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Complex regulation of CREB-binding protein by homeodomain-interacting protein kinase 2

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Abstract

CREB-binding protein (CBP) and p300 are transcriptional coactivators involved in numerous biological processes that affect cell growth, transformation, differentiation, and development. In this study, we provide evidence of the involvement of homeodomain-interacting protein kinase 2 (HIPK2) in the regulation of CBP activity. We show that HIPK2 interacts with and phosphorylates several regions of CBP. We demonstrate that serines 2361, 2363, 2371, 2376, and 2381 are responsible for the HIPK2-induced mobility shift of CBP C-terminal activation domain. Moreover, we show that HIPK2 strongly potentiates the transcriptional activity of CBP. However, our data suggest that HIPK2 activates CBP mainly by counteracting the repressive action of cell cycle regulatory domain 1 (CRD1), located between amino acids 977 and 1076, independently of CBP phosphorylation. Our findings thus highlight a complex regulation of CBP activity by HIPK2, which might be relevant for the control of specific sets of target genes involved in cellular proliferation, differentiation and apoptosis.

Keywords: transcriptional regulation; coactivator; CBP; protein kinase; HIPK2.

Abbreviations: BDNF, brain-derived neurotrophic factor; CBP, CREB-binding protein; C/EBP, CCAAT/enhancer binding protein; CRD1, cell cycle regulatory domain 1; CREB, cAMP-response element binding protein; CtBP, C-terminal-binding protein; DYRK, dual-specificity tyrosine-regulated kinases; HIPK2, homeodomain-interacting protein kinase 2; KD, kinase dead; JNK, Jun N-terminal kinase; Pin1, peptidyl-prolyl-cis-trans-isomerase; TGF-β, Transforming growth factor beta.

1. Introduction

Homeodomain-interacting protein kinase 2 (HIPK2) is a serine/threonine kinase belonging to the subfamily of dual-specificity tyrosine-regulated kinases (DYRK) [1, 2]. It was initially identified as a corepressor that potentiates the repressor activities of NK homeoproteins and was cloned by its ability to interact with Nkx-1.2 [3]. Since then, HIPK2 has been shown to be a pleiotropic regulator of cell death, having proapoptotic and antiproliferative effects in several cell types. Apoptosis evoked by ultraviolet irradiation requires activation of HIPK2 that, in turn, phosphorylates p53 at Ser46 [4, 5]. Activation of p53 is known to rely on a phosphorylation-acetylation cascade that increases its site-specific DNA-binding [6]. The transcriptional coactivators CREB-binding protein (CBP) or p300 act as acetylating enzymes in this cascade. HIPK2 is necessary *in vivo* for efficient p300/p53 co-recruitment onto apoptotic promoters, and p53 modifications both at Ser46 and Lys382 are necessary for p53 apoptotic transcription [7]. Consistent with the foregoing, phosphorylation at Ser46 by HIPK2 has been shown to augment CBP-dependent acetylation and the transcriptional activity of p53; moreover, the Ser46Ala mutant of p53 is not acetylated by CBP at Lys382 [4]. However, despite the absence of phosphorylable Ser46 residue, the transactivating potential of this point mutant is still strongly enhanced by cotransfection of HIPK2 in reporter assays [4], thus suggesting a putative direct effect of HIPK2 on CBP.

HIPK2 promotes apoptosis through p53-independent pathways as well. For example C-terminal-binding protein (CtBP) is phosphorylated by HIPK2 at Ser422, and this phosphorylation targets CtBP to degradation and contributes to the UV-induced apoptotic response [8, 9]. TGFβ-induced Jun N-terminal kinase (JNK) activation and apoptosis is also positively regulated by HIPK2 [10]. Furthermore, HIPK2 participates in a cascade leading to the phosphorylation induced degradation of the proto-oncogene product c-Myb [11].

Interestingly, HIPK2's role can also be anti-apoptotic. For instance, HIPK2 is required for TGF-β-mediated survival of midbrain dopamine neurons [12]. Moreover, HIPK2-induced phosphorylation of the transcription factor CREB at Ser271 favors CBP recruitment and expression of brain-derived neurotrophic factor (BDNF), thus decreasing SH-SY5Y neuroblastoma cells apoptosis induced by the DNA damaging agent etoposide [13].

Recently a modeling approach has revealed a role of HIPK2 in adipocyte differentiation, and experiments aimed at confirming this prediction have indeed shown that the expression level of HIPK2 increases 10 fold during adipocytic differentiation [14]. Furthermore, the white adipose tissue deposits of HIPK2 knock-out mice are smaller and the size of the adipocytes is reduced in these animals. The necessity of HIPK2 in this context is equally well demonstrated in 3T3 fibroblast culture, being an established *in vivo* model of adipocytic differentiation [15]. Furthermore, HIPK2 collaborates with the transcription factor AML-1 and the coactivator p300 to increase transcription at AML-1-dependent promoters. AML-1 is phosphorylated by HIPK2 and subsequently targets p300 to phosphorylation by the same kinase [16]. In accordance with these results, double transgenic mice lacking HIPK2 and HIPK1 show deficits in hematopoiesis, angiogenesis and vasculogenesis [16].

The coactivators CBP and p300 are believed to interact with hundreds of different transcription factors (for review, see Refs [17, 18]). Both coactivators have an intrinsic acetyltransferase activity, which allow them to acetylate histones, and thereby modify chromatin structure, as well as transcription factors like p53, as already mentioned. CBP and p300 also function as adaptors between tissue- and sequence-specific transcription factors and the general transcriptional machinery, and are thus believed to activate gene transcription by recruiting basal transcription factors. Although CBP and p300 are known to be phosphorylated in various conditions, relatively little is known about the protein kinases that target these coactivators, their phosphorylation sites and the effect of these post-translational modifications on CBP and p300 functions.

In this study, we report that HIPK2 interacts with and phosphorylates CBP at multiple sites. We show that HIPK2 phosphorylates a cluster of five serines and thus induces a mobility shift of a CBP C-terminal fragment. We demonstrate that HIPK2, but not its kinase-dead mutant, increases the transactivating potential of CBP. However, this activation of CBP by HIPK2 does not seem to be due to its phosphorylation, but rather through the action of this kinase on factors repressing CBP functions.

2. Materials and methods

2.1. Plasmids

pcDNA3-CBP-2×FLAG, pcDNA3-CBP$_{1680-2441}$-2×FLAG, pcDNA3-CBP$_{1680-1892}$-2×FLAG, pcDNA3-CBP$_{1893-2441}$-2×FLAG, and the Gal4-LUC (luciferase) reporter were described in Kovács et al. [19]. To construct the expression plasmids for the CBP fragments, PCR-generated DNA sequences encoding CBP amino acids 2-250, 251-450, 451-682, 683-900, 901-1100, 1101-1231, 1232-1711, 1893-2160, 2161-2441, and 1680-2160 were inserted into a modified pcDNA3 vector containing sequences encoding a nuclear localization sequence at the N-terminus and 2 FLAG epitopes at the C-terminus. Individual or combined serine to alanine mutations at position 2361, 2363, 2371, 2376, and 2381 were introduced in pcDNA3-CBP$_{1680-2441}$-2×FLAG with the QuikChange site-directed mutagenesis kit (Stratagene). To construct the expression plasmids for the various Gal4-CBP fusion proteins, full-length CBP cDNA or PCR-generated DNA sequences encoding CBP amino acids 1-450, 1-682, 1-900, 1-1100, 1-1231, 1232-1711, and 1232-2441 were inserted into a modified pcDNA3 vector containing sequences encoding the Gal4 DNA binding domain (amino acids 1 to 147) at the N-terminus and 2 FLAG epitopes at the C-terminus. pcDNA3-Gal4-CBP$_{1-682+977-1076}$-2xFLAG was constructed by subcloning a PCR-amplified DNA sequence encoding CBP amino acids 977-1076 into pcDNA3-Gal4-CBP$_{1-682}$-2xFLAG. Mutant pcDNA3-Gal4-CBP-2xFLAG with the 5 serine to alanine mutations was generated by swapping a C-terminal restriction fragment with the counterpart bearing the mutations that was taken from mutant pcDNA3-CBP$_{1680-2441}$-2×FLAG. pCMV-SPORT6-HIPK2 (cDNA clone MGC:36065, IMAGE:5368577) driving the expression of mouse HIPK2 was obtained from MRC geneservice (Cambridge, UK). The kinase-dead mutant pCMV-SPORT6-HIPK2 (K221R) was generated from pCMV-SPORT6-HIPK2 with the QuikChange site-directed mutagenesis kit (Stratagene). pCMV-HIPK2-FLAG is a kind gift of S. Soddu (Regina Elena Cancer Institute, Rome, Italy), and was described in D'Orazi et al. [5]. To produce the 10 purified GST-CBP fusion proteins used in the in vitro kinase assays, DNA sequences encoding CBP amino acids 2-250, 251-450, 451-682, 683-900, 901-1100, 1101-1231, 1232-1711, 1680-1892, 1893-2160, and 2161-2441 were transferred from the corresponding pcDNA3-CBP-2×FLAG constructs into pGEX-KG or pGEX-4T3 (Pharmacia). Mutant pGEX-CBP$_{2161-2441}$-

2×FLAG with the 5 serine to alanine mutations between amino acids 2361 and 2381 was constructed by cloning into pGEX-4T3 a fragment encoding CBP amino acids 2161-2441 and 2 FLAG epitopes, which was amplified by PCR using as a template pcDNA3-$CBP_{1680-2441}$-2×FLAG with the 5 serine to alanine mutations. All the constructs were verified by sequencing. Restriction maps and sequences of all the constructs described above are available upon request.

2.2. Cell culture and transfection assays

HEK 293T cells (American Type Culture Collection, CRL-11268) were cultured on gelatin-coated plates in high glucose GLUTAMAX™ Dulbecco's modified Eagle's medium (Invitrogen) containing 10% heat-inactivated newborn calf serum (Invitrogen), 100 U/ml penicillin G, and 100 µg/ml streptomycin sulfate (Invitrogen). Calcium phosphate transfection assays were performed according to an improved protocol described by Jordan et al. [20]. Briefly, HEK 293T cells were seeded at 8 x 10^5 cells per 60-mm plate, and 24 hr later, were transiently transfected with 8 or 9 µg of total DNA. The cell culture medium was changed 8hr later, and, 24 hr after transfection, cellular lysates were prepared for luciferase assays or immunoblot analysis.

