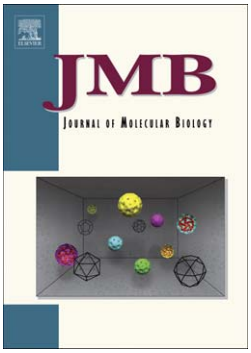


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# Arsenic-induced Activation of the Homeodomain Interacting Protein Kinase 2 (HIPK2) to cAMP-response element binding protein (CREB) Axis

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Running title: The HIPK2-CREB and PKA-CREB axes

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## ABSTRACT

CREB (cAMP-response element binding protein) plays key transcriptional roles in cell metabolism, proliferation, and survival. Ser133 phosphorylation by protein kinase A (PKA) is a well-characterized CREB activation mechanism. HIPK2 (homeodomain interacting protein kinase 2), a nuclear serine/threonine kinase, activates CREB through Ser271 phosphorylation; however, the regulatory mechanism remains uncharacterized. Transfection of CREB in HEK293 cells together with the kinase demonstrated that HIPK2 phosphorylated CREB at Ser271 but not Ser133, likewise PKA phosphorylated CREB at Ser133 but not Ser271, suggesting two distinct CREB regulatory mechanisms by HIPK2 and PKA. In vitro kinase assay revealed that HIPK2 as well as HIPK1 and HIPK3 directly phosphorylated CREB. Cells exposed to 10 μM sodium arsenite increased the stability of HIPK1 and HIPK2 proteins leading to CREB activation via Ser271 phosphorylation. Phospho-Ser271 CREB showed facilitated interaction with the TFIID subunit coactivator TAF4 assessed by immunoprecipitation. Furthermore, a focused gene array between cells transfected with CREB alone and CREB plus HIPK2 over empty vector-transfected control displayed 14- and 32-fold upregulation of CCNA1 (cyclin A1), respectively, while no upregulation by HIPK2 alone. These results suggest that the HIPK2-phospho-Ser271 CREB axis is a new arsenic-responsive CREB activation mechanism in parallel with the PKA-phospho-Ser133 CREB axis.


Key words: ATF1, HIPK1, CCNA1, CBP, TAF4

## Abbreviations

HIPK, homeodomain-interacting protein kinase

PKA, protein kinase A

CREB, cyclic AMP-responsive element-binding protein

ATF1, Activating transcription factor 1

CBP, CREB binding protein

TAF4, Transcription initiation factor TFIID subunit 4

CCNA1, Cyclin-A1

## INTRODUCTION

The CREB (cAMP-response element binding protein) transcription factor is a stimulus-induced phosphorylated protein regulated by a wide variety of cell signaling pathways such as growth factors, calcium, G-protein-coupled receptors, and neurotrophic factors [1]. Dysfunction and deregulation of CREB can cause cancer development and loss of long-term memory due to disorder of cell proliferation and neuronal plasticity [2-4]. For instance, CREB overexpression promotes leukemia cell growth and survival [5], and mutations in RSK2 (ribosomal S6 kinase 2) responsible for CREB phosphorylation or a coactivator of CREB, CBP (CREB binding protein) cause physical abnormalities and mental retardation, Coffin-Lowry syndrome [6] and Rubinstein-Taybi syndrome [7], respectively. Furthermore, deficiency of CREB in mouse impairs early development and disrupts neuron survival leading to neurodegeneration [8-11]. Thus CREB plays a key role in cell survival and integrity in various tissues.

CREB consists of four regulatory domains; two glutamine-rich domains (Q1 and Q2), a kinase-inducible domain (KID), and a basic leucine zipper DNA-binding domain (bZIP) [1]. The amino acid sequence is highly conserved in two other CREB family members ATF1 (activating transcription factor 1) and CREM (cAMP response element modulator) in both mouse and human. CREB plays a key role in transcriptional regulation of numerous genes via CRE (cAMP-response element) [12,13], where CREB activity is modulated by phosphorylation in conjunction with recruitment of cofactors such as p300/CBP [14-16], TAF4 (Transcription initiation factor TFIID subunit 4, also called $TAF_{II}130$) [17-19], and TORCs/CRTCs (Transducers of regulated CREB activity/CREB-regulated transcription coactivators) [20,21], through KID, Q2, and bZIP domains, respectively. Several kinases including protein kinase A (PKA) and $Ca^{2+}$/calmodulin

dependent protein kinase (CaMK) in cAMP/$Ca^{2+}$ signaling pathways are involved in CREB phosphorylation at Ser133 [22] that recruits p300/CBP to activate CREB-inducible gene [14,23]. Disruption of phosphorylation at Ser133 by expressing a CREB mutant containing a Ser133 to Ala substitution in mice caused somatotroph hypoplasia and dwarfism [24] and dilated cardiomyopathy [25]. However, given that the status of CREB Ser133 phosphorylation was not always correlated with CREB transcriptional function [12,26,27], and that individual CREB-inducible genes recruit distinct transcriptional coregulators and mechanisms [28,29], additional events along with CREB Ser133 phosphorylation seems to be involved in CREB activation in a stimulus-specific manner.

The nuclear HIPK (homeodomain interacting protein kinase) family, composed of HIPK1, HIPK2, and HIPK3, is a Ser/Thr protein kinase that shares evolutionary conserved domains with the DYRK (dual-specificity tyrosine-phosphorylated and regulated kinase) family [30,31]. In addition to these nuclear HIPK1-3, HIPK4 is a cytoplasmic and much smaller HIPK family member containing a different kinase domain from HIPK1-3[32]. Among the HIPK family members, HIPK2 has been most studied and characterized as a DNA-damage responsive kinase serving as both pro-apoptotic and anti-apoptotic functions in response to genotoxic and oxidative cell damage [33-37]. We recently found that HIPK2 phosphorylates CREB at Ser271, and ATF1 at Ser198, conserved amino acid sequences[38,39]. However, molecular characterization of HIPK2-phosphorylated CREB was limited. In this study, we attempted to uncover the role of CREB phosphorylation at Ser271 induced by HIPK2 on CREB transcriptional activity.

## RESULTS

### No cross-regulation of CREB/ATF1 phosphorylation by HIPK2 and PKA

In order to characterize the roles of HIPK2 and other nuclear HIPK family members in CREB and ATF1 regulation, we developed rabbit polyclonal antibodies that recognize the region containing the human phospho-Ser271 CREB and phospho-Ser198 ATF1 (Fig. 1a). Given the fact that PKA is one of the major protein kinases that phosphorylate CREB at Ser133 and ATF1 at Ser63 (Fig. 1a), we used a commercially available phospho-specific CREB Ser133 (cross-reactive to phospho-Ser63 ATF1) antibody as a control of the following experiments. First, to verify the specificity of our phospho-Ser271 CREB and phospho-Ser198 ATF1 antibodies, we transiently cotransfected HIPK2 (wild type (wt) or kinase dead) with CREB [wt, Ser133 to Ala mutant (S133A), Ser271 to Ala mutant (S271A)] or ATF1 [wt or Ser198 to Ala mutant (S198A)] into HEK293 cells, and phosphorylated CREB was detected by Western blotting with these phospho-specific antibodies. As shown in Fig. 1b, overexpression of wt CREB alone was enough to increase Ser271 phosphorylated CREB (lane 2), suggesting the presence of active CREB Ser271 kinase(s) in the cells. The phosphorylation of CREB Ser271 was enhanced by cotransfection of wt HIPK2 (lane 3) but not by kinase dead HIPK2 (lane 6). S133A mutant CREB was also efficiently phosphorylated by HIPK2 (lane 4) but S271A mutant CREB was not phosphorylated (lane 5). Likewise, phosphorylation of transfected wt ATF1 was detected by our phospho-Ser198 ATF1 antibody (Fig. 1c, lane 2) and it was enhanced by cotransfection with HIPK2 (Fig. 1c, lanes 5), while S198A mutant ATF1 was not phosphorylated (Fig. 1c, lanes 3 and 6). These results suggest that HIPK2 induces phosphorylation of CREB at Ser271 and ATF1 at Ser198, and that our phospho-specific CREB and ATF1 antibodies are working in Western blotting.

