Identification of Upstream Kinases by Fluorescence Complementation Mass Spectrometry

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Supporting Information

ABSTRACT: Protein kinases and their substrates comprise extensive signaling networks that regulate many diverse cellular functions. However, methods and techniques to systematically identify kinases directly responsible for specific phosphorylation events have remained elusive. Here we describe a novel proteomic strategy termed fluorescence complementation mass spectrometry (FCMS) to identify kinase–substrate pairs in high throughput. The FCMS strategy employs a specific substrate and a kinase library, both of which are fused with fluorescence complemented protein fragments. Transient and weak kinase–substrate interactions in living cells are stabilized by the association of fluorescence protein fragments. These kinase–substrate pairs are then isolated with high specificity and are identified and quantified by LC–MS. FCMS was applied to the identification of both known and novel kinases of the transcription factor, cAMP response element-binding protein (CREB). Novel CREB kinases were validated by in vitro kinase assays, and the phosphorylation sites were unambiguously located. These results uncovered possible new roles for CREB in multiple important signaling pathways and demonstrated the great potential of this new proteomic strategy.

INTRODUCTION

Protein kinase activities determine the phenotypes of all cells including cancer cells.† To dissect cellular signaling pathways, it is critical to identify direct relationships between kinases and their substrates. To date, several systems biology approaches have been applied to identify kinase substrates on a large scale.2−4 However, there are few high throughput methods available to identify, given a specific substrate, the upstream kinases that phosphorylate it and regulate its activity. Attempts have been made to apply affinity pull-down methods in which an immobilized substrate is used to adsorb potential interacting kinases from cell lysates.5,6 However, this strategy has considerable limitations because it is well established that many interactions between kinases and their substrates are weak and transient. Shokat and co-workers have devised clever substrate-trapping methods to convert transient enzyme–substrate interactions into covalent complexes.7,8 However, this technique has been demonstrated only with a model peptide as the substrate and has not been applied to the discovery of novel upstream kinases for their natural substrates in living cells. To date, the upstream protein kinases directly responsible for thousands of phosphorylation events known from the phosphoproteome remain to be discovered.

Here we present a general proteomic strategy, termed fluorescence complementation mass spectrometry (FCMS), to identify the upstream kinases of a given phosphoprotein. This approach uses fluorescent protein fragments as originally developed for the bimolecular fluorescence complementation (BiFC) assay. BiFC is widely used for the visualization of protein–protein interactions in living cells.9 In this technique, a fluorescent protein is split into two fragments, each of which is fused to one of two putative interacting proteins, which are then coexpressed in cells. Once the proteins interact, the fluorescent protein fragments are brought together to form a complex that emits fluorescence. Once formed, this fluorescent complex is stable in vivo, which makes BiFC a unique method to study transient or weak protein–protein interactions.10,11 Here, we selected the fluorescent protein Venus and split it into N-terminal (VN) and C-terminal (VC) fragments, which were fused to a substrate and to a library of kinases, respectively. The substrate and kinases were coexpressed in mammalian cells. Any transient interaction between a kinase and its substrates will be stabilized by the association of the fluorescent protein...
fragments. Instead of detection of fluorescence signal and dependence on fluorescence-activated cell sorting (FACS) in BiFC, FCMS specifically isolates kinase−substrate pairs for mass spectrometric analyses to identify, in a single experiment, multiple kinases capable of interacting with a single substrate.

We chose the transcription factor cAMP response element-binding protein (CREB) as a model substrate for the identification of its upstream kinases. CREB can be phosphorylated on multiple sites, and its activity is highly regulated by several known upstream kinases. CREB binds to specific DNA sequences called cAMP response elements (CRE) and, once phosphorylated, interacts with coactivators to alter gene transcription. Although several protein kinases are known to participate in the stimulus-induced phosphorylation of CREB, evidence for direct in vivo interactions between these kinases and CREB is often lacking. Furthermore, the complete repertoire of kinases capable of phosphorylating and regulating CREB activity is unknown. Given the important roles of CREB-mediated gene transcription in multiple disease states including neuropsychiatric disorders and cancer, this is an important question to explore. Finally, as a transcription factor, CREB lies at the base of multiple cellular signaling cascades, which allows us to study its upstream kinases without interference from downstream kinases.

