

Supplementary Materials for

**Inhibition of PP1 Phosphatase Activity by HBx: A Mechanism for the Activation of Hepatitis B Virus Transcription**

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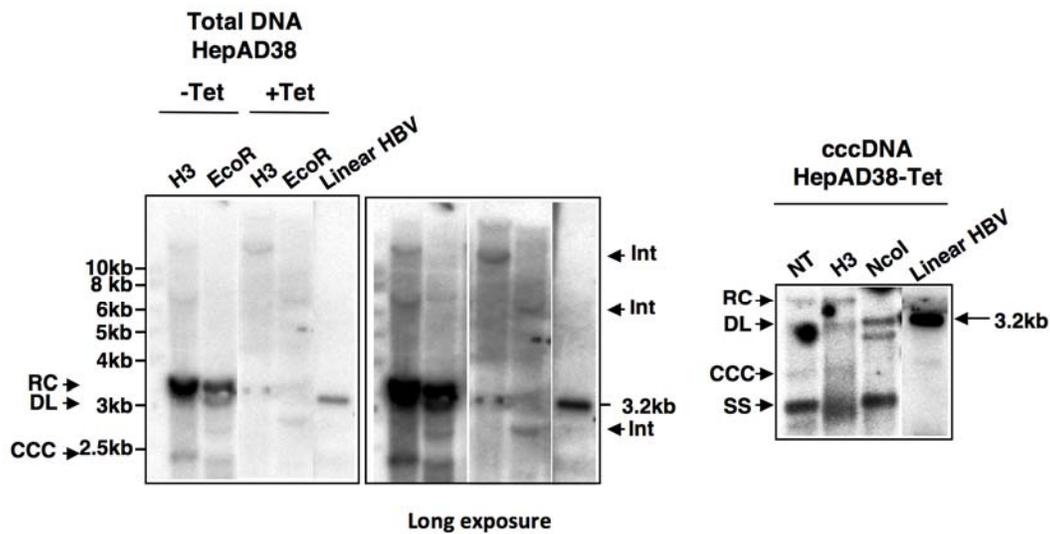
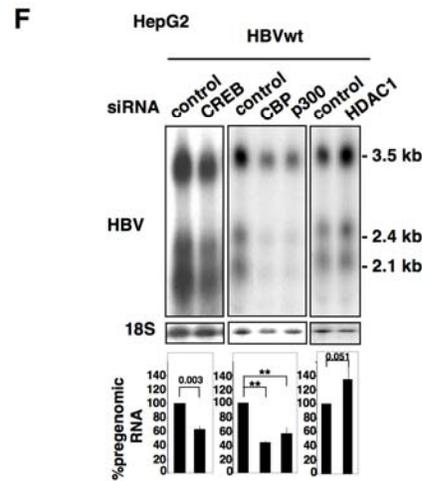
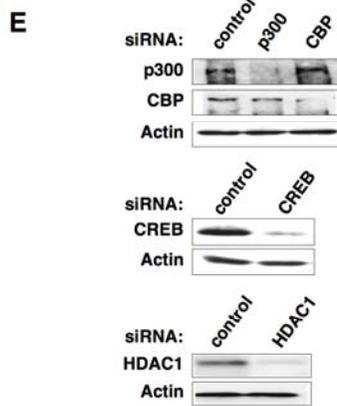
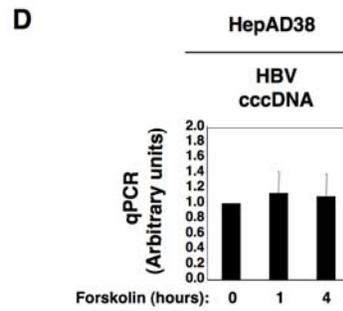
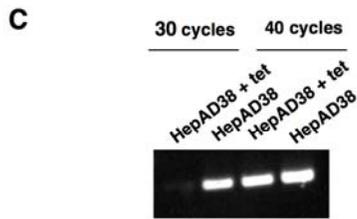
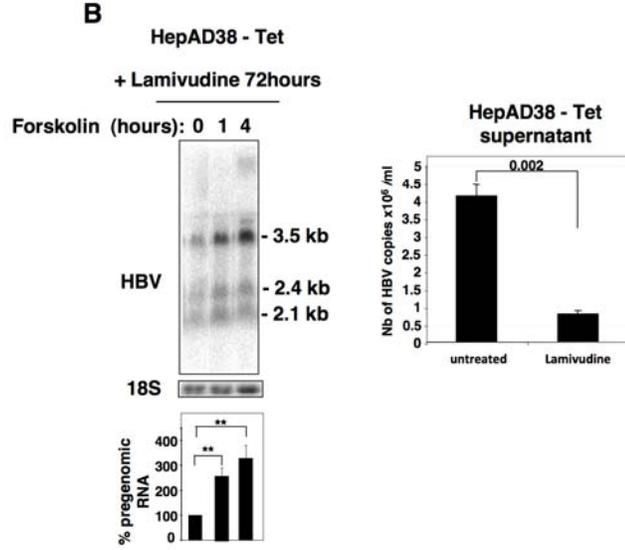
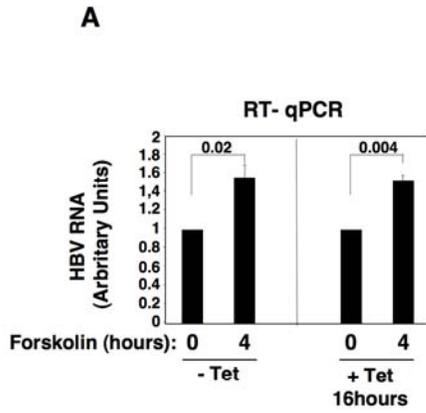


