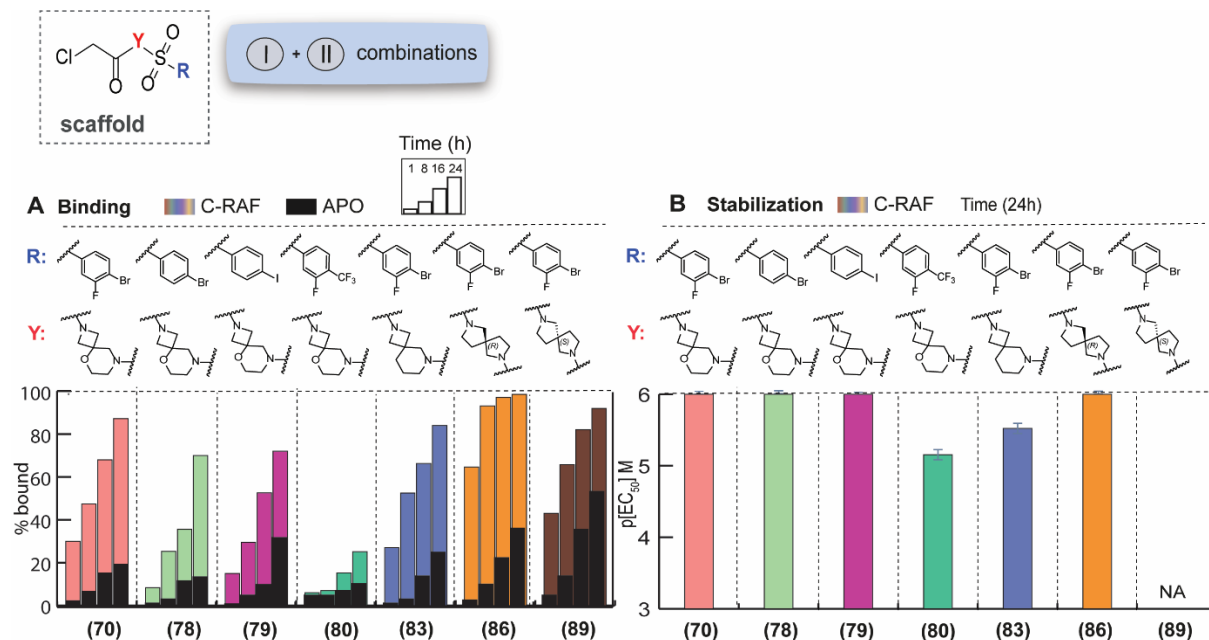


Figure S5.20: SAR of aryl and spiro/fused rings combinations

A) MS bar graphs sat 1 μ M. For each compound, time course experiments were performed with measurements at 1h, 8h, 16h and 24h. C-RAF259 data are shown with different colors for each compound, and apo data in black. **B)** Bar graphs of FA compound titration pEC₅₀ values after overnight incubation. C-RAF259 data are shown with different colors for each compound. Inactive compounds are described as non-applicable (NA).

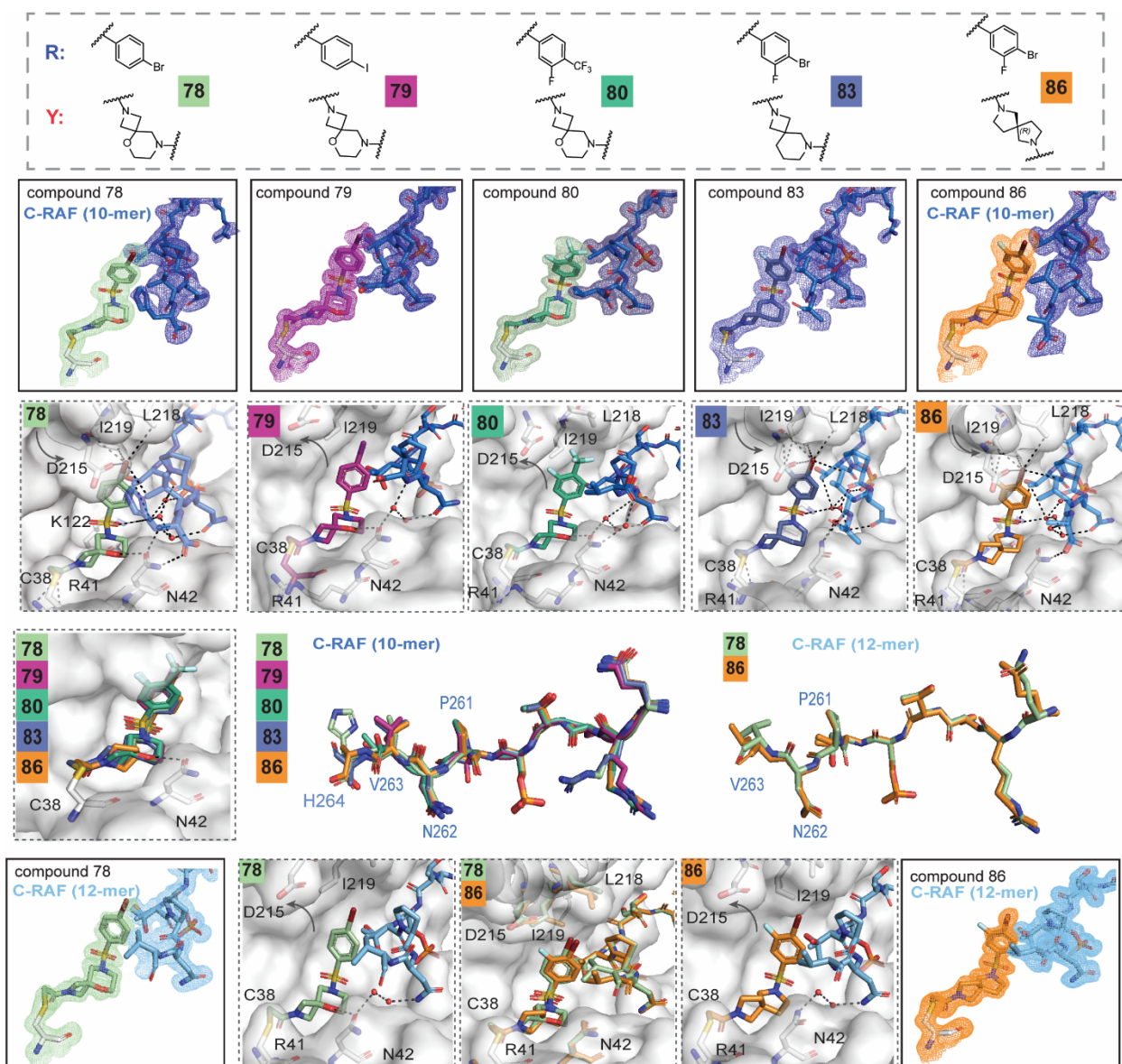


Figure S5.21: Crystal structures of 78, 79, 80, and 86 in complex with C-RAF pS259 10-mer peptide and 14-3-3 σ

Top: Densities and crystal structures of **78** (light green sticks), **79** (lilac), **80** (teal), **83** (sky blue), **86** (orange) in complex with C-RAF pS259 10-mer peptide and 14-3-3 σ . Close-up views on interacting amino acids. Interacting water molecules are shown as red spheres. 2Fo-Fc electron density maps (mesh) were contoured at 1 σ . Bottom: overlays, crystal structures of **78** (light green sticks) and **86** (orange) in complex with C-RAF pS259 12-mer peptide and 14-3-3 σ and close-up views of interacting amino acids.

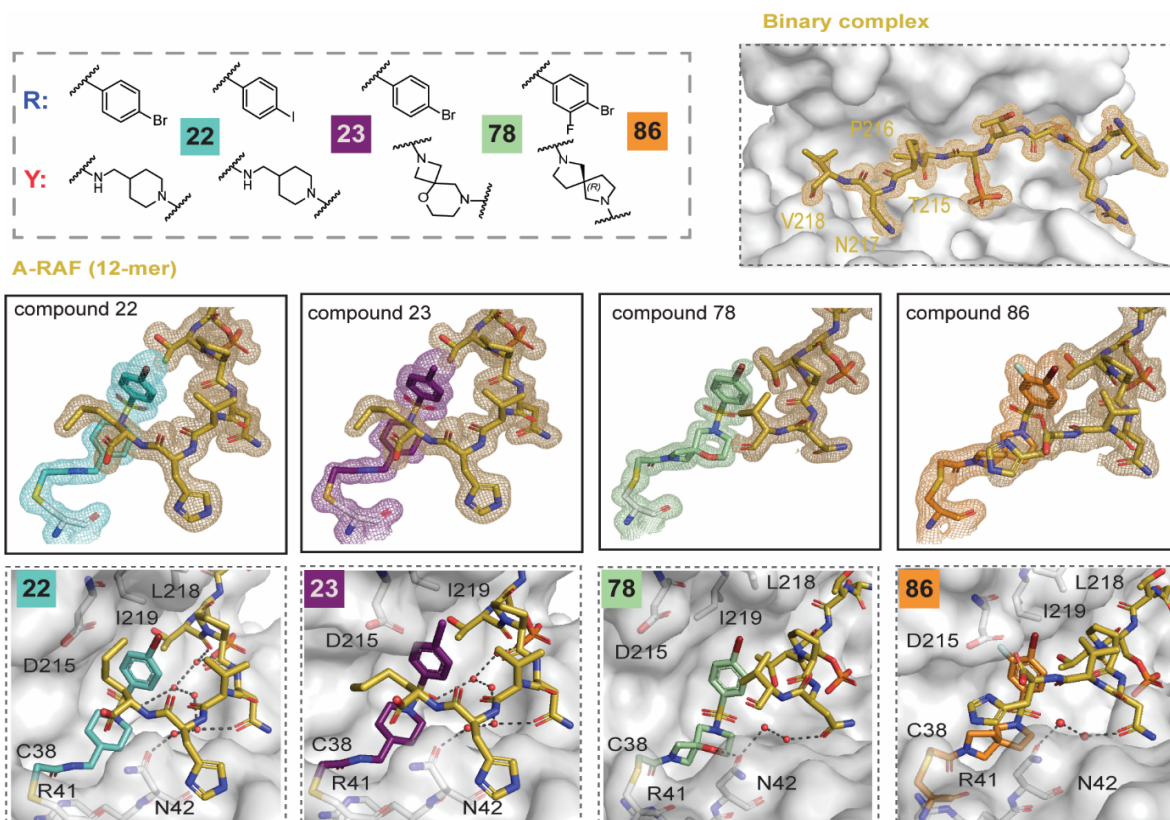


Figure S5.22: Densities and crystal structures with 14-3-3 σ /A-RAF pS214 12-mer peptide

Top right: binary complex. Middle and bottom: ternary complexes of **22** (turquoise), **23** (purple), **78** (light green) and **86** (orange) with A-RAF pS214 12-mer peptide and 14-3-3 σ . Close-up views on interacting amino acids. Interacting water molecules are shown as red spheres. 2Fo-Fc electron density maps (mesh) were contoured at 1 σ .

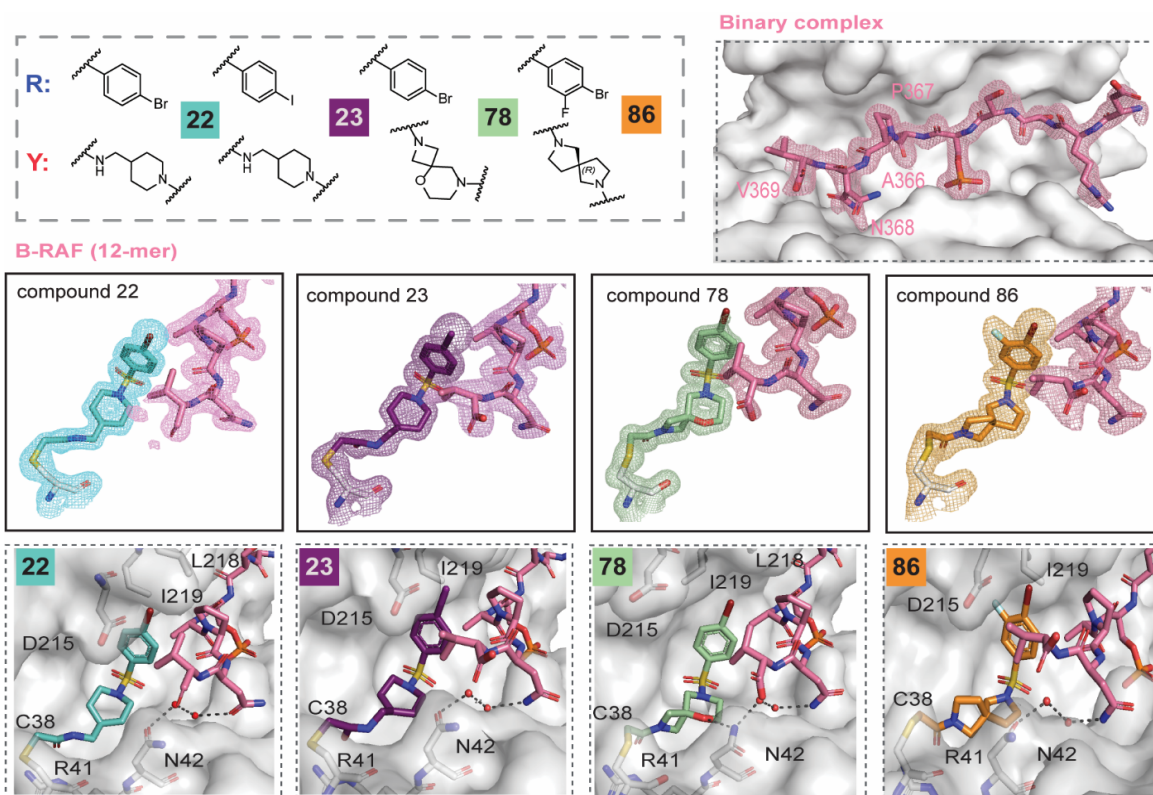


Figure S5.23: Densities and crystal structures with 14-3-3 σ /B-RAF pS365 12-mer peptide
 Top right: binary complex. Middle and bottom: ternary complexes of **22** (turquoise), **23** (purple), **78** (light green) and **86** (orange) with B-RAF pS365 12-mer peptide and 14-3-3 σ . Close-up views on interacting amino acids. Interacting water molecules are shown as red spheres. 2Fo-Fc electron density maps (mesh) were contoured at 1 σ .

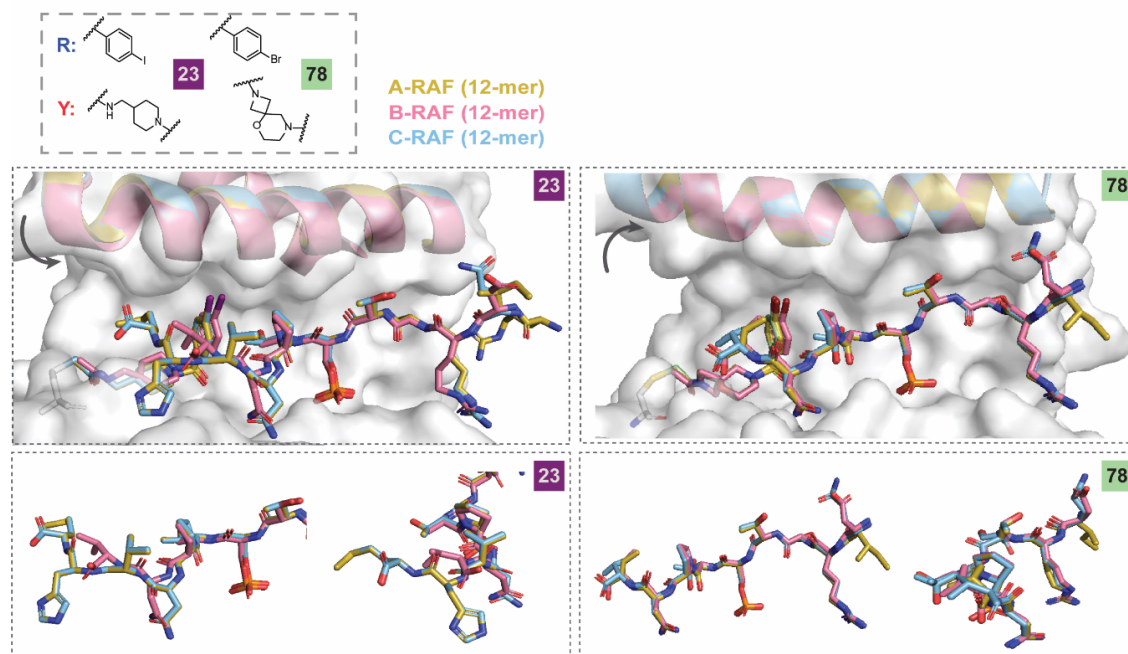


Figure S5.24: Overlays of ternary complexes
23 (purple) and **78** (light green) with A-RAF pS214, B-RAF pS365 and C-RAF pS259. Close-up views on peptide conformations.

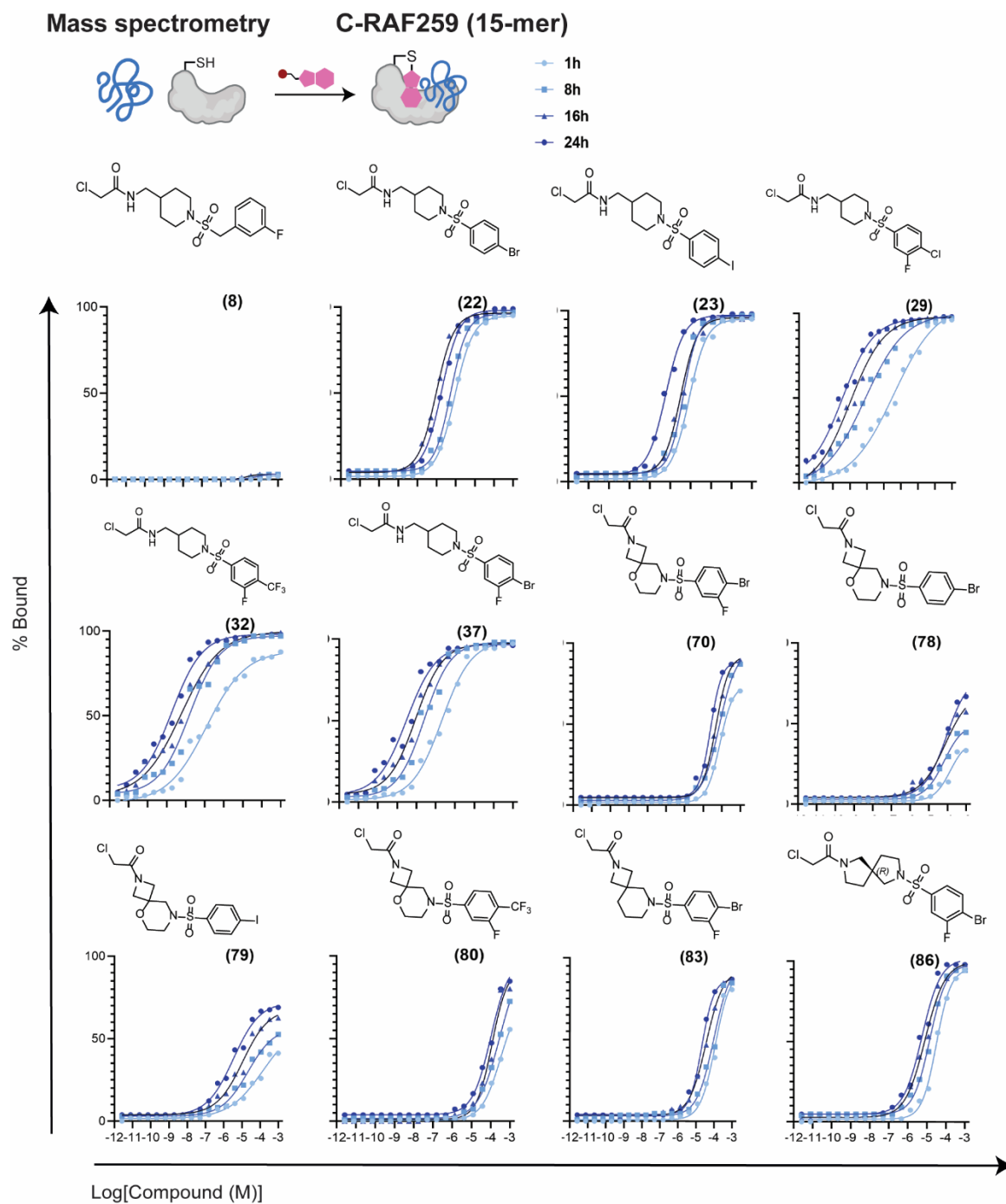


Figure S5.25: MS dose-response curves (C-RAF259 15-mer peptide)

C-RAF 259 (15-mer): Protein titrations

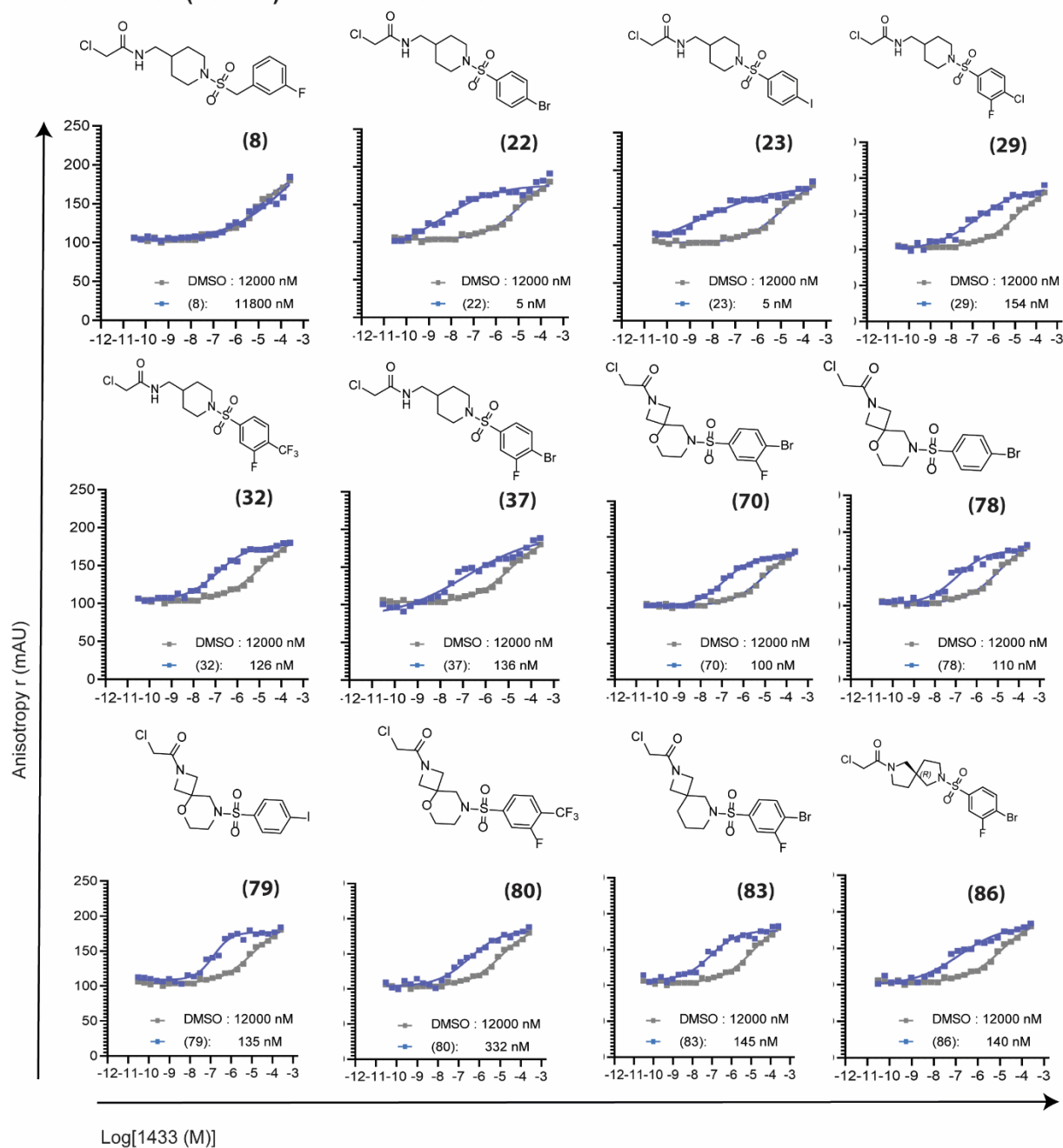


Figure S5.26: FA protein titrations (C-RAF259 15-mer peptide)

Mass spectrometry

A-RAF214 (15-mer)

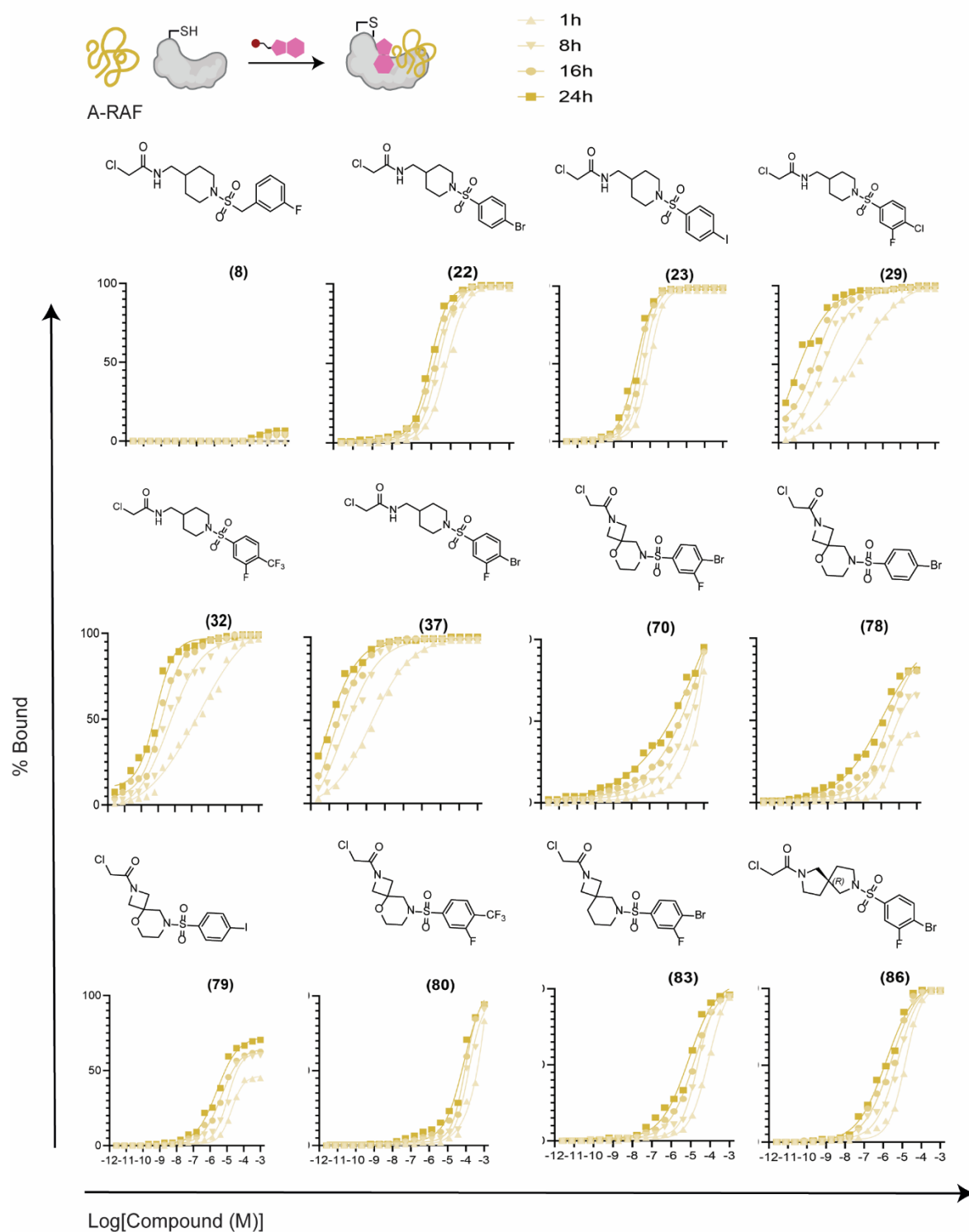


Figure S5.27: MS dose-response curves (A-RAF214 15-mer peptide)

A-RAF 214 (15-mer): Protein titrations

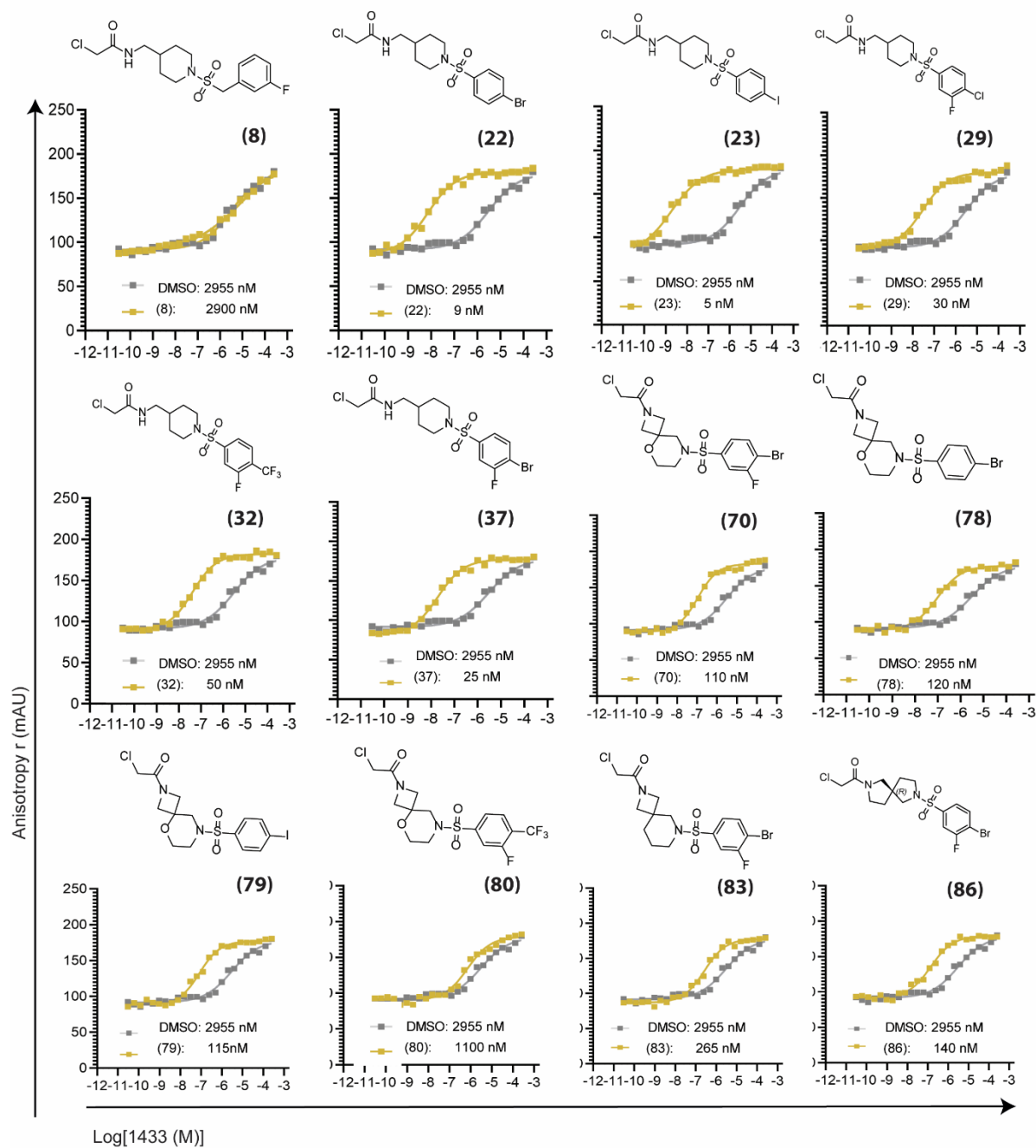


Figure S5.28: FA protein titrations (A-RAF214 15-mer peptide)

