Supporting Information to accompany

Conserved helix-flanking prolines modulate IDP:target affinity by altering the lifetime of the bound complex

Michael D Crabtree[1], Wade Borcherds[2,3], Anusha Poosapati[2,3], Sarah L Shammas[1,4*], Gary W Daughdrill[2,3*], Jane Clarke[1*]

[1]Department of Chemistry, University of Cambridge, Cambridge, UK, CB2 1EW. [2]Department of Cell Biology, Microbiology and Molecular Biology, University of South Florida, Tampa, Florida, USA. [3]Center for Drug Discovery and Innovation, University of South Florida, Tampa, Florida, USA. [4]Present address: Department of Biochemistry, University of Oxford, Oxford, OX1 3QU, UK. [*]Correspondence should be addressed to: S.L.S. (sarah.shammas@bioch.ox.ac.uk), G.W.D. (gdaughdrill@usf.edu) or J.C. (jc162@cam.ac.uk).

This Supporting Information comprises

Supplementary Materials and Methods

4 Supplementary Figures

4 Supplementary References

Supplementary Materials and Methods

Protein Expression and Purification

The kinase-inducible domain interacting protein (KIX) domain of mouse CBP (Uniprot 45481, residues 586–672) was expressed and purified as described previously[1]. Human p53 (Uniprot P04637, residues 1-73) and human MDM2 (Uniprot Q00987, residues 17-125) were expressed and purified as described previously[2,3].

Protein Expression and Purification for NMR

The synthetic gene for the transactivation domain of mouse MLL (Uniprot P55200, residues 2838 – 2869, with residue C2841 removed), fused with a gene for N-terminally histidine-tagged GB1 with a thrombin cleavage site (between the GB1 and MLL proteins), was obtained from Genscript. The gene was inserted into a modified version of the pRSET A vector. C41 DE3 *Escherichia coli* cells were grown to an OD_{600} of 0.4-0.6 at 37 °C in minimal media containing M9 salts and 100 mg/L ampicillin, with ^{13}C D-glucose and ^{15}N ammonium chloride (Cambridge Isotope Labortories) as the sole carbon and nitrogen sources. Protein expression was induced by addition of 1 mM isopropyl-beta-D-thiogalactopyranoside (IPTG). After incubating for 4 hours at 37 °C, cells were harvested by centrifugation. Cells were re-suspended in PBS, 25 mM imidazole, sonicated and centrifuged.

MLL was purified from the soluble fraction by affinity chromatography using a HisTrap column (GE Healthcare Life Sciences). Bound protein was eluted using a linear gradient of PBS 500 mM imidazole. Protein was diluted 5-fold before loading onto a HiTrap Q HP ion exchange column (GE Healthcare Life Sciences). His-GB1-MLL was separated from truncated versions using a linear gradient of 20 mM Tris 300 mM NaCl. Full length His-GB1-MLL eluted at ~ 200 mM NaCl. His-GB1-MLL was bound to Ni2+ agarose resin and exchanged into 100 mM sodium phosphate pH 7.4. MLL was cleaved from His-GB1 by overnight incubation with 100 units of thrombin. Thrombin was removed from the sample by filtration using a 20 kDa cutoff filter (GE Healthcare Life Sciences), with the flow through containing MLL. The identity and purity of MLL were confirmed by mass spectrometry (Department of Chemistry, University of Cambridge, UK) and amino acid analysis (Department of Biochemistry, University of Cambridge, UK).

Peptide Dye Labelling

Fluorescent labelling of p53 peptides by maleimide chemistry was achieved by substitution of a cysteine residue into the peptide sequence (D7C). This residue is N-terminal of the binding segment. During peptide purification DTT was used rather than beta-mercaptoethanol to avoid formation of adducts, and the N-terminal his-tag used for purification purposes was left attached to the peptide until after labelling to minimize aggregation problems. Following purification, the peptide was buffer exchanged into 50 mM tris pH 7.0 300 mM sodium chloride with 1 mM EDTA and frozen for storage. Peptide solution was diluted to 80 μM and reduced by incubation with 2 mM TCEP for 30 minutes at 37 °C. Alexa Fluor® 594 C5-maleimide, dissolved in DMSO at 11 mM concentration, was added dropwise to the solution to a final concentration of 110 μM, and labeling allowed to proceed for 4 hours under gentle agitation at room temperature. The labelled peptide solution was introduced to a 5 ml HisTrap column (GE Healthcare), which was then washed to remove free dye from the solution with p53 biophysical buffer. The peptide was retrieved from the column by incubation with 50 units of thrombin for 20 minutes to cleave the p53 peptide from the immobilized his-tag and subsequent elution with p53 biophysical buffer. Finally, thrombin was removed from the sample by filtration using a 20 kDa cutoff filter (GE Healthcare Life Sciences). Peptide purity was confirmed by mass spectrometry (Department of Chemistry, University of Cambridge, UK) and by SDS PAGE.

