Electronic Supplementary Information:

Functional phosphosite screening for targeted protein-protein interactions by combining phosphoproteomics strategies and mammalian two-hybrid assays

Mingquan Guo,[a,¶] Zanxian Xia,[a,¶] and Hong Ma [b*]

[a] Keck School of Medicine, University of Southern California, 1501 San Pablo St., Los Angeles, CA 90033
Tel: 626-500-8387. Fax: 425-427-0071. E-mail: mingquag@usc.edu
[b] Children's Hospital Los Angeles, 4650 West Sunset Boulevard, Los Angeles, CA 90027, USA
Tel: 425-503-8502. Fax: 425-427-0071. E-mail: hma@chla.usc.edu

Additional experimental details are **included** in this Supplementary Information.

1. Identification of phosphorylation sites from N-terminus of p300 and CBP by LC-MS/MS

Gel slices containing proteins of interest were subjected to trypsin digestion. One part of the tryptic digests was used to map the protein sequence before phosphopeptide enrichment by nano-LC-MS/MS. The remainder was diluted with loading solution (300 mg/ml lactic acid in 50% ACN and 2% TFA),[1] and absorbed onto the TiO_2 micro-column after equilibration of the TiO_2 micro-column with 10 μl of the loading solution. Two washes of the micro-column were performed with 10 μl of the loading solution, followed by an additional wash with 10 μl of 0.1% TFA in 50% ACN. The retained peptides were eluted with 10 μl of elution buffer (0.5% NH_4OH) and immediately acidified with formic acid for subsequent nano-LC-MS/MS analysis. The identification of phosphorylation sites was performed using LTQ XL ETD mass spectrometer (Thermo Fisher Scientific, CA).

Peptide mixtures were separated and analyzed by nano-LC–MS/MS. The nano-LC system was equipped with an auto-sampler, and a binary nano-LC pump (nano-LC 2D, Eksigent Technologies, Livermore, CA). Reversed-phase chromatography was performed by loading peptides using an automated sampler into two fused-silica capillary columns in tandem: a pre-column (OD 360μm, ID 300μm, 5 mm in length) packed with 5 micrometer C18 (LC Packing, Sunnyvale, CA), followed by an analytical column (OD 360μm, ID 75μm, 10cm in length) packed with 5 micrometer reversed-phase C18 packing material (New Objective, Woburn, MA). Columns were washed with 95% mobile phase A (0.1% formic acid in H_2O [v/v]) and 5% mobile phase B (98% ACN and 0.1% formic acid [v/v]). Subsequently, separation of peptides was carried out using a linear gradient elution from 5% mobile phase B to 60% mobile phase B in 55 min. Eluting peptides were analyzed with an ion trap hybrid mass spectrometer (LTQ XL ETD, Thermo Fisher Scientific). Source settings were as follows: spray voltage, 1.5 kV; capillary voltage, 35 V; ion transfer tube temperature, 200^{o}C. The mass spectrometer was operated in the data-dependent mode to automatically switch between MS and ion trap–MS/MS acquisition. Survey full-scan MS spectra (m/z 350–2000) were acquired. The five most intense ions were sequentially isolated for fragmentation in the linear ion trap using CID with a normalized collision energy setting of 35.0 or using ETD. All spectra were obtained in the positive ion mode.

Data acquired with the LTQ XL ETD mass spectrometer were searched against a custom made IPI database (ipi.Human.v3.18.fasta, containing 62000

entries and the sequences of p300/CBP (1-111) using Bioworks Software version 3.3). Search parameters included two missed cleavages from trypsin proteolysis and variable modifications including deamidation (N and Q), oxidation (M), and phosphorylation (S, T, and Y). The resultant CID spectra were filtered using the scores of Xcorr (1+ precursor ion >1.5, 2+ >2, and 3+ and above >2.5) with Delta CN over 0.08. The resultant ETD spectra were filtered using the scores of Sf over 0.75. All peptides with high-quality tandem mass spectra, were manually interpreted and confirmed. All tandem mass spectra were manually verified to assign the sequence and phosphorylation sites for all of the phosphopeptides identified in this study.