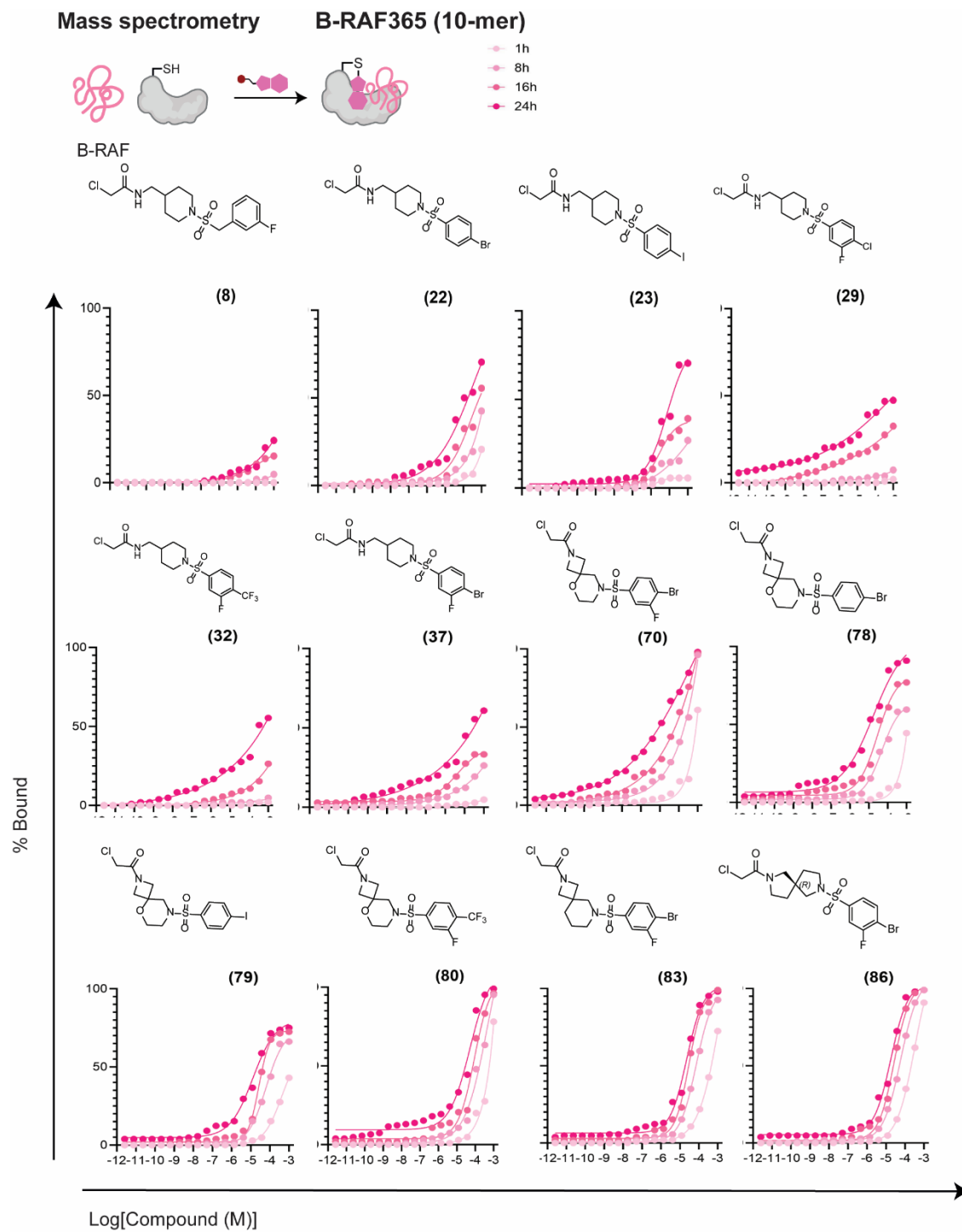


Figure S5.29: MS dose-response curves (B-RAF365 10-mer peptide)

B-RAF 365 (10-mer): Protein titrations

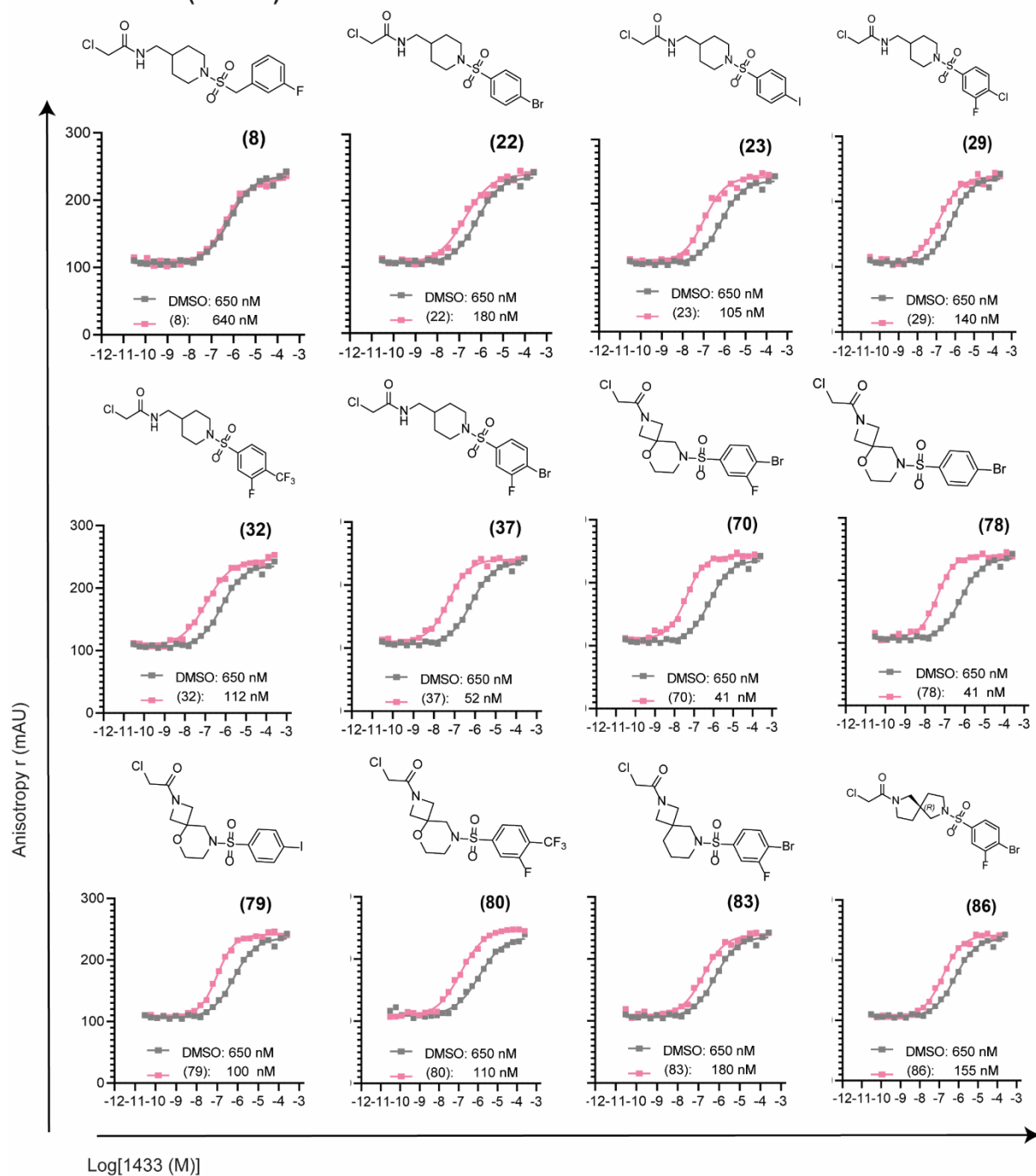


Figure S5.30: FA protein titrations (B-RAF365 10-mer peptide)

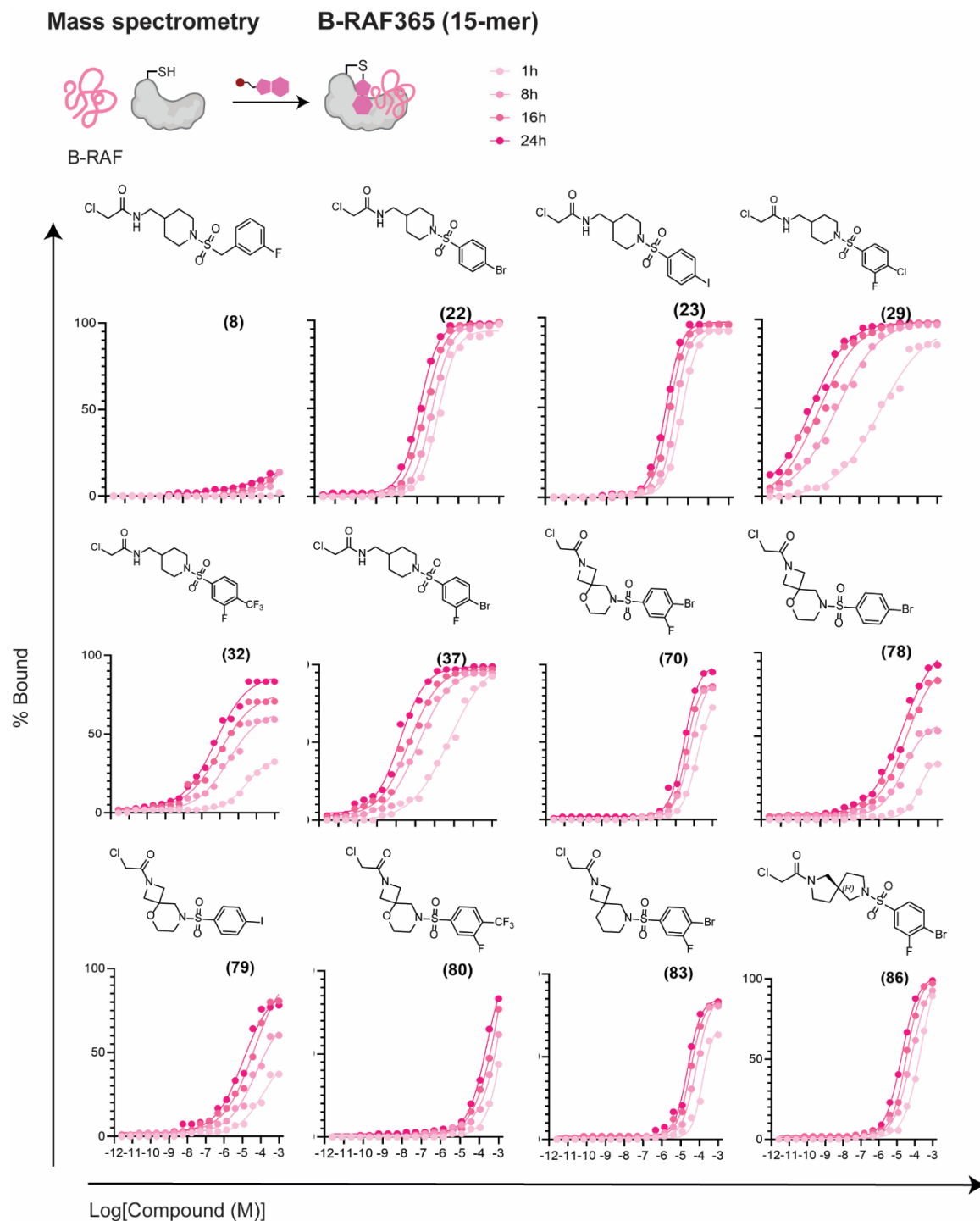


Figure S5.31: MS dose-response curves (B-RAF365 15-mer peptide)

B-RAF 365 (15-mer): Protein titrations

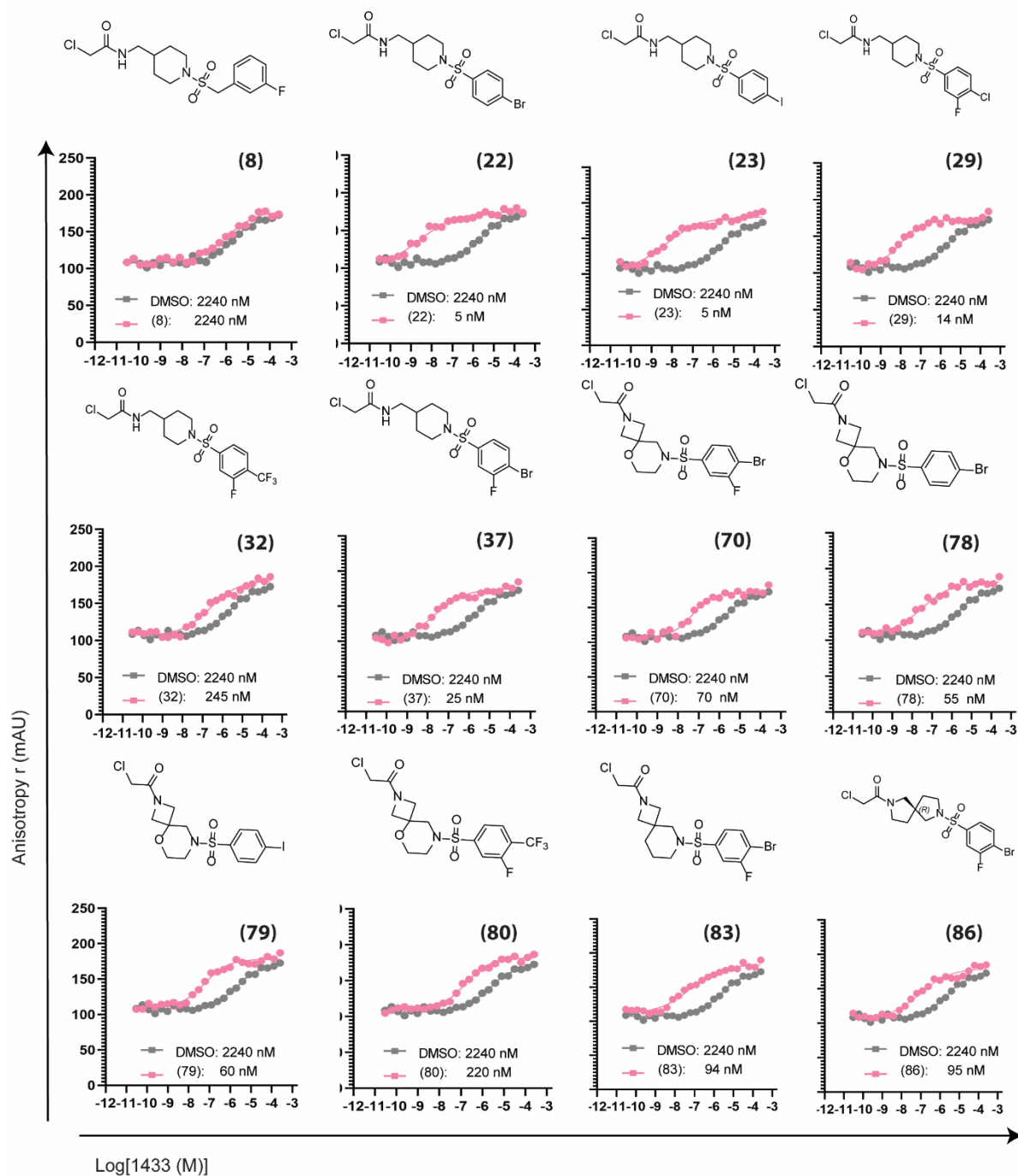


Figure S5.32: FA protein titrations (B-RAF365 15-mer peptide)

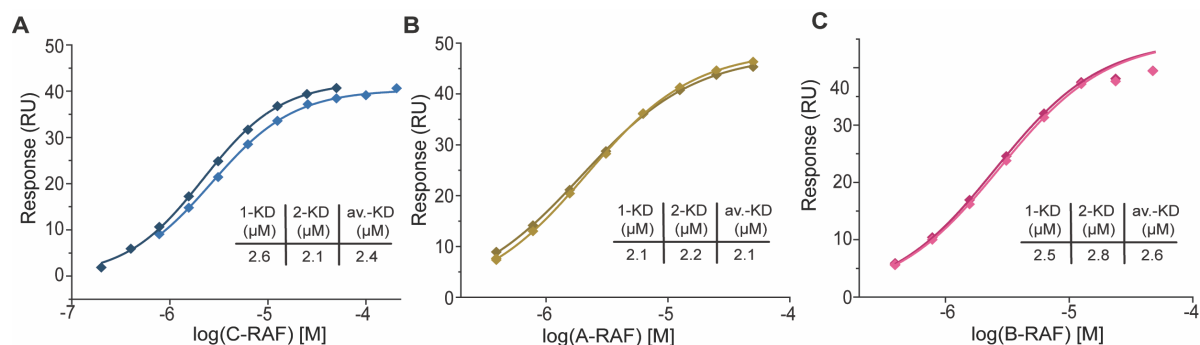


Figure S5.33: Affinity fitting of SPR data

Acetylated 12-mer A-, B-, C-RAF peptides binding to 14-3-3 σ captured on a chip (n=2). Response at equilibrium is plotted against peptide concentration to determine the affinity constant (only possible for binary interactions as for ternary interactions no equilibrium is reached during association).

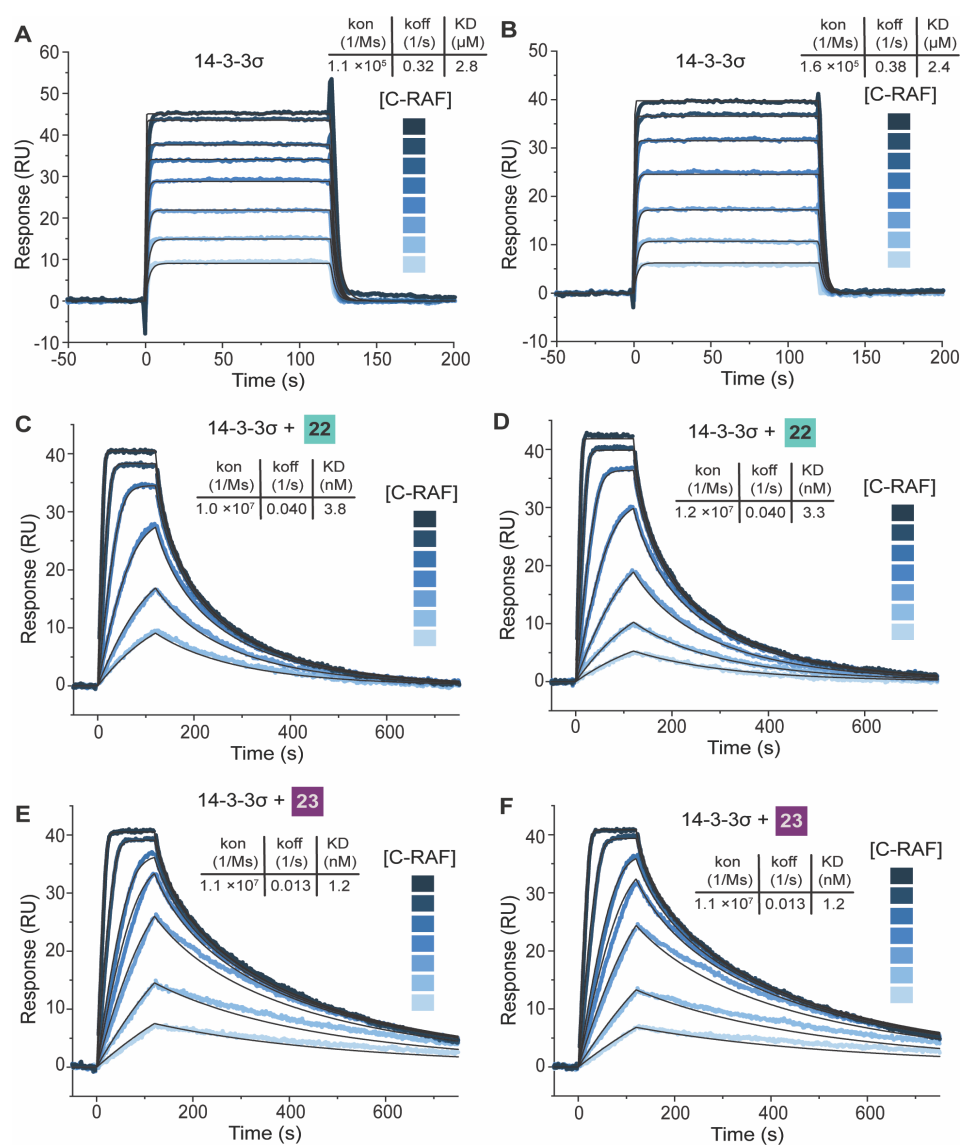


Figure S5.34: Kinetic fitting of C-RAF SPR data

Acetylated 12-mer C-RAF peptide binding to 14-3-3 σ captured on a chip without molecular glue and in the presence of compounds 22 and 23 (n=2).

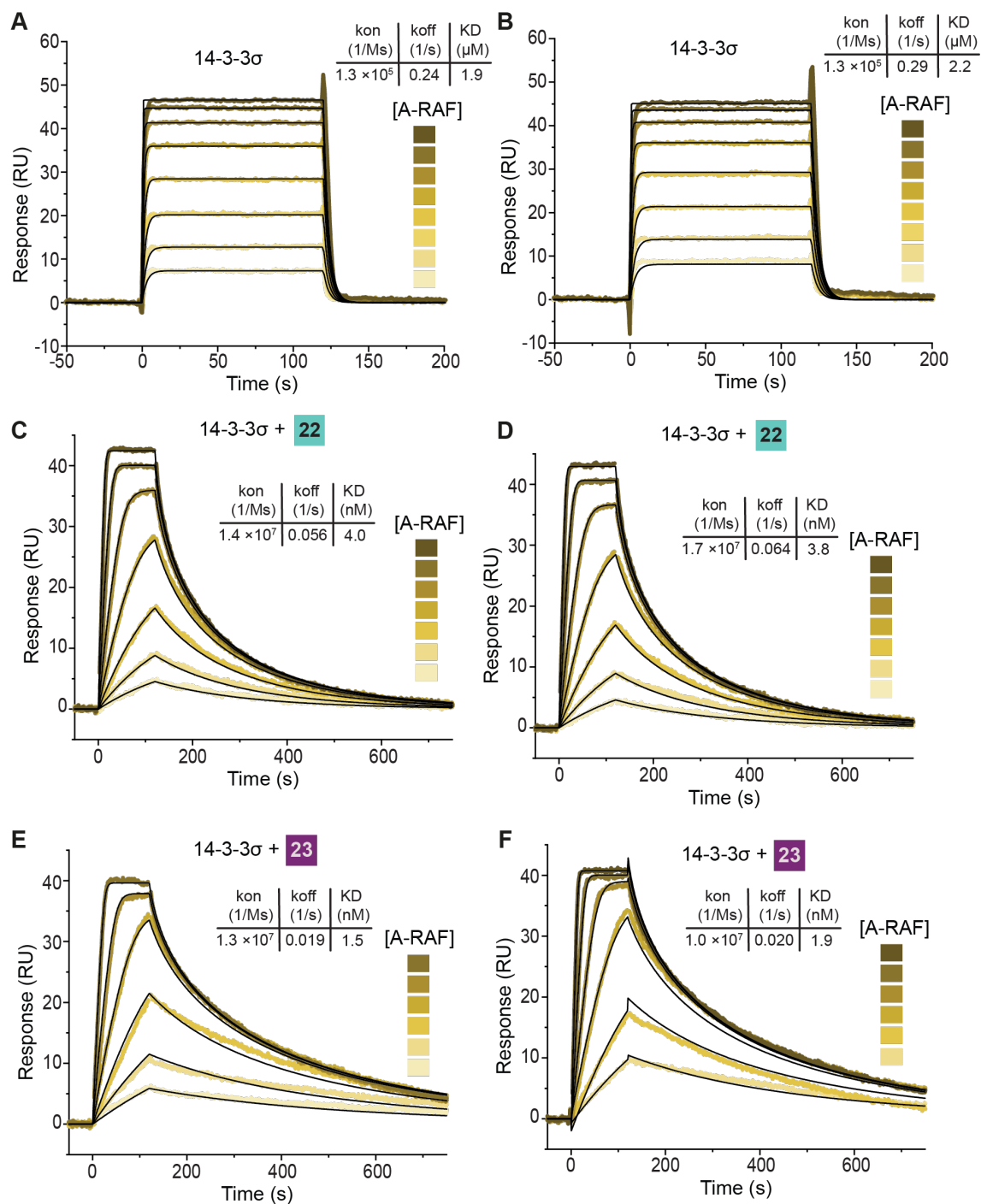


Figure S5.35: Kinetic fitting of A-RAF SPR data

Acetylated 12-mer A-RAF peptide binding to 14-3-3 σ captured on a chip without molecular glue and in the presence of compounds **22** and **23** (n=2).

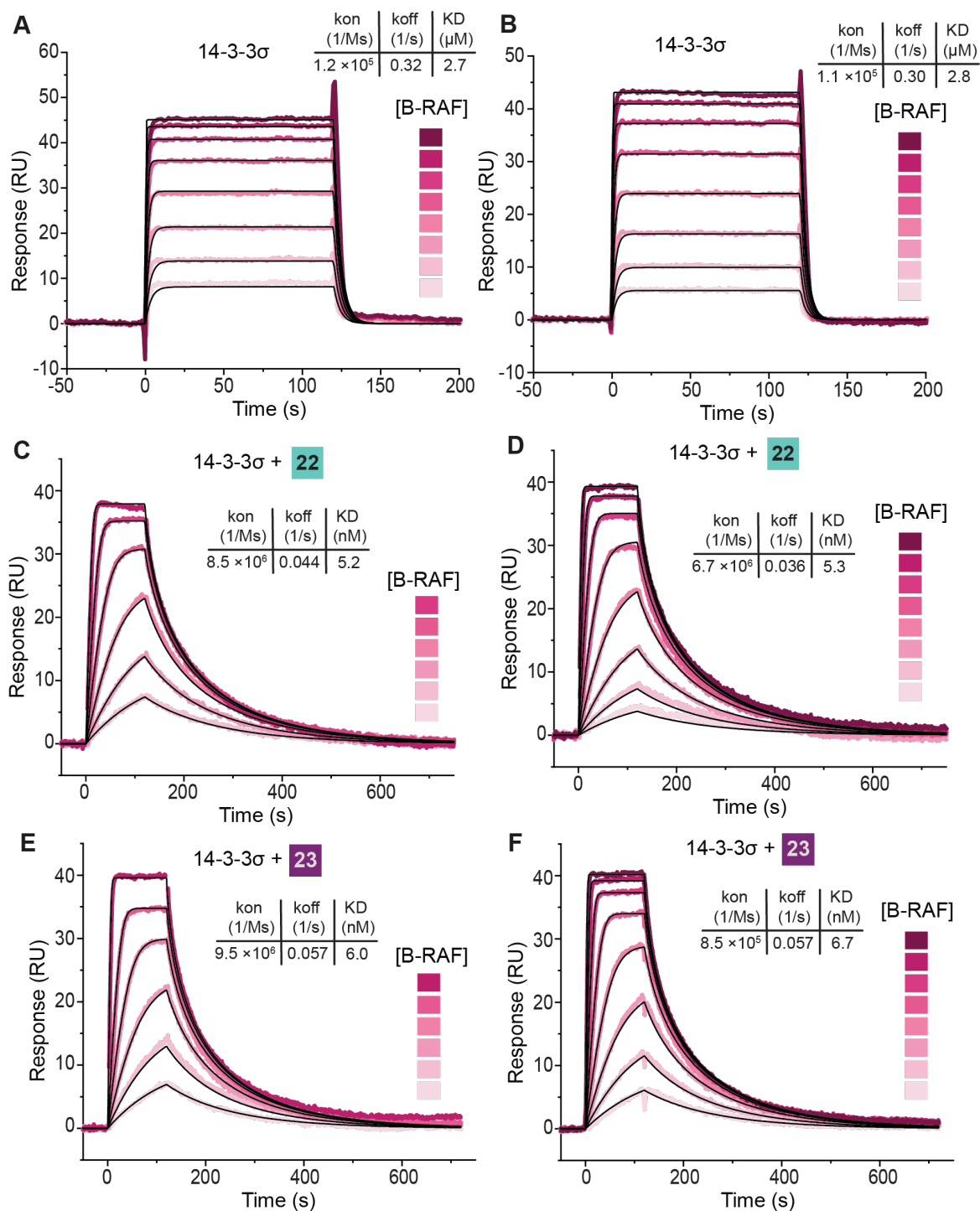


Figure S5.36: Kinetic fitting of B-RAF SPR data

Acetylated 12-mer B-RAF peptide binding to 14-3-3 σ captured on a chip without molecular glue and in the presence of compound **22** and **23** (n=2).