2.3. Preparation of cellular extracts

To perform experiments with recombinant proteins expressed in HEK 293T cells, whole cell extracts were prepared by lysing the cells in 50 mM Hepes pH 7.6, 250 mM NaCl, 0.2 mM EDTA, 0.5% NP-40, 1mM DTT, 10 µM NaF and 10 µM Na_3VO_4 for 30 min at 4°C. Then, the extracts were cleared by centrifugation at 16'000xg for 10 min. To prevent proteolysis, a protease inhibitor cocktail for mammalian tissue (Sigma) and calpain inhibitor were included in the lysis buffer.

2.4. Immunoblot analysis

After separation on SDS-polyacrylamide gels (SDS-PAGE), the proteins were transferred to PVDF membranes with a semi-dry blotting system (BioRad) for 45 min at 20 V as previously described [19]. Blots were blocked overnight at 4°C in TBST containing 10 mM Tris-HCl, 150

mM NaCl pH 7.4, and 0.05% Tween-20, supplemented with, 10% skim milk powder, and 1% BSA. Blots were subsequently incubated with a primary antibody in TBST plus 1% skim milk powder for 2 hr at room temperature. FLAG-tagged CBP fragments were detected with anti-FLAG M2 monoclonal antibody (Sigma), and HIPK2 as well as its kinase-dead mutant were monitored with a goat anti-HIPK2 polyclonal antibody (Santa Cruz). Vinculin was used as a high molecular weight protein loading control and was detected with a rabbit anti-vinculin monoclonal antibody (Abcam ab129002). Finally, PVDF membranes were incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies, and developed using chemiluminescence detection kits (enhanced chemiluminescence, Amersham, or SuperSignal® West Femto, Pierce).

2.5. GST proteins

GST fusion proteins were expressed in *Escherichia coli* (BL21-Codon Plus®, Stratagene) and purified over glutathione-Sepharose beads (Sigma).

2.6. In vitro kinase assays

Kinase assays were either performed with FLAG-tagged full-length HIPK2 purified by immunoprecipitation from transfected HEK 293T cells , or with a purified recombinant N-terminal fragment of human HIPK2 (amino acids 165-564, Upstate cell signaling solutions) according to the manufacturer's protocol. Recombinant HIPK2 (25 ng) was incubated with GST-CBP fragments (3 μM in Fig. 3*B* and 7 μM in Fig. 5*B*) in 25 μl kinase buffer (10 mM MOPS pH 7.0, 3 mM Tris-HCl pH 7.5, 8 mM NaCl, 22.5 mM Mg acetate, 0.4 mM EDTA, 3 mM sucrose, 1% glycerol, 0.01% β-mercaptoethanol, 0.1 mM DTT, 0.1 mg/ml BSA) in the presence of 10 μCi [γ-^{32}P]ATP and 90 μM unlabeled ATP for 20 min at 30°C. The reaction was stopped by the addition of 3 × SDS loading buffer, the products were resolved on a 12% SDS-PAGE, the gel was fixed, stained with Coomassie, dried, and ^{32}P-labeled proteins were detected by autoradiography.

2.7. Co-immunoprecipitation

HEK 293T cells were co-transfected with 7.5 μg of pSPORT6-HIPK2 and 7.5 μg of the appropriate CBP fragment or pcDNA3 as indicated in Fig. 4 using the abovementioned calcium phosphate method. Twenty-four hours later, cells were lysed in 50 mM Hepes pH 7.6, 150 mM NaCl, 0.2 mM EDTA, 0.5% NP-40, 10 μM NaF and 10 μM Na_3VO_4 for 30 min at 4°C. Then, the extracts were cleared by centrifugation at 16'000xg for 10 min. To prevent proteolysis, a protease inhibitor cocktail for mammalian tissue (Sigma) and calpain inhibitor were included in the lysis buffer. Whole cell extracts (1 mg) were incubated with 20 μl anti-FLAG agarose beads (Sigma) for 2 hours at 4 ºC with continuous agitation. Beads were then washed three times in lysis buffer, transferred to a new tube, and immunoprecipitated proteins were eluted by boiling in 30 μl SDS-loading buffer. To detect co-immunoprecipitated HIPK2, 10 μl of the supernatants were separated on a 6% SDS-polyacrylamide gel, whereas 10 μl were resolved on a precast 4-15% gradient SDS-polyacrylamide gel (BioRad) to detect the immunoprecipitated FLAG-tagged CBP fragments. HIPK2 and FLAG immunoblot analyses were performed as described above.

2.8. Luciferase Assays

Transfected cells were washed with PBS, lysed in 250 μl 1x Cell Culture Lysis Buffer (Promega), and centrifuged in a microcentrifuge for 2 min at 4˚C. To test the samples for luciferase activity, 20 μl of the 100×-diluted or undiluted supernatant was assayed in a Turner-Designs TD-20/20 luminometer using 100 μl of Luciferase Assay Reagent (Promega). Luciferase activity was normalized to total cellular protein (Bio-Rad Protein Assay). All experiments were performed in triplicate.

2.9. Statistical analysis

We determined statistical differences for the luciferase assays by two-way analysis of variance (ANOVA) with a Fisher Least Significant Difference (LSD) post hoc test.

3. Results

3.1. HIPK2 interacts with and phosphorylates CBP at multiple sites

We previously reported that the C-terminal region of CBP is phosphorylated by an unidentified kinase when it interacts with C/EBP family members [19]. Looking for kinases that could be involved in this process, we observed that cotransfection of HIPK2 retarded the band corresponding to full-length CBP in SDS-polyacrylamide gels (Figures 1A and 1B). These Western blot experiments show a CBP mobility shift only in the presence of HIPK2 and not with the kinase-dead (KD) mutant form of HIPK2 (K221R), thus suggesting that HIPK2 phosphorylates CBP. The HIPK2-induced mobility shift of full-length CBP is more obvious with the shortly exposed bands of Figure 1B. The C-terminal FLAG tag allowed for the visualization of naturally occurring C-terminal degradation products of full-length CBP that also showed a HIPK2-induced mobility shift (Figure 1A, upper panel, lane 2), which indicated that HIPK2 may phosphorylate the C-terminal portion of CBP. Indeed, HIPK2, but not the KD mutant, retarded the electrophoretic mobility of $CBP_{1680\text{-}2441}$ (Figure 1A) in a similar way to what was observed with the C/EBPs [19].

To uncover which part of CBP is modified by HIPK2, we generated several FLAG-tagged CBP fragments and verified whether their mobility was changed by pCMV-SPORT6-HIPK2 cotransfection (Figure 2). The following fragments showed a change in electrophoretic mobility: CBP5, CBP10, CBP9-10 (see the description of the fragments in Figure 2A). For an unknown reason, CBP9 was not expressed at detectable levels in eukaryotic cells, and thus could not be tested. These results suggested that in addition to the C-terminal part of CBP, the N-terminus can also be a substrate of HIPK2.

To show that the altered electrophoretic mobility of CBP fragments is indeed due to phosphorylation, we performed *in vitro* phosphorylation experiments with bacterially produced GST-CBP fragments and radiolabeled phosphate using either FLAG-tagged HIPK2 immunoprecipitated from lysates of transfected HEK 293T cells (Figure 3A), or human recombinant active HIPK2 (Figure 3B). In both experiments, the CBP fragments 4, 5, 9, and 10 were phosphorylated (Figures 3A and 3B). This further confirmed that the mobility shifts of CBP5 and CBP10 were probably due to phosphorylation that triggered profound conformational

changes in these CBP fragments. Furthermore, these *in vitro* phosphorylation experiments clearly showed that CBP4 and CBP9 are phosphorylated by HIPK2 as well. In contrast, no phosphorylation could be observed for the following fragments: CBP1-3, CBP6, CBP7 and CBP8 (Figures 3A and 3B). Together, these data indicate that CBP could be phosphorylated by HIPK2 mainly between amino acids 683-1100 and at the C-terminus between amino acids 1893-2441.

The interaction between HIPK2 and CBP has already been documented; however, the interacting domains of CBP have not been mapped in detail [4]. Given that our results had shown that CBP was phosphorylated at remotely situated sites, we asked whether HIPK2 contacted CBP at multiple regions. To address this question we co-transfected HEK 293T cells with equal amounts of pCMV-SPORT6-HIPK2 and various pcDNA3-CBP-2xFLAG constructs, immunoprecipitated FLAG-tagged CBP fragments and analyzed immunoprecipitates by western blot for the presence of HIPK2. We detected strong interaction between HIPK2 and full-length CBP, whereas HIPK2 was not present in immunoprecipitates of the negative control lacking FLAG-tagged CBP (Figure 4). CBP8-9 pulled down similar levels of HIPK2 as full-length CBP, whereas CBP8-9-10 and CBP9-10 showed somewhat weaker interaction. From these data, we inferred that the most important region for the interaction lie between amino acids 1893 and 2160, whereas the C-terminal fragment 2161-2441 rather inhibits the recruitment of HIPK2. Unfortunately, we could not test this hypothesis, because CBP9 could not be expressed in HEK 293T cells. CBP7, harboring the histone-acetyltransferase (HAT) domain, and CBP3, lying next to the N-terminal phosphorylation sites, both recruit HIPK2, however, to a weaker extent than the C-terminal fragments (Figure 4). These results thus suggest that HIPK2 interacts directly or indirectly with several regions of CBP. Moreover, HIPK2 is phosphorylating CBP at multiple sites, some of which could be within the interacting region (CBP9) and the others appear to be located within adjacent regions (CBP4, CBP5, CBP10).