To verify HIPK2 is a direct CREB and ATF1 kinase as well as to address whether HIPK2 and PKA share the phosphorylation sites in CREB and ATF1, recombinant HIPK2 or PKA was incubated with His-tagged CREB (wt, S133A, or S271A, Fig. 1d) or ATF1 (wt, S63A, S198A, Fig. 1e) *in vitro*, and phosphorylation was detected by Western blotting using phospho-specific CREB and ATF1 antibodies. The phospho-Ser271 CREB antibody detected wt and S133A mutant CREB incubated with HIPK2 (Fig. 1d top, lanes 2 and 5) but did not detect S271A mutant CREB (lane 8). The phospho-Ser133 CREB antibody detected none of these CREB samples incubated with HIPK2 (Fig. 1d, middle). Instead, it detected wt CREB and S271A mutant incubated with PKA (lanes 3 and 9) but failed to detect S133A mutant (lane 6). Interestingly, none of CREB proteins incubated with PKA gave rise to detectable Ser271 phosphorylated CREB (Fig. 1d top, lanes 3, 6, and 9). These results show that HIPK2 primarily phosphorylated CREB at Ser271 but not Ser133, likewise PKA phosphorylated CREB at Ser133 but not Ser271, suggesting that the axes of PKA-CREB and HIPK2-CREB are in parallel. This is also true in ATF1 phosphorylation, in which HIPK2 phosphorylated Ser198 of ATF1 wt and S63A but not S198A (Fig. 1e top, lanes 2, 5, and 8), while PKA but not HIPK2 phosphorylated ATF1 at Ser63 (Fig. 1e middle). Collectively, these results suggest that HIPK2 is a direct CREB Ser271 and ATF1 Ser198 kinase and that HIPK2 and PKA do not share the phosphorylation sites.

We next asked whether the phosphorylation of CREB and ATF1 is specific to HIPK2 or also catalyzed by two other nuclear HIPK family members, HIPK1 and HIPK3. First, we performed in vitro kinase assay by incubating His-tagged CREB or ATF1 protein (wt or phosphorylation-deficient Ser to Ala mutants) with recombinant HIPK1, HIPK2, HIPK3, or PKA, followed by

Western blotting with our phospho-Ser271 CREB or Ser198 ATF1 antibody. As shown in Fig. 1f, all three HIPKs phosphorylated wt and S133A mutant CREB but failed to phosphorylate CREB S271A mutant. Likewise, wt and S63A mutant ATF1, but not S198A mutant ATF1, were phosphorylated by HIPK1 and HIPK2 (Fig. 1g). In this condition, HIPK2 showed more efficient ATF1 phosphorylation than HIPK1, and HIPK3 showed little ATF1 kinase activity. However, we detected ATF1 Ser198 phosphorylation by HIPK3 when we used more recombinant HIPK3 in the kinase reaction (Fig. 1h). Furthermore, cotransfection of CREB or ATF1 together with HIPK1, HIPK2, or HIPK3 in HEK293 cells showed that all three HIPKs phosphorylated CREB at Ser271 (Fig. 1i) and ATF1 at Ser198 (Fig. 1j). These results suggest that CREB and ATF1 are phosphorylated not only by HIPK2 but also HIPK1 and HIPK3.

**Screening of CREB-regulated genes enhanced by HIPK2.**

We previously reported that HIPK2 activates CREB transcription function in CRE-luciferase reporter assays[39]. To identify CREB-regulated genes that are further induced by HIPK2, we analyzed mRNA expression in HEK293 cells transfected with control (empty vector), CREB alone, and CREB plus HIPK2 (Fig. 2a) using the cAMP/calcium signaling PCR array. This array contains 84 genes responsive to cAMP or calcium. When compared between control and CREB alone transfection, 5 genes were more than 2-fold upregulated (Fig. 2b) and 10 genes were more than 2-fold downregulated (Supplemental Table I). When compared control with CREB plus HIPK2, 14 genes were more than 2-fold upregulated (Fig. 2b) and 11 genes were more than 2-fold downregulated (Supplemental Table I). Four genes, *CCNA1* (cyclin A1), *PTGS2*, *JUNB*, and *SGK1*, were commonly upregulated in CREB transfection and cotransfection with HIPK2 (Fig. 2b), which was also confirmed by individual q-PCR (Fig. 2c). Among

commonly upregulated 4 genes, CCNA1 mRNA was significantly increased by cotransfection of CREB and HIPK2 compared to CREB alone transfection. In contrast, expression of *PTGS2*, *SGK1*, and *JUNB* genes were induced by CREB alone but not further increased by HIPK2 (Fig. 2c). We also verified expression of 9 genes only induced by CREB plus HIPK2 in the PCR array analysis (Fig. 2b). Among them, mRNA expression levels of only *TACR1*, *CALB1*, *NR4A2*, and *FOS* were highest by coexpression of CREB and HIPK2. Of note, HIPK2 alone showed no effect on these mRNA levels (Fig. 2c). These results suggest that Ser271-phosphorylated CREB induced by HIPK2 has target genes for transcriptional activation.

**Stabilization of endogenous HIPK2 protein by arsenic stress**

Among nuclear HIPK family members, HIPK2 was characterized as a stress-responsive protein kinase activated by ultraviolet, ionizing radiation, and DNA damaging chemicals [37], while HIPK1 and HIPK3 remain elusive [40]. Here we found that arsenic is an activator of HIPK2. To characterize the regulatory mechanism of HIPK2 along with HIPK1 and HIPK3 under arsenic stress, we expressed Flag-tagged HIPKs in HEK293 cells, treated them with sodium arsenite for 3 and 6 hr, and analyzed expression of HIPK proteins in the cytoplasmic and nuclear fractions by Western blotting. HIPK2 was characterized and understood as a nuclear protein kinase [30,41]; however, transfected Flag-HIPK2 expressed both in the cytoplasm and nucleus (Fig. 3a). In this condition, HIPK2 accumulated in the nucleus with concomitant decrease in the cytoplasmic HIPK2 following sodium arsenite treatment for 3 and 6 hr (Fig. 3a), suggesting the possibility of cytoplasmic to nuclear shuttling of transfected and overexpressed HIPK2. We also observed nuclear accumulation of HIPK1 (Fig 3a, middle) but not HIPK3 (Fig. 3a, right) with marginal decrease in the cytoplasmic HIPK1 and HIPK3 levels in this condition. To verify accumulation

of endogenous HIPK2 in cells exposed to sodium arsenite, SW480 cells which was reported to express relatively higher HIPK2 [41], were treated with sodium arsenite for 2.5, 5, and 10 hr and endogenous HIPK2 was detected by Western blotting using a HIPK2 monoclonal antibody kindly provided by the Dr. Schmitz's group [42]. Although basal levels of endogenous HIPK2 were undetectable in either the cytoplasm or nucleus in SW480, arsenic stress increased nuclear accumulation of HIPK2 in a time- and dose-dependent manner (Fig. 3b). We could not detect endogenous HIPK1 protein due to the lack of a good HIPK1 antibody. Given that HIPK2 protein expression is primarily regulated at the post-translational level [43] and that we did not observe expression changes in HIPK2 mRNA (HIPK1 and 3 mRNAs as well) after sodium arsenite treatment (Fig. 3c), these results suggest that the increased protein stability is involved in the nuclear accumulation of endogenous HIPK2 following arsenic treatment.

We next asked whether HIPK2 kinase activity is increased in cells treated with sodium arsenite or another environmental toxicant cadmium by immunoprecipitation of HIPK2 followed by *in vitro* kinase assay. However, we were not able to immunoprecipitate endogenous HIPK2 in SW480 cells enough to induce phosphorylation of myelin basic protein (MBP) or CREB as a substrate of *in vitro* kinase assays (not shown). As an alternative approach, we expressed Flag-HIPK2 in HEK293 cells, treated them with sodium arsenite or cadmium chloride for 1.5 hr, and immunoprecipitated Flag-HIPK2 with an anti-Flag antibody. After incubation of the immunoprecipitates with His-CREB protein as a HIPK2 substrate, we performed Western blotting with anti-phospho-Ser271 CREB antibody. As shown in Fig. 3d, both sodium arsenite and cadmium chloride activated HIPK2 kinase activity at 1.5 hr. Taken together, we concluded

that these environmental toxicants activate HIPK2 by enhancing HIPK2 kinase activity and HIPK2 protein nuclear accumulation.

**Endogenous CREB Ser271 and ATF1 Ser198 phosphorylation by sodium arsenite**

To address whether phosphorylation of endogenous CREB at Ser271 and ATF1 at Ser198 is actually induced by xenobiotic stress, SW480 cells were treated with sodium arsenite (Fig. 4a, left) or cadmium chloride (Fig. 4a, right) for 12 hr, and phosphorylation of CREB and ATF1 was detected by Western blotting with phospho-specific antibodies. Phosphorylation of CREB at Ser271 was induced by 10 μM sodium arsenite, and the higher concentration of sodium arsenite induced ATF1 phosphorylation at Ser198 along with phosphorylation of CREB at Ser133 and ATF1 at Ser63 (Fig. 4a, left). Similar results were obtained following cadmium chloride treatment (Fig. 4a, right). To characterize the role of arsenic-induced CREB phosphorylation in its transcriptional activity, we employed the GAL4-luciferase reporter assay by transfection of GAL4CREB-wt, -S133A, or -S271A mutant into SW480 cells followed by sodium arsenite treatment for 24 hr. Arsenic treatment caused 3-fold increase in wt CREB-mediated luciferase expression, while S271A mutation partially impaired CREB activation by arsenic treatment (Fig. 4b), suggesting that phosphorylation of CREB at Ser271 induced by arsenic is at least in part involved in the activation of CREB. S133A mutant CREB also showed partial impairment of CREB activation (Fig. 4b), consistent with the result that sodium arsenite treatment induced phosphorylation of CREB at Ser133 (Fig. 4a). In addition to SW480 cells, we employed stable HeLa GAL4-luciferase reporter (HLR) that showed more striking activation of CREB by sodium arsenite treatment (Fig. 4c) than SW480 (Fig. 4b), and knockdown of HIPK1-3 strongly reduced CREB-mediated luciferase expression (Fig. 4c). Taken all together, these results suggest that

arsenic stress stabilizes HIPK2 protein in the nucleus, induces phosphorylation of CREB at Ser271, and activates CREB transcription function.