A-RAF 582 (15-mer): Protein titrations

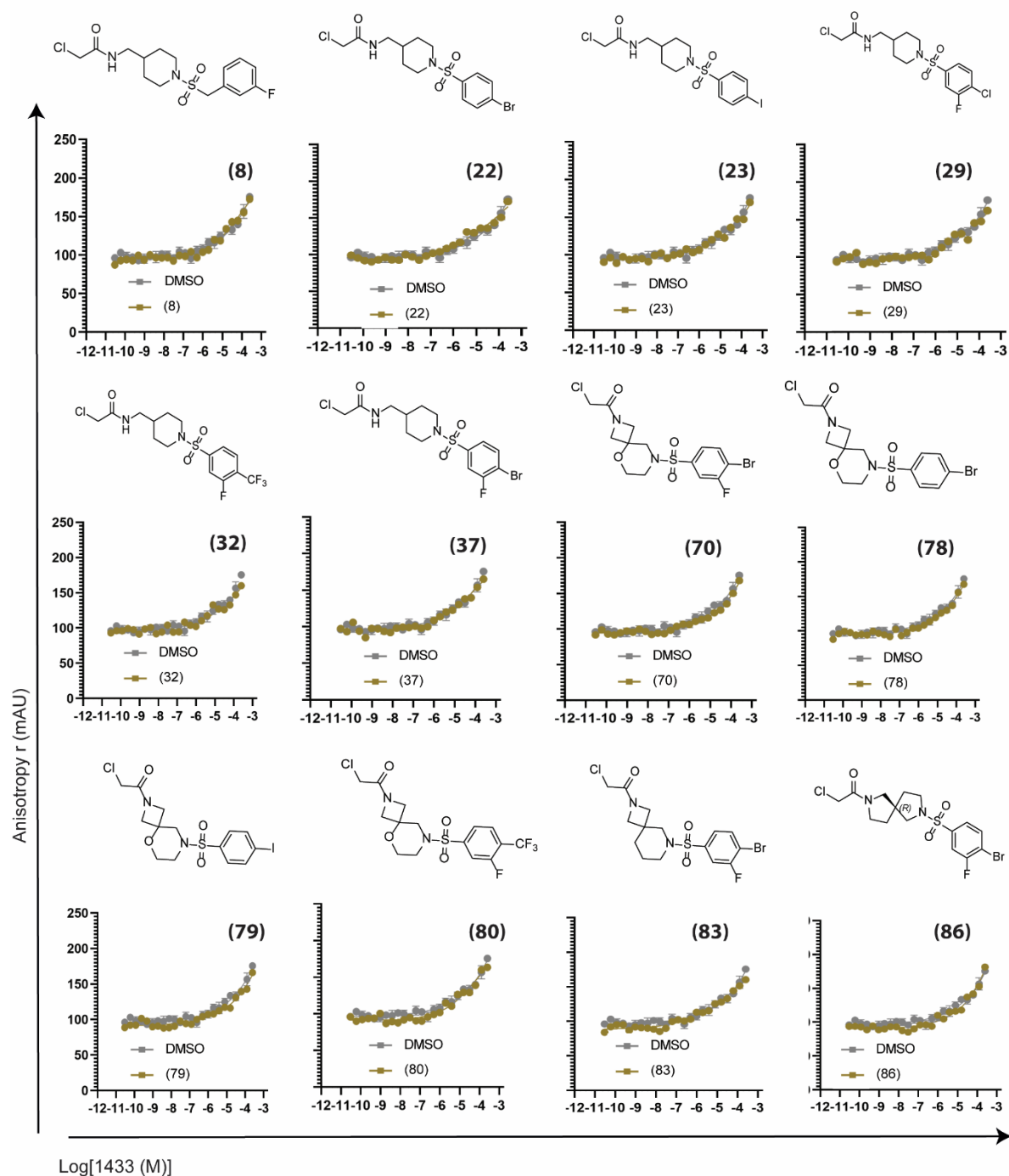


Figure S5.37: FA protein titrations (A-RAF582 15-mer peptide)

B-RAF 729 (15-mer): Protein titrations

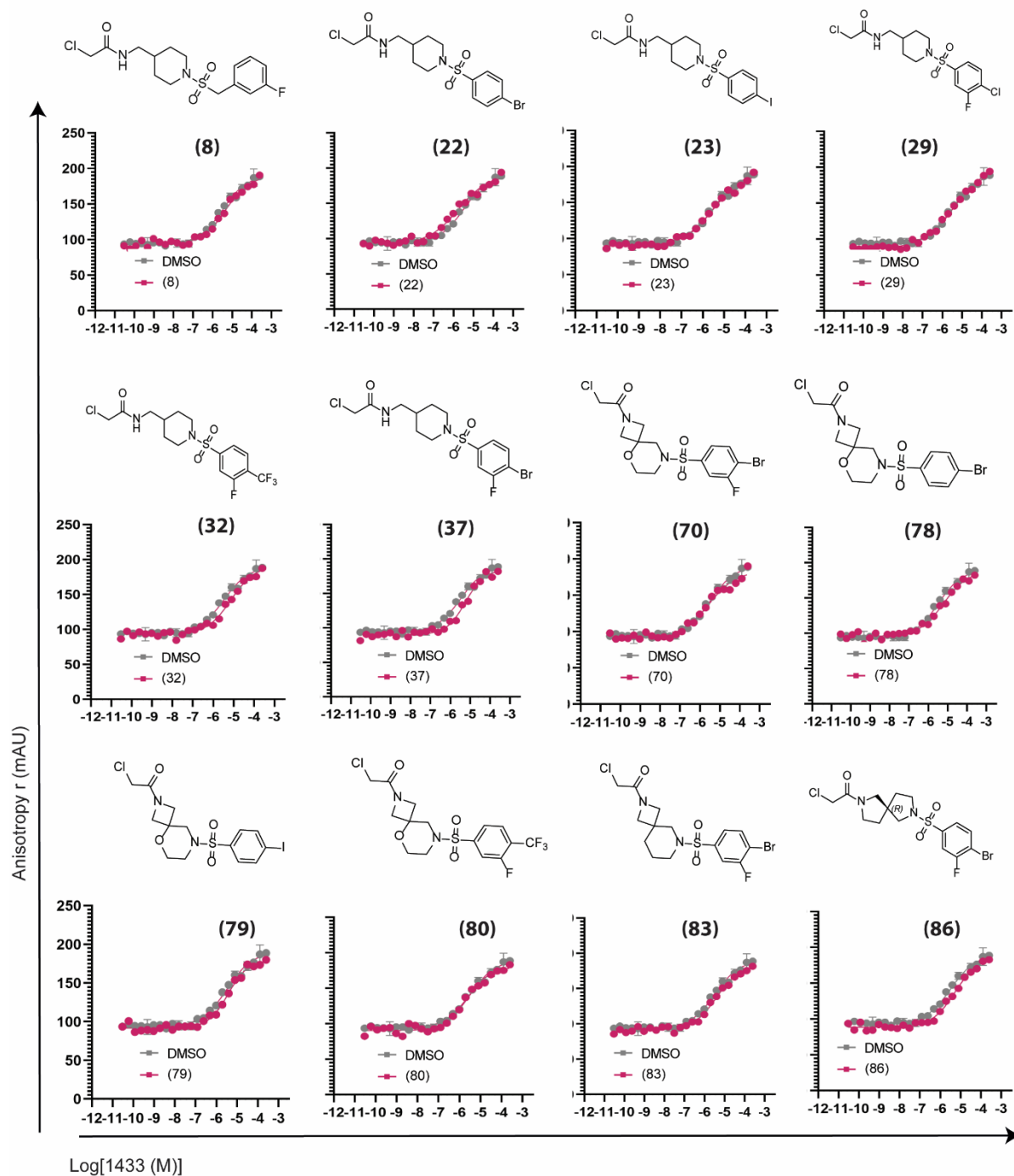


Figure S5.38: FA protein titrations (B-RAF729 15-mer peptide)

C-RAF 621 (15-mer): Protein titrations

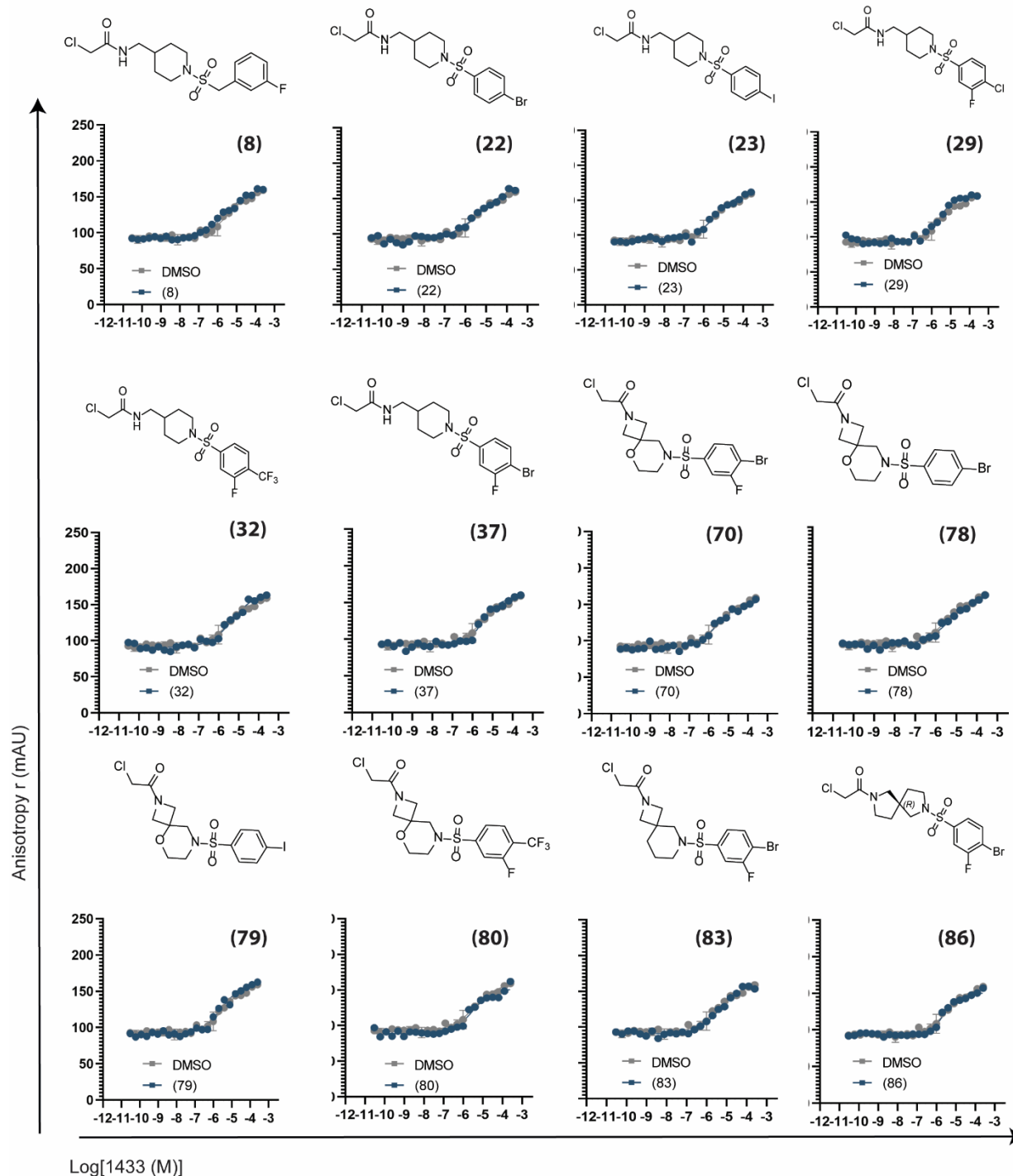


Figure S5.39: FA protein titrations (C-RAF621 15-mer peptide)

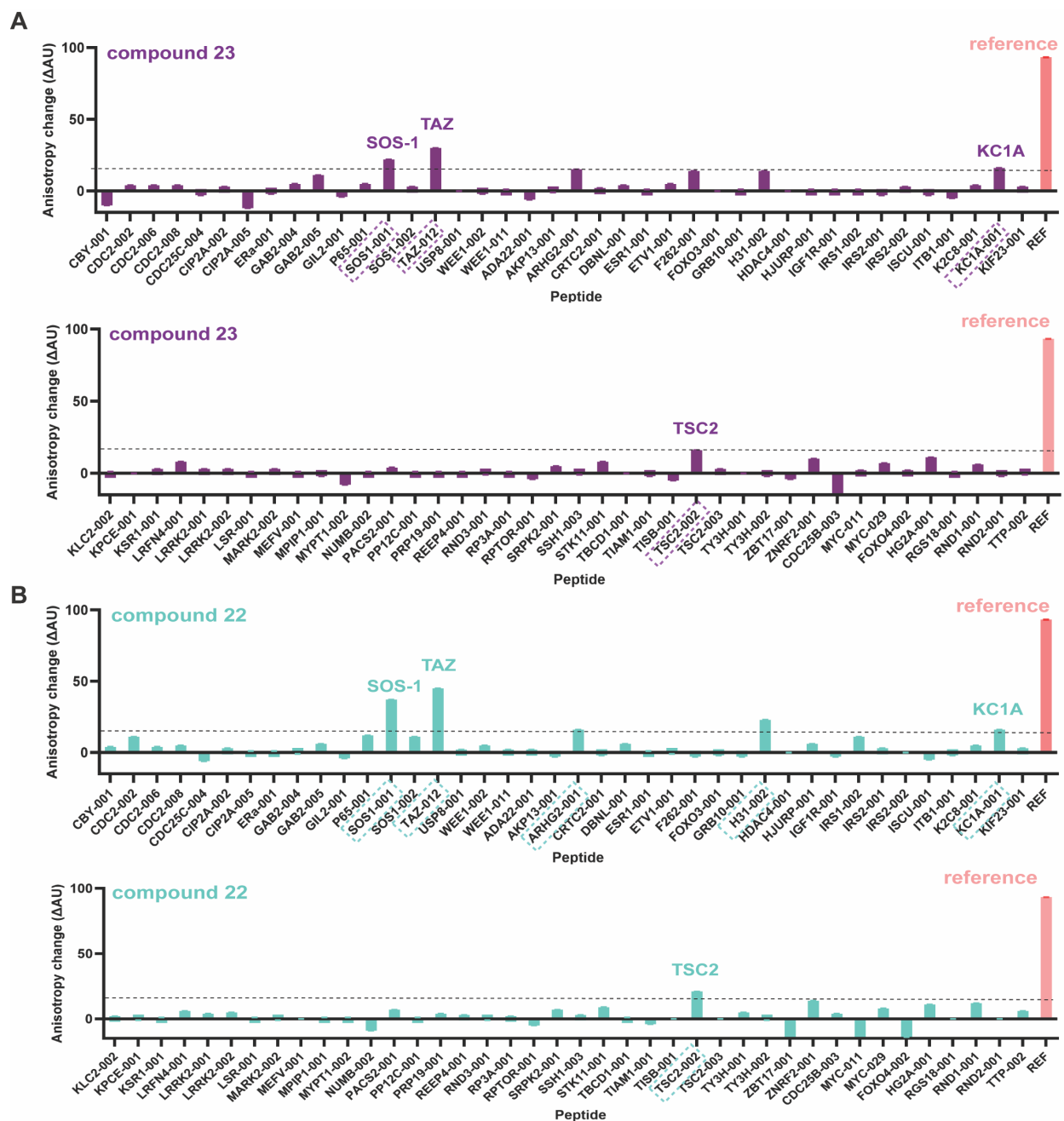


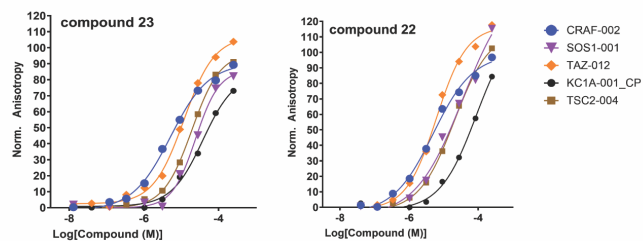
Figure S5.40: FA selectivity panel

Compounds **23** (A) and **22** (B) with 14-3-3 σ and 80 client peptides. Six client hits were identified: SOS1, TAZ, ARGH2, H31, KC1A and TSC2.

A

Peptide	Phosphosite	-2	-1	Phospho	+1	+2	+3
C-RAF	pS259	S	T	pS	T	P	N
A-RAF	pS214	S	T	pS	T	P	N
B-RAF	pS359	S	S	pS	A	P	N
SOS1	pS1161	P	E	pS	A	P	A
TAZ	pS89	S	H	pS	S	P	A
KC1A	pS218	R	T	pS	L	P	W
TSC2	pS1254	S	L	pS	V	P	A
ARGH2	pS886	R	R	pS	L	P	A
H31	pS29	R	K	pS	A	P	A

B



C

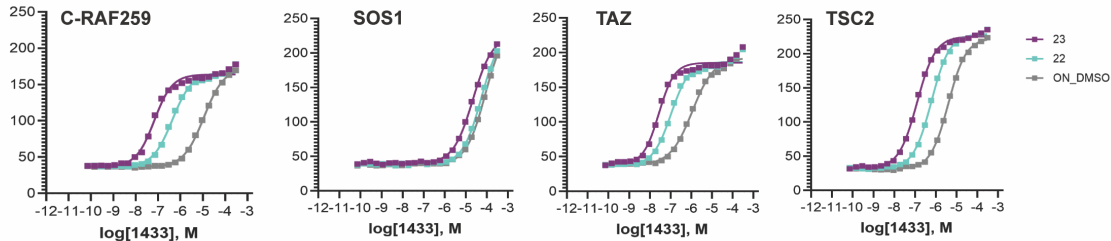


Figure S5.41: FA selectivity panel follow-up

A) Phospho-sites and sequences around the phospho-site for client peptides that emerged as hits. **B)** FA compound dose-response follow-up for the four common clients. **C)** FA protein titrations follow-up. EC_{50} values, $appK_d$ s and fold-stabilization are shown in tables S5.5 and S5.6.

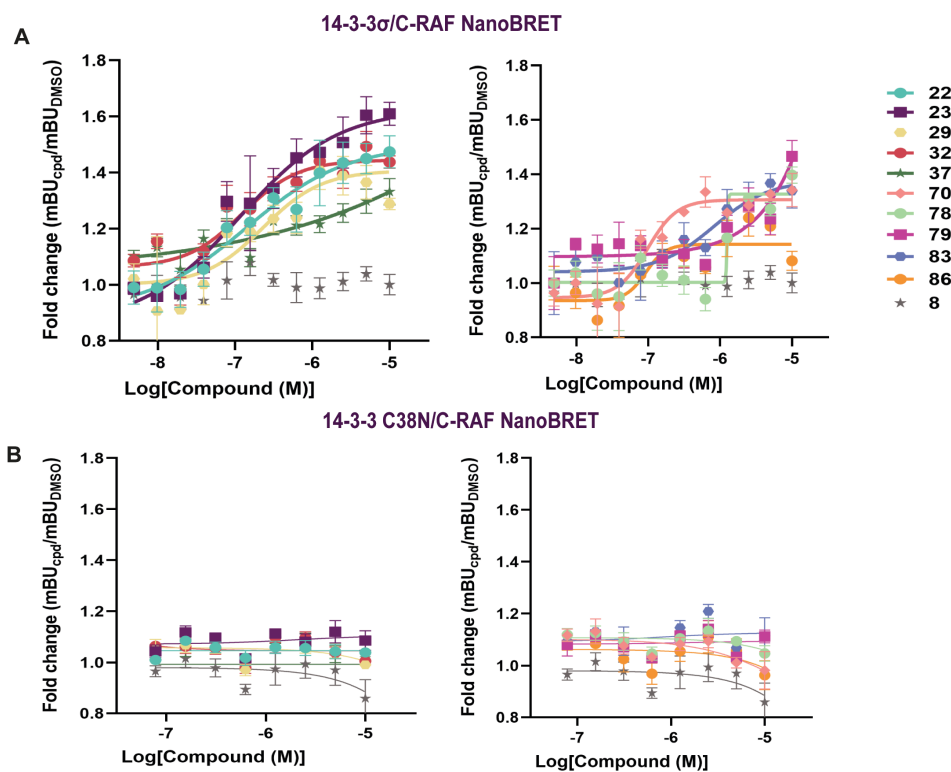


Figure S5.42: 14-3-3 σ /C-RAF NanoBRET

A) 14-3-3 σ /C-RAF NanoBRET dose-response data in HEK293T cells. Left: piperidine-containing compounds, right: spirocycles. **B)** 14-3-3 C38N/C-RAF NanoBRET dose-response data in HEK293T cells. Left: piperidine-containing compounds, right: spirocycles

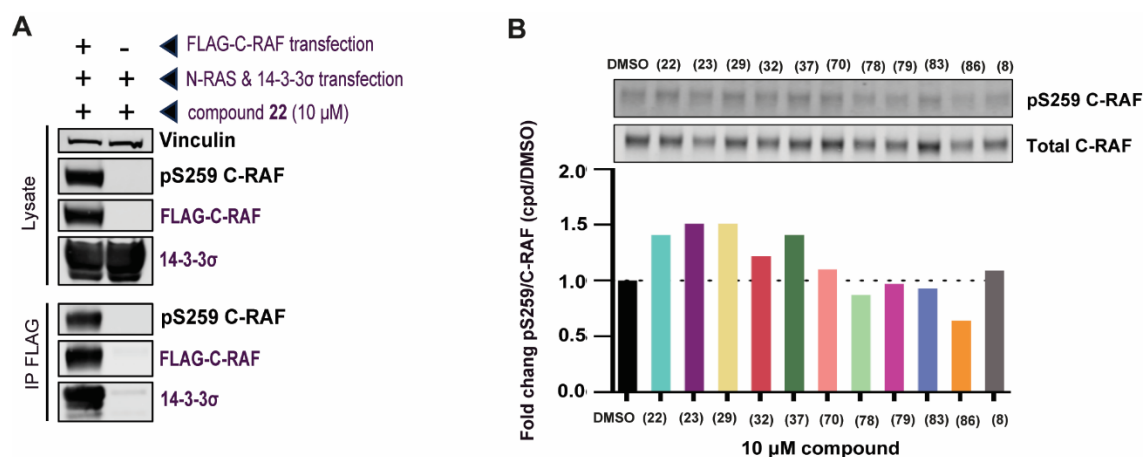


Figure S5.43: Stabilization of endogenous C-RAF

A) Co-IP for **22**, comparing endogenous and transfected C-RAF. **B)** Protection of phosphorylation of C-RAF pS259 site in MIA PaCa-2 cells.

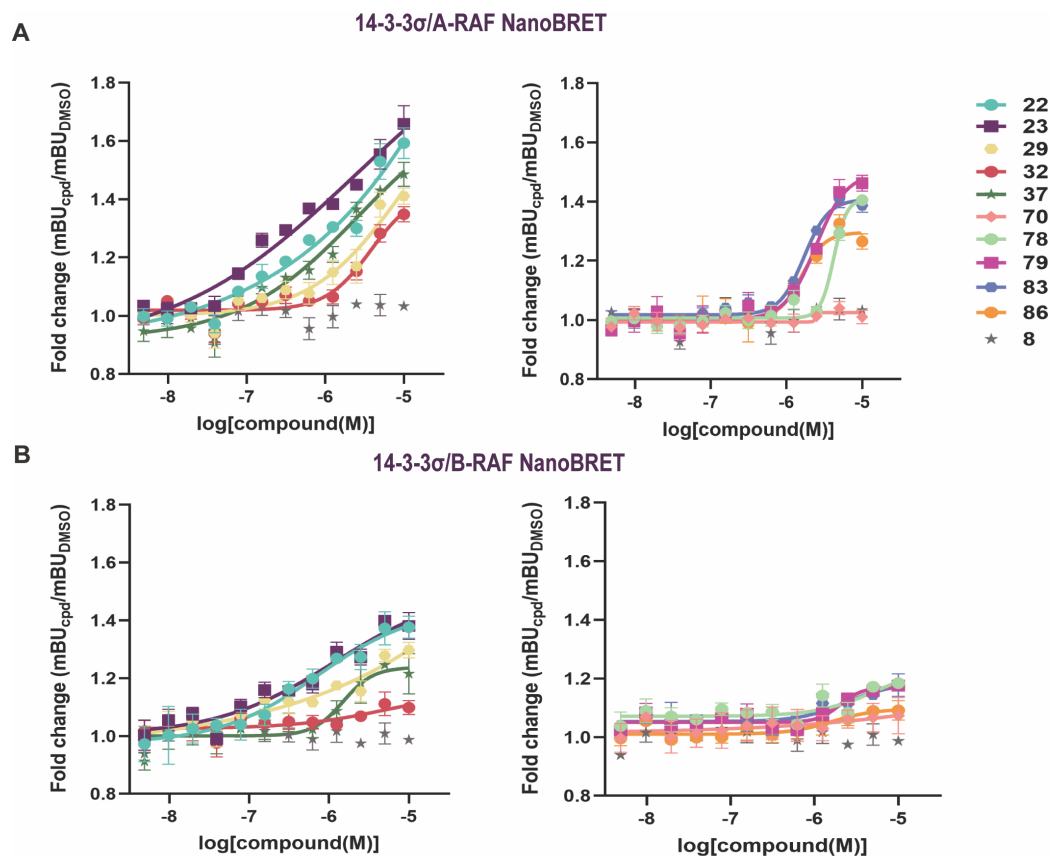


Figure S5.44: 14-3-3 σ /A-RAF and 14-3-3 σ /B-RAF NanoBRET

A) 14-3-3 σ /A-RAF NanoBRET dose-response data in HEK293T cells. Left: piperidine-containing compounds, right: spirocycles. **B)** 14-3-3 σ /B-RAF NanoBRET dose-response data in HEK293T cells. Left: piperidine-containing compounds, right: spirocycles.

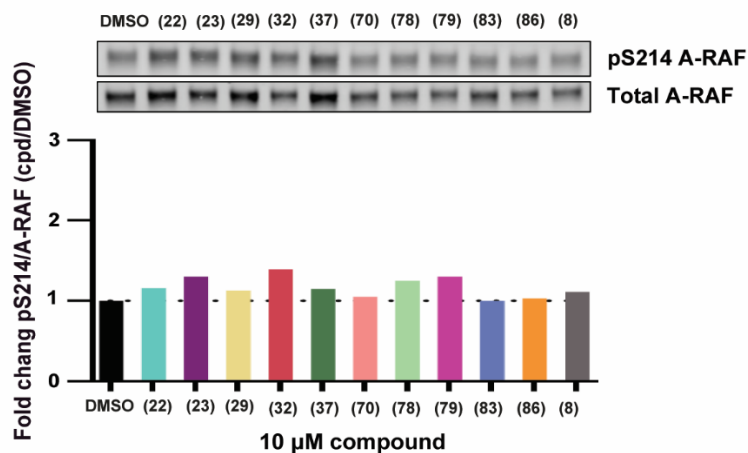


Figure S5.45: Protection of phosphorylation of A-RAF pS214 site in HEK293T cells
The assay was performed with the pS259 C-RAF antibody.

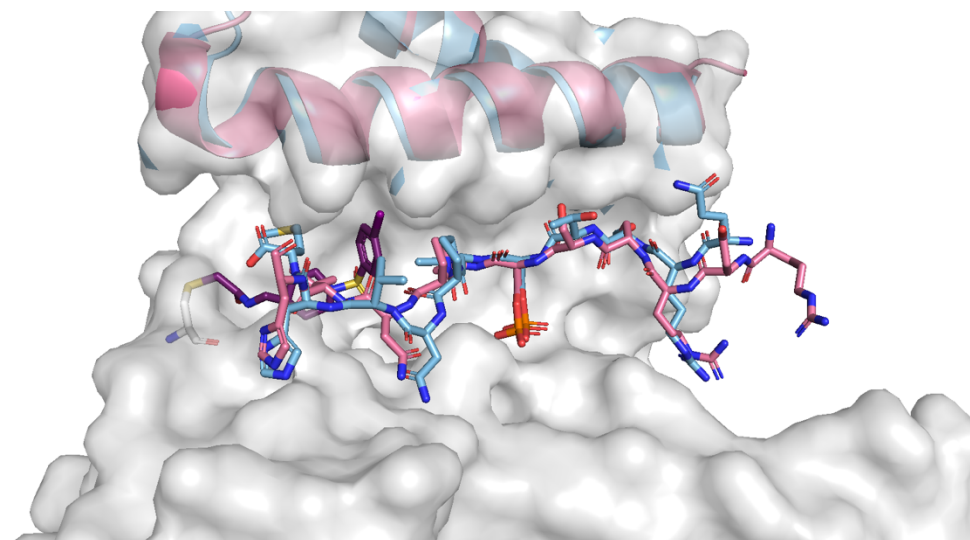


Figure S5.46: Overlay of 14-3-3/B-RAF cryoEM structure with the crystal structure of 14-3-3 σ /CRAF259/23

Compound **23** is shown as purple sticks, B-RAF365 as pink sticks and C-RAF259 as cyan sticks. Helix 9 is in the same position for both structures. PDB 6NYB

Supplementary Tables

Table S5.1: EC₅₀ values derived from FA compound titrations

In the presence of 10 nM of C-RAF259-FAM labeled peptide and 5 μ M 14-3-3 σ . Protein titrations with C-RAF-259-FAM for compounds with EC₅₀ < 20 μ M (10 nM of C-RAF259-FAM, 100 μ M compound, 250 μ M 14-3-3 starting concentration, 2-fold dilution). EC₅₀ and *app*K_d values refer to overnight measurements.

Compound No.	Compound titrations	Protein titrations		
	EC ₅₀ value C-RAF259 (μ M)	Apparent K _d (nM) (100 μ M compound)	K _d with DMSO reference	Fold stabilization (100 μ M compound)
(6)	>1000	NA	NA	NA
(7)	>1000	NA	NA	NA
(8)	>1000	6600 nM	6700 nM	1
(9)	>1000	NA	NA	NA
(10)	66 $\pm$ 4 μ M	NA	NA	NA
(11)	17 $\pm$ 3 μ M	400 nM	1325 nM	4
(12)	3 $\pm$ 1 μ M	190 nM	4200 nM	22
(13)	>1000	NA	NA	NA
(14)	82 $\pm$ 6 μ M	NA	NA	NA
(15)	87 $\pm$ 9 μ M	NA	NA	NA
(16)	> 150	NA	NA	NA
(17)	> 150	NA	NA	NA
(18)	17 $\pm$ 1 μ M	280 nM	4200 nM	15
(19)	13 $\pm$ 3 μ M	213 nM	3050 nM	14
(20)	>150	NA	NA	NA
(21)	5 $\pm$ 1.0 μ M	480 nM	4200 nM	9
(22)	1 $\pm$ 0.5 μ M	47 nM	8427 nM	179
(23)	1 $\pm$ 0.5 μ M	30 nM	8427 nM	280
(24)	14 $\pm$ 1 μ M	110 nM	2277 nM	21
(25)	57 $\pm$ 9 μ M	NA	NA	NA
(26)	26 $\pm$ 3 μ M	NA	NA	NA

	Compound titrations	Protein titrations		
Compound No.	EC ₅₀ value C-RAF259 (μM)	Apparent Kd (nM) (100 μM compound)	Kd with DMSO reference	Fold stabilization (100 μM compound)
(27)	>150 μM	NA	NA	NA
(28)	88 ± 2 μM	NA	NA	NA
(29)	1 ± 0.5 μM	100 nM	8427 nM	84
(30)	12 ± 2 μM	780 nM	2277 nM	3
(31)	8 ± 1 μM	1420 nM	2277 nM	2
(32)	4 ± 1 μM	140 nM	8427 nM	60
(33)	9 ± 1 μM	230 nM	3050 nM	13
(34)	> 1000	NA	NA	NA
(35)	> 1000	NA	NA	NA
(36)	25 ± 2 μM	240 nM	4200 nM	18
(37)	5 ± 1 μM	92 nM	8427 nM	92
(38)	2 ± 1 μM	218 nM	8427 nM	39
(39)	17 ± 3 μM	420 nM	4200 nM	7
(40)	2 ± 1 μM	320 nM	4200 nM	13
(43)	> 1000	NA	NA	NA
(46)	11 ± 1 μM	170 nM	8427 nM	49
(49)	47 ± 4 μM	NA	NA	NA
(52)	94 ± 9 μM	NA	NA	NA
(55)	> 1000	NA	NA	NA
(58)	12 ± 1 μM	450 nM	2700 nM	6
(61)	78 ± 7 μM	NA	NA	NA
(64)	15 ± 2 μM	1160 nM	2277 nM	2
(67)	14 ± 1 μM	250 nM	2700 nM	11
(70)	2 ± 1 μM	58 nM	5514 nM	95
(73)	> 1000	NA	NA	NA
(74)	> 1000	NA	NA	NA
(75)	> 1000	NA	NA	NA
(78)	2 ± 1 μM	40 nM	8427 nM	210
(79)	2 ± 1 μM	35 nM	8427 nM	240
(80)	9 ± 2 μM	420 nM	4600 nM	11
(83)	5 ± 2 μM	102 nM	8427 nM	83
(86)	1 ± 1 μM	28 nM	8427 nM	300
(89)	> 250	620 nM	5514 nM	9

Table S5.2: Mass spectrometry percentage (%) bound 1 μM compound to 14-3-3σ
14-3-3σ (100 nM) in the presence of C-RAF259 peptide (18 μM) or A-RAF214 peptide (16 μM) or B-RAF365 peptide (5 μM) after 1, 8, 16 and 24 hours. Peptide lengths are described in the tables below.