## RESULTS

The flowchart of fluorescence complementation mass spectrometry (FCMS) for the identification of upstream kinases is shown in Figure 1. To screen for potential upstream kinases, we constructed an expression library that potentially expresses 559 human kinases fused to the Venus C-terminal fragment VC. We devised two strategies to improve the specificity. First, a mutant or truncated substrate that does not interact with the kinases is used as a control, allowing stable isotope labeling based on amino acids in cell culture (SILAC) to be applied to quantitatively measure the interaction of kinases with the wild-type versus the mutated or truncated substrate. Second, GFP nanobody, which recognizes only the intact VN−VC complex and does not bind either VN or VC fragment alone, was used to purify the kinase−substrate complexes for mass spectrometric analyses.

Figure 1. Flowchart of FCMS to identify upstream kinases. Wild type substrate (SUB) or mutated substrate (mSUB) in BiFC vector Myc-VN155 and human kinase cDNA expression library in HA-VC155 vector are cotransfected to different SILAC cells. Cells are combined, and protein complexes are immunoprecipitated with GFP nanobody. Proteins on beads are reduced, alkylated, and tryptic digested, and the resulting peptide samples are analyzed by LC−MS for both protein identification and quantitation.
**Figure 2.** CREB fragment generation and validation. (a) CREB sequence structure and the constitution of CREB truncates. The glutamine rich domains Q1 and Q2 and the kinase-inducible domain (KID) constitute the transcription activation domain of CREB, while the basic region and the leucine zipper domain form the dimerization and DNA-binding region of the protein. Two truncated CREB fragments are Q1-KID (Q1K) and Q2-Basic domain-Leucine zipper (Q2L). Both were cloned into BiFC vector Myc-VN155 separately. (b) Expression of CREB mutants. Expression of Myc-Q1K-VN155 and Myc-Q2L-VN155 in 293T cells was examined with anti-Myc antibody. Note that Myc-Q1K-VN155 is 39 kDa and Myc-Q2L-VN155 is 41 kDa. (c) Fluorescence microscope imaging of BiFC assay of Myc-Q1K-VN or Myc-Q2L-VN cotransfected with HA-PKA-VC. (d) Western blotting against anti-Flag and anti-HA showed that when Flag-CREB-VN or HA-PKA-VC expressed alone, GFP nanobody did not capture either of them but instead captured the VN-VC complex.

**Specific Isolation of Kinase–Substrate Complexes.** In a typical BiFC experiment, the formation of a BiFC complex is typically quantified by measuring the fluorescence intensities of the complexes and of the intact fluorescent protein in the same cells using either fluorescence microscopy or flow cytometry. The ratio of the fluorescence intensities can be used to confirm the formation of specific BiFC complexes. However, such BiFC-based screening requires the use of fluorescence-activated cell sorting (FACS) to isolate highly homogeneous cells in which BiFC complexes have formed. This procedure is laborious and not compatible with MS-based experiments due to the limited quantity of material that can be isolated via FACS. We sought to develop a highly specific method to capture kinase–substrate complexes for MS analyses. After screening multiple anti-GFP antibodies,18 we identified an engineered single-chain anti-GFP antibody, GFP nanobody, that specifically recognized BiFC complexes, but not individual fragments. GFP nanobody (or nanotrap) was originally developed to isolate GFP, YFP, Venus, and Citrine with high affinity.17 Compared with traditional antibodies, GFP nanobody has a smaller size and higher affinity and can survive in harsh conditions such as high salt, low/high pH, and high temperature.19 To demonstrate its specificity, we expressed Flag-CREB-VN155/HA-PKA-VC155 and Flag-CREB-VN155/HA-PKA-VC155 in the cells separately and together. Then protein complexes were immunoprecipitated with GFP nanobody and analyzed by both Western blotting and MS. Both results indicated that the GFP nanobody captured fusion proteins.
proteins only when the VN−VC fragments of Venus had reassociated (Figure 2d). Since the GFP nanobody-based isolation is based on a high affinity interaction,19 we applied stringent washes to reduce nonspecific binding while preserving specific interactions. Different stringent washing conditions were examined. Western blotting and mass spectrometric analyses were applied to identify the optimum washing condition that includes the use of RIPA buffer, 5 M NaCl, and 500 mM glycine (pH 4.0) to remove nonspecific bindings while preserving VN−VC complexes. We reported the initial results in 2014,18 and high specificity of GFP nanobody has been also reported recently by a separate research group to identify interacting proteins of the epidermal growth factor receptor (EGFR) family member ERBB2.20

