

# Supporting Information

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## SI Text

**Kinase Expression and Purification.** p38 $\alpha$  MAPK and PRAK kinase were expressed with His<sub>6</sub>-fusion tags in *Escherichia coli* BL21 (DE3) [DNAY] cells. Cells were grown at 37 °C and overexpression was induced at an OD<sub>600</sub> of 0.7 by addition of 1 mM IPTG, at 15 °C for ~16 h. The cells were lysed in buffer containing 50 mM Tris pH 8, 200 mM NaCl, 1% Triton, 10 mM imidazole, 5 mM  $\beta$ -mercaptoethanol, 0.1 mM PMSF, and EDTA-free protease inhibitor tablets (Roche, 1 tablet per 50 mL). The crude lysate was centrifuged and the supernatant loaded onto a Ni-NTA (Qiagen) column equilibrated with lysis buffer. The column was washed with 50 mM Tris buffer (pH 8, containing 200 mM NaCl, 0.1% Tween 20, 20 mM imidazole, 5 mM  $\beta$ -mercaptoethanol, 0.1 mM PMSF), and the kinases were eluted with two column volumes of elution buffer (washing buffer with 250 mM imidazole). The proteins were dialyzed into buffer containing 10 mM Tris, pH 8, and 5 mM  $\beta$ -mercaptoethanol and stored on ice until use.

**Phosphorylation of p53 Using p38 $\alpha$  MAPK and PRAK.** Kinase reactions were performed on a 1-mL to 10-mL scale using ~250  $\mu$ M p53 transcriptional activation domain (TAD) and 10  $\mu$ M–50  $\mu$ M of kinase in 50 mM Hepes pH 7.5, 0.1 mM PMSF, 1 mM NaF, 0.1 mM NaVO<sub>4</sub>, 0.1 mM  $\beta$ -glycerol phosphate, 10 mM MgCl<sub>2</sub>, 1 mM DTT, and 10 mM ATP. p38 $\alpha$  was used to phosphorylate Ser33 and Ser46, PRAK to phosphorylate Ser37, and the p53 TAD was incubated with both kinases to obtain the triply phosphorylated peptide. Reaction mixtures were incubated overnight at 30 °C, and the phosphorylated peptides were purified by reverse phase HPLC using a C18 column. The molecular weights were confirmed by MALDI-TOF and incorporation of the phosphate group at the correct site was verified by NMR using <sup>15</sup>N-labeled samples and observing the chemical shifts of phosphorylated residues.

**Measurement of Dissociation Constants.** Competition fluorescence anisotropy was used to measure  $K_d$  for TAZ1 and TAZ2 complexes to avoid complexities arising from the presence of a weak secondary binding site (1). <sup>1</sup>H-<sup>15</sup>N HSQC chemical shift titrations were used to monitor binding to KIX and NCBP, rather than isothermal titration calorimetry (ITC) as in our previous work (1), because some of the phosphorylated p53 peptides exhibit extremely small heat changes upon binding, making it difficult to determine accurate  $K_d$  values. Use of fluorescence anisotropy to measure  $K_d$  for the KIX and NCBP domains was impractical because of the large quantity of phosphorylated p53 peptides needed to compete out the labeled p53 TAD due to the low binding affinity.

**Determination of  $K_d$  by Fluorescence Anisotropy.** Fluorescence spectra were recorded with 594-nm excitation and 620-nm emission wavelengths. A competition titration method was used, in which dye-labeled p53(13-61)D57C (designated as ligand L1) bound to TAZ1 or TAZ2 ( $M$ ) was competed out by titration with unlabeled, phosphorylated p53 peptide ( $L2$ ). The concentrations of total bound and unbound L1 and  $M$ , i.e.,  $[L1_T]$  and  $[M_T]$ , were kept constant. Changes in dye fluorescence anisotropy were monitored as a function of  $[L2_T]$  using an ISS-PC1 photon-counting steady-state spectrofluorometer. The competition ligand binding data were analyzed by nonlinear least squares fitting in Mathematica (Wolfram Research, Inc.) using the following equation:

$$Y = (\gamma Y_{ML1} + Y_{L1}) / (1 + \gamma),$$

where  $Y$  is the observed fluorescence anisotropy of the bound ( $ML1$ ) and unbound ( $L1$ ) dye-labeled p53, and  $\gamma = ([ML1]) / ([L1_T] - [ML1])$ . As previously described (2),  $[ML1]$  is given by

$$[ML1] = ([L1_T] \{ 2\sqrt{a^2 - 3b \cos(\theta/3)} - a \}) / (3K_{L1} + \{ 2\sqrt{a^2 - 3b \cos(\theta/3)} - a \}),$$

where  $\theta = \arccos(-2a^3 + 9ab - 27c) / (2\sqrt{(a^2 - 3b)^3})$ ,  $a = K_{L1} + K_{L2} + [L1_T] + [L2_T] - [M_T]$ ,  $b = K_{L1}K_{L2} + K_{L1}([L2_T] - [M_T]) + K_{L2}([L1_T] - [M_T])$ ,  $c = -[M_T]K_{L1}K_{L2}$ , and  $K_{L1}$  and  $K_{L2}$  are the dissociation constants for binding of ligands L1 and L2, respectively. Values of  $Y_{ML1}$  were obtained experimentally to reduce the number of fitting parameters,  $Y_{L1}$  and  $K_{L2}$  were fit, and  $K_{L1}$  values were determined through separate titrations of labeled p53 with TAZ2 or TAZ1. Single-ligand titration data were analyzed using the model described previously (1). The  $K_{L1}$  values for binding of dye-labeled p53(13-61)D57C to TAZ2 and TAZ1 were 8.5 nM and 190 nM, respectively. These dissociation constants are smaller than determined previously by NMR (1) for binding of p53(13-61), suggesting that the fluorescent dye contributes to binding; however, because dissociation constants for the phosphorylated peptides were determined by competition, the dye interaction does not influence the measured  $K_d$  values in Table 1. For the competition experiments, unlabeled p53 peptides with various phosphorylation patterns were titrated into 25 nM dye-labeled p53 and 700 nM TAZ2 and changes in fluorescence anisotropy were recorded. A similar procedure was used for TAZ1, with 50 nM labeled p53 and 3  $\mu$ M TAZ1.

1. Ferreón JC, et al. (2009) Cooperative regulation of p53 by modulation of ternary complex formation with CBP/p300 and HDM2. *Proc Natl Acad Sci USA* 106:6591–6596.

2. Wang ZX (1995) An exact mathematical expression for describing competitive binding of two different ligands to a protein molecule. *FEBS Lett* 360:111–114.