

Epigenetic Silencing of CCAAT/Enhancer-binding Protein δ Activity by YY1/Polycomb Group/DNA Methyltransferase Complex*

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Human CCAAT/enhancer-binding protein δ (CEBPD) has been reported as a tumor suppressor because it both induces growth arrest involved in differentiation and plays a crucial role as a regulator of pro-apoptotic gene expression. In this study, *CEBPD* gene expression is down-regulated, and “loss of function” alterations in *CEBPD* gene expression are observed in cervical cancer and hepatocellular carcinoma. Suppressor of zeste 12 (SUZ12), a component of the polycomb repressive complex 2 (PRC2), silences *CEBPD* promoter activity, enhancing the methylation of exogenous *CEBPD* promoter through the proximal CpG islands. Moreover, this molecular approach is consistent with the opposite mRNA expression pattern between *SUZ12* and *CEBPD* in cervical cancer and hepatocellular carcinoma patients. We further demonstrated that Yin-Yang-1 (YY1) physically interacts with SUZ12 and can act as a mediator to recruit the polycomb group proteins and DNA methyltransferases to participate in the *CEBPD* gene silencing process. Taking these results into consideration, we not only demonstrate the advantage of SUZ12-silenced *CEBPD* expression in tumor formation but also clarify an *in vivo* evidence for YY1-mediated silencing paths of SUZ12 and DNA methyltransferases on the *CEBPD* promoter.

CEBPD³ is one of the CCAAT/enhancer-binding protein family members that function as transcription factors acting in

tissue differentiation, metabolism, and immune responses (1). All of the family members contain a highly conserved basic leucine zipper domain for dimerization and a basic domain for DNA binding at the C terminus. The genes for six C/EBP members have been cloned to date from several species as follows: *CEBPA* (C/EBP, R_cEBP-1), *CEBPB* (NF-IL6, LAP, CRP2, NF-M), *CEBPG* (Ig/EBP-1), *CEBPD*, *CEBPE* (CRP-1), and *CEBPZ* (CHOP-10, GADD153) (1). The C/EBP family members recognize similar DNA sequences in their target genes and form homo- or heterodimers with other C/EBPs, as well as with transcription factors of the NF-κB and Fos/Jun families (2).

Previous studies have shown that CEBPD participates in controlling adipogenesis and the acute phase response to inflammatory stimuli (3). Mouse CEBPD is low in most cell types and tissues; however, it is rapidly induced by stimulators, such as interleukin-6 (4), lipopolysaccharide (5), interferon-α, interferon-γ (6), tumor necrosis factor-α (7), and epidermal growth factor (EGF) (8). CEBPD has recently been implicated in cell cycle regulation, its mRNA and protein levels being highly induced in mouse mammary epithelial cells upon serum and growth factor withdrawal (9). Overexpression of CEBPD inhibits the growth of human prostate cancer and erythroleukemia cells, thus exhibiting decreased cyclin D1, cyclin E, and hyperphosphorylated retinoblastoma protein levels accompanied by an increased p27 expression (10, 11). CEBPD is also involved in regulating the pro-apoptotic gene expression during mammary gland involution (12). On the other hand, the phenomena of genomic instability and centrosome amplifications are found in *Cebpd*-depleted primary embryonic fibroblasts (13). These results suggest that CEBPD plays an important role in inducing growth arrest and could serve as a tumor suppressor.

Recent reports reveal alterations in structure or expression levels of C/EBP family members in a variety of human cancers. For example, down-regulation of tumor suppressor CEBPA is seen in several human malignancies, including acute myelogenous leukemia and lung cancer through epigenetic modulation (14). CEBPB is overexpressed in carcinogen-induced skin tumorigenesis (15) and breast cancer (16). Moreover, the “loss of function” alterations of CEBPD and the promoter methylation of the *CEBPD* gene have been observed in primary human

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[§]The abbreviations used are: CEBPD, CCAAT/enhancer-binding protein δ; PRC, polycomb group; EGF, epidermal growth factor; PBS, phosphate-buffered saline; HCC, hepatocellular carcinoma; PMA, phorbol 12-myristate 13-acetate; RT, reverse transcription; DTT, dithiothreitol; MOPS, 4-morpholinepropanesulfonic acid; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; siRNA, small interfering RNAs; ChIP, chromatin immunoprecipitation; DAPA, DNA affinity precipitation assay; DNMT,

DNA methyltransferase; PRC, polycomb repressive complex; 5-azaC, 5-aza-cytidine; ES, embryonic stem.

breast tumors (17, 18). However, the silencing mechanism of the *CEBPD* gene is unknown in tumorigenesis. Hypermethylation of CpG islands, an epigenetic event that is not accompanied by changes in DNA sequence, represents an alternative mechanism different from deletions or mutations to inactivate tumor suppressor genes. Recent evidence supports the notion that CpG island hypermethylation, via the silencing of key cancer-related genes, plays a major causal role in cancer (19, 20).

PcG proteins are epigenetic chromatin modifiers involved in cancer development and also in the maintenance of embryonic and adult stem cells. These regulators, first discovered in *Drosophila*, repress the *homeotic* genes controlling segment identity in the developing embryo (21). The PcG proteins form multiple PRCs, the components of which are conserved from *Drosophila* to humans (22). The PRCs are recruited to repress the transcriptional initiation sites and the polycomb-responsive element of target genes. Thus, these PRCs act through epigenetic modification of the chromatin structure to promote gene silencing (23). To date, five PRCs have been discovered, including PhoRC, PRC1, PRC2, PRC3, and PRC4. PRC2 and its related complexes, PRC3 and PRC4, contain three core components as follows: EZH2, SUZ12, and EED (23). PRC2 catalyzes trimethylation of histone 3 lysine 27 (H3K27), and this enzymatic activity is required for PRC2-mediated gene silencing. H3K27 methylation is thought to provide a binding surface for PRC1, which facilitates oligomerization, condensation of chromatin structure, and inhibition of nucleosome remodeling to maintain silencing (24, 25). *Enhancer of Zeste Homolog 2* (EZH2) has been reported as a H3K27 methyltransferase, and SUZ12 is essential for EZH2 histone methyltransferase activity (26). EZH2 and SUZ12 are downstream target genes of the pRb/E2F pathway and are essential for the proliferation of primary and tumor cells (27, 28). Moreover, EZH2 and SUZ12 are highly expressed in numerous human tumors. SUZ12 is also up-regulated by TCF4/β-catenin complexes and plays an important role in tumorigenesis of the colon (29).

In this study, we demonstrated that SUZ12 bound to CpG islands of the *CEBPD* 5'-flanking region to repress *CEBPD* transcription through coordination of two methylation paths, H3K27 trimethylation and DNA methylation. Consistent with our molecular approach, the opposite expression pattern of *CEBPD* silencing and SUZ12 overexpression was observed in human tumor samples of cervical cancer and hepatocellular carcinoma. Furthermore, we demonstrated that SUZ12 functions as a mediator involved in the interaction of YY1 and the recruitment of DNMTs on the *CEBPD* promoter. In view of this, it is here suggested that the epigenetic silencing effect of overexpressed SUZ12 can be recruited by YY1 to attenuate *CEBPD* transcription in tumorigenesis. This study demonstrates the molecular mechanism of *CEBPD* silencing and further provides a target for tumor therapy.

EXPERIMENTAL PROCEDURES

Materials—Human EGF was purchased from PeproTech (Rocky Hill, NJ). Phorbol 12-myristate 13-acetate (PMA) was purchased from Merck. 5-Aza-cytidine (5-azaC) was purchased from Sigma. The antibody against *CEBPD* and YY1 was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-

bodies against SUZ12 and H3K27 trimethylation were purchased from Upstate (Charlottesville, VA). Antibodies against SUZ12, EZH2, and H3K27 trimethylation for the chromatin immunoprecipitation assay were purchased from Abcam (Cambridge, UK). Antibody against β-actin was purchased from Sigma. Antibodies against DNMT1, DNMT3A, and DNMT3B were purchased from IMGENEX (San Diego, CA). TRIzol RNA extraction reagent, Lipofectamine 2000, Dulbecco's modified Eagle's medium, SuperScript™ III, and Opti-MEM medium were obtained from Invitrogen. Super-Therm DNA polymerase was purchased from Bertec Enterprise Co., Ltd. (Taipei, Taiwan). All oligonucleotides were synthesized by MDBio Inc. (Taipei, Taiwan). Fetal bovine serum was obtained from HyClone Laboratories (Logan, UT). A luciferase assay system was purchased from Promega (Madison, WI). pSilencer™ 3.1-H1 neo vector was sourced from Ambion (Austin, TX). The ON-TARGET plus SMARTpool siRNA for knockdown of YY1 was purchased from Dharmacon. The expression plasmid of FLAG-SUZ12 was a gift from Dr. Yi Zhang (Howard Hughes Medical Institute, Department of Biochemistry and Biophysics, Lineberger Comprehensive Cancer Center, University of North Carolina, Chapel Hill). The expression plasmid of YY1 was a gift from Dr. Pan-Chyr Yang (College of Medicine, National Taiwan University, Taiwan).

Cell Culture and Treatments—Human epidermoid carcinoma cell line, A431, and cervical epithelioid carcinoma cell line, HeLa, were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 100 µg/ml streptomycin, and 100 units/ml penicillin at 37 °C and 5% CO₂. Cells grew to 80% confluence in complete media and were then switched to serum- or mitogen-free media for 24 h. Human EGF (50 ng/ml), PMA (10 nm), or 5-azaC (10 µM) were subsequently added to cells for the designated times.

Tissue Samples—Eleven patients with cervical cancer and 12 patients with hepatocellular carcinoma (HCC) were surgically resected at the National Cheng Kung University Hospital and the National Taiwan University Hospital, respectively. Total RNA samples were extracted from tumor tissues and adjacent unaffected cervical or liver tissues. All tumor specimens from patients were obtained from surgically resected tissues that had previously been pathologically assessed at both the National Cheng Kung University Hospital and the National Taiwan University Hospital. The fresh tissue samples were immediately cut into small pieces, snap-frozen in liquid nitrogen, and stored in a deep freezer. Total RNA was extracted from the tumorous and paired non-tumorous tissue using the TRIzol reagent. The staging system of hepatocellular carcinoma was based upon previous reports (30, 31). Total proteins were homogenized in the lysis buffer (20 mM MOPS, 2% SDS, 20 mM DTT, 10% glycerol, 1 mM DTT, 10 mM NaF, 1 mM phenylmethylsulfonyl fluoride, 1 µg aprotinin/ml, 1 µg leupeptin/ml, 1 mM Na₃VO₄) using a tissue grinder, and 20 µg of total proteins were analyzed by Western blot.