Identification of CREB Kinases. We then applied FCMS to identify the upstream kinases interacting with CREB. Myc-Q1K-VN155 and the kinase library were transfected into SILAC heavy cells, while Myc-Q2L-VN155 and the library were transfected into SILAC light cells. After protein expression, cells were observed under fluorescence microscopy. As we expected, Myc-Q1K-VN155 interacted with kinases, leading to the emission of a fluorescence signal, while any fluorescence signal from Myc-Q2L-VN155 expressing cells was too weak to be visible. Cells were harvested and lysed, and the GFP nanobody was used to isolate CREB−kinase complexes. The Western blot result was consistent with fluorescence imaging, showing the formation of VN−VC complexes primarily in Q1K-containing cells. Direct on-bead digestion with trypsin was performed to recover peptides for LC−MS analysis. Three biological replicate experiments were conducted for reciprocal SILAC experiments. The SILAC result, shown in Figure 3a, revealed that the ratio of the majority of endogenous proteins centers to 1:1 (i.e., they are found at equivalent levels in the heavy- and light-isotope labeled samples) while the ratios of kinases deviated greatly from 1:1. Kinases that prefer binding with Q1K rather than with Q2L are potential CREB-interacting kinases. The protein ratios of the reciprocal SILAC experiments were clustered to generate a heat map (Figure 3b). At the bottom of the heat map, proteins have higher ratios in group 1 (heavy cells with Myc-Q1K-VN, light cells with Myc-Q2L-VN), and lower ratios in group 2 (heavy cells with Myc-Q2L-VN, light cells with Myc-Q1K-VN). The majority of kinases were found in this area, which is consistent with our expectation that this method can identify specific interacting CREB kinases. One-tailed one sample t tests were conducted for each triplicate experiment. Kinases with p < 0.01 and an abundance change of at least 4-fold were chosen as the candidates with highest confidence (Figure 3c). From overlapping results of 3 reciprocal SILAC experiments, 23 protein kinases were identified (Table 1). Among the candidate protein kinases, 7 are known CREB kinases, representing almost half of all previously known CREB kinases identified in different systems and cellular states. These 7 known CREB kinases are cAMP-dependent protein kinase catalytic subunit alpha (PRKACA),21 LIM domain kinase 1 (LIMK1),22 calcium/calmodulin-dependent protein kinase type 1 (CaMKI),23 ribosomal protein S6 kinase (RPS6KA1),24 protein kinase D1 (PRKD1),25 glycogen synthase kinase-3 alpha (GSK3A),26 and S′ AMP-activated protein kinase (AMPK).27,28 Several different subunits of AMPK were found in reciprocal SILAC lists.

We selected six kinases, brain-selective kinase 2 (BRSK2), mitogen-activated protein kinase kinase 1 (MAP4K1), PIM2, cyclin-dependent protein kinase 3 (CDK3), cyclin-dependent protein kinase 4 (CDK4) and cyclin-dependent protein kinase 6 (CDK6), all of which were reported to be associated with CREB but never implicated as...
In living cells by stabilizing and capturing the kinase to thousands of sites of protein phosphorylation, there is a lack of many important biological events including the cataloging of proteome-wide studies. Although CDK3 phosphorylated CREB on S133, BRSK2 phosphorylated CREB on S98 while PIM2 and CDK3 phosphorylated CREB. Consequently, we generated CREB phosphorylation sites. The autoradiography detection showed that, after S98A mutation, BRSK2 did not phosphorylate CREB; on the other hand, after mutating 133S to 133A, PIM2 and CDK3 could not phosphorylate CREB.

