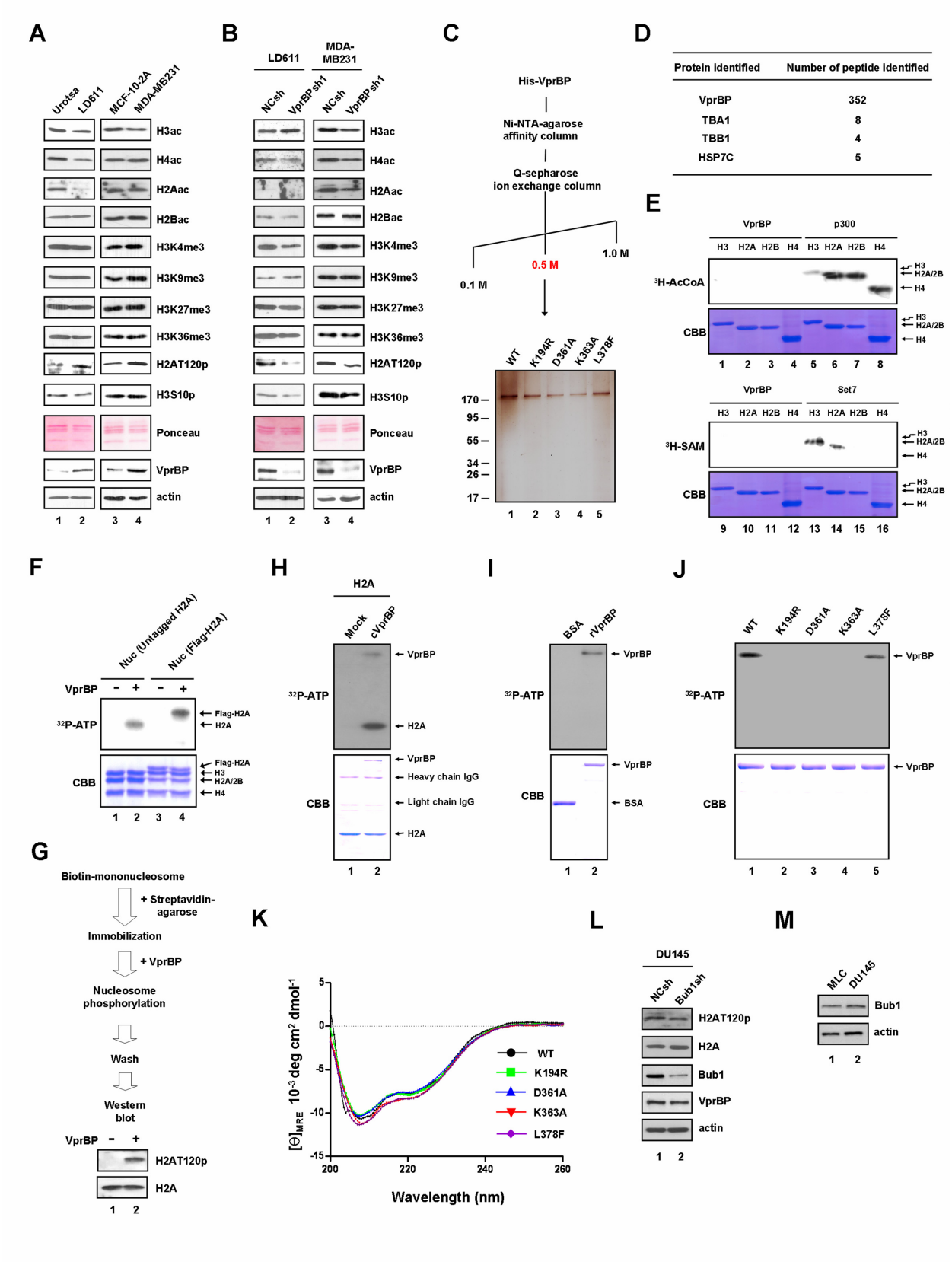
A
Urotsa
LD611
MCF-10-2A
MDA-MB231
H3ac
H4ac
H2Aac
H2Bac
H3K4me3
H3K9me3
H3K27me3
H3K36me3
H2AT120p
H3S10p
Ponceau
VprBP
actin
1 2 3 4
B
LD611
MDA-MB231
NCsh
VprBPsh1
NCsh
VprBPsh1
H3ac
H4ac
H2Aac
H2Bac
H3K4me3
H3K9me3
H3K27me3
H3K36me3
H2AT120p
H3S10p
Ponceau
VprBP
actin
1 2 3 4
C
His-VprBP
Ni-NTA-agarose affinity column
Q-sepharose ion exchange column
0.1 M
0.5 M
1.0 M
WT
K194R
D361A
K363A
L378F
170
95
55
34
26
17
1 2 3 4 5
D
Protein identified
Number of peptide identified
VprBP 352
TBA1 8
TBB1 4
HSP7C 5
E
VprBP
p300
H3 H2A H2B H4 H3 H2A H2B H4
3H-AcCoA
CBB
H3
H2A/2B
H4
1 2 3 4 5 6 7 8
VprBP
Set7
3H-SAM
9 10 11 12 13 14 15 16
F
Nuc (Untagged H2A)
Nuc (Flag-H2A)
VprBP - + - +
32P-ATP
Flag-H2A
H2A
CBB
H3
H2A/2B
H4
1 2 3 4
G
Biotin-mononucleosome
+ Streptavidin-agarose
Immobilization
+ VprBP
Nucleosome phosphorylation
Wash
Western blot
VprBP - +
H2AT120p
H2A
1 2
H
H2A
Mock
cVprBP
32P-ATP
VprBP
H2A
CBB
Heavy chain IgG
Light chain IgG
1 2
I
BSA
rVprBP
32P-ATP
VprBP
CBB
BSA
1 2
J
WT
K194R
D361A
K363A
L378F
32P-ATP
VprBP
CBB
1 2 3 4 5
K
[Θ]MRE 10-3 deg cm2 dmol-1
5
0
-5
-10
-15
200
220
240
260
Wavelength (nm)
WT
K194R
D361A
K363A
L378F
L
DU145
NCsh
Bub1sh
H2AT120p
H2A
Bub1
VprBP
actin
1 2
M
MLC
DU145
Bub1
actin
1 2