Plasmid Transfection and Reporter Gene Assay—Cells were re-plated 24 h before transfection at an optimal density in 2 ml of fresh culture medium in a 6-well plastic dish. They were then transfected with plasmids by Lipofectamine 2000 according to the manufacturer's instructions. The total amount of DNA for

each experiment was matched with the empty vector. The Opti-MEM media were changed to conditional medium, with or without stimulators after 6 h, and incubated for 15 h. The luciferase activities in cell lysates were measured by the luciferase assay system as per the manufacturer's instructions.

Reverse Transcription (RT)-PCR—Total RNA was isolated from cells using the TRIzol RNA extraction reagent. Three μ g of the isolated RNA was subjected to reverse transcription with SuperScriptTM III. Briefly, 1 μ l of reverse transcription product, 1 unit of Super-Therm polymerase, 1× Super-Therm buffer, and 1 μ l of 10 mM dNTP were mixed with primer pairs for SUZ12, CEBPD, GAPDH, or S26 in a total volume of 25 μ l. PCR was performed in a temperature-gradient thermocycler (ASTEC), initially heated to 94 °C for 2 min, followed by the Touch Down PCR program, 22 cycles at 94 °C for 30 s, annealing for 1 min (the annealing temperature is reduced by 1 °C per 2 cycles from 65 to 55 °C), 72 °C for 1 min, and a final 72 °C for 10 min. Specific primers used for SUZ12 are as follows: 5'-CTTAC-ATGTCTCATCGAAACTCC-3' and 5'-GGCTGGAAGCTCT-TCATTGACA-3'; for human CEBPD, 5'-AGCGCAACAAACAT-CGCCGTG-3' and 5'-GTCGGGTCTGAGGTATGGGT-3'; for glyceraldehyde-3-phosphate dehydrogenase (GAPDH), 5'-CCATCACCATCTTCCAGGAG-3' and 5'-CCTGCTTCAC-CACCTCTTG-3'; for S26, 5'-CCGTGCCTCCAAGATGAAAG-3' and 5'-GTTCGGTCTTGCAGGGCTTCAC-3' were used for the RT-PCR analysis. PCR was halted at the exponential phase of the amplified genes, 30 cycles for SUZ12, 28 cycles for CEBPD, 23 cycles for GAPDH, and 22 cycles for S26. The PCR products were separated by electrophoresis in 2% agarose gel and made visible with ethidium bromide staining. The intensity of the PCR fragment photographed was determined with the IS-1000 digital imaging system (Alpha Innotech, San Leandro, CA). The SUZ12 and CEBPD mRNA levels were determined by the ratio of signal intensity of SUZ12 or CEBPD to that of GAPDH or S26 measured by One-dimensional Image Analysis software (Eastman Kodak Co.) and scored as high (ratio >1.0) or low (ratio <1.0).

Western Blot Analysis—Cells were lysed in the modified RIPA buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Nonidet P-40, 0.25% sodium deoxycholate, 1 mM DTT, 10 mM NaF, 1 mM phenylmethylsulfonyl fluoride, 1 μ g of aprotinin/ml, 1 μ g of leupeptin/ml, and 1 mM Na₃VO₄) to be analyzed. Following lysis, the lysates were resolved on an SDS containing 10% polyacrylamide gel, transferred to polyvinylidene difluoride nylon membrane, and probed with specific antibodies at 4 °C overnight. The specific bands were detected by horseradish peroxidase-conjugated antibody and revealed by an enhanced chemiluminescence (ECL) Western blot system (Pierce).

siRNA Assay—The CEBPD knockdown was carried out as described by Wang *et al.* (32). Two knockdowns of SUZ12 oligonucleotides (25), SUZ12 si1 and SUZ12 si2, were synthesized according to the oligonucleotide design procedure manual (Ambion). It was as follows: 5'-GATCCGCCGGAAATTCCCGTCCCTCAAGAGAGGGACGGGAAATTCCGGGT-TTTTGGAAA-3' and 5'-GATCCGAGATGACCTGCATT-GCCCTCAAGAGAGGGCAATGCAGGTACATCTTTT-TTGGAAA-3'. The 5'- and 3'-ends of the oligonucleotide are

noncomplementary and formed the BamHI and HindIII restriction site overhangs that facilitated efficient directional cloning into the pSilencerTM 3.1-H1 neo vector. Cells were transfected separately with SUZ12 siRNA expression vectors or pSilencerTM 3.1 negative control vectors (siC) that encode a hairpin siRNA whose sequence was not found in the human genome data bases. After 24 h, transfectants were lysed to harvest total RNA or protein, and we performed RT-PCR or Western blot.

Sodium Bisulfite Modification of Genomic DNA/Methylation-specific PCR—Genomic DNA was isolated from cell lines, cervical cancer, or HCC samples using a DNeasy tissue kit (Qiagen). It was treated with sodium bisulfite, purified, and eluted in distilled water. The primers for the first PCR were as follows: forward primer, 5'-AAGAAAGAGGTTTGGAGTGTGGTAGAGG-3', and reverse primer, 5'-AACGAAACTATCACCTCGCTAACCCAACCC-3'. Initial PCR conditions were as follows: 94 °C for 3 min; 35 cycles at 94 °C for 30 s, 42 °C for 30 s, 72 °C for 1 min; subsequently followed by 6 min at 72 °C and cooled down to 4 °C. Following the first amplification, an aliquot of the initial PCR products was used as a template DNA for the nested PCR. The primers for the nested PCR were as follows: forward primer, 5'-TGGAGTGTGGTAGAGGGAGTGT-3', and reverse primer, 5'-TTCTAACCCGACTAACGTACACG-3'. Nested PCR conditions were as follows: 94 °C for 3 min; 36 cycles held at a temperature of 94 °C for 30 s, followed by 42 °C for 30 s and then 72 °C for 40 s; subsequently maintained at 72 °C for 6 min and then finally cooled down to 4 °C. The nested PCR products were subcloned into pT&A cloning vector (Yeastern Biotech Co., Taiwan) and sequenced with M13 forward and/or reverse primers by the Genomics BioSci&Tech. Co. For each sample, three separate clones were sequenced.

Chromatin Immunoprecipitation (ChIP) and re-ChIP Assay—The ChIP assay was carried out essentially as described by Wang *et al.* (8). Briefly, HeLa cells were treated with 1% formaldehyde for 15 min. The cross-linked chromatin was then prepared and sonicated to an average size of 300–500 bp. Equal 200 μ g of cross-linked protein/DNA lysates were divided and incubated with 5 μ g of specific antibodies or control rabbit IgG for each immunoprecipitation at 4 °C overnight. After cross-linking reversal, the immunoprecipitated chromatin was amplified by primers related to specific regions of the CEBPD genomic locus. Twenty μ g of cross-linked samples were reversed for equal input control. The primers were as follows: -1575(sense), 5'-GAGGCCGGCGAATCTCTTAAAGCCCCAGG-3', and -1151(antisense), 5'-TTTGAGGCCCGCATTCTGGTCTGCTTTGG-3'; -769(sense), 5'-CGTCTCCCCCATCTGCTCTGCTTTGG-3', and -447(antisense), 5'-TGGGAGAGGACCCGCGCGTCCAAGGAC-3'; -348(sense), 5'-CGAGGAGGTTCCAAGCCCAC-3', and +9(antisense), 5'-GGCTGTCACCTCGCTGGGCC-3'. For the re-ChIP assay, the first immune complex, initially washed twice with buffer, incorporated 50 mM Tris-HCl, pH 8.0, 0.1% SDS, 0.5% Nonidet P-40, 150 mM NaCl, and 2.5 mM EDTA. The complexes were then further washed three times with low salt buffer, consisting of 10 mM Tris-HCl, pH 8.0, and 0.1 mM EDTA, and resolved in 10 mM DTT at 37 °C, further diluted in ChIP dilution buffer, and then

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processed according to the same ChIP assay protocol using the indicated antibodies. Finally, the amplified DNA products were resolved by agarose gel electrophoresis and confirmed by sequencing. The PCR conditions were as follows: 94 °C for 3 min; 32 cycles of 94 °C for 40 s, 58 °C for 50 s, 72 °C for 1 min; and subsequently followed by 10 min at 72 °C. The amplified DNA products were resolved by agarose gel electrophoresis and confirmed by sequencing.

DNA Affinity Precipitation Assay (DAPA)—The 200 μg of nuclear extracts were incubated with 1 μg of biotinylated oligonucleotides with YY1 or mutant YY1 sites of CEBPD promoter in the presence of a DNA binding buffer containing 20 mM Hepes, pH 7.9, 15 mM NaCl, 0.1 mM KCl, 2 mM MgCl₂, 0.2 mM EDTA, 10% (v/v) glycerol, 1 mM DTT, 10 mM NaF, 1 mM phenylmethylsulfonyl fluoride, 1 μg of aprotinin/ml, 1 μg of leupeptin/ml, 1 mM Na₃VO₄, and 2 μg of poly(dI-dC). After 1 h of incubation at 4 °C, 40 μl of streptavidin-magnetic beads was added to the reaction mixture and the incubation continued for 1 h. The complexes were then precipitated by centrifugation and washed three times with DNA binding buffer before they were resolved by SDS-PAGE and subsequently analyzed by Western blot.

Soft Agar Assay—One ml of 0.3% agarose in complete growth medium containing 3000 HeLa cells bearing expression vectors of SUZ12, CEBPD, or CEBPD siRNA were seeded onto 1.5 ml of 0.6% agarose in 6-well plates. After 14–21 days, cells were stained with 0.05% crystal violet and the colonies photographed and scored for statistical analysis.