### Table 1. Identified Candidate Upstream Kinases of CREB

| gene name  | protein description                                      | previous knowledge                  |
|------------|----------------------------------------------------------|-------------------------------------|
| CDK3       | cyclin-dependent kinase 3                                 | regulates same family member of CREB |
| STK24      | serine/threonine-protein kinase 24                       |                                     |
| MAPK1      | mitogen-activated protein kinase 1                        | in the same pathway with CREB       |
| PHK        | phosphorylase b kinase                                   | in the same pathway with CREB       |
| CDK6       | cyclin-dependent kinase 6                                 | in the same pathway with CREB       |
| BRSK2      | serine/threonine-protein kinase BRSK2                     | in the same pathway with CREB       |
| TSSK2      | testis-specific serine/threonine-protein kinase 2         | its family member TTK2 is a CREB upstream kinase |
| RPS6KL1    | ribosomal protein S6 kinase like 1                        |                                     |
| TNNI3K     | serine/threonine-protein kinase TNNI3K                   |                                     |
| PHKB       | phosphorylase b kinase regulatory subunit beta            |                                     |
| CAMK1      | calcium/calmodulin-dependent protein kinase type 1        | CREB upstream kinase                |
| CDK4       | cyclin-dependent kinase 4                                 | in the same pathway with CREB       |
| RP56KA1 (p90RSK) | ribosomal protein S6 kinase | CREB upstream kinase                |
| LIMK1      | LIM domain kinase 1                                        | CREB upstream kinase                |
| CHEK2      | serine/threonine-protein kinase Chk2                      |                                     |
| MAP2K2     | dual specificity mitogen-activated protein kinase 2       | in the same pathway with CREB       |
| PRKACA     | cAMP-dependent protein kinase catalytic subunit alpha     | CREB upstream kinase                |
| EEF2K      | eukaryotic elongation factor 2 kinase                     | is a substrate of CREB kinase p90RSK|
| MAP4K1     | mitogen-activated protein kinase kinase kinase kinase     | in the same pathway with CREB       |
| PRKD1      | serine/threonine-protein kinase kinase kinase kinase      | CREB upstream kinase                |
| PIM2       | serine/threonine-protein kinase pim-2                     | in the same pathway with CREB       |
| PRKY       | putative serine/threonine-protein kinase PRKY             |                                     |
| GSK3a      | glycogen synthase kinase-3 alpha                          | CREB upstream kinase                |
| AMPK       | 5′-AMP-activated protein kinase                            | CREB upstream kinase                |

The five newly discovered CREB kinases all have some indirect connections to CREB according to previous studies. BRSK2 is a serine/threonine protein kinase of the CAMK group, and it plays a key role in polarization of neurons and axonogenesis,41,42 cell cycle progress,43 and insulin secretion.14 BRSK2 is phosphorylated and activated by liver kinase B1 (LKB1) and AMPK,34 which are also CREB kinases.27,28 Both BRSK2 and CREB are in the LKB1/AMPK pathway.27,34 The CREB phosphosite we have identified for BRSK2 is S98, a site that also can be phosphorylated by PRKD1.27,28 The sequence surrounding S98 is consistent with the substrate specificity of BRSK2 and is related in primary sequence to a previously reported phosphosite site on CREB.27,34 The biological function of this site still remains to be investigated.

Both CDK3 and PIM2 phosphorylate CREB on S133. CDK3 has been reported to phosphorylate and activate transcription factor ATF-1,27 which is in the same CREB/ATF family with CREB50,51 and which it phosphorylates on S63, a site with high sequence similarity to the region surrounding CREB S133. PIM2 is distantly related to the family of calcium/calmodulin-dependent protein kinases and has positive effects on cell cycle progression while inhibiting apoptosis. The substrate specificity of PIM2 matches well with the sequence of amino acids surrounding S133, and this site is the major site that regulates pairs. Using FCMS, we identified several known and novel kinases of CREB.
CREB activity. Kinases including PRKACA,\textsuperscript{46} ATM,\textsuperscript{47} PRKD1,\textsuperscript{25} and p90RSK\textsuperscript{38} are all reported to phosphorylate CREB on S133. The phosphorylation of this site is related to the regulation of apoptosis,\textsuperscript{49} differentiation,\textsuperscript{47} and its transcriptional activities.\textsuperscript{50–53} It is important in diseases such as prostate cancer,\textsuperscript{48} neuroblastoma,\textsuperscript{55} and many others.

