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Original Article

Enhancing chemotherapy sensitivity by targeting PcG via the ATM/p53 pathway

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Abstract: Histone modification and chromatin remodeling are important events in response to DNA damage, and Polycomb group (PcG) proteins, catalyzing H3K27 methylation, are involved. However, the biological function and mechanism of PcG in DNA damage are not fully understood. Additionally, downstream effectors in hepatocellular carcinoma (HCC) remain unclear. The present study investigated the biological and mechanistic roles of PcG in the DNA damage response induced by chemotherapeutic drugs in HCC. It was found that chemotherapy drugs, such as epirubicin (EPB) and mitomycin C (MMC), effectively blocked expression of PcG in *p53*-wild-type HepG2 cells but not in PLC/PRF5 and Hep3B cells with *p53* mutation or deletion. PcG-related target genes involved in DNA damage were identified, including *p53*, *Ataxia telangiectasia mutated* (*ATM*) and *Forkhead box O3* (*FOXO3*). Moreover, targeting PcG-induced p53 expression was associated with increased drug sensitivity in HCC cells. shRNA targeting *enhancer of zeste homolog 2* (*EZH2*) or its inhibitor GSK126 significantly promoted chemotherapeutic drug-induced genotoxicity and increased HepG2 cell chemosensitivity. Mechanistically, chromatin immunoprecipitation (ChIP) assays confirmed that PcG binds to the ATM promoter and inhibits its expression through covalent modification of H3K27me3. Herein, we establish a potential chemotherapy association with GSK126, and the findings suggest this link might represent a new strategy for increasing the sensitivity of HCC to chemotherapeutic agents.

Keywords: ATM, p53, Polycomb, DNA damage, chemotherapy sensitivity

Introduction

Hepatocellular carcinoma (HCC) is the main type of liver cancer [1]. Clinically, surgery and radio-/chemotherapy are currently the major therapeutic strategies. However, therapeutic effects are commonly limited by intrinsic or acquired resistance due to an abnormal repair response upon DNA damage induced by radio-/chemotherapy [2].

Several evolutionarily conserved SET (suppressor of variegation, Enhancer of Zeste, and Trithorax) domain-containing proteins have been shown to possess histone 3-specific methyltransferase (HMTase) activity. Interaction of these SET domain-containing factors with DNA results in dynamic alterations in chromatin conformation, which affect the accessibility of a variety of repair factors to the damaged site [3]. Upon DNA damage, Ku70 (Lupus Ku autoantigen protein p70) and BRCA1 (Breast cancer 1), which are the essential components of non-homologous end joining (NHEJ) and homologous recombination (HR), are recruited to DNA double-strand break (DSB) sites [4, 5]. It has been found that Lysine-specific histone demethylase 5B (KDM5B) is necessary for efficient DSB repair and recruitment of Ku70 and BRCA1, whereas KDM5B deficiency promotes spontaneous DNA damage, activates p53 signaling, and sensitizes cells to genotoxic insults [5]. Moreover, demethylation of histone H3 lysine 36 mediated by the interaction between fumarase and the histone variant H2A.Z at DSB regions can further increase accumulation of

Ku70-containing DNA-PK at DSB sites for NHEJ DNA repair and cell survival [6]. This evidence suggests that histone remodeling is essential for recruiting DNA damage response factors to damaged sites in the DNA damage response [7].

The Polycomb group (PcG) family comprises at least two distinct complexes named PRC1 (Polycomb repressive complex 1) and PRC2 (Polycomb repressive complex2) [8]. PRC1, the maintenance complex, includes the proteins B-lymphoma Moloney murine leukemia virus insertion region-1 (BMI1), Chromobox homolog 8 (CBX8), and others [9]. PRC2 is the initiation complex, and in humans, its core consists of EZH2, Suppressor of zeste 12 (SUZ12), and Embryonic extoderm development (EED). EZH2 specifically trimethylates histone 3 at lysine 27 (H3K27me3), and methylated H3K27 is recognized by PRC1 proteins to condense chromatin structure, leading to transcriptional repression of target genes [10]. Recent studies have reported that knockdown (KD) of CBX8 significantly reduces the efficiency of both homologous and nonhomologous recombination and increases the sensitivity of U2OS cells to ionizing radiation [11]. In addition, BMI1 is involved in the DNA damage response, and BMI1-knockout (KO) HCT116 cells are hypersensitive to UV exposure [12]. Poly ADP-ribose polymerase (PARP) is believed to be a DNA damage receptor that is activated upon identification of a site of DNA damage. Indeed, it was found that PARP recruits PcG to sites of DNA damage induced by UV and causes transcription inhibition and that PcG deficiency promotes radiation sensitivity in eukaryotic cells [13]. Moreover, it was reported that BMI1 decreases etoposide (ETOP)-induced G2/M checkpoint activation by reducing Nijmegen breakage syndrome 1 (NBS1)-mediated ataxia telangiectasia mutated (ATM) activation [14]. These results further suggest that PcGs are involved in the DNA damage response and are closely linked to chemo- or radio-sensitivity.

Although abnormal expression of EZH2 is well documented in HCC and promotes tumor development in both an H3K27me3-dependent and -independent manner [15-17], the potential action of EZH2 and its downstream effectors in regulating the DNA damage response in HCC is not well defined. Here, we report the biological and mechanistic importance of PcG in the DNA damage response with regard to epigenetic regulation of the classical ATM/p53 pathways in HCC.

Materials and methods

Chromatin immunoprecipitation assays

Chromatin immunoprecipitation (ChIP) assays were performed as previously described [18]. In brief, $1*10^6$ HepG2 cells were treated with 1% formaldehyde for 10 minutes at 37°C, followed by pulsed ultrasonication to shear cellular DNA. The ChIP assays were then carried out using the indicated antibodies according to the protocol of ChIP Assay Kit (Millipore). After overnight incubation with antibodies, protein A- or protein G-agarose beads were added. The cross-links between nuclear proteins and genomic DNA were reversed, and the DNA pulled down by the antibody was purified by phenol/chloroform extraction. The primer pair sequences and antibodies used in ChIP are listed in Supplementary Tables 1 and 2.