Concentration Determinations

Extinction coefficients of 12,009, 11,000 and 10,400 M^{-1} cm^{-1} at 280 nm were used for KIX, p53 and MDM2, respectively. Extinction coefficients of 92,000 M^{-1} cm^{-1} at 593 nm and 75,000 M^{-1} cm^{-1} at 490 nm were used for Alexa594 and FITC labelled peptides, respectively. Concentrations of unlabeled MLL peptides were determined from amino acid analysis (Department of Biochemistry, University of Cambridge, UK). Stock solutions were diluted by weight to ensure accurate concentrations in experiments.

Circular Dichroism

Circular dichroism scans were performed using a Chirascan CD spectrometer (Applied Photophysics). Experiments were performed at 5 °C for MLL using 1 or 2 mm path length cuvettes. The measured CD signal (mdeg) was converted to mean residual ellipticity, MRE (deg cm^2 $dmol^{-1}$). Helicity estimates were calculated using the method of Muñoz and Serrano[4]. For mixtures, equal concentrations of peptide and protein were mixed in a 1:1 ratio. The expected spectrum for the mixture in the absence of any structural change, was calculated from the mean average of the two individual spectra. Differential spectra were obtained by subtraction of the predicted from the measured spectra. The estimated concentration of complex ([MLL:KIX]) was calculated using Equation S1. To facilitate comparison of degree of structure gained during complex formation, the difference spectrum presented is per M of complex formed.

$$[\mathrm{MLL:KIX}] = \frac{\left([\mathrm{MLL}] + [\mathrm{KIX}] + K_d - \sqrt{(([\mathrm{MLL}] + [\mathrm{KIX}] + K_d)^2 - 4[\mathrm{MLL}][\mathrm{KIX}])}\right)}{2[\mathrm{MLL}]} \qquad \text{(S1)}$$

where [MLL] and [KIX] are the concentration of MLL and KIX, respectively, and K_d is the equilibrium dissociation constant for the interaction between MLL and KIX.

Equilibrium Binding Affinities

FITC labeled MLL (1 μM) was incubated with various concentrations of KIX. Sample temperature was maintained at 5 °C for MLL experiments. Anisotropy measurements were obtained using a Cary Eclipse Spectrophotometer (Varian) and fluorescence polarization accessory. Excitation and emission wavelengths for MLL peptides were 493 and 515 nm, respectively. Slit widths for excitation and emission were 5 and 10 nm, respectively. Anisotropy was calculated from fluorescence intensity measurements, I_{VV} and I_{VH}, and fit to obtain an estimate of the dissociation constant (K_d), as described previously[1].

p53: MEEPQSDPSVE**PP**LSQETFSDLWKLL**P**ENNVLSPLPSQAMDDLMLSPDDIEQWFTEDPGPDEAPRMPEAAPRV

Mdm2: SQIPASEQETLVRPKPLLLKLLKSVGAQKDTYTMKEVLFYLGQYIMTKRLYDEKQQHIVYCSNDLLGDLFG
VPSFSVKEHRKIYTMIYRNLVVVNQQESSDSGTSVSEN

MLL: (G)SDDGNIL**P**SDIMDFVLKNT**P**SMQALGESPES

KIX: (GS)GVRKGWHEHVTQDLRSHLVHKLVQAIFPTPDPAALKDRRMENLVAYAKKVEGDMYESANSRDEYY
HLLAEKIYKIQKELEEKRRSRL

Figure S1. Sequences for the proteins and peptides used in this study.

p53: Uniprot P04637, residues 1-73. Mdm2: Uniprot Q00987, residues 17-125. MLL: Uniprot Q03164, residues 2838 – 2869, with C2841 removed. KIX: Uniprot 45481, residues 586–672. Residues in brackets are remnants of protease cleavage and do not form part of the natural sequence. The helix-flanking prolines investigated in this work are shown in cyan.