MAP4K1 is reported to activate the c-JUN N-terminal kinase (JNK) pathway,\textsuperscript{56} a pathway that also mediates CREB phosphorylation and activation,\textsuperscript{37} but has not been reported previously to interact directly with CREB. Similarly, CDK6 has not been previously identified as a CREB kinase, although interesting roles for CDK6 and cyclin D in transcriptional regulation have recently come to light\textsuperscript{56} including the identification of cyclin D interactions with motifs in promoters recognized by CREB.\textsuperscript{57} It is interesting to speculate that CDK6 might phosphorylate and modulate the activity of CREB as a mechanism to regulate transcription.

Some other interesting kinases were also in our short list. Examples include mitogen-activated protein kinase 1 (MAPK1) and its upstream activator mitogen-activated protein kinase kinase 2 (MAP2K2). Both have previously been reported as functioning upstream of the activation of CREB, but not directly interacting with the substrate.\textsuperscript{32,58,59} These CREB kinases remain to be further verified.

FCMS is a novel proteomics method in several aspects. First, it works by stabilizing transient protein–protein interactions, enabling complexes to be isolated and analyzed by mass spectrometry. Previous studies showed that certain kinase–substrate interactions could be isolated using affinity purification while others could not.\textsuperscript{2,60} Before we applied FCMS as a high throughput strategy to identify substrates and their kinases, we compared the method with the traditional AP-MS method using a known kinase and substrate pair, PKA−CREB. We found that FCMS detected PKA−CREB interaction, while traditional AP-MS did not (data not shown). Second, this stabilization is specific, allowing us to focus on interacting kinase−substrate molecules of interest with low interference from other proteins in the lysate. This is especially advantageous when compared to current cross-linking-based AP-MS methods, in which the linker may nonspecifically cross-link all proteins within close proximity.\textsuperscript{61}

Third, we introduced an effective negative control to enhance further the high specificity of FCMS. Unlike normal negative controls in typical AP-MS experiments in which tag only is used, a fragment of the substrate itself or a mutant version in its native state can be employed. Using a negative control of similar protein size to the wild type substrate limits the potential size effect for protein–protein interactions. Lastly, we successfully captured assembled kinase−substrate pairs specifically in our purification process by using GFP nanobody. Instead of capturing all bait proteins no matter if binding to prey or not, as is the case when using tag-based methods, our method greatly reduces the sample complexity by capturing only interacting protein pairs. This is especially advantageous in proteomic studies since low abundant, but biologically significant, proteins often are difficult to identify in a protein mixture in the presence of large amounts of high abundant proteins.

The limitation of FCMS includes the use of an over-expression system which may introduce false positives. Activation of a kinase may also need a specific extracellular stimulation, which is difficult to implement in a high throughput experiment. As a high throughput screening method to identify upstream kinases, further validation of candidates is expected in different cellular systems. In addition, FCMS detects interacting kinases, not necessarily the upstream kinases, although in this study, CREB is a transcription factor, and we reason that few kinases are downstream of CREB signaling. Furthermore, one critical issue in FCMS is to make the mutant or segment of substrate with the disrupted interacting region between interactors as the negative control. In high throughput experiments, ideally a negative control should be equally effective for eliminating the identification of all nonspecific interactions. If there is a lack of knowledge related to generic interacting domains in the target proteins, it is recommended that one design and construct several mutant candidates at the same time, and compare them with the wild-type protein. For the case of CREB, we used two CREB segments that differentially interact with upstream kinases. After we tested the strategy, results in fluorescence imaging, Western blot, and quantitative mass spectrometry all confirmed our assumption that the C-terminal had significantly fewer interacting kinases than did the N-terminal. This can be a general strategy for any future high throughput FCMS experiment, in which the knowledge about the substrate’s kinase interaction domain or phosphorylation sites is limited.

\section*{METHODS}

\subsection*{Online Method and Additional Information.}
Methods and any associated references are available as Supporting Information. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium (http://proteomecentral.proteomexchange.org) with Project accession number PXD004739 via the PRIDE partner repository. Correspondence and requests for materials should be addressed to W.A.T.