Figure S1. Phosphorylation of H2A T120 by VprBP, related to Figure 1

(A) Chromatin was isolated from human bladder (LD611) and breast (MDA-MB231) cancer cell lines and their normal counterparts (Urotsa and MCF-10-2A). The levels of the indicated histone modifications were assessed by Western blotting as in Figure 1A.

(B) LD611 bladder and MDA-MB231 breast cancer cell lines were infected with a VprBP shRNA and examined for the indicated histone modifications by Western blotting.

(C) Recombinant VprBP proteins were expressed in Sf9 cells and purified as described under "Supplemental Experimental Procedures". The purity of the proteins used in this study was confirmed by SDS-PAGE and subsequent silver staining.

(D) The purity of the recombinant VprBP wild type purified in (C) was determined by mass spectrometry.

(E) Individual core histones were incubated with VprBP in the presence of [^{3}H]-AcCoA or [^{3}H]-SAM, and their modifications were determined by autoradiography. As positive controls, p300 (acetylating all four core histones) and Set7 (methylating H3K4) were included in the assays.

(F) In vitro kinase assays were performed as in Figure 1C, but using reconstituted nucleosomes containing untagged H2A (lanes 1 and 2) or Flag-tagged H2A (lanes 3 and 4).

(G) Mononucleosomes were immobilized on streptavidin-agarose beads and incubated with VprBP in the presence of 10 mM ATP. After extensive washing, intranucleosomal H2AT120p was accessed by immunoblotting with H2A and H2AT120p antibodies.

(H) In vitro kinase assays were performed with recombinant H2A and endogenous VprBP immunoprecipitated from DU145 cells.

(I) The recombinant VprBP (5 μg) was separated on an 8% SDS-PAGE gel, denatured with 6M guanidine HCl for 1 h and renatured for 16 h. To detect autophosphorylation of VprBP, the gel was soaked in kinase buffer in the presence of [γ-^{32}P]ATP (20 Ci/ml) for 1h, washed stringently for 2 h, dried, and visualized by autoradiography.

(J) Wild type and mutant VprBP proteins were run on a denaturing gel, refolded, and subjected to in situ kinase assay as in Figure S1I.

(K) CD values were determined using 1 μM VprBP proteins in the range of 200-260 nm. CD spectra are the average of 20 measurements.

(L) DU145 cells were infected with Bub1 shRNA, and the levels of H2AT120p were determined by Western blot analysis of cell extracts.

(M) Whole cell extract were prepared in MLC and DU145 cells, and total Bub1 expression levels were analyzed by Western blotting.