Cell proliferation assay

HCC cells were seeded in 96-well plates at 1500 cells/well and allowed to adhere overnight. The cells were then treated with various concentrations of epirubicin (EPB), mitomycin C (MMC), and GSK126 three times a week. After one week, cell proliferation was analyzed using CCK8 Cell Proliferation Assay Kit. The CCK8 reagent (10 μL) was added to each well and incubated for 1-4 hours. The number of viable cells was calculated by absorbance measurements at 450 nm. Each condition was assessed in triplicate.

Statistical analysis

All statistical analyses were performed using SPSS software version 13.0. Data for cell growth and clone formation assays and cell migration experiments were analyzed by two-tailed Student t tests. The results for parametric variables are expressed as means ± SEM. In all cases, $P < 0.05$ was considered statistically significant.

Results

Expression of PcG is activated in primary HCC

In our previous report, we found that expression of PcG to be activated in primary HCC specimens [17]. In the present study, western

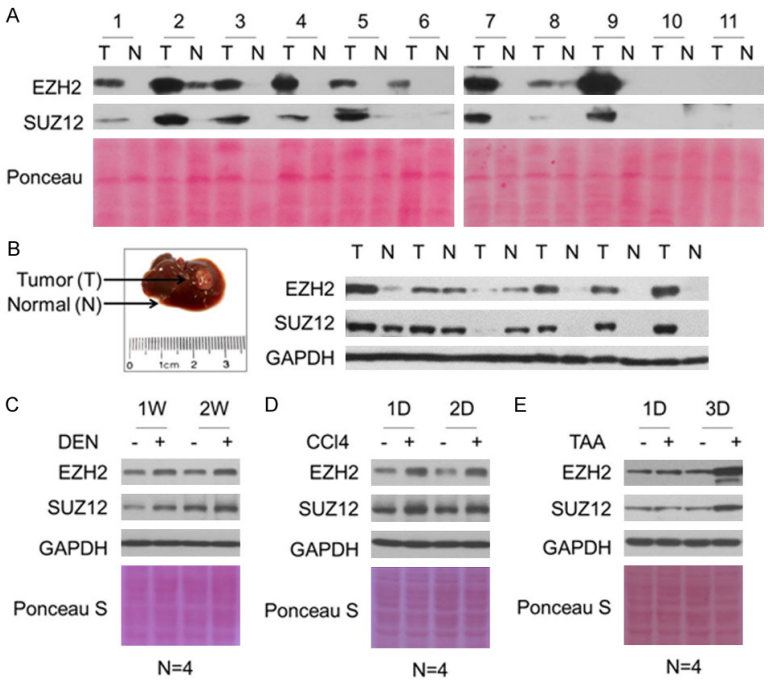

Figure 1. Expression of PcG is activated in both HCC and tumor-related animal models. A. Expression of EZH2 and SUZ12 was detected by western blotting in 11 paired samples of tumor (T) and adjacent tissues (N) from different HCC patients. B. Mice were injected with DEN (25 mg/kg, i.p.) at 15 days of age and were killed at 8 months after DEN injection. Representative liver image is shown on the left. Expression of EZH2 and SUZ12 in DEN-induced tumor (T) and normal (N) liver tissues of mice was detected by western blotting (on the right), n=6. C. C57BL/6 mice were treated with DEN (25 mg/kg, i.p.) at 6 weeks of age and killed at 1 or 2 weeks after DEN injection. Expression of EZH2 and SUZ12 in the liver was detected by western blotting. D. C57BL/6 mice were treated with CCl4 (2 mL/kg, i.p.) at 6 weeks of age and killed at 24 and 48 hours after CCl4 injection; expression of EZH2 and SUZ12 in the liver was detected by western blotting. E. C57BL/6 mice were treated with TAA (2 mL/kg, i.p.) at 6 weeks of age and killed at 24 or 72 hours after TAA injection; expression of EZH2 and SUZ12 in the liver was detected by western blotting.

blotting revealed robust up-regulation of EZH2 and SUZ12 in primary HCC specimens compared with adjacent tissues (**Figure 1A**). In addition, ectopic activation of PcG components was observed in several HCC cell lines (Supplementary Figure 1). Diethylnitrosamine (DEN), a liver-specific DNA damage carcinogen that specifically induces poorly differentiated primary HCC nodules within 8 to 9 months in mice, was applied in establishment of an HCC model [18, 19]. Using this HCC mouse model [18], we found that expression of EZH2 and SUZ12 was markedly up-regulated in cancerous tissue compared with adjacent normal tissue (**Figure 1B**). Furthermore, transient exposure of DEN stimulated expression of EZH2 and SUZ12 at one or two weeks *in vivo* (**Figure 1C**). To examine the early stage of HCC development, another well-established, liver-specific damage and inflammation mouse model was applied [20, 21], and we found that expression of EZH2 and SUZ-12 proteins to be robustly activated by CCL4 or thioacetamide (TAA) exposure at the indicated times (**Figure 1D** and **1E**). These observations suggest that expression of PcG is responsive to genotoxic insults in the liver.

Chemotherapy drugs repress expression of PcG in HepG2 cells

EPB and MMC are broad-spectrum anticancer drugs that primarily act through genome toxicity. It has been reported that chemotherapy tolerance in various types of liver cancer cells is associated with an abnormal DNA damage response [22]. To provide further evidence of the potential role of PcG in the DNA response induced by chemotherapy drugs, we treated HepG2 (*p53* wild-type) cells with MMC and EPB. Western blot analysis indicated that following activation of ATM, a marker of DNA damage, MMC and EPB clearly blocked expression of PcG, including PRC2 (SUZ12, EZH2) and PRC1 (BMI1, CBX8), in a dose-dependent manner (**Figure 2A**). Furthermore, expression of PcG proteins gradually decreased in a time course-dependent manner in HepG2 cells upon exposure to MMC or EPB (**Figure 2B**). These data suggest that the PcG pathway might serve as a target for chemotherapeutic drugs. As the p53 pathway is a key checkpoint for DNA damage repair, we assessed whether p53 is involved in chemotherapy-induced PcG inhibition. Unexpectedly, we found increased expression of PcG in PLC/PRF5 (*p53* mutant) cells exposed to MMC, whereas no obvious changes were observed in Hep3B (*p53* null) cells (**Figure 2C** and **2D**).

