Correction

BIOCHEMISTRY

The authors note that the author name Sang-Ho Park should instead appear as Sangho Park. The corrected author line and author contributions footnote appear below. The online version has been corrected.

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Combinatorial regulation of a signal-dependent activator by phosphorylation and acetylation

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In the fasted state, increases in catecholamine signaling promote adipocyte function via the protein kinase A-mediated phosphorylation of cyclic AMP response element binding protein (CREB). CREB activity is further up-regulated in obesity, despite reductions in catecholamine signaling, where it contributes to the development of insulin resistance. Here we show that obesity promotes the CREB binding protein (CBP)-mediated acetylation of CREB at Lys136 in adipose. Under lean conditions, CREB acetylation was low due to an association with the energy-sensing NAD\(^+\)-dependent deacetylase SirT1; amounts of acetylated CREB were increased in obesity, when SirT1 undergoes proteolytic degradation. Whereas CREB phosphorylation stimulated an association with the KIX domain of CBP, Lys136 acetylation triggered an interaction with the CBP bromodomain (BRD) that augmented recruitment of this coactivator to the promoter. Indeed, coincident Ser133 phosphorylation and Lys136 acetylation stimulated the formation of a ternary complex with the KIX and BRD domains of CBP by NMR analysis. As disruption of the CREB:BRD complex with a CBP-specific BRD inhibitor blocked effects of CREB acetylation on target gene expression, our results demonstrate how changes in nutrient status modulate cellular gene expression in response to hormonal signals.

CREB | CBP | SirT1 | acetylation | phosphorylation

In the fasted state, increases in circulating catecholamines promote a metabolic shift from carbohydrate to fat burning by stimulating lipolysis, in part via the protein kinase A (PKA)-mediated activation of hormone sensitive lipase (HSL). Cyclic adenosine monophosphate (cAMP) also promotes the expression of cellular genes via the PKA-mediated phosphorylation of cyclic AMP response element binding protein (CREB). CREB activity is increased in the settings of dietary and genetic obesity, where it appears to promote the recruitment of macrophages into white adipose (1). Reducing adipocyte CREB activity by overexpression of a dominant negative CREB polypeptide called A-CREB or by targeted disruption of the CREB coactivator CRTC3 blocks macrophage recruitment and protects against the development of insulin resistance (2).

In the setting of overnutrition and obesity, adipocytes secrete proinflammatory cytokines (3) that not only stimulate recruitment of innate immune cells but also promote adipocyte insulin resistance (4–6).

Adipocyte-derived tumor necrosis factor alpha (TNF\(\alpha\)) has been shown to promote insulin resistance, for example, by decreasing expression of the energy-sensing deacetylase SirT1 (7–9) and thereby up-regulating proinflammatory genes (10).

Phosphorylation of CREB at Ser133 increases its activity by promoting an interaction of the kinase-inducible domain (KID; amino acids 100–160) in CREB with the KIX domains of CBP and p300 (11). CREB activity also appears to be modulated by acetylation at Lys136 (12, 13). Although it is highly conserved through evolution, Lys136 does not appear to participate directly in the association of KID with the KIX domain. Indeed, the effects of acetylation on CREB activity appear cell context-dependent; in F9 teratocarcinoma cells, acetylation was found to inhibit CREB activity, whereas acetylation increased CREB activity in hepatocytes.

Here we evaluate the role of acetylation in modulating CREB activity following its phosphorylation at Ser133. We examine the effects of phosphorylation and acetylation, alone and in combination, on the interaction of CREB with CBP and p300. Our results suggest how changes in nutrient status modulate effects of hormonal stimuli on the activity of a signal-dependent activator.

