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Profiling the Dynamic Interfaces of Fluorinated Transcription Complexes for Ligand Discovery and Characterization

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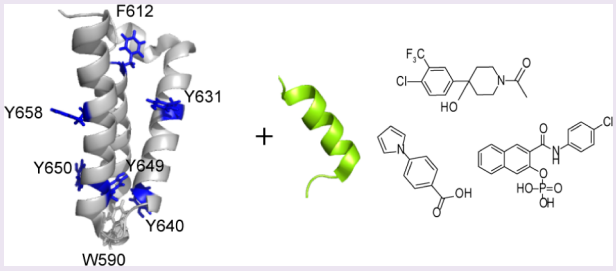
Supporting Information

ABSTRACT: The conformationally dynamic binding surfaces of transcription complexes present a particular challenge for ligand discovery and characterization. In the case of the KIX domain of the master coactivator CBP/p300, few small molecules have been reported that target its two allosterically regulated binding sites despite the important roles that KIX plays in processes ranging from memory formation to hematopoiesis. Taking advantage of the enrichment of aromatic amino acids at protein interfaces, here we show that the incorporation of six ^{19}F-labeled aromatic side chains within the KIX domain enables recapitulation of the differential binding footprints of three natural activator peptides (MLL, c-Myb, and pKID) in complex with KIX and effectively reports on allosteric changes upon binding using 1D NMR spectroscopy. Additionally, the examination of both the previously described KIX protein−protein interaction inhibitor Napthol-ASE-phosphate and newly discovered ligand 1-10 rapidly revealed both the binding sites and the affinities of these small molecules. Significantly, the utility of using fluorinated transcription factors for ligand discovery was demonstrated through a fragment screen leading to a new low molecular weight fragment ligand for CBP/p300, 1G7. Aromatic amino acids are enriched at protein−biomolecule interfaces; therefore, this quantitative and facile approach will be broadly useful for studying dynamic transcription complexes and screening campaigns complementing existing biophysical methods for studying these dynamic interfaces.



There is mounting interest in dissecting the architectural features and regulatory mechanisms of transcriptional coactivator complexes due to the role such complexes play in disease pathways and the accompanying desire to target them pharmacologically.[1−3] However, these dynamic interfaces are challenging to study and are often characterized as undruggable. In many instances, transcription complex assembly and function occurs through the interaction of structurally dynamic regions of the constituent proteins, with the resulting allosteric changes upon complex formation informing the function.[3,4] A foundational example is the KIX domain of the master coactivator CBP/p300, a domain that is targeted by more than a dozen transcriptional activators, including MLL, CREB, and c-Myb, through two allosterically regulated protein binding sites[5] Dysregulation of these protein−protein interactions contributes to cancer and neurological disorders,[6−8] prompting characterization of native KIX binding interactions and the resulting structural reorganizations.[9−11] However, standard screening methods have led to a very limited group of molecules that target this flexible protein domain, motivating a reevaluation of both ligand discovery and characterization strategies.[12−16]

Because side chain reorganization plays an important role in the binding events of KIX and other conformationally dynamic proteins,[17] we sought a side-chain-detected method that would be sensitive and facile enough for both characterization and screening purposes.[18] The employment of ^{19}F-labeled aromatic amino acids appeared an attractive approach due to the signal sensitivity of ^{19}F (83% as sensitive as ^{1}H), the environmental hypersensitivity of ^{19}F chemical shifts, and lack of background signals leading to simplified 1D NMR spectra.[19−21] In addition, aromatic amino acids such as tyrosine are enriched at protein binding interfaces.[22,23] 1D-Protein-observed ^{19}F NMR spectroscopy has been a versatile biochemical tool for studying protein folding and function.[19−21] In addition, several studies have reported changes in the ^{19}F NMR spectra of proteins in the presence of natural ligands or known drugs;[24−28] however, concerns have been raised that the unfavorable relaxation properties of fluorine-labeled proteins would prohibit its usage in NMR screening applications.[29,30] Indeed, there are no reports of 1D ^{19}F NMR spectroscopy of proteins for the characterization of small molecule protein−protein interaction inhibitors and no reports of ligand discovery/screening applications. However, we were encouraged by the fact that many transcription factors possess large regions of structural disorder leading to faster internal motion compared to many

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globular proteins.[31] We thus hypothesized that incorporation of a small subset of side chain labeled residues at the dynamic binding surfaces of KIX would enable characterization of native and small molecule complexes with this domain and, most importantly, screening applications to discover new ligands.

