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# Inactivation of CREB mediated gene transcription by HDAC8 bound protein phosphatase

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## Abstract

CREB activation via phosphorylation at serine 133 and resulting CREB mediated gene expression is a critical event which can have a significant effect on many cellular processes, including cell survival and plasticity. CREB can be activated by many kinases, for example, it can be phosphorylated by PKA, MAPK, and CaMKIV. The various signaling pathways leading to CREB activation have been extensively studied. On the other hand, CREB is inactivated by PP1 through dephosphorylation at S133 and not much attention has been paid to this aspect of the signaling pathway. It was shown recently that PP1 can be targeted to CREB, for efficient dephosphorylation, through PP1 binding protein HDAC1. In this study, we found that another class-I HDAC family protein, HDAC8, localized in the nucleus of HEK293 cells and also bound to both CREB and PP1. Expression of recombinant HDAC8 results in decreased CREB activation and CREB mediated gene transcription in response to forskolin application. Our study thus elucidated that more than one class-I HDAC family members can regulate the duration of CREB mediated gene transcription.

### Keywords

PP1; HDAC8; CREB; CRE-Luciferase reporter

CREB mediated gene transcription is critical for numerous cellular processes as diverse as glucose metabolism, cell survival and neuronal plasticity which are critical for learning and memory. Cells can respond to a variety of extra-cellular signals and activate CREB through phosphorylation at serine 133 by an array of kinases [1–6], including PKA, CaMKIV, and Msk. Phosphorylated CREB at S133 results in a conformational change in CREB which can then interact with CBP [7–9], which complexes with DNA transcriptional machinery through physical interaction with RNA helicase protein [6]. Thus activated CREB can bring RNA polymerase to the vicinity of genes with promoters having CREB binding consensus sequences or CREB responsive element (CRE), leading to new protein production.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2008.11.135.

Pharmacologic studies have established that PP1 is the CREB phosphatase in both non-neuronal [10] and neuronal cells [11]. The duration of CREB activation or the amount of gene transcription is thus also determined by the effect of PP1 activity on CREB phosphorylation [10, 11]. It was determined that rapid inactivation of CREB by PP1 will lead to no gene transcription [11–13] while much delayed CREB inactivation might lead to extra unwanted new protein synthesis which is not desirable and could lead to potential damage to cellular functions.

Even though signaling pathways of CREB activation by kinases have been extensively studied, the basic biological mechanism underlying CREB inactivation by non-discriminate phosphatase PP1 and its regulation is still poorly understood. In contrast to kinases, PP1 does not have good substrate specificity. PP1 interaction with different binding proteins not only localizes PP1 to different cellular compartments, it can also deliver PP1 to close proximity of potential substrates, increasing PP1 substrate specificity and catalytic efficiency. We have shown before that neurabin, a synaptic PP1 binding protein, localized PP1 to synaptic spines, determining its synaptic substrate specificity in synaptic transmission and plasticity [14–16]. On the other hand, the nuclear inhibitor of PP1 (NIPP1) and other PP1 binding proteins localize PP1 to nucleus [17–20]. Recent report indicates that another PP1 nuclear binding protein HDAC1 is critical for PP1's efficient action on CREB [17], elucidating again the critical importance of PP1 targeting by interacting proteins in achieving PP1 catalytic specificity.

Prior analysis of class-I HDAC proteins (HDAC1–3) indicates that both HDAC1 and HDAC2 bind to CREB while HDAC3 does not [17]. Moreover, only HDAC1 binds to PP1 while HDAC2 and HDAC3 does not bind to PP1 [21]. However, none of these earlier reports analyzed the new class-I HDAC member, HDAC8. In this study, we found that HDAC 8 binds to both CREB and PP1, inactivating CREB activation and decreasing CREB mediated gene transcription. Our study thus adds another critical regulator in the inactivation of CREB mediated gene transcription.