Results

Based on the ability of adipocyte CREB to promote insulin resistance in white adipose tissue (WAT) in the context of dietary and genetic obesity (1), we examined effects of proinflammatory cytokines on CREB activity in 3T3L1 adipocytes. Exposure to TNF\(\alpha\) augmented CREB reporter activity in cells coexposed to forskolin (FSK) (Fig. 1A). TNF\(\alpha\) exposure enhanced CREB activity over a large subset of target genes by transcriptome-wide analysis (Fig. 1B). Overexpression of a dominant negative CREB polypeptide (A-CREB) disrupted TNF\(\alpha\) effects, confirming the importance of CREB in this setting (Fig. 1C). Notably, TNF\(\alpha\) treatment did not augment CREB phosphorylation in cells exposed to FSK (Fig. 1D), suggesting that CREB may undergo additional regulatory modifications in response to this cytokine.

In mass spectrometry studies of HEK293T cells expressing epitope-tagged CREB, we identified CREB peptides containing phosphorylated and acetylated amino acids 100–160, in agreement with a recent report (11). This domain contains the KID and KIX motifs of CREB and CBP, respectively, which mediate association with endogenous CREB (12). Phosphorylation at Ser133 increased CREB activity, whereas acetylation at Lys136 increased CREB activity. Coexpression of A-CREB reduced TNF\(\alpha\)-dependent CREB activation; the A-CREB polypeptide was unable to recruit CBP to CREB at Ser133, highlighting a significant role for CREB phosphorylation in TNF\(\alpha\)-dependent CREB activation. Accumulation of CREB and CBP was enhanced in 3T3L1 adipocytes when CREB Ser133 was phosphorylated, indicating a potential role for phosphorylation in adipocyte development. Consistent with these findings, CREB Ser133 phosphorylation was increased with proinflammatory cytokines (3) that not only stimulate recruitment of innate immune cells but also promote adipocyte insulin resistance (4–6).

Significance

Catecholamines regulate adipocyte function in part by CREB activation. Obesity displays enhanced cyclic AMP response element binding protein (CREB) activity despite reduced catecholamine signals. We report that obesity promotes CREB binding protein (CBP)-mediated CREB acetylation at Lys136 in the adipose tissue by means of reduced SirT1 levels. CREB acetylation promotes binding with the bromodomain (BRD) of CBP, which can also bind phosphorylated Ser133, through its KIX domain, upon hormonal signals. This double recognition is reflected in potentiated CBP recruitment at promoters when CREB is doubly modified. Structural data show the formation of a ternary complex between CBP BRD and KIX domains when bound to phospho-Ser133 and acetylated-Lys136 CREB. Disruption of the BRD:CREB interaction with a small molecule reduced the enhanced CREB activity associated with acetylation.


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an acetyl-lysine residue at Lys136 (Fig. S1A). Using a synthetic acetyl-Lys136 CREB peptide (amino acids 129–146) as immunogen, we developed an acetylation-specific CREB antiserum. Amounts of acetyl (Lys136) CREB were increased in 3T3L1 cells following exposure to TNFα (Fig. 1D). CREB acetylation was also up-regulated in the context of dietary or genetic obese (ob/ob) mice (Fig. 1F and Fig. S1C).

By contrast with effects of obesity, exposure to the cAMP agonist FSK had no apparent effect on CREB acetylation in mouse embryo fibroblasts (MEFs) (Fig. 2A), prompting us to consider the involvement of protein deacetylases in this process. Although inhibition of the class I or IIa HDACs with Trichostatin A (TSA) had no effect, treatment with SirT1 antagonist Ex527 increased amounts of acetylated CREB in WT MEFs by Western blot assay (Fig. 2A and Fig. S1B). Moreover, knockout of SirT1 in MEFs further increased amounts of (Lys136) acetylated CREB (Fig. 2A and Fig. S1 B and D). Pointing to a direct role for this deacetylate in regulating CREB acetylation, we identified SirT1 in immunoprecipitates of CREB by mass spectrometry analysis (Fig. S1A). Consistent with previous reports showing that SirT1 undergoes proteolytic degradation in obesity and in response to TNFα (7–9), SirT1 protein amounts were down-regulated in TNFα-treated 3T3L1 cells and in WAT from ob/ob and high-fat-fed mice (Fig. 1 D and E and Fig. S1C).