As a first step, we tested if 1D ^{19}F NMR spectroscopy could differentiate the binding modes of known peptides targeting the allosterically regulated binding sites within the KIX domain. Aromatic amino acids (Phe, Tyr, and Trp) are highly enriched at protein−protein interfaces,[22,23] especially for α-helix-protein interactions such as those that occur along both of the KIX binding sites. Six of the seven aromatic amino acid residues present in KIX play critical roles in the binding behavior and structure of this conformationally dynamic domain (Figure 1A).

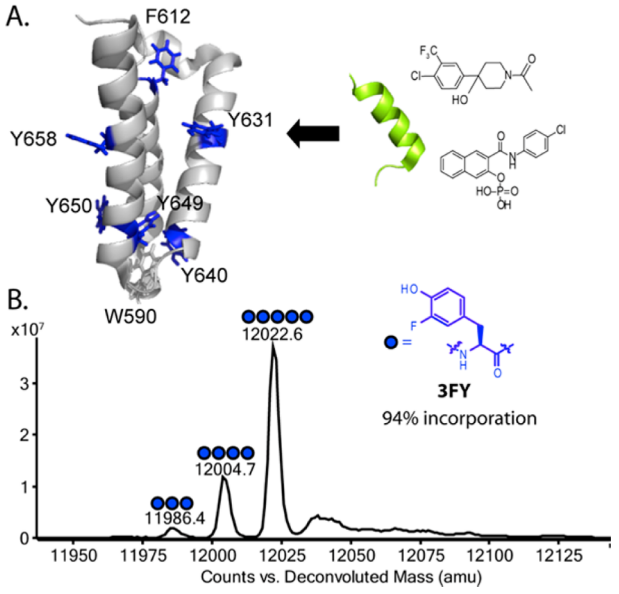

Figure 1. KIX interacting small molecules and peptides. (A) A ribbon diagram of the CBP KIX domain (586−672) is shown in gray (PDB ID: 2AGH). Five tyrosine residues (Y631, Y640, Y649, Y650, Y658), phenylalanine (F612), and tryptophan (W590) are available for substitution with fluorinated variants. Blue side chains were labeled in this study. (B) ESI mass spectrogram of a 3FY-KIX sample (19:6:1 ratio, of 3FY-labeled KIX, 94%).

F612 is in van der Waals contact with natural activator MLL.[5] Additionally, three of the five tyrosine residues make some of the most important contacts with endogenous ligands MLL (Y631)[9] and the phosphorylated kinase inducible domain of CREB, pKID (Y650, Y658). Y649 is in close proximity to bound pKID.[10] The remaining residue, Y640, structurally stabilizes KIX through a cation-pi interaction with R600.[32] Tyrosine and phenylalanine were therefore ideal choices as spectroscopic probes for ligand binding and monitoring long-range structural changes to KIX.

We incorporated 3FY into KIX using an auxotrophic bacterial strain, DL39(DE3). Protein expression conditions were optimized using tyrosine-depleted synthetic media with 3FY (0.05 mg/mL) resulting in >90% 3FY incorporation as judged by mass spectrometry (Figure 1B). Circular dichroism (CD) and fluorescence polarization (FP) were used to assess the effect of 3FY incorporation on KIX structure and function. The data were consistent with a well-folded 3FY-KIX construct with minimally affected function (Supplementary Figures S1, S2).[33]

^{19}F NMR resonance dispersion is enhanced by the hypersensitivity of the ^{19}F nucleus to differences in chemical environment provided by protein tertiary structure.[21] Consistent with this environmental sensitivity, five well-resolved 3FY resonances with an approximately 5 ppm difference in chemical shift were observed in the ^{19}F NMR spectrum at 25 °C (Figure 2A), with an increase in resonance dispersion at