Compound No.	A-RAF214 (15mer)				B-RAF365 (10mer)				C-RAF259 (11mer)			
	1h	8h	16h	24h	1h	8h	16h	24h	1h	8h	16h	24h
(8)	0	0	0	0	0	0	2.5	2.5	0	0	1.9	2.9
(22)	54.1	75	81.4	88.5	1	1.9	1.9	11.6	61	85.5	94.8	98.5
(23)	92	95.5	97.5	97.5	0	1.4	2.4	5.6	55.6	78.5	88.9	91
(29)	79	96.5	97	97	0	1.4	11.4	21.2	20.5	38.1	57.1	80.1
(32)	58.2	82.1	94.3	95.2	0	1	3.4	15.9	14.1	21.6	39.3	74.1
(37)	90.9	98	99	99	0	4.2	7	17.3	48.7	77.1	95.2	98
(70)	5.2	12.6	18.5	32.3	2.4	9.9	19.3	32.2	30	47.5	68	87.2
(78)	2.9	9	15.5	28	1	5.2	11.9	23	8.3	25.3	35.6	70
(79)	4.7	8.2	15.2	23.5	0	1.9	5.1	14.1	15	29.5	52.6	72
(80)	1.9	3.8	6.5	9	1	4.7	11.4	18.6	6	7	15.2	25.1
(83)	4.7	9.5	16.5	24.5	1.9	4.2	8.6	12.6	27	52.5	66.3	84
(86)	11.3	21.7	35.2	44.5	1	5.2	9.9	11.8	64.6	93.1	97.1	98.5

Compound No.	A-RAF214 (15mer)				B-RAF365 (15mer)				C-RAF259 (15mer)			
	1h	8h	16h	24h	1h	8h	16h	24h	1h	8h	16h	24h
(8)	0	0	0	0	0	0	0.9	3.8	0	0	0	0
(22)	54.1	75	81.4	88.5	39	56	71	83	48	58.9	84.5	89.3
(23)	92	95.5	97.5	97.5	12.8	21.2	36.2	46.7	50	66	72	91
(29)	79	96.5	97	97	45.5	87.8	93.9	96.2	56.8	85.9	92.2	95.3
(32)	58.2	82.1	94.3	95.2	4.7	23.1	36.4	51.6	59.7	86.7	89.9	94.4
(37)	90.9	98	99	99	32.1	69.5	81.7	92.7	64.3	91.3	92	94.4
(70)	5.2	12.6	18.5	32.3	1.4	2.9	3.4	4.3	2	3.8	5.6	5.6
(78)	2.9	9	15.5	28	1.9	9.8	16.8	21.8	2	4.7	14	15
(79)	4.7	8.2	15.2	23.5	3.4	8.3	15.2	19.2	7.4	9	16.8	24.5
(80)	1.9	3.8	6.5	9	0	1.9	2.9	4.3	1.9	1.9	3.8	5.6
(83)	4.7	9.5	16.5	24.5	1.4	2.9	2.9	6.5	2.9	4.7	8.3	9.09
(86)	11.3	21.7	35.2	44.5	2.9	3.4	5.2	7.3	7.4	16.7	18.5	23.1

Table S5.3: Protein titrations in the presence of 10 nM FAM-labeled peptides

A-RAF214, B-RAF365 or C-RAF259, 100 μ M compound, 250 μ M 14-3-3 σ starting concentration, 2-fold dilution. Apparent Kd (*appKd*) values refer to overnight measurements.

No.	<i>A-RAF 214</i> (15mer)		<i>B-RAF 365</i> (10mer)		<i>C-RAF 259</i> (10mer)		
	<i>App Kd</i>	fold stab.	<i>App Kd</i>	fold stab.	<i>App Kd</i>	<i>App Kd DMSO</i>	fold stab.
(8)	NA	NA	NA	NA	NA	6700 nM	NA
(22)	9 nM	328	180 nM	4	47 nM	8427 nM	179
(23)	5 nM	591	105 nM	6	30 nM	8427 nM	280
(29)	30 nM	99	140 nM	5	100 nM	8427 nM	84
(32)	50 nM	59	112 nM	6	140 nM	8427 nM	60
(37)	25 nM	118	52 nM	13	92 nM	8427 nM	92
(70)	110 nM	27	41 nM	15	58 nM	5514 nM	95
(78)	120 nM	25	41 nM	15	40 nM	8427 nM	210
(79)	115 nM	26	100 nM	6	35 nM	8427 nM	240
(80)	1100 nM	3	110 nM	6	420 nM	4600 nM	11
(83)	265 nM	11	180 nM	4	102 nM	8427 nM	83
(86)	140 nM	21	155 nM	4	28 nM	8427 nM	300
DMSO	2955 nM	-	650 nM	-	-	-	-

No.	<i>A-RAF 214</i> (15mer)		<i>B-RAF 365</i> (15mer)		<i>C-RAF 259</i> (15mer)		
	<i>App Kd</i>	fold stab.	<i>App Kd</i>	fold stab.	<i>App Kd</i>	<i>App Kd DMSO</i>	fold stab.
(8)	NA	NA	NA	NA	NA	12000 nM	NA
(22)	9 nM	328	5 nM	448	5 nM	12000 nM	2400
(23)	5 nM	591	5 nM	448	5 nM	12000 nM	2400
(29)	30 nM	99	14 nM	160	154 nM	12000 nM	78
(32)	50 nM	59	245 nM	9	126 nM	12000 nM	95
(37)	25 nM	118	25 nM	89	136 nM	12000 nM	88
(70)	110 nM	27	70 nM	32	100 nM	12000 nM	120
(78)	120 nM	25	55 nM	40	110 nM	12000 nM	110
(79)	115 nM	26	60 nM	37	135 nM	12000 nM	90
(80)	1100 nM	3	220 nM	10	332 nM	12000 nM	36
(83)	265 nM	11	94 nM	24	145 nM	12000 nM	83
(86)	140 nM	21	95 nM	24	140 nM	12000 nM	85
DMSO	2955 nM	-	2240 nM	-	12000 nM	-	-

Table S5.4: Compound titration follow-up from the selectivity panel

100 nM FAM-labeled peptides: C-RAF259, SOS1, TAZ, KC1A, TSC2, 14-3-3 σ concentration at EC₂₀ for each protein-peptide complex, 250 μ M compound starting concentration, 3-fold dilution. EC₅₀ values refer to overnight measurements.

No.	C-RAF259	SOS1	TAZ	KC1A	TSC2
(22)	5 μ M	35.7 μ M	6.2 μ M	87.4 μ M	20.3 μ M
(23)	5.1 μ M	24.2 μ M	11.7 μ M	38.1 μ M	19.2 μ M

Table S5.5: Protein titration follow-up from the selectivity panel

10 nM FAM-labeled peptides: C-RAF259, SOS1, TAZ, TSC2, 100 μ M compound, 300 μ M 14-3-3 σ starting concentration, 2-fold dilution. Apparent K_d (*appKd*) values refer to overnight measurements.

	C-RAF259		SOS1		TAZ		TSC2	
No.	<i>App Kd</i>	fold stab.	<i>App Kd</i>	fold stab.	<i>App Kd</i>	fold stab.	<i>App Kd</i>	fold stab.
(22)	400 nM	28	54 μ M	1.5	105 nM	11	600 nM	7
(23)	68 nM	162	21 μ M	4	27 nM	41	100 nM	40
DMSO	11 μ M	-	84 μ M	-	1.1 μ M	-	4 μ M	-

Table S5.6: Cell data for 14-3-3/C-RAF

NanoBRET, co-IP, pS259 protection of phosphorylation in HEK293T and MIA PaCa-2 cells. NA = non-applicable, NT = not tested.

No.	14-3-3/C-RAF NanoBRET EC ₅₀ (μ M)	14-3-3/C-RAF NanoBRET fold stab.	14-3-3/C-RAF co-IP fold change	C-RAF pS259 protection fold change (HEK293T)	C-RAF pS259 protection fold change (MIA PaCa-2)
(22)	0.2	1.5	5.1	2.4	1.4
(23)	0.18	1.6	5.3	3.2	1.5
(29)	0.14	1.3	NT	2.2	1.5
(32)	0.1	1.4	NT	1.9	1.2
(37)	NA	1.3	NT	2.4	1.4
(70)	0.12	1.3	NT	2.1	1.1
(78)	1.2	1.4	1.6	2.3	0.9
(79)	NA	1.5	NT	1.3	1.0
(83)	1.1	1.4	NT	1.3	0.9
(86)	0.3	1.2	NT	1.0	0.6

Table S5.7: Cell data for 14-3-3/A-RAF and 14-3-3/B-RAF

NanoBRET and pS214 protection of phosphorylation in HEK293T cells. NA = non-applicable, NT = not tested.

No.	14-3-3/A-RAF NanoBRET fold stab.	14-3-3/B-RAF NanoBRET fold stab.	A-RAF pS214 protection fold change (HEK293T)
(22)	1.6	1.4	1.2
(23)	1.7	1.4	1.3
(29)	1.4	1.3	1.1
(32)	1.4	1.1	1.4
(37)	1.5	1.3	1.2
(70)	1.0	1.1	1.1
(78)	1.4	1.2	1.3
(79)	1.5	1.2	1.3
(83)	1.4	1.2	1.0
(86)	0.3	1.2	1.0

References

1. Dhillon, A.S., Hagan, S., Rath, O., and Kolch, W. (2007). MAP kinase signalling pathways in cancer. *Oncogene* 26, 3279–3290. <https://doi.org/10.1038/sj.onc.1210421>.
2. Fernandez-Medarde, A., and Santos, E. (2011). Ras in Cancer and Developmental Diseases. *Genes & Cancer* 2, 344–358. <https://doi.org/10.1177/1947601911411084>.
3. Davies, H., Bignell, G.R., Cox, C., Stephens, P., Edkins, S., Clegg, S., Teague, J., Woffendin, H., Garnett, M.J., Bottomley, W., et al. (2002). Mutations of the BRAF gene in human cancer. *Nature* 417, 949–954. <https://doi.org/10.1038/nature00766>.
4. Zhang, W. (2015). BRAF inhibitors: the current and the future. *Current Opinion in Pharmacology* 23, 68–73. <https://doi.org/10.1016/j.coph.2015.05.015>.
5. Drosten, M., and Barbacid, M. (2020). Targeting the MAPK Pathway in KRAS-Driven Tumors. *Cancer Cell* 37, 543–550. <https://doi.org/10.1016/j.ccell.2020.03.013>.
6. Kondo, Y., Ognjenović, J., Banerjee, S., Karandur, D., Merk, A., Kulhanek, K., Wong, K., Roose, J.P., Subramaniam, S., and Kuriyan, J. (2019). Cryo-EM structure of a dimeric B-Raf:14-3-3 complex reveals asymmetry in the active sites of B-Raf kinases. *Science* 366, 109–115. <https://doi.org/10.1126/science.aay0543>.
7. Park, E., Rawson, S., Li, K., Kim, B.-W., Ficarro, S.B., Pino, G.G.-D., Sharif, H., Marto, J.A., Jeon, H., and Eck, M.J. (2019). Architecture of autoinhibited and active BRAF-MEK1-14-3-3 complexes. *Nature* 575, 545–550. <https://doi.org/10.1038/s41586-019-1660-y>.
8. Martinez Fiesco, J.A., Durrant, D.E., Morrison, D.K., and Zhang, P. (2022). Structural insights into the BRAF monomer-to-dimer transition mediated by RAS binding. *Nat Commun* 13, 486. <https://doi.org/10.1038/s41467-022-28084-3>.
9. Cutler, R.E., Stephens, R.M., Saracino, M.R., and Morrison, D.K. (1998). Autoregulation of the Raf-1 serine/threonine kinase. *Proc. Natl. Acad. Sci. U.S.A.* 95, 9214–9219. <https://doi.org/10.1073/pnas.95.16.9214>.

10. Chong, H., and Guan, K.-L. (2003). Regulation of Raf through Phosphorylation and N Terminus-C Terminus Interaction. *Journal of Biological Chemistry* 278, 36269–36276. <https://doi.org/10.1074/jbc.M212803200>.
11. Tzivion, G., Luo, Z., and Avruch, J. (1998). A dimeric 14-3-3 protein is an essential cofactor for Raf kinase activity. *Nature* 394, 88. <https://doi.org/10.1038/27938>.
12. Garnett, M.J., Rana, S., Paterson, H., Barford, D., and Marais, R. (2005). Wild-Type and Mutant B-RAF Activate C-RAF through Distinct Mechanisms Involving Heterodimerization. *Molecular Cell* 20, 963–969. <https://doi.org/10.1016/j.molcel.2005.10.022>.
13. Aitken, A. (2006). 14-3-3 proteins: a historic overview. *Semin Cancer Biol* 16, 162–172. <https://doi.org/10.1016/j.semcancer.2006.03.005>.
14. Sluchanko, N.N. (2020). Reading the phosphorylation code: binding of the 14-3-3 protein to multivalent client phosphoproteins. *Biochemical Journal* 477, 1219–1225. <https://doi.org/10.1042/BCJ20200084>.
15. Roskoski, R. (2010). RAF protein-serine/threonine kinases: Structure and regulation. *Biochemical and Biophysical Research Communications* 399, 313–317. <https://doi.org/10.1016/j.bbrc.2010.07.092>.
16. Wellbrock, C., Karasarides, M., and Marais, R. (2004). The RAF proteins take centre stage. *Nat Rev Mol Cell Biol* 5, 875–885. <https://doi.org/10.1038/nrm1498>.
17. Lavoie, H., and Therrien, M. (2015). Regulation of RAF protein kinases in ERK signalling. *Nat Rev Mol Cell Biol* 16, 281–298. <https://doi.org/10.1038/nrm3979>.
18. García-Alonso, S., Mesa, P., Ovejero, L.D.L.P., Aizpurua, G., Lechuga, C.G., Zarzuela, E., Santiveri, C.M., Sanclemente, M., Muñoz, J., Musteanu, M., et al. (2022). Structure of the RAF1-HSP90-CDC37 complex reveals the basis of RAF1 regulation. *Molecular Cell* 82, 3438-3452.e8. <https://doi.org/10.1016/j.molcel.2022.08.012>.

19. Okamoto, K., and Sako, Y. (2023). Two Closed Conformations of CRAF Require the 14-3-3 Binding Motifs and Cysteine-Rich Domain to be Intact in Live Cells. *Journal of Molecular Biology* 435, 167989. <https://doi.org/10.1016/j.jmb.2023.167989>.
20. Young, L.C., Hartig, N., Boned Del Río, I., Sari, S., Ringham-Terry, B., Wainwright, J.R., Jones, G.G., McCormick, F., and Rodriguez-Viciano, P. (2018). SHOC2-MRAS-PP1 complex positively regulates RAF activity and contributes to Noonan syndrome pathogenesis. *Proc Natl Acad Sci U S A* 115, E10576–E10585. <https://doi.org/10.1073/pnas.1720352115>.
21. Liao, N.P.D., Johnson, M.C., Izadi, S., Gerosa, L., Hammel, M., Bruning, J.M., Wendorff, T.J., Phung, W., Hymowitz, S.G., and Sudhamsu, J. (2022). Structural basis for SHOC2 modulation of RAS signalling. *Nature* 609, 400–407. <https://doi.org/10.1038/s41586-022-04838-3>.
22. Rushworth, L.K., Hindley, A.D., O'Neill, E., and Kolch, W. (2006). Regulation and Role of Raf-1/B-Raf Heterodimerization. *Molecular and Cellular Biology* 26, 2262–2272. <https://doi.org/10.1128/MCB.26.6.2262-2272.2006>.
23. Venkatanarayan, A., Liang, J., Yen, I., Shanahan, F., Haley, B., Phu, L., Verschueren, E., Hinkle, T.B., Kan, D., Segal, E., et al. (2022). CRAF dimerization with ARAF regulates KRAS-driven tumor growth. *Cell Reports* 38, 110351. <https://doi.org/10.1016/j.celrep.2022.110351>.
24. Müller, E., Bauer, S., Stühmer, T., Mottok, A., Scholz, C.-J., Steinbrunn, T., Brännert, D., Brandl, A., Schraud, H., Kreßmann, S., et al. (2017). Pan-Raf co-operates with PI3K-dependent signalling and critically contributes to myeloma cell survival independently of mutated RAS. *Leukemia* 31, 922–933. <https://doi.org/10.1038/leu.2016.264>.
25. Hatzivassiliou, G., Song, K., Yen, I., Brandhuber, B.J., Anderson, D.J., Alvarado, R., Ludlam, M.J.C., Stokoe, D., Gloor, S.L., Vigers, G., et al. (2010). RAF inhibitors prime wild-

- type RAF to activate the MAPK pathway and enhance growth. *Nature* **464**, 431–435.
<https://doi.org/10.1038/nature08833>.
26. Morgan, C.W., Dale, I.L., Thomas, A.P., Hunt, J., and Chin, J.W. (2021). Selective CRAF Inhibition Elicits Transactivation. *J. Am. Chem. Soc.* **143**, 4600–4606.
<https://doi.org/10.1021/jacs.0c11958>.
27. Poulikakos, P.I., Zhang, C., Bollag, G., Shokat, K.M., and Rosen, N. (2010). RAF inhibitors transactivate RAF dimers and ERK signalling in cells with wild-type BRAF. *Nature* **464**, 427–430. <https://doi.org/10.1038/nature08902>.
28. Heidorn, S.J., Milagre, C., Whittaker, S., Nourry, A., Niculescu-Duvas, I., Dhomen, N., Hussain, J., Reis-Filho, J.S., Springer, C.J., Pritchard, C., et al. (2010). Kinase-Dead BRAF and Oncogenic RAS Cooperate to Drive Tumor Progression through CRAF. *Cell* **140**, 209–221. <https://doi.org/10.1016/j.cell.2009.12.040>.
29. Molzan, M., Kasper, S., Röglin, L., Skwarczynska, M., Sassa, T., Inoue, T., Breitenbuecher, F., Ohkanda, J., Kato, N., Schuler, M., et al. (2013). Stabilization of physical RAF/14-3-3 interaction by cotylenin A as treatment strategy for RAS mutant cancers. *ACS Chem Biol* **8**, 1869–1875. <https://doi.org/10.1021/cb4003464>.
30. Kenanova, D.N., Visser, E.J., Virta, J.M., Sijbesma, E., Centorrino, F., Vickery, H.R., Zhong, M., Neitz, R.J., Brunsveld, L., Ottmann, C., et al. (2023). A Systematic Approach to the Discovery of Protein–Protein Interaction Stabilizers. *ACS Cent. Sci.* **9**, 937–946.
<https://doi.org/10.1021/acscentsci.2c01449>.
31. Konstantinidou, M., Visser, E.J., Vandenboorn, E., Chen, S., Jaishankar, P., Overmans, M., Dutta, S., Neitz, R.J., Renslo, A.R., Ottmann, C., et al. (2023). Structure-Based Optimization of Covalent, Small-Molecule Stabilizers of the 14-3-3 σ /ER α Protein–Protein Interaction from Nonselective Fragments. *J. Am. Chem. Soc.* **145**, 20328–20343.
<https://doi.org/10.1021/jacs.3c05161>.

32. Wolter, M., Valenti, D., Cossar, P.J., Hristeva, S., Levy, L.M., Genski, T., Hoffmann, T., Brunsveld, L., Tzalis, D., and Ottmann, C. (2021). An Exploration of Chemical Properties Required for Cooperative Stabilization of the 14-3-3 Interaction with NF- κ B—Utilizing a Reversible Covalent Tethering Approach. *J. Med. Chem.* 64, 8423–8436.
<https://doi.org/10.1021/acs.jmedchem.1c00401>.
33. Shinada, N.K., De Brevern, A.G., and Schmidtke, P. (2019). Halogens in Protein–Ligand Binding Mechanism: A Structural Perspective. *J. Med. Chem.* 62, 9341–9356.
<https://doi.org/10.1021/acs.jmedchem.8b01453>.
34. Milroy, L.-G., Brunsveld, L., and Ottmann, C. (2013). Stabilization and inhibition of protein-protein interactions: the 14-3-3 case study. *ACS Chem Biol* 8, 27–35.
<https://doi.org/10.1021/cb300599t>.
35. Vickery, H.R., Virta, J.M., Konstantinidou, M., and Arkin, M.R. (2024). Development of a NanoBRET assay for evaluation of 14-3-3 σ molecular glues. *SLAS Discovery*, 100165.
<https://doi.org/10.1016/j.slasd.2024.100165>.
36. Fabian, J.R., Vojtek, A.B., Cooper, J.A., and Morrison, D.K. (1994). A single amino acid change in Raf-1 inhibits Ras binding and alters Raf-1 function. *Proc. Natl. Acad. Sci. U.S.A.* 91, 5982–5986. <https://doi.org/10.1073/pnas.91.13.5982>.
37. Hobbs, G.A., Der, C.J., and Rossman, K.L. (2016). RAS isoforms and mutations in cancer at a glance. *Journal of Cell Science* 129, 1287–1292. <https://doi.org/10.1242/jcs.182873>.
38. Sanclemente, M., Francoz, S., Esteban-Burgos, L., Bousquet-Mur, E., Djurec, M., Lopez-Casas, P.P., Hidalgo, M., Guerra, C., Drosten, M., Musteanu, M., et al. (2018). c-RAF Ablation Induces Regression of Advanced Kras/Trp53 Mutant Lung Adenocarcinomas by a Mechanism Independent of MAPK Signaling. *Cancer Cell* 33, 217–228.e4.
<https://doi.org/10.1016/j.ccell.2017.12.014>.
39. Hallenbeck, K.K., Davies, J.L., Merron, C., Ogden, P., Sijbesma, E., Ottmann, C., Renslo, A.R., Wilson, C., and Arkin, M.R. (2018). A Liquid Chromatography/Mass Spectrometry

Method for Screening Disulfide Tethering Fragments. *SLAS Discovery* 23, 183–192.

<https://doi.org/10.1177/2472555217732072>.

Chapter 6

Restoring the 14-3-3/CRAF regulatory interaction in Noonan Syndrome using molecular glues

Abstract

Noonan Syndrome (NS) is a RASopathy, a developmental disorder that derives from the dysregulation of the MAPK pathway. NS results from modestly activating mutations in various proteins. To date, NS-specific therapeutic options are lacking. Trametinib, a MEK inhibitor has shown promising results for certain NS-complications. CRAF activity represents a key NS regulatory node that has not been exploited. CRAF exists in closed/inactive and open/dimeric/active states, governed by the adaptor protein 14-3-3. The 14-3-3/CRAF pS259 complex adopts a closed, inactive conformation in which CRAF does not bind to RAS or to other RAFs. Activating mutations upstream of CRAF reduce S259 phosphorylation and thereby activate CRAF. NS mutations on CRAF occur at residues surrounding S259 (CRAF^{NS}). Here, we quantify how these mutations impair 14-3-3/CRAF, both through decreased phosphorylation levels (64-97%) and decreased binding affinity to 14-3-3 (2- to over 12-fold compared to CRAF^{WT}). We also explore the potential of restoring homeostasis in NS by using molecular glues (MGs) to enhance the 14-3-3/CRAF inhibitory complex. We report that mutant effector proteins upstream of CRAF reduce pS259 levels and that MGs restore phosphorylation to a wildtype level. The MGs also stabilize 14-3-3/CRAF^{NS} interactions to varying degrees (2 to >100x) and strengthen the levels of pS259 phosphorylation up to 4.6-fold, leading to decreased activation of CRAF by NRAS and decreased formation of the active CRAF kinase dimers. Ultimately, inhibition of the CRAF activation mechanism leads to decreased phosphorylation of the downstream target ERK to similar extents as Trametinib in three different NS variants (activation of the phosphatase SHOC2 and CRAF mutations S257L and V263A). These results demonstrate the scope and limitations of stabilizing mutation-weakened complexes. They also reveal a novel therapeutic strategy for NS and related RASopathies.

Significance Statement

CRAF kinase, a key component of the MAPK signaling pathway, is 'auto'inhibited by phosphorylation (p) at S259, which induces binding to the adaptor protein 14-3-3 and formation of an inactive complex. We recently discovered molecular glues that stabilize this 14-3-3/CRAF pS259 complex, preventing CRAF activation. Here, we demonstrate that these molecular glues also restore 14-3-3/CRAF complexes in the context of a RASopathy called Noonan Syndrome (NS). NS mutations upstream of CRAF lower pS259 levels; NS mutations in CRAF itself reduce the binding affinity and by inhibit phosphorylation to impair the interaction with 14-3-3. Molecular glues rescued pS259 levels, increased 14-3-3/CRAF complexes, and inhibited pathway activity. This approach provides a new therapeutic opportunity for restoring homeostasis in RASopathies

Introduction

Protein-protein interactions (PPIs) are fundamental to nearly every biological process, governing the spatial and temporal regulation of cellular signaling, metabolism, and structural organization.¹ By enabling transient or stable associations between proteins, PPIs act as molecular switches or scaffolds to modulate the activation or inhibition of signaling pathways. Disruption or dysregulation of these interactions is a common feature in a wide range of diseases, including cancer, neurodegeneration, infectious diseases, and developmental disorders.² Importantly, PPIs also represent a growing frontier in drug discovery, offering opportunities to modulate protein function by inhibiting or stabilizing PPIs.^{3–5}

One of the most tightly regulated signaling networks reliant on PPIs is the mitogen-activated protein kinase (MAPK) pathway, which plays a central role in cell proliferation, differentiation, and survival. Within this cascade, dynamic PPIs orchestrate the assembly and activation of kinase complexes in response to extracellular cues.^{6–9} A critical player in this process is the hub protein 14-3-3, a phospho-binding scaffold protein that regulates the function and subcellular localization of hundreds of client proteins.^{10–12} The seven mammalian 14-3-3 isoforms are 69–88% conserved, allowing the isoforms to overlap in their functions.^{13–16} In particular, 14-3-3 binds to RAF kinases through phosphoserine motifs to maintain an autoinhibited conformation or promote active dimerization, depending on the cellular context.^{10–12,17} In CRAF, 14-3-3 engages two phosphorylated serine residues, pS259 and pS621, to modulate its activation state.^{18–22} When 14-3-3 is bound to pS259, CRAF is contained in its “auto”inhibited state which secludes the RAS binding domain (RBD) in CRAF and prevents activation by RAS.^{20,21,23} This inhibitory interaction between 14-3-3 and CRAF also sterically blocks CRAF dimerization, the active kinase form.²³ In the activation mechanism of CRAF, the holophosphatase SHOC2 complex dephosphorylates CRAF pS259 to reduce binding to 14-3-3.^{24,25} Hyperphosphorylation of CRAF can prevent its activation by upstream growth factors or activated RAS and is an important component in the negative feedback of ERK signaling.²⁶

Germline mutations that affect components of the MAPK pathway underlie a group of developmental syndromes collectively known as RASopathies.^{27,28} These disorders include Noonan syndrome (NS), Costello syndrome, cardiofaciocutaneous (CFC) syndrome, and neurofibromatosis type 1 (NF1), among others that cause hypo- or hyper-activation of the MAPK pathway. Although each condition has distinct features, they often share overlapping characteristics such as craniofacial abnormalities, cardiac defects, growth delays, skin abnormalities, and an increased risk of certain cancers.^{18,28–30} While RASopathies arise from subtle dysregulation of MAPK signaling -- often resulting in a 2- to 10-fold increase in pathway activity, compared to up to 600-fold in cancer -- their developmental impact is profound due to the sensitivity of embryonic tissues to MAPK signaling.³¹ Studying RASopathies provides valuable insights into the physiological thresholds of MAPK activity, deepening our understanding of how pathway dynamics influence development and disease.

Noonan syndrome is the most prevalent RASopathy, occurring in approximately 1 in 2,000 live births.^{19,30,32} This heterozygous, autosomal dominant disorder arises from mutations in genes encoding proteins that regulate MAPK signaling. The most frequently mutated genes include PTPN11 (encoding Shp2, ~50% of cases), SOS1 (~15%), and CRAF (~8%; Fig 6.1).³³ Mutations in CRAF (also known as RAF1) is the third most mutated protein in NS with mutations clustering around the pS259 site that impair the binding to 14-3-3.^{31,33} These mutations, collectively referred to as CRAF^{NS} here, weaken the autoinhibitory interaction between CRAF and 14-3-3 by reducing phosphorylation at pS259 and altering the local binding motif, leading to an increase in CRAF kinase activity. Clinically, these mutations are frequently associated with hypertrophic cardiomyopathy (HCM), a serious cardiac manifestation that contributes significantly to morbidity and mortality in NS patients carrying CRAF^{NS} mutations.^{18,30}

Despite the promiscuity of 14-3-3, recent work demonstrates the feasibility of developing molecular glues (MGs) that selectively stabilize specific 14-3-3/client interactions.^{4,34,35} These MGs bind cooperatively at the composite interface formed by 14-3-3

and its target protein, thereby enhancing the binding affinity and stabilizing the PPI.^{34,35} The discovery of MGs that preferentially stabilize the 14-3-3 σ /CRAF^{WT} interaction offers a promising therapeutic avenue, particularly in the context of RASopathies like NS, where loss of this interaction leads to pathogenic MAPK hyperactivation. Here, we demonstrate that, indeed, MGs developed for 14-3-3 σ /CRAF^{WT} lead to inhibition of the NS pathway activated either by upstream mutations or by mutations in the 14-3-3 σ /CRAF^{NS} complex itself. Unlike traditional kinase inhibitors, MGs offer a nuanced strategy to fine-tune signaling by restoring regulatory protein complexes.^{36–38} As such, molecular glue stabilization of the 14-3-3/CRAF interaction represents a compelling approach to rebalance MAPK signaling in NS and related RASopathies, potentially restoring developmental homeostasis without broadly suppressing MAPK activity.

Results

Molecular glue enhances CRAF^{WT} phosphorylation in NS mutant backgrounds

First, we evaluated mutations that occur upstream of CRAF to assess the effects of stabilizing the 14-3-3 σ /CRAF^{WT} regulatory interaction as a mode of modulating the MAPK pathway in a NS context. Transfected HEK293T cells containing known NS mutations in Shp2 or SOS1 were treated with the previously validated 14-3-3 σ /CRAF^{WT} MG, compound **23** (Fig S6.1). The 14-3-3 σ /CRAF^{WT} MGs target a native cysteine only present in the σ isoform of 14-3-3. We evaluated the most common mutations (Shp2: N308S, N308D, Q510P; SOS1: R552G, R522S), which together account for NS variants in approximately 65% of patients. NS mutations in Shp2 cluster in domains that are involved in the regulation of phosphatase activity.³⁷ These mutations stabilize Shp2 in its active conformation to constitutively activate MAPK signaling.³⁷ SOS1 regulates RAS activation, and NS mutations destabilize its inhibitory conformation to cause hyperactivation of RAS.^{38,39}

We monitored CRAF pS259 as evidence of 14-3-3s/CRAF^{WT} complex stabilization and pathway modulation²⁴ (Fig 6.2A, B; Fig S6.2). By western blot, minimal effects from the NS mutations were observed in the basal state of CRAF pS259; for instance, SOS1^{R552G} decreased the basal level of phosphorylation of CRAF pS259 by 14% compared to the control without SOS1 transfection. Upon treatment with compound **23**, the intensity of the pS259 increased at least 1.5-fold across all SOS1 and SHP2 mutations compared to the basal level (“no mutant”; Table 6.1). For SOS1^{R552G}, compound **23** increased CRAF pS259 by 2.0-fold compared to DMSO control (1.8-fold compared to the no-mutant control).

