

Epigenetically Down-Regulated Acetyltransferase PCAF Increases the Resistance of Colorectal Cancer to 5-Fluorouracil¹



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Abstract

Only 10%–20% of colorectal cancer (CRC) patients observe effective responses to 5-fluorouracil (5-FU) based chemotherapy. We used real-time PCR array and Western blot analysis to examine the expression alteration of acetyltransferases and deacetylases in 5-FU resistant CRC cell lines as compared to their respective parental CRC cell lines. Unlike other acetyltransferases and deacetylases, we found that the expression of acetyltransferase P300/CBP-associated factor (PCAF) is consistently decreased in three 5-FU resistant CRC cell lines. Similarly, knockdown of PCAF in HCT116 CRC parental cell line also increases the resistance to 5-FU and attenuates 5-FU-induced apoptosis. Mechanistically, we demonstrated that increased binding of trimethylated histone H3K27 in the promoter region of PCAF attenuated its transcription in 5-FU resistant HCT116/5-FU cells. Decreased PCAF impairs the acetylation of p53 and attenuates the p53-dependent transcription of p21, which results in the increased cyclin D1 and phosphorylation of Retinoblastoma 1. Conversely, overexpression of PCAF in CRC cell lines increases p21 and their susceptibility to 5-FU *in vitro* and *in vivo*. However, knockdown of p21 abolishes the beneficial effects of PCAF overexpression on increasing the sensitivity of HCT116/5-FU cells to 5-FU. Also, the reduced intensity of PCAF immunostaining was observed in the precancerous lesion, and microarray data from the public database further demonstrated the association between PCAF down-regulation and poor survival outcome. Our data suggest that PCAF-mediated p53 acetylation is an essential regulatory mechanism for increasing the susceptibility of CRC to 5-FU.

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Introduction

Colorectal cancer (CRC) is one of the most frequent malignant neoplasms worldwide, being the second most common in males and the third in females, and ranking the fourth and third leading causes of cancer deaths among males and females, respectively [1]. Although more and more patients are diagnosed at earlier stages and the overall survival rate of CRC is increased slightly in recent years, about 50% of the CRC patients will ultimately progress to metastatic colorectal cancer (mCRC) [2].

5-fluorouracil (5-FU) is one of the first-line chemotherapy agents for CRC and inoperable mCRC [3,4]. Unfortunately, only 10% to 20% of CRC patients observed effective responses to 5-FU-based chemo-treatment [5,6], and patients who initially responded to 5-FU eventually become resistant [7]. Although several tumor markers for 5-FU-based chemotherapy responses have been suggested, such as p53, thymidylate synthase, dihydropyrimidine dehydrogenase, and thymidine phosphorylase, no consistently valid predictors of drug response in CRC have been established to date [8]. Therefore, it is compelling to reveal the mechanism of 5-FU resistance, which is essential for identifying novel tumor markers and developing target therapies. Recently, several studies suggested that 5-FU resistance may be reversed by increased histone acetylation, resulting in an improved therapeutic outcome in CRC [9]. 5-FU may affect the acetylation of histone or non-histone proteins either by regulating the expression of acetyltransferases or deacetylases or by influencing their activities [10,11].

Histone acetylation is required for regulating gene transcription by promoting or repressing DNA replication activity [12,13]. Acetyltransferases transfer an acetyl moiety to lysine side chains on histone or non-histone proteins and change the stability, localization, and DNA binding capability of acetylated proteins [14]. Conversely, deacetylases remove acetyl groups from lysine residues and thereby not only regulate transcription but also affect other types of post-translational modifications (PTM) such as methylation, ubiquitination and sumoylation [15]. There are two major deacetylase families, histone deacetylase (HDACs) and NAD-dependent protein deacetylases (Sirtuins) [16,17].

PCAF (p300/CBP-associated factor), also named as lysine acetyltransferase 2B (KAT2B), is the first discovered mammalian histone acetyltransferase and belongs to the GNAT (GCN5-related N-acetyltransferase) family. PCAF-mediated acetylation of H3K9, H3K14, and H3K18 facilitates the transcription of downstream genes [18]. PCAF also regulates the acetylation of non-histone proteins, such as p53 [19–23], PTEN [24] and c-Myc [25]. PCAF has been reported to be involved in the tumorigenesis of many types of cancer, but the underlying mechanism by which PCAF regulates the pathogenesis of various tumors is still paradoxical. On the one hand, PCAF promotes hepatocellular carcinoma cell apoptosis and functions as a repressor [26], and PCAF is able to suppress tumorigenicity of gastric cancer cells and inhibits cell cycle [27]. On the other hand, PCAF-primed EZH2 acetylation promotes the progression of lung adenocarcinoma [28], and PCAF-mediated Akt1 acetylation enhances glioblastoma cells proliferation [29]. Still, the biological roles of PCAF in CRC remain unclear.

Here, we found PCAF decreased in 5-FU resistant CRC cell lines and elucidated the roles of 5-FU caused epigenetic changes in modulating the transcription of PCAF. Furthermore, we demonstrated the contribution of decreased PCAF to the resistance of CRC cells to 5-FU through p53-mediated p21 expression mechanism.