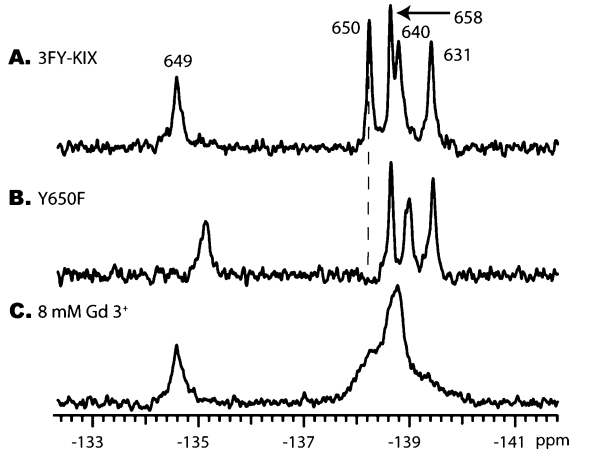

Figure 2. Resonance assignment of 3FY-KIX at 25 °C. (A) ^{19}F NMR spectrum of 94% 3F-Y-labeled KIX. (B) Representative spectrum of a tyrosine to phenylalanine point mutant, Y650F, for resonance assignment. (C) ^{19}F NMR spectrum of 3FY-KIX in the presence of 8 mM Gd-EDTA.

lower temperatures (see Supplementary Figure S3). Three of the five 3FY resonances were assigned using Tyr to Phe single point mutations (Y650F, Y658F, and Y640F). The spectra of each KIX mutant was similar to the parent sequence with the exception of one missing resonance per mutant (Figure 2B, Supplementary Figure S4). A paramagnetic line-broadening agent (Gd-EDTA) was used to assign the remaining two resonances as Y631 and Y649, the most and least solvent-exposed residues, respectively. The resonance at −134.6 ppm was minimally affected (Figure 2C). Protection from broadening is consistent with a solvent-inaccessible residue, Y649. In contrast, the resonance at −139.4 ppm experienced a high degree of broadening at 8 mM Gd-EDTA,[34] consistent with the surface-exposed residue Y631 (Figure 2C and Supplementary Figure S5).

With assignments in hand, attention first turned to using 1D ^{19}F NMR to define interactions between KIX and native ligands and the resulting conformational effects. ^{19}F NMR binding assays were carried out with the unlabeled variants of the transcriptional activation domain peptides used for FP binding studies (pKID29, MLL15, and c-Myb25).[9−11] Although the NMR structure of pKID-bound KIX reveals direct contact of residues Y658 and Y650 with pKID and that Y649 is in close proximity, amide-observed ^{1}H−^{15}N HSQC experiments show only Y658 exhibiting a significant chemical shift change.[35] ^{19}F NMR experiments enabled detection of these side chain contacts, as Y658 and Y650 are significantly perturbed. The resonance for Y649, which is in close proximity to pKID, was also affected, whereas the distal residue Y640 does not show a detectable alteration in chemical shift (Figure 3B and Supplementary Figure S6). In these experiments both the bound and unbound populations were observed, indicating that ligand binding was in the slow exchange regime ($\Delta\delta$ = 0.2 ppm,

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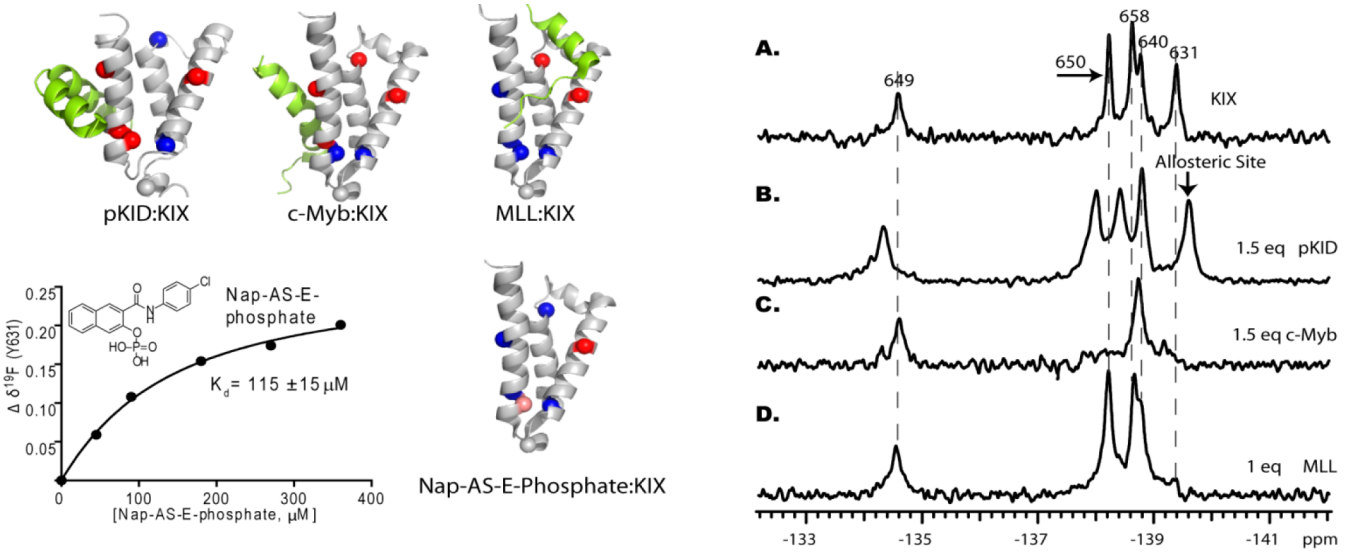

