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The Oncogenic Polycomb Histone Methyltransferase EZH2 Methylates Lysine 120 on Histone H2B and Competes Ubiquitination[1,2]

Masaharu Kogure[*,†], Masashi Takawa[*], Vassiliki Saloura[‡], Kenbun Sone[‡], Lianhua Piao[‡], Koji Ueda[§], Reem Ibrahim[*], Tatsuhiko Tsunoda[¶], Masanori Sugiyama[†], Yutaka Atomi[†], Yusuke Nakamura[‡] and Ryuji Hamamoto[*,‡]

*Laboratory of Molecular Medicine, Human Genome Center, Institute of Medical Science, The University of Tokyo, Minato-ku, Tokyo, Japan; [†]Department of Surgery, Kyorin University School of Medicine, Mitaka, Tokyo, Japan; [‡]Section of Hematology/Oncology, Department of Medicine, The University of Chicago, Chicago, IL; [§]Laboratory for Biomarker Development, RIKEN, Minato-ku, Tokyo, Japan; [¶]Laboratory for Medical Informatics, RIKEN, Yokohama, Kanagawa, Japan

Abstract

The histone methyltransferase enhancer of zeste 2 (EZH2) is known to be a polycomb protein homologous to *Drosophila* enhancer of zeste and catalyzes the addition of methyl groups to histone H3 at lysine 27 (H3K27). We previously reported that EZH2 was overexpressed in various types of cancer and plays a crucial role in the cell cycle regulation of cancer cells. In the present study, we demonstrated that EZH2 has the function to monomethylate lysine 120 on histone H2B (H2BK120). EZH2-dependent H2BK120 methylation in cancer cells was confirmed with an H2BK120 methylation-specific antibody. Overexpression of EZH2 significantly attenuated the ubiquitination of H2BK120, a key posttranslational modification of histones for transcriptional regulation. Concordantly, knockdown of EZH2 increased the ubiquitination level of H2BK120, suggesting that the methylation of H2BK120 by EZH2 may competitively inhibit the ubiquitination of H2BK120. Subsequent chromatin immunoprecipitation–Seq and microarray analyses identified downstream candidate genes regulated by EZH2 through the methylation of H2BK120. This is the first report to describe a novel substrate of EZH2, H2BK120, unveiling a new aspect of EZH2 functions in human carcinogenesis.

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Introduction

Enhancer of zeste 2 (EZH2) belongs to polycomb group (PcG) protein and is a member of the polycomb repressor complex 2 (PRC2) that methylates histone H3 at lysine 27 (H3K27), a repressive mark that maintains epigenetic silencing of genes. EZH2 is active only when it is associated with other PRC2 core components embryonic ectoderm development (EED), suppressor of zesta 12 homolog (SUZ12), and RBBP4 (retinoblastoma binding protein 4; RbAp48). The PRC2 complex is responsible for repressing of a large number of genes that are essential for development and differentiation. PcG proteins specify positional information such as antero-posterior patterning, through activating or repressing the stable state of *Hox* gene expression. In addition to these well-established functions in embryonic development,

Address all correspondence to: Ryuji Hamamoto, PhD, Section of Hematology/Oncology, Department of Medicine, The University of Chicago, 5835 S Cottage Grove Ave, MC2115 Chicago, IL 60637. E-mail: rhamamoto@medicine.bsd.uchicago.edu

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a series of studies have suggested that PcG proteins may influence both Hox-dependent and Hox-independent downstream pathways that control cell proliferation. We previously demonstrated that EZH2 was overexpressed in various types of human cancer, and its overexpression was correlated with a negative outcome in patients with non-small-cell lung carcinoma (NSCLC) after surgical resection [1]. Importantly, selective inhibitors targeting EZH2 have recently developed, and they showed the growth-inhibitory effects of tumor cells [2,3]. These results indicate that EZH2 is a promising target for development of cancer treatment. Although the transcriptional regulation mechanism by EZH2 through methylation of H3K27 has already been well studied, additional functions of EZH2 through methylation of other substrates still remained unclear.

Posttranslational modification of the four core histones is a commonly important process during the regulation of gene activation and repression. Histone modifications are also involved in various cellular processes, including DNA damage response and alternative splicing. Histone H2B is one of the four core histones involved in chromatin formation in eukaryotic cells. Featuring a main globular domain and a long N-terminal tail, H2B is involved with the structure of the nucleosomes of the "beads on a string" structure. As posttranslational modifications of histone H2B, acetylation, phosphorylation, ubiquitination, and sumoylation have already been reported [4–8]. Among these modifications, histone H2B lysine 120 monoubiquitination (H2BK120ub) is known to be a key histone modification that plays critical roles in the transcriptional regulation as well as higher order chromatin organization in many species [9]. H2BK120ub is associated with a high level of gene expression in human cells [10]. This histone modification is also induced after DNA damage and has been indicated to have a critical role in the maintenance of replication-dependent histone mRNA 3′-end processing [11]. The human ring finger protein 20 (RNF20)/RNF40 complex is the major H2B E3 ligase [12]. At the structure level, monoubiquitination of H2BK120 interferes with compaction of chromatin, resulting in open chromatin fibers that display greater accessibility to transcription factors and their coregulators [13]. Several studies implicate H2BK120ub in developmental processes including that optical embryonic stem cell differentiation requires dynamic changes in H2B ubiquitination patterns in a timely and well-coordinated manner [7,14].

In this study, we identified that the histone methyltransferase EZH2 methylates H2BK120 and competitively inhibits ubiquitination of this lysine residue in cancer cells. This is the first report describing identification of histone H2B as a novel substrate of EZH2 and demonstrating the biologic significance of the histone H2B methylation in human carcinogenesis.

Materials and Methods

Cell Culture

CCD-18Co, HFL1, HCT116, SW480, RT4, MCF7, HeLa, and 293T cells were from American Type Culture Collection (ATCC, Manassas, VA) in 2001 and 2003 and tested and authenticated by DNA profiling for polymorphic short tandem repeat (STR) markers. SBC5, RERF-LC-AI, and Alexander cells were from Japanese Collection of Research Bioresources (JCRB) in 2001 and tested and authenticated by DNA profiling for polymorphic STR markers.

All cell lines were grown in monolayers in the following appropriate media: Dulbecco's modified Eagle's medium for RERF-LC-AI and 293T cells; Eagle's minimum essential medium for CCD-18Co, SBC5, MCF7, and HeLa cells; Leibovitz's L-15 for SW480 cells; McCoy's 5A medium for RT4 and HCT116 cells; RPMI 1640 medium for Alexander cells; and Ham's F-12K Medium for HFL1 cells supplemented with 10% FBS and 1% antibiotic/antimycotic solution (Life Technologies, Carlsbad, CA). All cells were maintained at 37°C in humid air with 5% CO_2 condition (CCD-18Co, HFL1, SBC5, RERF-LC-AI, HCT116, SW480, Alexander, RT4, MCF7, HeLa, and 293T) or without CO_2 (SW480). Cells were transfected with FuGENE 6 (Roche Applied Science, Penzberg, Germany) according to the manufacturer's protocols [15,16].

Antibodies

The following primary antibodies were deployed: anti-FLAG [M2; Sigma-Aldrich (St Louis, MO); dilution used in Western blot (WB): 1:5000, immunocytochemistry (ICC): 1:100], anti-EZH2 (NCL-L-EZH2; Leica, Solms, Germany; dilution used in WB: 1:500), anti–histone H2B (ab1790; Abcam, Cambridge, United Kingdom; dilution used in WB: 1:1000), anti–Ubiquityl-Histone H2BK120 (No. 5546; Cell Signaling Technology, Danvers, MA; dilution used in WB: 1:1000, ICC: 1:50), and anti–actin, beta (ACTB; No. 4967; Cell Signaling Technology; dilution used in WB: 1:1000). The anti–K120-methylated H2B antibody [Sigma-Aldrich; dilution used in WB: 1:500, ICC: 1:100, immunohistochemistry (IHC): 1:5000] was produced in rabbit immunized with a synthetic peptide.

In Vitro *Methyltransferase Assay*

For the *in vitro* methyltransferase assay, recombinant Histone H2B (No. 14-410; Millipore, Billerica, MA) was incubated with EZH2 enzyme complex (No. 51004; BPS Bioscience, San Diego, CA) using 2 μCi of S-adenosyl-L-[methyl-^{3}H] methionine (Perkin-Elmer, Waltham, MA) as the methyl donor in a mixture of 10 μl of methylase activity buffer (50 mM Tris-HCl at pH8.8, 10 mM DTT, and 10 mM $MgCl_2$), for 1 hour at 30°C. Proteins were resolved on a 5% to 20% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) gel (Ready Gel; Bio-Rad Laboratories, Hercules, CA) and visualized by MemCode Reversible Stain (Thermo Fisher Scientific, Waltham, MA) and fluorography.

Mass Spectrometry

Histone H2B samples reacted with EZH2 were separated on SDS-PAGE and stained with Coomassie brilliant blue. The excised Histone H2B bands were reduced in 10 mM tris(2-carboxyethyl) phosphine (Sigma-Aldrich) with 50 mM ammonium bicarbonate (Sigma-Aldrich) for 30 minutes at 37°C and alkylated in 50 mM iodoacetamide (Sigma-Aldrich) with 50 mM ammonium bicarbonate for 45 minutes in the dark at 25°C. Trypsin GOLD (Promega, Fitchburg, WI) solution was added with the enzyme-to-protein ratio at 1/50 (wt/wt) and incubated at 37°C for 16 hours. The resulting peptides were extracted from gel fragments and separated on a 0.1 × 200 mm homemade C_{18} column using 45-minute linear gradient from 2% to 35% acetonitrile in 0.1% formic acid with flow rate at 200 nl/min. The eluting peptides were analyzed with QSTAR Elite QqTOF system (AB Sciex, Framingham, MA) in the smart information-dependent acquisition mode of the Analyst QS software 2.0 (AB Sciex). The acquired mass spectrometry (MS) and tandem mass spectrometry (MS/MS) peak lists were analyzed with in-house Mascot server version

2.4.01 (Matrix Science, Boston, MA) to identify peptide sequences. We finally accepted the assigned peptides with expectation value less than 0.05 as the positive identification in Mascot Database search.

Western Blot Analysis

Samples were prepared from the cells lysed with CelLytic M Cell Lysis Reagent (Sigma-Aldrich) [17], and whole-cell lysates or immunoprecipitation (IP) products were transferred to nitrocellulose membrane. Protein bands were detected by incubating with HRP-conjugated antibodies (GE Healthcare, Little Chalfont, United Kingdom) and visualizing with Enhanced Chemiluminescence (GE Healthcare). We declare that our blots were evenly exposed in each membrane and that the blots are not clopped to the bands.

Immunocytochemistry

Cultured cells were fixed in 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) at room temperature for 30 minutes, permeabilized in 0.1% Triton X-100 (Sigma-Aldrich) for 3 minutes on ice, and blocked with BLOCK ACE (Yukijirushi, Sapporo, Japan) for 1 hour at room temperature. Fixed cells were incubated with primary antibodies overnight at 4°C. Then incubated with Alexa Fluor–conjugated second antibody (Life Technologies) and observed using a Leica confocal microscopy [18,19].