Immunofluorescence Analysis—HeLa cells were grown on sterile glass coverslips for 18–24 h. The coverslips were removed from media and washed with 1× PBS and then fixed in 3.7% formaldehyde for 10 min. Cells were permeabilized in 2% fetal bovine serum, 0.4% Triton X-100, PBS for 15 min at 25 °C and then incubated for 2 h with SUZ12 (1:200), YY1 (1:50), DNMT1 (1 μg/ml), DNMT3A (2 μg/ml), or DNMT3B (1 μg/ml) antibodies. After three washes in 0.2% Triton X-100/PBS, cells were incubated with Alexa Fluor 488 goat anti-mouse or Alexa Fluor 568 goat anti-rabbit secondary antibody (1:200, Invitrogen) for 1 h at 25 °C. Cells were thoroughly washed in 0.2% Triton X-100/PBS, counter-stained, and mounted on glass slides with ProLong Gold antifade reagent with 4',6-diamidino-2-phenylindole (Invitrogen) for immunofluorescence microscopy analysis.

RESULTS

CEBPD Induces Cell Death and Inhibits Cell Proliferation and Transformation—CEBPD has been suggested as a potential tumor suppressor. However, the CEBPD-exerted anticancer role involved in tumorigenesis is not well studied. To investigate the biological function of CEBPD in cancer cells, especially in cervical cancer and HCC, several fundamental approaches, including pro-apoptotic assay, foci assay, and a soft agar assay were performed. Induction of CEBPD by an inducible expression system resulted in sub-G₁ accumulation in HeLa cells (Fig. 1A). The overexpression of CEBPD reduced foci formation, but its domain-negative mutant, lacking the transactivation domain of CEBPD, reversed this repressive effect of cell proliferation in HepG2 cells (Fig. 1B). Furthermore, the cell transfor-

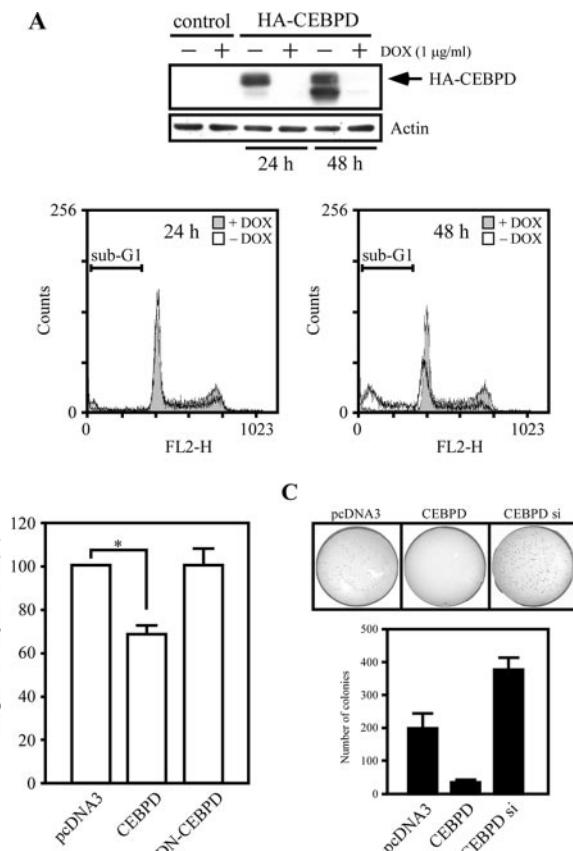


FIGURE 1. CEBPD induces cell death but attenuates cell proliferation and transformation. *A*, cells were transfected with pTRE2-hyg-HA-CEBPD plasmid in HeLa-off cell line. After 24 or 48 h of growth in the complete culture medium, cell lysates were harvested and analyzed by Western blot (*top panel*) or fixed with ethanol and then stained with propidium iodide followed by fluorescence-activated cell sorter analysis (*bottom panel*). DOX, doxycycline. *B*, HepG2 cells were transfected with wild type CEBPD (HA-CEBPD), CEBPD dominant negative (DN-CEBPD), or control expression vectors. After 12 h of growth in the complete culture medium, 1000 cells were cultured in a 10-cm dish for 1 week. The colonies were detected by methyl blue staining, and the number of colonies was then counted for statistical analysis. Values were relative cell number ± S.E. Percentage of colony number means the colony number of different overexpression conditions compared with the pcDNA3 control. *C*, HeLa cells were transfected with control, CEBPD, or CEBPD-knockdown (CEBPD si) expression vectors. After 24 h, a soft agar assay was performed with these transfectants and grown for 14–21 days. The quantitative results of colony numbers were indicated as the means ± S.D. of triplicate samples.

mation assay demonstrated that CEBPD could inhibit cell transformation, whereas the loss of CEBPD expression enhanced the transformation activity (Fig. 1C). These results suggest that the anticancer roles of CEBPD are involved in the pro-apoptotic regulation and inhibition of cell growth and transformation.

DNA Methyltransferase Inhibitor Reverses CEBPD Expression—A change in the methylation pattern of CpG islands has been identified in cancer cells and results in the silencing of important tumor suppressor genes. Evidence demonstrates that CEBPD is silenced in breast cancer, but the mechanism has not been characterized (17, 18). To identify whether the CEBPD gene locus contains CpG islands, the sequences (including CEBPD promoter and gene) from -1600 to +1000 (Fig. 2A, *bottom panel*) were analyzed using the EBI website CpGPlot program. The CEBPD gene locus carried a high percentage of C

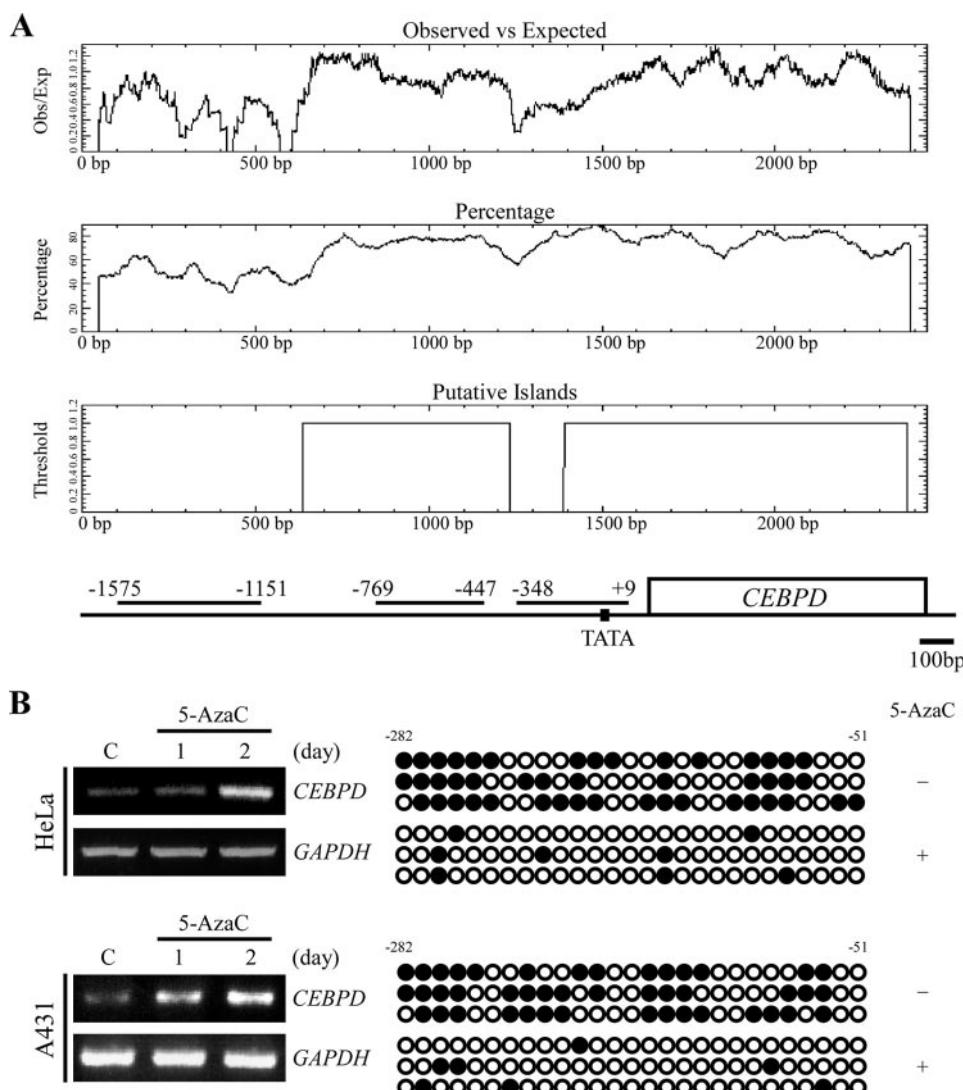


FIGURE 2. 5-AzaC reactivates endogenous CEBPD gene expression. *A*, sequence locations between -1600 and $+1000$ bp of the CEBPD gene were predicated by the EBI website CpGPlot program. The correlated positions of amplified products of three different primer pairs were represented in the bottom panel. *B*, HeLa and A431 cells were treated with $10 \mu\text{M}$ 5-azaC. Total RNAs were harvested at the indicated times to detect the CEBPD and GAPDH transcripts by RT-PCR (left panel). The CpG methylation status of CEBPD promoters in HeLa and A431 cells was performed by methylation-specific PCR analysis. The numbers, -282 and -51 , mean the distance from the transcription start site of the CEBPD gene. A total of 27 CpGs are represented by circles within the $-282/-51$ region of CEBPD promoter. The filled circles and open circles represent the methylated and nonmethylated CpG dinucleotides, respectively (right panel).

or G nucleotides, and two putative CpG islands were found around -960 to -360 bp and -210 to $+880$ bp of the input sequence (Fig. 2A). To investigate whether the DNA methylation plays a functional role in CEBPD transcription, two cervical cancer cell lines, including HeLa and A431, were treated with a DNA methyltransferase inhibitor 5-azaC. The endogenous CEBPD mRNA expression was able to be restored with 5-azaC treatment (Fig. 2B, left panel). This result indicates that DNA methylation plays a role in CEBPD gene silencing. Our previous study demonstrated that the proximal region of the CEBPD promoter is important for its transcriptional activation (8). To verify that DNA methylation occurs within this proximal region of CEBPD promoter in these cells, bisulfite sequencing was performed. There were highly methylated CpG dinucleotides in the CEBPD promoter region between

-282 to -51 bp in HeLa and A431 cells. The site-specific methylated CpG dinucleotides decreased after 5-azaC treatment accompanied CEBPD induction (Fig. 2B, right panel).