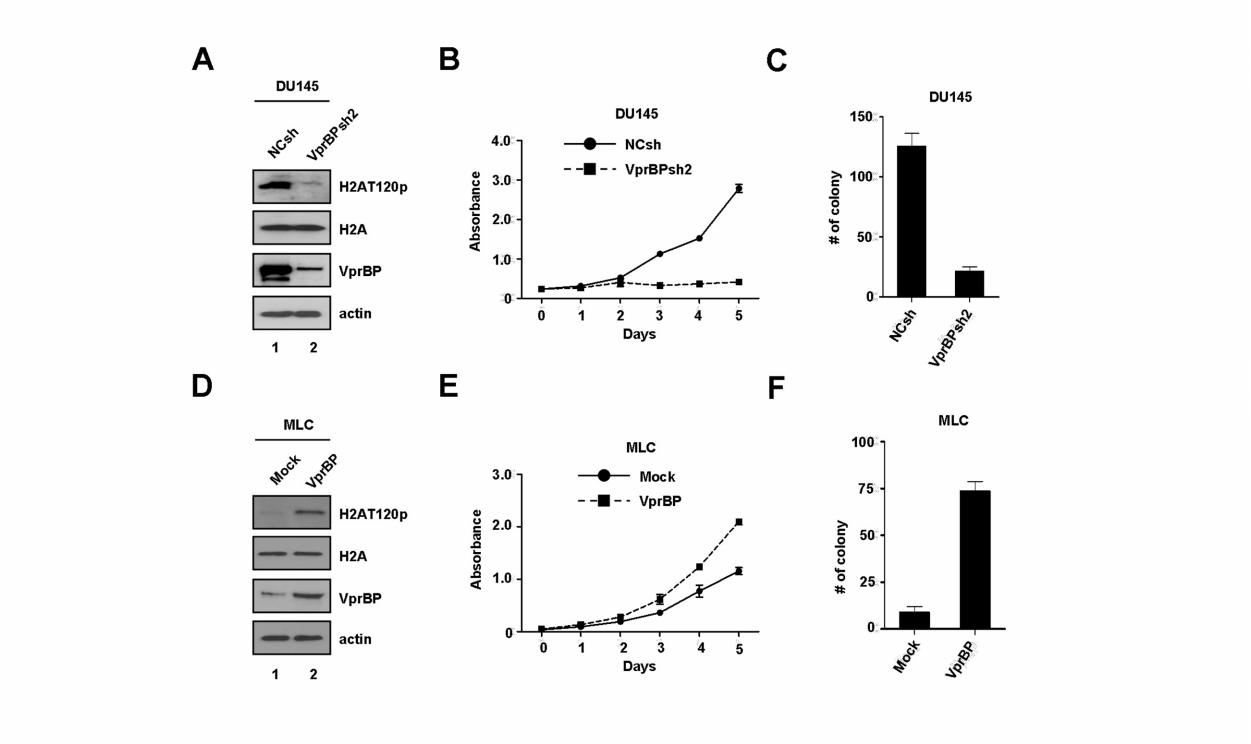

Figure S2. Effects of knockdown and overexpression VprBP on cell proliferation, related to Figure 2

(A) DU145 cancer cells were depleted of VprBP using another shRNA, as confirmed by Western blotting.

(B) DU145 cells depleted of VprBP in (A) were subjected to MTT assays over a period of 5 days. Results are the means ± S.D. of three experiments performed in triplicate.

(C) DU145 cells depleted of VprBP in (A) were subjected to colony formation assays as described in Figure 2D. Data represent the means ± S.D. of three independent experiments.

(D) MLC cells were infected with lentiviruses expressing VprBP, and the levels of VprBP and H2AT120p were assessed by Western blotting.

(E) VprBP was overexpressed in MLC cells as in (D), and its effects on cell proliferation were determined by MTT assay over a period of 5 days. Average and standard deviation are shown for three independent experiments.

(F) Colony formation assays were carried out as in (C), but using MLC cells overexpressing VprBP. Average and standard deviation of three independent experiments are shown.