Immunohistochemical Analysis

The K120 monomethylation status of histone H2B in clinical tissues were examined by immunohistochemical analysis [1,17]. EnVision+ kit/HRP kit (Dako, Carpinteria, CA) was applied, and slides of paraffin-embedded lung tumor specimens were processed under high pressure (125°C, 30 seconds) in antigen-retrieval solution, high pH 9 (S2367; Dako), treated with peroxidase-blocking regent, and then treated with protein-blocking regent (K130, X0909; Dako). Tissue sections were incubated with a rabbit anti-H2BK120me1 polyclonal antibody, followed by HRP-conjugated secondary antibody (Dako). Antigen was visualized with substrate chromogen (Dako Liquid DAB Chromogen; Dako). Finally, tissue specimens were stained with Mayer haematoxylin (Hematoxylin QS; Vector Laboratories, Burlingame, CA) for 20 seconds to discriminate the nucleus from the cytoplasm. The intensity of H2BK120 methylation was evaluated using the following criteria: strong positive (scored as 2+), brown staining in >50% of tumor cells completely obscuring cytoplasm; weak positive (1+), any lesser degree of brown staining appreciable in tumor cell cytoplasm; and absent (scored as 0), no appreciable staining in tumor cells. Cases were accepted as strongly positive if two or more investigators independently defined them as such. Detailed clinical information of lung and colony tissues is described in Tables W1 and W2.

siRNA Transfection

Small interfering RNA (siRNA) oligonucleotide duplexes were purchased from Sigma-Aldrich for targeting the human *EZH2* transcript. siNegative control (siNC) was used as a control siRNA. The siRNA sequences are described in Table W3. siRNA duplexes (100 nM final concentration) were transfected into bladder and lung cancer cell lines with Lipofectamine 2000 (Life Technologies) [20].

Immunoprecipitation

Transfected 293T or SBC5 cells were lysed with CelLytic M Cell Lysis Reagent (Sigma-Aldrich) containing a complete protease inhibitor cocktail (Roche Applied Science). In a typical immunoprecipitation reaction, 300 μg of whole-cell extract was incubated with an optimum concentration of primary antibody. After the beads had been washed three times in 1 ml of TBS buffer (pH 7.6), proteins that bound to the beads were eluted by boiling in Lane Marker Reducing Sample Buffer (Thermo Fisher Scientific).

Chromatin Immunoprecipitation–Seq

Chromatin immunoprecipitation (ChIP) assays were performed using ChIP Assay kit (Millipore) according to the manufacturer's protocol. Briefly, the fragment of EZH2 and chromatin complexes was immunoprecipitated with anti-H2BK120me1 (Sigma-Aldrich) and anti-FLAG (M2; Sigma-Aldrich) antibodies 36 or 48 hours after transfection with siRNAs and pCAGGS-n3FC (Mock) or pCAGGS-n3FC-EZH2 (3× FLAG-EZH2) vectors, respectively. ChIP-DNA samples were end repaired, A tailed, and finally ligated with DNA adaptors. Subsequently, samples were size fractionated (175-225 bp) on a 2% tris-borate-EDTA (TBE) agarose gel. Fractionated ChIP-DNA samples were amplified using KAPA Library Preparation Kit (Kapa Biosystems, Woburn, MA). After a final polymerase chain reaction amplification step (18 cycles), the resulting DNA libraries were analyzed by an Agilent Technologies 2100 Bioanalyzer (Santa Clara, CA), and DNA libraries were sequenced on Illumina GAIIx (San Diego, CA).

ChIP-Seq Data Analysis

Sequencing data were acquired from the Illumina GAIIx sequencer [21]. ChIP-Seq reads were aligned using Burrows-Wheeler Aligner software (version 0.62-r126) to the human genome (University of California, Santa Cruz, CA; hg19) with default settings [22]. Burrows-Wheeler Aligner's default mismatch criteria for creating the mismatches was set up that mismatch allowance was 3%, which means that allowance of InDels (insert and deletion) was at least more than 3 bp in 100-bp reads. When same reads mapped to the same genomic position, a maximum of two reads mapping to the same position were used. For enriched-region (peak) identification (peak calling), we used the Model-based Analysis of ChIP-Seq (MACS; Harvard University, Boston, MA; version 1.4.1) algorithm [23,24]. We set up a band with 300 bp, *P* value of 1.00e-06, model fold between 10 and 30 (default) to call peaks representing enriched H2BK120me1 marks and EZH2-located regions. To prospect the peak detection of enhancer region, tags detected were aligned in each gene set within 10 kbp upstream from transcription start sites or 10 kbp downstream from transcription terminal sites. We used the statistical software DESeq (European Molecular Biology Laboratory, Hamburg, Germany), an R package, to normalize the number of tags detected in each gene region and calculated the ratio of samples and controls [25]. Normalized intensity value of genes was ordered as a heatmap using hierarchical clustering in Cluster 3.0 software (Stanford University, Stanford, CA).

Microarray Hybridization and Statistical Analysis

Microarray analysis to identify downstream genes was described previously [20,26,27]. Purified total RNA was labeled and hybridized onto Affymetrix GeneChip U133 Plus 2.0 oligonucleotide arrays (Affymetrix, Santa Clara, CA) according to the manufacturer's instructions. Probe signal intensities were normalized by robust multichip average (RMA) and Quantile (using R and Bioconductor).

ChIP Assay

ChIP assays were performed using ChIP Assay kit (17-295; Millipore) according to the manufacturer's protocol. Briefly, the fragment of K120-ubiquitinated H2B and chromatin complexes in HEK293 cells was immunoprecipitated with an anti–Ubiquityl-Histone H2BK120 antibody. After DNA fragments bound to K120-ubiquitinated H2B were eluted out, an aliquot was subjected to quantitative real-time polymerase chain reaction reactions. Protein A Agarose/Salmon Sperm DNA (16-157; Millipore) was used as a negative control. Primers were designed near the transcriptional start site of each gene, and their sequence information is shown in Table W4.

Results

EZH2 Methylates Lysine 120 on Histone H2B

To identify a novel substrate of EZH2, we conducted *in vitro* methyltransferase assays using recombinant histone proteins as well as nonhistone proteins that play important roles in human carcinogenesis. Consequently, we identified that EZH2 appeared to methylate histone H2B in addition to histone H3 (Figure W1). To validate this result, we performed *in vitro* methyltransferase assays using different amount of EZH2 enzymes and confirmed histone H2B methylation by EZH2 in a dose-dependent manner (Figure 1*A*). Subsequently, we conducted liquid chromatography (LC)-MS/MS

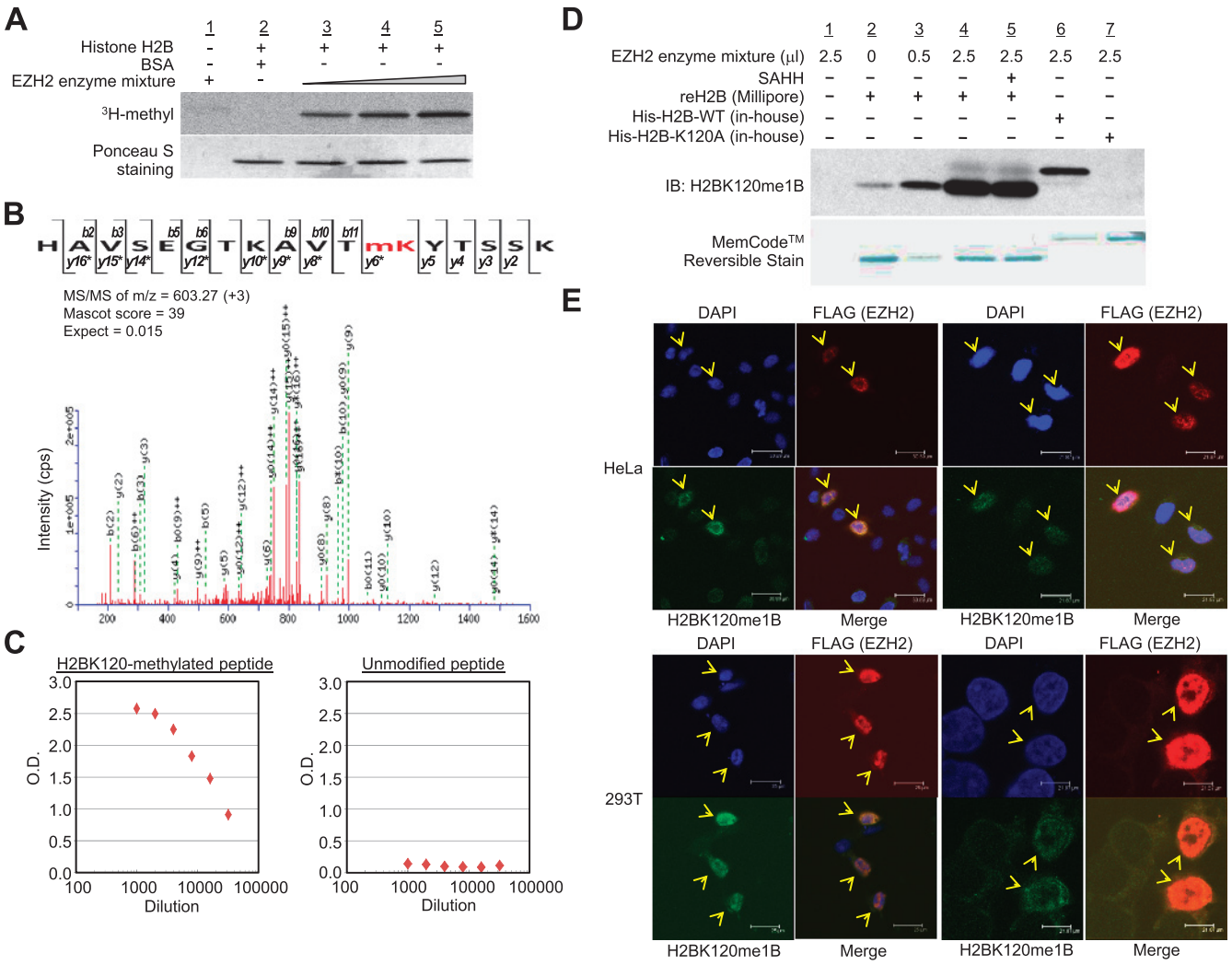

Figure 1. EZH2 methylates lysine 120 of histone H2B both *in vitro* and *in vivo*. (A) *In vitro* methyltransferase assay of H2B. Recombinant H2B and ^{3}H-SAM were incubated in the presence or absence of EZH2 enzyme complex (No. 51004; BPS Bioscience), and the reaction products were analyzed by SDS-PAGE, followed by fluorography (upper panel). The membrane was stained with Ponceau S (Sigma-Aldrich; lower panel). BSA was used as a control. (B) The MS/MS spectrum corresponding to the monomethylated H2B 109-125 peptide. The 14-Da increase of the lysine 120 residue was observed, demonstrating the monomethylated lysine 120. Score and Expect show Mascot Ion Score and Expectation value in Mascot Database search results, respectively. (C) Determination of the titer and specificity of the anti-monomethylated K120 H2B antibody analyzed by ELISA. (D) Validation of an anti-monomethylated K120 H2B antibody. Recombinant H2B-WT or H2B-K120A proteins and ^{3}H-SAM were incubated in the presence or absence of EZH2 enzyme complex, and the reaction products were analyzed by SDS-PAGE, followed by Western blot analysis with an anti-monomethylated K120 histone H2B antibody (upper panel) and stained with MemCode Reversible Stain (lower panel). SAHH, S-adenosyl-L-homocysteine hydrolase. (E) Immunocytochemical analysis of HeLa and 293T cells. Cells were stained with an anti-FLAG antibody [M2; Sigma-Aldrich; Alexa Fluor 594 (red)], an anti-H2BK120me1 antibody [Sigma-Aldrich; Alexa Fluor 488 (green)] and 4′,6′-diamidine-2′-phenylindole dihydrochloride [DAPI (blue)].