PcG Proteins Bind to CEBPD Promoter in Vivo—Previous studies have shown that PRC complexes play important roles in maintaining the silent state of target genes. Methylation of CpG islands is the most important cause in PcG protein-mediated gene silencing by recruiting DNMTs (33). Our data revealed that DNA methylation was involved in CEBPD silencing, so we next examined whether the epigenetic modifiers, PcG proteins, participated in the CEBPD gene silencing by means of a ChIP assay. The immunoprecipitated DNA products were amplified by PCR with specific primers corresponding to $-1575/-1151$, $-769/-447$, and $-348/+9$ on the CEBPD gene locus (Fig. 3A, top panel). We have shown that SUZ12 and EZH2 can specifically bind to the $-769/-447$ and $-348/+9$ regions of the CEBPD gene locus (Fig. 3A, bottom panel). Because PcG proteins are responsible for the H3K27 trimethylation, we further investigated the methylation regions of H3K27 on the CEBPD promoter. As expected, H3K27 also could be trimethylated in the $-769/-447$ and $-348/+9$ regions. Taken together, these results indicate that SUZ12 and EZH2 do indeed bind to the CpG islands of the proximal CEBPD promoter, and this binding activity is

coincident with H3K27 trimethylation.

SUZ12 Plays a Repressive Role in CEBPD Gene Transcription—As shown in Fig. 3A, SUZ12 could be recruited to the CEBPD promoter *in vivo*, so our research then turned to identifying the SUZ12 effect on CEBPD gene transcription. To clarify this issue, HeLa or A431 cells were transfected with different amounts of SUZ12 expression vectors and the level of CEBPD mRNA examined by RT-PCR assay. As shown in Fig. 3B, increasing SUZ12 expression attenuated the CEBPD mRNA expression in a dose-dependent manner. To verify that the repressive effect of SUZ12 was through promoter regulation, SUZ12 expression vectors were co-transfected with reporter vector bearing the promoter region of the CEBPD gene for a reporter assay. Consistent with the RT-PCR results, overexpression of SUZ12 repressed the CEBPD promoter activity in a

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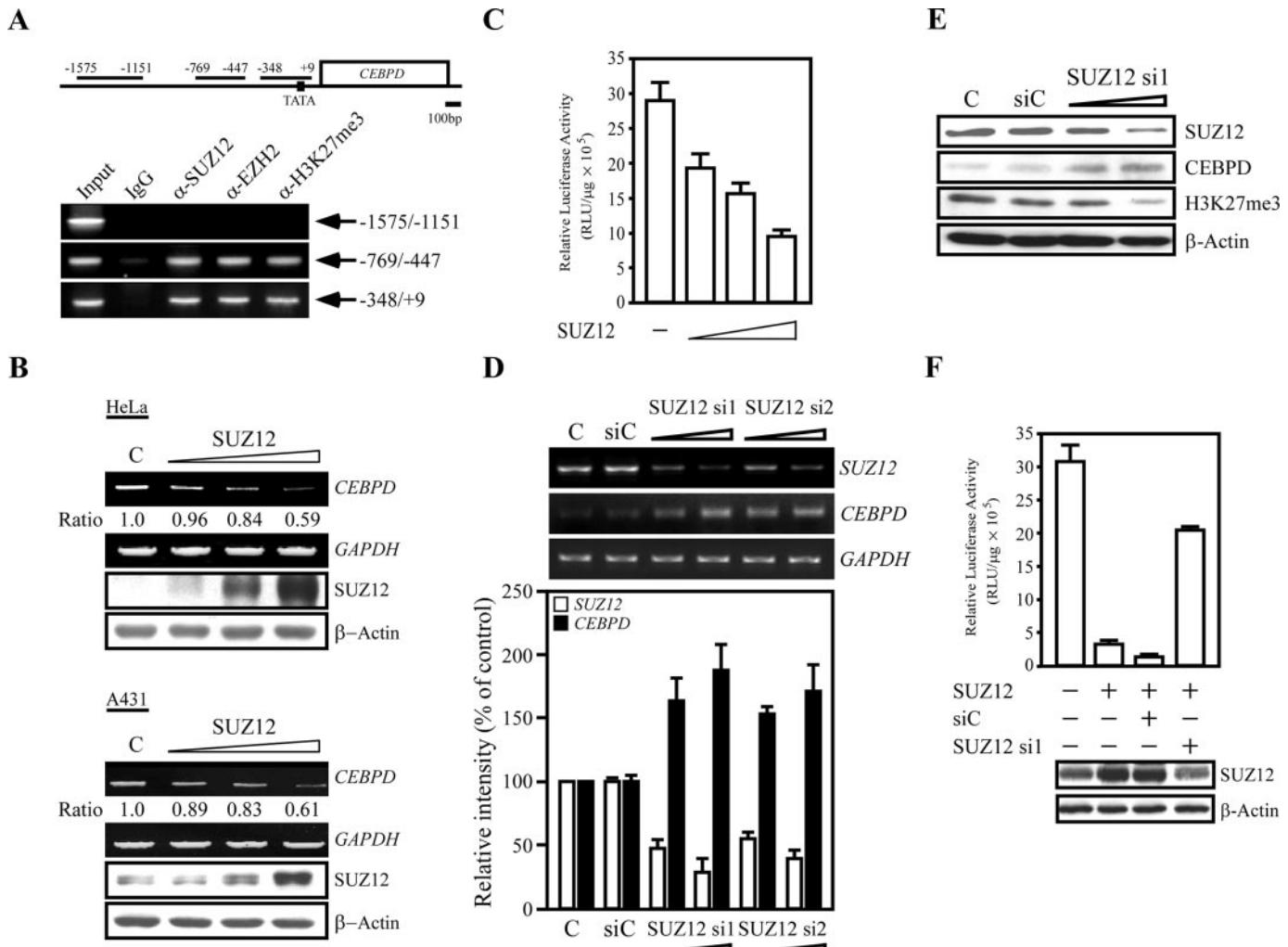


FIGURE 3. SUZ12 binds to CpG islands of the CEBPD promoter and represses CEBPD transcription through this promoter. *A*, SUZ12 and EZH2 bind to CEBPD promoter in vivo. Chromatin of HeLa cells was separately immunoprecipitated with specific antibodies, control IgG (IgG), SUZ12, EZH2, or H3K27me3, then amplified by PCR with different primers as indicated in the top panel. *B*, overexpressed SUZ12 decreases endogenous CEBPD gene expression in a dose-dependent manner. A431 or HeLa cells were transfected with different amounts of SUZ12 expression vectors. After 24 h, total RNA or protein of transfectants were harvested to perform RT-PCR or Western blot analysis. *C*, overexpression of SUZ12 represses CEBPD promoter activity. HeLa cells were co-transfected with the reporter vector bearing CEBPD promoter (-1000 to +18 bp) and various doses of SUZ12 expression vectors. After 24 h, cell lysates of transflectants were harvested for a luciferase assay. *D* and *E*, loss of SUZ12 expression increases CEBPD gene expression. The transfections of siC, SUZ12 si1 or SUZ12 si2, were performed in HeLa cells. It shows the transcriptional products of SUZ12 and CEBPD genes by RT-PCR analysis (*D*). The image density of three independent experiments was determined by a densitometer and normalized with GAPDH. The cell lysates were harvested after 24 h of transflectants and analyzed by Western blot with indicated antibodies (*E*). *F*, decrease of SUZ12 expression enhanced CEBPD promoter activity. The reporter vector carrying CEBPD promoter (-1000 to +18 bp) was co-transfected with expression vectors as indicated in HeLa cells. After 24 h, the cell lysates were harvested for luciferase assay. *C*, control.

dose-dependent manner (Fig. 3C). Furthermore, to ensure that SUZ12 indeed played a negative role in CEBPD transcription, a loss-of-function approach, an siRNA assay, was performed. Two specific sequences targeted to the coding region of SUZ12 were inserted into pSilencer 3.1-H1 neo vector (SUZ12 si1 and si2). Despite both SUZ12 si1 and si2 displaying the ability to attenuate SUZ12 mRNA expression, the SUZ12 si1 demonstrated a higher degree of efficiency with regard to this silencing effect. Therefore, the SUZ12 down-regulation was clearly linked to the increase of CEBPD mRNA and protein expression by the SUZ12 si1 (Fig. 3, *D* and *E*). In addition, we also validated the level of H3K27 trimethylation decrease following SUZ12 silencing (Fig. 3E). This was consistent with later results showing that the knockdown of SUZ12 reversed SUZ12-mediated inhibition of CEBPD promoter activity, whereas the siRNA

control (siC) did not (Fig. 3F). Summarily, these results demonstrated that the increases of SUZ12 and H3K27 trimethylation negatively regulate CEBPD gene expression.

SUZ12 Increases the Methylation Level of CEBPD Promoter in Vivo—This work shows that SUZ12 binds to the CEBPD promoter region, inhibiting CEBPD gene expression through SUZ12 overexpression and an increase of H3K27 trimethylation. Therefore, it became an interesting issue to address whether SUZ12 acted as a mediator in the two methylation paths of DNA and H3K27 contributing to CEBPD gene silencing. To clarify whether SUZ12 could function in the DNA methylation of the CEBPD promoter, an exogenous SUZ12-coupled DNA methylation assay was performed. The luciferase reporter vector bearing the CEBPD promoter was co-transfected with control or SUZ12 expression vectors in HeLa cells.