Based on its proximity to Ser133, Lys136 may modulate CREB activity by altering the phosphorylation of CREB (13). Arguing against this notion, however, disruption of SirT1 activity, either by exposure to EX527 inhibitor or by knockout of SirT1 in MEFs, had no effect on the kinetics or apparent stoichiometry of CREB phosphorylation in cells treated with FSK (Fig. 2A and Fig. S1D). We considered that the relative stoichiometry of Lys acetylation in MEFs could be too low to modulate cellular Ser133 phosphorylation detectably. To address this possibility, we performed in vitro phosphorylation studies using either unmodified or Lys136 acetylated synthetic CREB peptides (amino acids 129–148). Following incubation with purified PKA, acetylated and nonacetylated CREB peptides were comparably phosphorylated over a 100-fold range of peptide concentration. These results demonstrate that Lys136 acetylation does not alter the affinity of PKA for CREB (Fig. 2B). Moreover, in overexpression experiments in which p300 was able to acetylate WT but not a nonacetylable CREB mutant (Lys136Arg), the cAMP-induced Ser133 phosphorylation was comparable between the two overexpressed constructs used (Fig. S1E).

Considering that CREB associates with the KIX domains of CBP and p300 following its phosphorylation at Ser133, we reasoned that these HATs might mediate the acetylation of CREB at Lys136. Supporting this notion, knockout of both CBP and p300 reduced amounts of acetylated CREB as well as p53 almost completely (Fig. 2C). In line with the nuclear localization of CREB, immune-reactive acetyl (Lys136) CREB was also confined to the nucleus; this staining was blocked by pretreatment of acetyl antibodies against this notion, knockin mutations in p300 and CBP KIX domains that block their interaction with phospho (Ser133) CREB (14) had no effect on Lys136 acetylation of CREB (Fig. 2C). Taken together,
these results indicate that CREB is constitutively acetylated at Lys136 by p300 and CBP and deacetylated by SirT1.

Based on the sizable difference in amounts of acetyl (Lys136) CREB between WT and SirT1 mutant cells, we evaluated effects of this modification on target gene expression in these cells. CREB reporter activity was elevated in SirT1−/− MEFs (Fig. 3A) compared with WT MEFs following exposure to FSK. Reexpression of SirT1 in the SirT1−/− MEFs (Fig. 3A) reduced CREB activity to WT levels, indicating that the differences in CREB activity are indeed due to SirT1. CREB Lys136 acetylation appeared reduced after overexpression of SirT1 in SirT1−/− MEFs (Fig. S1F).

In gene-profiling studies, knockout of SirT1-potentiated effects of FSK on a large subset of CREB target genes, most notably the cytokine IL6 (Fig. 3B). Although the amplitudes of target gene expression were different, the dynamics appeared comparable between both cell types (Fig. S2A). In particular, the kinetics of CREB dephosphorylation were nearly identical between SirT1−/− and WT cells (Fig. S2B).

Having seen that FSK increases CREB phosphorylation comparably in WT and SirT1 mutant cells, we examined whether the subsequent recruitment of CBP/p300 is also similar between both cell types. In ChIP studies, exposure to FSK increased phospho-CREB amounts on the IL6 and DUSP1 promoters to the same extent in WT and SirT1 mutant cells (Fig. 3C and Fig. S2D). By contrast, amounts of p300/CBP over the promoter were substantially elevated in SirT1−/− relative to control cells.
following exposure to FSK, suggesting that CREB acetylation may enhance its interaction with CBP/p300. Arguing against a role for the KIX domain in this setting, acetylation of CREB at Lys136 did not further increase the association with KIX in vitro (Fig. S2C).