**Interaction of Ser271 phosphorylated CREB with TAF4 ($TAF_{II}130$).**

CREB transcriptional activity is regulated by coactivators such as CBP through Ser133 phosphorylation [14,15] and TAF4 through a Q2 glutamine-rich domain[17-19]. To address whether these interactions are affected by phosphorylation of CREB at Ser271, we cotransfected CREB (wt, S271E and S133E phosphomimetic mutants) and CBP or TAF4 in HEK293 cells and performed immunoprecipitation/Western blotting to assess their interactions. First, to characterize whether S271E CREB is a conformational mimic of phosphor-Ser271 CREB, we transiently transfected wt or S271E CREB and carried out immunoprecipitation with the phospho-Ser271 CREB antibody. Indeed, phosphomimetic S271E mutant CREB was successfully immunoprecipitated by the phospho-S271 CREB antibody and also directly detected by Western blotting (Fig. 5a). By contrast, phosphomimetic S133E mutant CREB was neither immunoprecipitated (Fig. 5a) nor detected by Western blotting (Fig. 5b) with a phospho-Ser133 CREB antibody, in which a positive control sample prepared from cells transfected with wt CREB and treated with forskolin (FSK) showed increased phospho-Ser133 CREB in the same Western blot (Fig. 5b). These results are consistent with the report that S133E phosphomimetic CREB is not equivalent to phosphorylated CREB at Ser133 [44]. This fact did not allow us to compare S271E CREB to S133E CREB for their interactions with CBP or TAF4. After transfection of CREB S271E alone or cotransfection with HA-CBP (Fig. 5c) or HA-TAF4 (Fig. 5d), cell lysates were immunoprecipitated with HA antibody followed by Western blotting with a CREB antibody. Although phosphomimetic S271E CREB showed no changes in its interaction

with CBP compared to wt CREB (Fig. 5c), it showed increased interaction with TAF4 compared with wt CREB (Fig. 5d). We also detected that the immunoprecipitate of TAF4, a subunit of the TFIID complex, contains endogenous TATA-binding protein (TBP), another component of the TFIID complex (Fig. 5d). Furthermore, we verified the interaction between TAF4 and phosphorylated CREB at Ser271 by cotransfection with HIPK2. After transfection of wt CREB and HA-TAF4 with or without HIPK2, cell lysates were immunoprecipitated with HA antibody followed by Western blotting with anti-phospho-Ser271 CREB or CREB antibody. HIPK2 cotransfection induced phosphorylation of CREB at Ser271 and increased immunoprecipitation of TAF4 (Fig. 5e). These results suggest that phosphorylation of CREB at Ser271 may utilize distinct mechanisms of CREB activation from Ser133 phosphorylation through different interactions with coactivators.

## DISCUSSION

In addition to the canonical CREB phosphorylation at Ser133 in the KID (kinase inducible domain), the KID is known to be phosphorylated at multiple sites by casein kinases during cell cycle [45], by CaMKII in calcium influx [46], and ATM (ataxia-telangiectasia-mutated) under genotoxic stress [47]. However, these phosphorylation sites in the KID serve as the inhibitory modification of CREB transcriptional activity [47]. HIPK phosphorylation sites of CREB at Ser271 and ATF1 at Ser198 are adjacent to the C-terminal from these KID phosphorylation clusters and localized between the second glutamine-rich (Q2) and basic/b-zip domains. Recently, phosphorylation of CREB at Ser270 and Ser271 by cyclin-dependent kinase 1 (CDK1) during cell cycle G2/M was reported [48]. This seems to be a little different from CREB phosphorylation by HIPK2 because we previously shown that HIPK2 is able to phosphorylate CREB only at Ser271 (ATF1 only at Ser198) [39]. No phosphorylation of CREB at Ser270 by HIPK2 is consistent with the fact that HIPK2 belongs to the DYRK (dual-specificity tyrosine-regulated kinase) family [49] that preferentially phosphorylates Ser/Thr flanked by Pro [37]. CREB Ser270 is adjacent to Ala269 and Ser271 and not conserved in ATF1 (Fig. 1a). It was previously reported that DYRK1 and DYRK3 phosphorylate and activate CREB during neuronal differentiation in hippocampal progenitor cells [50] and hematopoietic cell survival [51], respectively. Interestingly, both DYRK1 and DYRK3 in these studies were shown to phosphorylate CREB at Ser133 either directly [51] or through PKA-dependent pathways [50]. It is noteworthy that our results suggest that there is no cross-regulation of CREB and ATF1 phosphorylation by HIPK2 and PKA; namely, HIPK2 cannot phosphorylate CREB Ser133 (ATF1 Ser63) and PKA cannot phosphorylate CREB at Ser271 (ATF1 Ser198) (Fig. 1). Therefore, PKA to CREB Ser133 and HIPK2 to CREB Ser271 axes are in parallel, and external

stimuli to activate the HIPK2-CREB Ser271 axis may be more specific to environmental and xenobiotic stressors such as ultra violet (UV), ionizing irradiation, DNA-damaging chemicals that are known to activate HIPK2 [37], and some metalloids and metals such as arsenic and cadmium shown in this study.

Catalytic activity of HIPK2 is regulated by autophosphorylation of Tyr354 in mouse HIPK2 (equivalent to Tyr361 in human HIPK2) in the activation loop [52,53] and Thr880/Ser882 (in human HIPK2) [54], along with HIPK2 protein stability via the ubiquitin E3 ligase Siah1 [55] and WSB-1 [56]. Our observation of HIPK2 protein accumulation in the nucleus following arsenic exposure (Fig. 3) may be mediated through these mechanisms involving dissociation of HIPK2-Siah1 or -WSB1 E3 ligase complex. In addition to HIPK2 protein stabilization, HIPK2 catalytic activity was enhanced at earlier time point (90 min) after treatment with arsenic or cadmium (Fig. 3d). Thus, we understand that phosphorylation of CREB at Ser271 can be induced by HIPK2 when it is activated by two mechanisms; autophosphorylation and/or increased nuclear accumulation of HIPK2 in a toxicant- and exposure time-dependent manner. HIPK1 and HIPK3 may be activated through the similar mechanisms. The autophosphorylated human HIPK2 Tyr361 in the activation loop is highly conserved in HIPK1 (Tyr352) and HIPK3 (Tyr359) and shares the mechanism of kinase activation via autophosphorylation [57]. HIPK1 was also stabilized under arsenic stress (Fig. 3a) and may be regulated by the same or related HIPK2 E3 ligases, but further study will be necessary to elucidate the commonality of protein stability regulation among the HIPK family.

To understand the mechanism of transcriptional regulation by phospho-Ser271 CREB, we tried to compare phosphomimetic Ser271 to Glu CREB (S271E) to wt CREB or phosphomimetic Ser133 to Glu CREB (S133E) in their abilities to interact with coactivator proteins. Our phosphomimetic CREB S271E was indistinguishable from HIPK2-phosphorylated CREB at Ser271 in terms of its retarded migration in SDS-PAGE and successful immunoprecipitation and Western blotting with the phospho-Ser271 CREB antibody (Fig. 5a). In contrast, CREB S133E was not recognized with a widely used phospho-Ser133 CREB antibody in our immunoprecipitation and Western blotting (Fig. 5a). Later we realized that the Ser133 to Glu substitution in CREB was previously characterized as harboring a more flexible KID and is conformationally different from phosphorylated CREB at Ser133 [44]. Therefore, we compared S271E with wt CREB for their interactions with CBP and TAF4, in which S271E CREB exhibited no significant changes for interaction with CBP but enhanced interaction with TAF4 (Fig. 5c-d). This was confirmed by the interaction between HIPK2-induced Ser271 phosphorylated CREB and TAF4 (Fig. 5e).

The activation mechanisms of CREB-regulated genes seem to be diverse. Given the fact that CREB dimerizes with other b-zip family members including ATF1 [58], both of which are subject to phosphorylation at multiple Ser/Thr sites by the canonical and stress signaling pathways leading to preferential recruitment of a specific set of coactivators including CBP/p300 and TORC2 (transducer of regulated CREB) [29,59]. In addition, HIPK2 phosphorylates p300 [60] and CBP [61] that activate p300 and CBP transcriptional coactivator functions. Although we did not observe enhanced interaction between CREB S271E and CBP in transfected cells,

activation of HIPK2 by arsenic and DNA stress may not only facilitate CREB-TAF4 interaction but also activate p300/CBP through multiple sites of phosphorylation as reported [60,61].