## Materials and methods

### Materials.

Forskolin was purchased from Sigma (Saint Louis, MO). Lipofectamine 2000 transfection reagent and a monoclonal antibody against epitope tag V5 were obtained from Invitrogen. Monoclonal antibody against HDAC8 was obtained from Sigma. The monoclonal anti-HA (hemagglutinin) antibody was purchased from Convance. Anti-CREB antibody was supplied by Zymed, and anti-phospho CREB antibody was supplied by Upstate. Protein G PLUS-Agarose and anti-PP1 antibody was obtained from Santa Cruz Biotechnology. DAPI nucleic acid stain was obtained for Molecular Probes. V5-HDAC8 and FLAG-HDAC1/2 are gifts of Dr. Stefan Kass (Janssen Research Foundation, Belgium) and Tso-Pang Yao (Duke University, USA), respectively.

### Cell culture and plasmids transfection.

HEK293 cells (from ATCC) were cultured in high glucose DMEM medium containing 10% fetal bovine serum and 1% penicillin/streptomycin. HEK293 cells, plated in 6-well plates grown to 80–90% confluency 18–20 h after plating, were transfected with 1.5 μg of DNA encoding HA-CREB or V5-HDAC8 as indicated using lipofectamine 2000. Assays were performed 36–48 h after transfection. For CREB activation and CRE-mediated luciferase reporter expression experiments, transfected cells were starved overnight in FBS-free medium.

### Western blotting, co-immunoprecipitation (co-IP) and pull-down assays.

Proteins were subjected to electrophoresis on SDS–PAGE, and then the separated proteins were transferred electrophoretically to polyvinylidene difluoride (PVDF) membranes, which were subsequently blocked in PBS plus 0.05% Tween 20 and 5% (w/v) non-fat milk. The membranes were then incubated with primary antibody at 4 °C overnight or 2 h at room temperature. After washing with PBST, the membranes were incubated with appropriate secondary antibodies for 1hr at room temperature. The immunoreactive proteins were visualized with chemiluminescence (GE healthcare UK limited). The membranes were stripped using Western Re-probe (G-Biosciences) reagent and re-probed with indicated antibodies.

HEK293 cells were lysed in 500 μl RIPA buffer (10 mM sodium phosphate pH 7.5, 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, and 0.1% SDS) plus protease inhibitor cocktail (Roche) for 1.5 h at 4 °C. After centrifugation at 13,000g for 20 min, the supernatant was divided into two aliquots with equal volume, one aliquot was incubated with normal mouse serum (NMS), and the other aliquot was incubated with 2 μg of indicated antibodies, together with 30 μl 1:1 slurry of protein G PLUS-aga-rose beads at 4 °C for 4 h. The beads were washed 3 times with RIPA buffer prior to solubilizing the immunoprecipitates in SDS sample buffer and analysis on SDS–PAGE.

One percent of Triton X-100 soluble cell lysates from transfected or non-transfected HEK293 cells were used for pull-down by microcystin-conjugated sepharose beads. Protein phosphatases and their binding proteins are eluted by SDS sample buffer and elutes were subject to Western blot analysis. The membranes were probed with HDAC8 or V5 antibody and re-probed with anti-PP1 antibody.

## Results and discussion

### HDAC8 forms complex with CREB in HEK293 cells

Two days after transfection of V5-HDAC8 and HA-CREB, HEK 293 cells were lysed for immunoprecipitation (IP) experiments. As shown in Fig. 1A, anti-HA antibody immunoprecipitated (IPed) HA-CREB efficiently (bottom panel). Interestingly, V5-HDAC8 (the band below IgG) was readily detected in HA-CREB immune complexes in a duplicate blot. IP with normal mouse serum (NMS) did not precipitate either V5-HDAC8 or HA-CREB (the band in the NMS column of the bottom panel is heavy chain of IgG, which is the same size as HA-CREB). Our results thus indicate that anti-HA antibody specifically pulled

down both HA-CREB and V5-HDAC8, suggesting that the expressed recombinant CREB and HDAC8 can interact with each other in HEK293 cells.