We noticed that Acetyl-Lys136 in CREB conforms to an optimal binding site for the bromodomains (BRDs) of p300 and CBP (Ω+[+]AcK-ψ-ψ; Tyr-Arg-Lys-Ile-Leu) (15). We evaluated the potential role of this interaction in potentiating CREB activity using a CBP/p300-specific BRD inhibitor called ischemin. In transient assays of HEK293T cells, exposure to FSK increased the activity of a WT GAL4 CREB construct, which contains the CREB activation domain (amino acids 1–286) fused to the GAL4 DNA-binding domain, to a greater extent than acetylation-defective (Lys136Arg) GAL4 CREB (Fig. 4A). Similarly, coexpression of CBP also potentiated WT GAL4-CREB activity, but less so the Lys136Arg mutant. Pretreatment with ischemin decreased WT GAL4-CREB activity in response to FSK treatment or CBP overexpression; however, it had no effect on GAL4 CREB (Lys136Arg) activity. In keeping with its effects in reporter assays, ischemin also blocked the TNFα-dependent potentiation of Il6 and Ptgs2 genes in 3T3 L1 cells exposed to FSK, demonstrating the importance of the CBP/p300 BRDs for these effects (Fig. 4B).

CREB stimulates cellular gene expression, following its phosphorylation at Ser133, via a kinase-inducible transactivation domain called KID (16). To determine whether acetylated CREB binds to the CBP BRD, we performed NMR studies. Following addition of acetylated KID peptide (AcKID), the NMR signals of a number of amino acid residues in the CBP BRD exhibited significant changes in chemical shift that are indicative of direct interaction between the BRD and AcKID (Fig. S3A). By contrast,
addition of unmodified KID peptide caused little change in the BRD spectrum (Fig. S3B), confirming the importance of Lys136 acetylation for binding. The residues that exhibit the greatest NMR signal perturbations upon addition of AcKID map to the acetyl-lysine-binding site in the CBP BRD structure. Binding of acetyl-lysine peptides to the CBP bromodomain is typically weak, with dissociation constants in the 100–400 μM range (15, 17). NMR titration experiments were performed to confirm that ischemin inhibits the interaction between the BRD and AcKID (Fig. S3C). Addition of ischemin to the complex of AcKID with BRD causes the BRD cross-peaks to shift toward their positions in the ischemin:BRD complex, confirming that ischemin and AcKID compete for the acetyl-lysine-binding site (Fig. S3C).

To determine the importance of the BRD for the association of CBP/p300 with CREB, we performed coimmunoprecipitation studies. Under basal conditions, WT CREB associated with epitope-tagged p300; this interaction was increased following exposure to FSK (Fig. S5D). By contrast with WT CREB, the interaction of p300 with nonacetylatable Lys136Arg CREB was substantially weaker. Indeed, deletion of the BRD in p300 disrupted the interaction with CREB under basal conditions and following stimulation with FSK.

Based on the ability of CBP to recognize phosphorylated or acetylated forms of KID via its KIX and BRD domains, respectively, we wondered whether it could recognize doubly modified (phospho-Ser133, acetyl-Lys136) KID (AcKID). Ser133 phosphorylation did not perturb binding to the BRD: addition of AcKID caused the same chemical shift changes and broadening in the BRD spectrum as binding of AcKID. This was confirmed by the reverse experiment, in which equimolar BRD was added to 15N-labeled AcKID. Upon addition of the BRD, the NMR signals of several residues in the BRD interaction region of AcKID (S133, Y134, R135, AcK136, and I137) exhibited exchange broadening that is indicative of complex formation (Fig. 4C, Left). Residues in other regions of the BRD-bound AcKID peptide remain disordered, as indicated by the narrow chemical shift dispersion in the 1H dimension. In contrast, upon addition of KIX, the cross-peaks of several AcKID residues surrounding the Ser133 phosphorylation site (S129, R130, pS133, Y134, I137, and S142) exhibit greatly increased chemical shift dispersion (Fig. 4C, Center), consistent with formation of helical secondary structure upon binding to the pKID KIX complex (11). The NMR spectrum of 15N-AcKID in the presence of equimolar KIX and BRD provides direct evidence for ternary complex formation (Fig. 4C, Right). Cross-peaks for residues S129, R130, pS133, and S142 are observed at the same positions as in spectra of the binary AcKID-KIX complex. However, cross-peaks for pS133, Y134, R135, K136, and I137 exhibit exchange broadening, as they did in the binary AcKID-BRD complex. Because binding of pKID to KIX is in slow exchange on the NMR time scale (11, 18), the specific broadening of NMR signals associated with residues located near acetyl-Lys136 provides direct evidence for binding of the BRD to the AcpKID-KIX complex. Thus, the NMR data show that the KIX domain and the BRD do not compete for binding to AcKID and that acetylation of Lys136 functions to recruit the BRD to the pKID-KIX complex.