Fig. S1. Analysis of integrated HBV DNA and cccDNA in HepAD38 cells. HepAD38 cells were grown with or without tetracycline and used to either extract total DNA (left panel) or to isolate cccDNA (right panel). Total DNA was digested with Hind III (H3) or EcoRI (Eco1) and analyzed by Southern blot hybridization with a HBV probe using 50 pg of unit length HBV DNA as control (left panel). A longer exposure is shown. Alternatively, cccDNA was specifically extracted from HepAD38 cells and analyzed by enzymatic digestion using HindIII and NcoI (NT, no restriction) and blot hybridization (right panel). Nomenclature of HBV DNA species: int, integrated viral DNA; RC, relaxed circular, DL, double strand linear, CCC, covalently closed circular, SS, single strand DNA.



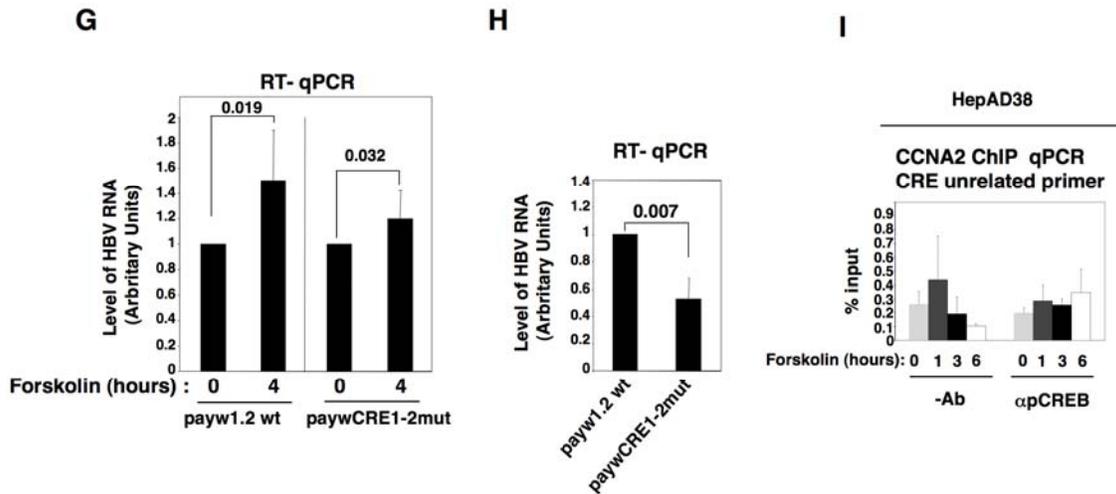


Fig. S2. Role of CREB in HBV DNA transcription. (A) HepAD38 cells were treated or not with tetracycline before forskolin treatment. HBV transcription was analyzed by RT-qPCR. P values shown were determined by Student's *t*-test. *n*=3 experiments. (B) RNA from HepAD38 cells grown without tetracycline and treated with 2  $\mu$ M lamivudine for 3 days was analyzed by Northern blotting after treatment with forskolin. The amount of the pgRNA in untreated cells was set at 100% (left panel). Error bars are SD. *n*=3 experiments. \*\**p*<0.01, One-way ANOVA with Dunnett post hoc test. Virus production in the supernatant of HepAD38 cells treated or not with lamivudine was quantified. P values shown were determined by Student's *t*-test. *n*=3 experiments. (C) Input sample from ChIP experiment from Fig. 1D were subjected to 30 or 40 cycles of PCR amplification using HBV-ChIP primers. (D) HepA38 cells grown without tetracycline were treated with forskolin before isolation of nuclear DNA. qPCR was carried out using primers that amplified HBV cccDNA or the *CCNA2* promoter to normalize the DNA samples. There was no significant difference after forskolin treatment compared to the control. *P*=0.34 by One-way ANOVA analysis. (E) HepAD38 cells were transfected with control Scr siRNA or specific siRNA directed against

CBP or p300, CREB or HDAC1 as indicated. The amounts of p300, CBP, CREB, and HDAC1 were analyzed by Western blotting using specific antibodies. Actin was used as loading control. (F) Northern blot analysis of HBV transcripts isolated 7 days after transfection of HepG2 cells with the payw1.2 wt plasmid (5  $\mu$ g) and control or specific siRNA as indicated (top panel). The amount of pgRNA in cells transfected with control siRNA was set at 100% (bottom panel). Statistical differences were assessed using either t-test (P values are shown) or one-way ANOVA with Dunnett post hoc test (\*\* $p < 0.01$ ). (G) HepG2 cells transfected with either payw1.2 wt vector or paywCRE1-2 mut vector were grown in presence of lamivudine for 48 hours before adding forskolin. HBV transcription was analyzed by RT-qPCR. The relative amount of HBV RNA at time 0 was set to 1. P values were determined by the Student's *t*-test.  $n=3$  replicates. (H) HepG2 cells transfected with either payw1.2 vector or paywCRE1-2 mut vector were grown in presence of lamivudine. HBV transcription was analyzed by RT-qPCR. P values were calculated with t-test.  $n=4$  experiments. (I) ChIP assays were carried as in Fig. 1F with primers that amplified sequences located 10 kb upstream the *CCNA2* promoter region. Immunoprecipitation using anti-pCREB shows no significant difference after forskolin stimulation compared to control by one-way ANOVA analysis.