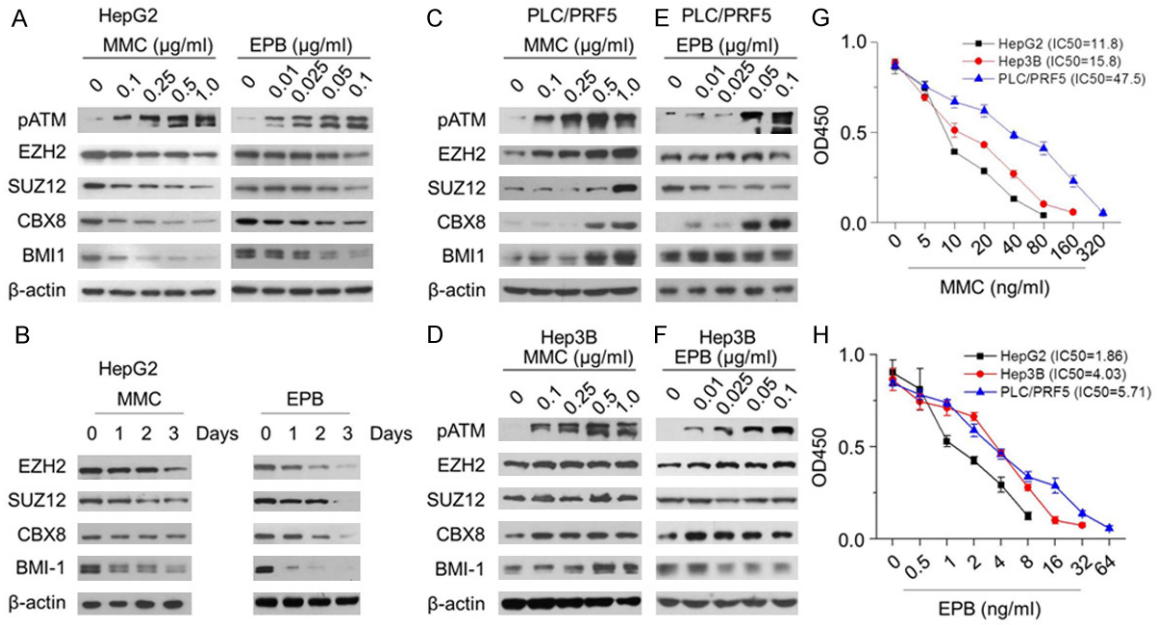

Figure 2. Expression of PcG is down-regulated upon chemotherapy treatment in a p53-dependent manner. A. HepG2 cells were treated with MMC or EPB at the indicated concentrations for 72 hours; expression of PcG and ATM is shown. B. HepG2 cells were exposed to MMC (1.0 μg/mL) or EPB (0.1 μg/mL) on the indicated days. The protein levels of PcG and ATM were detected by western blotting. C, E. Expression of PcG and ATM in PLC/PRF/5 cells treated with MMC and EPB at the indicated concentrations for 72 hours is shown. D, F. Hep3B cells were exposed to MMC or EPB at the indicated concentrations, and the protein levels of PcG were detected by western blotting. G, H. Subconfluent cultures of HepG2, Hep3B and PLC/PRF/5 cells were exposed to increasing concentrations of EPB and MMC, and the cell proliferation capacity was measured by the cholecystokinin-8 (CCK8) assay. The values are the means of three independent experiments.

Similar results were obtained using PLC/PRF5 and Hep3B cells exposed to EPB (**Figure 2E** and **2F**). These findings suggest that regulation of PcG expression upon DNA damage induced by MMC and EPB is dependent on the p53 pathway in HCC cells. Phenotypically, cell toxicity assays revealed HepG2 cells to be more sensitive than Hep3B and PLC/PRF5 cells to either MMC or EPB (**Figure 2G** and **2H**). These results suggest that chemosensitivity in HCC is at least partly related to inhibition of PcG expression in a p53-dependent manner.

Inhibition of EZH2 increases chemotherapy drug genotoxicity in HCC

To explore a functional implication of PcG in the DNA damage response in HCC, we detected the formation of γH2A.X foci in HepG2 cells stably transfected with either vectors or constructs expressing shRNAs that specifically target *EZH2* after EPB or MMC treatment. We found that exposure of either EPB or MMC increased the presence of phosphorylated histone H2A.X (γH2A.X), a DSB marker, at 1 or 6 hours (**Figure 3A-C**). KD of *EZH2* did not markedly affect the basal level of γH2A.X but did strikingly increase the level of γH2A.X foci in HepG2 cells exposed to EPB or MMC at the indicated times (**Figure 3D-F**). The significant difference in the DNA damage response between the *EZH2* shRNA group versus the control group treated with chemotherapy drugs is highlighted in Supplementary Figure 2. These results indicate that interfering with EZH2 expression contributes to DNA damage accumulation upon exposure to chemotherapy drugs.

PcG regulates the p53/ATM pathway

p53 mutation is a major genetic event in HCC. We previously reported that expression of PcG was negatively correlated with p53 in HCC and that PcG regulates *p53* transcription in an H3K27me3-independent manner [16]. Consistent with this, mRNA expression of *p53* was clearly elevated by substantial KD of *CBX8*, *BMI1*, *EZH2* or *SUZ12* with shRNA (**Figure 4A** and **4B**). In addition, *CDKN2A* and *FOXO3*, which are linked to the p53 apoptotic pathway,