Discussion
Like other signaling pathways, cAMP stimulates cellular gene expression with burst-attenuation kinetics: rates of transcriptional peak after 30 min, diminishing thereafter to near-baseline levels within 4–6 h. CREB target gene expression closely mirrors the level of CREB Ser133 phosphorylation, suggesting that the relative recruitment of CBP/p300 is rate-limiting for transcriptional induction. Supporting this notion, mutations in the KID that reduce KIX binding decrease cellular gene expression, whereas mutations that increase the KID:KIX interaction up-regulate it (19, 20). The current work extends these findings by showing that the amplitude of the transcriptional response to cAMP is further modulated by acetylation of CREB in response to nutrient signals.

In contrast with another study, we found that Lys136 acetylation has no apparent effect on its phosphorylation by PKA in vitro (13). Indeed, the dynamics of CREB phosphorylation and dephosphorylation in response to FSK are comparable between SirT1−/− MEFs and WT cells. Lys136 acetylation appears to increase CREB activity globally, but certain inflammatory response genes such as IL6 appear to be more robustly up-regulated. Although the mechanism underlying the preferential induction of these CREB target genes is unclear, we imagine that acetylated CREB could be either selectively recruited to these promoters or it could synergize with adjacent factors on the promoter. In line with this idea, CREB acetylation was increased in adipocytes following exposure to TNFα, where it stimulated the expression of IL6 and other cytokine genes. Future studies should reveal whether CREB acetylation increases cooperativity with NF-κB on these promoters.

We found that CREB Lys136 acetylation is catalyzed primarily, if not exclusively, by CBP/p300. Surprisingly, the phosphorylation-dependent interaction between phospho (Ser133) CREB and CBP/p300 is not required for CREB acetylation at Lys136, suggesting that CREB may associate with other domains of CBP/p300 in addition to the KIX domain. Indeed, we found that CREB acetylation promotes its association with the BRD of CBP. This association appears important in modulating CREB activity because treatment with the BRD small-molecule inhibitor ischemin reduced target gene expression.

Remarkably, phospho (Ser133)-acetyl (Lys136) CREB is capable of forming a ternary complex with the CBP KIX and BRD domains, indicating that CBP can discriminate between singly and doubly modified CREB (Fig. 4D). As a result, amounts of CBP recruited to the promoter in response to cAMP agonist are increased when CREB is both phosphorylated and acetylated. Thus, depending on cellular energy status, the transcriptional response to cAMP may be further modified by SirT1, which modulates CREB activity. Although our results point to an important role for the CBP/p300 BRDs in this setting, we imagine that CREB may stimulate cellular gene expression through its interaction with other bromodomain proteins as well.

Methods

Animal Studies. High-fat diet-fed ob/+ and ob/ob mice were purchased from Jackson Laboratories. All animal studies were performed for two weeks before performing the studies. All animal studies were performed with the approval of the Institutional Animal Care and Use Committee (IACUC) at the Salk Institute.

Antibody Preparation. Antiserum was raised in rabbit using a synthetic peptide encoding (Ac)Lys136Cys146 CREB (129–148) coupled to keyhole limpet hemocyanin via maleimide. Coupling method, preparation of immunogen, and immunization schedule were as previously described in detail (21). Selected bleeds were purified using a column containing (Ac)K31/Cys146 CREB (129–148) covalently linked to an agarose resin. The antibody-containing fraction was passed over a second column containing Cys148 CREB (129–148) covalently linked to an agarose resin, and the flow-through was collected to obtain purified, highly selective antiserum against acetyl-Lys136 CREB.