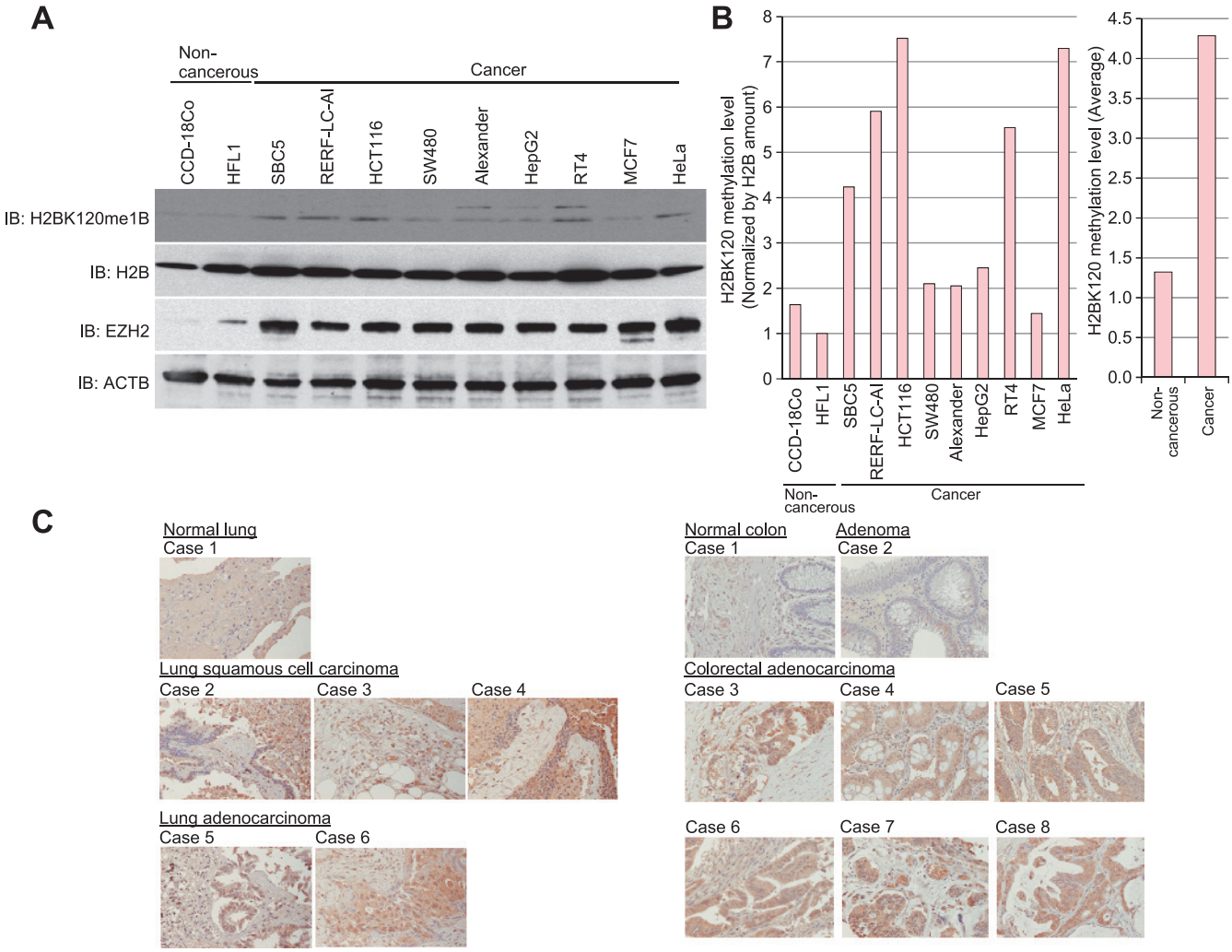

Figure 2. H2BK120 methylation is increased in cancer cells. (A) Validation of H2BK120 methylation status in various cell lines. Lysates from noncancerous cell lines (CCD-18Co and HFL1) and cancer cell lines (SBC5, RERF-LC-AI, HCT116, SW480, Alexander, HepG2, RT4, MCF7, and HeLa) were immunoblotted with anti-monomethylated K120 H2B, anti–histone H2B (ab1790; Abcam), anti-EZH2 (NCL-L-EZH2; Leica), and anti-ACTB (No. 4967; Cell Signaling Technology) antibodies. Information of certificated cell lines is described in Table W4. (B) Quantitative analysis of H2BK120 methylation levels. X-ray films were scanned with GS-800 calibrated densitometer (Bio-Rad Laboratories). The intensity of each H2BK120 monomethylation signal was normalized by the corresponding H2B signal and averaged. (C) Tissue microarray images of lung and colon tissues stained by standard immunohistochemistry for the methylation status of H2BK120. Clinical information for each section is represented in Tables W1 and W2. All tissue samples were purchased from BioChain (Newark, CA). Original magnification, ×200.

analysis to define the methylation site on H2B and found that H2BK120 was monomethylated by EZH2 (Figure 1*B*). We then generated anti–H2BK120-methylated antibodies using an H2BK120-methylated peptide (Figure W2). Among four different lots of the antibodies we obtained, we confirmed by slot blot analysis that H2BK120me1B showed the best quality of the result (Figure W3). In addition, ELISA analysis using this H2BK120-methylation–specific antibody clearly indicated that this antibody recognized the K120-methylated peptide but did not react to the unmethylated peptide, supporting the high specificity of this antibody against H2BK120 methylation (Figure 1*C*). To further verify the quality of the H2BK120-methylation antibody, we prepared both wild-type H2B (H2B-WT) and K120-substituted H2B (H2B-K120A) recombinant proteins and performed *in vitro* methyltransferase assays using EZH2. The H2BK120-methylation signal was increased in a dose-dependent manner, and the signal was completely diminished when H2B-K120A protein was used as a substrate (Figure 1*D*). Using the methylation-specific antibody, we performed immunocytochemical analysis after transfection of EZH2 vectors into HeLa cells to confirm EZH2-dependent H2BK120 methylation *in vivo* and detected the strong staining of methylated H2BK120 in EZH2-overexpressing cells (Figure 1*E*). Taken together, EZH2 can methylate H2BK120, and this methylation is also observed *in vivo*.

H2BK120 Monomethylation Is Increased in Cancer Cells

Because we previously found that the histone methyltransferase EZH2 was overexpressed in various types of cancer [1], we examined whether the methylation level of H2BK120 was enhanced in human cancer cells. Firstly, we prepared cell extracts of two noncancerous cell lines (CCD-18Co and HFL1) and nine cancer cell lines (SBC5, RERF-LC-AI, HCT116, SW480, Alexander, HepG2, RT4, MCF7,

and HeLa; Table W5), and conducted Western blot analysis using the specific antibody (Figure 2*A*). As we expected, the methylation levels of H2BK120 in cancer cell lines expressing high levels of EZH2 were higher than in noncancerous cell lines in which EZH2 expression was very low (Figures 2*B* and W4). We subsequently conducted immunohistochemical analysis of lung and colon tissues (one normal lung, three lung squamous cell carcinomas, and two lung adenocarcinomas as well as one normal colon, one colon adenoma, and six colorectal adenocarcinomas) using the methylation-specific antibody and found higher methylation levels of H2BK120 in cancer cells than normal cells (Figure 2*C*, *left* for lung and *right* for colon).

H2BK120 Monomethylation Competitively Inhibits Monoubiquitination

As H2BK120 was shown to be monoubiquitinated and play a crucial role in the transcriptional regulation, we examined the relationship between H2BK120 methylation and ubiquitination [9]. Immunocytochemial analysis was conducted after FLAG-tagged EZH2 expression vectors were transfected into HeLa cells. EZH2-transfected cells showed significantly lower signals of H2BK120 ubiquitination than control cells (Figure 3*A*). This result indicated that EZH2-dependent H2BK120 methylation appears to competitively inhibit the ubiquitination. In addition, we knocked down the expression of EZH2 in SBC5 cells and conducted the Western blot analysis using purified histone samples to validate the relationship between H2BK120 methylation and ubiquitination. Consistently, H2BK120 methylation status of purified histone H2B proteins was increased after knockdown of EZH2 (Figure 3*B*). Furthermore, we examined the status of H2BK120 ubiquitination in normal and cancer cells and confirmed that ubiquitination levels in cancer cells were lower than those in noncancerous cells (Figure W5). These results reveal that the histone methyltransferase EZH2 is likely to inhibit the