The focused cAMP/calcium signaling PCR array in Fig. 2 shed light on genes upregulated by CREB and those showing the trend of further augmentation by HIPK2. *c-FOS*, Calbindin including *CALB1*, and NR4A orphan nuclear receptors including *NR4A2* are involved in neuronal cell survival against neuropathological stress such as ischemia [62-64]. *TACR1* (Tachykinin Receptor 1) known as neurokinin 1 receptor (NK1R) modulates morphine reward and anxiety behavior in the mouse amygdala neurons [65]. The recent study showed that HIPK2 protein level was downregulated by elevated amount of Amyloid β (Aβ), a hallmark of Alzheimer's disease [66]. These results suggest the possibility of impairment of HIPK2-CREB axis in neurodegeneration.

The activity of CREB is also associated with cancer progression via the induction of downstream genes including cyclins such as cyclin A1 and cyclin D1 [67]. Cyclin A1 is a regulator of G1/S or G2/M cell cycle progression [68,69] and upregulation of cyclin A1 has been implicated as a driver of progression of various cancer types including bladder cancer, prostate cancer, and acute myeloid leukemia [70-72]. We compared the overall survival of the bladder urothelial carcinoma patients between two groups with high cyclin A1 expression (top 25 %) and low expression (bottom 25 %) using TCGA database and cBioPortal [73,74]. The overall survival of high cyclin A1 group was significantly shorter than low cyclin A1 group (Supplemental Figure 1a), and the overall survival of high HIPK2 group showed the tendency of shorter span compared to low HIPK2 group (Supplemental Figure 1b). We also found the positive correlation between

cyclin A1 and HIPK2 mRNA levels (Spearman correlation: 0.291, P value=2.74e-09) in the bladder urothelial carcinoma (Supplemental Figure 1c). Given the association of arsenic exposure and increased bladder cancer risk [75] along with our observation of predominant upregulation of cyclin A1 (CCNA1) by HIPK2 and CREB (Fig. 2), the arsenic-activated HIPK2-phosphoSer271CREB-cyclin A1 axis may be involved in arsenic-induced bladder cancer development and malignancy.

In summary, the pathway of HIPK2 leading to phosphorylation of CREB at Ser271 is a new CREB activation mechanism in parallel with the canonical PKA-phospho-Ser133 CREB axis. Among representative cAMP/calcium-regulated 84 genes, cyclin A1 mRNA is predominantly induced by HIPK2-induced phospho-Ser271 CREB. This work also provides evidence that arsenic and cadmium induces CREB Ser271 phosphorylation, in which the catalytic activity and/or stability of HIPK2 was increased. The potential mechanism of the transcriptional activation of CREB via Ser271 phosphorylation is its enhanced interaction with TAF4 and associated TFIID complex.

## MATERALS AND METHODS

### Cell culture

HEK293 cells, human embryonic kidney cell line transformed with adenovirus 5 DNA (ATCC, CRL1573, Manassas, VA) were cultured in Eagle's minimum essential media (MEM) supplemented with 2 mM L-glutamine, Eagle's BBS, 0.1 mM nonessential amino acids, 1 mM sodium pyruvate, and 10 % FBS (Mediatech, Manassas, VA). SW480 cells, human colon adenocarcinoma cell line provided by Dr. J. Ninomiya-Tsuji, were cultured in Dulbecco's modified Eagle's medium (DMEM) with 2 mM L-glutamine and 10 % FBS. The PathDetect HeLa Luciferase Reporter (HLR) cells (#800050, Agilent, La Jolla, CA) were cultured in DMEM with 2 mM L-glutamine and 10 % FBS. Cells were cultured at 37°C, 5% CO2 in a humidified atmosphere.

### Plasmids, DNA Transfection, and siRNA Transfection

pCMVCREB, pFA2-CREB (wild type (wt) and point mutants), and pCMVATF1 plasmids carry a cDNA for full-length human CREB and ATF1, respectively, cloned into the SmaI site of pCMV vector and the EcoRI/BglII site of pFA2 vector (Stratagene, Santa Clara, CA). pQE-CREB, pQE-ATF1, and pCMV-PKA plasmids were described previously[38]. pCMVFlag-HIPK1 and pCMVFlag-HIPK3 were constructed by cloning the full-length human HIPK1 cDNA (pCMV6-XL4HIPK1, SC127889, OriGene, Rockville, MD) and HIPK3 cDNA (a gift from Dr. William Hahn and David Root, pDONR223-HIPK3, #23467, Addgene, Cambridge, MA) into the EcoRI/EcoRV site and the EcoRI/SalI site of pCMVFlag vector, respectively. pCMVFlag-HIPK2 plasmids (wt and kinase dead (kd) mutant K221R) were kindly provided by Dr. J. Ninomiya-Tsuji. pFR-luc ($GAL4_5$-TATA-luciferase) and pRL-SV40 (SV40 promoter-Renilla

luciferase) reporter plasmids were purchased from Stratagene and Promega, respectively. pcDNA3β-Flag-CBP-HA (N-terminal Flag, C-terminal HA-tagged mouse CBP, a gift from Dr. Tso-Pang Yao, plasmid #32908) and CMV-HA-hTAF4 (N-terminal HA-tagged human TAF4, a gift from Dr. Naoko Tanese, plasmid #17983) were obtained from Addgene. Transient DNA transfection was carried out by electroporation (X-Cell, Bio-Rad Laboratories, Hercules, CA) with an optimized preset condition by Bio-Rad for HEK293 cells (square wave, 25 msec, 110 V). Cells were suspended in 100 μL of media containing plasmid DNA in a cuvette with a 0.2-cm gap. For the assays of protein-protein interactions, pCMVCREB plus pcDNA3β-Flag-CBP-HA or CMV-HA-hTAF4 plasmids were cotransfected into 80 % confluent HEK293 cells using polyethylenimine (MW-25K, Aldrich) (final concentration: 5 μg/mL). For luciferase reporter assays, plasmid DNA transfection into SW480 cells was carried out with TransIT-X2 Transfection Reagent (Mirus Bio LLC, Madison, WI) according to the manufacturer's instructions. Mixture of pFR-Luc, pFA2 or pFA2-CREB (wt, S133A, or S271A), and pRL-SV40 as a transfection internal control, was simultaneously transfected into SW480 cells in a 24-well plate. For knockdown of HIPKs, siRNA for human HIPK1 (SI00288008, Qiagen), human HIPK2 (J-003266-12, GE Healthcare Dharmacon Inc., CO), human HIPK3 (SI00287805, Qiagen, Valencia, CA), or Non-Targeting siRNA (D-001210-01, GE Healthcare Dharmacon Inc.) was transfected into HLR cells by Lipofectamine RNAiMax (Thermo Fisher Scientific, Rockford, IL).

**Phospho-Ser271 CREB and phospho-Ser198 ATF1 antibodies**

Two rabbits each were immunized once a week for five weeks with KLH-conjugated phospho-Ser271 CREB peptide (VMASS$^{phospho}$PALPTQPAE) or phospho-Ser198 ATF1 peptide (PGTVVMTS$^{phospho}$PVTLTSQ), and the antibody titers from three batches of week-6, -8, and -10

were tested by ELISA using 200 ng of the antigen phosphopeptide and 5,000 fold dilution of sera (IMGENEX/Novus Biologicals, Littleton, CO). The 10-week sera, which showed the highest titer in ELISA as well as Western blotting, was used throughout this study. For Western blotting, both CREB and ATF1 phospho-antibodies were 500~1,000-fold diluted with 5% skim milk in 0.1% Tween/TBS (Tris-buffered saline) and incubated at 4$^{o}$C overnight. We also used anti-phospho-CREB (Ser271) antibody (CP4161, ECM Biosciences, Versailles, KY).