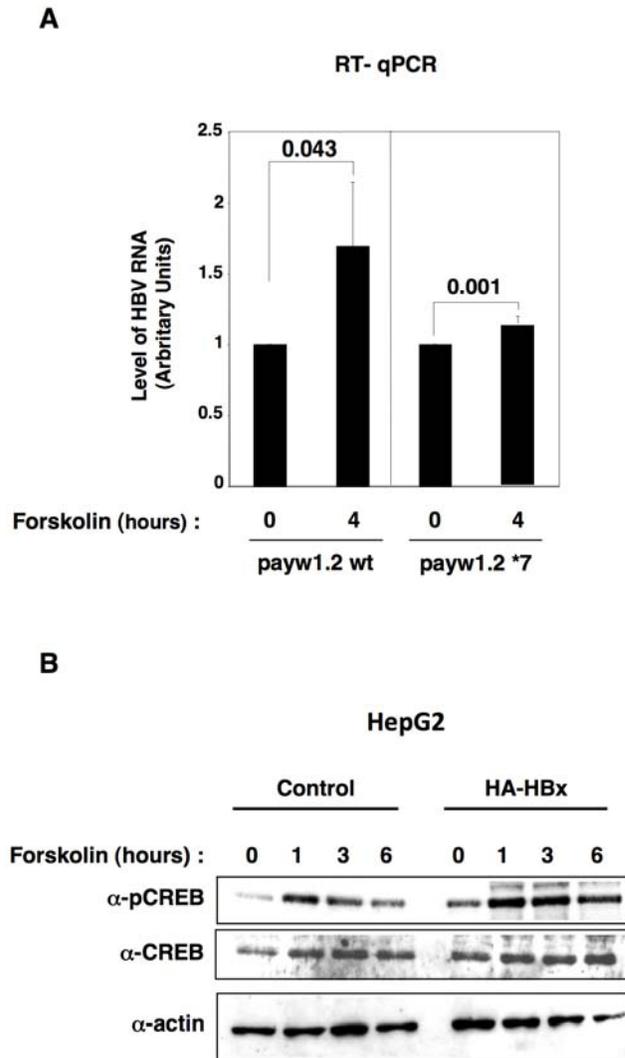


Fig. S3. HBx prolongs CREB phosphorylation in HepG2 cells. (A) HepG2 cells were transfected with payw1.2 or payw1.2\*7 HBV vectors and treated with forskolin for 4 hours before total RNA was extracted. HBV transcription was analyzed by RT-qPCR. The amount of HBV pgRNA at time 0 for the wild-type and HBx-deficient HBV vector was set at 1. Results are the average of four independent experiments. P values are calculated with t-test (B) Western blot analysis of endogenous pCREB and total CREB in whole cell extracts from HepG2 cells transfected with an HBx expression vector or empty vector. Cells were treated with forskolin and harvested at the indicated time points. Actin was used as loading control.