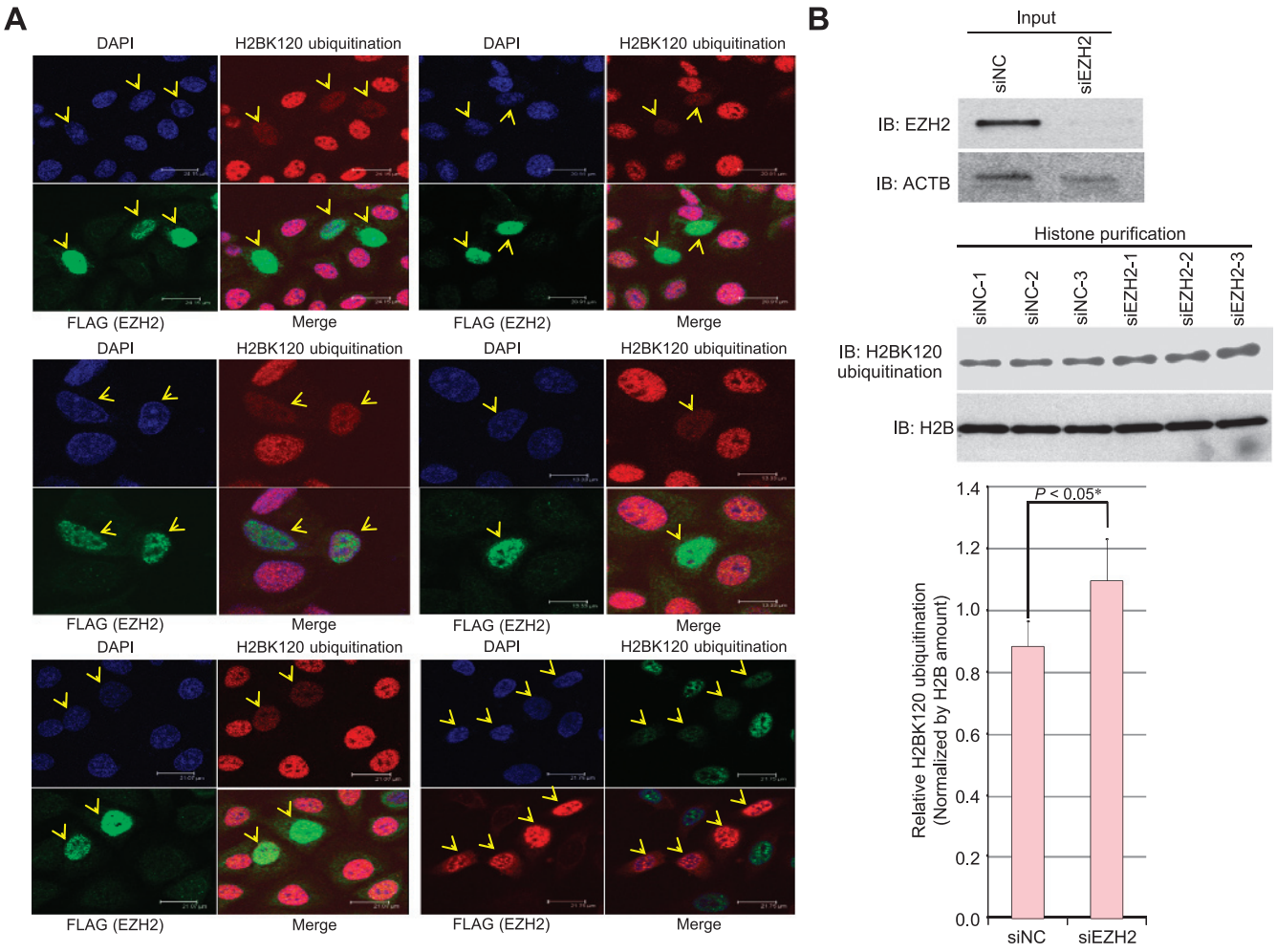

Figure 3. H2BK120 methylation competitively inhibited the ubiquitination. (A) Immunocytochemical analysis of HeLa cells. Cells were stained with an anti–Ubiquityl-Histone H2BK120 antibody (No. 5546; Cell Signaling Technology), an anti-FLAG antibody (M2; Sigma-Aldrich), and 4′,6′-diamidine-2′-phenylindole dihydrochloride [DAPI (blue)]. Alexa Fluor 488 (green) and Alexa Fluor 594 (red) antibodies were used as secondary antibodies. HeLa cells were fixed in 4% paraformaldehyde 24 hours after transfection of FLAG-tagged EZH2 expression vectors. (B) Effects of EZH2 knockdown on the ubiquitination of H2BK120 in SBC5 cells. SBC5 cells were lysed 48 hours after treatment with siNC and siEZH2. Samples were immunoblotted with anti-EZH2 (NCL-L-EZH2; Leica, upper) and anti–Ubiquityl-Histone H2BK120 (middle) antibodies. Expressions of ACTB (No. 4967; Cell Signaling Technology) and H2B (ab1790; Abcam) were the internal controls. The ubiquitination level of H2BK120 was calculated by GS-800 (Bio-Rad Laboratories, lower). Results are the means ± SD of three independent experiments. The *P* value was calculated using Student's *t* test.

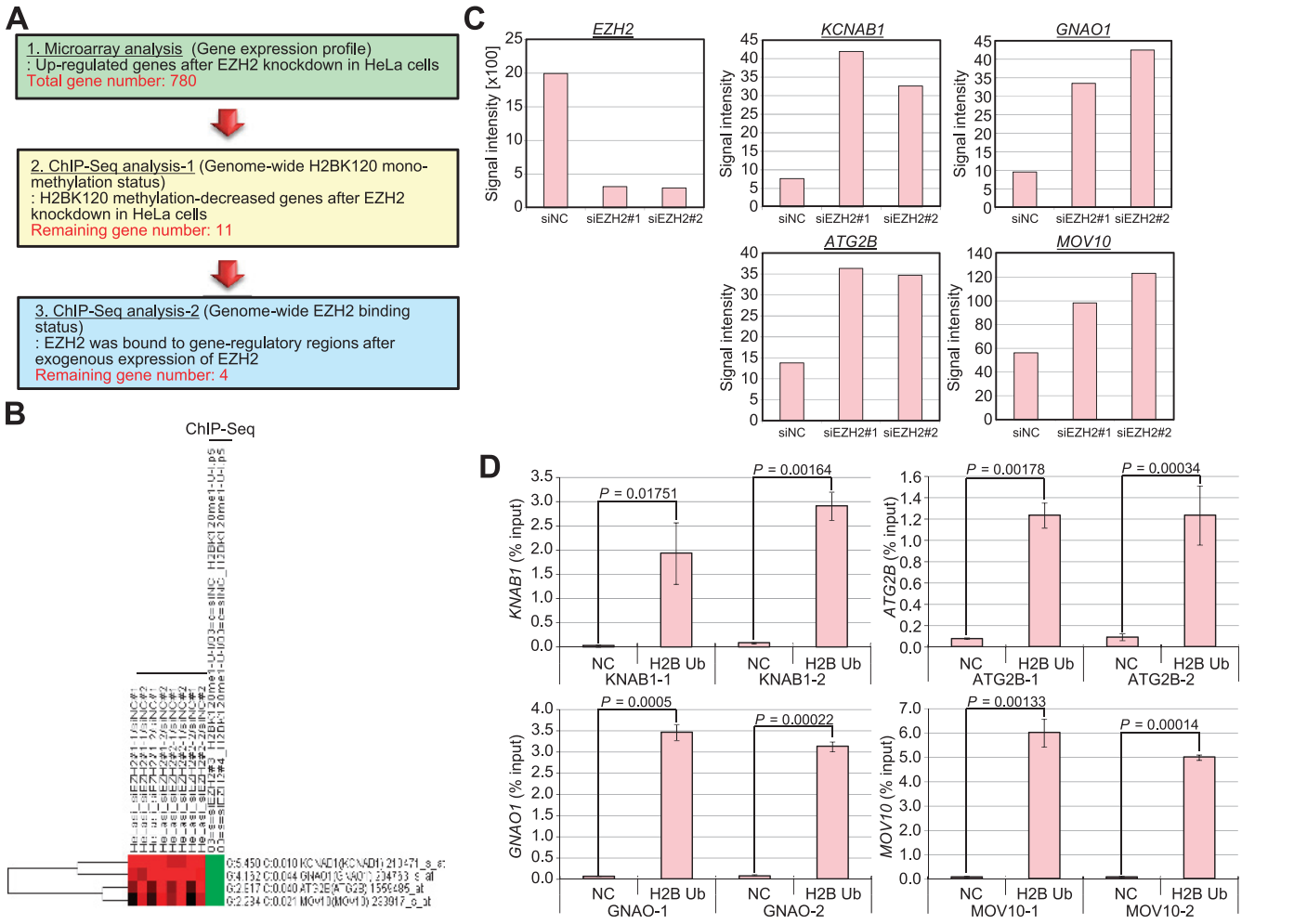

Figure 4. Direct downstream genes of EZH2 through the methylation of H2BK120. (A) Schematic diagram of the strategy to identify direct downstream genes of EZH2. Detailed procedures of ChIP-Seq and microarray analyses were described in Materials and Methods section. 1) Gene expression profile analysis was conducted using the Affymetrix GeneChip system after knockdown of EZH2 in HeLa cells. Seven hundred eighty genes were identified to be downregulated by EZH2 knockdown (>2.0). 2) Chip-Seq analysis to examine the methylation status of histone H2BK120 monomethylation in gene-regulatory regions. Among 780 genes identified by microarray analysis, the methylation status was decreased in 11 genes. 3) ChIP-Seq analysis to identify binding regions of EZH2 proteins after exogenous expression of EZH2. Finally, four genes remained as direct downstream genes of EZH2 through the methylation of H2BK120. (B) The heatmap of four genes directly regulated by EZH2 through the inhibition of histone H2BK120 methylation. Affymetrix GeneChip data (microarray analysis) and ChIP-Seq data were combined. G, the expression ratio of each target gene between siEZH2-treated and control cells (siEZH2/Control). C, the ratio of methylation status in the regulatory region of each gene (siEZH2/Control). Probe Set ID (HG-U133_Plus_2.na33.annotation) is shown with gene symbol. (C) Expression levels of *EZH2*, *KCNAB1*, *GNAO1*, *ATG2B*, and *MOV10* in HeLa cells were analyzed by Affymetrix GeneChip system. (D) ChIP analysis using primer pairs of downstream candidate genes as described under Materials and Methods section. Cross-linked and sheared chromatin was immunoprecipitated with an anti–Ubiquityl-Histone H2BK120 antibody (No. 5546; Cell Signaling Technology). Protein A Agarose/Salmon Sperm DNA (Millipore) was used as a negative control. Results are the means ± SD of three independent experiments and shown as a percentage of the input chromatin. The *P* value was calculated using Student's *t* test.

ubiquitination of H2BK120 in a competitive manner through the methylation of the lysine residue.

Mechanisms in Transcriptional Regulation through EZH2-Dependent H2BK120 Methylation

As it is known that H2BK120 ubiquitination plays a crucial role in the transcriptional regulation, we next examined the transcriptional regulation mechanism of EZH2-dependent H2BK120 methylation in cancer cells. To identify direct downstream genes of EZH2 through the methylation of H2BK120, we conducted cDNA microarray and ChIP-Seq analyses and combined the data. At first, cDNA microarray analysis using the Affymetrix GeneChip system was performed, and expression levels of 780 genes were increased after treatment with siEZH2 in HeLa cells (Figure 4*A*, more than two times compared with control). Then, we planned to do ChIP-Seq analysis to examine the genome-wide H2BK120 monomethylation status. Firstly, we validated the quality of the antibody whether it is available for immunoprecipitation or not. FLAG-conjugated wild-type H2B (H2B-WT) and K120-substituted H2B (H2B-K120A) expression vectors were

transfected into 293T cells, and cell lysates were immunoprecipitated with anti-H2BK120 methylation antibodies, followed by Western blot analysis using an anti-FLAG antibody (Figure W6*A*). Among three lots of anti-H2BK120 methylation antibodies, H2BK120me1B showed the best quality as well as slot blot analysis. To evaluate the availability of this antibody for the immunoprecipitation of endogenous H2BK1200-methylated proteins, Western blot analysis using the H2BK120me1B antibody was performed after immunoprecipitation of SBC5 cell lysates using the same antibody (Figure W6*B*). Subsequently, we confirmed the specific signal of K120-methylated H2B proteins, implying that this antibody is also available for the immunoprecipitation of endogenous K120-methylated histone H2B. On the basis of this result, ChIP-Seq analysis was conducted after treatment with siNC (negative control) and two independent siEZH2s, and among 780 genes selected by microarray analysis, the methylation status of gene-regulatory regions of 11 genes was significantly decreased (Figure 4*A*). In addition, we also examined the genome-wide EZH2-binding status to identify the gene-regulatory regions that EZH2 was bound by ChIP-Seq analysis. Finally, four genes (*KCNAB1*, *GNAO1*, *ATG2B*, and *MOV10*) remained as direct downstream genes of EZH2 through the methylation of H2BK120 after combining the data of microarray and ChIP-Seq analyses (Figure 4*B*). Detailed expression profile data of the four genes were shown in Figure 4*C*. These results reveal that EZH2-dependent H2BK120 methylation can regulate the transcription of downstream genes, and it is a novel mechanism of human carcinogenesis mediated by EZH2 overexpression.