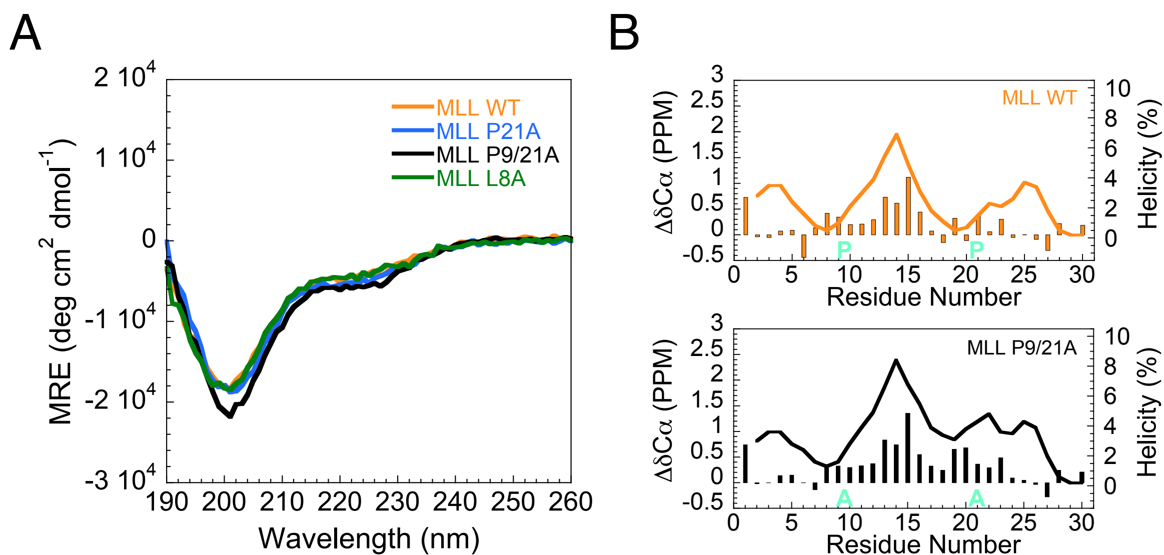

Figure S2. Helix-flanking prolines modulate residual structure in MLL.

(A) CD spectra for MLL peptides. Spectra are the average of three independent experiments.

(B) Cα random coil chemical shift deviations (ΔδCα, bars) and percentage helicity estimates (line), as determined with the d2D method. The position and identity of helix-flanking residues investigated in this work are shown in cyan.