We then performed IP experiment on total soluble cell lysates from untransfected HEK293 cells. Endogenous HDAC8 protein was IPed by HDAC8 antibody, and CREB protein was readily detected in HDAC8 immunocomplexes, but not in control normal mouse serum immunocomplexes (Fig. 1B). In the reciprocal IP experiment with anti-CREB antibody (Fig. 1C), CREB protein was IPed, and HDAC8 protein was also readily co-IPed as shown in the duplicate blot. Our results thus convincingly demonstrated the assembly of CREB/HDAC8 complex in HEK293 cells.

## HDAC8 and CREB all are localized in nucleus

CREB is a known transcription factor, localized in nucleus. We confirmed this and also determined the cellular localizations of HDAC8 by performing immunofluorescence experiments. Non-transfected HEK293 cells and HEK293 cells transiently expressing V5-HDAC8 were stained with anti-CREB, anti-HDAC8 or anti-V5 antibody. They were counterstained with nucleic acid stain DAPI. As shown in supplemental Fig. 1A, CREB was localized in the nucleus and well co-localized with DAPI staining, consistent with the CREB known nucleus localization pattern. Immunostaining of endogenous HDAC8 indicates that HDAC8 is concentrated in nucleus, co-localized well with DAPI staining (Supplemental Fig. 1C), consistent with V5-HDAC8 staining result (Supplemental Fig. 1B). Our results thus indicate that endogenous CREB, HDAC8 as well as V5-HDAC8 are all localized in the nucleus, consistent with their postulated role in gene transcription.

However, there is a recent report that HDAC8 is localized in the cytosol in smooth muscle cells, binding to microtubule associated proteins, and has non-transcription related functions [22]. This is not what we observed in HEK293 cells because HDAC8 is clearly concentrated in the nucleus. While there are some faint staining of both HDAC8 and V5 in cytosol, HDAC8 is clearly mainly concentrated in nucleus. So even though we cannot rule out HDAC8 cytosolic expression in HEK293 cells, it would exist at a much lower expression level when compared with that in the nucleus.

## HDAC8 complexes with PP1 in HEK293 cells

It has been shown [17, 21] that HDAC family proteins show diversity in whether they can bind to PP1, a known CREB phosphatase in both neuronal and non-neuronal cells. We thus determined whether the new class-I HDAC family member, HDAC8, can complex with PP1. We used microcystin-conjugated sepharose beads to pull-down endogenous PP1 (Fig. 2A). HDAC2 is not, but HDAC1 is, co-pulled down with PP1 from HEK293 cells expressing recombinant FLAG-HDAC2 or FLAG-HDAC1, respectively (Fig. 2A). This is consistent with literature data [17, 21] that HDAC2 does not, but HDAC1 does, bind to PP1. Interestingly, in cells expressing V5-HDAC8, HDAC8 is co-pulled down with PP1 (Fig. 2A), indicating that recombinant HDAC8 can complex with PP1. Additionally, we performed microcystin pull-down on non-transfected HEK293 cells and we found that endogenous HDAC8 was also pulled down (Fig. 2B), indicating the endogenous HDAC8-PP1 complex association in HEK293 cells.