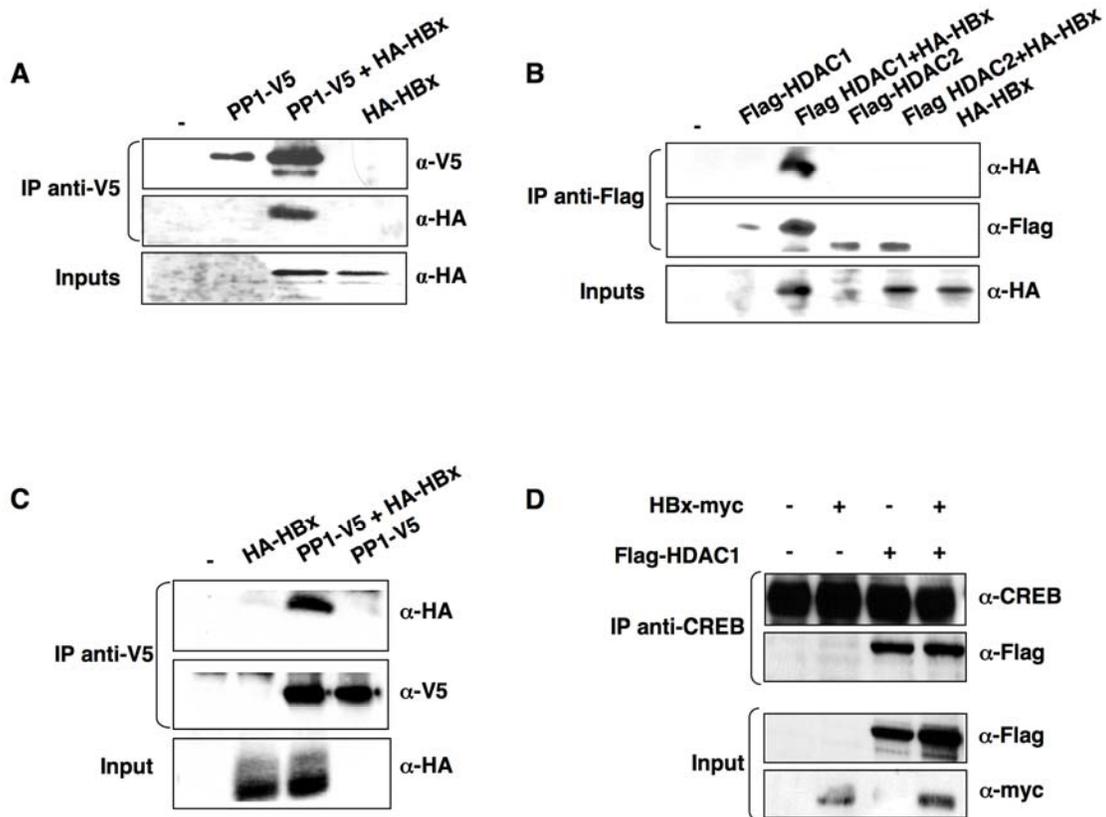
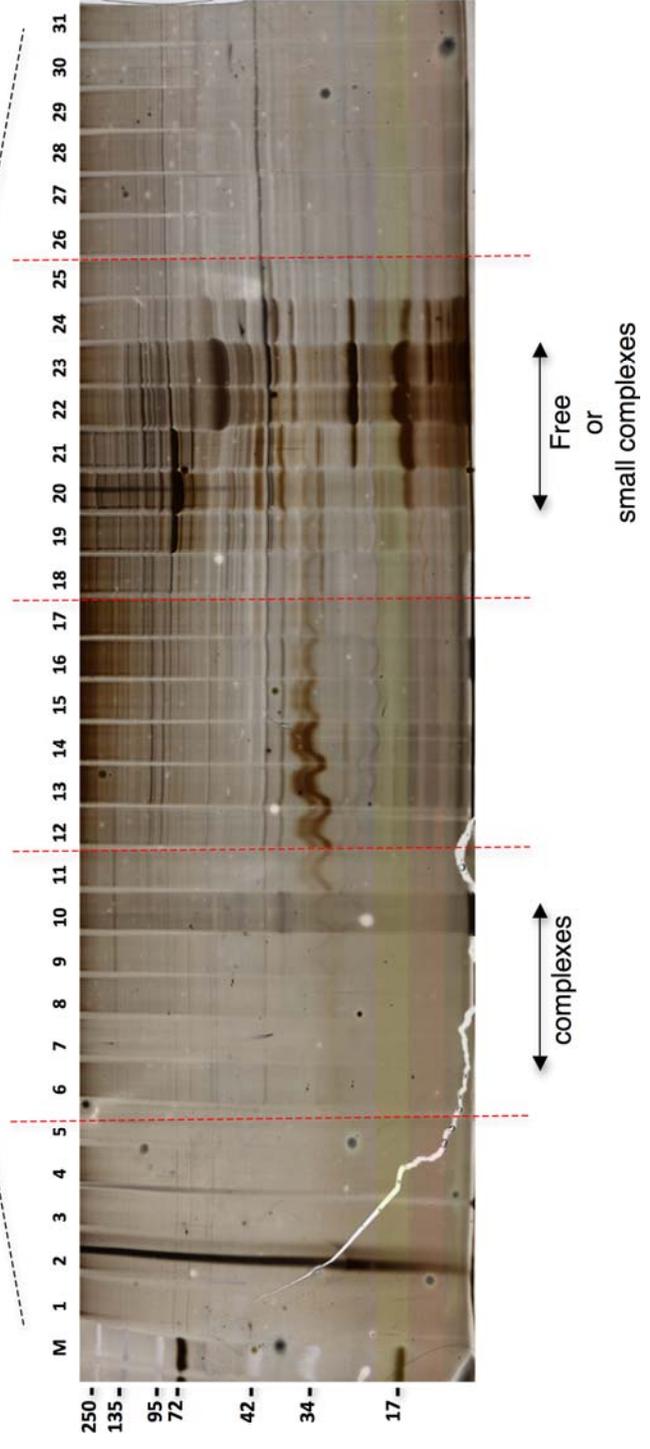
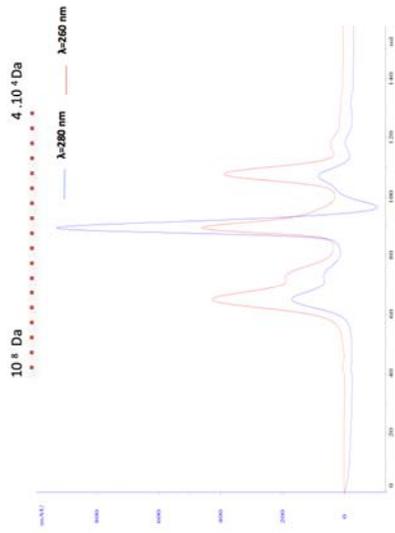


Fig. S4. HBx interacts with PP1 and HDAC1. (A) HEK293 cells were cotransfected with empty vector or with different combinations of vectors encoding V5-His-PP1 or HA-HBx and total extract was immunoprecipitated with anti-V5 antibody and analyzed by Western blotting using anti-HA or anti-V5 antibodies. (B) Anti-Flag immunoprecipitates from HEK293 cells cotransfected with a combination of empty vector, HA-HBx, Flag-HDAC1, or Flag-HDAC2 were subjected to SDS-PAGE followed by Western blotting with anti-HA or anti-FlagM2 antibodies. The amount of HA-HBx in the total lysate was determined by Western blotting (input). (C) Anti-V5 immunoprecipitates from Huh7 cells cotransfected with empty vector or with different combinations of vectors encoding V5-His-PP1 or HA-HBx were analyzed by Western blotting using anti-HA or anti-V5 antibodies. (D) Anti-CREB immunoprecipitates from HeLa cells cotransfected with empty vector or different combination of Flag-HDAC1 and HBx-myc plasmids were analyzed by Western blotting using anti-Flag M2 antibody to

determine recovery of Flag-HDAC1. The amounts of Flag-HDAC1 and HBx-myc in the input are shown.