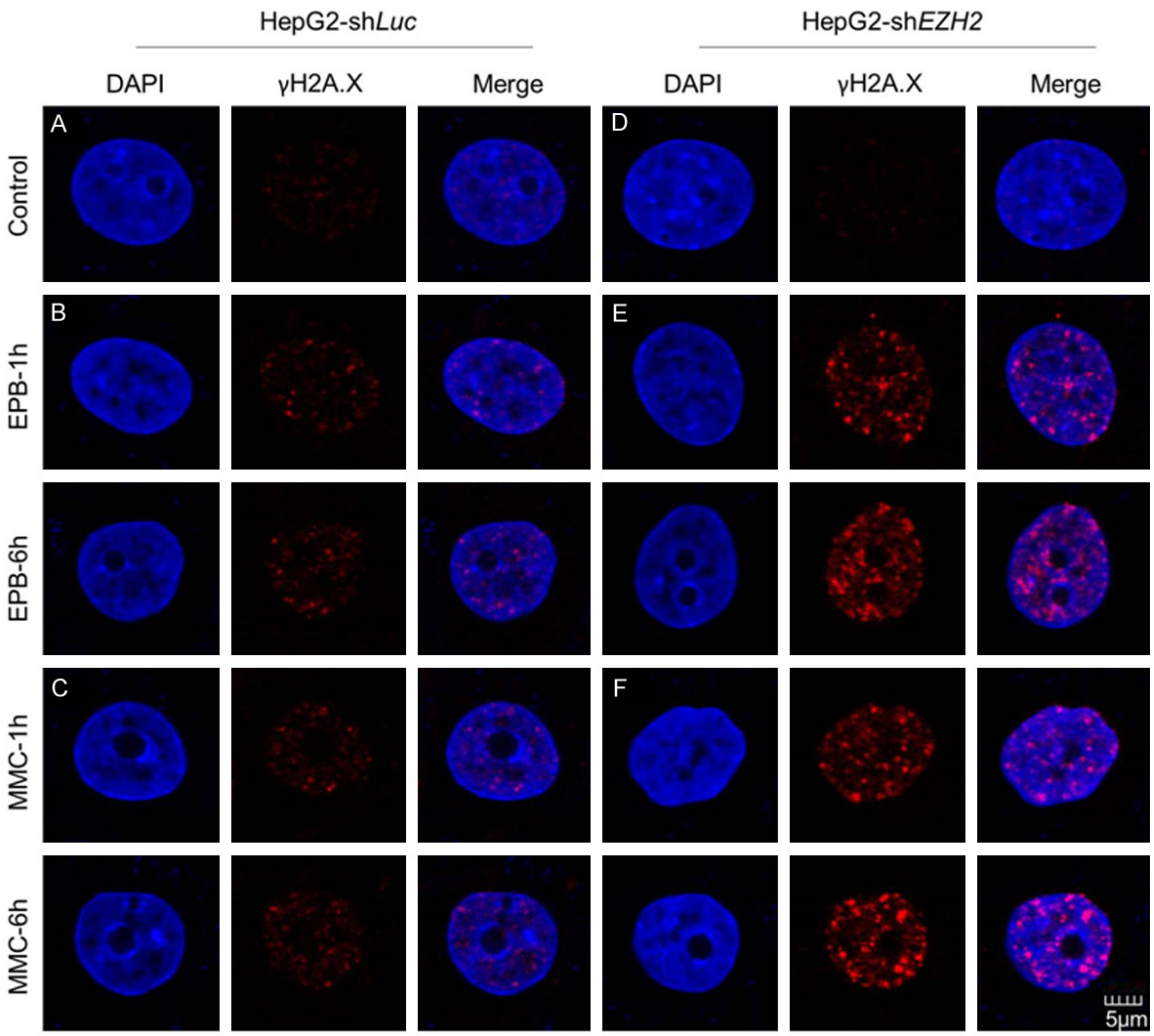

Figure 3. Knockdown of *EZH2* is involved in DNA damage and leads to increased chemosensitivity. Serum-starved shLuc- or shEZH2-transfected HepG2 cells were treated with 0.1 μg/mL EPB or 1.0 μg/mL MMC and harvested at the indicated time points. Nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI; color-inverted to blue), and γH2A.X is shown in red; images were captured by immunofluorescence (IF), 1600-fold. Scale bar, 5 μm.

were also dramatically stimulated in HepG2 cells with PcG silencing (**Figure 4A** and **4B**). DNA lesions, especially DSBs, activate the ATM-p53 and ATM-Chk2 pathways to induce cell cycle arrest and apoptosis [23]. Interestingly, we found that KD of either *EZH2* or *SUZ12* modestly increased mRNA levels of *ATM* (**Figure 4C** and **4D**). Furthermore, reduction of *EZH2*, *BMI1*, and *CBX8* by shRNA effectively increased expression of wild-type and phosphorylated ATM, which is associated with activation of p53 expression (**Figure 4E**). These results indicate that PcG function in the DNA damage response occurs at least in part via regulation of the ATM/p53 pathway in *p53*-wild-type HCC.

The ATM/p53 pathway is epigenetically regulated by PcG in HCC

ATM, one of the most important factors in the DNA damage response, is immediately activated by DSBs via autophosphorylation at serine-1981 (ATM-pS1981). However, transcriptional regulation of ATM by certain mediators in HCC has not been fully characterized. Thus, we further explored the mechanism of transcriptional regulation of ATM by PcG. As important epigenetic regulators, PcG proteins regulate target gene transcription through H3K27 remodeling at specific promoter loci [16]. Interestingly, in our previous ChIP-on-chip

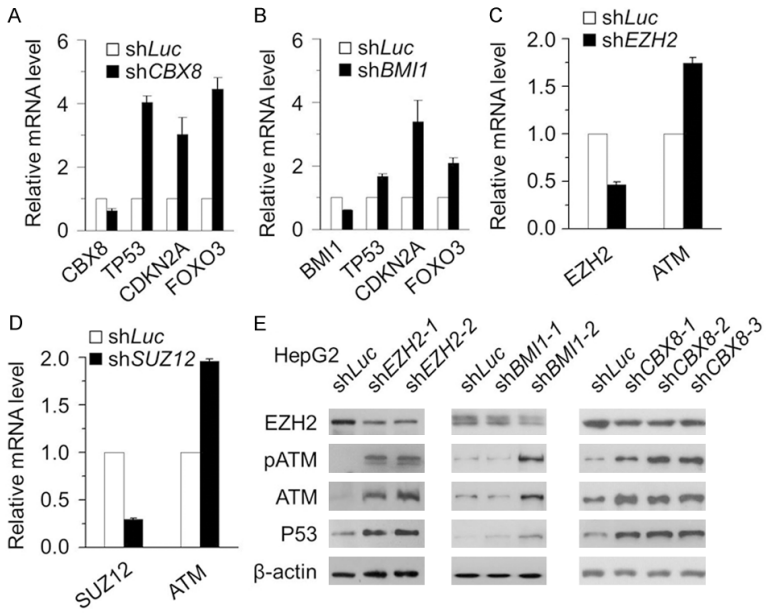

Figure 4. Interfering with PcG activates the p53/ATM pathway. A, B. mRNA levels of TP53, CDKN2A and FOXO3 were measured by quantitative reverse transcription-polymerase chain reaction (qRT-PCR) in HepG2 cells transfected with shLuc, shCBX8, or shBMI1. C, D. mRNA levels of *ATM* were measured by qRT-PCR in shLuc, shEZH2 or shSUZ12 HepG2 cells. E. Total ATM, p-ATM, and p53 levels were detected by western blotting in shLuc, shCBX8, shBMI1, shEZH2 or shSUZ12 HepG2 cells.