Discussion

Histone H2B is a core histone, and so far, acetylation, phosphorylation, ubiquitination, and sumoylation have already been reported as posttranslational modifications of this protein. In the present study, we identified that lysine 120 of histone H2B is methylated by EZH2 and that the methylation competitively inhibits the

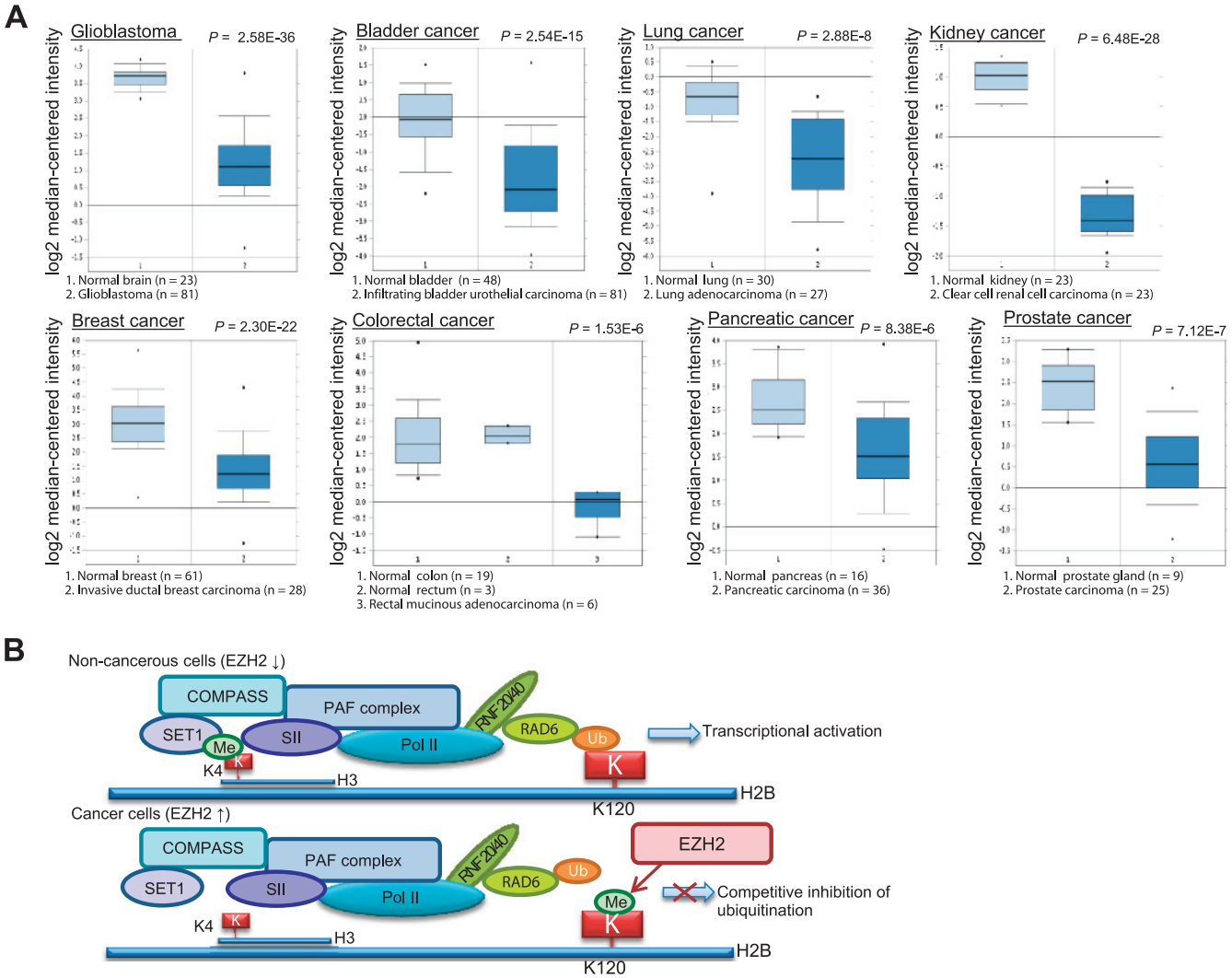

Figure 5. EZH2-dependent transcriptional regulation mechanism through H2BK120 methylation. (A) Expression of *KCNAB1* in cancer tissues. Expression levels of *KCNAB1* are downregulated in various types of cancer. Gene expression data in Oncomine (University of Michigan, Ann Arbor, MI) were analyzed. The thick bars in the boxes are average expression levels, and the boxes represent 95% of the samples. The error bars are above or below the boxes, and the range of expression levels is enclosed by two dots. (B) Proposed model for regulation of H2BK120 posttranslational modification during human carcinogenesis.

ubiquitination of H2BK120. This is the first report to describe the function of histone H2B methylation.

In mammals, H2BK120 monoubiquitination was reported to be preferentially associated with highly transcribed genes [10]. H2BK120 monoubiquitination can cooperate with facilitates chromatin transcription (FACT) and the polymerase associated factor (PAF) complex to regulate transcription elongation by RNA polymerase II [28] and can also facilitate DNA repair [29] and mRNA 3′ end processing [11] in human cells. In addition, Vitaliano-Prunier et al. recently reported that H2B ubiquitination in yeast cells plays a role in mRNA export from the nucleus into the cytoplasm [30]. Importantly, there are several reports describing the relationship between H2BK120 ubiquitination and cancer. The H2B deubiquitinase ubiquitin specific peptidase 22 (USP22) is part of a gene signature predictive of a cancer stem cell tumor phenotype of aggressive growth, metastasis, and therapy resistance [31]. RNF20, the E3 ubiquitin ligase for monoubiquitination of H2BK120, is a putative tumor suppressor [32]; its down-regulation in human cells promotes migration, anchorage independence, and carcinogenesis [32,33]. Furthermore, it was recently reported that H2B ubiquitination levels were decreased in advanced and metastatic breast cancer, parathyroid tumors, and seminoma [34–36]. These data imply that H2BK120 ubiquitination appears to possess tumor-suppressive functions, and its dysfunction causes malignant alteration of human cells.

In this study, we demonstrated that the histone methyltransferase EZH2 methylated H2BK120 and competitively inhibited the ubiquitination and that in cancer cells, the methylation level was increased, and the ubiquitination was decreased at lysine 120 of histone H2B. From these results, H2BK120 ubiquitination shows tumor-suppressive functions, and the oncogenic polycomb histone methyltransferase EZH2 competitively inhibits the ubiquitination through the methylation of H2BK120, which may result in the reduction of tumor-suppressive effects. In addition, we identified direct downstream genes of EZH2 through the methylation of H2BK120 on the basis of ChIP-Seq and microarray analyses (Figure 4*A*). Importantly, ChIP analysis indicated that H2BK120 ubiquitination levels of downstream candidate genes were significantly high in nontumor cells, implying that these genes may be transcriptionally activated in nontumor cells through the H2BK120 ubiquitination (Figure 4*D*). Among direct downstream candidates we identified, expression levels of *KCNAB1* and *GNAO1* are significantly decreased in various types of tumor tissues (Figures 5*A* and W7). *KCNAB1* is a voltage-gated potassium channel β subunit and modulates the activity of the pore-forming α subunit [37,38]. It has already been reported that some components of potassium channel serve as tumor suppressors. *KCTD11* (potassium channel tetramerization domain containing 11) is a novel tumor suppressor gene that inhibits cell growth and is mapping on human chromosome 17p13.2 [39]. According to the research using a panel of 177 human tumor samples and their normal matching samples representing 18 different types of cancer, down-regulation of KCTD11 protein level is a diffusely common event in tumorigenesis [40]. In addition, the putative tumor suppressor *KCNRG* (potassium channel–regulating gene) encodes a protein with a high homology to the tetramerization domain of voltage-gated K+ (Kv) channels [41]. This protein appears to interfere with the normal assembly of the K+ channel proteins by binding to their tetramerization domain, thereby causing the suppression of Kv currents [42]. Because Kv channels are involved in the proliferation of tumor cells [43] and normal lymphocytes [44], while being upregulated in neoplastic hematopoietic cells [45], *KCNRG* seems to exert a tumor suppressor effect [41]. On the contrary, *GNAO1*, a member of the signal-transducing guanine nucleotide-binding (G) protein family, has been implicated in ion channel regulation [46], and mutation of *GNAO1* was reported to cause the malignant alteration of cells [47,48]. *GPRC5a*, a family gene of *GNAO1*, is reported as a tumor suppressor gene, and knockout of GPCR5 leads to NF-κB activation in airway epithelium and promotes lung tumorigenesis [49]. Furthermore, according to expression profile analysis, expression levels of *ATG2B*, one of the autophagy-related genes, in several types of tumor tissues were much lower than those in corresponding normal tissues (Figure W8). Frameshift mutations of ATG2B were found in gastric and colorectal cancers [50]. The truncation mutants of ATG2B may inactivate its autophagy function and/or autophagic cell death, which resembles a typical loss-of-function mutation. These data imply that *KCNAB1*, *GNAO1*, and *ATG2B* are likely to be deregulated in cancer and work as tumor suppressors. Taken together, the oncogenic histone methyltransferase EZH2 is likely to contribute to human carcinogenesis based on the transcriptional regulation of downstream genes through not only H3K27 methylation but also H2BK120 methylation. In line with this, we also conducted the ChIP-Seq analysis and found that *GNAO1*, *ATG2B*, and *MOV10* were regulated by H2BK120 methylation, but the *KCNAB1* gene appears to be regulated by both H2BK120 methylation and H3K27 methylation (Table W6). This implies that some gene is likely to be synergistically regulated by two different histone marks.

All the while, histone H3-K27 has been only the known target of EZH2-dependent methylation, and the importance of EZH2 in human carcinogenesis was described only focusing on H3K27 methylation. In this study, we identified H2BK120 to be a novel target of EZH2-dependent methylation and downstream candidates through the novel methylation site (Figure 5*B*). Because EZH2 is overexpressed in various types of cancer and it plays a critical role in the growth regulation of cancer cells, EZH2 is recognized as an important target of anticancer treatment. In fact, chemical compounds, which inhibit methyltransferase activity of EZH2, have recently been developed as anticancer drugs. According to our findings, we need to take care of H2BK120 methylation besides H3K27 methylation to develop efficient anticancer treatment targeting EZH2, and the identification of this novel methylation site must contribute to unveil the multifunctions of EZH2 in human carcinogenesis. Furthermore, additional functional analysis of H2BK120 methylation may elucidate the significance of biologic function of this methylation, including disease like cancer, and the importance for diagnosis, prognosis, and treatment for cancer.