### Inhibitory effect of HDAC8 on CREB activation

We then explored the functional significance on CREB of HADC8 proteins in being able to complex with both CREB and PP1. To this end, we initiated CREB activation by incubating the HEK293 cells with 10 μM forskolin for a period ranging from 0 h to 6 h. HEK293 cells were transfected with either empty vector or HDAC8 encoding plasmids. Total cell lysates were then assayed for CREB activation by determining the CREB phosphorylation status at Ser133 using site-specific phosphor-CREB antibody. In control HEK293 cells transfected with empty vector, forskolin dramatically increased CREB phosphorylation at Ser133, peaked at 1 h, and then the phosphorylation decreased gradually overtime (3 and 6 h, Fig. 3A). However, in cells expressing V5-HDAC8, the CREB phosphorylation was decreased significantly at basal state, 1 h forskolin treatment and subsequent time points (Fig. 3A and B; $p < 0.05$). Total CREB did not show detectable changes in response to forskolin treatment and transfection. V5-HDAC8 expression is approximately similar for different time points. Our results thus suggest that HDAC8 binds to both CREB and PP1, bringing PP1 to the close proximity of CREB to dephosphorylate it at Ser133.

### Inhibitory effect of HDAC8 on CRE-mediated luciferase reporter expression

We have shown that HDAC8 has inhibitory effect on forskolin-induced CREB activation, and then we studied whether it has inhibitory effects in CRE-driven gene transcription. We co-transfected HEK293 cells with CRE-Luciferase construct and one of these constructs: empty vector or V5-HDAC8. After incubating the cells with 10 μM forskolin for 3 h, HEK293 cells were lysed and CRE-Luciferase expression was examined by Western blotting using luciferase antibody. CRE-Luc expression in HEK293 cells expressing V8-HDAC8 was decreased significantly to 91.37 ± 2.23% ($p < 0.01$) compared to HEK293 cells transfected with empty vector (Fig. 4). These results demonstrated that HDAC8 has inhibitory effect in CREB mediated gene transcription.

An emerging theme in both kinases and especially phosphatases is that targeting of these enzymes to close proximity of phosphor–proteins, through interaction with binding proteins, increases their substrate specificity and catalytic efficiency. For example, targeting of PKC by AKAP-79 selectively shift the dose dependence for PKC phosphorylation of GluR1 at S831 by 20 fold [23], making PKC a much more efficient and physiological relevant kinase in modulation of GluR1 current [23]. Protein phosphatase-2B (PP2B, also called calcineurin) needs targeting by AKAP79 for its physiological modulation on calcium channel function as well [24]. As to PP1, there are more than 100 binding proteins identified so far [25] and most of them exhibit a consensus motif for binding to PP1: R/K-X(0, 1)-I-X-F. However, examination of HDAC8 and HDAC1 sequence did not yield a R/K-X(0, 1)-I-X-F motif, nor a new type of FXX[R/K]X[R/K] motif [26]. But HDAC8 could potentially contain tandem of two new types of PP1 binding motif, RXXQ-[VIL]-[KR]-x-[YW] and K-[27]-ILK motifs [28], in its extreme C terminus (KIQQILNY and KGNLK). Future work is needed to confirm that HDAC8 complexes with PP1 through these two putative PP1 binding motifs.

CREB is activated by various kinases via phosphorylation at Ser133, which induces a conformational change in CREB and results in interaction with CBP and CREB mediated

gene transcription. Kinase pathways activating CREB have been extensively studied while mechanistic information on how CREB is inactivated is still lacking. Even though it is known that PP1 is the CREB phosphatase inactivating CREB, little was known until recently about the targeting mechanism of how PP1 is brought in to dephosphorylate CREB efficiently [17]. Our study now shows that HDAC1 homologue, HDAC8, can also target PP1 to the close physical proximity of CREB, suggesting that cells might use either one of them depending on the cellular expression level of HDAC1 and HDAC8. Interestingly, a close homologue of HDAC1, HDAC2, can only bind to CREB, but not PP1. We are currently testing the hypothesis that endogenous HDAC2 could exert a dominant interference effect on the roles of HDAC1 and HDAC8 in inactivating CREB. It suffices to say, dynamics of CREB activation and inactivation in response to extra-cellular activating signals could be drastically different in different cell types with different expression levels of HDAC1, 8 and 2.