assay (GSE52300) [17], we found EZH2 and SUZ12 to be present at *ATM* promoter loci (Supplementary Figure 3). To elucidate the mechanistic importance of PcG in regulating ATM expression, we performed ChIP assays using six distinct pairs of ChIP primers for the *ATM* promoter (**Figure 5A**). Using an H3K27me3-specific antibody, we demonstrated that H3K27me3 was present at the *ATM* promoter in HepG2 cells (**Figure 5B**). Importantly, KD of *EZH2* by shRNA led to a significant decrease in the level of H3K27me3 at the *ATM* promoter (**Figure 5B**). Next, we treated HepG2 cells with 5 μM GSK126 (a potent, highly selective S-adenosyl-methionine-competitive, small molecule inhibitor of EZH2 methyltransferase activity). ChIP assays showed H3K27me3 at the *ATM* promoter, the levels of which were modestly reduced by GSK126 at days 3 post-treatment (**Figure 5C**). Therefore, we conclude that *ATM* transcription is directly regulated by EZH2-mediated H3K27me3 modification in HCC cells.

Targeting PcG increases the chemosensitivity of HCC cells

To further evaluate the biological importance of PcG in the DNA damage response, the combination of a PcG inhibitor and certain chemicals was applied. First, HepG2 cells were treated with KU55933, a potent inhibitor of ATM, GSK126 or MMC, for 12 hours. As expected, exposure to MMC or KU55933 notably increased γH2A.X foci in HepG2 cells (**Figure 6A**). Importantly, formation of γH-2A.X foci was also strikingly increased by GSK126 treatment in HepG2 cells (**Figure 6A**), suggesting that specifically targeting global H3K-27me3 by GSK126 substantially provoked the DNA damage response in HCC cells. To confirm whether GSK126 effectively increased sensitivity cytotoxins, HCC cells were treated with GSK126 and/or chemotherapeutic drugs. CCK8 assays indicated that GSK126 treatment strikingly increased the sensitivity of HepG2 cells to EPB or MMC (**Figure 6B** and **6C**, Supplementary Figure 4A and 4B). These results indicate that GSK126 directly induces cytotoxic effects in HCC cells and further increases the sensitivity of tumor cells to chemotherapeutic drugs. A similar tendency was also found with *EZH2* KD in HepG2 cells treated with EPB and MMC (**Figure 6F** and **6G**). In contrast, no obvious synergistic effect was found with PLC/PRF/5 cell exposure to GSK126 combined with chemotherapy (**Figure 6D** and **6E**, Supplementary Figure 4C and 4D). These findings further demonstrate that PcG promotes DNA damage in a manner that is dependent on the p53 pathway.

Discussion

The present study confirmed the biological and mechanistic importance of PcG in regulating the DNA damage response in HCC. Here, we show that *EZH2* shRNA or specific inhibition of chromatin H3K27me3 by GSK126 significantly promoted EPB- or MMC-induced DNA damage, resulting in elevated sensitivity to chemotherapy. Mechanistically, *ATM* promoter loci were occupied by H3K27me3 in response to PcG, which led to gene silencing. Furthermore, EPB or MMC significantly inhibited expression of

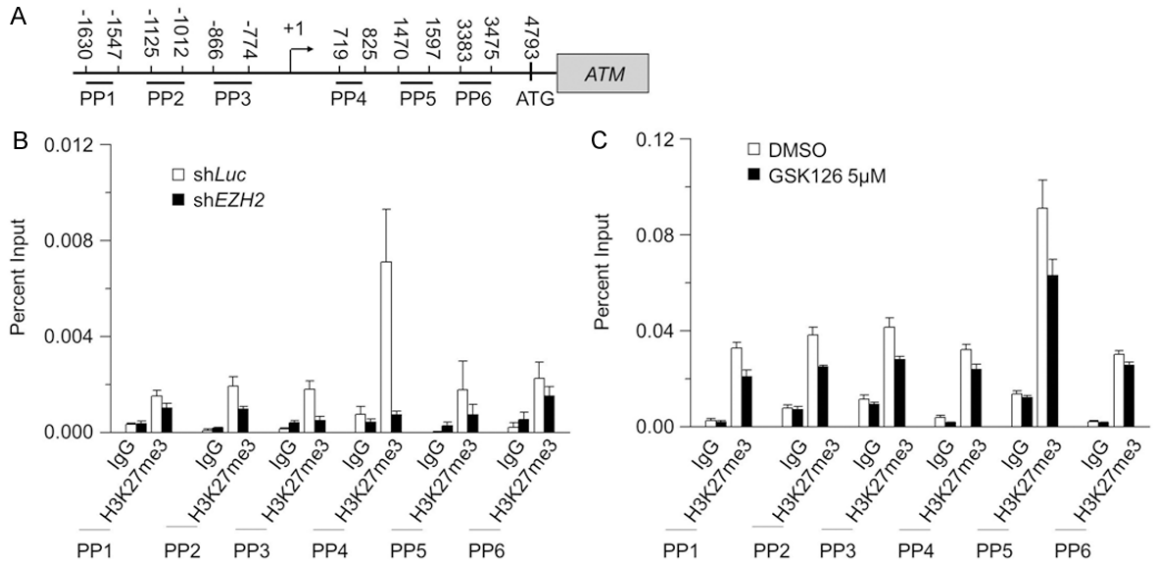

Figure 5. PcG epigenetically regulates ATM in HCC cells. A. A schematic representation of human ATM and primer pairs (PPs) used for ChIP assays. B. ChIP assays were performed using an antibody against H3K27me3 in HepG2 cells carrying shLuc and shEZH2. C. ChIP assays were performed using an antibody against H3K27me3 in HepG2 cells treated with 5 μM GSK126. Rabbit IgG served as a negative control.