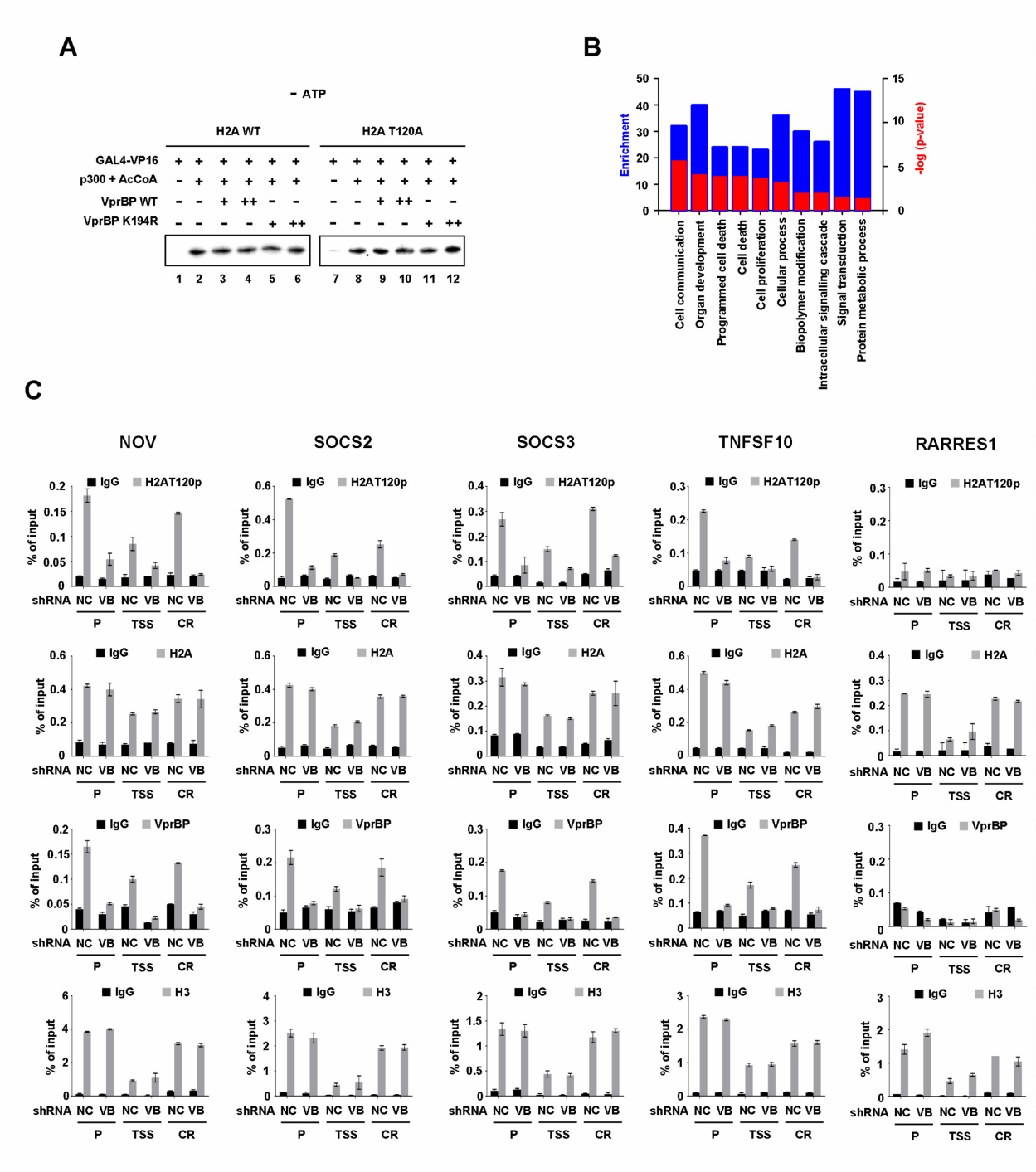

Figure S3. H2AT120p is required for the transrepression activity of VprBP, related to Figure 3

(A) In vitro transcription assays were performed using chromatin templates containing wild type or T120-mutated H2A as in Figure 3A, but in the absence of ATP.

(B) The 292 genes identified as being upregulated upon VprBP knockdown from the microarray analysis were subjected to functional enrichment analysis using DAVID. The blue and red bars represent the number of enriched genes and p-values for the enrichment, respectively.

(C) ChIP assays of four VprBP target genes (*NOV*, *SOCS2*, *SOCS3* and *TNFSF10*) and one control gene (*RARRES1*) were performed as in Figure 3E using the indicated antibodies. The precipitated DNA was quantified by qPCR with the primers listed in Supplemental Experimental Procedures. Results represent the means ± S.D. of three independent experiments.