SUZ12-mediated Silencing Regulation of CEBPD Expression

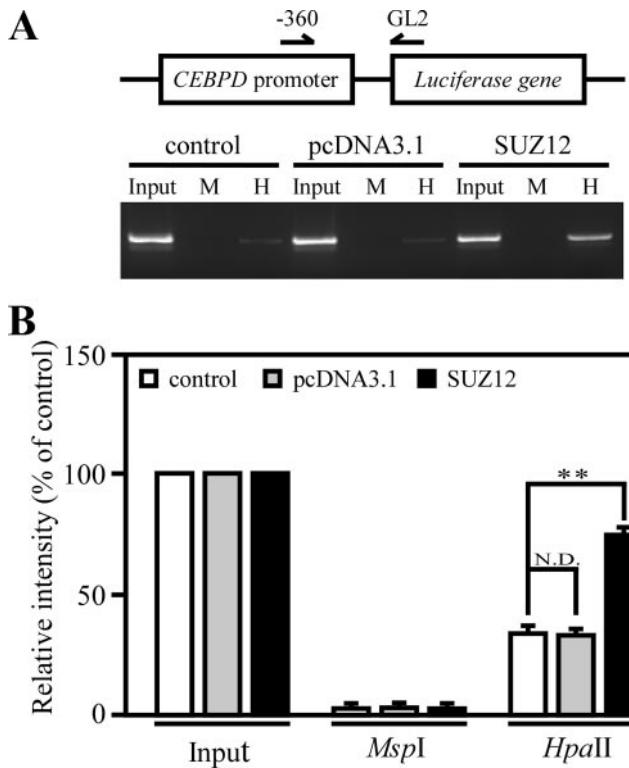


FIGURE 4. SUZ12 can increase DNA methylation level of CEBPD promoter. *A*, *M* and *H* designate *Msp*I and *Hpa*II restriction enzymes, respectively. *Input* means the control of undigested DNA. The reporter vector of pGL2-Basic carrying CEBPD promoter (-1000 to +18 bp) was co-transfected with SUZ12 expression vector, pcDNA3.1, or none. After 24 h, the exogenous CEBPD promoter plasmids were extracted from transfectants and then digested with restriction enzymes. A specific CEBPD promoter region primer, -360, and a specific luciferase gene primer, GL2, were performed by PCR amplification. *B*, data shown means \pm S.D. of three independent experiments in *A*. Setting the image intensity of control *Input* to be standard as 100, the remaining intensity values were normalized compared with the standard. Statistical analysis was performed by Student's *t* test (**, $p \leq 0.01$). N.D. = no difference.

Later the CEBPD reporter plasmids were extracted. The reporter DNAs were digested by restriction enzymes, *Msp*I or *Hpa*II, and then PCR amplification was subsequently performed with a specific primer on the CEBPD promoter, -360, and a specific luciferase gene primer, GL2, for this CpG methylation assay. *Hpa*II digestion is blocked by internal CpG methylation; however, *Msp*I was able to digest both methylated and nonmethylated CpG in their recognition sequence CCGG. If DNA methylation occurs at a CpG within the recognition sequence CmGG, where *m* is methyl cytosine, then a PCR product of ~400-bp is expected in samples digested with *Hpa*II but not with *Msp*I. The methylation status of CEBPD promoter was highly increased in SUZ12-transfected HeLa cells, as judged from the presence of the ~400-bp product after *Hpa*II digestion but not *Msp*I digestion (Fig. 4, *A* and *B*).

SUZ12 and EZH2 Attenuate Mitogen-induced CEBPD Expression—CEBPD plays a role in differentiation induction and pro-apoptotic activity. The mitogenic stimulators, EGF and PMA, not only induce CEBPD expression but also play important roles for tumorigenesis. This raises an important issue of whether this Pcg protein-induced methylation activity disturbed the mitogenic factors, inducing CEBPD transcription. Both ChIP and reporter assays were performed to clarify

this issue. Compared with the positive control of phospho-CREB (Fig. 5*A*, top panel) (8), the binding activities of SUZ12 and EZH2 on the CEBPD promoter were attenuated in either EGF or PMA treatment (Fig. 5*A*). Furthermore, reporter assays showed that the overexpression of SUZ12 or EZH2 could attenuate EGF- or PMA-induced CEBPD promoter activity (Fig. 5*B*). These results suggest that an aberrant increase of SUZ12 or EZH2 would reduce the mitogenic response of the CEBPD gene transcription. This in turn would prevent the counteractive effect of CEBPD-exerted anticancer activity in tumorigenesis.

Opposite Expression of SUZ12 and CEBPD mRNA in Human Cervical Cancer and HCC—Formation of PRC complexes via SUZ12 and EZH2 shows gene silencing in development and tumorigenesis. Similar to EZH2, SUZ12 has been identified as an E2F target gene and is up-regulated in a number of different human tumors, including tumors of the colon, breast, and liver (27, 29). On the other hand, our results suggest that CEBPD participates in negative regulation of cell growth and anti-apoptosis. However, whether the phenomenon of CEBPD gene silencing occurs in other tumors, aside from breast tumors, is uncertain. To link and clarify whether the connection between overexpression of SUZ12 and CEBPD gene silencing has clinical significance in tumorigenesis, 11 pairs of cervical cancer and 12 pairs of HCC with adjacent non-tumorous tissue were analyzed by RT-PCR. SUZ12 was overexpressed in 10 of 11 tumorous samples (90%), and CEBPD was down-regulated in 10 of 11 samples (90%) in cervical cancer samples (Fig. 6*A*), respectively. Among the 10 samples of SUZ12 overexpression in tumors, nine samples expressed low level CEBPD, revealing a concordance rate of 90%. We further verified this phenomenon in HCC tumors. Twelve clinical samples of HCC patients were analyzed. Among these, 10 cases (83.3%) showed concurrent high level SUZ12 and low level CEBPD in tumors (Fig. 6*B*). Hence, we suggest that SUZ12 plays a functional role in tumorigenesis by silencing CEBPD gene expression in clinical samples. To further assess the correlation between epigenetic changes of CEBPD promoter methylation and its mRNA expression, bisulfite sequencing was performed on these primary tumor isolates. The cervical cancer and HCC with opposite expression patterns of SUZ12 and CEBPD mRNA were selected to verify the methylation status of the CEBPD promoter region (Fig. 6, *C* and *D*). In clinical analysis, a highly methylated CpG island was detected within the -282/-51 region of the proximal CEBPD promoter. A hypermethylation pattern, showing 70.7% of 27 CpG dinucleotides, was observed in the tumor portion of cervical cancer patients; however, only 7.1% of 27 CpG dinucleotides were methylated in the normal portion of the same patients. Similar to cervical cancer patients, 64.8% of 27 CpG dinucleotides in the tumor region and 9.6% of 27 CpG dinucleotides of the normal portion of HCC were detected in a methylated status (Fig. 6*E*).

CEBPD Can Repress the SUZ12-induced Tumorigenic Phenotype—Previously, we demonstrated that induction of CEBPD could induce apoptosis by sub-G₁ checking and inhibit cell proliferation by the foci assay (Fig. 1, *A* and *B*). We also showed that overexpressed CEBPD could reduce colony numbers and that colony numbers were reversed upon CEBPD down-regulation in the soft agar assay (Fig. 1*C*). As mentioned

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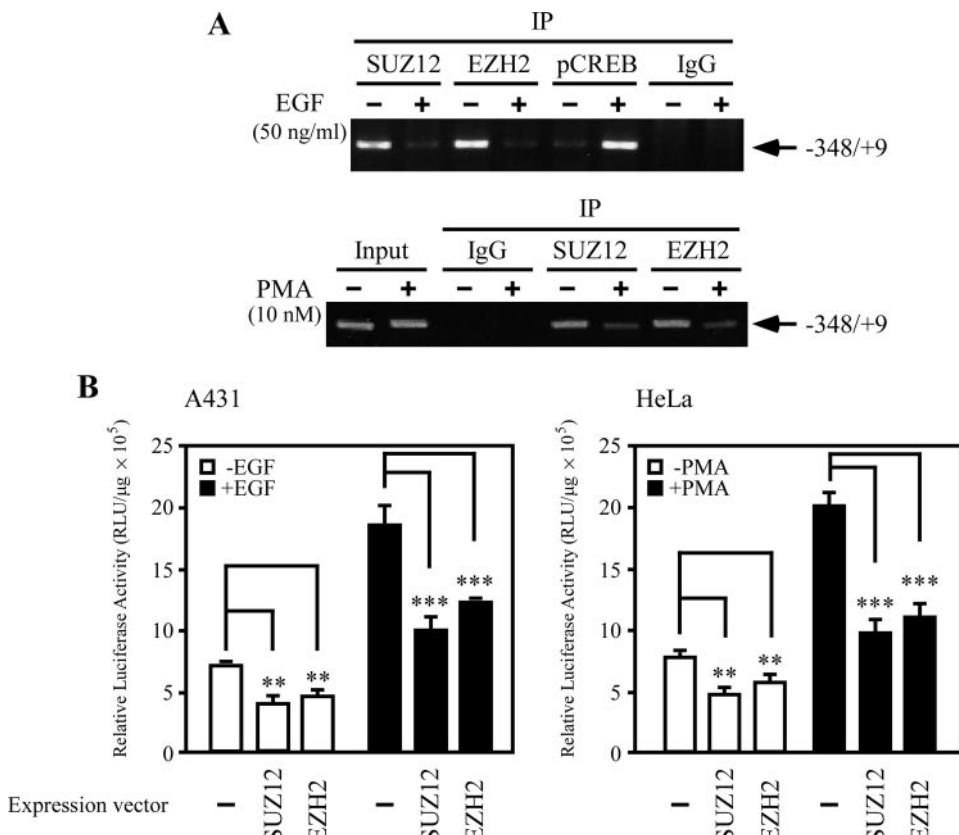


FIGURE 5. Increase of SUZ12 and EZH2 negatively regulate PMA-induced CEBPD promoter activity. *A*, SUZ12 and EZH2 substantially lost their DNA binding activity in PMA- and EGF-treated cells. Chromatin was immunoprecipitated (IP) with specific antibodies, control IgG (IgG), SUZ12, EZH2, or pCREB after 1 h of PMA treatment in HeLa cells or 1 h of EGF treatment in A431 cells. The precipitated DNAs then were amplified by PCR with -348 and $+9$ on the CEBPD gene locus. *B*, overexpressed SUZ12 and EZH2 attenuate EGF- or PMA-induced CEBPD promoter activity. The reporter vector of pGL2-Basic carrying CEBPD promoter (-1000 to $+18$ bp) was co-transfected with SUZ12 or EZH2 expression vectors in A431 or HeLa cells. After 6 h of transfection, cells were treated with EGF or PMA, and the cell lysates later harvested for luciferase assay. Statistical analysis was performed by Student's *t* test (**, $p \leq 0.01$; ***, $p \leq 0.001$).

above, SUZ12 is highly expressed in tumors and is considered as an oncogene facilitating tumor formation. To clarify the advantage of increasing SUZ12 and the effect of SUZ12-disregulated CEBPD in cancer cells, the soft agar assay was performed. As shown in Fig. 6*F*, part of the colonies in the SUZ12-overexpressed cells showed larger sizes and penetrated the bottom gel of the dish, although they did not show significantly increased colony numbers. It might imply that SUZ12 could facilitate cancer cell invasion. Additionally, current data show that SUZ12 plays an important role in the down-regulation of the CEBPD gene and that it is also correlated to clinical cancer samples. Moreover, the exogenous cytomegalovirus promoter-driven CEBPD expression vector, independently regulated by SUZ12, was capable of substantially inhibiting colony formation (Fig. 6*F*). In conjunction, these results confirm that CEBPD functions in suppressing tumor formation and its silence could provide an opportunity for tumorigenesis.