Cell Culture. Hek293T, 3T3L1CARα, and MEF cells were cultured in 4.5g/L glucose DMEM containing 10% (vol/vol) FBS (HyClone) and 100 mg/mL penicillin–streptomycin. The 3T3L1CARα were differentiated as previously described (22). Briefly, cells were allowed to grow. Two days after reaching confluency (day 0), medium was replaced with induction medium [DMEM, 10% (vol/vol) FBS, 100 mg/mL penicillin–streptomycin, 500 μM isobutylmethylxanthine, 1 μM Dexamethasone, 5 μg/mL Insulin, 1 μM Roglitazone]. Induction medium was left for 3 d and then replaced by differentiation media [DMEM, 10% (vol/vol) FBS, 100 μg/mL penicillin–streptomycin, 1 μg/mL Insulin, 1 μM Roglitazone]. Differentiation media was changed every 2 d, and experiments were performed on day 12. Recombinant mouse TNFα (Biologend) was added to the media for two consecutive days.
ChIP assays were performed as described, with minimal modification (23). Briefly, cells were cross-linked in 1% formaldehyde. After 10 min of cross-linking, formaldehyde was inactivated with glycine. Nuclei were isolated using hypotonic lysis buffer, and chromatin was sonicated 8 times for 10 s at 50% output. Chromatin was precleared and incubated with the indicated antibodies overnight in the presence of protease, phosphatase, and HDAC inhibitors. Precipitated chromatin was purified and quantified by qPCR (Lightcycler 480; Roche), relative to input chromatin. ChIP primer sequences are listed in Table S1.

**Gene Expression.** Cells were treated with Forskolin (10 μM) or vehicle for 60 min. Cellular RNA was extracted using RNeasy Kit (Qiagen), and cDNA was generated using First Strand cDNA Synthesis Kit for RT-PCR (Roche). cDNA was quantified on a Lightcycler 480 instrument (Roche). mRNA amounts were normalized relative to the expression of the housekeeping gene Rpl27 (L32). Primer sequences are listed in Table S1. For global analysis of gene expression, cells were treated with Forskolin (10 μM) or vehicle for 60 min, and total RNA was extracted using RNeasy Kit (Qiagen). For gene array studies, RNA was converted to cDNA and hybridized to GeneChip Mouse Gene 1.0 ST Arrays (Affymetrix). Arrays were normalized and analyzed using Affymetrix Expression Console v. 1.1 software. For RNAseq, total RNA was extracted, and RNAseq library was prepared using Illumina’s Miseq Reagent Kit v3. The cDNA library was run on an Illumina Miseq instrument.

**In Vitro Kinase Assay.** In vitro kinase assays were performed using CREB peptides (amino acids 129–148), either acetylated at Lys136 or unmodified, as substrate. Peptides were diluted in kinase buffer (50 mM Tris, 10 mM MgCl₂, 1 mM NaVO₃, 0.2 mM ATP, 1 mM DTT, and 1 μCi γ-[³²P]ATP), and reaction was initiated by addition of purified PKA (Sigma) and deacetylases inhibitors (10 mM sodium butyrate, 20 mM Nicotinamide). Proteins were quantified using BCA reagents (Thermo Scientific) and separated using SDS/PAGE. For pulldown experiments, GST proteins were bacterially expressed and purified.

**Statistics.** All experiments were performed on at least three independent occasions. Results are presented as the average ± SD of technical triplicates. Unpaired, two-tailed Student’s t test was used to compare groups, and P < 0.05 was considered significant (P < 0.05; **P < 0.01; ***P < 0.001).