Figure 3. 1D ^{19}F NMR binding experiments. (Left) Binding sites for three natural activators with KIX and small molecule Napthol-ASE-phosphate. Red balls refer to significantly perturbed residues upon binding (PDB IDs: 2AGH, 1KDX), and pink indicate significant but lower levels of chemical shift perturbation. Binding isotherm for Napthol-ASE-phosphate binding of KIX is shown for perturbation of the resonance for Y631. (Right) ^{19}F NMR spectra of 3FY-KIX (25−55 μM) in the (A) absence and presence of (B) pKID, (C) c-Myb, and (D) MLL. Dashed lines indicate position of 3FY resonances for unbound KIX.

k_{ex} < 148 s^{-1}), consistent with previous reports.[36] Binding of pKID to KIX also allosterically regulates MLL binding,[5] and ^{19}F NMR experiments revealed perturbation of the 3FY resonance for Y631 in the MLL site upon pKID binding (Figure 3B and Supplementary Figure S6) supporting an allosteric effect at this site.

Myb and pKID target overlapping but distinct sites on KIX due to their different binding orientations.[35] In ^{19}F NMR experiments with c-Myb, the fluorine resonances for Y658, Y650, and Y631 are the most significantly broadened (intermediate exchange). Binding of c-Myb also allosterically regulates the MLL binding site of KIX,[5] and ^{19}F NMR binding studies revealed a perturbation of Y631 (Figure 3D, Supplementary Figure S7). Little effect on the resonances for Y649 and Y640 were observed (Figure 3C, Supplementary Figure S7). In contrast, the resonance for the side chain of Y649 is strongly perturbed upon pKID binding ($\Delta\delta$ = 0.2 ppm) likely due to the close proximity of Y649 to the C-terminal helix of pKID.[10] From these results we conclude that, even with only five 3FY probes, we are able to distinguish distinct binding footprints for two ligands with overlapping binding sites (Figure 3).

The second activator binding site of KIX is targeted by the transcriptional activation domain of MLL. The NMR structure of a ternary complex of MLL and c-Myb simultaneously bound to KIX reveals close contact between MLL and the Y631 side chain.[9] A 1D ^{19}F NMR spectrum of KIX in complex with MLL shows that Y631 is the only resonance significantly perturbed, with a second population growing in under the resonance assigned to Y650, indicating binding in the slow/intermediate exchange regime (Figure 3D, Supplementary Figure S8). A single MLL binding site probe could be limiting for detecting protein−ligand interactions. Therefore, a second binding-site probe was investigated. The aromatic amino acid F612 makes extensive hydrophobic contacts with the side chains of MLL (e.g., F2852 and V2853).[9] We expressed a KIX protein that was singly labeled at position 612 with 4-fluorophenylalanine (4FF). The appearance of a new shifted resonance at −116.2 ppm upon MLL binding supported the sensitivity of this probe (Supplementary Figure S9). Because c-Myb and pKID each enhance MLL binding allosterically,[9] we also examined the F612-containing KIX in complex with these two peptides. Surprisingly, only binding of c-Myb perturbs this 4FF resonance (Supplementary Figures S10 and S11). This differential effect suggests distinct structural differences conferred upon the binding of each of the peptides underlies the similar binding cooperativity, although further studies will be necessary to fully define this effect. Nonetheless, the F612 to 4FF mutation is clearly a valuable probe for detecting changes in the MLL binding surface and complements the 3FY-containing KIX.