PcG Proteins and DNMTs Can Be Recruited to CEBPD Promoter Region through YY1—SUZ12/EZH2 complex has been suggested as a regulator of CpG island methylation (22, 34). This study demonstrates that the SUZ12/EZH2 complex indeed regulates methylation paths, H3K27 and DNA, of the CEBPD promoter. However, this SUZ12/EZH2 complex

requires recruitment by DNA-binding proteins to exert their silencing effects. YY1 has been suggested to interact with PcgGs (35, 36); however, the effective targets of YY1/PcgGs-mediated gene silencing in tumorigenesis remain unknown. To verify the possibility that SUZ12 functions as a mediator and is involved in the interaction of YY1 and recruitment of DNMTs on the CEBPD promoter, an immunocytochemistry analysis was first performed to determine the co-localization of SUZ12, YY1, and DNMTs in HeLa cells. Endogenous SUZ12, YY1, DNMT1, DNMT3A, and DNMT3B can be observed in the nucleus, aside from DNMT3B, which also showed a cytosolic location (Fig. 7*A*). Their locations were merged to demonstrate that the SUZ12 can co-localize with YY1, DNMT1, DNMT3A, and DNMT3B in the nucleus. To further address whether YY1 can specifically recruit the PcgGs/DNMTs onto the CEBPD promoter, we identified two putative YY1-binding sites corresponding to the CpG islands of the CEBPD promoter (Fig. 7*B*). To determine the specific binding of YY1, SUZ12, EZH2, and DNMTs on the two YY1 motifs, YY1-1 and YY1-2, of the CEBPD promoter, a

DAPA experiment was performed. The results showed that SUZ12, EZH2, DNMT1, DNMT3A, and DNMT3B could be recruited by these two YY1-binding motifs but not by either of their specific mutant controls, YY1-1 mut and YY1-2 mut. Furthermore, we performed *in vivo* DNA binding assay, ChIP and re-ChIP, to prove that these epigenetic regulators could bind to the CpG islands of the CEBPD promoter, $-769/-447$ and $-348/+9$, rather than the $-1575/-1151$, which contain no putative YY1-binding site (Fig. 7, *C* and *D*). To further elucidate that YY1 plays a critical role in recruiting Pcg proteins and DNMTs to CEBPD promoter region, a ChIP assay was performed by the transfected lysates containing the wild type or the double mutation of YY1-binding motifs of CEBPD promoter reporters. Deficiency of double YY1 motifs loses the bindings of SUZ12, EZH2, DNMT1, DNMT3A, and DNMT3B on CEBPD promoter (Fig. 7*E*). However, the mutation of deficient YY1 motifs can enhance the recruitment of p300 onto CEBPD promoter. It indicates that the loss of YY1 binding can facilitate the transcriptional activation of CEBPD gene (Fig. 7*E*). To sum up, these findings indicate that Pcg proteins, YY1 and DNMTs, form protein complexes that bind to the CEBPD promoter through YY1-binding motifs.

YY1 Represses CEBPD Gene Expression through Promoter Methylation—YY1 plays a functional role in the recruitment of PcgS and DNMTs to CpG islands of the *CEBPD* promoter. Furthermore, to ensure that YY1 indeed plays a central role in repressing *CEBPD* transcription, a knockdown approach using YY1 siRNA was performed. The increases of *CEBPD* mRNA and methylation status of *CEBPD* promoter clearly coincided with the YY1 down-regulation (Fig. 7F). In addition, we also performed the exogenous DNA methylation assay to re-confirm that the YY1 is involved in the regulation of *CEBPD* promoter methylation. The *CEBPD* reporters bearing wild type or mutation of YY1-motifs were co-transfected with control or SUZ12 expression vectors in HeLa cells. Compared with the co-transfector of SUZ12 expression vector and wild type *CEBPD* reporter, the methylation status of *CEBPD* promoter was significantly decreased in the co-transfector of SUZ12 expression vector and YY1 motif mutants (Fig. 7, G and H).

Recruitment of SUZ12 through YY1 Represses CEBPD Transcription—To further confirm whether YY1 was involved in the SUZ12-mediated *CEBPD* silencing, the reporter of the *CEBPD* promoter was co-transfected with the YY1 or knockdown of the SUZ12 expression vectors to address this issue. The YY1 alone repressed *CEBPD* promoter activity, but the repressive effect was reversed by the knockdown of SUZ12 expression (Fig. 8A). To determine whether YY1 played critical roles in the regulation of the *CEBPD* promoter, the *CEBPD* reporter, bearing a mutation of the double YY1-binding motifs, *CEBPD-dmYY1*, was generated by site-directed mutagenesis. In comparison with wild type *CEPBD* reporter, the *CEBPD-dmYY1* showed an enhanced reporter activity (Fig. 8B, compare 1st and 3rd lanes). Furthermore, the *CEBPD-dmYY1* also partially lost the SUZ12-induced inhibitory effect of *CEBPD* reporter activity. This suggests that both YY1 motifs play a crucial role in regulating SUZ12-mediated *CEBPD* gene silencing. Taken together, YY1 not only functions as a repressor but is also involved in the SUZ12-mediated repression of *CEBPD* transcription.

DISCUSSION

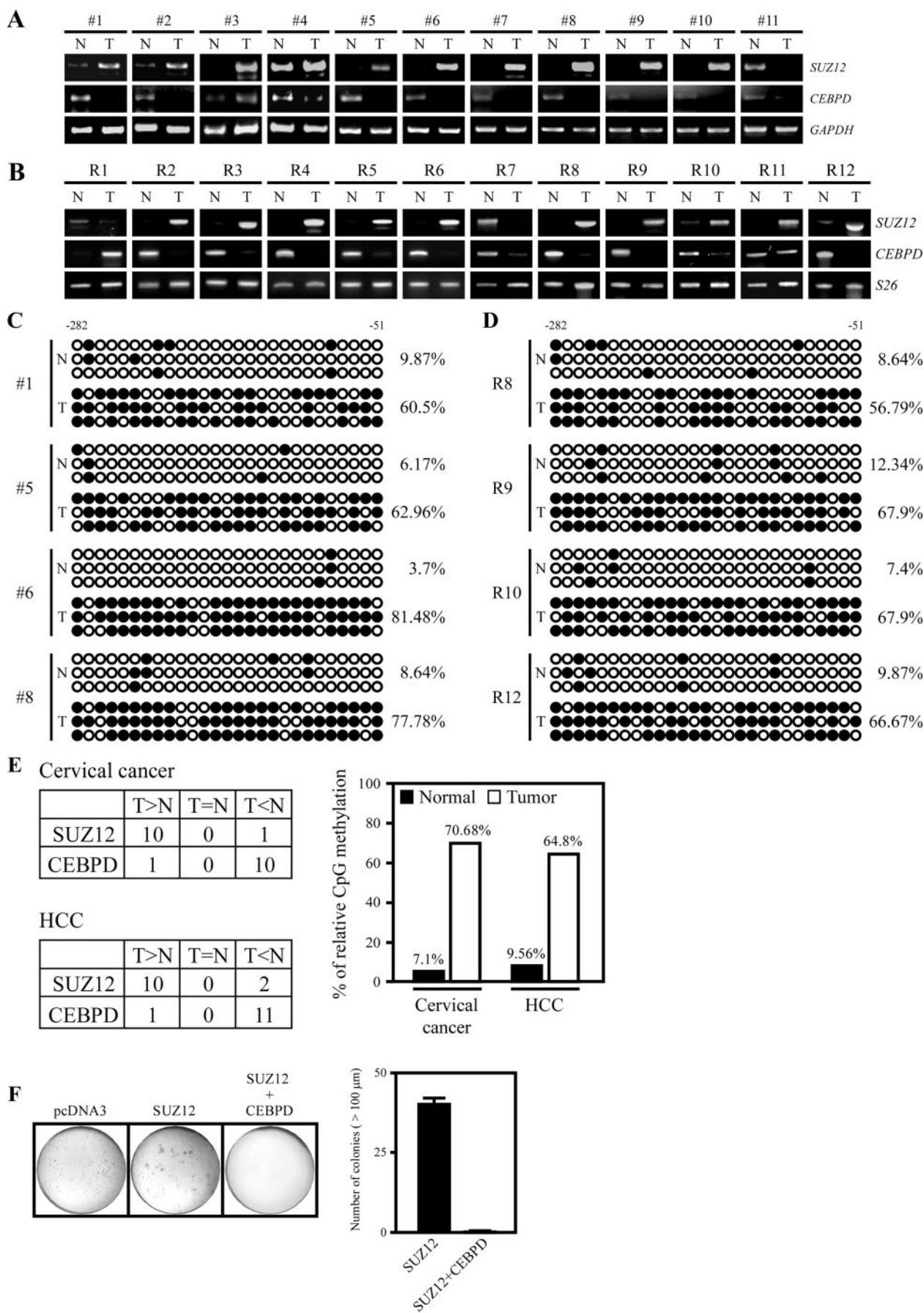
Epigenetic gene silencing is critical during development and plays a role in several cell fate processes, including maintenance of pluripotency, differentiation, and cancer progression (20, 34). In addition, aberrations in the DNA methylation patterns are also recognized as a hallmark of human cancer. Recent studies suggest that promoter hypermethylation of tumor suppressors results in the consequence of gene silencing. One of the most characteristic changes is the hypermethylation of CpG islands of tumor suppressor genes associated with their transcriptional silencing. Several tumor suppressor genes, such as *BRCA1*, p16 *INK4a*, and *VHL* and putative tumor suppressor genes, such as p14 *ARF* and *GSTP1*, have been shown to be silenced by hypermethylation of their promoter regions (37–41). In addition to DNA methylation, more and more studies focus on the impact of covalent modifications of the histone core in the nucleosome structure of gene regulation. One recent study demonstrated that the Pcg proteins directly control DNA methylation (33). However, the *in vivo* evidence was controlled by this aberrant epigenetic remodeling and target

genes still less known, especially in tumorigenesis. Here, our study shows that SUZ12 mediated silencing target *CEBPD* and further clarifies that an oncogene inhibits a tumor suppressor through the regulation of epigenetic silencing in tumors.