3.2. Phosphorylation of five serines at the C-terminus of CBP is responsible for the mobility shift of $CBP_{1680-2441}$ induced by HIPK2

To identify residues targeted by HIPK2 at the C-terminus of CBP, we took advantage of the fact that phosphorylated $CBP_{1680-2441}$ is strongly retarded in SDS-polyacrylamide gels. Within

this C-terminal fragment, we mutated to an alanine several serines and threonines that were followed by a proline, and monitored by Western blot the corresponding changes in the migration of $CBP_{1680-2441}$ in the presence of HIPK2. Among the various putative phosphorylation sites that were tested, we found a cluster of five serines, located at positions 2361, 2363, 2371, 2376 and 2381, whose mutation to alanine almost completely abolished the HIPK2-induced mobility shift of $CBP_{1680-2441}$ (Figure 5A). Individual point mutations had either no effect (1,2,4) or a marginal one (3,5), whereas the combination of the point mutations in two groups (1,2 and 3,4,5) decreased the mobility shift of the fragment by approximately a half. Combining the two groups of point mutations nearly eliminated CBP mobility shift showing that phosphorylation at these five phosphoacceptor sites is responsible for the robust conformational change of $CBP_{1680-2441}$ (Figure 5A, lower panel). *In vitro* phosphorylation of wild-type (wt) or mutant (5S→A) GST-$CBP_{2161-2441}$ by human recombinant active HIPK2 confirmed that the mutation of the five clustered serines decreased CBP phosphorylation (Figure 5B). However, the remaining phosphorylation of the mutant also indicated that HIPK2 might phosphorylate other serine or threonine residues in $CBP_{2161-2441}$, although it is possible that *in vitro* phosphorylation with purified proteins might not reflect the physiological conditions that would occur in a cellular context.

3.3. HIPK2 enhances the transcriptional activity of CBP mainly by counteracting the repressive action of the CRD1 domain

Having found which HIPK2 phosphorylation sites were responsible for the mobility shift of $CBP_{1680-2441}$, we next tested whether the transcriptional activity of CBP was increased in the presence of HIPK2 and whether the five serines at the C-terminus that induce a conformational change were actually playing a role in this activation. To this end, we examined the transactivating potential of Gal4-CBP in the presence or in the absence of cotransfected HIPK2, using a Gal4-LUC reporter plasmid (Figure 6). HIPK2 clearly stimulated the transcriptional activity of CBP through a process that required its kinase activity, because the kinase-dead (KD) mutant form of HIPK2 was ineffective. Nonetheless, the activity of the Gal4-CBP construct, in which the 5 serine to alanine mutations had been introduced at the C-terminus, was not different

from the wild-type Gal4-CBP construct, suggesting that these phosphorylation sites do not play a major functional role, at least in these experimental conditions.

To further determine through which region of CBP HIPK2 is stimulating its transcriptional activity, we first split the molecule into N-terminal and C-terminal halves. The transactivating potential of Gal4-CBP_{1-1231} was increased nearly 50 fold by HIPK2 and was comparable to the stimulated activity of Gal4-CBP_{FL}, whereas that of Gal4-$CBP_{1232-2441}$ showed only a 5 fold increase (Figure 7). Gal4-$CBP_{1232-1711}$, a fragment encompassing the histone acetyltransferase domain was activated by HIPK2 only approximately 2 fold. To analyze the strong effect that HIPK2 exerted on the transcriptional activity of Gal4-CBP_{1-1231}, we then generated several N-terminal fragments of CBP fused to the Gal4 DNA-binding domain. Gal4-CBP_{1-450} and Gal4-CBP_{1-682} showed weak and strong transactivating potential, respectively. Neither of these fragments was activated by cotransfection of HIPK2 (Figure 7). The basal transcriptional activity of Gal4-CBP_{1-682} was comparable to the activity of Gal4-CBP_{1-1231} in the presence of HIPK2, suggesting the existence of a repressor domain between 683 and 1232 that could be neutralized by HIPK2. We further defined the position of this repressor domain by observing that Gal4-CBP_{1-900} and Gal4-CBP_{1-1100} showed high and low basal activity, respectively, and that only Gal4-CBP_{1-1100} was activated by HIPK2 (Figure 7). These observations strongly suggested that the repressor domain contained within $CBP_{900-1100}$ was presumably identical to the CRD1 domain first described in p300 [21]. We have shown that $CBP_{900-1100}$ is indeed phosphorylated by HIPK2 (Fig. 2 and 3), but a close examination of the murine CBP sequence corresponding to the p300 CRD1 domain revealed no putative HIPK2 phosphorylation site between amino acids 977 and 1076, where the CRD1 domain is located, whereas 10 putative phosphorylation sites are present in $CBP_{900-1100}$, mostly between amino acids 900 and 977. Instead of trying to identify which serine and threonine residues of $CBP_{900-1100}$ are phosphorylated by HIPK2, we first determine whether the phosphorylation of this CBP fragment by HIPK2 was indeed required to lift CRD1-mediated inhibition. To this end, we fused Gal4-CBP_{1-682} to the CBP amino acids 977-1076 containing the CRD1 domain but devoid of any putative HIPK2 phosphorylation sites, and as expected, we observed a strong repressive effect of the CRD1 adjunction (Figure 8). Much to our surprise, however, HIPK2 completely abolished the repression exerted by the CRD1 domain. The much weaker activation by the kinase-dead (KD) HIPK2 mutant showed that the kinase activity of HIPK2 is required to counteract the effect of CRD1, even though this domain of CBP

does not contain any putative phosphorylation site for this kinase. Taken together, these results provide evidence that HIPK2 mainly enhances the transcriptional activity of CBP by counteracting the repressive action of the CRD1 domain through a mechanism that appears to be independent of CBP phosphorylation.

4. Discussion

The present study provides evidence that HIPK2 interacts with several domains of the coactivator CBP and phosphorylates it at multiple sites located between amino acids 683-1100 and between 1893-2441 at the C-terminus. We identified five HIPK2 phosphorylation sites at position 2361, 2363, 2371, 2376, and 2381 that are responsible for the mobility shift of $CBP_{1680-2441}$. However, we could not relate the phosphorylation of these five serines to the stimulation of the transcriptional activity of Gal4-CBP by HIPK2. Instead, we showed that HIPK2 activates Gal4-CBP mainly by lifting the repressive effect of the CRD1 domain located between amino acids 977 and 1076. Our data suggest that this derepression is not dependent on CBP phosphorylation, but rather on the phosphorylation of other factors acting in the repression pathway mediated by the CRD1 domain. Taken together, these findings identify CBP as a new substrate of HIPK2 and suggest a complex regulation of CBP activity by this kinase.

The HIPK2-mediated phosphorylation of CBP identified in the present study is accompanied by profound conformational changes as evidenced by the altered electrophoretic mobility of $CBP_{901-1100}$, $CBP_{1680-2441}$, and full-length CBP. We identified five serine residues as phosphoacceptors, all of them being followed by a proline in agreement with the fact that HIPK2 is a proline-directed serine/threonine kinase. Conformational changes at phosphorylated SP or TP sites often occur as a result of prolyl-cis-trans isomerisation and require the enzymatic activity of Pin1 (peptidyl-prolyl-cis-trans-isomerase) [22]. Therefore, the conformational change at the C-terminus of CBP might rely on Pin1. In this case, the phosphorylation of CBP by HIPK2 would be a prerequisite for the Pin1-mediated conformational change. Further studies are needed to determine whether Pin1 is implicated in this process.

We previously observed similar mobility shifts of full-length CBP and $CBP_{1680-2441}$ upon C/EBPα, -β, -δ co-expression, and phosphatase treatment of $CBP_{1680-2441}$ showed that its shift was indeed due to phosphorylation [19]. Interestingly, the C/EBP-induced mobility shift of $CBP_{1680-2441}$ was not anymore observed when serines 2361, 2363, 2371, 2376, and 2381 were replaced by alanines (data not shown). These findings suggest that HIPK2 could be the kinase recruited and activated by C/EBPs that phosphorylates the C-terminus of CBP. This is further supported by a report that identified HIPK2 as the kinase that phosphorylates p300, the functional homolog of CBP, when it interacts with C/EBPβ [23]. Recently, on the basis of

previous work from our laboratory identifying homology boxes in C/EBP proteins [19] the structure of a putative complex between C/EBPε and p300 has been described using the crystal structure of a chimeric protein containing a segment from each interacting partner [24]. It has been proposed that HIPK2 could be a third partner in a complex containing C/EBPβ and p300, and that v-Myc inhibits the activity of C/EBPβ supposedly by interfering with p300 phosphorylation, by binding to C/EBPβ and displacing HIPK2 from the ternary complex [23]. This report also demonstrated that HIPK2 phosphorylates serine 2280 of p300, which was previously involved with other putative phosphorylation sites in the mobility shift of the C-terminal part of p300 induced by C/EBPβ [25]. This serine does not correspond to any of the five serines of CBP that were identified in the present study. While we were trying to identify the phosphorylation sites responsible for the C/EBP-induced mobility shift of $CBP_{1680-2441}$, we replaced by alanines all the serines and threonines corresponding to mutA and mutB (Ser2280) of p300 [25], but this did not decrease the mobility shift of this CBP fragment (data not shown). Therefore, it appears that the conformational change of the C-terminal part of CBP and p300 is not induced by the homologous HIPK2 phosphorylation sites in these two coactivators, although the amino acids sequences responsible for the mobility shift of p300 [25] and CBP (the 5 serines identified in the present study) are well conserved in both coactivators.

In addition to the identification of phosphorylation sites within the C-terminal part of CBP, we showed that HIPK2 phosphorylates $CBP_{683-900}$ and $CBP_{901-1100}$. These two adjacent regions contain 17 SP and TP sites, thus rendering the identification of the HIPK2 phosphorylation sites difficult. In a study showing that HIPK2 forms a complex with, and phosphorylates AML1 and p300, Aikawa and collaborators described 11 putative HIPK2 phosphorylation sites in the corresponding region of p300 [16]. However, these authors did not formally identify which of these sites are actually phosphorylated by HIPK2. The mutation of these 11 sites to alanines significantly reduced the activation of Gal4-p300 by HIPK2, although the mobility shift of the mutant protein did not seem to be different from wild-type Gal4-p300. Eventually, the activation of Gal4-p300 by HIPK2 was totally abolished when 23 serines and threonines were replaced by alanines at 3 different clusters in p300 [16]. We did not follow the same systematic mutational approach to identify the functional HIPK2 phosphorylation sites in CBP, because we were afraid that the introduction of so many point mutations might change the properties of CBP in an artifactual way. Moreover, our data of Fig. 8, which show that HIPK2 is counteracting the

repressive effect of the CRD1 domain that is devoid of phosphorylation site, suggest that HIPK2 is activating CBP independently of its phosphorylation.