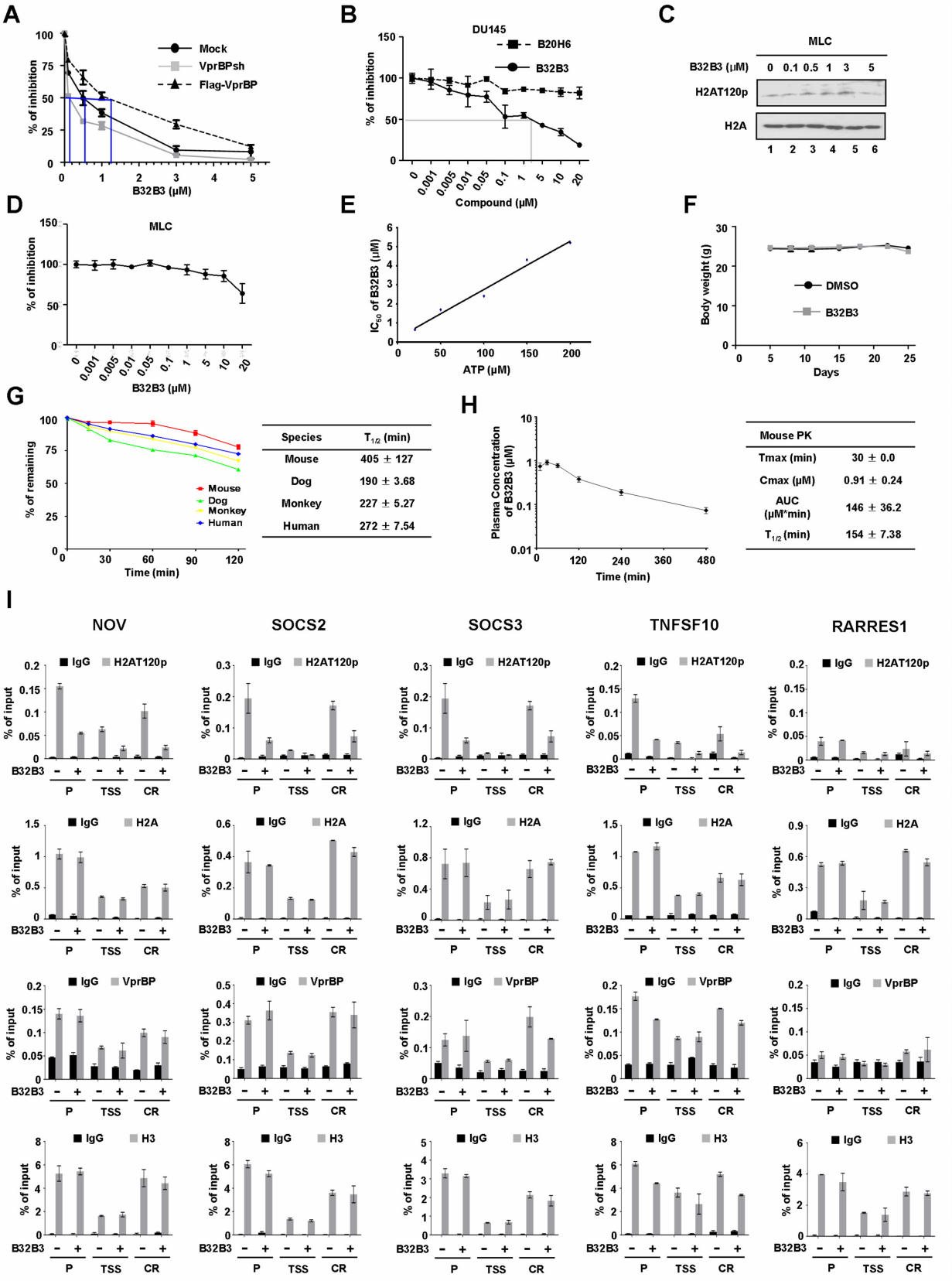
A
% of inhibition
Mock
VprBPsh
Flag-VprBP
B32B3 (μM)
B
DU145
B20H6
B32B3
% of inhibition
Compound (μM)
C
MLC
B32B3 (μM) 0 0.1 0.5 1 3 5
H2AT120p
H2A
1 2 3 4 5 6
D
MLC
% of inhibition
B32B3 (μM)
E
IC50 of B32B3 (μM)
ATP (μM)
F
Body weight (g)
DMSO
B32B3
Days
G
% of remaining
Mouse
Dog
Monkey
Human
Time (min)
Species
T1/2 (min)
Mouse 405 ± 127
Dog 190 ± 3.68
Monkey 227 ± 5.27
Human 272 ± 7.54
H
Plasma Concentration of B32B3 (μM)
Time (min)
Mouse PK
Tmax (min) 30 ± 0.0
Cmax (μM) 0.91 ± 0.24
AUC (μM*min) 146 ± 36.2
T1/2 (min) 154 ± 7.38
I
NOV
SOCS2
SOCS3
TNFSF10
RARRES1
IgG
H2AT120p
H2A
VprBP
H3
% of input
B32B3
P
TSS
CR

Figure S4. Characterization of B32B3 as a small-molecule VprBP kinase inhibitor, related to Figure 4.

(A) DU145 cells were infected with mock lentiviruse, VprBP-expressing shRNA lentivirus or Flag-VprBP-expressing lentivirus, and treated with the indicated concentrations of B32B3 for 24 h. Changes in H2AT120p as the results of B32B3 treatment were determined by the quantitative estimates of Western band intensity. Results represent the means ± S.D. of three independent experiments.

(B) DU145 cells were treated with the indicated concentrations of B32B3 and B20H6 for 72 h. Cell viability was measured by the MTT assay and was normalized to cells not exposed to the compounds. Data represent the means ± S.D. of three independent experiments.

(C) MLC cells were grown in the presence of the indicated concentrations of B32B2 for 24 h, and subjected to immunoblotting with H2AT120p and H2A antibodies.

(D) MLC cells were treated with B32B3 at the indicated doses for 24 h, and cell viability was assessed by MTT assay. Average and standard deviation of three independent experiments are shown.

(E) VprBP-mediated H2AT120p was analyzed in the presence of increasing concentrations of ATP and B32B3.

(F) Body weights of vehicle or B32B3-treated mice were monitored during the treatment period. Mean body weights ± S.E.M. are shown.

(G) B32B3 was spiked into the indicated blank plasma to a concentration of 10 μM. The stability of B32B3 in plasma was determined by LC-MS/MS at the indicated time points. Results are shown as the mean of three independent experiments ± S.D.

(H) B32B3 was administered to mice (n = 5) at a dose of 5 mg/kg. Blood samples were collected at the indicated time points, and the concentration of B32B3 in plasma was analyzed by HPLC-coupled mass spectrophotometry. The data are shown as the mean ± S.D.