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Fig. S1. Effect of CREB acetylation at Lys136 on phosphorylation at Ser133. (A) CREB (Top) and SirT1 (Bottom) peptides recovered from immunoprecipitates of Halo-tagged CREB expressed in HEK293T cells. (Top) CREB peptide containing acetylated lysine at Lys136. (B) Effect of class I and IIa inhibitor TSA on CREB acetylation in WT and SirT1−/− MEFs. Amounts of acetylated histone (H3K27, H3K9) shown. (C) Immunoblot of Epididymal white adipose tissue from normal chow or high-fat-diet–fed mice (one animal per lane). Amounts of acetylated (Lys136) CREB shown. (D) Effect of SirT1 deletion on CREB phosphorylation in response to increasing concentrations of FSK. Phosphorylation of LKB1 at the PKA phosphorylation site Ser431 also shown. (E) Immunoblot showing acetylated (Lys136) levels of overexpressed WT CREB or Lys136Arg mutant. HA-tagged p300 and CREB constructs were overexpressed in HEK293T cells, stimulated with FSK and immunoprecipitated. (F) Immunoblot comparing effects of SirT1 knockout or overexpression on amounts of acetylated (Lys136) or phosphorylated (Ser133) CREB in cells exposed to FSK and phosphodiesterase inhibitor IBMX as indicated. WT and SirT1−/− cells indicated.
Fig. S2. Effect of CREB acetylation on dynamics of target gene activation. (A) Time course analysis of Il6 and Ptgs2 mRNA accumulation in WT or SirT1 mutant MEFs following stimulation with FSK for times indicated. (B) Effect of acetylation on Ser133 phosphorylation in time course stimulation with FSK in WT and SirT1−/− MEFs. Amounts of SirT1, phosphorylated Ser133 CREB, acetylated Lys136 CREB, and total CREB are shown. (C) Effect of acetylation of full-length recombinant CREB on Ser133 phosphorylation and interaction with the KIX domain of CBP/p300. (Left) Immunoblot showing amounts of acetylated (Lys136) CREB recovered following incubation of CREB with CBP HAT domain. Effect of incubation with PKA on Ser133 phosphorylation also shown. (Right) GST pulldown assay showing effect of acetylation and phosphorylation, alone and in combination on binding of CREB to the KIX domain. (D) ChIP assay of WT and SirT1−/− MEFs showing recruitment of CREB, P-CREB, and CBP to the Dusp1 promoter under basal conditions and following exposure to FSK.
Fig. S3. Effect of acetylation on binding of CREB to the BRD of CBP. (A and B) $^{15}$N-HSQC spectrum of the $^{15}$N-labeled BRD following incubation with acetylated KID peptide (A) or unmodified KID (B) at 1:1 mol ratio. The BRD residue numbers are shown for cross-peaks that are significantly perturbed by binding of AcKID. (C) Region of the $^1$H-$^{15}$N HSQC spectrum of $^{15}$N-BRD (200 μM) showing the effects of binding AcKID and ischemin. The spectrum of the free BRD is shown in black, that of the AcKID-BRD complex is shown in blue, and the spectrum of the ischemin-bound BRD is shown in red. Spectra in cyan, green, and orange show the effects of titrating increasing amounts of ischemin into the AcKID-BRD complex. The molar ratio of the BRD, AcKID, and ischemin is shown according to the color of the spectra. (D) Immunoblot showing amounts of WT or acetylation-defective (K136R) CREB recovered from immunoprecipitates of V5 epitope-tagged p300. Relative binding of CREB to WT and BRD mutant p300 shown.
Table S1. Sequences of the primers used

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|                         | Rev 5' –TCTGGTGAAGGCAAGATCG-3' | Rev 5' –CTCGGGTTTTCGCCAGCT-3' |
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<th>DUSP1</th>
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<td>Rev 5' –GTTGGGCTGATTGGAAACCT-3'</td>
<td>Rev 5' –CGTTTTGTTTGGTTGAAATGGCAC-G-3'</td>
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|                         | Rev 5' –CAGGCGCTAGACCCCGAGAGG-3' | Rev 5' –CGTTTTGTTTGGTTGAAATGGCAC-G-3' |
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