Shp2 and SOS1 indirectly activate CRAF, so we next analyzed a mutation in SHOC2 (SHOC2^{S2G}) that is directly responsible for dephosphorylating CRAF pS259. SHOC2 is the regulatory subunit of the phosphatase complex, and the S2G mutation leads to increased phosphatase activity.⁴⁰⁻⁴² Transfection of SHOC2^{S2G} decreased the CRAF pS259 level by 37% (Fig 6.2C, D). Treatment with compound **23** restored the CRAF pS259 to the WT phosphorylation level (1.9-fold increase). In summary, these sets of NS mutations in SHOC2, Shp2, and SOS1 had minimal-to-measurable effects on phosphorylation of CRAF pS259. In all cases, compound **23** enhanced CRAF pS259, likely through stabilization of 14-3-3 σ /CRAF-pS259 complex.

CRAF NS mutations directly impair the regulatory 14-3-3/CRAF interaction

Mutations in CRAF account of a third most common cause of NS with mutations tending to occur around the regulatory pS259 site to impair the inhibitory interaction with 14-3-3. As such, we evaluated known CRAF^{NS} mutations that lie N- and C-terminal to the pS259 (R256S, S257L, P261A, and V263A). Of the CRAF^{NS} mutants, the S257L mutation is the most common CRAF mutation in NS.^{18,30,45} The 14-3-3/CRAF^{NS} regulatory interaction is weakened through a loss of binding and loss of phosphorylation at pS259.³¹ This was confirmed via fluorescence anisotropy (FA) binding analysis and immunoblotting for pS259 (Fig 6.3).

In the FA binding assay, 14-3-3 σ was titrated with FAM-labeled phosphorylated CRAF peptides that mimic the 14-3-3 binding region to ensure the effects on the binding affinity were solely due to the mutations and not the phosphorylation status of pS259. The CRAF^{NS} mutants weakened the binding affinity with increased K_d values from 3.2-fold to >100-fold compared to CRAF^{WT} (Fig 6.3A). The mutations more proximal to the pS259, S257L and P261A, exhibited greater increases in the K_d values (67-fold and >100-fold, respectively). Whereas the mutations further away from the pS259, R256S and V263A, increased the K_d value 7.6-fold and 3.2-fold, respectively. To assess the basal phosphorylation status, the CRAF^{NS} mutants were transfected in HEK293T cells that have an endogenous inactive MAPK pathway. These mutations decreased the amount of pS259 compared to CRAF^{WT}, but phosphorylation was still present unlike the CRAF^{S259A} mutant (Fig 6.3B, C). The loss in phosphorylation at pS259 caused the CRAF kinase to become hyperactivated compared to CRAF^{WT}, shown through a drastic increase in phosphorylation of the downstream target pERK (Fig 6.3B, D). These CRAF^{NS} mutants resulted in a loss of binding and phosphorylation to weaken the inhibitory interaction with 14-3-3 to cause MAPK hyperactivation.

Biochemical 14-3-3 σ /CRAF^{NS} stabilization and structural insights

Because the CRAF^{NS} mutations impaired the binding affinity with 14-3-3 σ , we aimed to restore the interaction to that of the 14-3-3 σ /CRAF^{WT} utilizing the previously optimized MGs. To understand the effects on the binding affinity, FA experiments were conducted as aforementioned with the addition of DMSO or MG (100 μ M) to obtain the apparent K_d ($appK_d$) and fold-stabilization ($\Delta appK_d$) values. Chemically similar compounds **22** and **23** were the most efficacious of the MGs when tested against 14-3-3 σ /CRAF^{WT}; whereas compound **78** was more CRAF-selective over other 14-3-3 clients, but less potent compared to **22** and **23** (Fig S6.1).

The MGs (**22**, **23**, and **78**) restored the $appK_d$ values of all of the CRAF^{NS} mutants back to the binding affinity of CRAF^{WT} except for P261A that was only minimally affected (Fig 6.4A-D, Fig S6.3). Overall, compounds **22** and **23** stabilized the 14-3-3 σ /CRAF^{NS} protein-peptide complexes more than compound **78** (Fig 6.4A-D, Table 6.3). For example, the fold-stabilizations for compounds **22** and **23** for S257L were 236x and 425x, respectively, compared to 44x for compound **78**. For the V263A mutant, all the MGs tested stabilized the 14-3-3 σ /CRAF^{V263A} interaction greater than 1,000x (μ M to nM). The Val to Ala mutation was a minimal change compared to the other mutations (R -> S, S -> L, and P -> A), although the mutation occurred in the region of the peptide crucial for forming interactions with the MGs. To further understand the binding of the MGs to the 14-3-3 σ /CRAF^{NS} interface, we turned to x-ray crystallography.

Crystal structures of the ternary complexes (14-3-3 σ /CRAF^{NS}/MG) were solved to elucidate any differences across the CRAF^{NS} mutations compared to CRAF^{WT}. From the crystal structures with compound **23**, differences arose in the backbone of the CRAF peptides in the C-terminus, closest to the MG binding pocket (Fig 6.4E-H). Both of the N-terminal mutants, R256S and S257L, showed differences in the peptide overlay close to the mutations, but the C-termini of the peptides confirmed the same conformation as the WT peptide (maximum 1.0 Å shift at the +4 Val). On the N-terminus of the peptides at the α carbon of the mutations, R256S shifted 1.8 Å and S257L shifted 5.2 Å from the corresponding α carbons in the WT peptide. The V263A mutant differed the greatest from the CRAF^{WT} peptide C-terminus with the C-terminus shifted towards compound **23**. This shift started after the +2 Pro where the main chain α carbon on the +3 Asn differed by 2.0 Å that caused the main chain α carbon the +4 residue to move 2.9 Å from the WT structure and the β carbon of the +4 Ala (V263A mutated residue) to be 4.2 Å away from the comparable the γ carbon of the +4 Val (WT; Fig 6.4H). The other MGs (**22** and **78**) exhibited minimal to no change in the C-terminus, similar to the **23** with R256S and S257L mutant peptides although the MG conformations shifted slightly (Fig S6.4).

14-3-3 σ /CRAF^{NS} stabilization in cells

The efficacies of the MGs against full-length CRAF^{NS} mutants interacting with 14-3-3 σ were confirmed using a proximity-based bioluminescence resonance energy transfer (NanoBRET) assay to probe the 14-3-3 σ /CRAF PPI in cells. In the assay set up, 14-3-3 σ was fused to the energy acceptor, HaloTag, and the energy donor, Nanoluciferase (Nluc), was conjugated to the CRAF^{NS} mutant proteins. This format allowed for direct comparison of the 14-3-3/CRAF interaction between the biochemical CRAF^{NS} peptide model results and full-length proteins containing the mutations and reduced phosphorylation at pS259 reflecting cellular levels. The NanoBRET assay recapitulated the weakened binding of 14-3-3 σ and CRAF^{NS} mutants via a decrease in BRET signal, exhibiting similar trends of impairment between the mutants (Fig S6.5). This supports that the weakened PPI is caused by both altering the binding motif with mutations (FA binding experiments) as well as loss of phosphorylation needed to recognize 14-3-3 (pS259). Utilizing the NanoBRET assay, we set out to measure the restoration of the mutant PPIs to the WT interaction in cells.

All three MGs were evaluated against the four CRAF^{NS} mutants and were expected to increase the BRET signal, suggesting increased PPI formation. As anticipated, **22** and **23** significantly increased the BRET signal of all four CRAF^{NS} mutants at 20 μ M (Fig 6.5A, B). However, **22** did not restore the S257L or P261A mutant PPIs, the most impaired PPIs of those tested, to the WT interaction (Fig 6.5B). Compound **23** did restore the S257L mutant PPI to the WT level (Fig 6.5B). The CRAF-selective compound **78** exhibited the least stabilization (fold-increase in BRET) for CRAF^{WT} and CRAF^{NS} mutants; although **78** was able to significantly increase S257L and V263A, only the V263A PPI was restored (Fig 6.5C). Compound **23** outperformed compounds **22** and **78** by restoring three of the four 14-3-3 σ /CRAF^{NS} mutant interactions to the basal inhibitory 14-3-3 σ /CRAF^{WT} interaction level. The EC₅₀ values for compound **23** ranged from 3.7 to 7.7 μ M across the four CRAF^{NS} mutants with maximum fold-increases of 1.2- to 1.7-fold (Table 6.3, Fig S6.5). The compounds were less potent against the

14-3-3 σ /CRAF^{NS} interaction compared to 14-3-3 σ /CRAF^{WT} but were similarly or more efficacious than CRAF^{WT} as seen through the increase in the BRET signal for each PPI (CRAF^{WT}: EC₅₀ = 0.2 – 1.8 μ M, 1.1 – 1.4-fold increase).

After the stabilization of full-length 14-3-3 σ /CRAF^{NS} mutant PPIs was assessed, the protection of pS259 was analyzed upon MG treatment because the regulatory interaction between 14-3-3 and CRAF requires pS259 (Fig 6.5D-F, Fig S6.6). Again, the basal phosphorylation of pS259 was reduced in the CRAF^{NS} mutants compared to CRAF^{WT}, with S257L and P261A exhibiting the greatest reduction in pS259. Treatment with compounds **22**, **23**, and **78** increased the intensity of phosphorylation of pS259 in the CRAF^{NS} mutants and CRAF^{WT}. CRAF^{WT} was already highly phosphorylated at pS259 due to the endogenous inactive MAPK pathway in HEK293T cells, resulting in a larger population of CRAF^{WT} in the inhibited state bound to 14-3-3 (pS259). The CRAF^{NS} mutants decreased the inhibited CRAF population and allowed for greater pS259 enhancement/protection. Phosphorylation of pS259 with S257L increased the most of the CRAF^{NS} mutants with all the MGs tested (**23**: 2.1-fold, **22**: 2.2-fold, and **78**: 1.9-fold). Whereas pS259 only increased up to 1.6-fold with R256S, the overall CRAF^{NS} mutant whose pS259 state was least impacted by MG treatment. The western blot analysis confirmed that stabilization of the 14-3-3 σ /CRAF^{NS} interaction enhanced the phosphorylation state of pS259 to restore the regulatory PPI.

Inhibition of the CRAF kinase activation mechanism

In its activation mechanism, CRAF is first recruited to plasma membrane by RAS for activation, followed by dimerization of CRAF to form the active kinase^{20,21,46} (Fig 6.6A). To understand the effects of 14-3-3 σ /CRAF^{NS} stabilization on CRAF's activation mechanism, we used NanoBRET assays to evaluate these additional CRAF PPIs with compound **23** as it performed overall best in the previous assays. First, we analyzed the transition state through the interaction between CRAF^{NS} (Nluc) and the activator NRAS (HaloTag). Each CRAF^{NS} mutant

showed increased NRAS/CRAF^{NS} interaction compared to CRAF^{WT}, suggesting an increase in the kinase activation state caused by the NS mutations (Fig 6.6B). However, only S257L and V263A showed significant inhibition of the NRAS/CRAF^{NS} interaction with MG treatment (Fig S6.7). The S257L mutant significantly decreased the NRAS/CRAF^{S257L} inhibition with compound **23** with an IC₅₀ value of 0.12 μ M (p-value < 0.01, Fig 6.6C). Compared to a mutation in CRAF that prevents the interaction with NRAS (R89L), there was only partial inhibition, 57%, to recover the inactive WT state (Fig 6.6C, D). Compound **23** reduced the transition state of NRAS recruiting and activating CRAF^{NS} through stabilization of the 14-3-3 σ /CRAF^{NS} complex.

Next, we characterized the active CRAF dimer state by probing the CRAF^{WT}/CRAF^{NS} dimerization since NS is heterozygous disorder and cells carry both WT and mutant CRAF.³⁰ In this NanoBRET assay, HaloTag was conjugated to CRAF^{WT} and Nluc to CRAF^{NS} to monitor the dimerization. Across all of the CRAF^{NS} mutants, there was an increase in the dimerization compared to inactive CRAF^{WT} that recapitulated the binding, phosphorylation, and NRAS/CRAF^{NS} results in the basal state (Fig 6.6E). Treatment of compound **23** with all four of the CRAF^{NS} mutants resulted in significant decreases in the CRAF^{WT}/CRAF^{NS} interaction (Fig S6.7). However, only partial recovery back to the WT inactive level. The most common mutation, S257L, had a 40% reduction in CRAF dimerization compared to WT (IC₅₀ value 0.11 μ M; Fig 6.6F, G). This supports that stabilization of regulatory 14-3-3 σ /CRAF^{NS} complex with compound **23** prevented the critical steps in the activation mechanism to form the active CRAF kinase dimer.

Finally, we assessed the kinase activity of CRAF by measuring the phosphorylation of the downstream target pERK using a Lumit Immunoassay with a split luciferase target conjugated to secondary antibodies to monitor total and phosphorylated protein.⁴⁷ This assay format allowed for multiple doses and replicates to be tested with ease compared to a traditional immunoblot methods and reduced the time compared to other immunoassays. In the Lumit assay, the mutations that showed the greatest efficacy with compound **23** were assessed; the

upstream mutation SHOC^{S2G}, CRAF^{S257L}, and CRAF^{V263A} (Fig 6.6H). Samples were treated with DMSO, compound **23** (10 μ M), or the MEK inhibitor Trametinib as a control. Trametinib is a current MEK inhibitor treatment for NS patients however shows significant toxicity in the clinic.^{32,48} Trametinib (100 nM) decreased the phosphorylation of pERK for all three mutants. Compound **23** decreased pERK by 70% in the SHOC2^{S2G} background, more than Trametinib (50%), with overnight treatment (p-values < 0.001). For the CRAF^{NS} mutants tested, **23** inhibited phosphorylation of pERK compared to DMSO to a similar extent as Trametinib for CRAF^{V263A} (p-value < 0.05) but did not significantly decrease pERK in the CRAF^{S257L} background (p-value = 0.075). Across the assays measuring the activation of CRAF kinase, compound **23** decreased the activation of the CRAF kinase through inhibition of NRAS recruitment and CRAF dimerization to prevent the kinase cascade to phosphorylate pERK.

Discussion

This work showcases the use of molecular glues to rescue weakened PPIs in dysregulated states as a method to restore homeostasis. This was done through the study of the RASopathy Noonan Syndrome and targeting the regulatory 14-3-3 σ /CRAF interaction in the MAPK pathway. The disorder arises through a variety of mutations that can occur in upstream activators of CRAF or directly in CRAF to cause an impaired 14-3-3/CRAF PPI. Through stabilization of this PPI with MGs, we showed restored binding of the 14-3-3 σ /CRAF^{NS} complex biochemically and in cells, phosphorylation protection of the necessary phospho-residue required for the interaction, and downstream effects of inhibiting the kinase activity of CRAF through this novel method of targeting a regulatory PPI.

In the inhibition of the CRAF activation mechanism and downstream effects, only partial inhibition was achieved. Various reasons could be at play here such as other RAS isoforms able to activate CRAF, RAF heterodimers forming, and exclusively targeting the 14-3-3 σ isoform with cysteine reactive MGs. The seven isoforms of 14-3-3 share clients and thus our MGs are only

targeting a proportion of the 14-3-3/CRAF population. Because NS is a heterozygous disorder, only the CRAF^{WT}/CRAF^{NS} dimers were analyzed to more closely reflect the disease state. The study of other RAS/CRAF interactions and CRAF heterodimers is an avenue to explore for future study with pan 14-3-3 MGs. More advanced models (iPSCs, animal models, etc) would also provide additional evidence to tease apart the required amount of stabilization needed to overcome the pathology of Noonan syndrome. Mutant selective molecular glues are another alternative route for future exploration in recovering weakened PPIs in disease. Taken together, the study presented here provides a foundation for restoring homeostasis in dysregulated disease states using native PPI stabilization.

Materials and Methods

Molecular Cloning and Mutagenesis

CRAF^{NS} mutant plasmids were generated from CRAF^{WT} plasmid containing either a N-terminal FLAG or NanoLuc tag using Q5 site-directed mutagenesis (NEB KLD enzyme kit M0554) and expressed in NEB 5α competent *E. coli* (NEB C2987).

Protein Expression and Purification

14-3-3σ was expressed and purified as previously described (full-length for mass spectrometry and fluorescence anisotropy assays, ΔC for crystallography).^{4,34,35} The 14-3-3σ with an N-terminal His6 tag were expressed in RosettaTM 2(DE3) pLysS competent *E. coli* (Novagen) from a pPROEX HTb expression vector and transformed following the manufacturer's protocol. Single colonies were used to inoculate 25 mL LB starter cultures for overnight incubation (37°C, 250 rpm). Starter cultures were then added to 1.5 L TB medium. Expression was induced at OD₆₀₀ ~2 with 750 μM IPTG and cultures expressed overnight at 30°C, 250 rpm. Cells were

harvested at 6,500 rpm and resuspended in lysis buffer (50 mM HEPE pH 7.5, 500 mM NaCl, 20 mM imidazole, 10% glycerol, 1 mM TCEP) before sonication. Protein was purified using Ni-affinity chromatography (Ni-NTA Agarose, Invitrogen; Wash buffer: 50 mM HEPES pH 7.5, 500 mM NaCl, 20 mM imidazole, 1 mM TCEP; Elution buffer: 50 mM HEPES pH 7.5, 500 mM NaCl, 500 mM imidazole, 1 mM TCEP). Purified proteins were analyzed by SDS-PAGE and Q-ToF LC/MS. Following a buffer exchange (25 mM HEPES pH 7.5, 150 mM NaCl, 1 mM TCEP), protein was concentrated to 500 μ M and flash-frozen for storage at 80 °C.

14-3-3 σ Δ C was truncated after T231 to enhance crystallization and after the first Ni-affinity chromatography column, the construct was treated with TEV protease to cleave off the His6 tag during dialysis (25 mM HEPES, pH 7.5, 200 mM NaCl, 5% glycerol, 10 mM MgCl₂, 250 μ M TCEP) overnight at 4 °C. The flow-through of a second Ni-affinity column was subjected to a final purification step by size exclusion chromatography (Superdex 75 pg 16/60 size exclusion column (GE Life Science) (SEC buffer: 25 mM HEPES pH 7.5, 100 mM NaCl, 10 mM MgCl₂, 250 μ M TCEP). The protein was concentrated to ~60 mg/mL, analyzed for purity by SDS-PAGE and Q-ToF LC/MS and aliquots flash-frozen for storage at -80 °C.

Peptides

Fluorescein-labeled (5-FAM) peptides used in fluorescence anisotropy assays were purchased from Elim Biopharmaceuticals, Inc. (Hayward, CA). Peptides for X-ray crystallography were purchased from GenScript Biotech Corp.

Peptides for fluorescence anisotropy:

CRAF^{WT} pS259 (15-mer): 5-FAM-SQRQRST{pS}TPNVH MV-NH₂

CRAF^{R256S} pS259 (15-mer): 5-FAM-SQRQSST(pS)TPNVH MV-NH₂

CRAF^{S257L} pS259 (15-mer): 5-FAM-SQRQRLT(pS)TPNVH MV-NH₂

CRAF^{P261A} pS259 (15-mer): 5-FAM-SQRQRST(pS)TANVHMH-NH₂

CRAF^{V263A} pS259 (15-mer): 5-FAM-SQRQRST(pS)TPNAHMH-NH₂

Peptides for crystallography:

CRAF^{R256S} pS259 (11-mer): Ac-QSST(pS)TPNVHM-NH₂

CRAF^{S257L} pS259 (11-mer): Ac-QRLT(pS)TPNVHM-NH₂

CRAF^{V263A} pS259 (11-mer): Ac-QRST(pS)TPNAHM-NH₂

Fluorescence Anisotropy Protein Titrations

FA experiments were conducted as previously described.³⁴ Briefly, 14-3-3 protein was serially diluted (2-fold) in buffer (10 mM HEPES pH 7.5, 150 mM NaCl, 0.1% Tween20) at a starting concentration of 250 μ M in black, flat-bottom 384-well assay plates (Corning 3575). 5-FAM labeled peptides were added to a final concentration of 100 nM (final sample volume of 25 μ L). Assay plates were incubated at room temperature with gentle rocking. The fluorescence anisotropy was measured after 30 minutes to determine the binding affinity (K_d) for each peptide/14-3-3 complex using a Molecular Devices iD5 SpectraMax plate reader (filter set λ_{ex} : 485 $\pm$ 20 nm; λ_{em} : 535 $\pm$ 25 nm). K_d values were obtained using a four-parameter logistic model (4PL) in GraphPad Prism. Assays were performed in triplicate.

For experiments with compound, the previous protocol was followed with these modifications: buffer (10 mM HEPES pH 7.5, 150 mM NaCl, 0.1% Tween20, 0.1% BSA, final sample volume 10 μ L (black, flat-bottom 384-well assay plate, Greiner Bio-One #784900), and 100 μ M compound (or same % v/v DMSO). Fluorescence was measured at 1 hr and 24 hr.

X-ray Crystallography

Crystallography was performed as previously described.^{34,35} The 14-3-3 σ Δ C protein, Ac-CRAF, and compounds (50 mM stock in DMSO) were dissolved in complexation buffer (25 mM HEPES

pH=7.5, 2 mM MgCl₂ and 100 μM TCEP) and mixed in a 1:2:3 or 1:3:5 molecular stoichiometry (protein:peptide:compound) with a final protein concentration of 11 or 12 mg/mL. The complex was set-up for sitting-drop crystallization after overnight incubation at 4 °C, in a custom crystallization liquor (0.05 M HEPES (pH 7.1, 7.3, 7.5, 7.7), 0.19 M CaCl₂ 24-29% PEG400, and 5% (v/v) glycerol). Crystals grew within 10-14 days at 4 °C. Crystals were fished and flash-cooled in liquid nitrogen. X-ray diffraction (XRD) data were collected at the European Synchrotron Radiation Facility (ESRF Grenoble, France, beamline ID23-1, ID30A-3/MASSIF-3, or ID23-2). Initial data processing was performed at ESRF using autoPROC after which pre-processed data was taken towards further scaling steps, molecular replacement, and refinement.

Data was processed using CCP4i2 suite (version 7.1.010). After indexing and integrating the data, scaling was done using AIMLESS. The data was phased with MolRep, using PDB 3IQU as template. The presence of co-crystallized ligands was verified by visual inspection of the Fo-Fc and 2Fo-Fc electron density maps in COOT (version 0.9.3). If electron density corresponding to the co-crystallized ligand was present, its structure and restraints were generated using AceDRG. After building in the ligand, model rebuilding and refinement was performed using REFMAC5. The PDB REDO server (pdb-redo.edu) was used to complete the model building and refinement. The images were created using the PyMOL Molecular Graphics System (Schrödinger LLC, version 2.4.1). See SI table 1 for data collection and refinement statistics.

Cell culture

HEK293T were purchased from ATCC (CRL-3216) and maintained as recommended in DMEM, high glucose (Gibco) supplemented with 10% Fetal Bovine Serum (FBS), 1% penicillin/streptomycin, and 1% non-essential amino acids.

Western Blot

HEK293T cells were seeded in 6-well plates (Corning 3516) and transiently transfected 1:1 of HA-14-3-3 σ :FLAG-CRAF^{NS}. After at least 24-hours, media was replaced with DMEM (4% FBS) and treated with compound at 10 μ M (or same % v/v DMSO) for 24 hours. Cells were lysed with Triton X-100 buffer (50 mM Tris pH 8.0, 150 mM NaCl, 5 mM MgCl₂, 1% Triton X-100, phosphatase inhibitor, protease inhibitor). Cell lysate was clarified at 15,000 rpm for 10 minutes. Anti-phospho-CRAF pS259 (Invitrogen 44-502), anti-C-RAF (BD Biosciences 610152), anti-phospho-ERK (Cell Signaling Technology 4376), anti-ERK (Santa Cruz Biotechnology 514302), and anti-Vinculin (Cell Signaling Technology 13901S) antibodies were used for protein detection. Antibodies were used at a 1:1,000 dilution except anti-Vinculin (1:2,000) and anti-ERK (1:500). Western blots were imaged on a LI-COR imaging system and analyzed using Image Studio (version 5.2.5).

NanoBRET Assays

NanoBRET assays were performed as previously described.⁴⁹ All NanoBRET assays were performed in HEK293T cells. Compounds were tested in a 2-fold dilution series starting at 20 μ M. CRAF^{NS}-NanoLuc (C-terminal tag)/14-3-3-HaloTag (C-terminal tag) plasmids were transfected at a 1:10 ratio. NRAS/CRAF NanoBRET was performed at a 1:1:1 ratio of CRAF^{NS}-NanoLuc (C-terminal tag), HaloTag-NRAS (N-terminal tag), and HA-14-3-3 σ . CRAF^{NS}/CRAF NanoBRET used CRAF^{NS}-NanoLuc/HaloTag-CRAF^{WT} with HA-14-3-3 σ at a 1:1:1 ratio. Data was analyzed as previously described.⁴⁹ Briefly, milliBRET units (mBU) were calculated as a ratio of acceptor/donor and mean mBU values were corrected by subtracting the mean mBU of controls with no HaloTag ligand. Fold change was calculated by dividing mBU_{compound} by mBU_{DMSO control}. For NRAS/CRAF NanoBRET, 100% was considered the DMSO mBU and 0% the mBU value of NRAS/CRAF^{R89L}. Statistical significance determined via unpaired t-test.

Lumit Immunoassay

Lumit immunoassays were performed as according to the manufacturer (Promega W1331). Briefly, HEK293T cells were transfected in a 1:1 ratio of HA-14-3-3 σ :FLAG-CRAF^{NS} in 10% FBS DMEM (Gibco). 24 hours after transfection, cells were re-seeded in 384-well plates (10,000 cells per well). Cells were treated with compound in a 2-fold dilution series, starting at 10 μ M, or LY3009120 (100 nM; Selleck Chemicals S7842) or Trametinib (100 nM; Selleck chemicals S2673) for 24 hours before performing the lumit protocol. After compound treatment, media was replaced with 10 μ L media supplemented with fluorogenic live cell GF-AFC substrate (50 μ M) for normalization purposes. Cells were incubated for 30 minutes before proceeding. The protocol was optimized for the antibodies used: digitonin (0.1%) lysis buffer, 100 ng/ μ L pERK antibody (Cell Signaling Technology 4376), 100 ng/ μ L ERK antibody (Santa Cruz Biotechnology 514302), and 0.1 μ L/well of secondary antibodies (SmBiT mouse, LgBiT rabbit). The ratio of cell lysate:antibody:substrate was 2:2:1. Cells were lysed for 40 minutes at 800 rpm before incubation with the antibody mix for 90 minutes (2 minutes at 400 rpm prior to incubation). After antibody incubation, substrate was added, incubated for 2 minutes at 400 rpm. Luminescence was then read on an EnVision Xcite 2105 plate reader. Luminescence was normalized against the total fluorescence from the GF-AFC (Luminescence/Total fluorescence). Values were then normalized to DMSO treated cells (experimental/DMSO). Data was analyzed using GraphPad Prism. Data was collected in 3 biological replicates and averaged. Statistical significance determined via unpaired t-test.

Figures

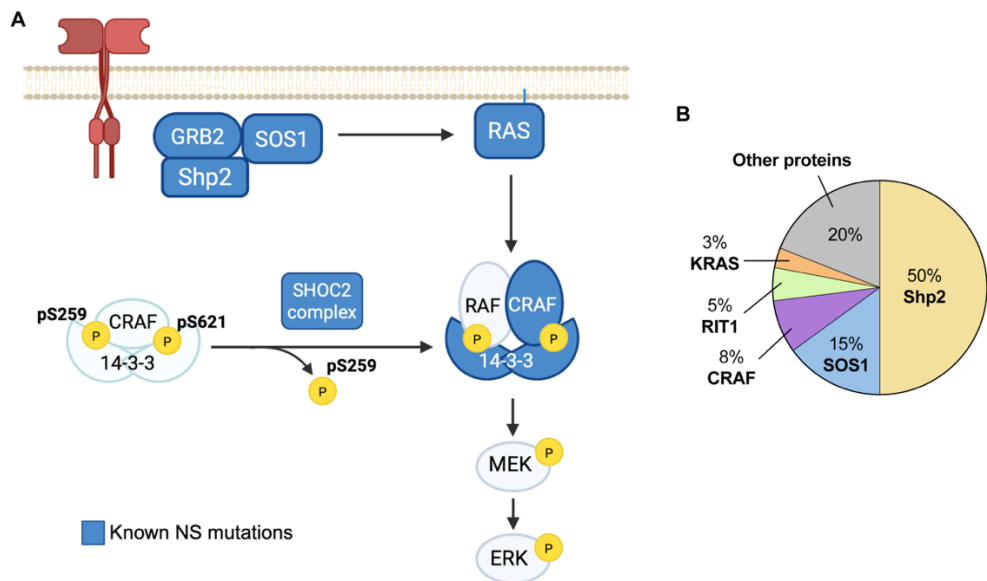


Figure 6.1: Noonan Syndrome mutations dysregulate the MAPK pathway
A) MAPK pathway. Highlighted proteins in dark blue have known NS mutations. **B)** Distribution of proteins mutated in NS.