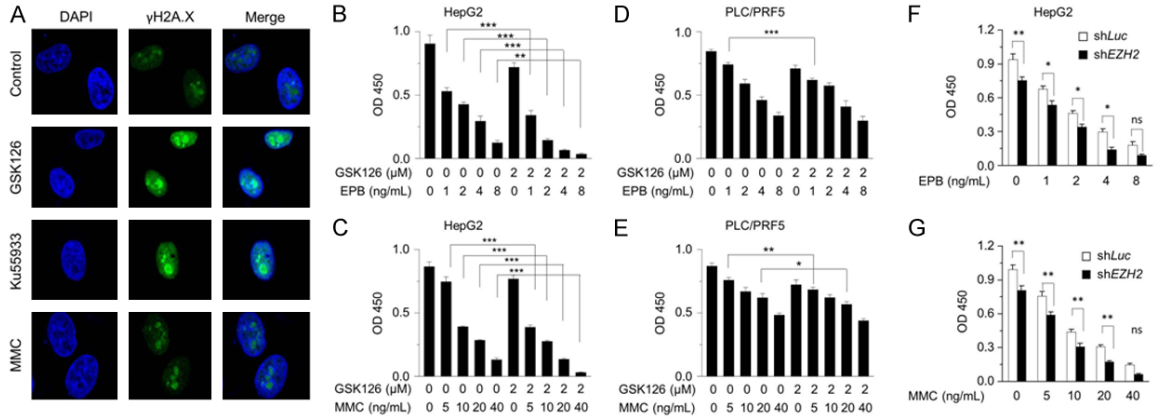

Figure 6. The EZH2 inhibitor GSK126, combined with chemotherapy, further inhibits proliferation of HepG2 cells and increases chemosensitivity in a p53-dependent manner. A. Serum-starved HepG2 cells were treated with 10 μM GSK126, 10 μM KU55933 or 1.0 μg/mL MMC for 12 hours. DAPI (blue), γ-H2A.X (green), and merged images were detected by IF, 1600-fold. B-E. Subconfluent cultures of HepG2, Hep3B and PLC/PRF/5 cells were co-treated with increasing concentrations of GSK126 and EPB or MMC, and cell proliferation was measured by the cholecystokinin-8 (CCK8) assay. The values are the means of three independent experiments. F and G. Serum-starved shLuc or shEZH2 HepG2 cells were treated with 0.1 μg/mL EPB or 1.0 μg/mL MMC, and cell proliferation was measured by the CCK8 assay. The values are the means of three independent experiments.

PcG family proteins in *p53*-wild-type HepG2 cells but not in Hep3B or PLC/PRF5 cells with *p53* deletion or mutation. These findings suggest that the cytotoxicity of chemotherapeutic drugs is mediated at least in part through PcG regulation, which is dependent on wild-type p53. In other words, targeting PcG could be an effective strategy for increasing the sensitivity of cancer cells through the ATM/p53 pathway.

Mutation of *EZH2* in *Caenorhabditis elegans* and deletion of PcG proteins in mammals results in a DNA damage-sensitive phenotype [24]. ChIP sequencing data demonstrate that

EZH2 is present at the promoters of DNA damage-response genes [25]. These findings highlight an interesting mechanism between DNA damage and PcG. As one of the most important effectors in response to DNA damage and epigenetic regulators, PcG-mediated regulation of p53 has drawn much attention. It has been reported that PcG represses p53 expression in human HCC cells, and it was reported that an increased level of PcG correlates with down-regulation of p53 in multiple tumors, including HCC [16, 26-28]. However, the mechanism underlying this correlation remains largely unknown. Recently, it was reported that as a key negative regulator of the tumor suppressor p53, Mouse double minute 2 homolog (Mdm2), is recruited to target gene promoters by EZH2 and enhances PcG-dependent repressive chromatin modifications. Mdm2 also cooperates in gene repression with the PRC1 protein RING1B, an H2AK119 ubiquitin ligase [29]. Moreover, as components of PRC1, it was found that BMI1 and RNF2/RING1B heterodimerize via their N-terminal RING domains to form an active E3 ubiquitin ligase that targets p53 for degradation. Unlike Mdm2, this RNF2/RING1B E3 ligase only degrades p53 in selective cell lines, such as those from germ-cell tumors, including HCT116 cells [30]. These findings could be used to partially interpret the observed suppressive effect of PcG on p53. Nonetheless, it is unclear whether p53 is required for PcG-mediated suppression. Indeed, it has been reported that p53 recruits both Histone deacetylases (HDACs) and PcG to the *Arf* locus to repress its expression. Binding of both HDAC and PcG to *Arf* is disrupted by inactivation of p53 and can be restored in *p53*-null mouse embryonic fibroblasts (MEFs) by reintroduction of wild-type but not mutant *p53*. This repression constitutes a second feedback loop in p53 regulation [31]. These results may partially explain why PcG expression is inhibited by chemotherapeutic agents in *p53*-wild-type HCC cells. Chemotherapy drugs induce the DNA damage response by activating the p53 pathway, and increasing p53 further inhibits expression of PcG.

Of note, expression of p53 upon DNA damage can be repressed by PcG in an H3K27me3-independent manner [16], whereas activation of ATM appears to occur in an H3K27me3-dependent manner. In the present study, ChIP assays confirmed that PcG bound to the *ATM* promoter with enrichment of H3K27me3 modification in HepG2 cells. These results suggest that transcription of *ATM* is directly regulated by EZH2-mediated H3K27me3 and further regulates expression of p53 in HCC cells. From this perspective, ATM might act as the mediator or bridge for this indirect regulation between PcG and p53 upon DNA damage in HCC cells. Moreover, this is the first report of epigenetic regulation of ATM by PcG, which could provide a promising strategy for designing and applying novel chemotherapy treatment.