\section*{ASSOCIATED CONTENT}

\subsection*{Supporting Information}

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L.Z. and W.A.T. participated in the planning, data generation, and interpretation; L.Z., J.A., and W.-H.W. participated in detailed experimental planning and data interpretation; G.S. contributed in the preparation of the CREB protein; R.L.G. and C.-D.H. participated in data interpretation; L.Z. and W.A.T. wrote the manuscript.

\section*{Notes}

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REFERENCES

(1) Hunter, T. Signaling—2000 and beyond. Cell 2000, 100, 113–127.
(2) Bodenmiller, B.; Wanka, S.; Kraft, C.; Urban, J.; Campbell, D.; Pedrioli, P. G.; Gerrits, B.; Picotti, P.; Lam, H.; Vitek, O.; Brusniak, M. Y.; Roschitzki, B.; Zhang, C.; Shokat, K. M.; Schlapbach, R.; Colman-Lerner, A.; Nolan, G. P.; Nesvizhskii, A. I.; Peter, M.; Loewith, R.; von Mering, C.; Aebersold, R. Phosphoproteomic analysis reveals interconnected system-wide responses to perturbations of kinases and phosphatases in yeast. Sci. Signaling 2010, 3, r4.
(3) Shah, K.; Shokat, K. M. A chemical genetic screen for direct v-Src substrates reveals ordered assembly of a retrograde signaling pathway. Chem. Biol. 2002, 9, 35–47.
(4) Xun, L.; Wang, W. H.; liuk, A.; Hu, L.; Galan, J. A.; Yu, S.; Hans, M.; Geahlen, R. L.; Tao, A. W. Sensitive kinase assay linked with phosphoproteomics for identifying direct kinase substrates. Proc. Natl. Acad. Sci. U. S. A. 2012, 109, 5615–5620.
(5) Xiao, Y.; Wang, Y. Global discovery of protein kinases and other nucleotide-binding proteins by mass spectrometry. Mass Spectrom. Rev. 2016, 35, 601–619.
(6) Ji, J. H.; Hwang, H. I.; Lee, H. J.; Hyun, S. Y.; Kang, H. J.; Jang, Y. J. Purification and proteomic identification of putative upstream regulators of polo-like kinase-1 from mitotic cell extracts. FEBS Lett. 2010, 584, 4299–4305.
(7) Maly, D. J.; Allen, J. A.; Shokat, K. M. A mechanism-based cross-linker for the identification of kinase-substrate pairs. J. Am. Chem. Soc. 2004, 126, 9160–9161.
(8) Statsuk, A. V.; Maly, D. J.; Seeliger, M. A.; Fabian, M. A.; Biggs, W. H., 3rd; Lockhart, D. J.; Zarrinkar, P. P.; Kuriyan, J.; Shokat, K. M. Tuning a three-component reaction for trapping kinase substrate complexes. J. Am. Chem. Soc. 2008, 130, 17568–17574.
(9) Hu, C. D.; Chinenov, Y.; Kerppola, T. K. Visualization of interactions among bZIP and Rel family proteins in living cells using bimolecular fluorescence complementation. Mol. Cell. 2002, 9, 789–798.
(10) Pusch, S.; Harashima, H.; Schnittger, A. Identification of kinase substrates by bimolecular complementation assays. Plant J. 2012, 70, 348–356.
(11) Pusch, S.; Dissmeyer, N.; Schnittger, A. Bimolecular-fluorescence complementation assay to monitor kinase-substrate interactions in vivo. Methods Mol. Biol. 2011, 779, 245–257.
(12) Johannessen, M.; Moens, O. Multisite phosphorylation of the cAMP response element-binding protein (CREB) by a diversity of protein kinases. Front. Biosci., Landmark Ed. 2007, 12, 1814–1832.
(13) Shawwitz, A. J.; Greenberg, M. E. CREB: A Stimulus-Induced Protein Kinase Cascades in Transcriptional Regulation. J. Biol. Chem. 2007, 282, 14777−14787.
(14) Hur, E.-M.; Zhou, F.-Q. GSK3 signaling in neural development. Nat. Rev. Neurosci. 2010, 11, 539−551.
(15) Thomson, D. M.; Herway, S. T.; Fillmore, N.; Kim, H.; Brown, J. D.; Barrow, J. R.; Winder, W. W. AMP-activated protein kinase phosphorylates transcription factors of the CREB family. J. Appl. Physiol. 2008, 104, 429−438.
(16) Gu, Y.; Lin, S.; Li, J. L.; Nakagawa, H.; Chen, Z.; Jin, B.; Tian, L.; Ucar, D. A.; Shen, H.; Lu, J.; Hochwald, S. N.; Kaye, F. J.; Wu, L. Altered LKB1/CREB-regulated transcription co-activator (CRTC) signaling axis promotes esophageal cancer cell migration and invasion. Oncogene 2012, 31, 469−479.
(17) Zheng, D.; Cho, Y. Y.; Lau, A. T.; Zhang, J.; Ma, W. Y.; Bode, A. M.; Dong, Z. Cyclin-dependent kinase 3-mediated activating transcription factor 1 phosphorylation enhances cell transformation. Cancer Res. 2008, 68, 7650−7660.
(18) Hummel, E.; Cole, T. J.; Blendy, J. A.; Ganss, R.; Aguzzi, A.; Schmid, W.; Beermann, F.; Schultz, G. Targeted mutation of the CREB gene: compensation within the CREB/ATF family of transcription factors. Proc. Natl. Acad. Sci. U. S. A. 1994, 91, 5647−5651.
(19) Johannessen, C. M.; Johnson, L. A.; Piccioni, F.; Townes, A.; Frederick, D. T.; Donahue, M. K.; Narayan, R.; Flaherty, K. T.; Wargo, J. A.; Root, D. E.; Garraway, L. A. A melanocyte lineage program confers resistance to MAP kinase pathway inhibition. Nature 2013, 504, 138−142.
(20) Li, L.; Fan, D.; Wang, C.; Wang, J. Y.; Cui, X. B.; Wu, D.; Zhou, Y.; Wu, L. L. Angiotensin II increases peroxisome proliferation-activated receptor α expression via Ras/p38 MAPK/CREB and ERK1/2/TGF-beta1 pathways in cardiac fibroblasts. Cardiovasc. Res. 2011, 91, 80−89.
(21) Moro, T.; Ogawara, T.; Chikuda, H.; Ikeda, T.; Ogata, N.; Maruyama, Y.; Komori, T.; Hoshi, K.; Chung, U. I.; Nakamura, K.; Okayama, H.; Kawaguchi, H. Inhibition of Cdk6 expression through p38 MAP kinase is involved in differentiation of mouse prechondrocyte ATDC5. J. Cell. Physiol. 2005, 204, 927−933.
(34) Bright, N. J.; Carling, D.; Thornton, C. Investigating the Regulation of Brain-specific Kinases 1 and 2 by Phosphorylation. J. Biol. Chem. 2008, 283, 14946–14954.