Pcg proteins were originally considered to function in the development of *Drosophila* (42) and were further proved to play important roles in the embryonic development of vertebrates (34). A recent report indicates that an increase of EZH2 in cancer cells generates variant PRCs that may result in gene silencing (20). SUZ12 and EZH2 belong to the core components of PRC2 and have been reported as E2F1 target genes. Overexpression of EZH2 has been linked with several tumors, and it has also been proven to be an oncogene (43). Moreover, whether the rest of the PRC2-associated Pcg proteins, such as SUZ12, are also overexpressed and function as oncogenes in tumorigenesis has not been well characterized. We first clarified that SUZ12 is frequently overexpressed in cervical cancer and HCC, and we suggest that it also could be a potential therapeutic target in these two tumors. On the other hand, *CEBPD* is thought to be a tumor suppressor. Overexpression of *CEBPD* can induce apoptosis, reduce proliferation, and inhibit colony formation of the soft agar assay in this study (Fig. 1). Furthermore, SUZ12-deregulated *CEBPD* expression significantly blocks SUZ12-induced cell transformation (Fig. 6F). Therefore, these results suggest that the *CEBPD* gene silencing provides an opportunity for tumorigenesis, especially in the SUZ12-involved tumorigenesis.

The bisulfite sequencing of the *CEBPD* promoter in cancer cell lines and clinical samples of cervical cancer in HCC patients demonstrate the presence of site-specific CpG methylation occurring in the proximal region of the *CEBPD* promoter (Fig. 2B, right panel, and Fig. 6, C and D). These data suggest that the hypermethylation of the *CEBPD* promoter indeed both occurs and correlates with its silence in tumor samples. In the exogenous transfection experiment, we showed that SUZ12 can increase methylation status between the –360 to +10 of *CEBPD* promoter region (Fig. 4, A and B). Furthermore, the overexpression of SUZ12 or EZH2 can inhibit the *CEBPD* induction by external stimulation (Fig. 5B). These data suggest that SUZ12 possesses the ability to intermediate histone methyltransferase and DNMTs for histone and CpG DNA methylation. Comparing the normal and tumor portions of the same patient, the majority of CpG islands of the proximal *CEBPD* promoter exhibited highly site-specific methylation, and this DNA modification coincides with decreasing levels of *CEBPD* mRNA and an increasing level of *SUZ12* mRNA in these clinical tumor samples (Fig. 6, A–D). Methylation-induced suppression of gene transcription is thought to occur either by direct interference with the binding of transcription factors or through the action of methyl-CpG-binding proteins, which trigger a cascade of chromatin modifications that result in a condensed chromatin structure. Alterations in nucleotides that flank the enhancer-binding sites have been previously shown to influence transcriptional activator binding and transcriptional activation (32, 44). Two Sp1 motifs and a CRE motif play important roles in the transcriptional activation of the *CEBPD* gene (8), which is also located

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within or nearby the analyzed 27 CpG dinucleotides. The CpG dinucleotides of Sp1-1, 5'-GGGGCGG-3' and the Sp1-2, 5'-GGGGCGG-3' on the *CEBPD* promoter are separately located on the 15th and 27th CpG dinucleotides. Not one cervical cancer sample and only 8.3% of HCC samples showed a detectable methylated-Sp1-CpG pattern in the normal portion of the SUZ12-overexpressed cancer patients. However, 83.3% of cervical cancers and 54.2% of HCCs displayed a methylated Sp1-CpG pattern in the tumor section of the same cancer patients. This suggests that a high coincidence represents the fact that overexpression of SUZ12 parallels the silence of the *CEBPD* expression in samples of patients with tumors. However, some RNA samples of patients were not consistent with our evidence, such as higher SUZ12 with higher *CEBPD* or lower SUZ12 with lower *CEBPD*, which might result from other transcriptional regulation factors.

Some evidence shows that *CEBPD* is silenced in breast cancer and leukemia; however, the molecular mechanism of gene silencing has not yet been adequately characterized (17, 18, 45). This phenomenon of *CEBPD* gene silencing is also observed in cervical cancer and HCC. Our current results show that SUZ12 not only alters the H3K27 methylation status but also increases the DNA methylation level to mediate *CEBPD* gene silencing (Figs. 3E and 4A). PRC2 target genes in human ES cell have been reported (46). Three important transcription factors, OCT4, SOX2, and NANOG, were identified to co-occupy a significant subset of these genes. Therefore, whether YY1 can collaborate with OCT4, SOX2, or NANOG and play a functional role in *CEBPD* transcription needs to be further examined. According to the ChIP-CHIP profile in that study, the binding of SUZ12 was detectable on the *CEBPD* promoter region before inducing differentiation. We further found that the promoter regions of other C/EBP family members, except CEBPA, were not involved in SUZ12-mediated epigenetic regulation. This suggests that the SUZ12-EZH2 complex can selectively regulate a group of genes to contribute to tumor progression, especially in *CEBPD* or possibly in *CEBPA*. It has been reported that YY1 can interact with PcG proteins (36). However, these YY1-PcG complex-mediated downstream targets are still unknown. Undifferentiated ES cells clearly have the capacity to *de novo* methylate DNA (47), like tumor cells, and levels of both DNMT3A and -3B are very high in ES cells (48). We showed that YY1 plays an important role in the *CEBPD* silencing through recruiting SUZ12 and DNMTs to the CpG islands of the *CEBPD* promoter (Figs. 7 and 8). Although three DNMTs, DNMT1, DNMT3A, and DNMT3B, could bind to the SUZ12-responsive regions of the *CEBPD* promoter *in vivo* (Fig. 7, C and D), the detailed function for each DNMT association with the YY1-PcG complex needs to be further dissected in this chro-

matin remodeling. DNMT3A and DNMT3B were suggested to participate in *de novo* DNA methylation. In contrast, DNMT1 shows high preference for the maintenance of DNA methylation. However, an increasing number of studies suggest that DNMT1, DNMT3A, and DNMT3B functionally cooperate in both maintenance and *de novo* methylation of DNA. DNMT1 associates with DNMT3A/3B *in vivo* through its N-terminal region (49). DNMT1, DNMT3A, and DNMT3B cooperatively regulate cytosine methylation in CpG dinucleotides in mammalian genomes, and this provides an epigenetic basis for gene silencing and maintenance of genome integrity (50, 51). In addition, the PcG protein, EZH2, not only functions as a histone 3 methyltransferase but also interacts with DNMT1 and DNMT3A/3B to participate in the DNA methylation (33). As shown in Fig. 7, our *in vitro* and *in vivo* data suggest that YY1 plays an important role in the scenario of PcG-DNMTs complex-mediated epigenetic regulation of the *CEBPD* promoter. Moreover, Fig. 8B shows that the mutation of double YY1 motifs enhances *CEBPD* promoter activity, while also losing the SUZ12-repressive effect on *CEBPD* promoter. However, several fundamental questions still need to be further examined. For instance, which methylation path, histone methylation or DNA methylation, could occur first through this YY1-mediated epigenetic regulation has yet to be identified.

An alternative approach is to induce cancerous cells to behave like normal cells through what is known as "differentiation therapy" (52). It is based on the hypothesis that resident adult stem cells could be a cancer inducer within the human body, of which there is increasing evidence to date (53). Induction of *CEBPD* functions in many kinds of cell differentiations, such as mammary gland (54), adipocyte (55), monocyte (56), myofibroblast (57), and osteoblast differentiation (58). Several studies demonstrate that *CEBPD* plays a functional role in differentiation and as a growth arrest inducer (9, 11). Except for the PcG protein-mediated methylations of *CEBPD* promoter in tumorigenesis, this study also provides a good model for further analysis of the co-regulation between PcG proteins and *CEBPD*-involved cell differentiation. Our preliminary data suggest that *CEBPD* could repress *Bcl-2*, *cyclin D1*, *HMGA1*, and *Jun*, but activate *PPAR γ* and *MET*.⁴ On the other hand, YY1 functions in the genomic imprinting have been reported (59). We demonstrate that YY1 is able to recruit PcG proteins and DNMTs onto the *CEBPD* promoter (Fig. 7). Thus, the PcG protein-associated DNMTs and exertion of the *de novo* DNA methylation effect may also be involved in the YY1-regulated epigenetic regulation during differentiation or tumorigenesis. This suggests that PcG protein-mediated epigenetic regulation

⁴ J. M. Wang, unpublished data.

FIGURE 6. Opposite expression pattern of SUZ12 and CEBPD in human cervical cancer and HCC. A and B, RT-PCR results of surgical biopsies from 11 patients with cervical cancer and 12 patients with HCC, respectively. *N* and *T* denote the normal and tumor areas, respectively, of the same patients. C and D, genomic DNA was extracted from cervical cancer or HCC samples and subjected to methylation-specific PCR analysis and sequencing. E, table shows the quantitative results of RT-PCR analysis from patients with cervical cancer or HCC (left panel) and the corresponding percentage of CpG island methylation of the proximal *CEBPD* promoter (right panel). F, overexpression of *CEBPD* blocks SUZ12-induced transformation activity. HeLa cells were transfected with control, SUZ12, or *CEBPD* expression vectors. After 24 h, a soft agar assay was performed with these transfectants, which were allowed to grow for another 14–21 days. The number of colonies with sizes $\leq 100 \mu\text{m}$ as well as the mean \pm S.D. of the colony sizes of triplicate samples are indicated. The average colony size of the control vector is about 100 μm .