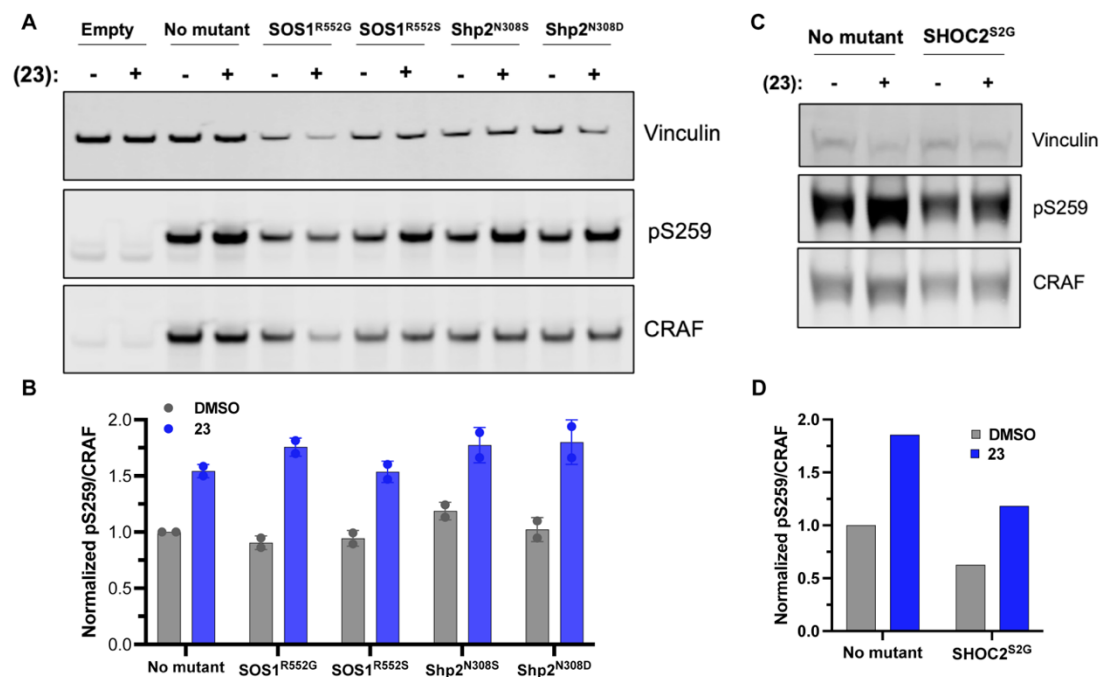


Figure 6.2: CRAF pS259 phosphorylation protection in upstream NS mutant backgrounds
A) Western blot of SOS1 and Shp2 mutations with and without overnight treatment with compound **23** in HEK293T cells. **B)** Quantification of pS259/CRAF intensity. Normalized to “no mutant” background (with 14-3-3 σ and CRAF transfection, but no SOS1 or SHP2 transfection). **C)** Western blot of SHOC2 mutant background with and without overnight treatment with compound **23** in HEK293T cells. **D)** Quantification of pS259/CRAF intensity. Normalized to “no mutant” background.

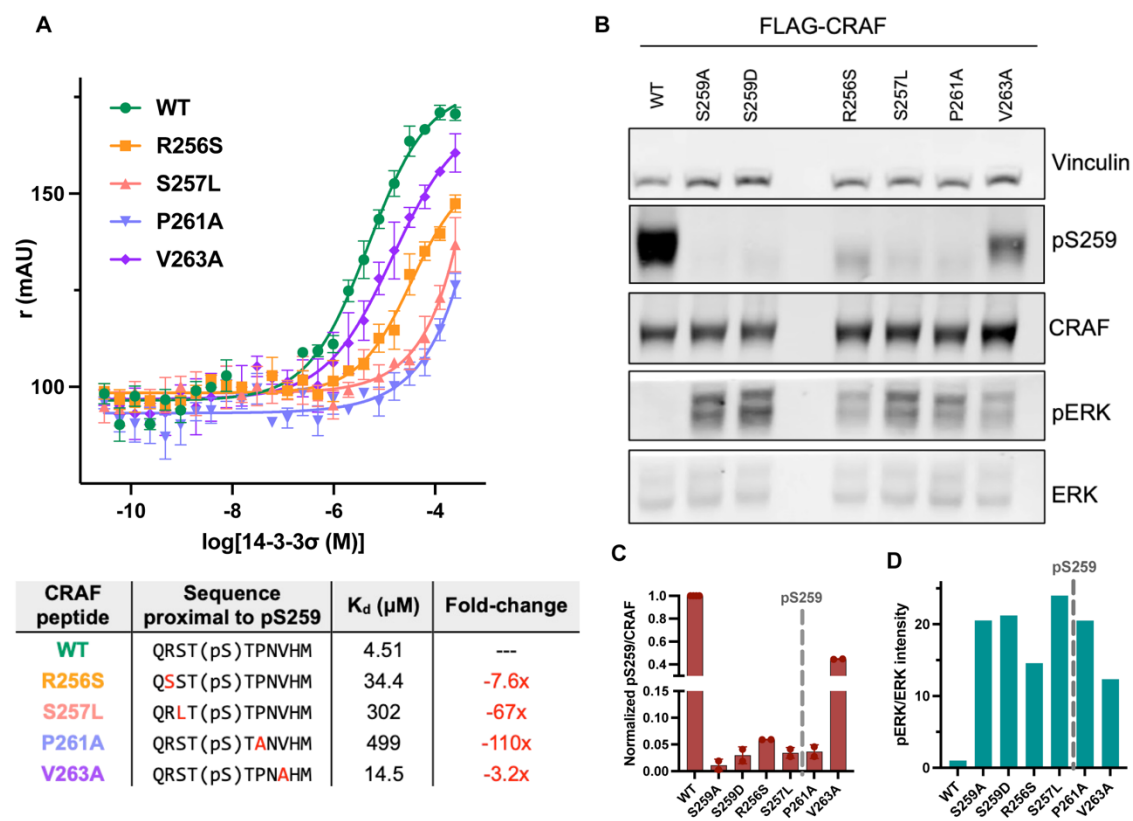


Figure 6.3: CRAF mutational effects on binding affinity and phosphorylation

A) FA protein titration of 14-3-3 σ protein with CRAF phospho-peptides (average $\pm$ SD, $n=3$). Mutations in the binding motif weakened the binding affinity to 14-3-3 σ . **B)** Western blot comparing CRAF^{NS} mutations to WT, S259A, and S259D for pS259 and pERK. **C)** Quantification of pS259/CRAF intensity. Normalized to WT intensity. **D)** Quantification of pERK/ERK intensity.

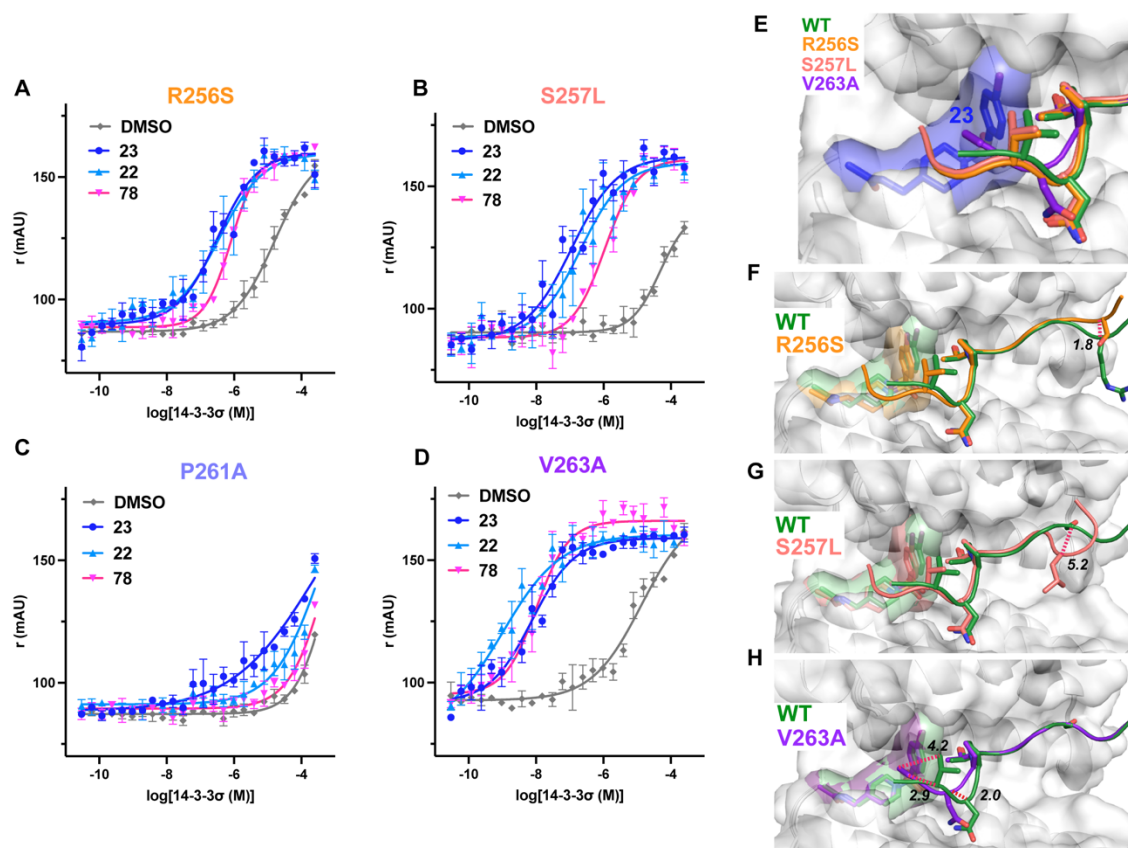


Figure 6.4: Biochemical stabilization of 14-3-3 σ /CRAF^{NS} protein-peptide complexes
A-D) FA protein titrations of 14-3-3 σ and CRAF^{NS} peptides with compound (100 μ M) or DMSO (average $\pm$ SD, n = 3). Fold-stabilizations derived from the *appK_d* values. **E-H)** Crystal structures of ternary complexes 14-3-3 σ /CRAF/compound 23. 14-3-3 σ represented as grey surface. **E)** Overlay of CRAF^{NS} mutant peptides with CRAF^{WT}. 23 shown in blue. **F)** R256S (orange) overlay with WT (green). **G)** S257L (salmon) overlay with WT (green). **H)** V263A (purple) overlay with WT (green).

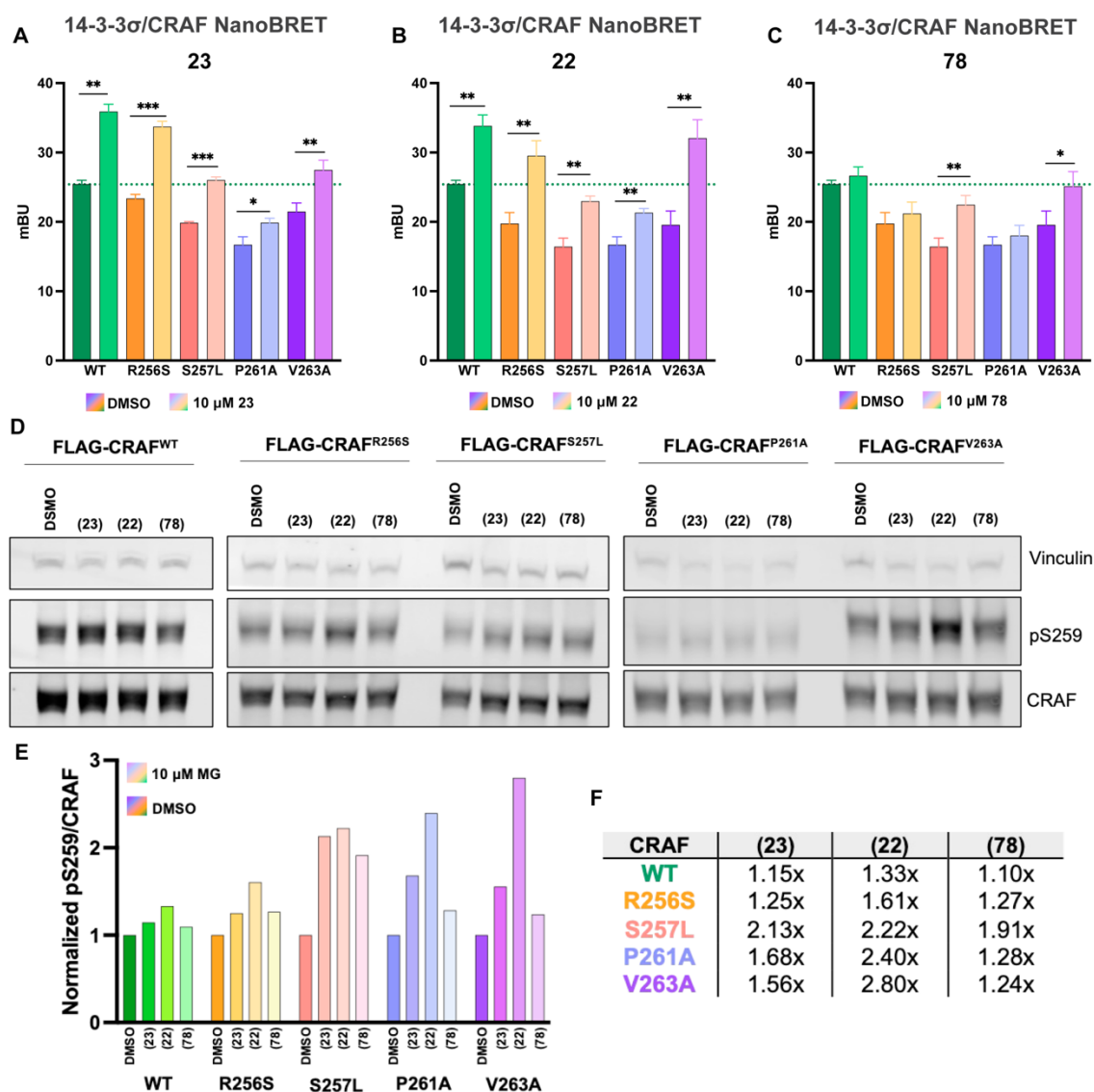


Figure 6.5: 14-3-3 σ /CRAF stabilization in cells

A-C) 14-3-3 σ /CRAF NanoBRET. Comparison of DMSO treated samples (basal level) to the maximum fold-increase in BRET signal with each compound (average $\pm$ SD, $n = 3$; t-test, p-value: * < 0.05 , ** < 0.01 , *** < 0.001 . **D)** Western blot of compound effects on pS259. **E-F)** Quantification of pS259/CRAF intensity and fold-increase upon compound treatment compared to DMSO. Normalized to corresponding CRAF DMSO samples.

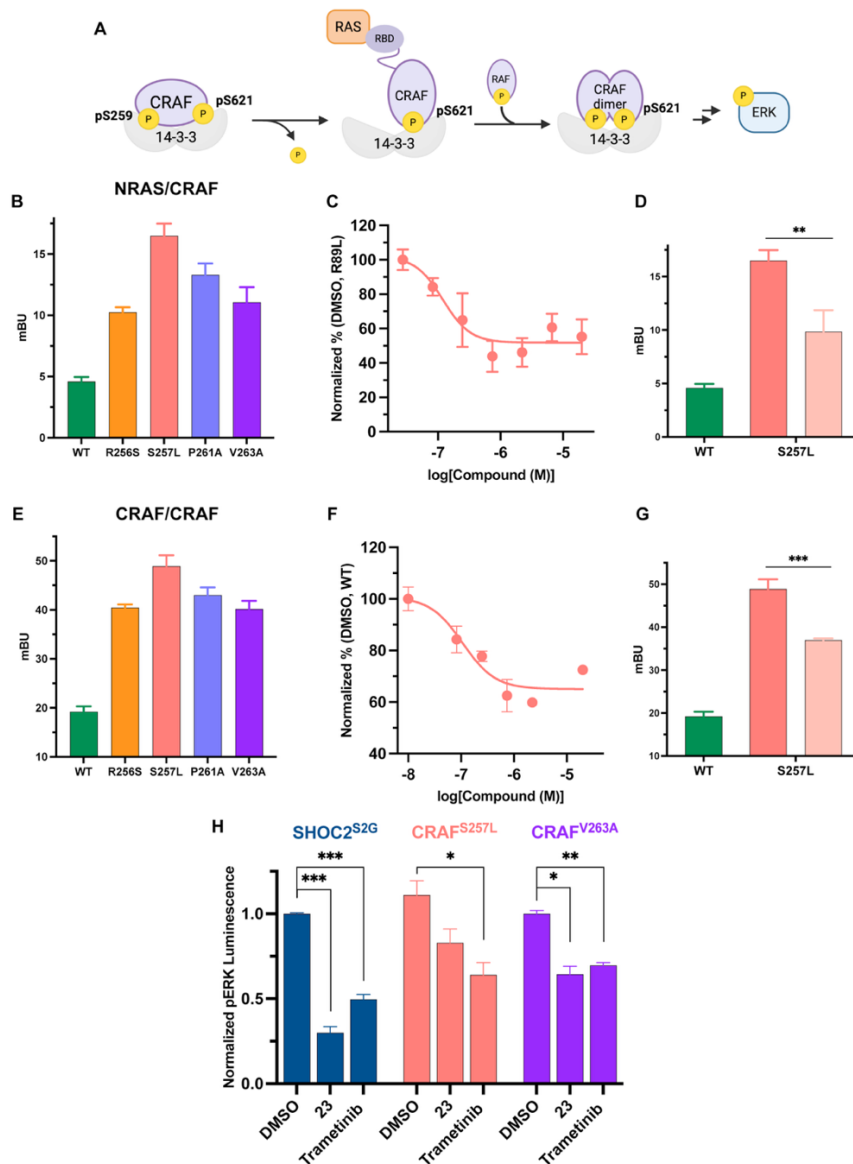


Figure 6.6: Inhibition of CRAF kinase activation through 14-3-3 σ /CRAF^{NS} stabilization

A) Schematic of 14-3-3/CRAF mechanism from the inhibited state bound to 14-3-3 at pS259 to the transition state of CRAF activation by RAS and the final active CRAF kinase dimer. **B)** NRAS/CRAF NanoBRET comparison of basal interaction of the transition activation state (average $\pm$ SD, $n = 3$). **C)** NRAS/CRAF^{S257L} NanoBRET dose-response of **23**, normalized to DMSO and a CRAF^{R89L} mutant that does not bind NRAS (average $\pm$ SD, $n = 3$). **D)** Bar graph of NRAS/CRAF^{S257L} BRET signal comparing non-activated CRAF^{WT} (green) to the hyperactive CRAF^{S257L} (basal level, dark salmon) and maximum decrease with compound **23** (light salmon), p -value < 0.01 (t-test, average $\pm$ SD). **E)** CRAF/CRAF NanoBRET comparison of basal interaction of the active kinase dimer (average $\pm$ SD, $n = 3$). **F)** CRAF^{WT}/CRAF^{S257L} NanoBRET dose-response of **23**, normalized to DMSO and the inactive CRAF^{WT} state (average $\pm$ SD, $n = 3$). **G)** Bar graph of CRAF^{WT}/CRAF^{S257L} BRET signal comparing the inactive CRAF^{WT} state (green) to the hyperactive CRAF^{S257L} (basal level, dark salmon) and maximum decrease with **23** (light salmon), p -values < 0.001 (t-test, average $\pm$ SD). **H)** Lumit Immunoassay measuring pERK of different NS mutant backgrounds (SHOC2^{S2G}: navy; CRAF^{S257L}: salmon; CRAF^{V263A}: purple) comparing DMSO, **23** (10 μ M), and Trametinib (100 nM). T-test, p -value: * < 0.05 , ** < 0.01 , *** < 0.001 . Average $\pm$ SD, $n = 3$.

Tables

Table 6.1: Upstream NS mutations pS259 protection

	- (23)	+ (23)
Western Blot 1 (Fig 2A)		
No mutant	1	1.5
SOS1 ^{R552G}	0.91	1.8
SOS1 ^{R552S}	0.94	1.5
Shp2 ^{N308S}	1.2	1.8
Shp2 ^{N308D}	1.1	1.8
Western Blot 2 (Fig 2C)		
No mutant	1	1.9
SHOC2 ^{S2G}	0.6	1.2

Table 6.2: FA experimental values of 14-3-3 σ /CRAF^{NS} peptides

AppK_d values with MGs (100 μ M) and fold-stabilizations (Δ *appK_d*).

	DMSO		(23)		(22)		(78)	
	<i>AppK_d</i> (μ M)	Δ <i>appK_d</i>	<i>AppK_d</i> (μ M)	Δ <i>appK_d</i>	<i>AppK_d</i> (μ M)	Δ <i>appK_d</i>	<i>AppK_d</i> (μ M)	Δ <i>appK_d</i>
WT	12	--	0.005	> 1000x	0.005	> 1000x	0.110	110x
R256S	15.9	--	0.318	50x	0.369	43x	0.818	19x
S257L	48.0	--	0.113	425x	0.203	236x	1.10	44x
P261A	770	--	290	2.7x	380	2.0x	600	1.3x
V263A	13.5	--	0.008	> 1000x	0.001	> 1000x	0.010	> 1000x

Table 6.3: NanoBRET EC₅₀ values and fold-stabilization

	(23)		(22)		(78)	
	EC ₅₀ (μ M)	Max fold-increase	EC ₅₀ (μ M)	Max fold-increase	EC ₅₀ (μ M)	Max fold-increase
WT	0.18	1.4	0.2	1.3	1.2	1.1
R256S	7.71	1.4	~20	1.5	--	1.1
S257L	3.71	1.3	~20	1.4	~20	1.4
P261A	6.13	1.2	6.93	1.3	~20	1.1
V263A	3.78	1.7	6.57	1.9	~20	1.5

Table 6.4: CRAF^{NS} fold-change in pS259 protection

	(23)	(22)	(78)
WT	1.2	1.3	1.1
R256S	1.3	1.6	1.3
S257L	2.1	2.2	1.9
P261A	1.7	2.4	1.3
V263A	1.6	2.8	1.2

Supplemental Figures

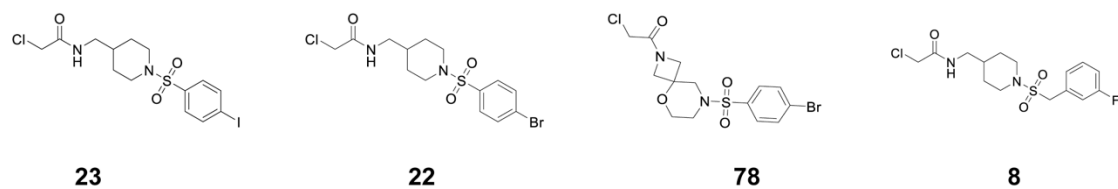


Figure S6.1: Chemical structures of 14-3-3 σ /CRAF molecular glues

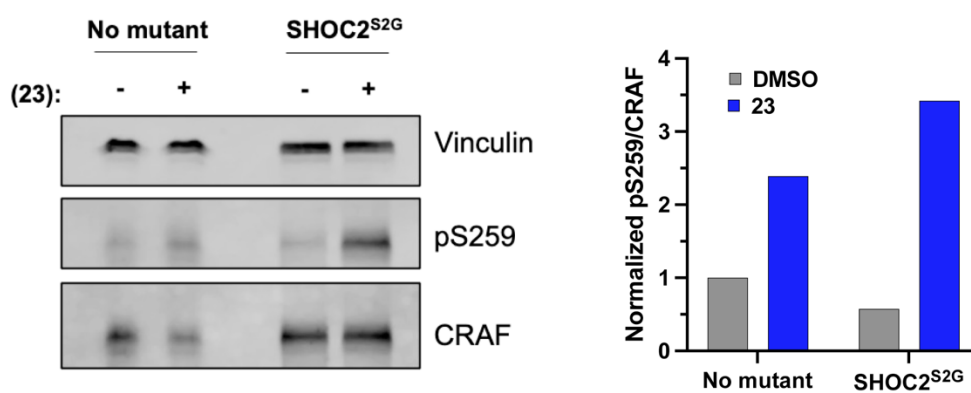
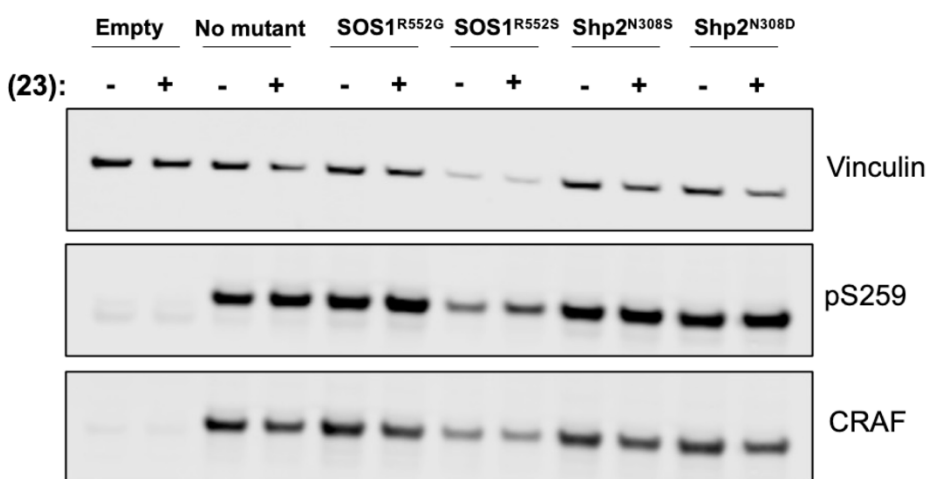


Figure S6.2: Upstream MAPK mutations western blot replicates

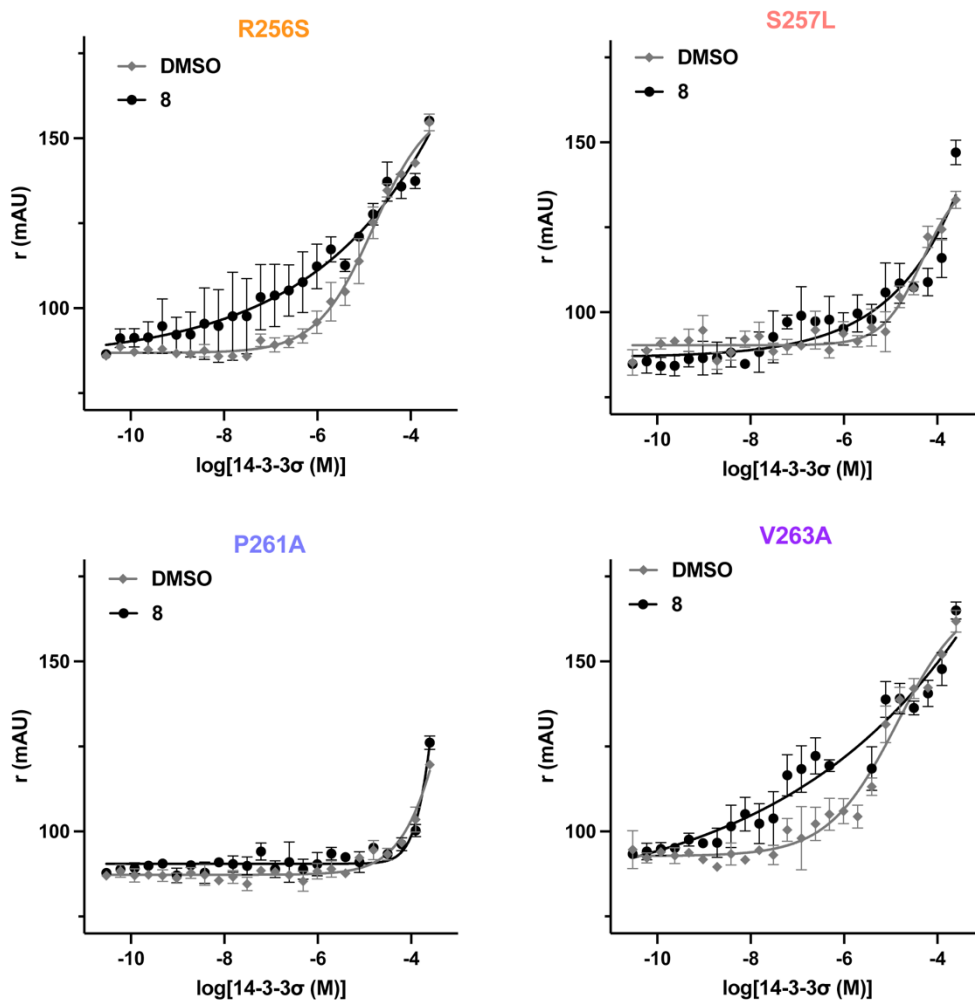


Figure S6.3: FA protein titrations of negative control molecular glue

14-3-3σ protein titration with CRAF^{NS} peptides and negative control compound **8** (100 μM) or DMSO (v/v). Average ± SD, n=3

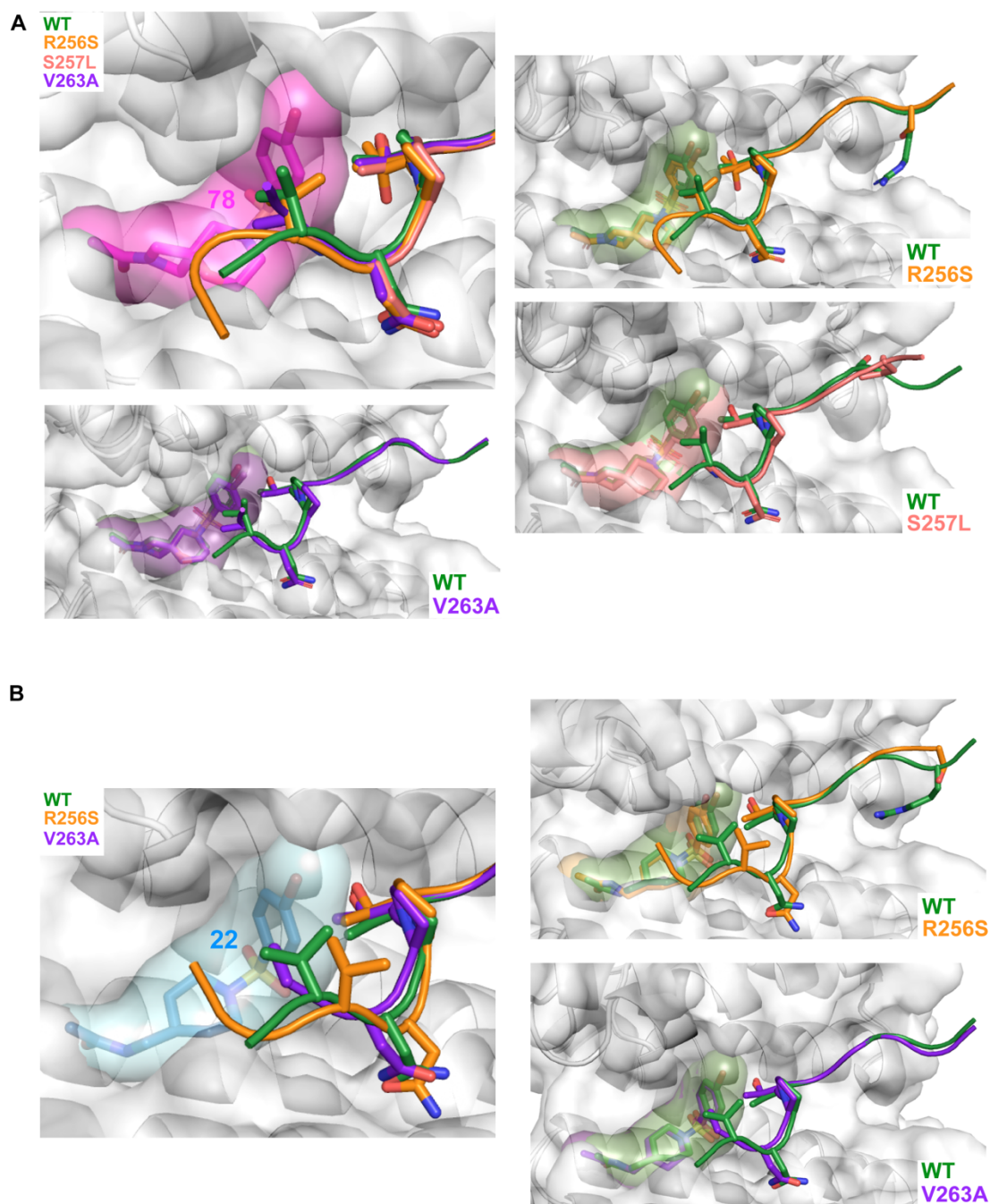


Figure S6.4: Crystal structures of compounds 78 and 22

14-3-3 σ shown as grey surface R256S shown in orange, S257L shown in salmon, V263A shown in purple, WT shown in green. Compounds conformations shown in corresponding colors. CRAF^{NS} ternary complexes overlaid with CRAF^{WT} complexes (14-3-3 σ crystallized with CRAF^{WT} shown in green cartoon).