In short, we have demonstrated that a new class-I HDAC family protein, HDAC8, can bind to both CREB and PP1, and function to inactivate CREB mediated gene transcription. Our study hence has added valuable results on the new class-I HDAC protein, HDAC8, in its CREB binding and PP1 targeting properties and the resultant effects on CREB activation via its phosphorylation at S133.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## Acknowledgments

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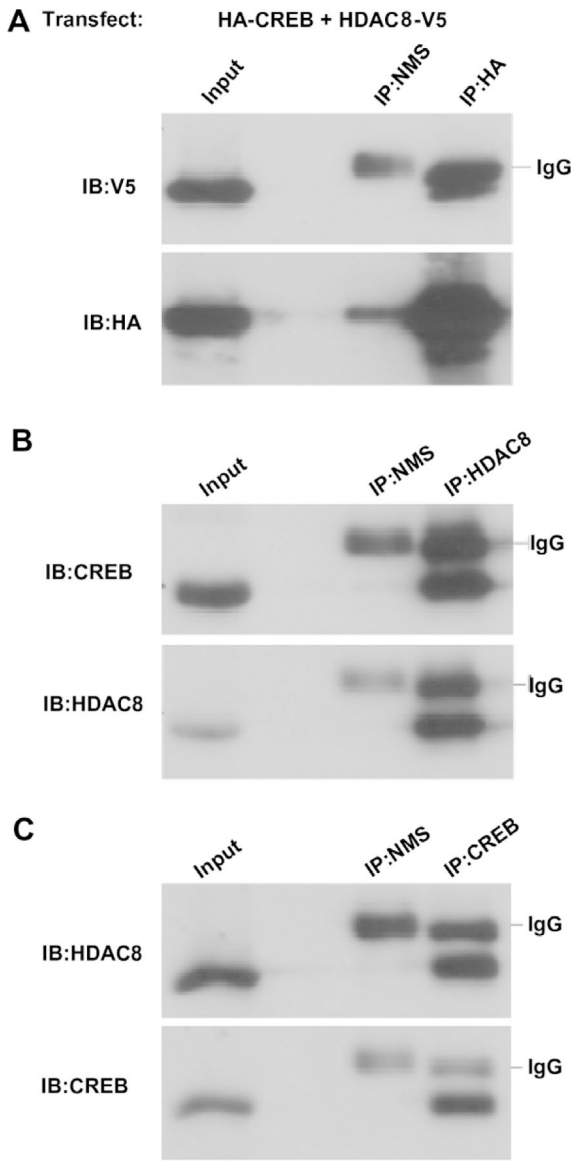


**Fig. 1.**

Assembly of CREB-HDAC8 complex in HEK293 cells. (A) Recombinant HDAC8 co-IPes with recombinant CREB. HEK293 cells cotransfected with HA-CREB and HDAC8-V5 were IPed using anti-HA antibody (IP: HA) (with normal mouse serum (IP: NMS) as negative control) as described in Materials and methods. The immunoprecipitates were immunoblotted (IB) with anti-V5 antibody to detect co-IP. Input indicates 10% of material used for IP experiment (same in B and C). (B,C) Endogenous CREB complexes with endogenous HDAC8. (B) Endogenous HDAC8 proteins were IPed using anti-HDAC8 antibody (with NMS used as control). The immunoprecipitates were immunoblotted with anti-CREB antibody to detect co-IP. The membrane was stripped and re-probed with anti-HDAC8 antibody to confirm successful IP. (C) Reverse IP experiment to those in (B). endogenous CREB proteins were IPed using anti-CREB antibody (with NMS as control). The immunoprecipitates were immunoblotted with anti-HDAC8 antibody to detect co-IP.

The membrane was stripped and re-probed with anti-CREB antibody to confirm successful IP.