**A**



**B**

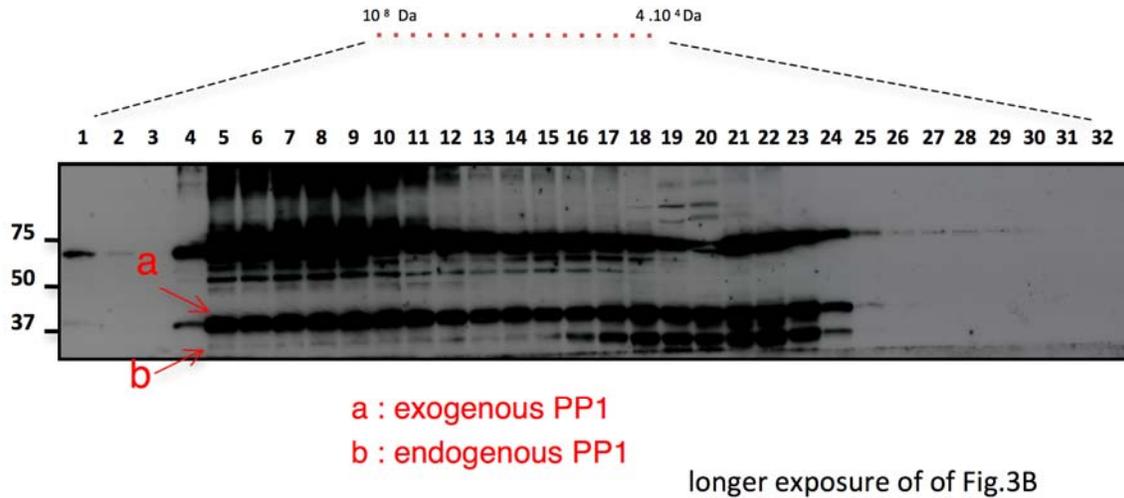


Fig. S5. Endogenous PP1 elutes with HDAC1 and HA-HBx in high-molecular weight fractions. (A) Total cell lysate from HEK293 cells transfected with HA-HBx, Flag-HDAC1, and V5-His-PP1 was analyzed by gel filtration chromatography followed by SDS-PAGE and visualized by silver staining (see Fig. 3B for Western blots). (B) Longer exposure of Fig. 3B to show detection of endogenous PP1.