Although 1D ^{19}F NMR spectroscopy proved to be a sensitive technique for characterizing the relatively large surface area complexes between KIX and its native ligands, it remained an open question if the binding of small molecule ligands, with significantly smaller surface area and weaker binding affinity, could be observed by this method. Therefore we next tested the ability to both detect and quantify the binding interactions for libraries of low molecular weight small molecules to assess the feasibility for screening applications.

We first examined the KIX ligand Naphthol-ASE-phosphate, a PPI inhibitor that blocks formation of the KIX·pKID complex.[12] In a 1D ^{19}F NMR experiment a perturbation of Y631 in the MLL site with a large change in chemical shift (0.2 ppm) was identified. Fast exchange data from small molecule titration (Figure 3) provided a K_d determination of 115 ± 15 μM, comparable to the reported 90 μM K_i.[8] A small but significant change in Y649 near the pKID binding site was also observed.[33] In a separate experiment, the fluorine resonance of 4FF at F612 was minimally affected in the presence of Napthol-ASE-phosphate ($\Delta\delta$ < 0.04 ppm, Figure S13). A large perturbation of the fluorine resonance of Y631 and a small perturbation of resonance Y649 at the pKID site supports the report of Naphthol-ASE-phosphate inhibiting pKID:KIX interactions from a distal binding site.[12]

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We next applied ^{19}F NMR to characterize an entirely new KIX ligand, molecule 1-10 (Figure 4), that was identified *via* a

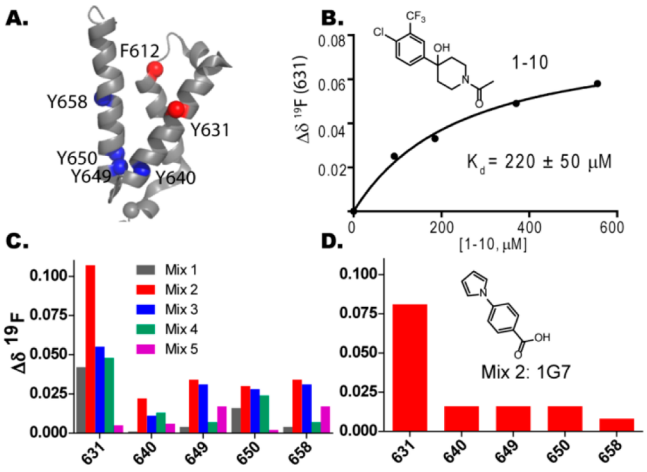

Figure 4. Discovery and characterization of new small molecule KIX complexes. (A) Residues with perturbed chemical shifts upon 1-10 binding are mapped onto KIX in red (PDB ID: 2AGH). (B) Binding isotherm for 1-10 with KIX is shown for perturbation of the resonance for Y631. (C) Chemical shift perturbations of aryl fluorine resonance from 42 μM 3FY-KIX in the presence of small molecule mixtures or (D) molecule 1G7 (500 μM).

tethered fragment screen directed at the MLL binding site but had not yet been characterized or reported.[37] Due to the format of the screen, we anticipated that 1-10 would bind in the MLL site but that the affinity would be modest at best. ^{19}F NMR confirmed binding of compound 1-10 to the MLL site *via* perturbation of fluorine resonances of Y631 and F612[33] (Figure 4B and Supplementary Figures S14, 15). ^{19}F NMR chemical shift perturbation data from titration of increasing amounts of 1-10 up to 15 equiv yielded a K_d of 220 ± 50 μM (Figure 4C). Binding was further confirmed *via* ^{1}H−^{15}N HSQC NMR (Supplementary Figure S20). Successful analysis of weak binding ligands and a small molecule fragment encouraged us to proceed with applying ^{19}F NMR as a fragment screening technique, a mode of screening that is gaining increasing attention for developing protein−protein interaction inhibitors and assessing druggability.[38]