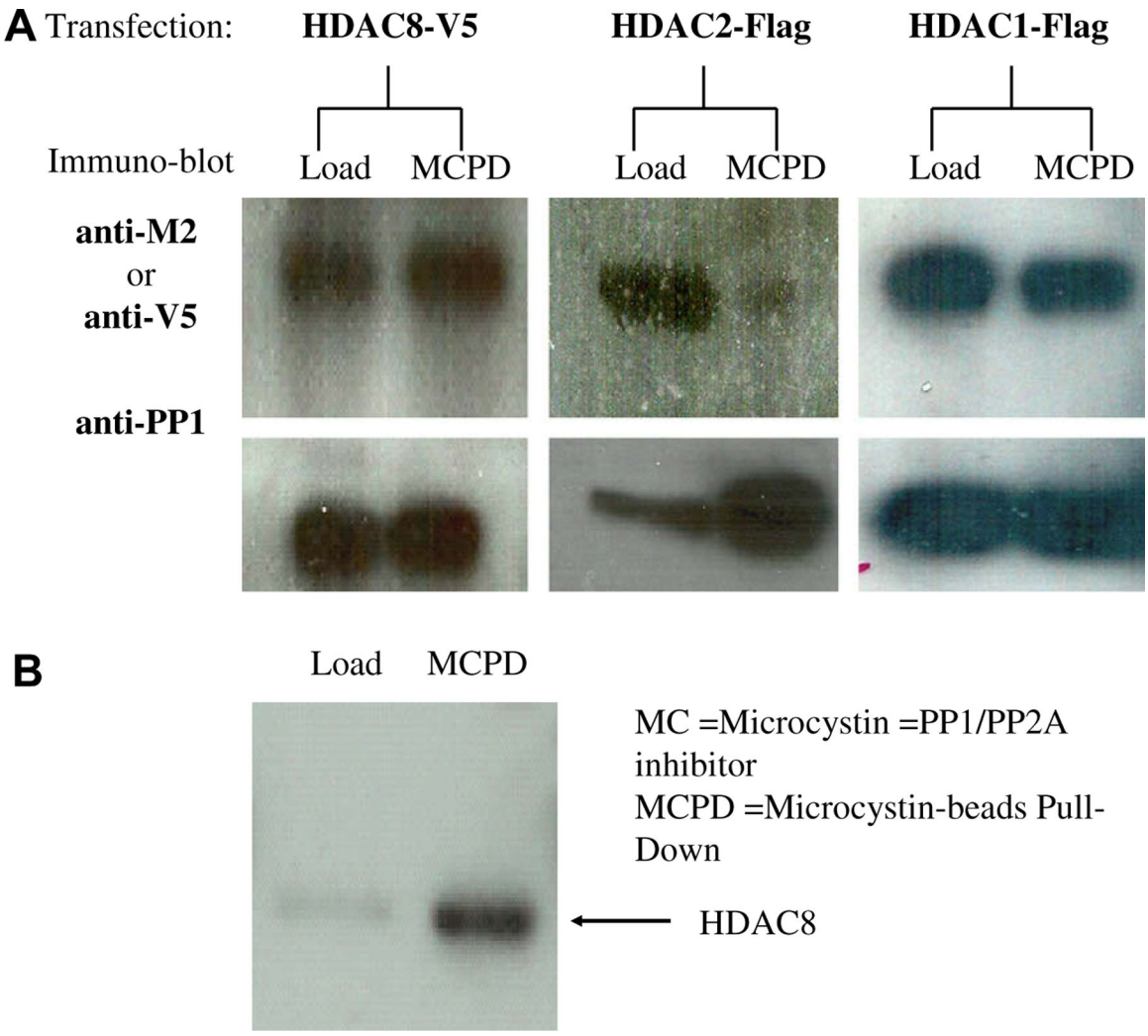


**Fig. 2.**

HDAC8 complexes with PP1. (A) HEK293 cells were transfected with FLAG-HDAC1/2 or HDAC8-V5. Soluble cell lysates were used for pull-down by microcystin (MC)-conjugated sepharose beads. PP1 and PP1 interacting proteins are eluted by SDS loading buffer and analyzed by Western blotting. The membranes were probed with anti-FLAG or V5 antibody. The blots were then re-probed with anti-PP1 antibody. (B) Soluble cell lysates from non-transfected HEK293 cells were subject to pull-down by microcystin-conjugated sepharose beads. Elutes were treated the same as in (A).