2. Purification of N-terminal fragments of recombinant p300 and CBP

Monolayer C2C12 cells cultured in the DMEM medium (Invitrogen, Carlsbad CA, supplemented with 10% FBS and 4.5g/L glucose) at ~70% confluence were transfected with plasmid expressing $p300^{1-111}$ or CBP^{1-111} fragment with BioT reagent (Bioland Scientific, Cerritos, CA) according to the manufacture's specification.[2] Approximately 48 hours later, cells were harvested and lysed on ice using M-PER Protein Extraction Reagent (Thermo Scientific, Rockford IL) in the presence of a protease inhibitor cocktail and phosphatase inhibitors I and II (Sigma-Aldrich, St. Louis, MO). Two-step affinity purification was performed as follows: at first, the cleared lysates were incubated with Ni-NTA beads (Qiagen Inc., Valencia CA) for 1 hr at 4 ^{o}C, and after wash, the purified proteins were eluted by 250 mM imidazole in a buffer (50 mM NaH_2PO_4, 300 mM NaCl, 10% glycerol, pH8.0); then, the eluates were further incubated with S-protein agarose

(EMD Chemicals Inc., Gibbstown, NJ) for 2 hr at 4^{o}C. The bound proteins were eluted by SDS-PAGE loading buffer and loaded to SDS-PAGE 4-20% precast gel (Invitrogen). Then silver staining was carried out by using the Silver Staining Kit (Invitrogen). Bands of interest were cut for mass spectrometry analysis.

3. Site-directed mutagenesis and mammalian two-hybrid assay for protein-protein interaction

Site-directed mutagenesis was performed for Ser12, Ser89 and Ser20/50/90 of p300 or for Ser77, Ser79 and Ser92 of CBP to generate corresponding Alanine mutants with QuikChange II Site-Directed Mutagenesis Kits (Agilent Technologies, Santa Clara, CA) following the manufacture's specification.

Interaction of p300 or CBP with β-catenin was determined using a commercial available mammalian two-hybrid reporter system (Promega, Madison, WI). The full-length human β-catenin fused with a VP16 tag at the C-terminus as "bait" was constructed into the pAct vector, producing pAct-β-catenin-VP16. Similarly, human p300 (1-662 residues) or CBP (1-682 residues) fragments which encompasses the N-terminus (1-111), CH1 and KIX domains as "prey" were cloned to the pBind vectors, fusing to a GAL4 tag, yielding pBind-p300-GAL4 or pBind-CBP-GAL4. The luciferase assay was performed according to the manufacture's recommendation. Briefly, each well (24-well plate) seeded with C2C12 cells at 70% confluence was cotransfected with equal amount (0.5 μg/well) of pAct-β-catenin-VP16, pBind-p300-GAL4 or pBind-CBP-GAL4, and the reporter plasmid pG5-Luc using Superfect transfection reagent (Qiagen Inc., Valencia, CA). To eliminate the trans-activation signal caused by p300 or CBP

fragment alone, interaction controls were included for each experiment by cotransfecting cells in the same manner except for replacing pAct-β-catenin-VP16 with empty pAct-VP16 plasmid. Approximately 48 hours after each transfection, the luciferase activity (*Firefly*) was detected and normalized by *Renilla* activity. Therefore, the degree of p300/β-catenin or CBP/β-catenin interaction was judged after subtracting the normalized value of the corresponding interaction control.

4. Immunoprecipitation and Western blotting

Endogenous interactions of p300 or CBP (and their mutants) with β-catenin were demonstrated by immunoprecipitating p300 or CBP from nuclear protein fraction (200 μg/sample) following the procedures previously described by Ma et al.[3] The immunocomplexes were separated on SDS-PAGE 4-20% gradient precast gel, transferred to Immobilon-P (PVDF) (Millipore, Bedford, MA), and immunoblotted with mouse monoclonal β-catenin (Santa Cruz Biotechnology, CA). The membranes were developed using ECL system (Amersham Pharmacia Biotech, UK). For Western blot analyses, whole cell lysates, cytosolic and nuclear fractions were prepared by using M-PER Protein Extraction Reagents (Thermo Scientific) and loaded equally (15-20 μg/well depending upon experiments). To ensure no contamination between the cytosolic and nuclear fractions, each sample was carefully examined for the presence of cytosolic (β-actin or α-tubulin) or nuclear markers (lamin A/C) (all antibodies from Santa Cruz Biotechnology, Santa Cruz, CA).

1. N. Sugiyama, T. Masuda, K. Shinoda, A. Nakamura, M. Tomita and Y. Ishihama, *Mol. Cell. Proteomics*, 2007, **6**, 1103.
2. K. H. Emami, C. Nguyen, H. Ma, D. H. Kim, K. W. Jeong, M. Eguchi, R. T. Moon, J. L. Teo, H. Y. Kim, S. H. Moon, J. R. Ha and M. Kahn, *Proc. Natl. Acad. Sci. U. S. A.*, 2004, **101**, 12682.
3. H. Ma, C. Nguyen, K. S. Lee and M. Kahn, *Oncogene*, 2005, **24**, 3619.