**Western blotting and Immunoprecipitation**

For Western blotting, whole cell lysates were prepared from cells lysed in lysis buffer containing 100 mM Tris (pH7.4), 100 mM NaCl, 1 % Triton X-100, 0.1 % SDS, 0.5 % deoxycholate, 10 % glycerol, 1 mM EDTA, 1 mM EGTA, 2 mM $Na_3VO_4$, 1 mM NaF, 20 mM $Na4P_2O_7$, 0.1 mM DTT, and protease inhibitor cocktail (Set I, Calbiochem EMD Millipore, Billerica, MA). Cytosolic and nuclear fractionations were isolated using a nuclear extraction kit (Active Motif, Carlsbad, CA). For immunoprecipitation, cells were lysed in PC+100 buffer (20 mM Hepes (pH 7.9), 100 mM KCl, 0.2 mM EDTA, 5 mM MgCl2, 0.1 % Nonidet P-40, 20 % glycerol, protease inhibitor cocktail, phosphatase cocktail) [23]. Protein concentration was determined using the Bio-Rad Protein Assay reagent (Bio-Rad). Cell lysates were incubated with anti-CREB (EMD Millipore, Billerica, MA), anti-phospho-CREB (Ser271) (ECM Biosciences), and protein G plus A agarose (Calbiochem, EMD Millipore) or HA antibody conjugated-agarose (Thermo Scientific, Rockford, IL). Immunoprecipitated proteins were separated on 7.5% or 10 % SDS-PAGE, transferred to polyvinylidene difluoride (PVDF) membrane (Immobilon-P, EMD Millipore or #88518, Thermo Scientific) and incubated at 4°C overnight with primary antibodies for our phospho-CREB (Ser271) or phospho-ATF1 (Ser198), phospho-CREB (Ser271) (ECM

Biosciences), CREB (sc-240X, Santa Cruz Biotechnology), ATF-1 (sc-243, Santa Cruz Biotechnology), phospho-CREB (Ser133) (#9191, Cell signaling Technology, Danvers, MA), HIPK2 kindly provided by Dr. Schmitz [42], Flag (F1804, Sigma, St. Louis, MO), HA (HA.11, Biolegend, Dedham, MA), LaminA/C (4C11, Cell Signaling Technology), Lamin B (NA12, Oncogene Research Products, MA), or β-actin (AC-15, Sigma Aldrich), and TBP (sc-421, Santa Cruz Biotechnology). After incubation with secondary antibodies conjugated with horseradish peroxidase, proteins were visualized using an ECL detection kit (HyGLO, Denville Scientific, Metuchen, NJ) or ECL prime (GE Healthcare).

**In vitro kinase assay**

His-tagged wt or mutant CREB/ATF-1 proteins were expressed in E. coli and purified through Ni-NTA agarose according to the instructions of pQE vector system (Qiagen). Purified His-CREB or -ATF1 was incubated with 30 ng of recombinant HIPK1, HIPK2, HIPK3 (SignalChem, BC, Canada) or 0.1 ng of PKA (Millipore) for 2-3 hr at 30°C in the kinase buffer (10 mM HEPES, pH 7.4, 5 mM MgCl2, 1 mM DTT) followed by Western blotting with anti-phospho-specific CREBSer271 or ATF1Ser198 antibody, or CREB Ser133 phospho-specific antibody (Cell Signaling Technology). To measure HIPK2 activity in cells under arsenic or cadmium exposure, pCMVFlag or pCMVFlagHIPK2 was transfected into HEK293 cells, treated with sodium arsenite or cadmium chloride for 1.5 hr, and whole cell lysates were immunoprecipitated with anti-Flag antibody, incubated with His-CREB protein at room temperature for 30 min, and subjected to Western blotting with anti-phospho-Ser271 CREB antibody.

**Luciferase reporter assay**

SW480 cells were plated into a 24-well plate using a half of cell suspension prepared from confluent cells in 100 mm dish. After 1 day, cells were transfected with luciferase reporter plasmids using TransIT-X2 reagent for 22-24 hr and the medium was replaced with fresh growth medium containing 0 or 50 μM of $NaAsO_2$. Cells were incubated for 24 hr, lysed in 50 μL of Passive lysis buffer (Promega, Madison, WI), and luciferase assays were performed using Dual Luciferase Assay kit and GloMax 20/20 (Promega). Firefly luciferase expression was normalized by Renilla luciferase activity. HLR cells were plated into a 24-well plate using a half of cell suspension prepared from confluent cells in 100 mm dish. After 1 day, cells were successively transfected with pFA2-CREB plasmid and siRNAs using TransIT-X2 reagent and Lipofectamine RNAiMax, respectively. After 24 hr, the medium was replaced with fresh growth medium containing 0 or 50 μM of $NaAsO_2$. Cells were incubated for 10 hr, lysed in 100 μL of Passive lysis buffer, and luciferase assays were performed using Luciferase Assay kit and GloMax 20/20. Firefly luciferase expression was normalized by protein concentration.

**RNA Purification and real time q-PCR**

Total RNA was purified using TRI reagent RT (Molecular Research Center, Cincinnati, OH) 24 hr after siRNA transfection. For real-time q-PCR, 300 ng of total RNA was reverse-transcribed with iScript cDNA Synthesis Kit (Rio-Rad). cDNAs were mixed with iTaq Universal SYBR Green Supermix (Bio-Rad) and specific primers for human HIPK2 (QT00051485, Qiagen), human β-actin and GAPDH (HHK-1, Real Time Primers, LLC, PA), or the other primers as listed below (Sigma). q-PCR was performed with the CFX96 qPCR System (Bio-Rad) and data was normalized with β-actin or GAPDH as an internal control gene. The following human primer sequences were used (5' to 3'):

HIPK1 forward primer: TCTCAGTGCCGGAACAAAAAC

HIPK1 reverse primer: CCCTCCAGGTCTGTAGACATATT

HIPK3 forward primer: TCACAAGTCTTGGTCTACCCA

HIPK3 reverse primer: CACATAGGTCCGTGGATAGTTTC

CALB1 forward primer: GGCTCCATTTCGACGCTGA

CALB1 reverse primer: GCCCATACTGATCCACAAAAGTT

CCNA1 forward primer: GAGGTCCCGATGCTTGTCAG

CCNA1 reverse primer: GTTAGCAGCCCTAGCACTGTC

FOS forward primer: CCGGGGATAGCCTCTCTTACT

FOS reverse primer: CCAGGTCCGTGCAGAAGTC

FOSB forward primer: GCTGCAAGATCCCCTACGAAG

FOSB reverse primer: ACGAAGAAGTGTACGAAGGGTT

JUNB forward primer: ACGACTCATACACAGCTACGG

JUNB reverse primer: GCTCGGTTTCAGGAGTTTGTAGT

NR4A2 forward primer: GTTCAGGCGCAGTATGGGTC

NR4A2 reverse primer: CTCCCGAAGAGTGGTAACTGT

PTGS2 forward primer: CTGGCGCTCAGCCATACAG

PTGS2 reverse primer: CGCACTTATACTGGTCAAATCCC

SGK1 forward primer: AGGATGGGTCTGAACGACTTT

SGK1 reverse primer: GCCCTTTCCGATCACTTTCAAG

TACR1 forward primer: CTAACACCTCGGAACCCAATC

TACR1 reverse primer: CCACAATGACCGTGTAGGCAG

**Human cAMP / $Ca^{2+}$ signaling pathway Finder $RT^2$ profiler PCR array**

The Human cAMP/$Ca^{2+}$ Pathway Finder RT² Profiler PCR Array contains 84 genes that are known to be responsive to cAMP or calcium elevation (Qiagen). Many of these genes are regulated by PKA-induced phosphorylation of CREB at Ser133 signaling to cAMP-response element along with serum-response element and calcium-response element. This PCR array was employed to identify the genes that are also regulated and even further activated by HIPK2-induced phosphorylation of CREB at Ser271. Total RNA was purified using TRI reagent RT (Molecular Research Center, Cincinnati, OH) 24 hr after transfection of pCMVCREB alone, pCMVFlag-HIPK2 alone, or both of pCMVCREB and pCMVFlag-HIPK2 into HEK293 cells. cDNA was reverse transcribed using the RT2 First Strand Kit (Qiagen) and the human cAMP/$Ca^{2+}$ Pathway PCR Array was performed with the CFX96 real-time PCR system (Bio-Rad) according to manufacturer's instructions. The array data was analyzed by the company's online software $RT^2$ Profiler PCR Array Data Analysis v3.5 (Qiagen). To normalize gene expression ($2^{-\Delta Ct}$) and determine the fold change between groups ($2^{-\Delta\Delta Ct}$), a housekeeping gene (GAPDH) was used as an internal control.

**Statistical analysis**

Data were analyzed statistically by two-tailed Student's t-test and one-way factorial analysis of variance (ANOVA), followed by the post hoc Tukey or Holm test.

**The Cancer Genome Atlas (TCGA) Database analysis**

The Clinical dataset of bladder urothelial carcinoma was obtained from the TCGA database and cBioPortal [73,74], including overall survival and mRNA expression z-Scores (RNA Seq V2

RSEM) of cyclin A1 and HIPK2. Cancer samples with top 25 % of cyclin A1 or HIPK2 mRNA level were identified as the groups of high cyclin A1 and/or high HIPK2, and samples with bottom 25% of cyclin A1 or HIPK2 levels were identified as the groups of low cyclin A1 and/or low HIPK2. Correlation between overall survival time and cyclin A1/HIPK2 was evaluated by Kaplan-Meier curve and log-rank test (Pairwise comparisons using Generalized Wilcoxon test, P value adjustment method: Holm).