(35) Wang, X.; Li, W.; Williams, M.; Terada, N.; Alessi, D. R.; Proud, C. G. Regulation of elongation factor 2 kinase by p90(RSK1) and p70 S6 kinase. EMBO J. 2001, 20, 4370–4379.

(36) Hu, M. C.; Qiu, W. R.; Wang, X.; Meyer, C. F.; Tan, T. H. Human HPK1, a novel human hematopoietic progenitor kinase that activates the JNK/SAPK kinase cascade. Genes Dev. 1996, 10, 2251–2264.

(37) Dong, C.; Davis, R. J.; Flavell, R. A. MAP kinases in the immune response. Annu. Rev. Immunol. 2001, 20, 55–72.

(38) Bansal, K.; Kapoor, N.; Narayana, Y.; Puzo, G.; Gilleron, M.; Balaji, K. N. PIM2 Induced COX-2 and MMP-9 expression in macrophages requires PI3K and Notch1 signaling. PLoS One 2009, 4, e4911.

(39) Hamerman, P. S.; Fox, C. J.; Cinalli, R. M.; Xu, A.; Wagner, J. D.; Lindsten, T.; Thompson, C. B. Lymphocyte transformation by Pim-2 is dependent on nuclear factor-kappaB activation. Cancer Res. 2004, 64, 8341–8348.