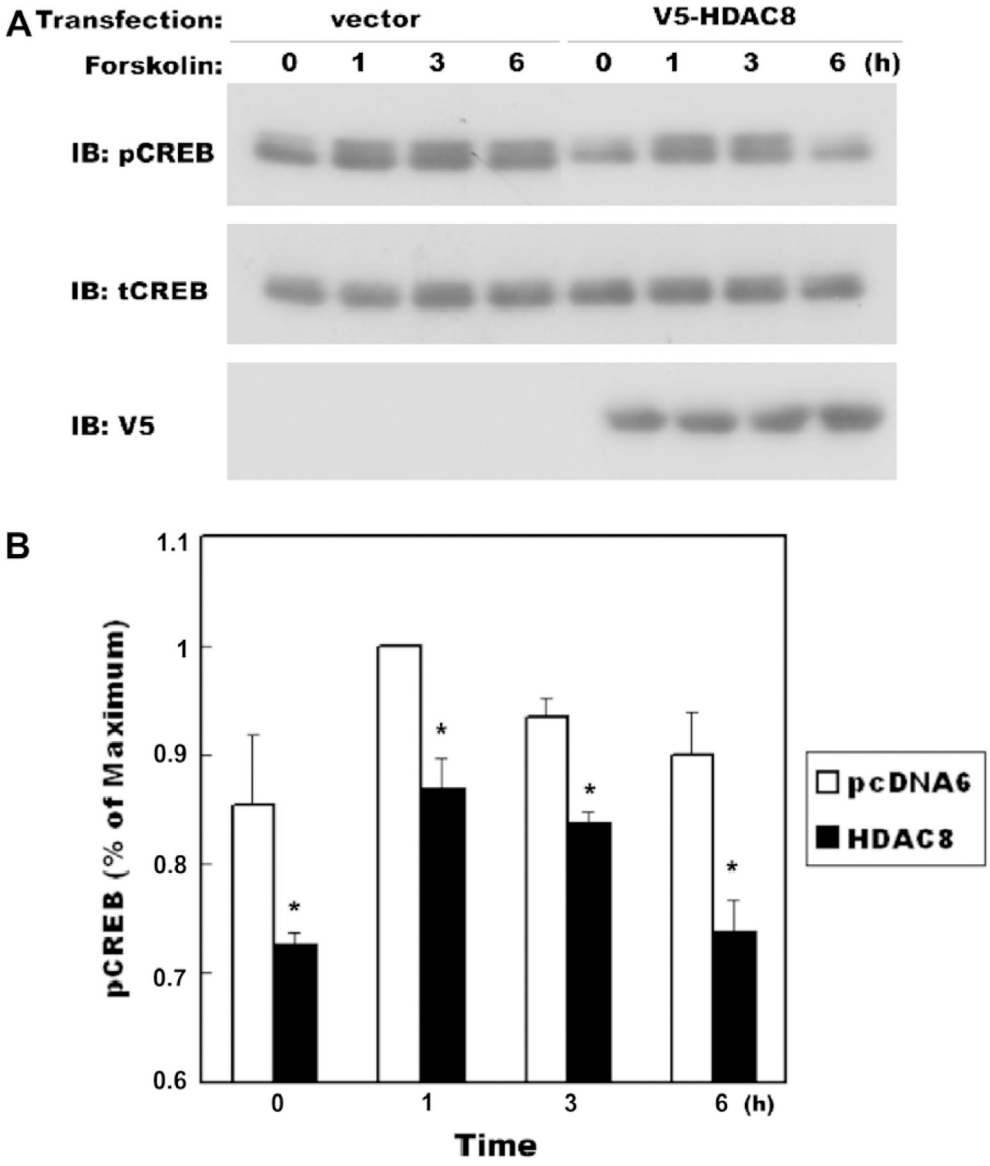


**Fig. 3.**

V5-HDAC8 decreases the forskolin-induced CREB activation in HEK293 cells. (A) HEK293 cells were transfected with plasmids encoding empty vector or V5-HDAC8 in 6-well plates. The transfected cells were re-plated to 12-well plates, to ensure equal protein levels, and starved in FBS-free medium overnight before stimulation. The cells were incubated in the absence or presence of 10 μM forskolin for indicated time (h) and the cells were then lysed on ice. Cell lysates were subjected to Western blotting analysis. The membranes were probed with anti-phospho-CREB antibody and then re-probed with anti-CREB antibody. The expression level of V5-HDAC8 was detected using