The CRD1-mediated inhibition of CBP and p300 is mechanistically similar, both coactivators can be relieved of the repression by cotransfection of p21 [21], and inhibition seems to involve the conjugation of SUMO to both p300 and CBP [25, 26]. However, slight differences exist in the manner that CRD1 exerts its effects on the coactivators. The SUMO-modified p300 recruits directly HDAC6 that decreases transcriptional activity [26], whereas SUMOylated CBP first recruits Daxx, which then brings HDAC2 to the complex [27].

To test whether HIPK2 neutralizes CRD1-mediated repression through phosphorylation, we generated the Gal4-$CBP_{1\text{-}682\ +\ 977\text{-}1076}$ mutant. This mutant contained the first 682 amino acids of CBP, which were shown to have a strong activity that was not influenced by HIPK2, fused to a region comprising the CRD1 domain, but devoid of HIPK2 phosphorylation sites. As expected, the activity of Gal4-$CBP_{1\text{-}682}$ was repressed by the adjunction of the CRD1 domain. Surprisingly however, HIPK2 relieved this repression although no putative phosphorylation site was present between amino acids 977 and 1076. The likely interpretation of these data is that HIPK2 is phosphorylating an upstream factor that acts on the CRD1 domain to derepress CBP.

5. Conclusions

In the present study, we show a complex interplay between HIPK2 and the coactivator CBP: (1) HIPK2 interacts with several domains of CBP; (2) HIPK2 phosphorylates CBP at multiple sites located between its KIX domain and bromodomain, and within the C-terminal activation domain; (3) phosphorylation by HIPK2 of a cluster of five serines at position 2361, 2363, 2371, 2376, and 2381 induces a conformational change and a mobility shift of $CBP_{1680\text{-}2441}$; (4) HIPK2 very potently increases the activity of CBP, however, this activation does not seem to depend on CBP phosphorylation, but rather on the inactivation of the CRD1 repression domain located between amino acids 977 and 1076. Given the multitude of transcription factors interacting with CBP and the versatile role of HIPK2, the present study raises the interesting possibility that HIPK2-mediated CBP regulation may influence the transcription of a large panel of genes involved in apoptosis or alternatively in differentiation and development.

Acknowledgments

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Figure legends

Fig. 1. HIPK2 alters the electrophoretic mobility of CBP. (A) Western blot analysis of full-length CBP (CBP_{FL}) or a C-terminal fragment ($CBP_{1680-2441}$) co-expressed with HIPK2 in HEK 293T cells. Equal amounts of pcDNA3-CBP-2xFLAG, or pcDNA3-$CBP_{1680-2441}$-2xFLAG and either pcDNA3 (-), pCMV-SPORT6-HIPK2 (+), or a kinase-dead mutant pCMV-SPORT6-HIPK2-K221R (KD) were co-transfected, and 24 hr later, cellular extracts were prepared and analyzed on a 6% SDS-PAGE, followed by Western immunoblotting using anti-FLAG (top) or anti-HIPK2 (bottom) antibodies. The position of full-length CBP or $CBP_{1680-2441}$ in the absence of HIPK2 is indicated by a black arrow, whereas the slow migrating forms of CBP are marked by empty arrows. The different migration of the KD mutant compared to HIPK2 is due to its inability to autophosphorylate. (B) To better visualize the alteration in electrophoretic mobility of full-length CBP, another independent experiment was performed as in (A). Cellular proteins were separated on a 6% SDS-PAGE and analyzed by Western immunoblotting using anti-FLAG, anti-HIPK2, and anti-vinculin antibodies. The 125 kDa cytoskeletal protein vinculin was used as a loading control. A short exposure of chemiluminescence immunodetection is shown in the top panel to better appreciate the electrophoretic mobility shift of full-length CBP in the presence of active HIPK2 (compare lane 2 to lanes 1 and 3).

Fig. 2. HIPK2 induces a mobility shift of two distinct domains of CBP. (A) Schematic representation of CBP and its most important domains, with the various CBP fragments used in this study (numbers between 2 and 2441 refer to CBP amino acids). CH1-3: Zinc finger domains; KIX: kinase inducible domain interacting domain; CRD1: cell cycle regulatory domain 1; BD: Bromodomain; HAT: Histone acetyltransferase domain; SID: SRC1 interaction domain; pQ: glutamine rich region; AD: activation domain. (B) HEK 293T cells were transfected with various FLAG-tagged CBP fragments along with pCMV-SPORT6-HIPK2 (+) or the kinase-dead mutant pCMV-SPORT6-HIPK2-K221R (KD) as indicated, and protein extracts were analyzed by SDS-PAGE and Western immunoblotting with anti-FLAG or anti-HIPK2 antibodies. Empty arrows indicate slow migrating forms of CBP fragments that are not visible in the presence of the kinase-dead HIPK2 mutant.

Fig. 3. *In vitro* phosphorylation of GST-CBP fragments. (A) Full-length FLAG-tagged HIPK2 was expressed in HEK 293T cells, immunoprecipitated with anti-FLAG agarose beads, and incubated with 2 μg of the various purified GST-CBP proteins in the presence of γ-^{32}P-ATP. Numbers 1 to 10 correspond to the fragments described in Figure 2. An extract of non-transfected HEK 293T cells was used in the control conditions (-). The top panels show the autoradiography of the Coomassie-stained gels displayed in the bottom panels. The position of the phosphorylated GST-CBP fragments is indicated by an empty arrow. The band above 150 kDa (^{32}P-HIPK2) corresponds to autophosphorylated full-length FLAG-tagged HIPK2. Coomassie-stained GST-CBP proteins are localized by arrowheads. (B) Human recombinant active HIPK2 (amino acids 165-564) was incubated with the GST-CBP proteins in the presence of γ-^{32}P-ATP. The position of the phosphorylated GST-CBP fragments is indicated by an empty arrow (top panel). The band above 50 kDa (^{32}P-rHIPK2) corresponds to autophosphorylated recombinant HIPK2. Coomassie-stained GST-CBP proteins are localized by arrowheads (lower panel).

Fig. 4. HIPK2 interacts with C-terminal fragments of CBP. HEK 293T cells were co-transfected with equal amounts of pCMV-SPORT6-HIPK2 and pcDNA3-CBP-2xFLAG (CBP_{FL}), or various FLAG-tagged CBP fragments. As a negative control, cells were transfected only with pCMV-SPORT6-HIPK2 (Ctrl). Cell lysates (5%, top panel) and immunoprecipitates with anti-FLAG agarose beads were analyzed by immunoblotting with anti-HIPK2 or anti-FLAG antibodies. Numbers 1 to 10 correspond to the fragments described in Fig. 2. Arrowheads indicate the position of full-length CBP or its various fragments.

Fig. 5. Serine to alanine mutation of five clustered residues abolishes the HIPK2-induced mobility shift of $CBP_{1680\text{-}2441}$. (A) Amino acid sequence of five putative HIPK2 phosphorylation sites in the C-terminal part of CBP. Serine 2361, 2363, 2371, 2376, and 2381 were numbered 1 to 5, respectively, to indicate the various individual or combined serine to alanine mutations in

$CBP_{1680-2441}$. Western blot analysis of wild-type (wt) or mutant C-terminal fragments ($CBP_{1680-2441}$) co-expressed with HIPK2 in HEK 293T cells. Equal amounts of wt or mutant pcDNA3-$CBP_{1680-2441}$-2xFLAG constructs and either pcDNA3 (-), pCMV-SPORT6-HIPK2 (+), or the kinase-dead mutant pCMV-SPORT6-HIPK2-K221R (KD) were co-transfected, and 24 hr later, cellular extracts were prepared and analyzed on a 6% SDS-PAGE, followed by Western immunoblotting using anti-FLAG antibody. The 125 kDa cytoskeletal protein vinculin was used as a loading control. The position of $CBP_{1680-2441}$ in the absence of HIPK2 (-) or in the presence of the kinase-dead mutant (KD) is indicated by a black arrow, whereas the slow migrating form is marked by an empty arrow. (B) *In vitro* phosphorylation of wild-type (wt) or mutant (5S→A) GST-$CBP_{2161-2441}$. GST-$CBP_{2161-2441}$ (GST-CBP10) proteins were incubated with γ-^{32}P-ATP in the presence (+) or in the absence (-) of human recombinant active HIPK2 (rHIPK2). The top panel shows the ^{32}P-labeled GST-CBP10 proteins, and the lower panel the Coomassie-stained gel. The serine to alanine mutation of the five putative HIPK2 phosphorylation sites reduces, but does not completely abolish, the *in vitro* phosphorylation of $CBP_{2161-2441}$. These results suggest that HIPK2 phosphorylates the C-terminal part of CBP on other sites than those involved in the induction of the mobility shift.

Fig. 6. HIPK2 increases the transcriptional activity of Gal4-CBP independently of Serine 2361-2381 phosphorylation. HEK 293T cells were transfected with 1 μg of Gal4-LUC, 2 μg of pcDNA3-Gal4-CBP-2xFLAG wild-type or with the five serine to alanine mutations (5S→A), and either 1.5 μg of pcDNA3 (-), pCMV-SPORT6-HIPK2 (+), or the kinase-dead mutant pCMV-SPORT6-HIPK2-K221R (KD). pcDNA3 was used to normalize the amounts of DNA to 8 μg for each condition. Cellular extracts were prepared 24 hr after transfection and analyzed for luciferase activity. Values were normalized for protein levels. Results are displayed as the mean ± SEM (n=3) relative luciferase activity. * $p<0.05$, *** $p<0.001$ vs respective condition w/o HIPK2 (two-way ANOVA, Fisher LSD test). The effect of 5S to A mutations on HIPK2-mediated CBP activation is not statistically significant ($p=0.318$).