Although the exact mechanism of anticancer drug resistance remains unknown, the strong DNA damage repair capability of cancer cells might be one explanation. Histone methylation is considered a pivotal characteristic in the choice of DNA damage repair pathway, therefore it might allow for the discovery and pharmacological intervention of cancer treatment. To date, a number of small molecule inhibitors targeting histone methyltransferases and demethylases have reached the first stages of clinical trials in cancer treatment [32, 33]. Moreover, several clinical trials of inhibitors targeting histone methylation (DOT1L, EZH2, G9a, SMYD2, LSD1, KDM5B, KDM6B and IDH inhibitors) in leukemia, non-Hodgkin's lymphoma (NHL) and solid tumors are already completed or ongoing [32-34]. Regarding the EZH2 inhibitor EPZ-6438 (tazemetostat), the first part of its clinical phase 1/2 trial in patients with advanced solid tumors or with relapsed or refractory B cell lymphoma has already been completed, with both favorable safety and tolerability (ClinicalTrials.gov identifier: NCT01897571). In addition, nine of the fifteen NHL patients in the group achieved an objective response, with the majority of adverse events evaluated at grade 1 or 2 [32]. Interestingly, preclinical data indicate synergism between EPZ-6438 and R-CHOP (rituximab, cyclophosphamide, doxorubicin, vincristine, and prednisone) (Epizyme, International Conference on Malignant Lymphoma (ICML), Recap Presentation June 22, 2015). In our study, combining *EZH2* shRNA or GSK126 effectively enhanced the sensitivity of HCC cells to chemotherapeutic drugs in the MMC or EPB treatment group, respectively. Our results suggest that GSK126 directly inhibits the growth of *p53*-wild-type HCC cells and increases the susceptibility of tumor cells to

chemotherapy drugs. Hence, our findings indicate that small molecule inhibitors targeting PcG might serve as sensitizers for *p53*-wild-type HCC chemotherapy. Indeed, targeting PcG could provide a promising method to prevent further tumor occurrence by this cocktail-type chemotherapy through regulation of the ATM/p53 pathway.

Methylation and other modifications of different histones and their performances are not individual but connected, and such factors have been indicated as acting in concert in a context-dependent manner in the DNA damage response. Therefore, targeting multiple histone modifiers with chemotherapy might be a promising future strategy for cancer therapy.

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Disclosure of conflict of interest

None.

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Supplementary material and methods

Cell culture

The human HCC cell lines HepG2 (*p53* wild-type) and Hep3B (*p53* null) were obtained from Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). PLC/PRF5 (human HCC with *p53* mutation) cells were obtained from China Center for Type Culture Collection (Wuhan, China). Cell culturing was performed as previously described [1, 2]. Briefly, cells were cultured in DMEM (Gibco), RPMI-1640 medium (Gibco), or F12 (Gibco) supplemented with 10% fetal bovine serum (FBS; HyClone) and 1% penicillin-streptomycin.

Western blotting and immunofluorescence

Western blotting and immunofluorescence (IF) were performed as previously described [3].

Real-time qRT-PCR

qRT-PCR was performed as previously described [3] using an ABI Step One detection system with the primers listed in Supplementary Table 2. qRT-PCR reactions were performed using SYBR Green (Roche Applied Science) technology and repeated at least three times.

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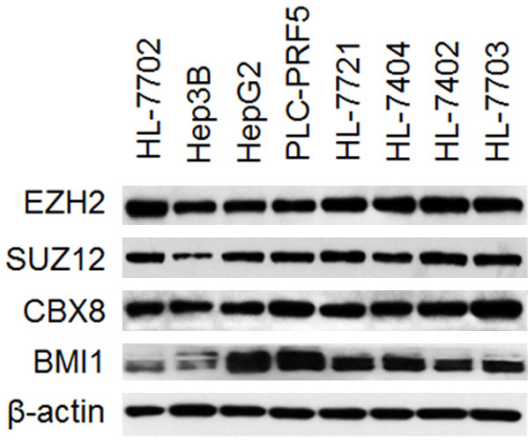
Supplementary Table 1. The list of primers used in the study

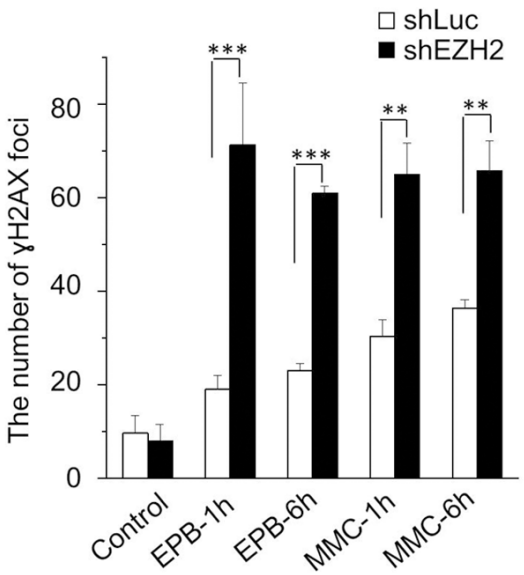
qRT-PCR Primers		
Gene name	Forward primer sequence (5' to 3')	Reverse primer sequence (5' to 3')
TP53	GCCGTCCCAAGCAATGGATGATTT	TCTGGCATTCTGGGAGCTTCATCT
EZH2	ACATGCGACTGAGACAGCTCAAGA	AGGATGTGCACAGGCTGTATCCTT
SUZ12	TTTCTCAGGGACCTACGTTGCAGT	AGGTTTGGCAATAGGAGCCGTAGA
BMI1	GCTGATGCTGCCAATGGCTCTAAT	TGCTGCTGGGCATCGTAAGTATCT
CBX8	TCGCAGAAGTACAGCACATGGGAA	TGGCTGAGTCACTTCGAAACTCGT
CDKN2A	ACCAGAGGCAGTAACCATGCC	CATGCCTGCTTCTACAAACCCACA
FOXO3	ACTCACTTAGCCACAGCGATGTCA	TGACCAAACTTCCCTGGTTAGGCT
ATM	AACTGTGAGCTGTCTCCATTAC	CTTCCGTAAGGCATCGTAACA
ChIP Primers		
Target name	Forward primer sequence (5' to 3')	Reverse primer sequence (5' to 3')
ATM PP1	GAACGGGTGTCCTACTGTAATC	GAGCTATCCTGTGCTCAAACA
ATM PP2	TTTGAGCTTTACCTGGCTCTC	ACTTGTCTGTCAGGGTGTATTG
ATM PP3	CTCTACACTGGACGACGTATTG	CCTTCTAATAACCCGCCCTTAT
ATM PP4	GCTTGGGCTCTGGAATCATA	GCTCACCACAGGTCAAGATTA
ATM PP5	TGCCAAGGGCAGAGTTATTT	CTTCCTACGAGCCTCTGAATTG
ATM PP6	TGAGGCTTGGTGTACGAATG	TTGGAAAGAAAGGGAGGTATGG