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Table W1. Characteristics of Lung Tissues.

Case No.	Age	Gender	Pathology	Grade	Stage (TNM)	Nature
Case 1	34	Male	Normal	Normal		
Case 2	58	Male	Squamous cell carcinoma	II	T2N0M0	Malignant
Case 3	42	Male	Squamous cell carcinoma	II	T2N0M0	Malignant
Case 4	57	Male	Squamous cell carcinoma	III	T2N0M0	Malignant
Case 5	62	Male	Adenocarcinoma	II	T2N0M0	Malignant
Case 6	73	Male	Adenosquamous carcinoma	III	T2N1M0	Malignant

Table W2. Characteristics of Colon Tissues.

Case No.	Age	Gender	Histology	Grade	Stage (TNM)	Anatomic Site
Case 1	49	Female	Normal			Colon
Case 2	65	Male	Adenoma			Colon
Case 3	51	Male	Adenocarcinoma	II	T3N1M0	Colon
Case 4	39	Female	Adenocarcinoma	I_II	T3N1M0	Colon
Case 5	49	Female	Adenocarcinoma	I_II	T3N0M0	Colon
Case 6	62	Female	Adenocarcinoma	I_II	T2N1M0	Colon
Case 7	60	Male	Adenocarcinoma	II	T2N1M0	Colon
Case 8	34	Male	Adenocarcinoma	I	T3N0M0	Colon

Table W3. siRNA Sequences.

siRNA Name	Sequence
siNC (cocktail)	
Target No. 1	Sense: 5′ AUCCGCGCGAUAGUACGUA3′
	Antisense: 5′ UACGUACUAUCGCGCGGAU 3′
Target No. 2	Sense: 5′ UUACGCGUAGCGUAAUACG 3′
	Antisense: 5′ CGUAUUACGCUACGCGUAA 3′
Target No. 3	Sense: 5′ UAUUCGCGCGUAUAGCGGU 3′
	Antisense: 5′ ACCGCUAUACGCGCGAAUA 3′
siEZH2 No. 1	Sense: 5′ CUAACCAUGUUUACAACUA 3′
	Antisense: 5′ UAGUUGUAAACAUGGUUAG 3′
siEZH2 No. 2	Sense: 5′ GACAGAAGAGGGAAAGUGU 3′
	Antisense: 5′ ACACUUUCCCUCUUCUGUC 3′

Table W4. Primer Sequences for ChIP Analysis.

Name	Sequence	Length	Tm (°C)	MW
KCNAB1 Set 1 (KCNAB1-1): Amplification Size = 123				
KCNAB1-ChIP-f1	TTC CGT GTT CGA AGA TAC CAC	21	59.5	6381.2
KCNAB1-ChIP-r1	AAC CTT ATC CTG CCA CAA AGC	21	59.5	6319.2
KCNAB1 Set 2 (KCNAB1-2): Amplification Size = 119				
KCNAB1-ChIP-f2	ATT TGT CAG AAG TGC TGG GAG G	22	62.1	6870.5
KCNAB1-ChIP-r2	TCT TTG AAT GTC AGT GAA CCA C	22	58.4	6709.4
GNAO1 Set 1 (GNAO1-1): Amplification Size = 139				
GNAO1-ChIP-f1	AGC CTC GGG TGT CAC ATA TTA G	22	62.1	6750.4
GNAO1-ChIP-r1	CTC AGG AAA CGC GAT GTG GTA G	22	64.2	6824.5
GNAO1 Set 2 (GNAO1-2): Amplification Size = 118				
GNAO1-ChIP-f2	ATT CCG ACC CAC TAC CAC ATC	21	61.2	6255.1
GNAO1-ChIP-r2	GGG CCG GCT CTC CAT CTT GTC	21	67.3	6365.2
ATG2B Set 1 (ATG2B-1): Amplification Size = 122				
ATG2B-ChIP-f1	TCG GAG CCG GAA CTG CTC CAG	21	67.3	6432.2
ATG2B-ChIP-r1	CTC CTG GCG CGT TCA CGA GAC	21	67.3	6383.2
ATG2B Set 2 (ATG2B-2): Amplification Size = 98				
ATG2B-ChIP-f2	CCA GGA TTA AGC GAG CGT ATG	21	61.2	6495.3
ATG2B-ChIP-r2	CCC CGC CTC ATT CAG GTA TTG	21	63.2	6333.2
MOV10 Set 1 (MOV10-1): Amplification Size = 149				
MOV10-ChIP-f1	TTC CCA CTG ACA TTG CAT TTC	21	57.5	6307.2
MOV10-ChIP-r1	AGG CCA CAC ACT CAA TCT ACG	21	61.2	6344.2
MOV10 Set 2 (MOV10-2): Amplification Size = 104				
MOV10-ChIP-f2	TTA CTG TGT ATC CTG GCA GAG C	22	62.1	6741.4
MOV10-ChIP-r2	CAT AAG GGT CAA AGA AGT TTG G	22	58.4	6847.6

Tm indicates melting temperature; MW, molecular weight.

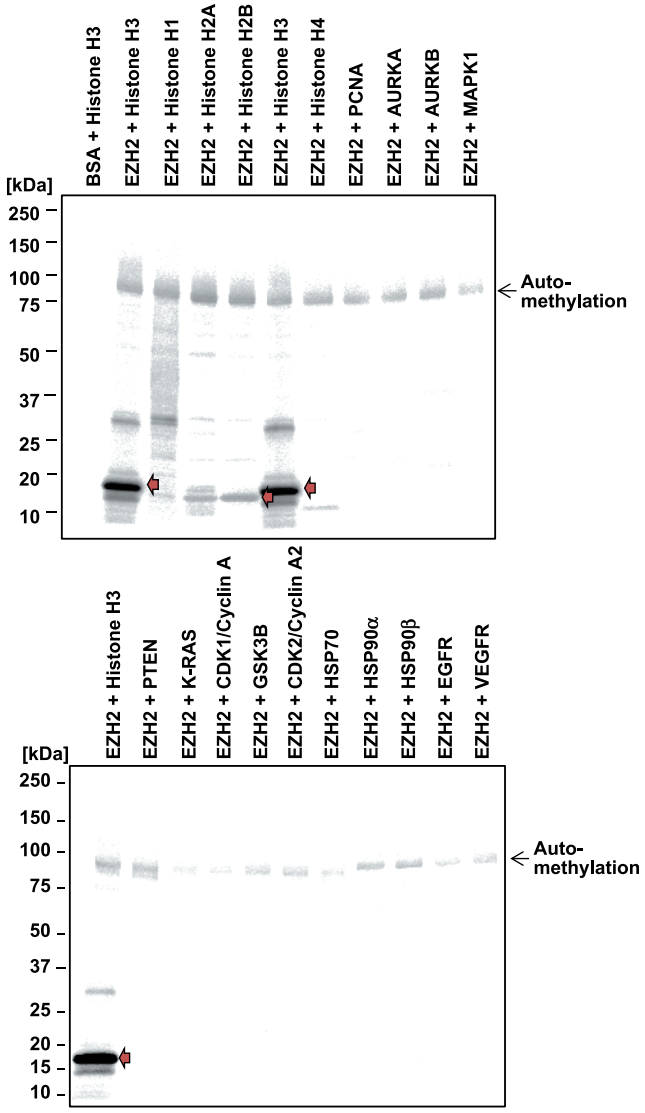

Figure W1. EZH2 methylates histone H2B. *In vitro* methyltransferase of EZH2 was performed using various types of recombinant proteins as substrates. Methylated proteins were visualized with fluorography.

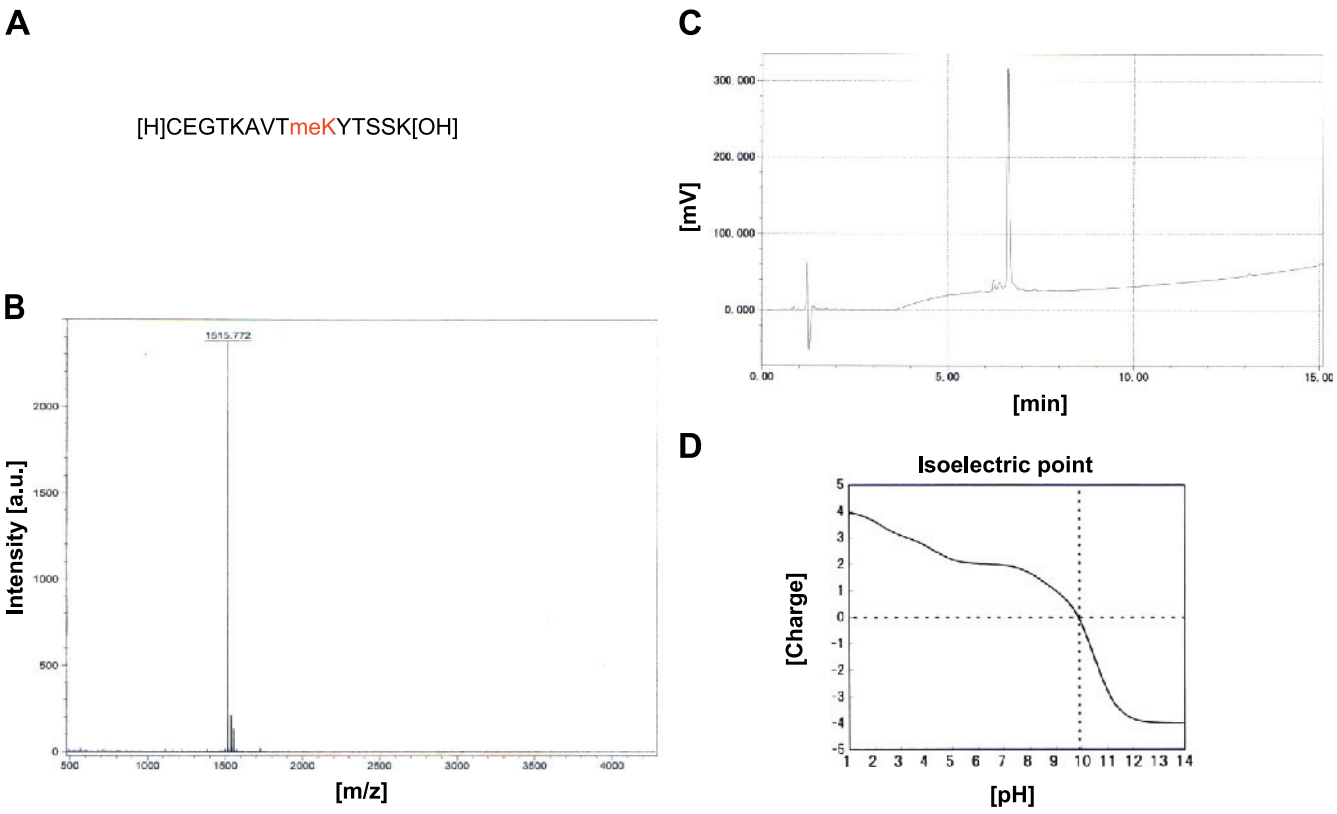

Figure W2. Quality check of peptides used as immunogens. (A) Amino acid sequence of the peptide. Ninth lysine residue is monomethylated. (B) Mass spectrum of the peptide. Bruker Daltonics flexAnalysis software (Billerica, MA) was used for the analysis. (C) Chromatogram of the peptide analyzed by the MultiStation LC-8020 system. (D) Isoelectric point figure of the peptide.