A) Compound **78** overlays. **B)** Compound **22** overlays.

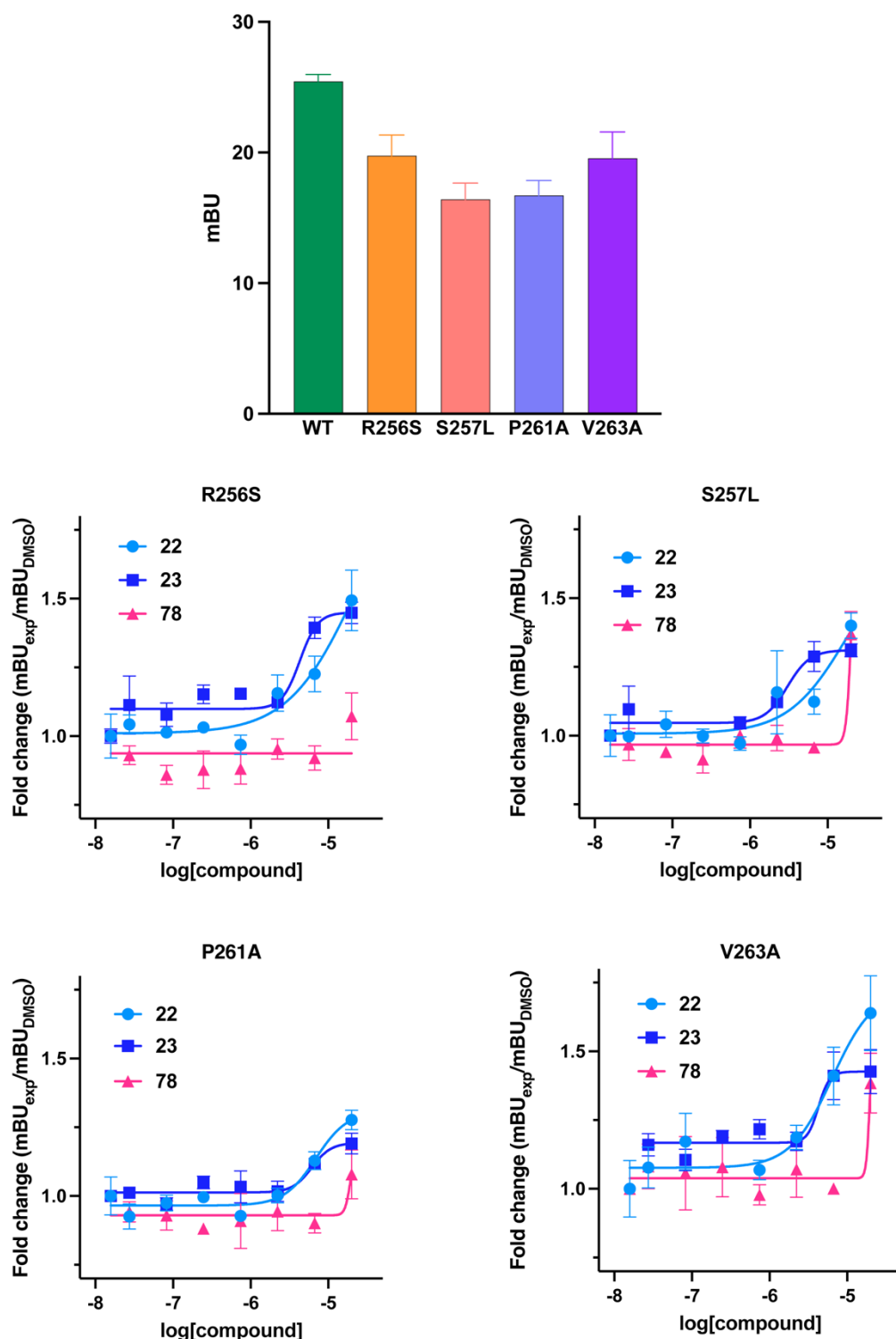


Figure S6.5: 14-3-3σ/CRAF^{NS} NanoBRET

Basal full-length PPI interaction comparison of 14-3-3σ with CRAF^{WT} and CRAF^{NS} mutants. Dose-response of fold-change in BRET signal for 14-3-3σ/CRAF^{NS}, 3-fold dilution, top concentration 20 μM (average ± SD, n=3).

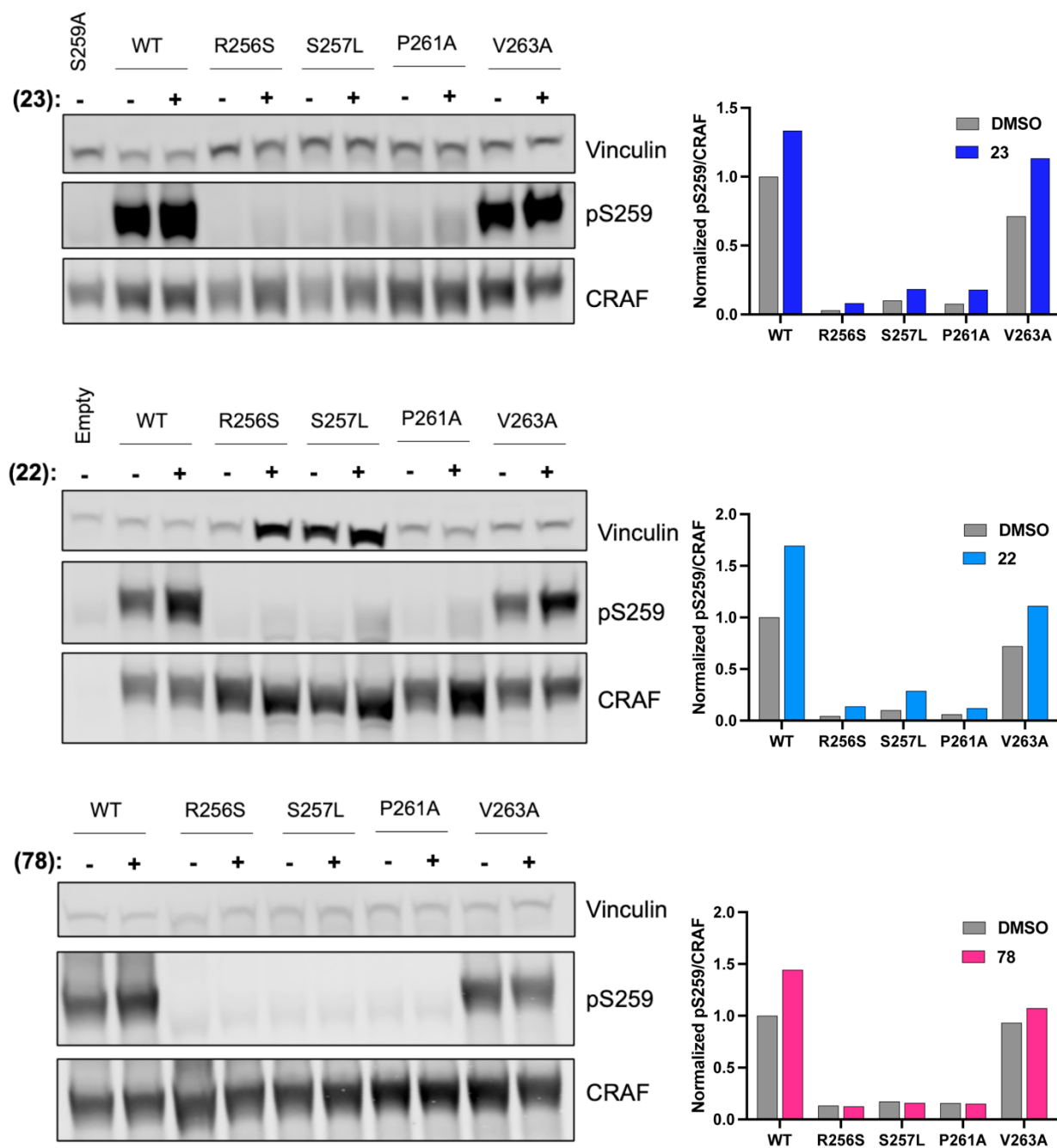


Figure S6.6: Western blot replicates of CRAF^{NS} pS259 protection with compound treatment
 Quantification of pS259/CRAF normalized to WT to store rescue of phosphorylation upon compound treatment.

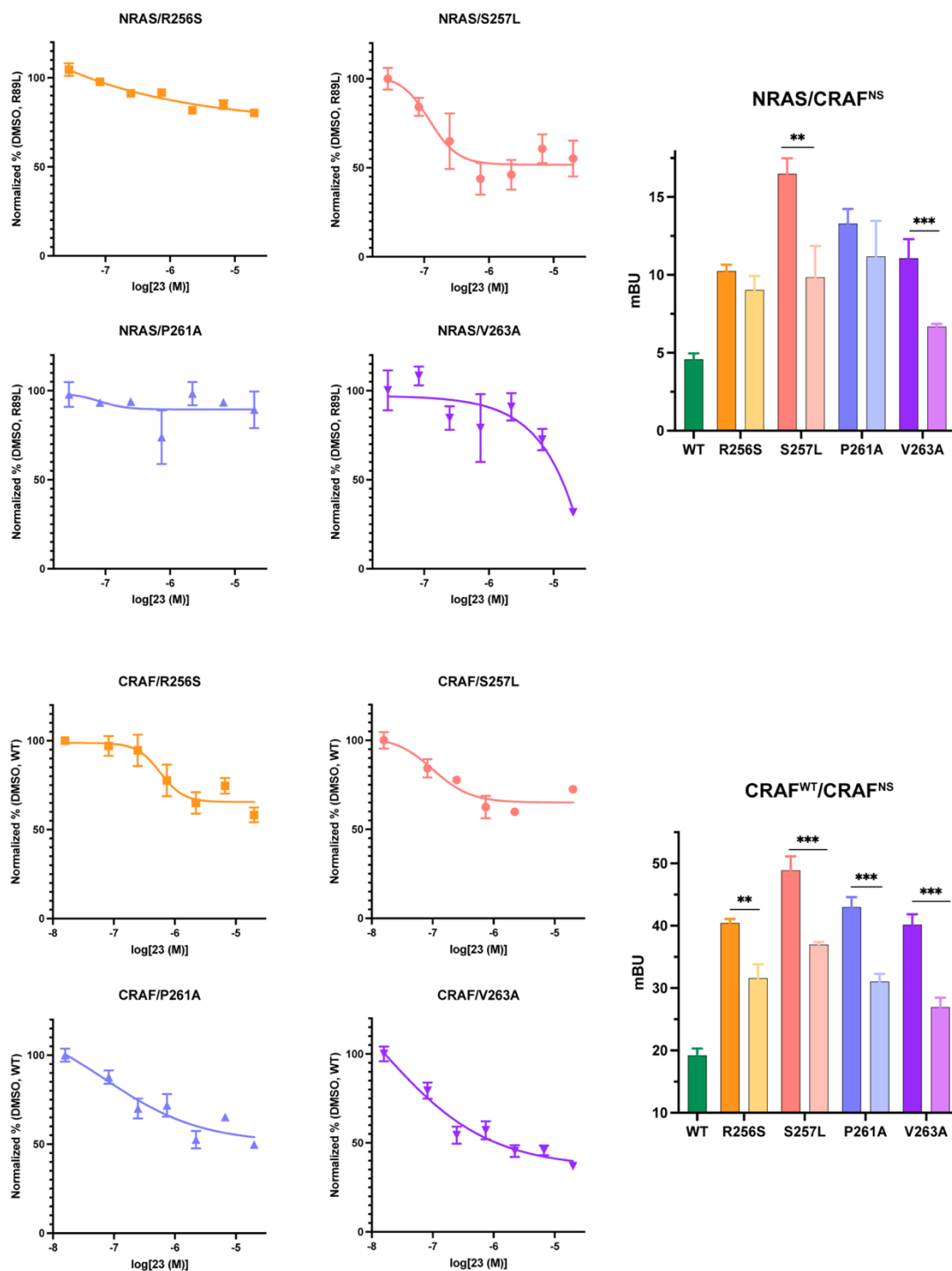


Figure S6.7: CRAF^{NS} activation inhibition

NRAS/CRAF^{NS} NanoBRET dose-response and bar chart showing basal level of transition state with hyperactive CRAF^{NS} mutants compared to maximum inhibition of NRAS/CRAF^{NS} interaction. CRAF/CRAF^{NS} NanoBRET dose-response and bar chart of basal level of active kinase dimer state of hyperactive CRAF^{NS} mutants and maximum inhibition of CRAF dimerization. Both compared to inactive WT state (green).

References

1. Zhong, M., Lee, G. M., Sijbesma, E., Ottmann, C. & Arkin, M. R. Modulating protein-protein interaction networks in protein homeostasis. *Curr Opin Chem Biol* **50**, 55–65 (2019).
2. Xiong, D., Lee, D., Li, L., Zhao, Q. & Yu, H. Implications of disease-related mutations at protein–protein interfaces. *Current Opinion in Structural Biology* **72**, 219–225 (2022).
3. Sijbesma, E. *et al.* Site-Directed Fragment-Based Screening for the Discovery of Protein-Protein Interaction Stabilizers. *J Am Chem Soc* **141**, 3524–3531 (2019).
4. Kenanova, D. N. *et al.* A Systematic Approach to the Discovery of Protein–Protein Interaction Stabilizers. *ACS Cent. Sci.* **9**, 937–946 (2023).
5. Raimundo, B. C. *et al.* Integrating fragment assembly and biophysical methods in the chemical advancement of small-molecule antagonists of IL-2: an approach for inhibiting protein-protein interactions. *J. Med. Chem.* **47**, 3111–3130 (2004).
6. Freed, E., Symons, M., Macdonald, S. G., McCormick, F. & Ruggieri, R. Binding of 14-3-3 proteins to the protein kinase Raf and effects on its activation. *Science* **265**, 1713–1716 (1994).
7. García-Alonso, S. *et al.* Structure of the RAF1-HSP90-CDC37 complex reveals the basis of RAF1 regulation. *Molecular Cell* **82**, 3438-3452.e8 (2022).
8. Garnett, M. J., Rana, S., Paterson, H., Barford, D. & Marais, R. Wild-type and mutant B-RAF activate C-RAF through distinct mechanisms involving heterodimerization. *Mol Cell* **20**, 963–969 (2005).
9. Hu, J. *et al.* Allosteric Activation of Functionally Asymmetric RAF Kinase Dimers. *Cell* **154**, 1036–1046 (2013).
10. Roskoski, R. RAF protein-serine/threonine kinases: Structure and regulation. *Biochemical and Biophysical Research Communications* **399**, 313–317 (2010).

11. Garnett, M. J., Rana, S., Paterson, H., Barford, D. & Marais, R. Wild-Type and Mutant B-Raf Activate C-Raf through Distinct Mechanisms Involving Heterodimerization. *Molecular Cell* **20**, 963–969 (2005).
12. Tzivion, G., Luo, Z. & Avruch, J. A dimeric 14-3-3 protein is an essential cofactor for Raf kinase activity. *Nature* **394**, 88 (1998).
13. Obsil, T. & Obsilova, V. Structural basis of 14-3-3 protein functions. *Semin Cell Dev Biol* **22**, 663–672 (2011).
14. Obsilova, V. & Obsil, T. The 14-3-3 Proteins as Important Allosteric Regulators of Protein Kinases. *Int J Mol Sci* **21**, E8824 (2020).
15. Pennington, K. L., Chan, T. Y., Torres, M. P. & Andersen, J. L. 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 (2018).
16. Johnson, C. *et al.* Bioinformatic and experimental survey of 14-3-3-binding sites. *Biochem J* **427**, 69–78 (2010).
17. Sluchanko, N. N. Reading the phosphorylation code: binding of the 14-3-3 protein to multivalent client phosphoproteins. *Biochemical Journal* **477**, 1219–1225 (2020).
18. Kobayashi, T. *et al.* Molecular and clinical analysis of *RAF1* in Noonan syndrome and related disorders: dephosphorylation of serine 259 as the essential mechanism for mutant activation. *Hum. Mutat.* **31**, 284–294 (2010).
19. Razzaque, M. A. *et al.* Germline gain-of-function mutations in *RAF1* cause Noonan syndrome. *Nat Genet* **39**, 1013–1017 (2007).
20. Dedden, D. *et al.* Cryo-EM Structures of CRAF2/14-3-32 and CRAF2/14-3-32/MEK12 Complexes. *J Mol Biol* **436**, 168483 (2024).
21. Park, E. *et al.* Architecture of autoinhibited and active BRAF-MEK1-14-3-3 complexes. *Nature* **575**, 545–550 (2019).

22. García-Alonso, S. *et al.* *Structure of the RAF1-HSP90-CDC37 Complex Reveals the Basis of RAF1 Regulation*. <http://biorxiv.org/lookup/doi/10.1101/2022.05.04.490607> (2022) doi:10.1101/2022.05.04.490607.
23. Martinez Fiesco, J. A., Durrant, D. E., Morrison, D. K. & Zhang, P. Structural insights into the BRAF monomer-to-dimer transition mediated by RAS binding. *Nat Commun* **13**, 486 (2022).
24. Young, L. C. *et al.* SHOC2-MRAS-PP1 complex positively regulates RAF activity and contributes to Noonan syndrome pathogenesis. *Proc Natl Acad Sci U S A* **115**, E10576–E10585 (2018).
25. Boned del Río, I. *et al.* SHOC2 complex-driven RAF dimerization selectively contributes to ERK pathway dynamics. *Proc. Natl. Acad. Sci. U.S.A.* **116**, 13330–13339 (2019).
26. Lake, D., Corrêa, S. A. L. & Müller, J. Negative feedback regulation of the ERK1/2 MAPK pathway. *Cell. Mol. Life Sci.* **73**, 4397–4413 (2016).
27. Riller, Q. & Rieux-Laucat, F. RASopathies: From germline mutations to somatic and multigenic diseases. *Biomedical Journal* **44**, 422–432 (2021).
28. Hebron, K. E., Hernandez, E. R. & Yohe, M. E. The RASopathies: from pathogenetics to therapeutics. *Disease Models & Mechanisms* **15**, dmm049107 (2022).
29. Orlova, A. A., Dadali, E. L. & Polyakov, A. V. Clinical and Genetic Characteristics of Noonan Syndrome and Noonan-like Diseases. *Russ J Genet* **56**, 540–547 (2020).
30. Tartaglia, M., Gelb, B. D. & Zenker, M. Noonan syndrome and clinically related disorders. *Best Pract Res Clin Endocrinol Metab* **25**, 161–179 (2011).
31. Molzan, M. *et al.* Impaired binding of 14-3-3 to C-RAF in Noonan syndrome suggests new approaches in diseases with increased Ras signaling. *Mol Cell Biol* **30**, 4698–4711 (2010).
32. Roberts, A. E., Allanson, J. E., Tartaglia, M. & Gelb, B. D. Noonan syndrome. *The Lancet* **381**, 333–342 (2013).

33. Saint-Laurent, C., Mazeyrie, L., Yart, A. & Edouard, T. Novel therapeutic perspectives in Noonan syndrome and RASopathies. *Eur J Pediatr* **183**, 1011–1019 (2023).
34. Konstantinidou, M. *et al.* Structure-Based Optimization of Covalent, Small-Molecule Stabilizers of the 14-3-3 σ /ER α Protein–Protein Interaction from Nonselective Fragments. *J. Am. Chem. Soc.* **145**, 20328–20343 (2023).
35. Markella Konstantinidou, *et al.* Scaffold-hopping for molecular glues: from fragments to cell-active probes targeting the 14-3-3/ER α complex. *Nat Commun* **in press**, (2025).
36. Konstantinidou, M. & Arkin, M. R. Molecular glues for protein-protein interactions: Progressing toward a new dream. *Cell Chem Biol* **31**, 1064–1088 (2024).
37. Maneiro, M. *et al.* PROTACs, molecular glues and bifunctionals from bench to bedside: Unlocking the clinical potential of catalytic drugs. *Prog Med Chem* **60**, 67–190 (2021).
38. Katz, L. S. *et al.* Molecular glues of the regulatory ChREBP/14-3-3 complex protect beta cells from glucolipotoxicity. *Nat Commun* **16**, 2110 (2025).
39. Tartaglia, M. *et al.* PTPN11 Mutations in Noonan Syndrome: Molecular Spectrum, Genotype-Phenotype Correlation, and Phenotypic Heterogeneity. *The American Journal of Human Genetics* **70**, 1555–1563 (2002).
40. Lepri, F. *et al.* SOS1 mutations in Noonan syndrome: molecular spectrum, structural insights on pathogenic effects, and genotype-phenotype correlations. *Hum. Mutat.* **32**, 760–772 (2011).
41. Zenker, M. *et al.* SOS1 is the second most common Noonan gene but plays no major role in cardio-facio-cutaneous syndrome. *Journal of Medical Genetics* **44**, 651–656 (2007).
42. Cordeddu, V. *et al.* Mutation of SHOC2 promotes aberrant protein N-myristoylation and causes Noonan-like syndrome with loose anagen hair. *Nat Genet* **41**, 1022–1026 (2009).
43. Komatsuzaki, S. *et al.* Mutation analysis of the SHOC2 gene in Noonan-like syndrome and in hematologic malignancies. *J Hum Genet* **55**, 801–809 (2010).

44. Gripp, K. W. *et al.* Expanding the SHOC2 mutation associated phenotype of noonan syndrome with loose anagen hair: Structural brain anomalies and myelofibrosis. *Am. J. Med. Genet.* n/a-n/a (2013) doi:10.1002/ajmg.a.36098.
45. Nakhaei-Rad, S. *et al.* Molecular and cellular evidence for the impact of a hypertrophic cardiomyopathy-associated RAF1 variant on the structure and function of contractile machinery in bioartificial cardiac tissues. *Commun Biol* **6**, 1–18 (2023).
46. Kondo, Y. *et al.* Cryo-EM structure of a dimeric B-Raf:14-3-3 complex reveals asymmetry in the active sites of B-Raf kinases. *Science* **366**, 109–115 (2019).
47. Hwang, B., Engel, L., Goueli, S. A. & Zegzouti, H. A homogeneous bioluminescent immunoassay to probe cellular signaling pathway regulation. *Commun Biol* **3**, (2020).
48. Garutti, M. *et al.* BRAF and MEK Inhibitors and Their Toxicities: A Meta-Analysis. *Cancers* **15**, 141 (2023).
49. Vickery, H. R., Virta, J. M., Konstantinidou, M. & Arkin, M. R. Development of a NanoBRET assay for evaluation of 14-3-3 σ molecular glues. *SLAS Discovery* 100165 (2024) doi:10.1016/j.slasd.2024.100165.

Chapter 7

Screening for Noonan mutant-selective 14-3-3/CRAF stabilizing fragments

Abstract

14-3-3 regulates the activity of the kinase CRAF in the MAPK pathway. The 14-3-3/CRAF inhibitory interaction (CRAF pS259) is impaired in the RASopathy Noonan Syndrome to cause dysregulation of the MAPK pathway and hyperactivation of CRAF. One mode of impairment is through mutations surrounding the pS259 site needed to bind to 14-3-3 in the inhibited state. Disease mutations in CRAF (CRAF^{NS}) weaken the binding affinity to 14-3-3. However, there are seven isoforms of 14-3-3 that vary in expression level and binding affinity to client proteins. In this work, the binding affinities of three 14-3-3 isoforms were compared with different CRAF^{NS} mutants to optimize the interaction conditions to screen for modulators of 14-3-3/CRAF^{NS}. Previously synthesized 14-3-3/CRAF^{WT} molecular glues (MGs) were tested against the CRAF mutants with the 14-3-3 σ isoform originally used in the testing of these MGs and a cysteine mutant of the 14-3-3 γ isoform to ensure MG efficacy between isoforms. This isoform comparison elucidated that the basal binding affinities differed between isoforms and shift the assay window between DMSO and MG treatment. Expanding upon the previous work from the Arkin lab to find client-selective stabilizers of 14-3-3/client PPIs using a mass spectrometry (MS) disulfide tethering screen, here mutant-selective stabilizing hits for 14-3-3/CRAF^{NS} for three different disease mutants of CRAF were identified for chemical optimization into cell active molecular glues.

Introduction

Protein-protein interactions (PPIs) play critical roles in regulating biological processes from signal transduction to apoptosis.^{1,2} Although previously perceived as “undruggable” or difficult to target, PPIs form induced pockets at the interaction interface to target and modulate PPIs.^{2,3} Targeting the interaction instead of a singular protein has promise of improved specificity.^{1,3–5} Previous work has explored systematically dissecting a protein’s interactome through the discovery and development of specific client protein modulators for the hub protein 14-3-3 with various client proteins.^{6–15} The work described here expands on this by exploring disease mutants of one of the client proteins, CRAF, to find mutant-selective modulators.

14-3-3 is a hub protein that binds phospho-residues in client proteins to regulate their activity.^{16–21} There are seven mammalian isoforms (ϵ , σ , γ , η , θ , β , ζ) of 14-3-3 that are 69-88% conserved.^{22,23} 14-3-3 proteins typically form heterodimers with only the σ isoform forming homodimers.²² The isoforms share client proteins as the majority of conserved residues lie in the client binding groove (Figure 7.1). Only the σ isoform contains the native cysteine at the edge of the binding groove (C38, Figure 7.2A). Due to the presence of this cysteine (C38), 14-3-3 σ is advantageous to for the discovery and development of molecular glues containing a cysteine reactive warhead.^{6,11,24} However, the σ isoform is less abundant in cells and binds client proteins more weakly than other 14-3-3 isoforms.^{17–19} Of the seven isoforms, 14-3-3 ζ has rich structural data with most of the previously published structures of 14-3-3 being the ζ isoform as well as being the predominant isoform expressed in cells.²² Whereas the γ isoform is known to have improved client binding affinities (lower K_d values) among the rest of the 14-3-3 isoforms and is highly expressed in most cells.^{16,25,26}

14-3-3 regulates the kinase CRAF by binding the phospho-residue S259 in the inhibited state.^{27–31} Mutations around the pS259 in CRAF impair the regulatory interaction with 14-3-3 to lead to hyperactivation of the MAPK pathway, causing the developmental disorder Noonan Syndrome (NS).^{32–35} Majority of these mutations flank the pS259 site.³² Molecular glues (MGs)

have been developed to stabilize 14-3-3 σ /CRAF^{WT} and have potency towards CRAF Noonan mutants (CRAF^{NS}; Chapters 5-6). Targeting the 14-3-3/CRAF inhibitory interaction could provide a novel therapeutic route for modulating the MAPK pathway.³⁶ Mutations C-terminal to the pS259 would affect the compound binding pocket as these residues make direct contacts with the MGs, suggesting mutant-selective MGs could improve potency and selectivity toward CRAF^{NS}.⁶ NS is also a heterozygous disorder, so patients have a mutant and wild-type allele.³⁷ Overstabilization of 14-3-3/CRAF by targeting both WT and mutant CRAF interactions could lead to overcorrection of the MAPK pathway, also suggesting a need for mutant-selective MGs.³³

The use of a mass spectrometry (MS) disulfide tethering screen to find hits that modulate PPIs has been used to find stabilizers of 14-3-3/client interactions.^{6,11,38} Stabilizing hits bind cooperatively to the composite surface of PPI at a cysteine of interest. The σ isoform of 14-3-3 has previously been used as it contains a native cysteine (C38) proximal to the natural product binding pocket.⁶ In the tethering screens, a model system of full-length 14-3-3 protein is used with peptides that mimic the 14-3-3 binding region of the client protein containing the phospho-residue as they similarly disordered. Hits from the tethering screens are validated and can be optimized to gain selectivity and potency to be turned into cell active compounds.^{7,8,15,39}

Using a MS disulfide tethering screen of ~2000 disulfide fragments, mutant-selective hits were identified for 14-3-3/CRAF^{NS} for the mutations S257L, P261A, and V263A in CRAF. The optimization of the screening conditions showcases the difference in 14-3-3 isoforms and use of engineered cysteines for screening. Optimized MGs were also tested against a native and engineered cysteine to ensure potency. The results from these screens provide a starting point for chemical optimization to develop mutant-selective MGs for 14-3-3/CRAF^{NS}.

Results

To screen for Noonan Syndrome (NS) mutant-selective stabilizers, the optimal 14-3-3 isoform was first determined because the mutations in CRAF decrease the binding affinity to 14-3-3 (increase K_d values). The binding affinities of CRAF with varying 14-3-3 isoforms were determined via fluorescence anisotropy (FA) protein titration experiments using WT and mutant CRAF peptides (all at 100 nM peptide). From these experiments, there was minimal to no improvement in the K_d values with the ζ isoform in comparison to the σ isoform (Table 7.1, Figure 7.2B-D). For example, the K_d values for CRAF^{V263A} with 14-3-3 σ was 11.0 μ M, compared to 0.934 μ M with 14-3-3 ζ . The largest improvement in the binding affinities was with the γ isoform where the K_d value for CRAF^{V263A} decreased 21x (σ : 11.0 μ M, γ : 0.525 μ M). The two mutant peptides that bound the least to 14-3-3 σ (S257L and P261A) resulted in the greatest increase in binding affinity with γ isoform, > 100 μ M to ~10 μ M (S257L: 9.45 μ M, P261A: 12.1 μ M). By identifying the 14-3-3 isoform with the overall lowest K_d values across the CRAF mutants, this allowed for the screening conditions to be optimized to use a lower concentration of CRAF^{NS} peptides (concentration of peptide ~2x K_d).

After determining the binding affinities for the CRAF peptides, cysteines were engineered at the residues of interest. From an alignment of 14-3-3 isoforms, the Asn residue at position 39 in 14-3-3 γ overlaid with C38 in 14-3-3 σ , so the 14-3-3 γ cysteine mutant was constructed lacking the C-terminal tail, 14-3-3 γ ^{N39C} Δ C (Figure 7.1). Another cysteine engineered at position 38 in 14-3-3 γ (S38C), to allow for comparison of fragments binding at residue on the edge or the adjacent residue outside of the binding groove (Figure 7.3A).

The binding affinities of the CRAF peptides were determined with these 14-3-3 γ cysteine mutants via FA protein titrations. Engineering in the cysteines for 14-3-3 γ affected the binding affinities for the CRAF peptides. The K_d values increased at least 5x compared to 14-3-3 γ ^{WT} (5-14x for CRAF^{NS}, >100x for CRAF^{WT}), except for the K_d value of CRAF^{S257L} that was relatively unchanged (9.45 μ M vs 6.51 μ M, 14-3-3 γ ^{WT} and 14-3-3 γ ^{N39C} Δ C, respectively; Table 7.1, Figure

7.3B). The binding affinities for 14-3-3 γ^{S38C} followed similar trends as 14-3-3 $\gamma^{N39C}\Delta C$ where the K_d value of CRAF S257L remained unchanged (10.8 μ M) and the K_d values of the remaining CRAF NS peptides increase 4-30x compared to 14-3-3 γ^{WT} (Table 7.1, Figure 7.3C).