Fig. 7. HIPK2 counteracts the inhibitory activity of the CRD1 domain of CBP located between the amino acids 977 and 1076. HEK 293T cells were transfected with 1 µg of Gal4-LUC, 2 µg of various pcDNA3-Gal4-CBP-2xFLAG constructs, as indicated, and either 1.5 µg of pcDNA3 (-), or pCMV-SPORT6-HIPK2 (+). pcDNA3 was used to normalize the amounts of DNA to 8 µg for each condition. Cellular extracts were prepared 24 hr after transfection and were diluted 100-fold for the analysis of luciferase activity, except for the HAT domain (1232-1711) and the C-terminal fragment (1232-2441) that have a moderate transcriptional activity. Values were normalized for protein levels. Results are displayed as the mean ± SEM (n=3) relative luciferase activity. * $p<0.05$, *** $p<0.001$ vs respective condition w/o HIPK2 (two-way ANOVA, Fisher LSD test).

Fig. 8. HIPK2 counteracts the repressive effect of the CRD1 domain that is devoid of HIPK2 phosphorylation site. HEK 293T cells were transfected with 1 µg of Gal4-LUC, 2 µg of pcDNA3-Gal4-CBP_{1-682}-2xFLAG or pcDNA3-Gal4-$CBP_{1-682\ +\ 977-1076}$-2xFLAG constructs, as indicated, and either 1.5 µg of pcDNA3 (-), pCMV-SPORT6-HIPK2 (+), or the kinase-dead mutant pCMV-SPORT6-HIPK2-K221R (KD). pcDNA3 was used to normalize the amounts of DNA to 8 µg for each condition. Cellular extracts were prepared 24 hr after transfection and analyzed for luciferase activity. Values were normalized for protein levels. Results are displayed as the mean ± SEM (n=3) relative luciferase activity. *** $p<0.001$ vs Gal-$CBP_{1-682\ +\ 977-1076}$ w/o HIPK2 condition (two-way ANOVA, Fisher LSD test). The CRD1 domain comprised between the amino acids 977 and 1076 of CBP does not contain any putative HIPK2 phosphorylation sites.

Figure 1

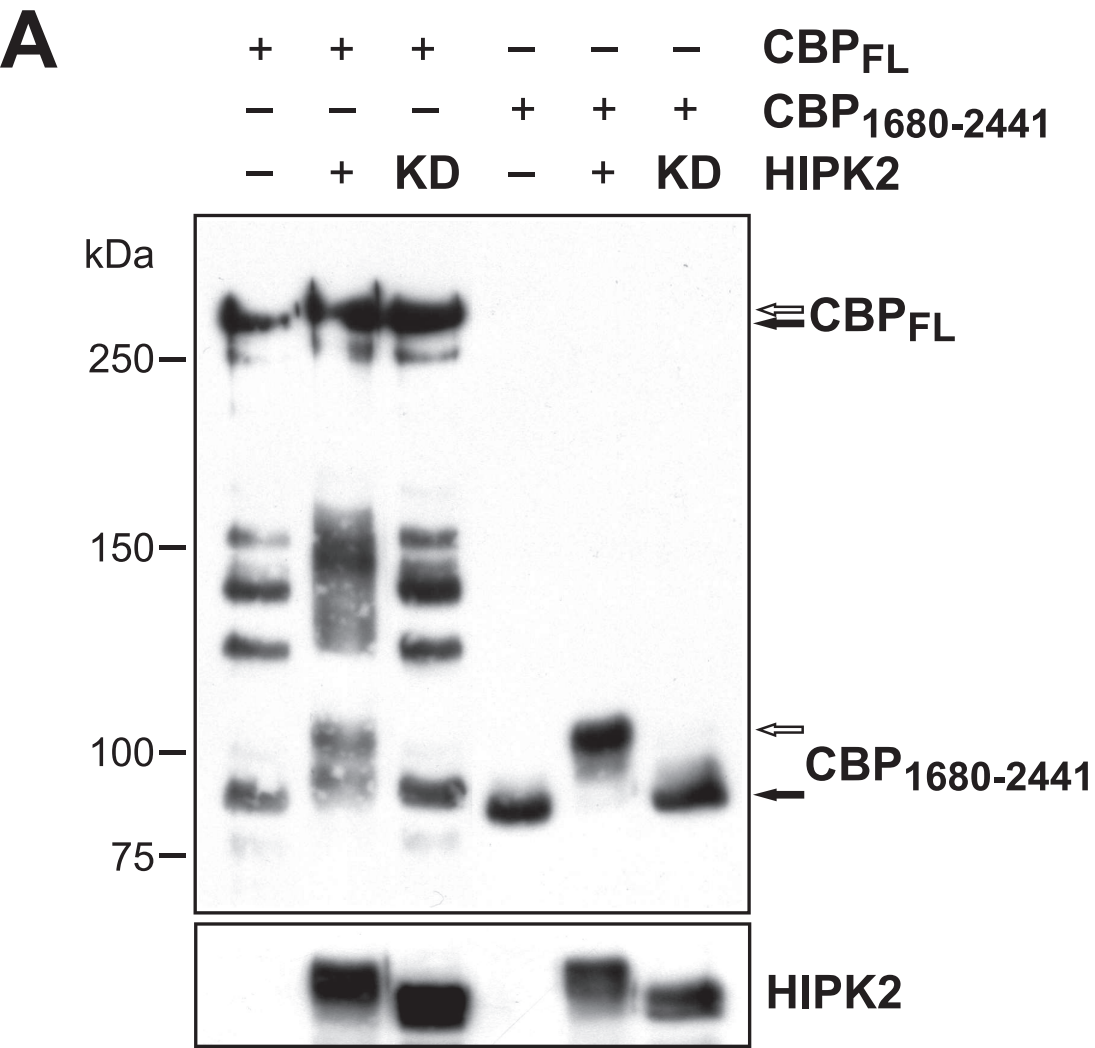
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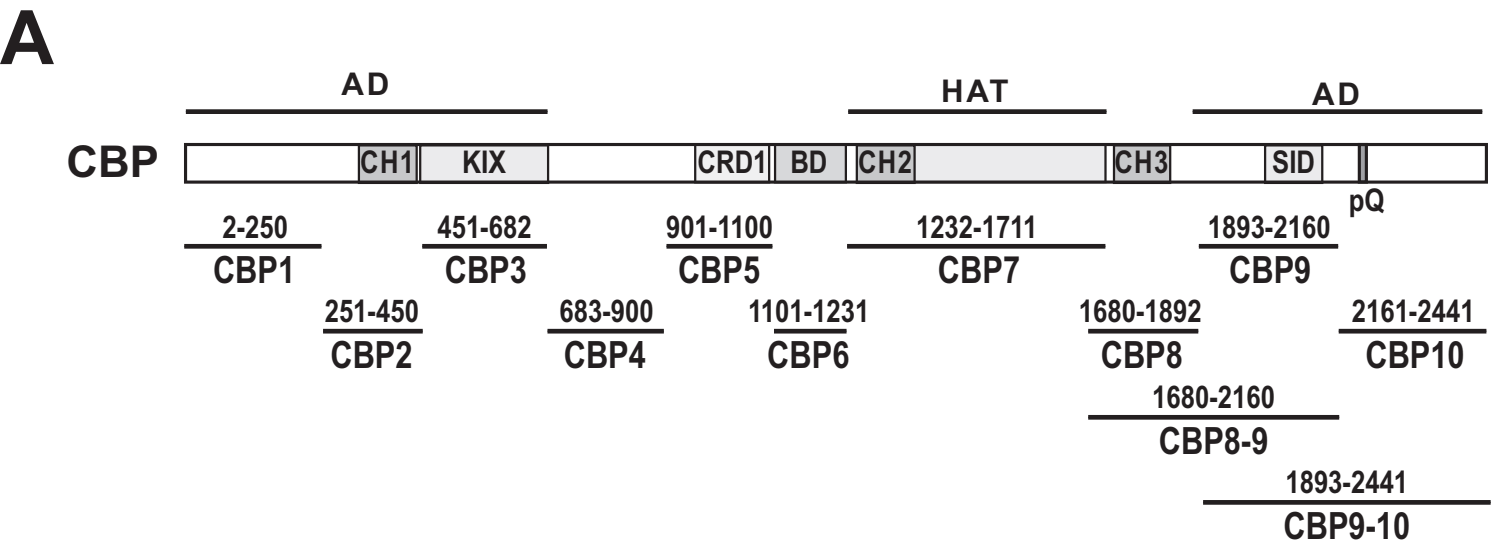
Figure 2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CBP5
1232-1711
CBP7
1893-2160
CBP9
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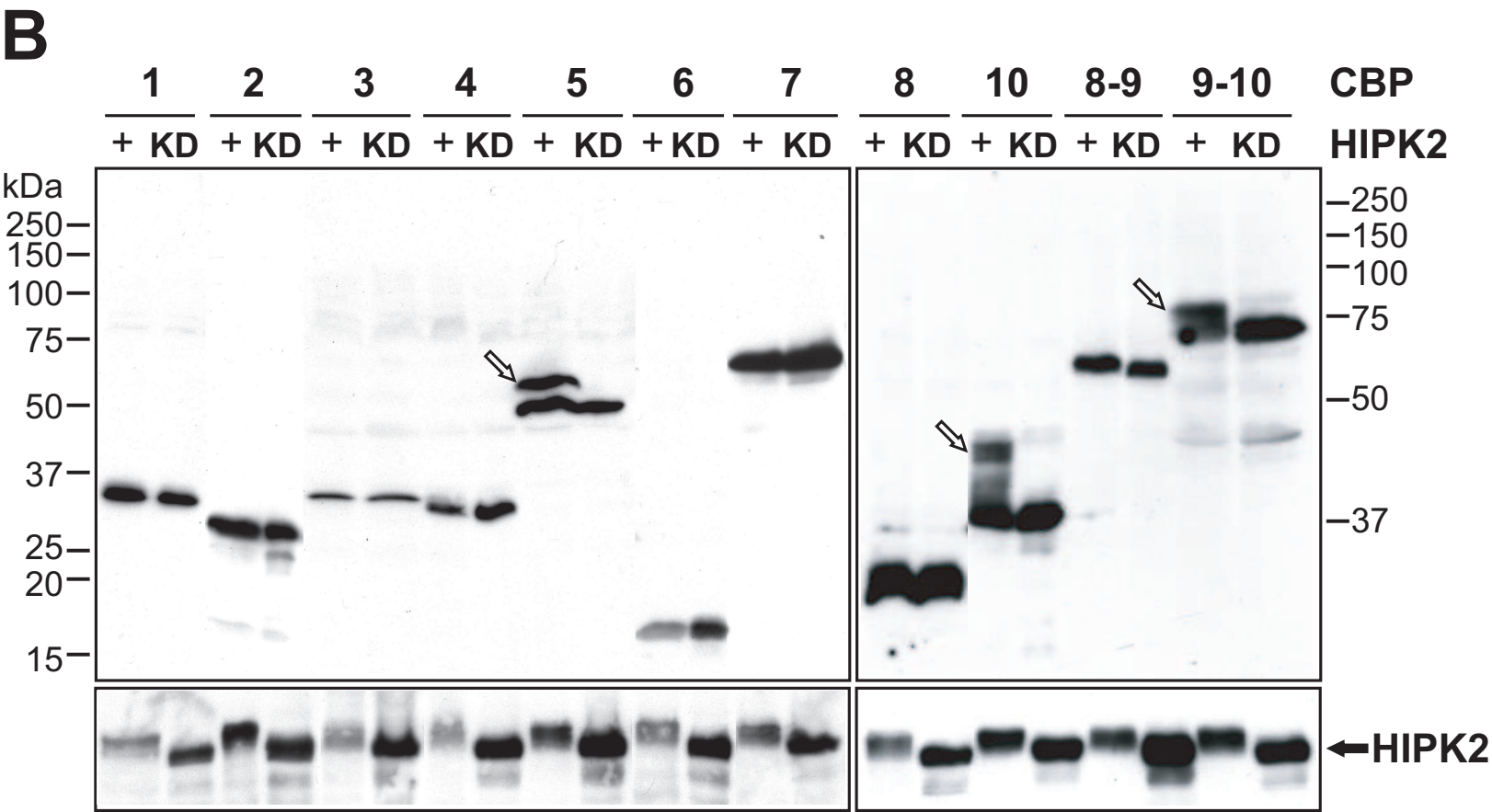
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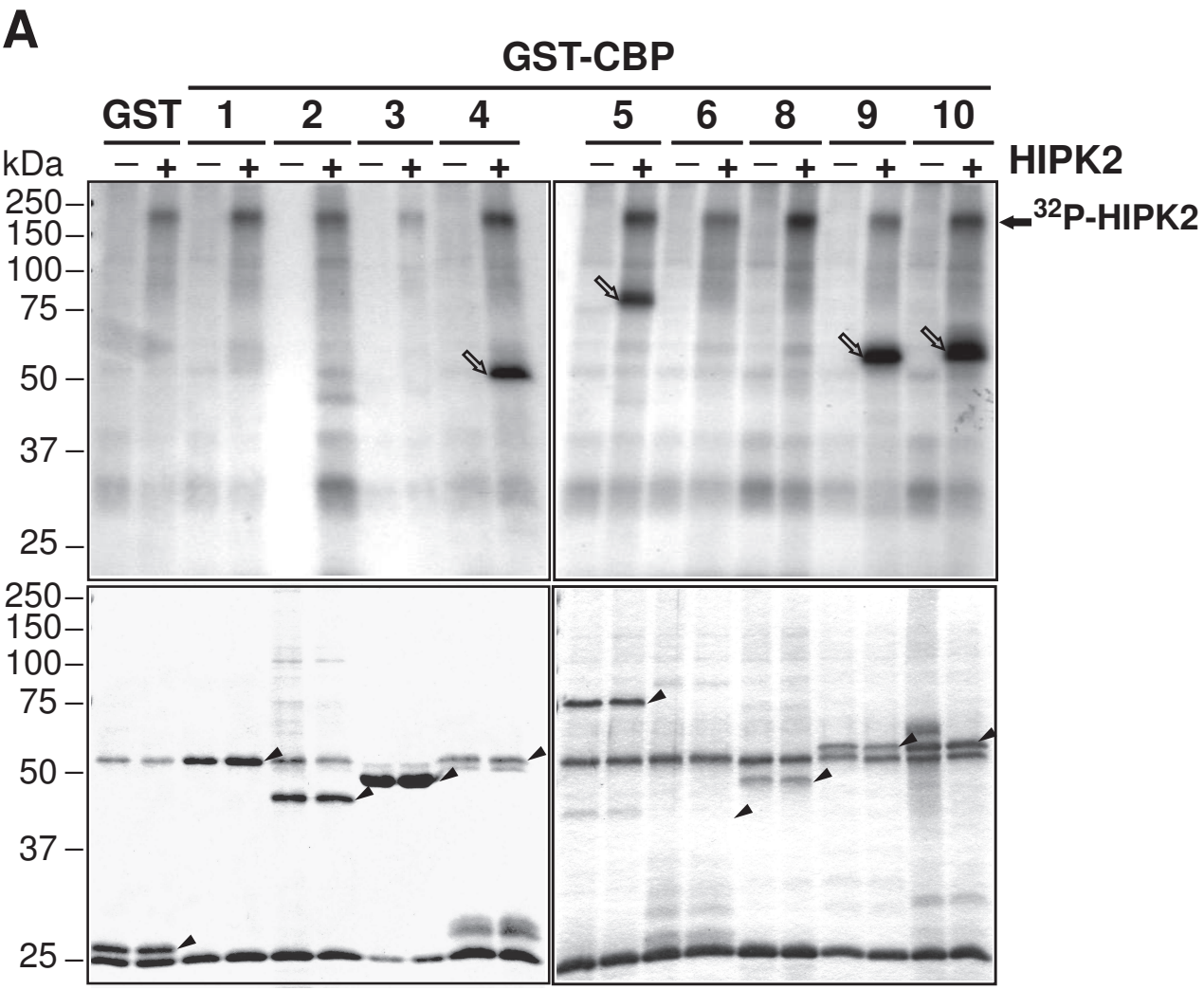
Figure 3