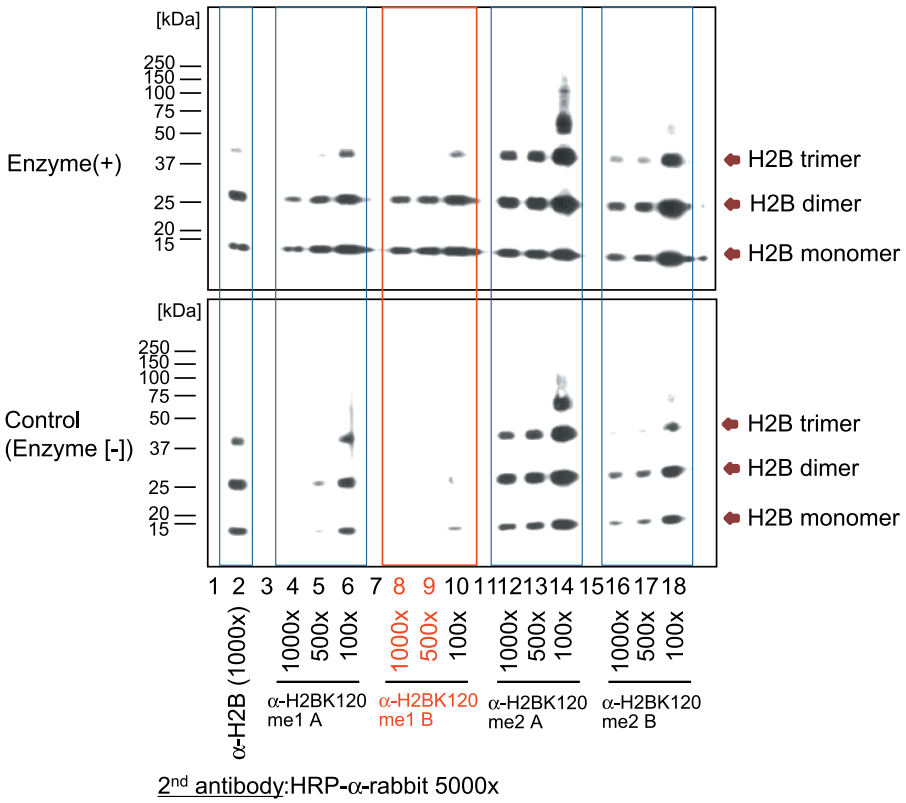

Figure W3. Slot blot analysis for the quality check of antibodies. *In vitro* methyltransferase assays were performed using recombinant EZH2 enzyme complex (No. 51004; BPS Bioscience) as an enzyme source. Samples were fractionated with SDS-PAGE and transferred to the nitrocellulose membranes. Slot blot analysis was conducted using anti-H2BK120 antibodies. An anti–histone H2B antibody was used as an internal control.

Table W5. Information of Certificated Cell Lines.

Name	Origin	Certification Institution	Tested Method	DNA Profile or Characteristics
CCD-18Co	human colonic fibroblast	ATCC	STR	Amelogenin: X CSF1PO: 8 D13S317: 12 D16S539: 12,13 D5S818: 12 D7S820: 8 THO1: 6,7 TPOX: 8,11 vWA: 15,17
HFL1	human fetal lung fibroblast	ATCC	STR	Amelogenin: X,Y CSF1PO: 10,12 D13S317: 11,12 D16S539: 9,11 D5S818: 12 D7S820: 9,10 THO1: 7,9 TPOX: 6,9 vWA: 17
SBC5	human small lung cancer	JCRB	STR	Amelogenin: X,Y TPOX: 9,12 CSF1PO: 10 D5S818: 10,11 D13S317: 8,10 D7S820: 8,11 D16S539: 12 vWA: 14,18 TH01: 6
RERF-LC-AI	human lung squamous cell carcinoma	JCRB	STR	Amelogenin: X,Y TPOX: 10,11 CSF1PO: 12 D5S818: 10.3 D13S317: 10 D7S820: 10,11 D16S539: 11 VWA: 17 TH01: 7,9
HCT116	human colorectal carcinoma	ATCC	STR	Amelogenin: X,Y CSF1PO: 7,10 D13S317: 10,12 D16S539: 11,13 D5S818: 10,11 D7S820: 11,12 THO1: 8,9 TPOX: 8,9 vWA: 17,22
SW480	human colorectal carcinoma	ATCC	STR	Amelogenin: X CSF1PO: 13,14 D13S317: 12 D16S539: 13 D5S818: 13 D7S820: 8 THO1: 8 TPOX: 11 vWA: 16
Alexander	human malignant liver cancer	JCRB	STR	D5S818: 12 D13S317: 11,12 D7S820: 9,11 D16S539: 13 vWA: 15,16 TH01: 7,8 Amelogenin: X TPOX: 8 CSF1PO: 10
RT4	human urinary bladder cancer	ATCC	STR	Amelogenin: X,Y CSF1PO: 10,12 D13S317: 8 D16S539: 9 D5S818: 11,12 D7S820: 9,12 THO1: 9,9.3 TPOX: 8,11 vWA: 14,17
MCF7	human breast adenocarcinoma	ATCC	STR	Amelogenin: X CSF1PO: 10 D13S317: 11 D16S539: 11,12 D5S818: 11,12 D7S820: 8,9 THO1: 6 TPOX: 9,12 vWA: 14,15
HeLa	human cervix carcinoma	ATCC	STR	Amelogenin: X,Y CSF1PO: 11,12 D13S317: 11,14 D16S539: 9,11 D5S818: 11,12 D7S820: 10,11 THO1: 8 TPOX: 8 vWA: 15

ATCC indicates American Type Culture Collection; JCRB, Japanese Collection of Research Bioresources.

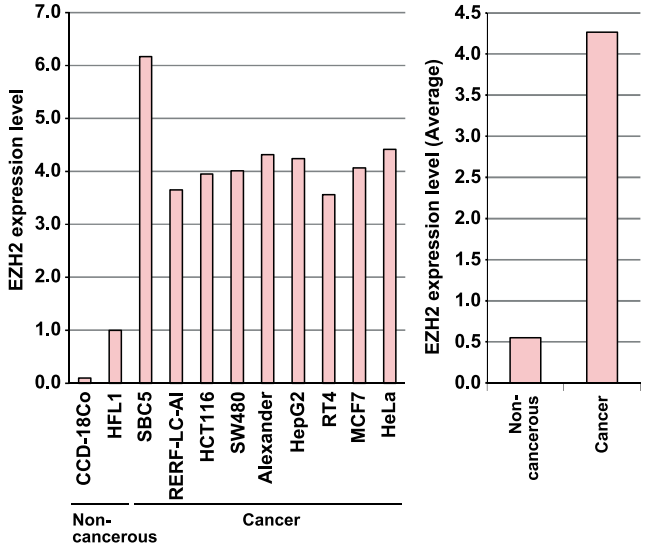

Figure W4. Quantitative analysis of EZH2 expression in various types of cell lines. X-ray films were scanned with GS-800 calibrated densitometer (Bio-Rad Laboratories). The intensity of each EZH2 signal was normalized by the corresponding ACTB signal and averaged.

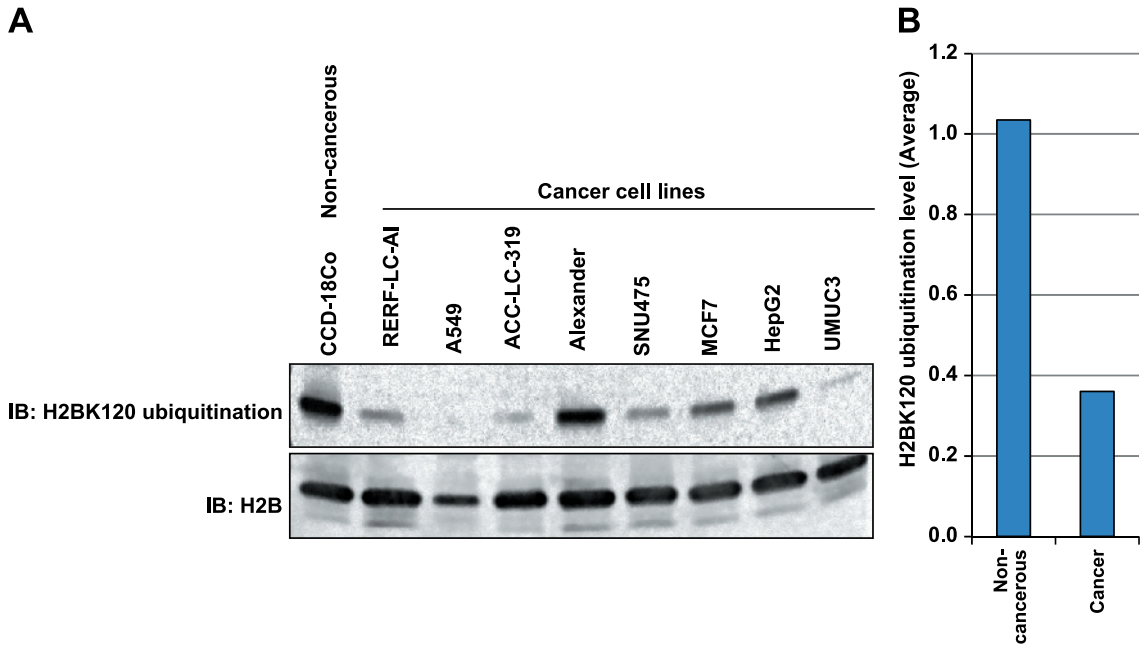

Figure W5. H2BK120 ubiquitination was decreased in cancer cells. (A) Validation of H2BK120 ubiquitination status in various cell lines. Lysates from a noncancerous cell line (CCD-18Co) and cancer cell lines (RERF-LC-AI, A549, ACC-LC-319, Alexander, SNU475, MCF7, HepG2, and UMUC3) were immunoblotted with anti–Ubiquityl-Histone H2BK120 antibody (No. 5546; Cell Signaling Technology) and anti–histone H2B (ab1790; Abcam) antibodies. (B) Quantitative analysis of H2BK120 ubiquitination levels. X-ray films were scanned with GS-800 calibrated densitometer (Bio-Rad Laboratories). The intensity of each H2BK120 ubiquitination signal was normalized by the corresponding H2B signal and averaged.