Compounds were optimized for CRAF to contain a chloroacetamide cysteine-reactive warhead to replace the disulfide. During the optimization of these compounds, they were validated using the 14-3-3 σ protein that contains the native C38. These compounds were tested against the 14-3-3 $\gamma^{N39C}\Delta C$ protein to confirm their potency towards an engineered cysteine in the γ isoform compared to the native cysteine in the σ isoform.

Comparing the fold-stabilization ($\Delta appK_d$), MG **23** stabilizes the 14-3-3 σ /CRAF NS complexes 3.2x to over 1000x compared to DMSO treated samples (Table 7.2, Figure 7.4). The CRAF NS mutants that lie further away from pS259, R256S and V263A, had $appK_d$ values of 16 and 14 μ M, R256S and V263A, respectively. Whereas the mutants that affected the binding affinity the most, S257L and P261A, were estimated to be high μ M K_d values with DMSO as the binding curves did not reach a plateau at high concentration of 14-3-3 σ . Upon treatment with **23**, the $appK_d$ values were sub μ M with R256S, S257L, and V263A. The P261A mutant was minimally stabilized, 3.2x. In comparison with 14-3-3 $\gamma^{N39C}\Delta C$, the DMSO $appK_d$ values improved to low μ M, except for P261A the $appK_d$ value was improved to 49 μ M (vs. >100 μ M with 14-3-3 σ). Treatment with **23** stabilized the 14-3-3 $\gamma^{N39C}\Delta C$ /CRAF NS complexes 22-400x with sub μ M $appK_d$ values for all CRAF NS mutants. The fold-stabilization decreased for the CRAF NS mutants with 14-3-3 $\gamma^{N39C}\Delta C$, except for P261A that increased (3.2x and 53x for 14-3-3 σ and 14-3-3 $\gamma^{N39C}\Delta C$, respectively) due to the binding affinities being more easily measurable in the FA assay with this 14-3-3 protein. The binding curves for **23** and 14-3-3 σ , overlay with the binding curves for DMSO with 14-3-3 $\gamma^{N39C}\Delta C$ for all the CRAF NS mutants except of V263A. The binding curves for DMSO with 14-3-3 σ exhibit the lower end of the range whereas the binding curves for **23** with 14-3-3 $\gamma^{N39C}\Delta C$ suggest the upper end of the range of the FA assays, providing the

maximum dynamic range. Although basal binding affinities were improved with 14-3-3 $\gamma^{N39C}\Delta C$, the fold-stabilization was limited due to assay window (Table 7.1, Figure 7.4).

The C-terminal CRAF mutants were first screened to find mutant-selective fragments because these residues are more proximal to the fragment binding pocket and change the composite surface the fragments bind than the N-terminal residues (Figure 7.2A). Of the N-terminal mutants, only CRAF $S257L$ was screened as it is the most common mutation in Noonan patients carrying a CRAF mutation. To find mutant-selective fragments, a site-directed disulfide tethering MS screen was used to identify fragments from a library of ~2000 disulfides that bound cooperatively to 14-3-3 and CRAF (Figure 7.5A). The screens were run in the presence and absence (apo) of CRAF NS peptides to separate hits into stabilizing fragments, neutral binders, or inhibiting fragments. The apo screens (only 14-3-3 protein) were compared between the 14-3-3 $\gamma^{N39C}\Delta C$ and 14-3-3 γ^{S38C} proteins. From the comparison, the majority of fragments bound both cysteine mutants similarly where only 1.5-1.8% of the fragments were unique hits for either 14-3-3 γ protein (Figure 7.5B). Since the fragment hits were not unique to the 14-3-3 construct, only the 14-3-3 $\gamma^{N39C}\Delta C$ screen results were analyzed for the CRAF NS peptides (CRAF NS screens also conducted with 14-3-3 γ^{S38C} , data not shown).

The 14-3-3 $\gamma^{N39C}\Delta C$ /CRAF NS screening conditions were similar to those previously described⁶ at concentration of CRAF NS peptide 2x the K_d except for P261A where the concentration of peptide was set to 30 μM due to the large K_d value (60 μM) and instrument limitations. The results from the screens of the CRAF NS mutants, the V263A mutant had a 3.1% hit rate of identifying stabilizing fragments compared to 6.0% for P261A and 6.7% for S257L (Figure 7.5C-E). The larger hit rate for S257L and P261 was likely due to the BME concentration needing to be further optimized for those CRAF NS mutant peptides. From the hits, mutant-selective fragments were identified: 0.48% V263A-selective, 1.7% P261A-selective, 1.2% S257L-selective, and 0.58% CRAF NS mutant-selective over CRAF WT out of the fragment library (Figure 7.5F). The mutation V263A likely had the least amount of selective hits due to the

mutation being minimal (Val vs. Ala). The Pro to Ala mutation eliminates a highly structure region of CRAF to cause a greater change to the compound binding pocket. Surprisingly, the S257L mutant peptide resulted in selective hits although the mutation lies N-terminal to the pS259 and is not proximal to the compound binding pocket. The S257L mutation may have caused conformational changes in the N-terminal region that triggered the C-terminal region to alter the binding pocket. From the comparison of CRAF^{NS} and CRAF^{WT} screening results, selective hits were discovered for all CRAF peptides screened.

Discussion

The binding affinity analysis of the 14-3-3 isoforms confirmed the γ isoform bound client proteins more tightly than the σ and ζ isoforms. Engineering a cysteine in 14-3-3 γ caused a loss in binding affinity but still exhibited improved binding affinities compared to the other isoforms. Impaired PPIs can be difficult to study, so the use of the 14-3-3 γ cysteine mutant provided measurable binding affinities.

The 14-3-3 γ cysteine mutant allowed for optimized screening conditions of the impaired 14-3-3/CRAF interaction because the CRAF^{NS} mutants weakened the inherent binding affinities. Mutant-selective hits were identified for the CRAF^{NS} mutants screened. Validated hits provide starting points for optimization of MGs through SAR of 14-3-3/CRAF^{NS} to improve mutant-selectivity and potency to target the site of interest at the composite interface between 14-3-3 and CRAF^{NS}. The results from these screens showcase how a cysteine can be engineered at a site of interest to identify fragments that bind. However, only 14-3-3 σ contains the native cysteine of interest and is not highly expressed in cells, so cysteine-reactive MGs would only target a small population of client protein bound to 14-3-3 σ . To overcome this, noncovalent MGs need to be developed to be efficacious towards all 14-3-3 isoforms such as the example of aldehydes targeting the conserved N42 residue.

This work provides insight into the discovery of mutant-selective molecular glues for 14-3-3/CRAF in Noonan Syndrome to gain efficacy towards CRAF disease mutants. By selectively targeting and stabilizing the impaired mutant 14-3-3/CRAF interaction, the hyperactivation of the MAPK pathway could be reserved and lead to a novel therapeutic route for Noonan patients.

Figures

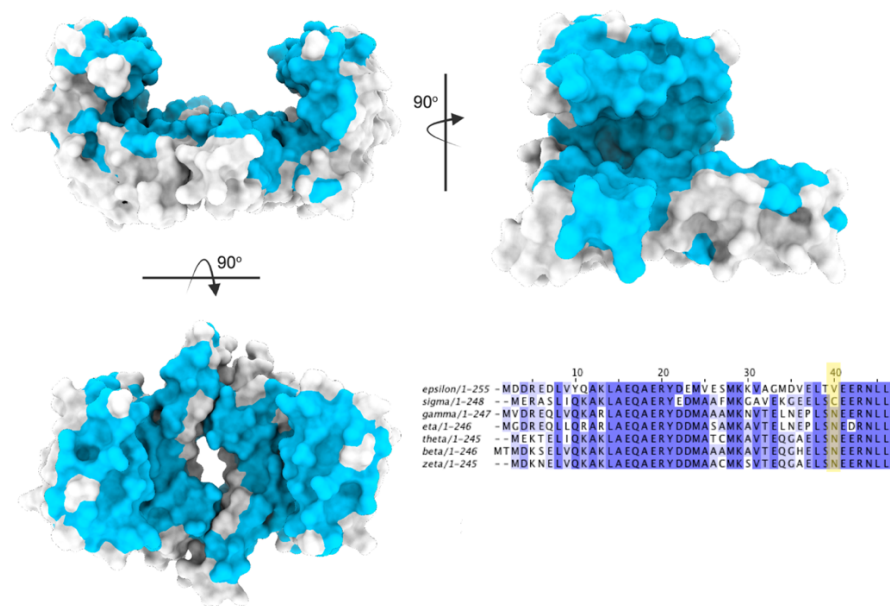


Figure 7.1: 14-3-3 isoform comparison

Crystal structure of 14-3-3 dimer (top left, bottom left) in white with conserved residues among the 7 isoforms (>80%) shown in blue. Front on view of 14-3-3 binding groove (top right). PDB 4IHL. Sequence alignment of 7 isoforms highlighting conserved residues, showing only the first ~45 residues. C38 in the ϵ isoform with corresponding residues in the other isoforms highlighted in yellow.

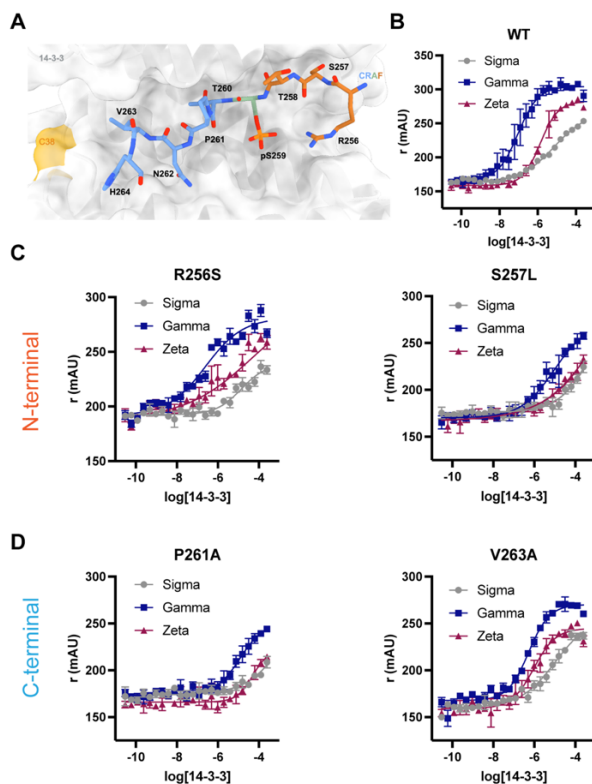


Figure 7.2: 14-3-3 isoform binding affinity comparison

A) Crystal structure of 14-3-3 (white surface) with C38 residue (14-3-3 σ) highlighted in yellow bound to CRAF peptide (N-terminal region in orange, C-terminal region in blue in regard to pS259). PBD: 3CU8. **B-D)** FA binding assays comparison the σ , γ , and ζ 14-3-3 isoforms between WT and mutant CRAF peptides. Data points represent mean $\pm$ SD, $n = 3$.

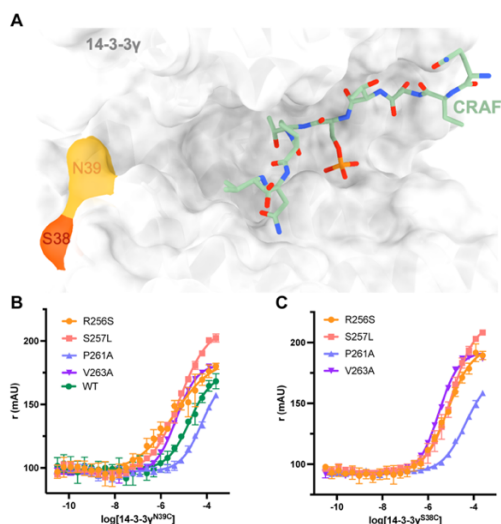


Figure 7.3: 14-3-3 γ cysteine mutant binding analysis

A) Crystal structure of 14-3-3 (white surface) and CRAF peptide (sage green). Residues in 14-3-3 γ , S38 and N39, that were mutated to cysteine highlighted in yellow and orange. **B, C)** FA binding assay of 14-3-3 γ cysteine mutants with CRAF mutant peptides. Data points represent mean $\pm$ SD, $n = 3$. **B)** 14-3-3 γ^{N39C} **C)** 14-3-3 γ^{S38C} .

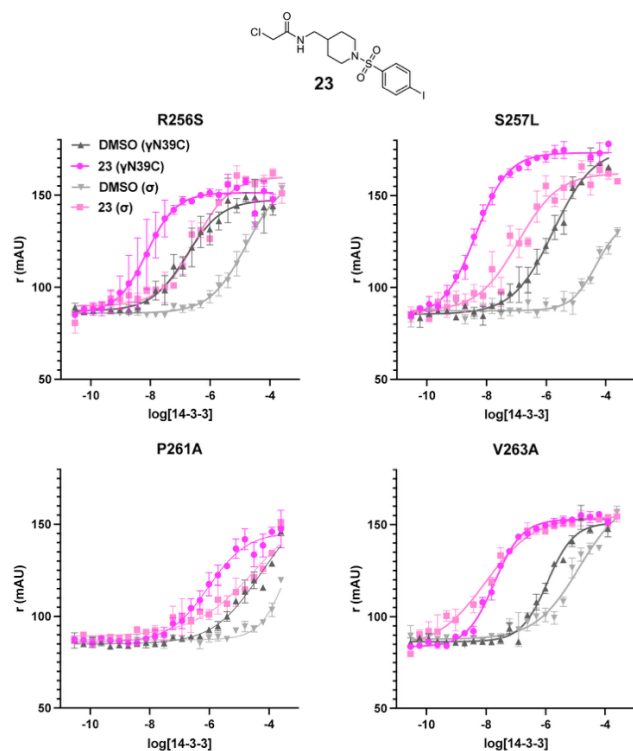


Figure 7.4: Binding affinity effects of molecular glue between 14-3-3 isoforms

Structure of molecular glue **23**. FA binding assays of CRAF^{NS} mutants comparing the 14-3-3^σ and 14-3-3^{N39C} ΔC proteins. Data points represent mean $\pm$ SD, n = 3.

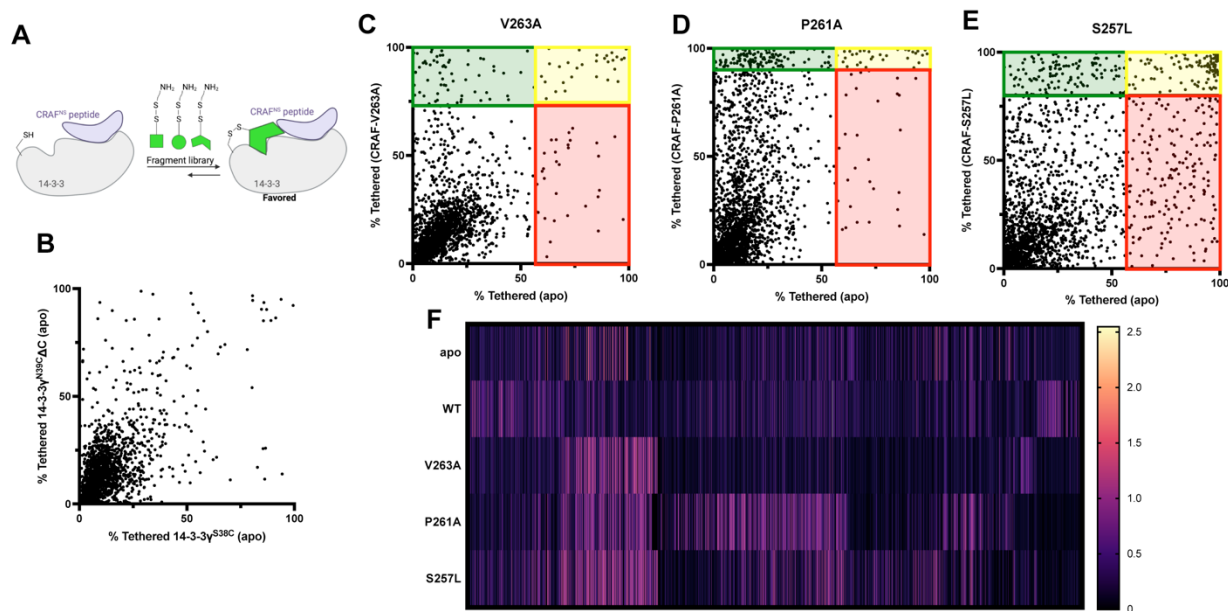


Figure 7.5: CRAF^{NS} mutant disulfide tethering screens

A) Schematic of disulfide tethering. **B)** Scatterplot comparing the results from the 14-3-3^{N39C} ΔC and 14-3-3^{S38C} apo screens. **C-E)** Scatterplots of CRAF^{NS} vs apo screens. Green quadrant = stabilizing hits, yellow quadrant = neutral binders, red quadrant = inhibiting hits. **F)** Heatmap of disulfide hits among CRAF^{NS} and CRAF^{WT}. % tethered normalized to 3SD value (threshold for hits) for each screen. Values $\geq$ 1.0 indicate hits at or above 3SD.

Tables

Table 7.1: Binding affinity comparison of CRAF peptides between 14-3-3 isoforms and cysteine mutants

	K_d (μM)				
Peptide	14-3-3σ	14-3-3ζ	14-3-3γ	14-3-3γ^{N39C}ΔC	14-3-3γ^{S38C}
WT	7.3	1.4	0.094	18	--
R256S	14	47	0.25	3.6	7.4
S257L	~200	~200	9.5	6.5	11
P261A	> 200	> 200	12	60	44
V263A	11	0.93	0.53	4.7	3.0

Table 7.2: Molecular glue effects on 14-3-3/CRAF^{NS} binding affinities

	14-3-3σ		14-3-3γ^{N39C}ΔC	
	<i>appK_d</i>^{DMSO} (μM)	<i>appK_d</i>⁸⁴⁸ (μM)	<i>appK_d</i>^{DMSO} (μM)	<i>appK_d</i>⁸⁴⁸ (μM)
R256S	16	0.32 (50x)	0.16 (97x)	0.0076 (22x)
S257L	48	0.11 (430x)	1.7 (28x)	0.0043 (400x)
P261A	410	32 (3.2x)	49 (8.2x)	0.93 (53x)
V263A	14	0.0076 (>1000x)	1.2 (12x)	0.019 (67x)

Materials and Methods

Protein Expression and Purification

The 14-3-3 proteins (σ, ζ, γ) with an N-terminal His6 tag were expressed in Rosetta™ 2(DE3) pLysS competent E. coli (Novagen) from a pPROEX HTb expression vector. Plasmids were transformed following the manufacturer's protocol. Single colonies were used to inoculate 25 mL LB starter cultures for overnight incubation (37°C, 250 rpm). Starter cultures were then added to 1.5 L TB medium. Expression was induced at OD₆₀₀ ~2 with 750 μM IPTG and cultures expressed overnight at 30°C, 250 rpm. Cells were harvested at 6,500 rpm and resuspended in lysis buffer (50 mM HEPE pH 7.5, 500 mM NaCl, 20 mM imidazole, 10% glycerol, 1 mM TCEP) before sonication. Protein was purified using Ni-affinity chromatography (Ni-NTA Agarose, Invitrogen; Wash buffer: 50 mM HEPES pH 7.5, 500 mM NaCl, 20 mM imidazole, 1 mM TCEP;

Elution buffer: 50 mM HEPES pH 7.5, 500 mM NaCl, 500 mM imidazole, 1 mM TCEP). Purified proteins were analyzed by SDS-PAGE and Q-ToF LC/MS. Following a buffer exchange (25 mM HEPES pH 7.5, 150 mM NaCl, 1 mM TCEP), protein was concentrated to 500 μ M and flash-frozen for storage at 80°C.

Cysteine residues were engineered into 14-3-3 γ (S38C and N39C), and the C-terminus (S235-N248) was removed from the 14-3-3 γ^{N39C} construct (14-3-3 $\gamma^{N39C}\Delta$ C) for future crystallographic studies.

Peptides

Peptides were purchased from Elim Biopharmaceuticals, Inc. (Hayward, CA). Fluorescein-labeled (5-FAM) peptides were used in fluorescence anisotropy assays.

Peptides for mass spectrometry:

CRAF^{S257L} pS259 (14mer): Ac-SQRQRLT(pS)TPNVHM-NH₂

CRAF^{P261A} pS259 (14mer): Ac-SQRQRST(pS)TANVHM-NH₂

CRAF^{V263A} pS259 (14mer): Ac-SQRQRST(pS)TPNAHM-NH₂

Peptides for fluorescence anisotropy:

CRAF^{WT} pS259 (15-mer): 5-FAM-SQRQRST{pS}TPNVHNV-NH₂

CRAF^{R256S} pS259 (15-mer): 5-FAM-SQRQSST(pS)TPNVHNV-NH₂

CRAF^{S257L} pS259 (15-mer): 5-FAM-SQRQRLT(pS)TPNVHNV-NH₂

CRAF^{P261A} pS259 (15-mer): 5-FAM-SQRQRST(pS)TANVHNV-NH₂

CRAF^{V263A} pS259 (15-mer): 5-FAM-SQRQRST(pS)TPNAHNV-NH₂

Fluorescence Anisotropy Protein Titrations

FA experiments were conducted as previously described in Chapter 5. Briefly, 14-3-3 protein was serially diluted (2-fold) in buffer (10 mM HEPES pH 7.5, 150 mM NaCl, 0.1% Tween20) at a

starting concentration of 250 μ M in black, flat-bottom 384-well assay plates (Corning 3575). 5-FAM labeled peptides were added to a final concentration of 100 nM (final sample volume of 25 μ L). Assay plates were incubated at room temperature with gentle rocking. The fluorescence anisotropy was measured after 30 minutes to determine the binding affinity (K_d) for each peptide/14-3-3 complex using a Molecular Devices iD5 SpectraMax plate reader (filter set λ_{ex} : 485 ± 20 nm; λ_{em} : 535 ± 25 nm). K_d values were obtained using a four-parameter logistic model (4PL) in GraphPad Prism. Assays were performed in triplicate.

For experiments with compound, the previous protocol was followed with these modifications: buffer (10 mM HEPES pH 7.5, 150 mM NaCl, 0.1% Tween20, 0.1% BSA, final sample volume 10 μ L ((black, flat-bottom 384-well assay plate, Greiner Bio-One #784900), and 100 μ M compound (or same % v/v DMSO). Fluorescence was measured at 1 hr and 24 hr.

Disulfide Tethering Mass Spectrometry Screening

Screens were ran as previously described in Chapter 2.⁶ The screens were run with either 14-3-3 γ^{S38C} or 14-3-3 γ^{N39C} Δ C (250 – 500 nM), peptide concentration of $\sim 2\times$ the K_d (determined by FA protein titration) or 30 μ M (maximum limit set for instrument), 250 μ M BME, and 200 μ M disulfide library fragments in buffer (10 mM Tris pH 8.0). Assay plates were incubated for 3 hours at room temp with gentle rocking and then read via LC/M (Waters Acquity UPLC/Xevo G2-HS Q-ToF mass spectrometer). Data collection and automated processing followed a previously described custom workflow.⁶

14-3-3 Protein	CRAF mutant peptide
14-3-3 γ^{S38C} (300 nM)	--
14-3-3 γ^{S38C} (300 nM)	S257L (22 μ M)
14-3-3 γ^{S38C} (300 nM)	P261A (30 μ M)
14-3-3 γ^{S38C} (300 nM)	V263A (3 μ M)
14-3-3 γ^{N39C} Δ C (500 nM)	--
14-3-3 γ^{N39C} Δ C (250 nM)	S257L (14 μ M)
14-3-3 γ^{N39C} Δ C (250 nM)	P261A (30 μ M)
14-3-3 γ^{N39C} Δ C (500 nM)	V263A (10 μ M)

References

1. Wells, J. A. & McClendon, C. L. Reaching for high-hanging fruit in drug discovery at protein-protein interfaces. *Nature* **450**, 1001–1009 (2007).
2. Lu, H. *et al.* Recent advances in the development of protein–protein interactions modulators: mechanisms and clinical trials. *Signal Transduct. Target. Ther.* **5**, 213 (2020).
3. Zhong, M., Lee, G. M., Sijbesma, E., Ottmann, C. & Arkin, M. R. Modulating protein-protein interaction networks in protein homeostasis. *Curr. Opin. Chem. Biol.* **50**, 55–65 (2019).
4. Andrei, S. A. *et al.* Stabilization of protein-protein interactions in drug discovery. *Expert Opin. Drug Discov.* **12**, 925–940 (2017).
5. Arkin, M. R., Tang, Y. & Wells, J. A. Small-Molecule Inhibitors of Protein-Protein Interactions: Progressing toward the Reality. *Chem. Biol.* **21**, 1102–1114 (2014).
6. Kenanova, D. N. *et al.* A Systematic Approach to the Discovery of Protein–Protein Interaction Stabilizers. *ACS Cent. Sci.* **9**, 937–946 (2023).
7. Konstantinidou, M. *et al.* Structure-Based Optimization of Covalent, Small-Molecule Stabilizers of the 14-3-3 σ /ER α Protein–Protein Interaction from Nonselective Fragments. *J. Am. Chem. Soc.* **145**, 20328–20343 (2023).
8. Visser, E. J. *et al.* Estrogen Receptor α /14-3-3 molecular glues as alternative treatment strategy for endocrine resistant breast cancer. Preprint at <https://doi.org/10.1101/2024.04.25.591105> (2024).
9. Somsen, B. A. *et al.* Reversible Dual-Covalent Molecular Locking of the 14-3-3/ERR γ Protein–Protein Interaction as a Molecular Glue Drug Discovery Approach. *J. Am. Chem. Soc.* **145**, 6741–6752 (2023).
10. Somsen, B. A. *et al.* Molecular basis and dual ligand regulation of tetrameric estrogen receptor α /14-3-3 ζ protein complex. *J. Biol. Chem.* **299**, 104855 (2023).
11. Sijbesma, E. *et al.* Site-Directed Fragment-Based Screening for the Discovery of Protein-Protein Interaction Stabilizers. *J. Am. Chem. Soc.* **141**, 3524–3531 (2019).

12. Andlovic, B. *et al.* Fragment-Based Interrogation of the 14–3–3/TAZ Protein–Protein Interaction. *Biochemistry* **63**, 2196–2206 (2024).
13. Soini, L. *et al.* Identification of molecular glues of the SLP76/14-3-3 protein–protein interaction. *RSC Med. Chem.* **12**, 1555–1564 (2021).
14. Katz, L. S. *et al.* Molecular glues of the regulatory ChREBP/14-3-3 complex protect beta cells from glucolipotoxicity. *Nat. Commun.* **16**, 2110 (2025).
15. Konstantinidou, M. *et al.* Scaffold-hopping for molecular glues targeting the 14-3-3/ER α complex. *Nat. Commun.* **16**, (2025).
16. Aghazadeh, Y. & Papadopoulos, V. The role of the 14-3-3 protein family in health, disease, and drug development. *Drug Discov. Today* **21**, 278–287 (2016).
17. Liu, J. *et al.* The role of 14-3-3 proteins in cell signalling pathways and virus infection. *J. Cell. Mol. Med.* **25**, 4173–4182 (2021).
18. Morrison, D. K. The 14-3-3 proteins: integrators of diverse signaling cues that impact cell fate and cancer development. *Trends Cell Biol.* **19**, 16–23 (2009).
19. Obsilova, V. & Obsil, T. The 14-3-3 Proteins as Important Allosteric Regulators of Protein Kinases. *Int. J. Mol. Sci.* **21**, E8824 (2020).
20. Pennington, K. L., Chan, T. Y., Torres, M. P. & Andersen, J. L. 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 (2018).
21. Stevers, L. M. *et al.* Modulators of 14-3-3 Protein-Protein Interactions. *J. Med. Chem.* **61**, 3755–3778 (2018).
22. Obsil, T. & Obsilova, V. Structural basis of 14-3-3 protein functions. *Semin. Cell Dev. Biol.* **22**, 663–672 (2011).
23. Aitken, A. 14-3-3 proteins: a historic overview. *Semin. Cancer Biol.* **16**, 162–172 (2006).
24. Sijbesma, E. *et al.* Exploration of a 14-3-3 PPI Pocket by Covalent Fragments as Stabilizers. *ACS Med. Chem. Lett.* **12**, 976–982 (2021).

25. Gardino, A. K., Smerdon, S. J. & Yaffe, M. B. Structural determinants of 14-3-3 binding specificities and regulation of subcellular localization of 14-3-3-ligand complexes: a comparison of the X-ray crystal structures of all human 14-3-3 isoforms. *Semin. Cancer Biol.* **16**, 173–182 (2006).
26. Sengupta, A., Liriano, J., Bienkiewicz, E. A., Miller, B. G. & Frederick, J. H. Probing the 14-3-3 Isoform-Specificity Profile of Protein-Protein Interactions Stabilized by Fusicoccin A. *ACS Omega* **5**, 25029–25035 (2020).
27. Tzivion, G., Luo, Z. & Avruch, J. A dimeric 14-3-3 protein is an essential cofactor for Raf kinase activity. *Nature* **394**, 88 (1998).
28. Dedden, D. *et al.* Cryo-EM Structures of CRAF2/14-3-32 and CRAF2/14-3-32/MEK12 Complexes. *J. Mol. Biol.* **436**, 168483 (2024).
29. Martinez Fiesco, J. A., Durrant, D. E., Morrison, D. K. & Zhang, P. Structural insights into the BRAF monomer-to-dimer transition mediated by RAS binding. *Nat. Commun.* **13**, 486 (2022).
30. Park, E. *et al.* Architecture of autoinhibited and active BRAF–MEK1–14-3-3 complexes. *Nature* **575**, 545–550 (2019).
31. Dedden, D. *et al.* Cryo-EM Structures of CRAF2/14-3-32 and CRAF2/14-3-32/MEK12 Complexes. *J. Mol. Biol.* **436**, 168483 (2024).
32. Molzan, M. *et al.* Impaired binding of 14-3-3 to C-RAF in Noonan syndrome suggests new approaches in diseases with increased Ras signaling. *Mol. Cell. Biol.* **30**, 4698–4711 (2010).
33. Pandit, B. *et al.* Gain-of-function RAF1 mutations cause Noonan and LEOPARD syndromes with hypertrophic cardiomyopathy. *Nat. Genet.* **39**, 1007–1012 (2007).
34. Fernandez-Medarde, A. & Santos, E. Ras in Cancer and Developmental Diseases. *Genes Cancer* **2**, 344–358 (2011).

35. Razzaque, M. A. *et al.* Germline gain-of-function mutations in RAF1 cause Noonan syndrome. *Nat. Genet.* **39**, 1013–1017 (2007).
36. Molzan, M. *et al.* Stabilization of physical RAF/14-3-3 interaction by cotylenin A as treatment strategy for RAS mutant cancers. *ACS Chem. Biol.* **8**, 1869–1875 (2013).
37. Roberts, A. E., Allanson, J. E., Tartaglia, M. & Gelb, B. D. Noonan syndrome. *The Lancet* **381**, 333–342 (2013).
38. Erlanson, D. A., Wells, J. A. & Braisted, A. C. Tethering: fragment-based drug discovery. *Annu. Rev. Biophys. Biomol. Struct.* **33**, 199–223 (2004).
39. Visser, E. J. *et al.* From Tethered to Freestanding Stabilizers of 14-3-3 Protein-Protein Interactions through Fragment Linking. *Angew. Chem. Int. Ed.* **62**, e202308004 (2023).

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