## FIGURE LEGENDS

**Figure 1. No cross-regulation of CREB/ATF1 phosphorylation by HIPK2 and PKA.**

a) Phosphorylation sites of human CREB and ATF1 by PKA and HIPK2. b) HIPK2 [wild type (wt), kinase dead (kd)] and CREB (wt, S133A, S271A) were cotransfected into HEK293 cells. Phosphorylation of CREB at Ser271 was analyzed by Western blotting with a phospho-Ser271 CREB antibody. c) wt HIPK2 and ATF1 (wt or S198A) were transfected into HEK293 cells. Phosphorylation of ATF1 at Ser198 was analyzed by Western blotting with a phospho-Ser198 ATF1 antibody. d) His-tagged recombinant CREB (wt, S133A, S271A) was incubated with recombinant HIPK2 or PKA. Phosphorylation of CREB at Ser271 and Ser133 was analyzed by Western blotting. e) His-tagged recombinant ATF1 proteins (wt, S63A, S198A) were incubated with recombinant HIPK2 or PKA. Phosphorylation of ATF1 was analyzed by Western blotting. f) His-tagged recombinant CREB proteins (wt, S133A, S271A) were incubated with 30 ng of recombinant HIPK1, HIPK2, or HIPK3, and phosphorylation of CREB at Ser271 was analyzed by Western blotting. g, h) His-tagged recombinant ATF1 proteins (wt, S63A, S198A) were incubated with 30 ng of recombinant HIPK1, HIPK2, or HIPK3 in g), or with 30 or 100 ng recombinant HIPK3 in h). Phosphorylation of ATF1 at Ser198 was analyzed by Western blotting. i, j) Flag-tagged HIPK plasmids (1, 2, and 3) were cotransfected with wt CREB in i) or wt ATF1 in j) into HEK293 cells, and phosphorylated CREB at Ser271 and ATF1 at Ser198 were analyzed by Western blotting.

**Figure 2. Screening of CREB-regulated genes enhanced by HIPK2.**

a) HEK293 cells were transfected with wt CREB, Flag-HIPK2, or both together and incubated for 24 hr. Whole cell lysates were prepared and phosphorylation of CREB at Ser271 was

analyzed by Western blotting. b) Total RNA was isolated from HEK293 cells used in a) that were transfected with wt CREB alone and wt CREB plus Flag-HIPK2. Gene expression was analyzed with human cAMP / $Ca^{2+}$ signaling pathway Finder $RT^2$ profiler PCR array (Qiagen). More than 2-fold upregulated genes are listed in the comparisons between control and CREB alone, and control and CREB plus HIPK2 (left). A gray or a white circle indicates the numbers 2-fold induced or repressed genes by CREB alone or CREB plus HIPK2, respectively, compared to control. Overlapping area indicates commonly regulated genes in two groups (left). c) Individual q-PCR was performed to analyze mRNA expressions of CALB1, CCNA1, FOS, JUNB, NR4A2, PTGS2, SGK2, and TACR1. Data are mean + s.d. (*$p<0.05$, #$p<0.01$ by one-way ANOVA followed by post hoc Holm test) of 3 to 5 independent experiments, normalized by mRNA expression level of GAPDH relative to control.

**Figure 3. Stabilization of endogenous HIPK2 protein in response to arsenic exposure.**

a) HEK293 cells were transfected with Flag-tagged HIPK2, HIPK1, or HIPK3, treated with 50 μM of $NaAsO_2$ for 0, 3, and 6 hr, and separated into cytoplasmic and nuclear fractions. Expression of Flag-tagged HIPKs was analyzed by Western blotting with anti-Flag antibody. Arrowheads indicate Flag-tagged HIPKs. b) SW480 cells were treated with 50 μM $NaAsO_2$ for 2.5, 5, or 10 hr (left) or 1, 10, 50, or 100 μM $NaAsO_2$ for 5 hr (right). Endogenous HIPK2 protein was analyzed in the cytoplasmic and nuclear fractions by Western blotting with a HIPK2 monoclonal antibody [42]. c) SW480 cells were treated with 1, 10, or 50 μM $NaAsO_2$ for 5 hr (left) or 10 μM for 10 hr. Expression of HIPK1-3 mRNA was analyzed by q-PCR. Data are mean + s.d. of three independent experiments, normalized by mRNA expression level of $\beta$-actin relative to control. d) Flag-tagged HIPK2 was transfected into HEK293 cells, treated with 50

μM $NaAsO_2$ or $CdCl_2$ for 1.5 hr. Whole cell lysates were immunoprecipitated with anti-Flag antibody, incubated with recombinant His-CREB protein and subjected to Western blotting with anti-phospho-Ser271 CREB antibody.

**Figure 4. Endogenous CREB Ser271 and ATF1 Ser198 phosphorylation in response to arsenic exposure**.

a) SW480 cells were treated with 10 and 50 μM of $NaAsO_2$ or $CdCl_2$ for 12 hr, and the nuclear fraction was subjected to Western blotting with antibodies of phospho-CREB Ser271, phospho-ATF1 Ser198, phospho-CREBSer133/-ATF1Ser63. Anti-CREB and -ATF1 Western blots are shown along with anti-lamin B Western as a nuclear fraction loading control. b) SW480 cells were transfected with pFR-Luc reporter together with pFA2 or pFA2-CREB (wt, S133A, or S271A) and pRL-SV40 Renilla luciferase as a transfection internal control. Cells were treated with 50 μM of $NaAsO_2$ for 24 hr and subjected to dual luciferase assays. Firefly luciferase expression was normalized by Renilla luciferase activity. Data are mean + s.d. (*$p<0.05$, #$p<0.01$ by one-way ANOVA followed by post hoc Tukey test) of four independent experiments relative to wt CREB without $NaAsO_2$ treatment. c) HeLa Luciferase Reporter (HLR) cells were transfected with pFA2-wt CREB plasmid with negative control siRNA (Con) and siRNAs targeting HIPK(1-3). Cells were treated with 50 μM of NaAsO2 for 10 hr and subjected to luciferase assays. Data are mean + s.d. (#$p<0.01$ by one-way ANOVA followed by post hoc Tukey test) of three independent experiments relative to untreated control. Expression of HIPK1-3 mRNA was analyzed by q-PCR. Data are mean + s.d. (#$p<0.01$ by unpaired two-tailed Student's t-test) of three independent experiments, normalized by mRNA expression level of GAPDH relative to control siRNA.

**Figure 5. Interaction of Ser271 phosphorylated CREB with CBP and TAF4 ($TAF_{II}130$)**

a) HA-CREB (wt, S271E, S133E) was transiently transfected into HEK293 cells, and whole cell lysates were immunoprecipitated with anti-phospho-Ser271 (ECM) or anti-phospho-Ser133 CREB antibody followed by Western blotting with anti-HA antibody. Expression of CREB (phosphorylated and total) along with β-actin (as a loading control) in whole cell lysate was analyzed by Western blotting (input). b) CREB (wt, S133E) was transiently transfected, and one set of cells transfected with wt CREB were treated with 10 μM forskolin for 13 hrs. Whole cell lysates were subjected to Western blotting with a phospho-Ser133 CREB or CREB antibody. β-actin as a protein loading control. c) CREB (wt, S271E) and HA-CBP plasmids were transfected into HEK293 cells. Whole cell lysates were prepared, and immunoprecipitated with anti-HA agarose followed by Western blotting with a CREB antibody and anti-HA antibody. Expression of CREB (total and phosphorylated) and HA-CBP along with β-actin in the whole cell lysate was analyzed by Western blotting (input). d) CREB (wt, S271E) and HA-TAF4 plasmids were transfected into HEK293 cells, and whole cell lysates immunoprecipitated with anti-HA agarose were subjected to Western blotting with anti-CREB, anti-TATA binding protein (TBP), and anti-HA antibodies. Whole cell lysates were subjected to Western blotting with anti-CREB, phospho-Ser271 CREB, phospho-Ser133 CREB, TBP, and β-actin antibodies. e) CREB(wt), HA-TAF4, and HIPK2 plasmids were transfected into HEK293 cells, and whole cell lysates immunoprecipitated with anti-HA-agarose were analyzed by Western blotting with antibodies for phospho-Ser271 CREB, CREB, and HA. Expression of CREB (total and phosphorylated) and HA-TAF4 along with β-actin in the whole cell lysate was analyzed by Western blotting (input).