Given the small surface area and weak binding propensities of fragment libraries, screens of such molecules represent a particular detection challenge. To assess the sensitivity of 1D ^{19}F NMR in this format, 3FY-KIX was used in a screen with 50 small molecule fragments (from a rule of 3 compliant commercial library) using mixtures of 10 compounds at concentrations of 500 μM. One mixture, Mix 2, showed a large shift of the resonance for Y631 in the MLL binding site (Figure 4C). Deconvolution of the mixture identified a pyrrole-substituted benzoic acid, 1G7, as the main contributor to the chemical shift perturbation (Figure 4D). Initial chemical shift information can be obtained within 5−10 min for mixtures of 10 compounds, and therefore screening of 50 compounds as well as deconvolution can be carried out rapidly in a single day. Few small molecule KIX ligands have been reported, and therefore the identification of a new ligand from this screen highlights the potential for using 1D ^{19}F NMR of aromatic side chains for both follow-up and initial screening.

In conclusion, we deployed 1D ^{19}F NMR for structurally characterizing and quantifying KIX−ligand interactions by achieving sufficient surface coverage by incorporating five to six ^{19}F NMR nuclei at dynamic protein−protein interaction sites. Our studies led to the discovery of two new small molecule KIX ligands, 1-10 and 1G7, and demonstrated for the first time the utility of ^{19}F protein-observed NMR for screening applications. An added benefit was the sensitive detection of differential modes of protein allostery, a common regulatory mechanism of transcriptional complexes and a useful feature to target for developing selective small molecules.[39] The ease of experimental setup (no solvent suppression), tolerance to a wide range of buffer conditions and temperature and speed of data acquisition (chemical shift estimates can be obtained in 5−10 min) and F−F NOEs[40] enables rapid characterization of protein−ligand complexes ranging from peptides to weak-binding small molecules, extending the utility of ^{19}F NMR of proteins into ligand discovery. Resonance broadening from chemical shift anistropy effects becomes more severe for large proteins (*i.e.*, long correlation times). However, the shorter correlation times observed for proteins with larger stretches of disorder,[31] including many transcription factors, as well as the introduction of new cryoprobe technology will be enabling for extending these studies to a broader set of transcription factors. This will thus complement existing HSQC assays (SAR by NMR)[41] and ligand-observed experiments.[29]

METHODS

Protein Expression and Purification of His$_6$-KIX (586−672). The DNA sequence encoding the KIX domain of mouse CBP (586−672) was cloned into a pRSETB vector incorporating a hexahistidine tag with a short linker fused to the N-terminus of KIX (pRSETBHIS6KIX, Supplementary Table S2) for protein expression resulting in a protein with the following sequence (linker residues are shown in lower case): mrgshhhhhhgmasGVRKGWHEHVTQDLRSHLVHKLVQAIFPTPDPAALKDRRMENLVAYAKKVEGDMY ESANSRDEYYHLLAEKIYKIQKELEEKRRSRL.

KIX was expressed as previously described.[42] The His-tagged protein was isolated using Ni-NTA beads (Qiagen) according to the manufacturer's instruction and eluted with 400 mM imidazole. Final purification was carried out by ion-affinity column chromatography on a Source S column, (GE Healthcare) in 50 mM phosphate buffer, pH 7.2 eluting with increasing concentrations of NaCl (0−0.6 M NaCl). Purified protein solutions were buffer exchanged into 10 mM sodium phosphate, 100 mM NaCl, 10% glycerol, 0.01% NP40, pH 6.8, using a PD-10 column (GE Healthcare) and stored at −80 °C.

Protein Expression and Purification of Fluorine-Labeled-KIX (586−672) Proteins. Fluorinated amino acids can be incorporated into KIX by two protocols. Protocol 1 uses the auxotrophic bacterial cell line DL39(DE3) and was optimized for high label incorporation. Protocol 2 uses small-molecule-induced auxotrophy of tryptophan, phenylalanine, and tyrosine by addition of glyphosate (1 mg/mL). Both methods were adapted from protocols detailed by Frieden *et al.*[20] Protocol 2 can be found in the Supporting Information.