(I) B32B3-treated DU145cells were subjected to ChIP analysis as in Figure 4I using indicated antibodies. Average and standard deviation are shown for three independent experiments.

Table S4. Inhibition of human kinases by B32B3, related to Figure 4

Kinase	IC_{50} (μM)	Kinase	IC_{50} (μM)
VprBP	0.6	DNAPK	> 10
AKT1	> 10	DYRK1	> 10
AKT3	> 10	EEF2K	> 10
ATM	> 10	GSK3β	> 10
ATR	> 10	HCK	> 10
AURKA	> 10	LCK	> 10
AURKB	> 10	PKM2	> 10
BCK	> 10	PKN2	> 10
BUB1	> 10	PRKCD	> 10
CDK2	> 10	PLK1	> 10
CDK7	> 10	PLK2	> 10
CDK9	> 10	RSK2	8.6
CDK18	> 10	RSK3	> 10
CHK1	> 10	STK25	> 10
CHK2	> 10	STK33	> 10
CSNK1D	> 10	SYK	> 10
CSK1E	> 10	VRK2	> 10

Supplemental Experimental Procedures

Cell culture, constructs and antibodies

MDA-MB231, LD611 and DU145 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS. MCF-10-2A cells were grown in a 1:1 mixture of DMEM and Ham's F12 supplemented with 20 ng/ml epidermal growth factor, 100 ng/ml cholera toxin, 0.01 mg/ml insulin, 500 ng/ml hydrocortisone, and 5% horse serum. Urotsa cells were grown in DMEM low glucose containing 10% FBS. MLC cells were grown in T medium containing 10% FBS. To express VprBP using the baculovirus system, VprBP cDNA was subcloned into the EcoRI and XhoI sites of pFASTBAC vector with an N-terminal His epitope. To generate VprBP mutants, VprBP cDNA was mutated by the QuikChange® II site-directed mutagenesis kit (Agilent Technologies) before the construction. For mammalian expression of VprBP wild type and mutants, the corresponding cDNAs were amplified by PCR and ligated into the EcoRI and SalI sites of lentiviral expression vector pLenti-Hygro (addgene) containing 5' FLAG coding sequence. For bacterial expression of human H2A proteins, H2A cDNA was inserted into the NdeI and BamHI sites of pET-11a or pET-11d vector in frame with FLAG sequences. Single- or multiple- residue substitutions in H2A were made by QuickChange kit and verified by DNA sequencing. Antibodies specific for H3ac, H4ac, H2Aac, H2Bac, H3K27me3, H3S10p and H2A were from Millipore; antibodies for H3K4me3, H3K9me3 and H2AT120p (for Western blotting) were from Active Motif; antibodies for H3K36me3 and H2AT120p (for immunostaining and ChIP) were from Abcam; antibody for VprBP was from Proteintech Group; antibody for Bub1 was from GeneTex; and antibody for actin was from Sigma.

Chromatin extraction

Cells were lyzed by suspending in buffer A (10 mM HEPES, pH 7.4, 10mM KCl, 1.5 mM $MgCl_2$, 0.34 M sucrose, 10% glycerol, 1 mM DTT, 5 mM β-glycerophosphate, 10 mM NaF, protease inhibitor, and 0.2% TritonX-100) and incubating on ice for 8 min. Nuclei were isolated by centrifugation (1,300 X g for 10 min at 4°C), and the supernatant was discarded. The resulting nuclei pellet was resuspended in buffer B (3 mM EDTA, 0.2 mM EGTA, 1 mM DTT, 5 mM β-glycerophosphate, 10 mM NaF, and protease inhibitor) and incubated for 30 min on ice. The

suspension was centrifuged (1,700 X g for 5 min at 4°C) and then the pellet was washed with buffer B three times. The chromatin pellet was sonicated in Laemli sample buffer.

Recombinant proteins

His-VprBP wild type and mutants were expressed using a baculovirus vector in insect (Sf9) cells. The expressed proteins were initially purified with Ni-NTA agarose (Novagen), and further purified with Q Sepharose (GE healthcare) column according to standard procedures. The purity and intactness of the recombinant VrpBP proteins were confirmed by quantitative LC-MS/MS and Western blotting. Recombinant histones were expressed in Escherichia coli Rosetta 2 (DE3) pLysS cells (Novagen) and purified as described previously (Dyer et al., 2004).

In-gel kinase assay

In-gel kinase assay was performed as described (Wooten, 2002) with minor modifications. Briefly, wild type and mutant VprBP proteins (5 μg) were resolved on an 8% SDS-PAGE gel, and the VprBP proteins in the gel were denatured in denaturation buffer (50 mM Tris-HCl, pH 8.0, 20 mM DTT and 6 M Guanidine HCl) for 1 h at room temperature and were renatured for 16 h at 4°C in renaturation buffer (50 mM Tris-HCl, pH 8.0, 5 mM DTT, 0.04% Tween-20, 100 mM NaCl and 5 mM $MgCl_2$). The kinase reaction was initiated in 15 ml of kinase buffer (25 mM Hepes, pH 7.4, 20 mM $MgCl_2$, 5 mM NaF, 1 mM DTT) containing 50 μM ATP and 20 Ci of [γ-^{32}P] ATP for 1 h at 30°C. The reaction was terminated by washing the gel with a fixing solution containing 10 mM sodium pyrophosphate and 5% trichloroacetic acid for 2 h. The gel was dried and subjected to autoradiography.