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Figure 1 Hashimoto K and Tsuji Y

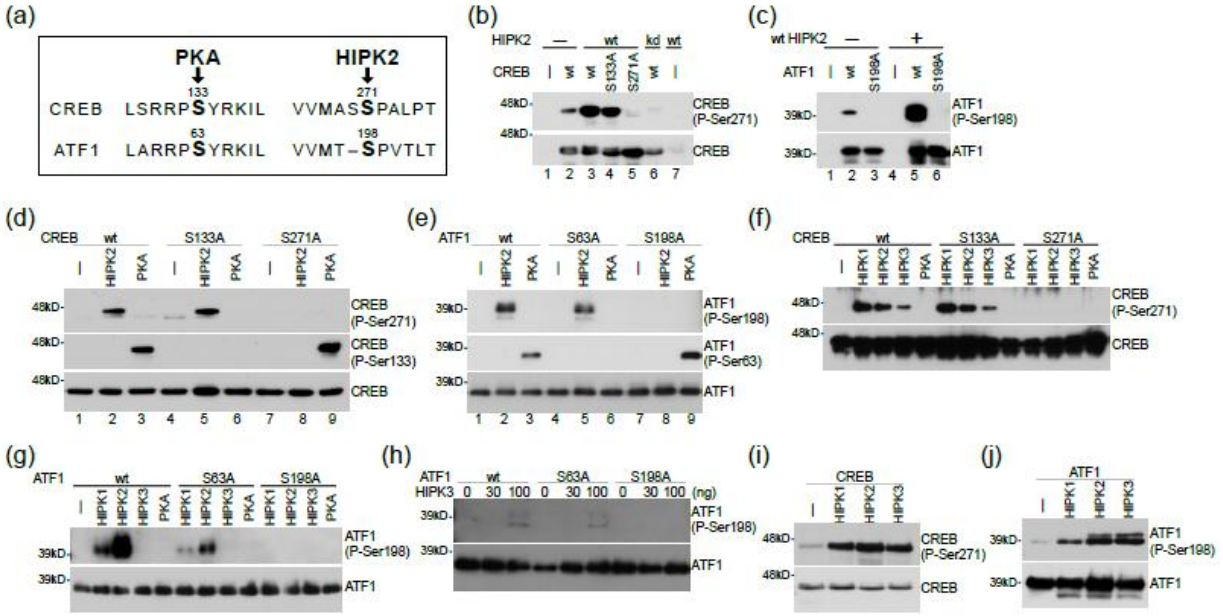
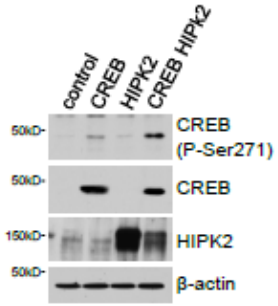


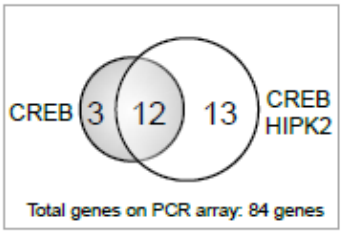
Figure 2 Hashimoto K and Tsuji Y

(a)




(b)

| > 2-fold upregulated genes | | | |
|---|---|---|---|
| CREB | | CREB & HIPK2 | |
| Symbol | Fold Change | Symbol | Fold Change |
| **CCNA1** | **7.4146** | **CCNA1** | **37.7036** |
| **PTGS2** | **7.3642** | **PTGS2** | **12.614** |
| **JUNB** | **2.2077** | TNF | 5.1396 |
| **SGK1** | **2.1447** | TACR1 | 4.5173 |
| DDIT3 | 2.0193 | CALB1 | 2.8891 |
| | | **INHBA** | 2.6157 |
| | | NR4A2 | 2.5543 |
| | | **SGK1** | **2.5476** |
| | | EGR1 | 2.459 |
| | | CYR61 | 2.4227 |
| | | FOSB | 2.3472 |
| | | FOS | 2.2819 |
| | | **JUNB** | **2.2369** |
| | | **IL6** | 2.196 |




(c)