Figure 3



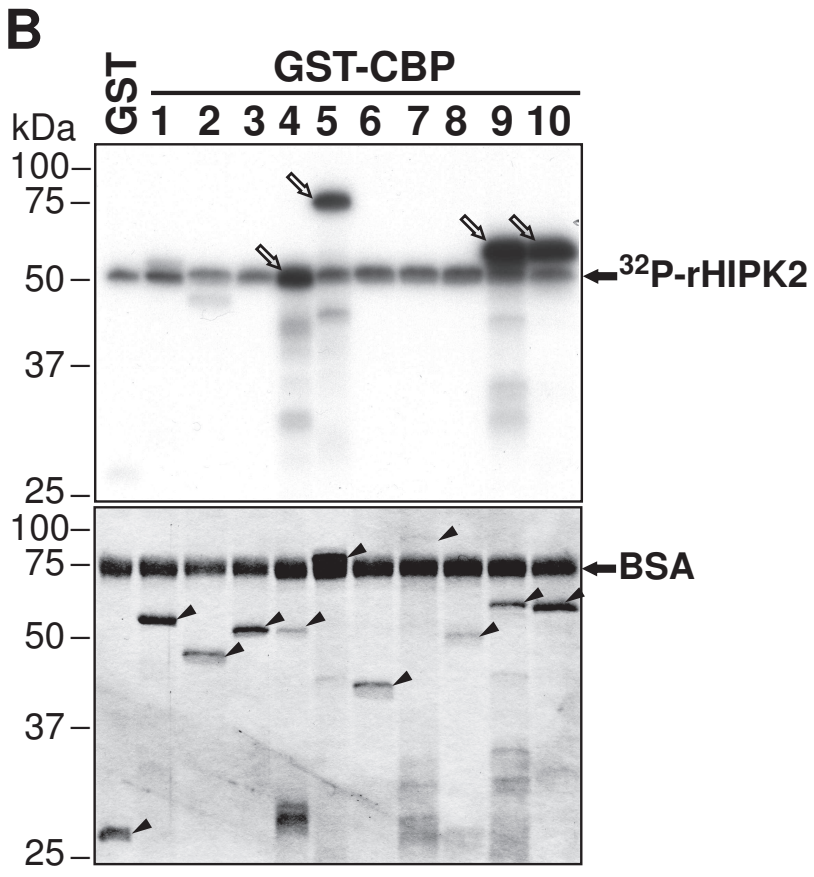
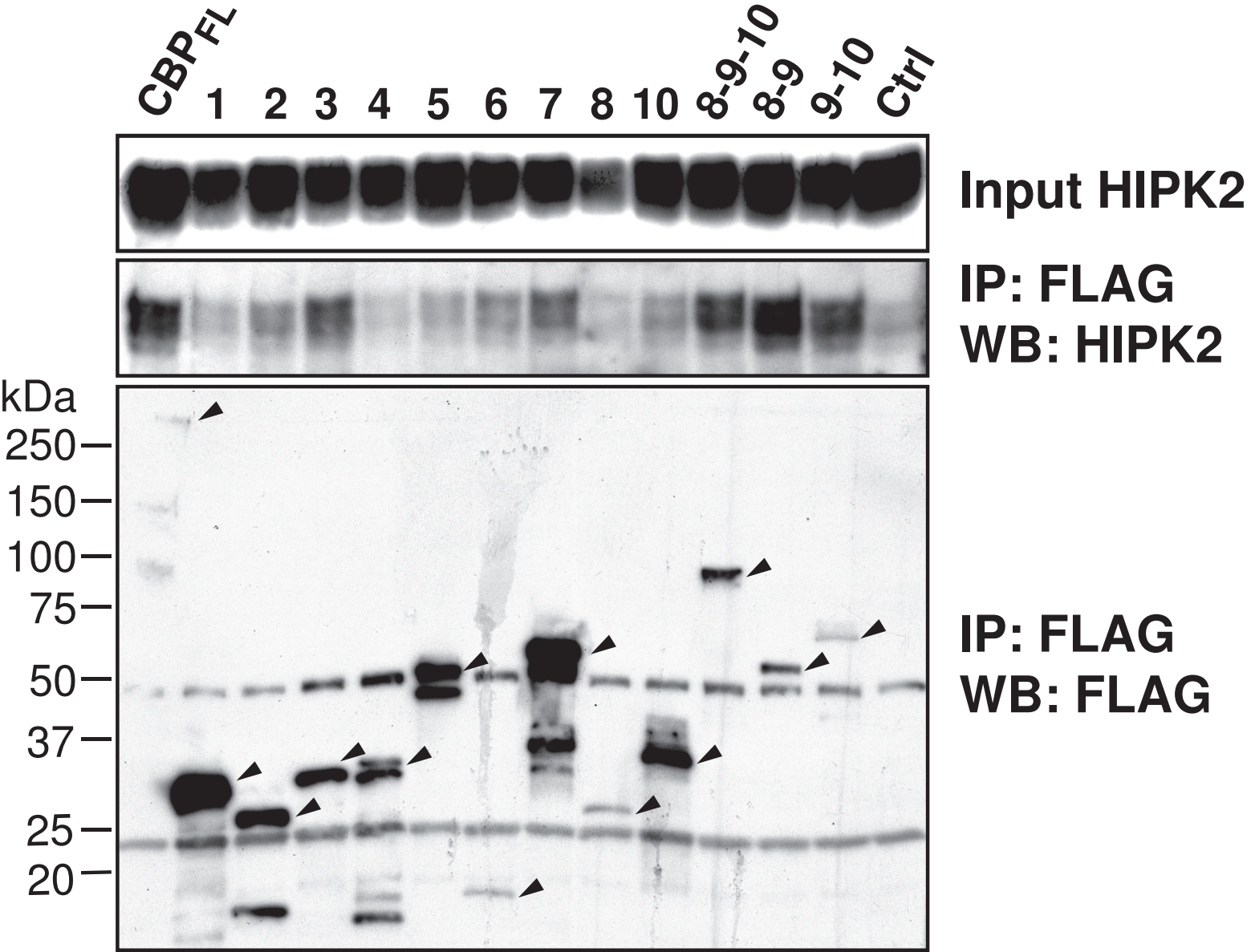