Circular dichroism (CD) spectroscopy

CD measurements were recorded using a Jasco J-810 spectropolarimeter with a 0.1 cm pathlength cuvette and a protein concentration of 1 μM. Circular dichroism spectra were obtained at 25 ^{o}C in phosphate buffer (10 mM sodium phosphate and 50 mM NaCl, pH 7.4). For each sample, 20 scans from 200 to 260 nm were averaged.

Immunostaining

The levels of VprBP and H2AT120p in tumors tissues were determined in FDA human tumor organ tissue microarray, which includes 16 types of organ cancer with matched or unmatched adjacent normal tissue (US Biomax, Inc). The formalin-fixed, paraffin-embedded sections were blocked by treating with blocking reagent (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.3% Triton X-100, and 5% normal goat serum) for 30 min at room temperature and incubated with VprBP and H2AT120p antibodies at 4 °C overnight. Immunodetection was performed using ABC reagent (Vectorstain). DAB (Vector Lab) was used for color development and hematoxylin (Sigma) was used for counterstaining. The intensity and distribution patterns of staining were evaluated by semiquantitative immunohistochemical assessment. The intensity of staining was graded from - to +++ (-, no staining; +, weak staining; ++, moderate staining; and +++, strong staining). The distribution of staining was classified from 0 to 3 (0, 0-20%; 1, 21-50%; 2, 51-80%; 3, 81-100%). For immunofluorescence of DU145 cells, cells were treated with DMSO or B32B3 (0.5 μM) for 24 h and fixed with 4% paraformaldehyde for 15 min. The corresponding samples were permeabilized with 0.3% Triton X-100 for 15 min and immunostained with H2AT120p antibody.

RNA interference

DNA oligonucleotides encoding VprBP shRNA1 (5'-CGAGAAACTGAGTCAAATGAA-3'), VprBP shRNA2 (5'- AATCACAGAGTATCTTAGA -3') and Bub1 shRNA (5'-CGAGGTTAATCCAGCACGTAT-3') were subcloned into pLKO.1-puro (Addgene) lentiviral vector according to standard procedures. To produce virus particles, 293T cells were cotransfected with the plasmids encoding VSV-G, NL-BH and the shRNAs. Two days after transfection, the soups containing the viruses were collected and used to infect cancer cells in the presence of polybrene (8 μg/ml).

Cell proliferation and colony formation assays

Cell proliferation was assessed by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay as previously described (Kim et al., 2012). To evaluate IC_{50} of compound B32B3 and B20H6, DU145 cells were treated with various concentrations (0.001, 0.005, 0.01, 0.05, 0.1, 1, 5, 10, and 20 μM) of compounds for 72 h and viability was measured by MTT assays. For soft agar colony formation assays, DU145 cells were treated with B32B3 (0.5, 1, and

3 μM). Cells were suspended in semisolid medium (DMEM 10% FBS plus 0.3% ultra pure noble agar) at concentrations of 2×10^5 cells/ml, added over a layer of 0.6% agar in RPMI on 35 mm plate and incubated for an additional 21 days. The colonies in each well were stained with 0.005% crystal violet in 20% ethanol, counted and photographed. All assays were run in triplicate, and results presented are the average of three individual experiments.

Microarray and qRT-PCR

Total RNA was isolated from mock- or VprBP-depleted cells using the TRIzol reagent according to the manufacturer's instructions (Invitrogen). Gene expression microarray experiments were conducted using a whole-genome expression array (Human HT-12 v4 Expression BeadChip, Illumina). This high density oligonucleotide array chip targets more than 47000 probe sequences derived from National Center for Biotechnology Information Reference Sequence (NCBI) RefSeq Release 38 (November 7, 2009) and other sources. Data were processed and analyzed by the ArrayPipe software (www.pathogenomics.ca/arraypipe). Genes whose expression level was increased or decreased by a factor of >1.7 after VprBP knockdown are listed in Tables S2 and S3. For qRT-PCR, total RNA was extracted as described for microarray and subjected to RT reactions with the use of PerfeCta® SYBR Green FastMix (Quanta BIOSCIENCES) and an iCycler IQ5 real time cycler (Bio-Rad). The specificity of the amplification reactions were monitored by melting curve analysis. Assays were normalized to β-actin mRNA levels. The following primers were used for qRT-PCR: BMF (5'-CTCAGCCGACTTCAGCTCTT-3' and 5'-AGCCAGCATTGCCATAAAAG-3'), NKX3-1 (5'-AGAAAGGCACTTGGGGTCTT-3' and 5'-TCCGTGAGCTTGAGGTTCTT-3'), NOV (5'-ACGAGCTTTTGTCTCCGAAA-3' and 5'-ACACCAGACAGCATGAGCAG-3'), OPN3 (5'-GATCCCTTTTGCAGCTTCTG-3' and 5'-TTTGGACCCATTGGTTTTGT-3'), SOCS2 (5'-AAAAGAGGCACCAGAAGGAA-3' and 5'-GTCCGCTTATCCTTGCACAT-3'), SOCS3 (5'-GCCACCTACTGAACCCTCCT-3' and 5'-ACGGTCTTCCGACAGAGATG-3'), TNFSF10 (5'-TTCACAGTGCTCCTGCAGTC-3' and 5'-ACGGAGTTGCCACTTGACTT), and TOB1 (5'-GGTGAAAAGGGACCAGTGAA-3' and 5'-TGGAGAGCTGGACACTGATG).