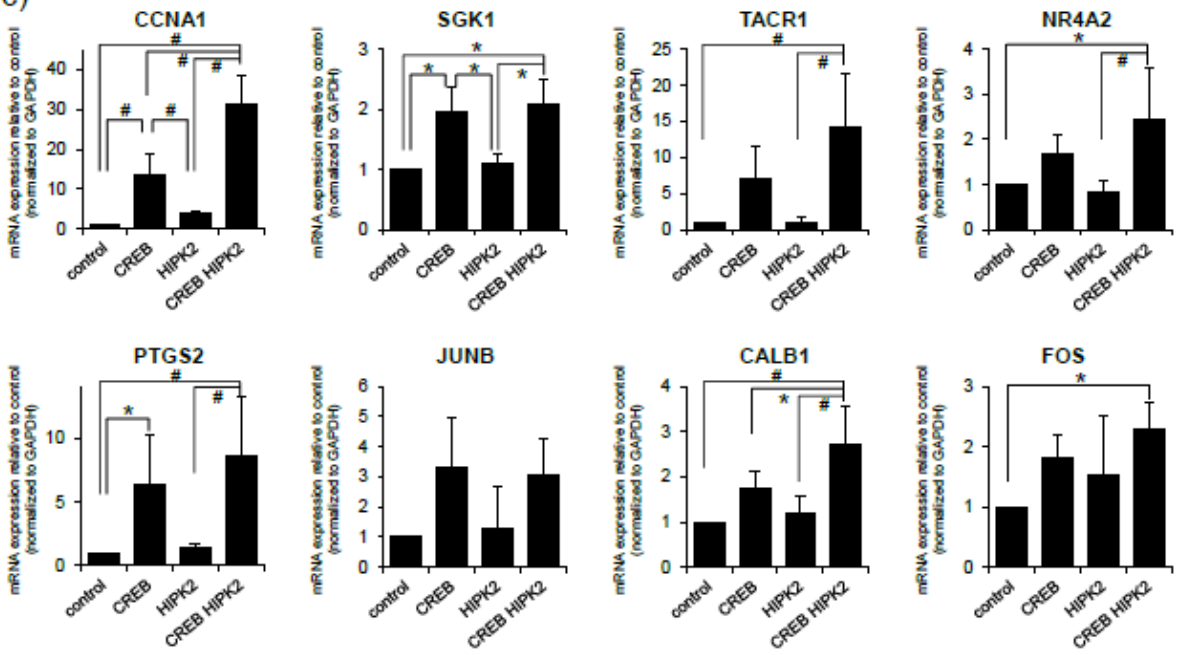


Figure 3 Hashimoto K and Tsuji Y

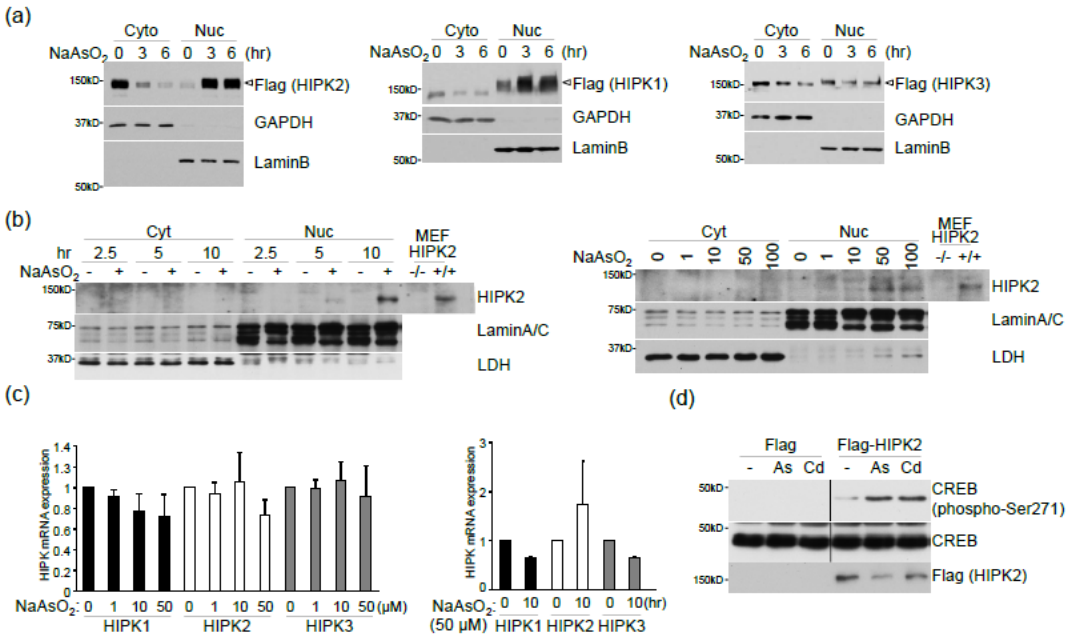


Figure 4 Hashimoto K and Tsuji Y

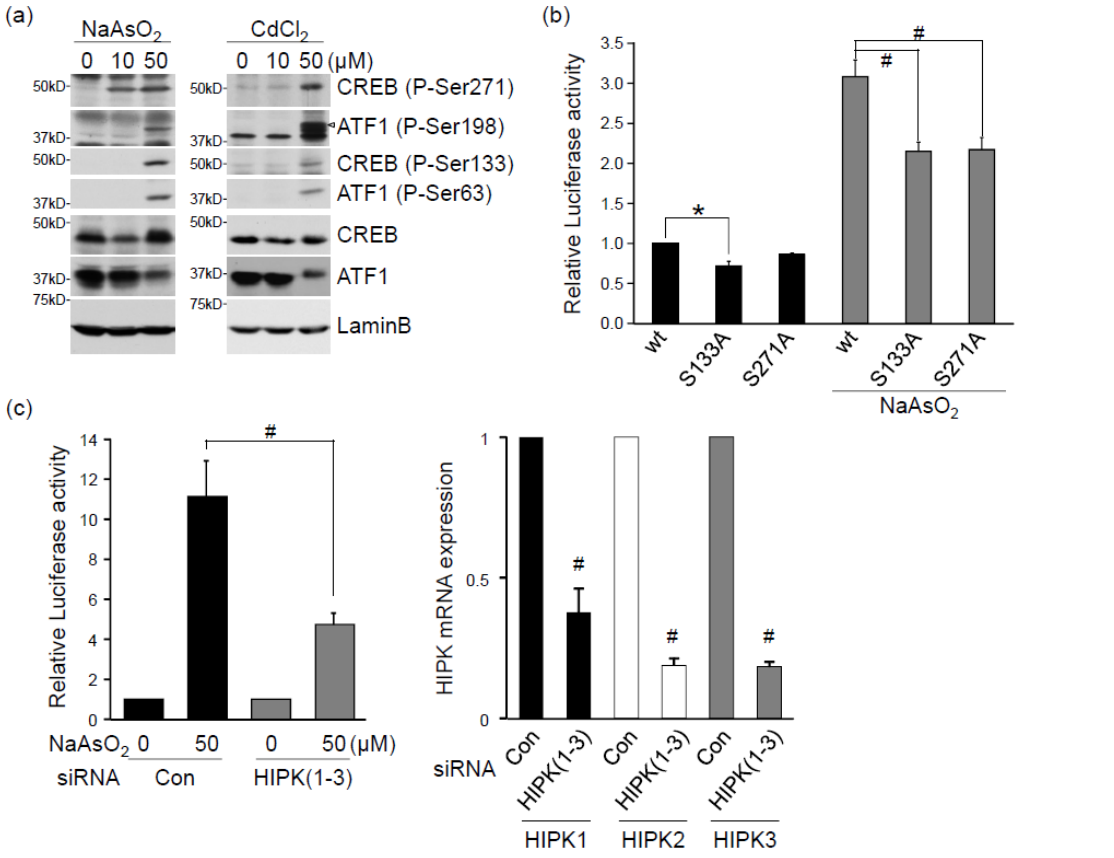
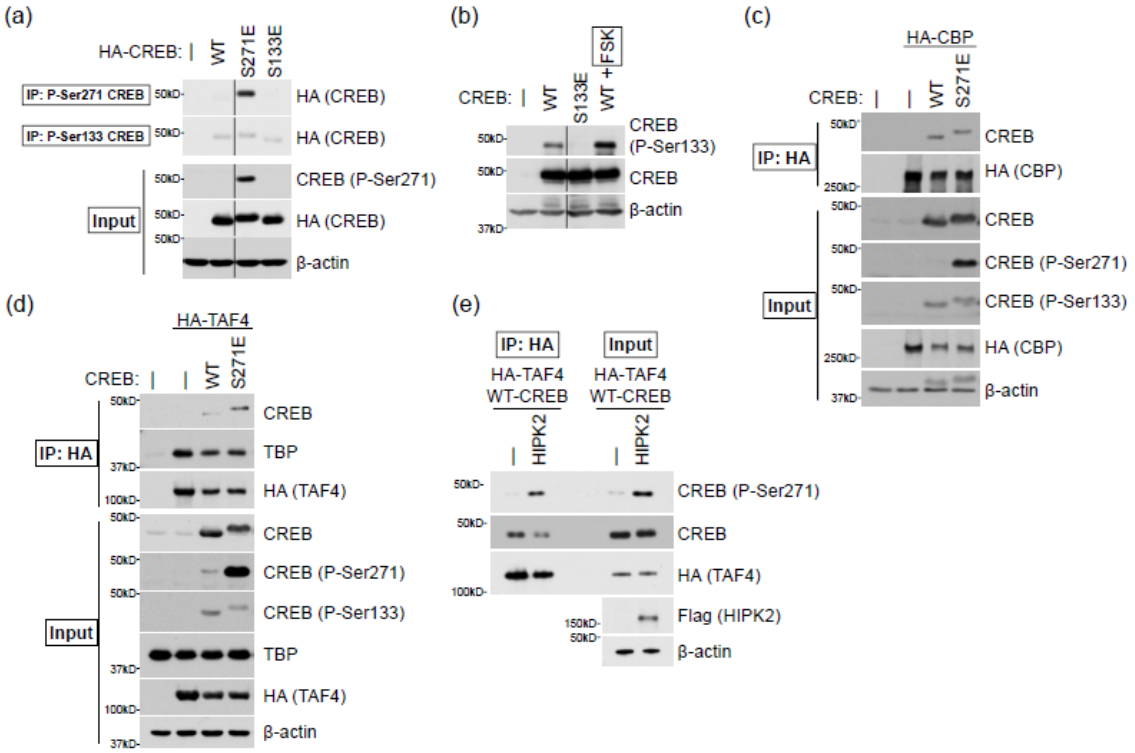
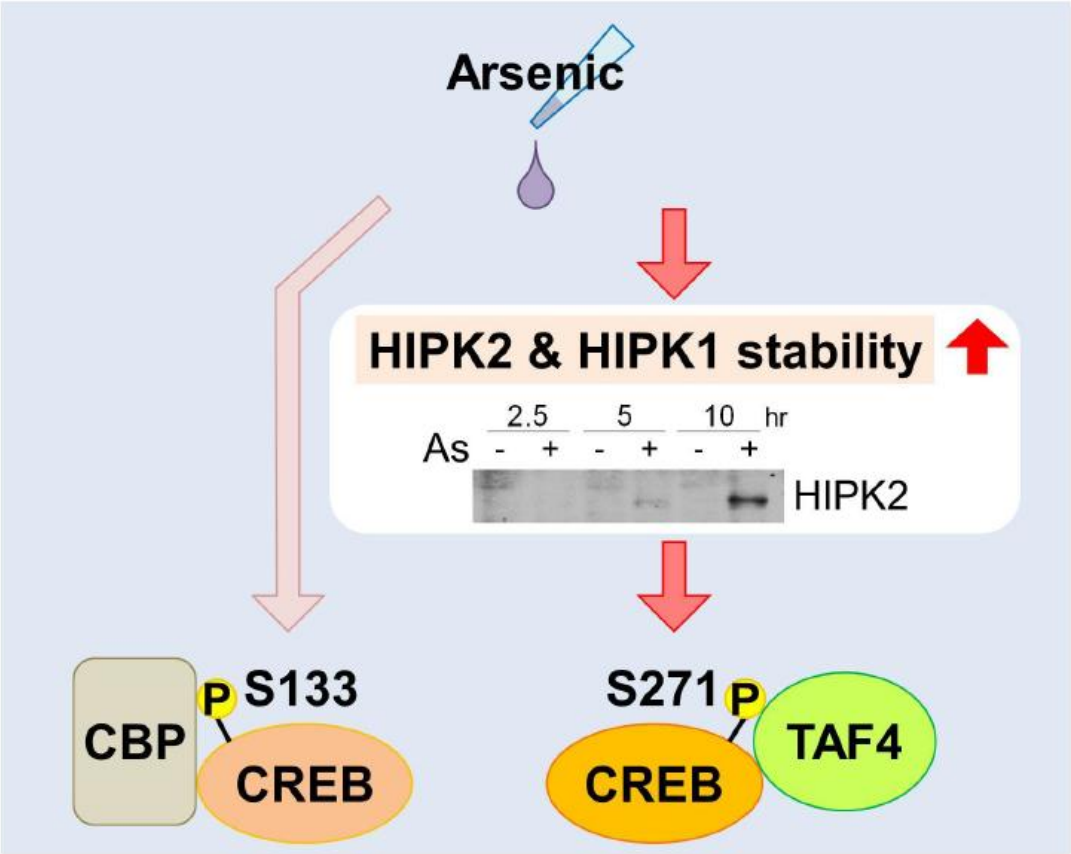


Figure 5 Hashimoto K and Tsuji Y






Graphical abstract

## Highlights

The mechanism of CREB activation via Ser271 phosphorylation remains uncharacterized.

Arsenic stabilizes HIPK2 and HIPK1, and induces phosphorylation of CREB at Ser271.

Phosphorylation of CREB at Ser271 enhances transcription of CREB-target genes.

Phosphorylation of CREB at Ser271 facilitates interaction with TAF4.

The HIPK2-Ser271CREB-TAF4 axis signals in parallel with the PKA-Ser133CREB-CBP axis.