3FY and 4FF Incorporation into KIX Using the Auxotrophic Cell Line DL39(DE3). *3FY-KIX.* The pRSETBHIS6KIX plasmid (Supplementary Table S2) and the pRARE plasmid (Novagen) were cotransformed into DL39(DE3) cells and grown overnight in 50 mL cultures of Lennox L. broth (Research Products International) supplemented with ampicillin (100 μg/mL) and chloramphenicol (34 μg/mL) at 37 °C, 250 rpm. Two 2 mL aliquots were spun down to remove Lennox L. Broth and resuspended in 100 mL of synthetic media reported by Muchmore *et al.*[43] (referred to here as -Y media,) containing all 20 amino acids except tyrosine and supplemented with ampicillin (100 μg/mL) and tyrosine (150 μM). Cells were grown to an OD of 0.7−1.0 (37 °C, 250 rpm). The cells were pelleted, washed 1 time with -Y media, and resuspended in 100 mL of -Y media plus 0.05 mg/mL 3-fluorotyrosine (Sigma-Aldrich). The bacteria were left to recover at 37 °C, 250 rpm for 3.5 h. Protein expression was induced

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with 0.5 mM IPTG for 3 h at 250 rpm at 37 °C. The His-tagged protein was purified as described above with protein yields up to 18 mg/L and stored at −80 °C in storage buffer, (10 sodium mM phosphate, 100 mM NaCl, 10% glycerol 0.01% NP40, pH 6.8) or NMR buffer, (20 mM HEPES, 100 mM NaCl, 0.2 mM EDTA, 5% D_2O, pH 7.2). Label incorporation percentage was determined using a Q-TOF HPLC−MS (Agilent) with electrospray ionization and ranged from 90 to 99%

4FF-KIX. 4FF-KIX was expressed using Protocol 1 with the following exceptions: DL39(DE3) cells were grown in 460 mL of synthetic media reported by Muchmore et al.[43] (referred to here as -F media) containing all 20 amino acids except phenylalanine and supplemented with ampicillin (100 μg/mL) and phenylalanine (110 μM). Cells were grown to an OD of 1.3, (37 °C, 250 rpm). The cells were pelleted, washed 1 time with -F media, and resuspended in 460 mL of -F media plus 4-fluorophenylalanine (125 μM, Sigma-Aldrich) and a minimal amount of phenylalanine (40 μM). The bacteria were left to recover at 37 °C, 250 rpm for 30 min. Protein expression was induced with 0.5 mM IPTG for 3 h at 250 rpm at 37 °C. The His-tagged protein was purified and characterized as described above; 85% 4FF incorporation.

1D ^{19}F NMR. *Parameters Used.* ^{19}F NMR data were acquired at 470 MHz on a Varian 500 spectrometer with a 5 mm PFG OneNMR Probe. Spectra were obtained without proton decoupling. Samples containing 20−55 μM 3FY or 4FF-labeled protein in 20 mM HEPES, 100 mM NaCl, 5% D_2O, pH 7.2 were prepared containing 50 μM sodium trifluoroacetate as a reference (−76.5 ppm) for binding assays. A T_1 value of 0.6 s was obtained by the inversion−recovery method. Measurement parameters included a relaxation delay time of 1 s for small molecules and c-Myb and an acquisition time of 0.3 s. A delay time of 1.5 s and a 0.5 s acquisition time was used in binding experiments with pKID and MLL. All experiments used a 9500 Hz sweep width, 15−20 Hz line-broadening was applied to each spectrum, and 1000−2500 transients were taken for each solution unless otherwise stated.

Ligand Binding Studies. Peptides and small molecules were titrated into the protein solution from a concentrated stock solutions of DMSO (20 mM for peptides and 100 mM for small molecules). Final DMSO concentrations were kept below 0.5% DMSO. For small molecule titrations 2000 scans were acquired to ensure good S/N resolution for improved fitting of the data. However, good chemical shift estimates can be readily acquired at 200−400 scans, 5−10 min for initial screening. A pilot fragment screen was carried out using a concentration of 42 μM 3FY-KIX in the presence of a mixture of 10 small molecule fragments with each molecule at 0.5 mM per sample mixture (5% DMSO). Molecule 1G7 was identified by rescreening small molecules as binary mixtures or as a single compound.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

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Notes

The authors declare no competing financial interest.

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