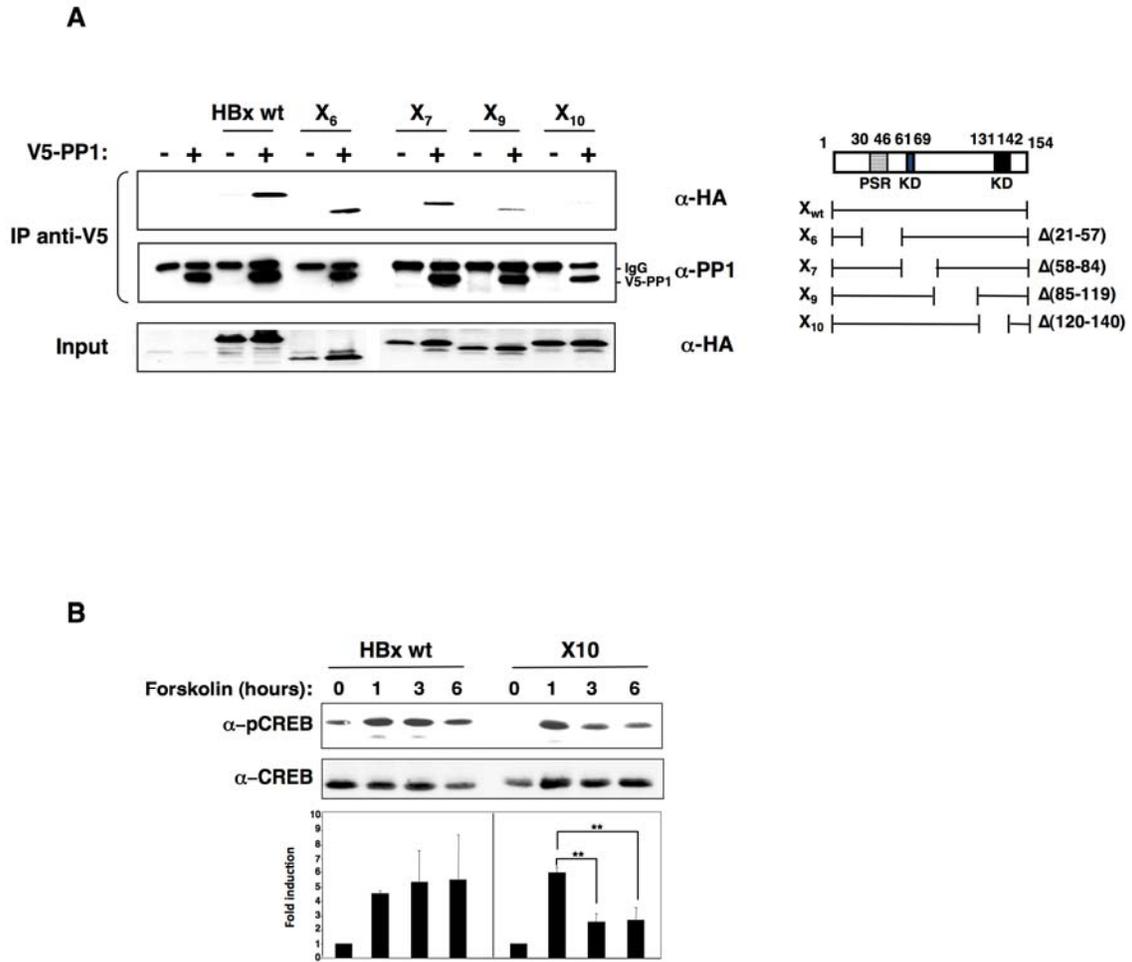


Fig. S6. The C-terminal region of HBx interacts with PP1 and is required for prolongation of CREB phosphorylation. (A) Anti-V5 immunoprecipitates from HEK293 cells cotransfected with V5-His-PP1 plasmid and either wild-type HBx or the indicated deletion mutants were immunoblotted with anti-HA antibody to detect HBx in the immune complexes. The amount of immunoprecipitated V5-His-PP1 was determined by immunoblotting with V5 antibodies. Wild-type HBx and deletion mutants were detected with anti-HA antibodies. Right panel: Schematic representation of the full-length wild-type HBx protein and in-frame HBx deletion mutants used for the coimmunoprecipitation experiment. (B) Western blotting analysis of endogenous pCREB and total CREB in HEK293 cells transfected with either wild-type HBx

or the X10 mutant and treated with forskolin. Cells were harvested at the times indicated. The pCREB signal was normalized to the CREB signal, then normalized to the signal at time 0. Values are the average of three separate experiments and are presented graphically relative to the amount of pCREB at time 0 (lower panel). \*\*P<0.001, by one-way ANOVA with Bonferroni post hoc test.

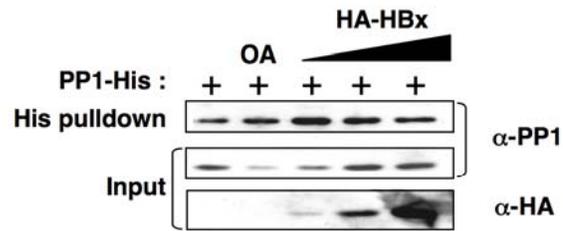


Fig. S7. Analysis of His-PP1 $\alpha$  elution and HA-HBx abundance by Western blot. HEK293 cells were transfected with V5-His-PP1 vector alone or with increasing amounts of HA-HBx plasmid and were treated or not with okadaic acid as indicated. Cell lysates were subjected to Ni-pulldown and His-PP1 proteins bound to the beads were eluted and analyzed by Western blotting using anti-PP1 antibody. The amounts of His-PP1 and HA-HBx in total lysates were analyzed by immunoblotting with anti-PP1 or anti-HA antibodies.