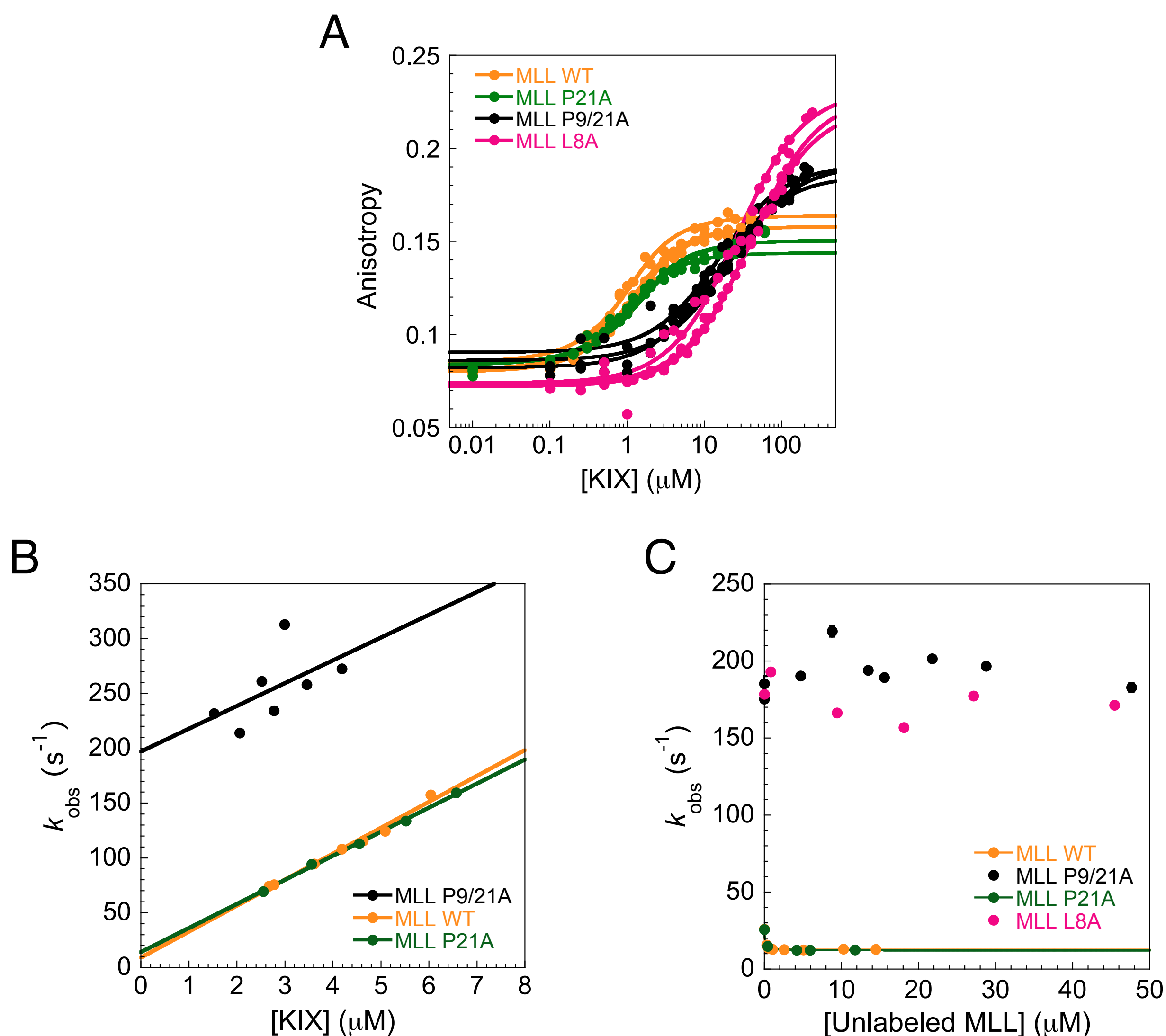

Figure S3. Thermodynamic and kinetic characterization of the interaction between MLL and KIX. (A) Equilibrium titration curves. Three independent curves for each peptide are shown. (B) Observed association rate constants obtained under pseudo-first order conditions, with KIX in excess. The association rate constant (k_{on}) is given by the gradient of the straight-line fit. (C) Observed dissociation rate constants, obtained from competition studies. The dissociation rate constant is given by the asymptote for MLL WT and P21A, and given by the average observed rate constants obtained when mixing with competitor, for MLL P9/20A and L8A.

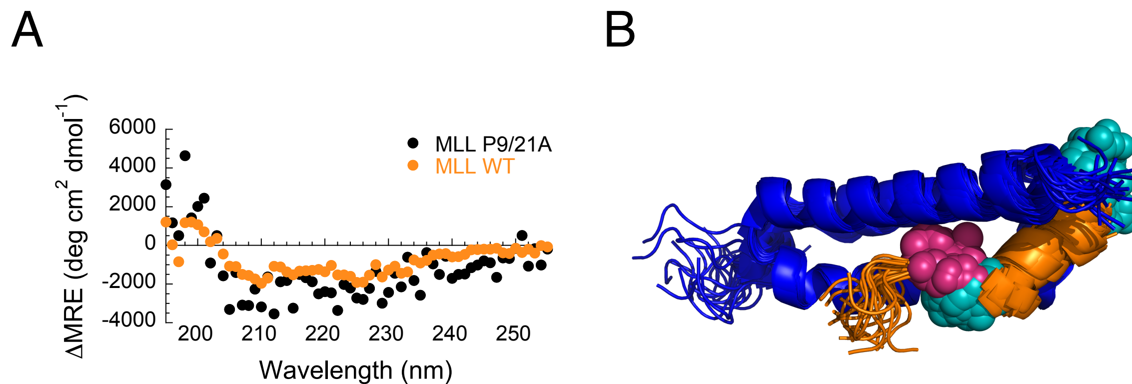

Figure S4. Structure of the MLL:KIX complex.

(A) The difference in MRE between the mean of free KIX and free MLL, and the MRE obtained when the two are mixed, as described in the Supplementary Methods. Spectra are corrected for the proportion of MLL in complex, therefore indicating the change in CD spectrum if all MLL was bound to KIX. (B) 20 lowest energy NMR structures of the MLL:KIX complex (PDB:2LXS, MLL in orange). Helix-flanking prolines are shown as cyan spheres. L8 is shown as a pink sphere.

Supplementary References

(1) Shammas, S. S. L., Travis, A. A. J., and Clarke, J. (2013) Remarkably fast coupled folding and binding of the intrinsically disordered transactivation domain of cMyb to CBP KIX. *J. Phys. Chem. B 117*, 13346–56.

(2) Borcherds, W., Theillet, F.-X., Katzer, A., Finzel, A., Mishall, K. M., Powell, A. T., Wu, H., Manieri, W., Dieterich, C., Selenko, P., Loewer, A., and Daughdrill, G. W. (2014) Disorder and residual helicity alter p53-Mdm2 binding affinity and signaling in cells. *Nat. Chem. Biol. 10*, 1000–2.

(3) Vise, P. D., Baral, B., Latos, A. J., and Daughdrill, G. W. (2005) NMR chemical shift and relaxation measurements provide evidence for the coupled folding and binding of the p53 transactivation domain. *Nucleic Acids Res. 33*, 2061–77.

(4) Muñoz, V., and Serrano, L. (1995) Elucidating the folding problem of helical peptides using empirical parameters. III. Temperature and pH dependence. *J. Mol. Biol. 245*, 297–308.