Supplementary Table 2. The list of reagents used in the study

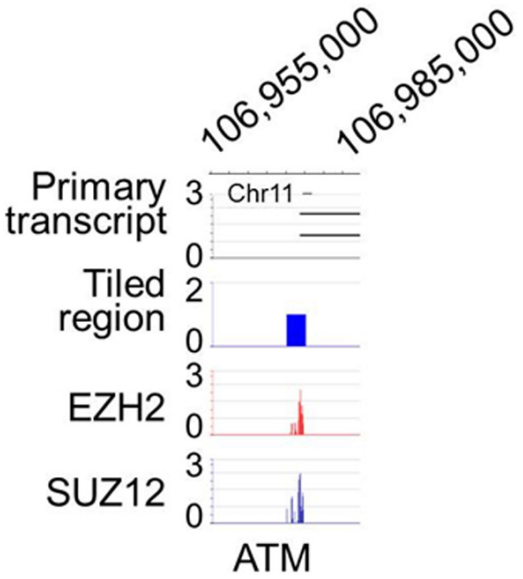
Antibodies			
Western blotting, IF			
Antigen	Host	Company	Cat#/code
EZH2	Rabbit	Cell signaling	#5246
CBX8	Rabbit	Sigma	R30545
SUZ12	Rabbit	Cell signaling	#3737
BMI1	Rabbit	Epitomics	#5590-1
P53	Mouse	Santa Cruz	sc-126
β-actin	Mouse	Santa Cruz	sc-47778
GAPDH	Rabbit	EPITOMICS	2251-1
p-H_2A(Ser139)	Mouse	Millipore	#05-636-1
P-ATM(S1981)	Rabbit	Abcam	Ab81292
p-H_2A(Ser139)	Rabbit	Cell signaling	#2577
ChIP			
H3K27me3	Rabbit	Millipore	#17-622
Small molecular inhibitors			
Name	Company		Cat#/code
GSK126	MedChem Express		HY-13470
KU-55933	Selleck		587871-26-9
MMC	Hisun Pharmaceutical		
EPB	Hisun Pharmaceutical		



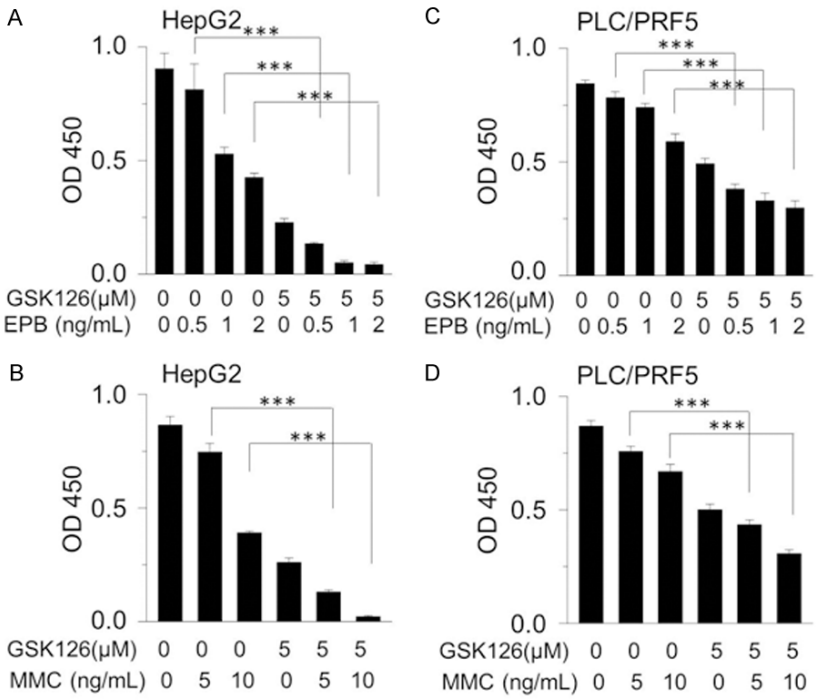
Supplementary Figure 1. Expression of PcG was analyzed in human hepatocellular carcinoma (HCC) cell lines by western blotting.



Supplementary Figure 2. An average (%) of DNA damaged (γH2AX-positive) cells was determined, and the results are shown in the diagram. Three biological replicates were performed; the error bars represent standard deviation, and the paired t-test was used. Significant *P* values between the EZH2 shRNA group and the control group when treated with chemotherapy drugs are highlighted.



Supplementary Figure 3. Genome-wide ChIP-on-chip assays using antibodies against EZH2 and SUZ12 in HepG2 cells, with IgG-ChIP as the negative control. An expanded view of the *ATM* promoter region used in ChIP-on-chip assays.



Supplementary Figure 4. The effect of simultaneous GSK126 and chemotherapy treatment of HCC cells. (A, C) Subconfluent cultures of HepG2 (A) and PLC/PRF/5 (C) cells were co-treated with GSK126 and increasing concentrations of EPB, and the extent of cell proliferation was measured by the cholecystokinin-8 (CCK8) assay. (B, D) HepG2 (B) and PLC/PRF/5 (D) cells were co-treated with GSK126 and increasing concentrations of MMC, and cell proliferation was measured by the CCK8 assay. The values are the means of three independent experiments.