Figure 4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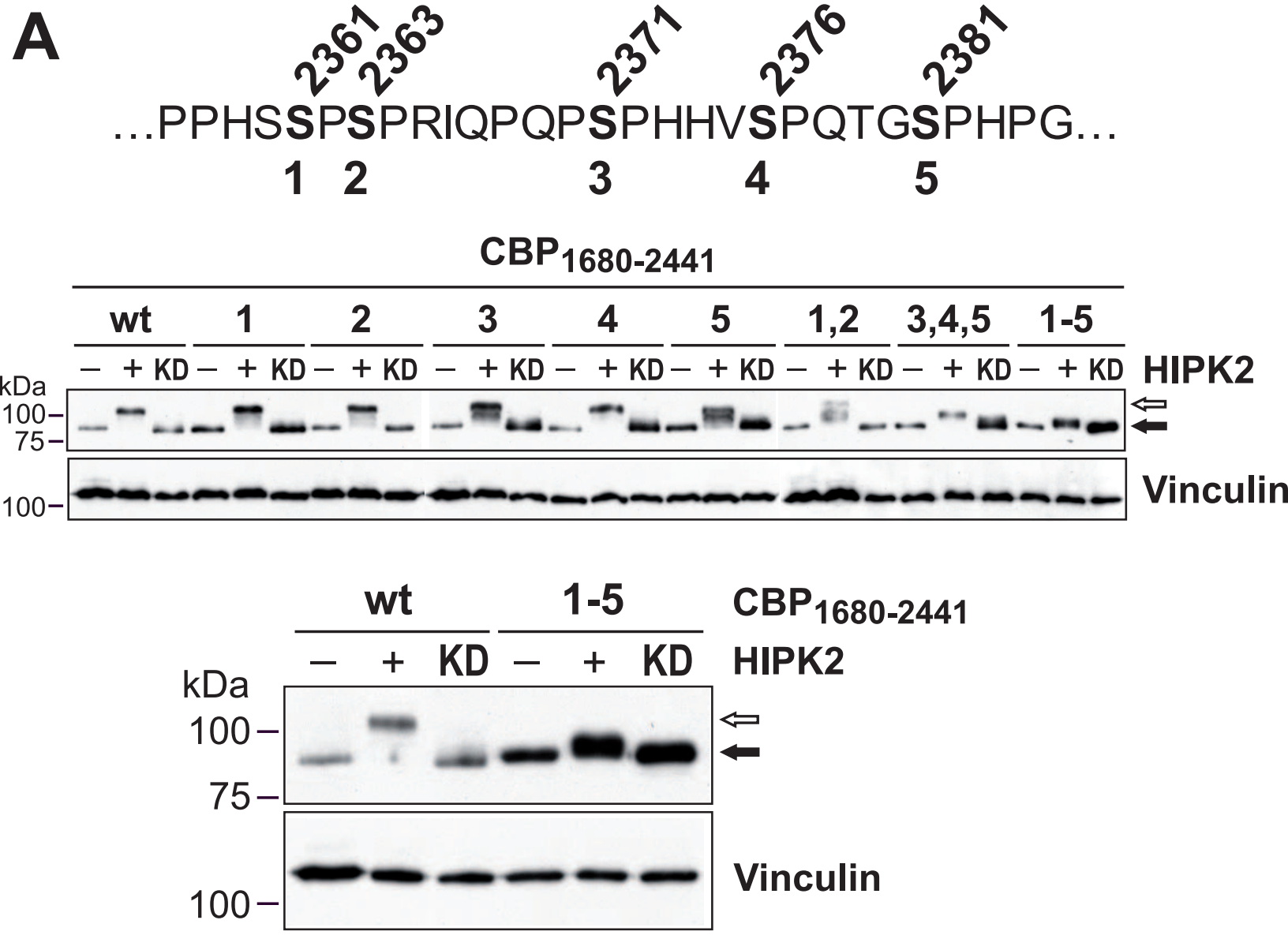
Revised Figure 5