anti-FLAG antibody. Shown is one representative experiment among three independent experiments. (B) Quantitation of all three experiments (*$p < 0.05$).

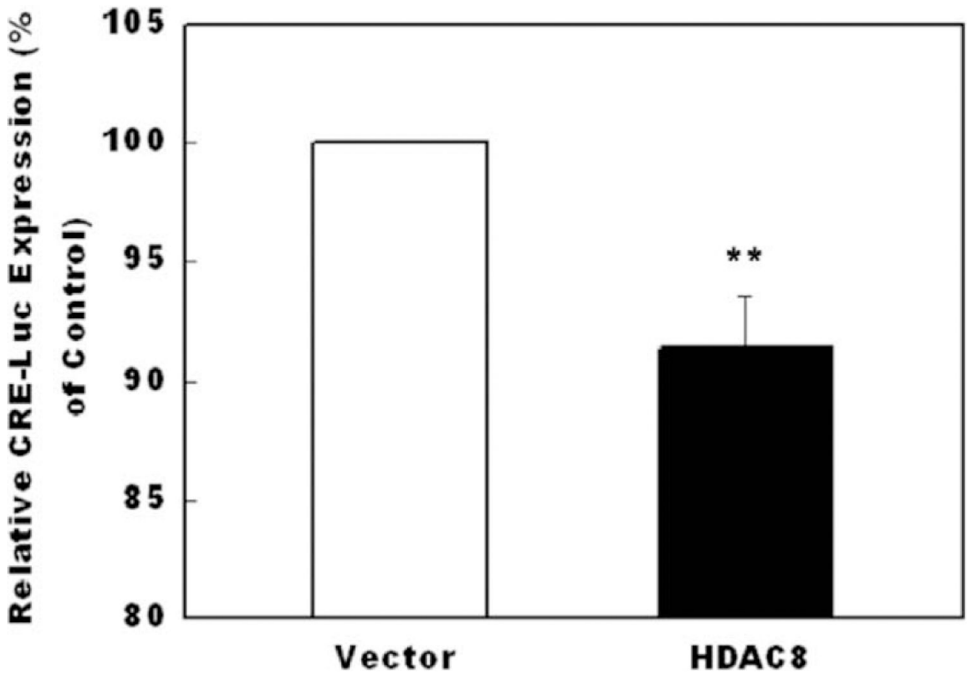


**Fig. 4.**

V5-HDAC8 expression inactivates CRE-driven luciferase reporter expression in HEK293 cells. HEK293 cells were transfected with 125 ng plasmids encoding empty vector or V5-HDAC8 plus 1 μg CRE-Luciferase in 12-well plates. The cells were silenced in FBS-free medium overnight before the cells were incubated in 10 μM forskolin for 3 h. Luciferase signals were analyzed as in Fig. 3 and quantitated using Metamorph software. Final data were represented as means ± SE ($n = 4$). $^{**}p < 0.01$ compared with vector control.