(40) Kaltschmidt, B.; Ndawe, D.; Korte, M.; Pothen, S.; Arbibe, L.; Prullage, M.; Pfeiffer, J.; Lindecke, A.; Staiger, V.; Israel, A.; Kaltschmidt, C.; Mémet, S. NF-κB Regulates Spinal Memory Formation and Synaptic Plasticity Through Protein Kinase A/CREB Signaling. Mol. Cell. Biol. 2006, 26, 2936–2946.

(41) Yao, P.; Wang, Z. B.; Ding, Y. F.; Ma, J. M.; Hong, T.; Pan, S. N.; Zhang, J. Regulatory network of differentially expressed genes in metastatic osteosarcoma. Mol. Med. Rep. 2015, 11, 2104–2110.

(42) Lilley, B. N.; Krishnaswamy, A.; Wang, Z.; Kishi, M.; Frank, E.; Sanes, J. R. SAD kinases control the maturation of nerve terminals in the mammalian peripheral and central nervous systems. Proc. Natl. Acad. Sci. U. S. A. 2014, 111, 138–143.

(43) Wang, Y.; Wang, J.; Liu, D.; Zhou, J.; Li, R.; Bai, M.; Chen, F.; Yu, L. BRSK2 is regulated by ER stress in protein level and involved in ER stress-induced apoptosis. Biochem. Biophys. Res. Commun. 2012, 423, 813–818.

(44) Chen, X. Y.; Gu, X. T.; Saijyn, H.; Wan, B.; Zhang, Y. J.; Li, J.; Wang, Y. L.; Gao, R.; Wang, Y. F.; Dong, W. P.; Najar, S. M.; Zhang, C. Y.; Ding, H. F.; Liu, J. O.; Yu, L. Brain-selective kinase 2 (BRSK2) phosphorylation on PTCAIRE1 negatively regulates glucose-stimulated insulin secretion in pancreatic beta-cells. J. Biol. Chem. 2012, 287, 30368–30375.

(45) Chen, X. Y.; Gu, X. T.; Saijyn, H.; Wan, B.; Zhang, Y. J.; Li, J.; Wang, Y. L.; Gao, R.; Wang, Y. F.; Dong, W. P.; Najar, S. M.; Zhang, C. Y.; Ding, H. F.; Liu, J. O.; Yu, L. Brain-selective kinase 2 (BRSK2) phosphorylation on PTCAIRE1 negatively regulates glucose-stimulated insulin secretion in pancreatic beta-cells. J. Biol. Chem. 2012, 287, 30368–30375.

(46) Parker, D.; Ferreri, K.; Nakajima, T.; LaMorte, V. J.; Evans, R.; Koerber, S. C.; Hoeger, C.; Montminy, M. R. Phosphorylation of CREB at Ser-133 induces complex formation with CREB-binding protein via a direct mechanism. Mol. Cell. Biol. 1996, 16, 694–703.

(47) Fernandes, N. D.; Sun, Y.; Price, B. D. Activation of the kinase activity of ATY by retinoic acid is required for CREB-dependent differentiation of neuroblastoma cells. J. Biol. Chem. 2007, 282, 16577–16584.

(48) Deak, M.; Clifton, A. D.; Luocoq, L. M.; Alessi, D. R. Mitogen- and stress-activated protein kinase-1 (MSK1) is directly activated by MAPK and SAPK/P38, and may mediate activation of CREB. EMBO J. 1998, 17, 4426–4441.

(49) Bonni, A.; Brunet, A.; West, A. E.; Datta, S. R.; Takasu, M. A.; Greenberg, M. E. Cell survival promoted by the Ras-MAPK signaling pathway by transcription-dependent and -independent mechanisms. Science 1999, 286, 1358–1362.

(50) Zhao, L.; Li, G.; Zhou, G. Q. SOX9 directly binds CREB as a novel synergism with the PKA pathway in BMP-2-induced osteochondrogenic differentiation. J. Bone Miner. Res. 2009, 24, 826–836.

(51) White, P. C.; Shore, A. M.; Clement, M.; McLaren, J.; Soeho, I.; Lam, E. W.; Brennan, P. Regulation of cyclin D2 and the cyclin D2 promoter by protein kinase A and CREB in lymphocytes. Oncogene 2006, 25, 2170–2180.