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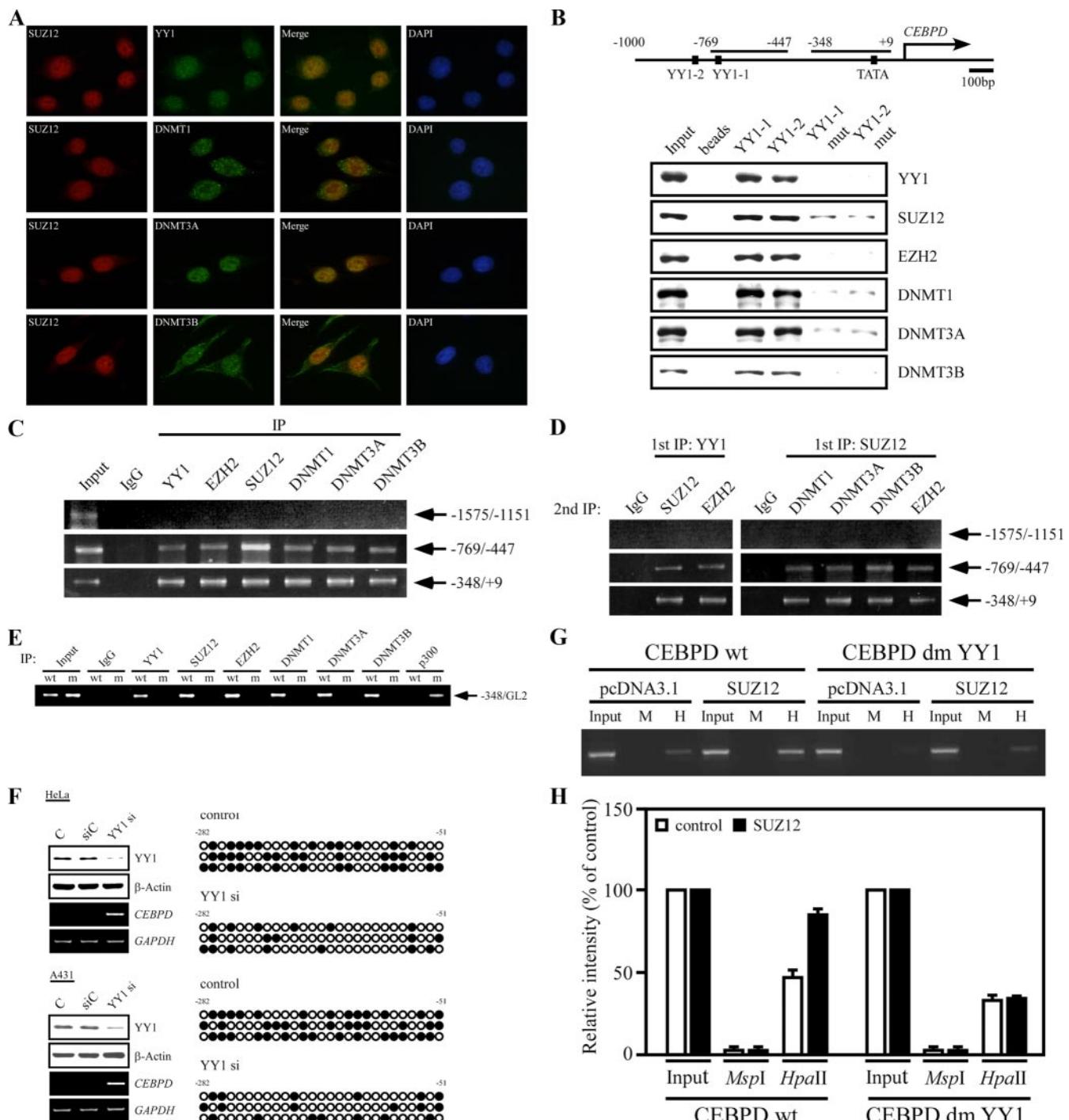
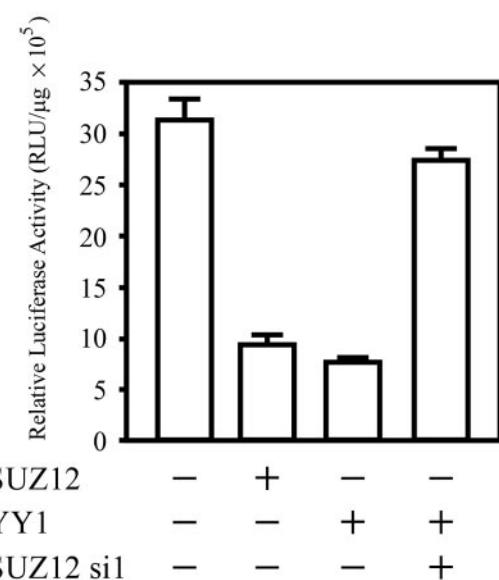


FIGURE 7. SUZ12 and DNMTs can be recruited to CEBPD promoter region through YY1-binding motifs. *A*, SUZ12 can co-localize with YY1 or DNMTs. HeLa cells were fixed with formaldehyde and followed by immunofluorescence analysis with SUZ12, YY1, or DNMTs antibodies. DAPI, 4',6-diamidino-2-phenylindole. *B*, YY1-binding motifs are important for the recruitment of Pcg proteins and DNMTs. Pcg proteins and DNMTs can be recruited by the YY1 motifs of the CEBPD promoter. ChIP was performed with oligonucleotides of two individual YY1-binding sites, YY1-1 and YY1-2, or their mutants, YY1-1mut and YY1-2mut, on the CEBPD promoter and then analyzed by Western blot with indicated antibodies. *C* and *D*, YY1, Pcg proteins, and DNMTs bind to the CEBPD promoter *in vivo*. *IP*, immunoprecipitated. The ChIP and re-ChIP assays were performed as described under “Experimental Procedures” with specific antibodies, control IgG (IgG), EZH2, SUZ12, YY1, DNMT1, DNMT3A, or DNMT3B and then amplified by PCR with specific primers as indicated. *E*, YY1 plays the crucial role in recruiting Pcg proteins and DNMTs to the CEBPD promoter. The wild type (wt) or mutation of double YY1 motifs, dm YY1, of the CEBPD reporter were transfected in HeLa cells, then ChIP assay was performed with -348 and GL2 primers. *F*, loss of YY1 expression increases CEBPD gene expression. The transfections of siC or siYY1 were performed in HeLa and A431 cells. Left panel shows the protein expression of YY1 by Western blot and transcriptional product of CEBPD gene by RT-PCR analysis. The CpGs methylation status of CEBPD promoter in HeLa and A431 cells was performed by methylation-specific PCR analysis. The numbers, -282 and -51, mean the distance from the transcription start site of the CEBPD gene. A total of 27 CpGs are represented by circles within the -282/-51 region of CEBPD promoter. The filled circles and open circles represent the methylated and nonmethylated CpG dinucleotides, respectively (right panel). *G*, loss of YY1 binding decreases DNA methylation level of CEBPD promoter. *M* and *H* designate *Msp*I and *Hpa*II restriction enzymes, respectively. *Input* means the control of undigested DNA. The reporter vector of pGL2-Basic carrying wild type (wt) or mutation of double YY1 motifs, dm YY1, of the CEBPD promoter (-1000 to +18 bp) was co-transfected with SUZ12 expression vector, pcDNA3.1 or none. After 24 h, the exogenous CEBPD promoter plasmids were extracted from transfectants for further assay. The PCR amplification was performed by specific primers of 360 and GL2. *H*, data shown means \pm S.D. of three independent experiments in *G*. The image intensity of control *Input* was set to be 100 as the standard, and the remaining intensity values were normalized to the standard. *C*, control.

A



B

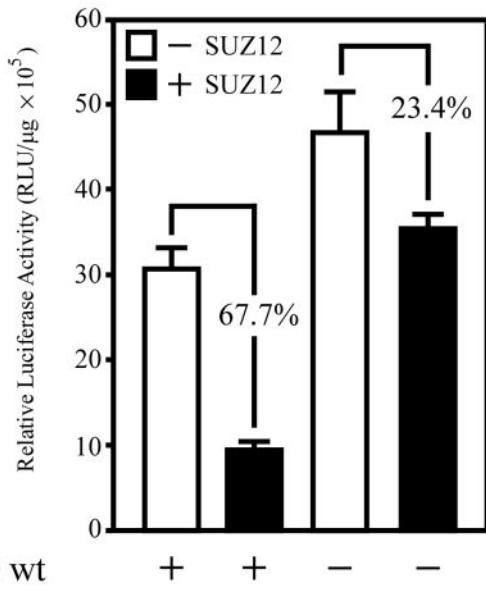


FIGURE 8. YY1 functions in the SUZ12-mediated repression of the *CEBPD* gene. *A*, YY1 is important for SUZ12-mediated repression of the *CEBPD* promoter activity. The reporter vector bearing *CEBPD* promoter (-1000 to $\sim +18$ bp) was co-transfected with expression vectors as indicated in HeLa cells. *B*, both enhancement of *CEBPD* promoter activity and attenuation of SUZ12-repressive activity occur in the mutation of double YY1 motifs on the *CEBPD* reporter. The wild type (wt) or mutation of double YY1 motifs, dm YY1, of the *CEBPD* reporter were co-transfected with expression vectors as indicated in HeLa cells.

not only regulates the *CEBPD* gene in its normal organic development, including cell differentiation but that it also induces hypermethylation of the *CEBPD* promoter to modulate and create a friendly environment for tumor progression. Several differentiation inducers such as retinoic acid or dexamethasone can activate *CEBPD* expression (60). Therefore, our study could be a good model to explain the anticancer effect for drug-induced differentiation and to generate a differentiation inducer that can reverse SUZ12/EZH2-mediated DNA methylation silencing for cancer therapy.

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