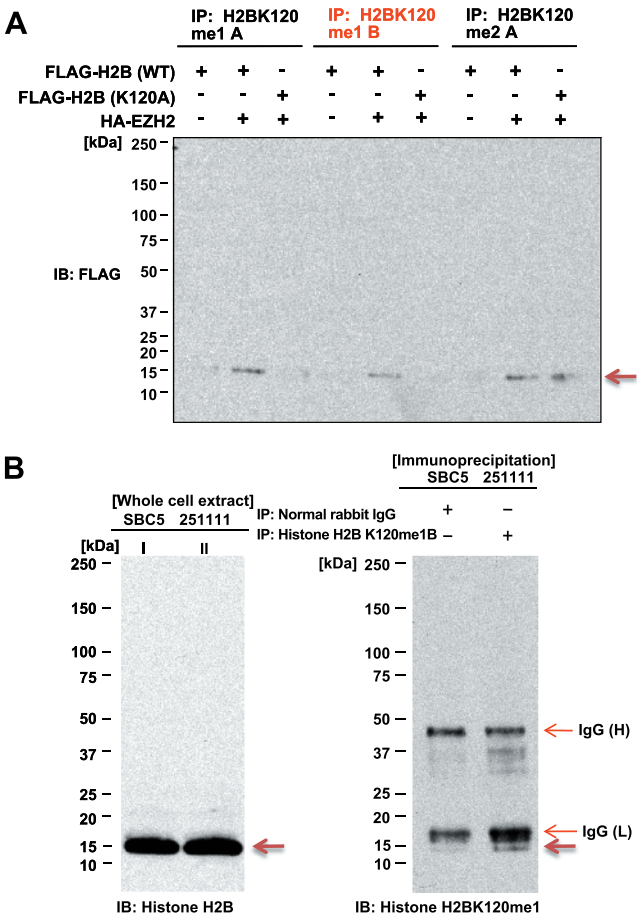

Figure W6. Validation of anti-H2BK120 methylation antibodies. (A) Wild-type FLAG-H2B (H2B-WT) and lysine 120–substituted FLAG-H2B expression vectors (K120A) were transfected into 293T cells. Cell lysates were immunoprecipitated with anti-H2BK120 methylation antibodies, and immunoblot analysis was performed using an anti-FLAG antibody (M2; Sigma-Aldrich). (B) SBC5 cells were lysed with CelLytic M, and cell lysates were immunoprecipitated with an anti-H2BK120 methylation antibody. Whole-cell extracts were blotted with an antihistone H2B antibody (Abcam; ab1790).

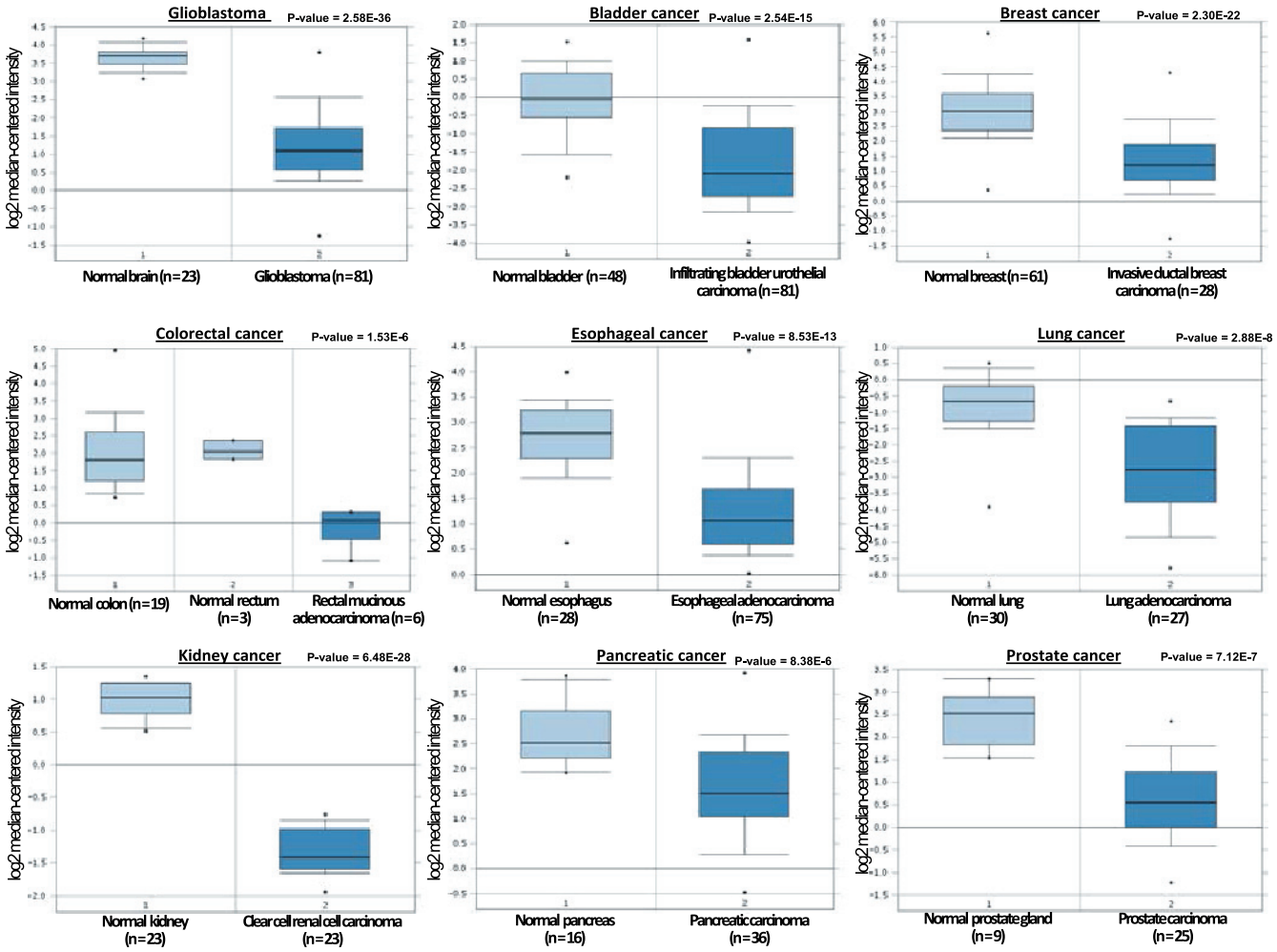

Figure W7. Expression of *GNAO1* in cancer tissues. Expression levels of *GNAO1* are downregulated in various types of cancer. Gene expression data in Oncomine were analyzed. The thick bars in the boxes are average expression levels, and the boxes represent 95% of the samples. The error bars are above or below the boxes, and the range of expression levels is enclosed by two dots.

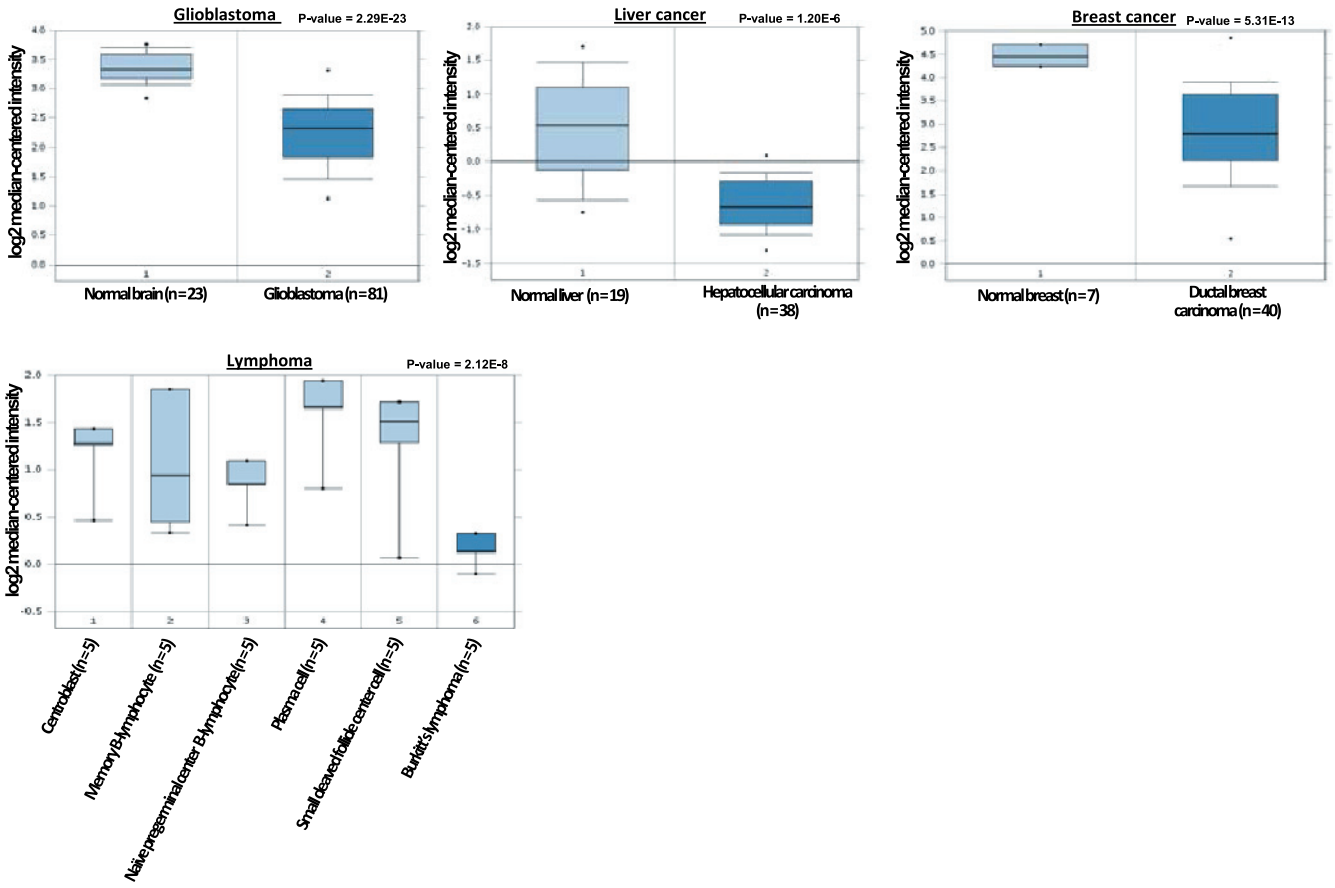

Figure W8. Expression of *ATG2B* in cancer tissues. Expression levels of *ATG2B* are downregulated in various types of cancer. Gene expression data in Oncomine were analyzed. The thick bars in the boxes are average expression levels, and the boxes represent 95% of the samples. The error bars are above or below the boxes, and the range of expression levels is enclosed by two dots.

Table W6. Comparison of H2BK120 Monomethylation and H3K27 Trimethylation Status in the Gene-Regulatory Regions of Downstream Candidates.

Gene Name	H2BK120me1*	H3K27me3*
KCNAB1	0.018	0.024
GNAO1	0.044	0.58435
ATG2B	0.04	NA†
MOV10	0.021	NA†

*Value is the ratio of tag number (siEZH2/siNC).
†NA; No significant methylation was detected in both siEZH2 and siNC samples.