Figure 5



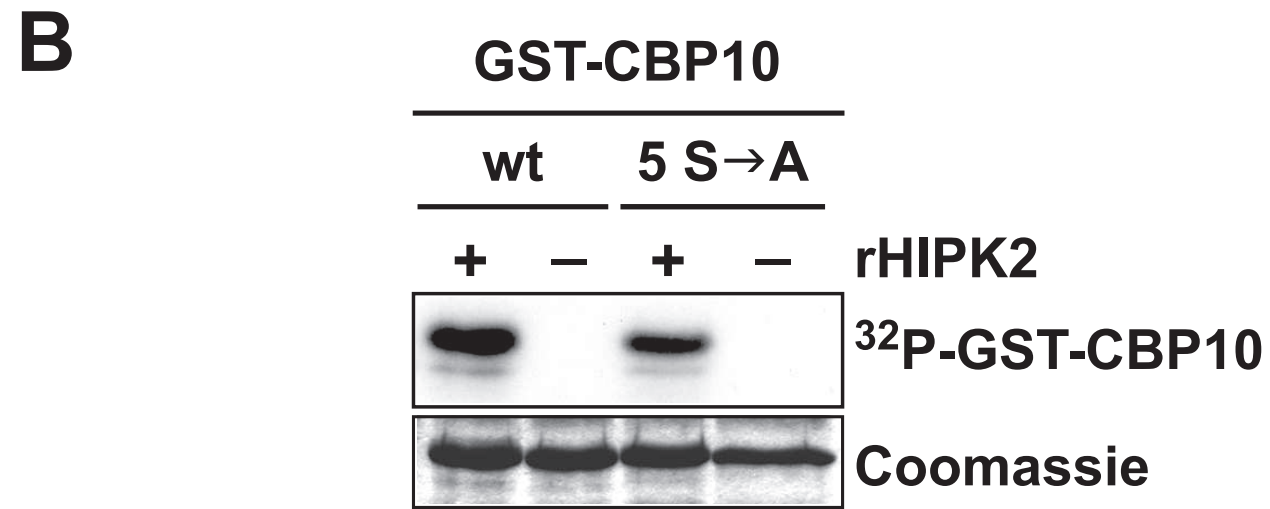


Figure 6

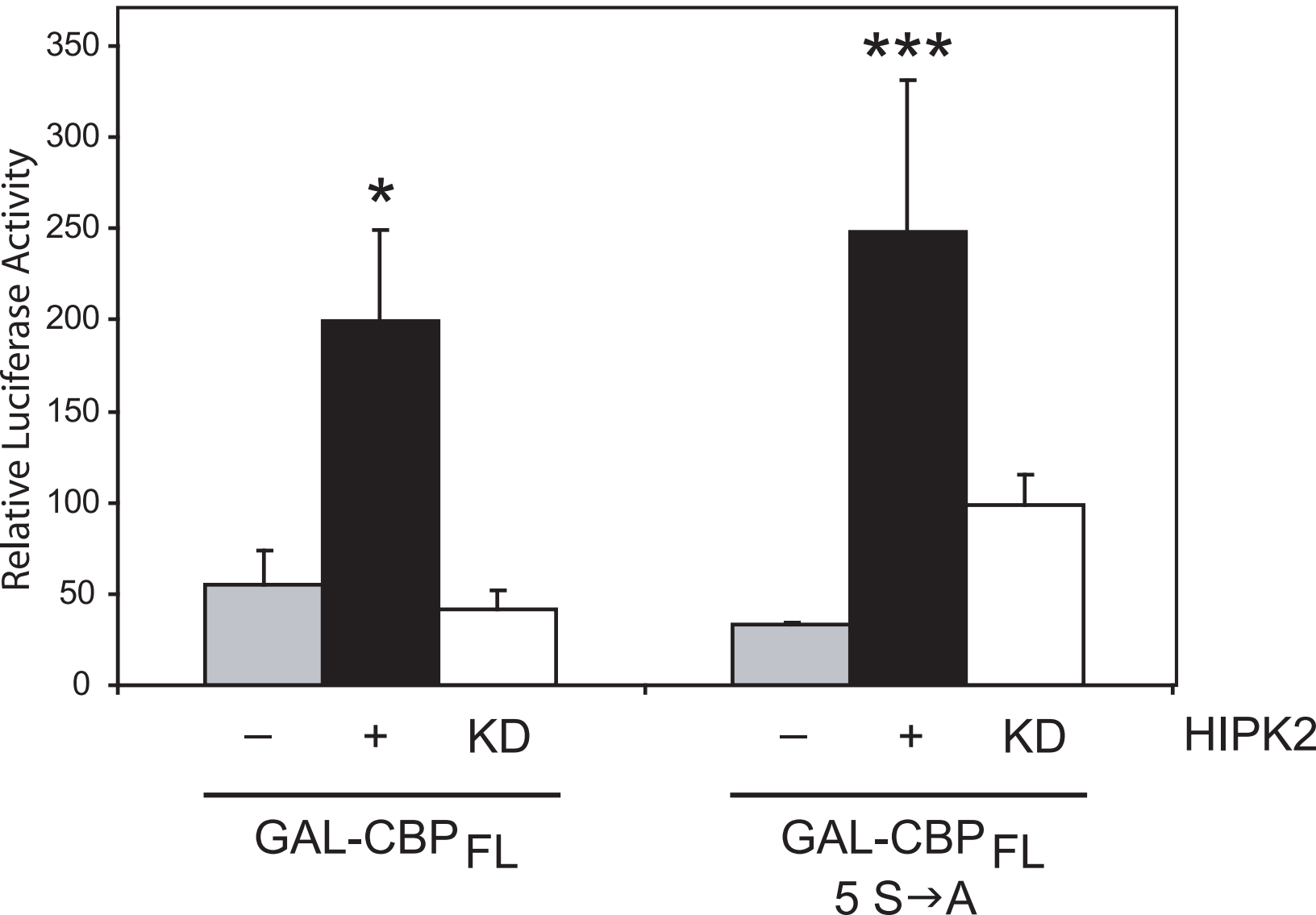

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Figure 7

Figure 7

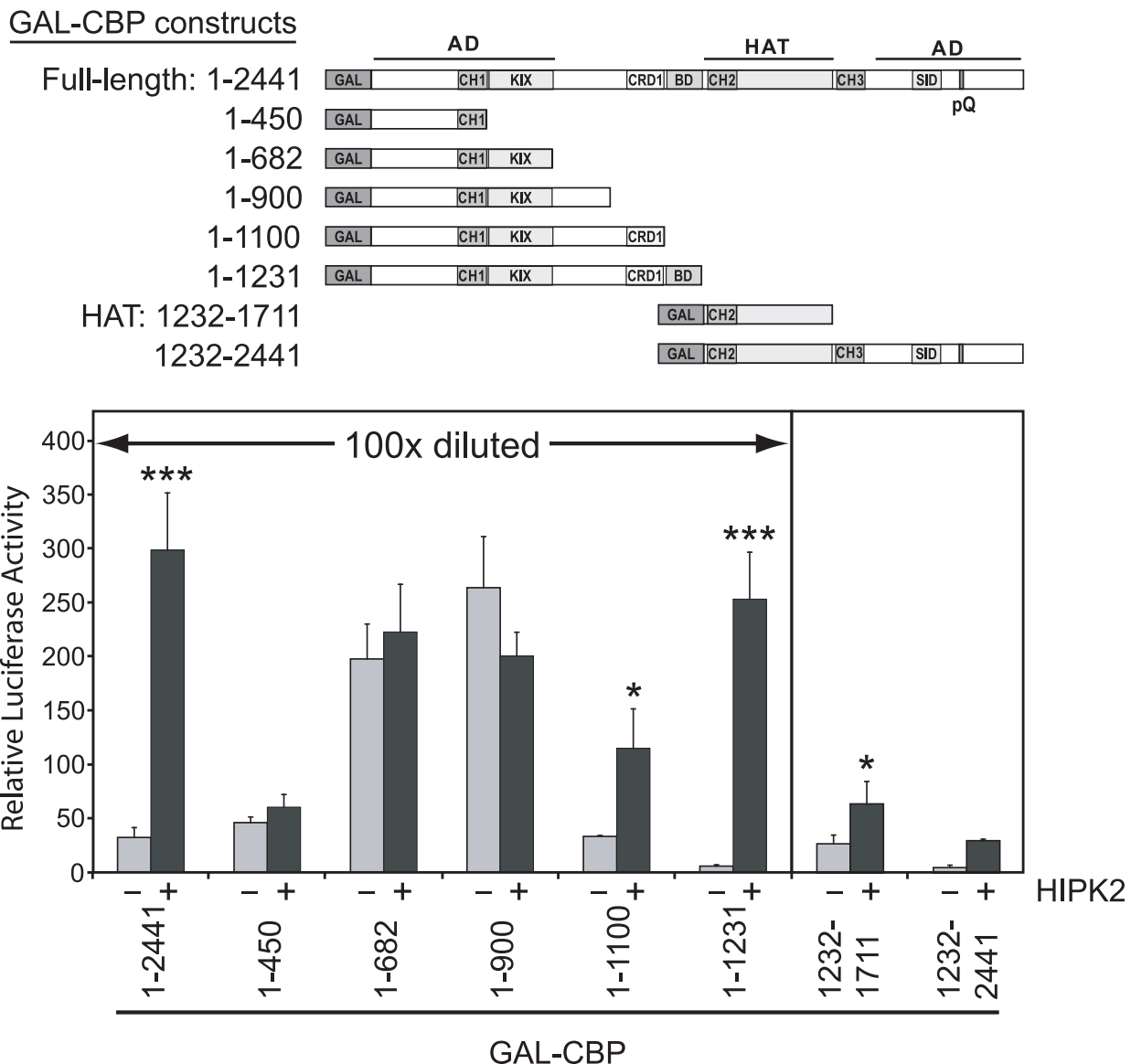
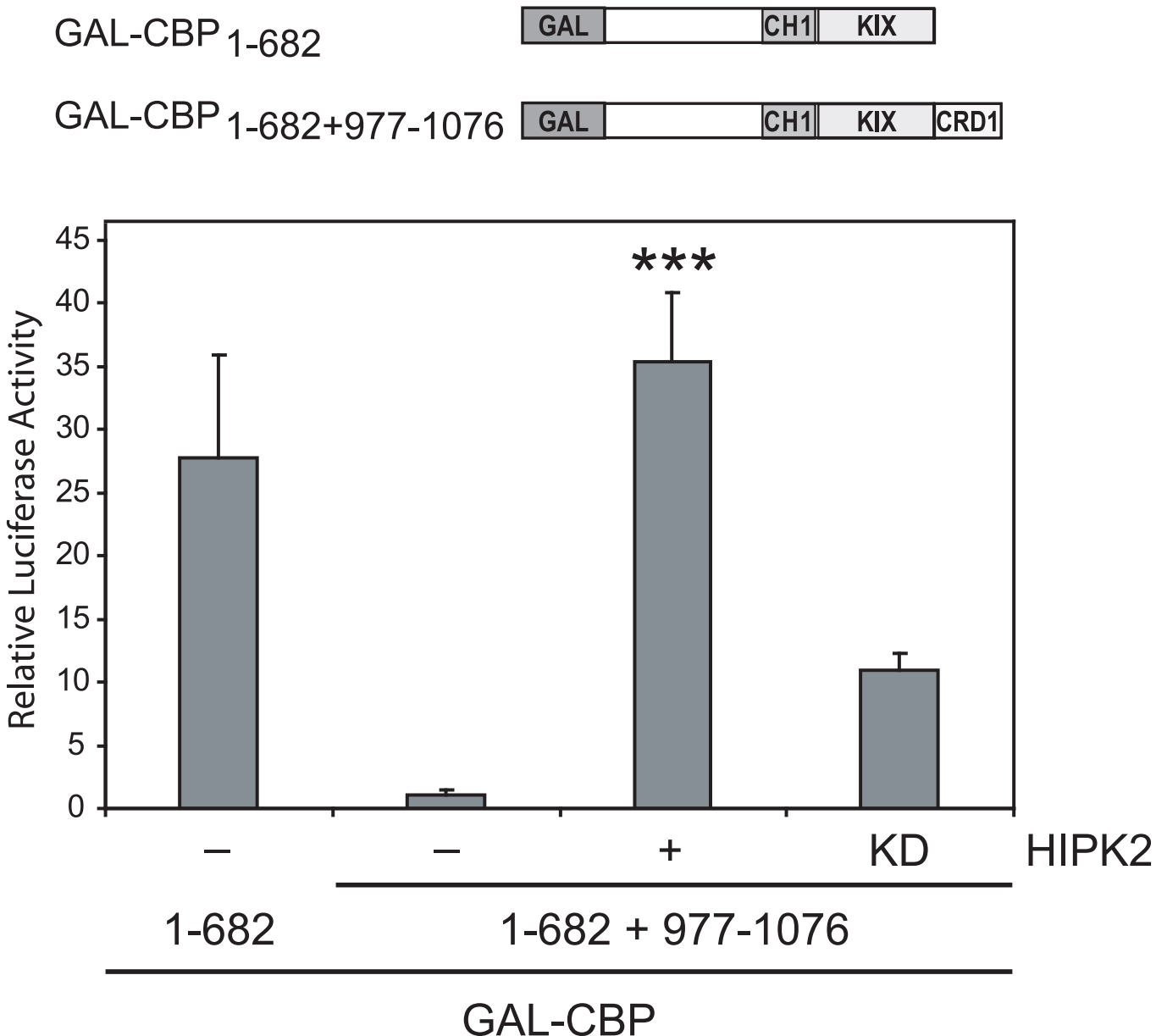


Figure 8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1-682 + 977-1076
GAL-CBP