Chromatin immunoprecipitation (ChIP)

Mock-depleted or VprBP-depleted DU145 cells were grown to 70-80% confluence, cross-linked with 1% formaldehyde for 10 min, and processed for ChIP as recently described (Kim et al., 2012). ChIP assays on B32B2-treated cells were performed in a similar manner, except that DU145 cells were treated with DMSO or 1 μM B32B3 for 24 h. All samples were run in triplicate and results were averaged. Sequences of the primers used for quantitative real time PCR (qPCR) are as follows: NOV (promoter, 5'-GCACCAGTGTTGAAGTGTGG-3' and 5'-GGCATGCTTGTCATCTCTCA-3'; TSS, 5'-GCCCTAAGGAGAGCAGCAC-3' and 5'-TTCGCTGTAGATTGGCACTG-3'; coding, 5'-CTGCTCATGCTGTCTGGTGT-3' and 5'-AGCTGCAGGAGAAGAGGTCA), OPN3 (promoter, 5'-TAGCTTGCACAAACCCTGTG-3' and 5'-TGTGGTTGCACAATCCCTAA-3'; TSS, 5'-GAAGGTGCCCAGCCAGTG-3' and 5'-GCCTGCTCTAGCCATTGTG-3'; coding, 5'-CAGGACTCCATTCCTGTGGT-3' and 5'-GGTTTCGTGCCTTGTTGAGT-3'), SOCS2 (promoter, 5'-GAAACGGGGTTGGCTGTAG-3' and 5'-GTCGCAATACACAGGCTTCA-3'; TSS, 5'-ATCCTCGAGGCTTTTGTGTG-3' and 5'-TCCCCCGTTAACGTTTAATTT-3'; coding, 5'-AGGATCTGGGGAGAAAGAGC-3' and 5'-GGGTCATGAGAGAAGGGTCA-3'), SOCS3 (promoter, 5'-CCGGAAATTCTCTCCTGCTA-3' and 5'-GGAGAGCTCGAGGTGGAAC-3'; TSS, 5'-CTCTCGTCGCGCTTTGTCT-3' and 5'-GGAGCAGGGAGTCCAAGTC-3'; coding, 5'-ATGGTCACCCACAGCAAGTT-3' and 5'-GCTGCACATTGGACTCAAAA-3'), and TNFSF10 (promoter, 5'-AAAATTAGCTGGGCATGGTG-3' and 5'-AACCTCCACCTCCCAGATTC-3'; TSS, 5'-GGGACAGTTGCAGGTTCAAT-3' and 5'-GGAGCACTGTGAAGATCACG-3'; coding, 5'-ATCCAAAGGGACTGGAGCTT-3' and 5'-GCTGCACATTGGACTCAAAA-3').

B32B3 stability assessment

To assess the metabolic stability of B32B3 in mouse, dog, monkey, and human plasma, the compound was spiked into blank plasma to a concentration of 10 μM. At 0, 15, 30, 60, 90, and 120 min time points, a 30 μl aliquot was collected and mixed with 270 μl acetonitrile solution for protein precipitation. After centrifugation, the supernatant was analyzed by LC-MS/MS. In vitro half-life of B32B3 in plasma was calculated using the slope (k) of the log-linear regression from the concentration remaining parent compound versus time.

$T_{1/2} = - \ln 2 / k$

Mouse pharmacokinetics of B32B3

Mouse pharmacokinetic study was carried out using mice (n=5) that were fasted for 12 h prior to and 2 h after dosing with B32B3 (5 mg/kg). Blood samples were collected from orbital sinus at 10, 30, 60, 120, 240, and 480 min post dose. The plasma was separated from blood samples by centrifugation and analyzed in the Agilent 1200 HPLC system coupled to Agilent 6460A QQQ mass spectrophotometer. The plasma concentration-time data were analyzed by non-compartmental analysis using WinNonlin version 4.1 (Pharsight).

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