Materials and Methods

Cell Culture

SW620, SW48, SW480 were obtained from ATCC. HCT116 p53^{+/+} and p53^{-/-} cells were generously provided by Dr. Bert Vogelstein's laboratory (Johns Hopkins School of Medicine). SW620/5-FU (SW620 5-FU resistant cell line) was generously provided by Dr. Kebin Liu's laboratory (Medical College of Georgia, Augusta University). HCT116/5-FU (HCT116 5-FU resistant cell line) and SW48/5-FU (SW48 5-FU resistant cell line) were derived from their parental HCT116 and SW48 cells by culturing cells with stepwise increasing doses of 5-FU. Cells were grown in Dulbecco's modification of Eagle's medium (DMEM, Life Technologies) with 10% (v/v) fetal bovine serum (Sigma-Aldrich), penicillin (100 U/ml), streptomycin (100 mg/ml). All cell lines were incubated at 37 °C in a humidified atmosphere containing 5% CO₂.

Immunohistochemistry (IHC) Staining and Scoring

Immunohistochemical analysis for PCAF status was performed on tissue microarrays from BioChain Institute using respective antibodies from Abcam (Anti-KAT2B / PCAF antibody, ab12188). The detection system used was 3,30-diaminobenzidine (DAB) from DAKO (Santa Clara, CA). Slides were counterstained using hematoxylin. Quantitative scoring of PCAF immunostaining was performed following previously published methods [30]. The quantitative results show the mean integrated optical density (IOD).

Clonogenic Survival Assay

Cells were transfected with non-silencing (NS) control siRNA, PCAF siRNA, empty vector plasmid or Flag-PCAF plasmid DNA. After 24 h transfection, 1000 cells were seeded in triplicate into a 60 mm culture dish. Then, cells were cultured in medium in the absence or presence of 5-FU at the indicated doses for 48 h. After a 2-week culture, the cell colonies were washed three times with phosphate buffered saline buffer (PBS), fixed in cold methanol for 15 min, and stained with Crystal Violet (Sigma-Aldrich, St. Louis, MO, USA) for 15 min at room temperature.

Cell Viability Assay

Cell viability was determined by CCK-8 assay according to the manufacturer's instructions. Cells were seeded in 96-well plates at 5000 cells/well. Then, cells were transfected with indicated siRNA or plasmid DNA. After 48 h treatment with 5-FU (2 µg/ml), the cells were incubated in 100 µl of medium and 10 µl of CCK-8 reagent for 2 h at 37 °C. The optical density was measured at 450 nm. Three replicates were prepared for each condition.

Cell Cycle Analysis

Cells cultured in 60 mm culture dishes were transfected with non-silencing (NS) control siRNA and PCAF siRNA. After 24 h transfection, cells were harvested by trypsinization, and fixed overnight with 70% cold ethanol at -20 °C. Fixed cells were stained with Propidium Iodide (PI)/RNase Staining Solution (#4087, Cell signaling technology, Danvers, MA) at room temperature in the dark for 30 min. BD LSRII flow cytometer was used to detect cell cycle. FlowJo software was used to calculate the percentages of cells in each cycle phase.

AO/EB Staining

Cells cultured in 24-well plates were transfected with non-silencing (NS) control siRNA or PCAF siRNA. After 24 h transfection, cells were treated with DMSO or 5-FU for 48 h. Then the cells were stained with acridine orange (AO, 100 mg/mL) and ethidium bromide (EB, 100 mg/mL). Images were taken with OLYMPUS fluorescence microscope immediately. The normal and early apoptotic cells were stained by AO to display bright green fluorescence, while the late apoptotic cells were stained by EB to display orange fluorescence.

Annexin V-FITC/ Propidium Iodide (PI) Staining

Cells cultured in 60 mm culture dishes were transfected with non-silencing (NS) control siRNA or PCAF siRNA. After 24 h transfection, cells were treated with DMSO or 5-FU for 48 h, and then harvested and stained with Annexin V-FITC/PI apoptosis kit (BD Biosciences) at room temperature in the dark for 15 min. BD LSRII flow cytometer was used to quantify the percentage of cells undergoing apoptosis. The total number of cells in the Q2 and Q4 quadrant was regarded as apoptotic cells.

Nuclear Protein Isolation

Nuclear proteins were extracted using a nuclear extraction kit (#10009277, Cayman chemical). Cells were washed twice with cold PBS and harvested in 500 μ l complete hypotonic buffer. After incubation for 15 min on ice, 100 μ l 10% NP-40 was added to the suspension. Supernatant was removed after centrifuging the suspension for 30s at $14,000 \times g$ at 4 °C. The pellet was re-suspended in 100 μ l complete nuclear extraction buffer and kept on ice for 15 min with vigorous 15 s vortex twice. The mixture was spun again for 10 min at $14,000 \times g$ at 4 °C. The supernatant was collected as nuclear extract and kept at -80 °C for analysis.

Real-Time Quantitative PCR (qPCR)

Total RNA was extracted using an RNeasy kit (QIAGEN). Using iScript cDNA synthesis kit (Bio-Rad, Los Angeles, CA), the cDNA was reverse-transcribed from 2 μ g total RNA. Real-time PCR was performed with Bio-Rad MyiQ detection system, using iTaq Universal SYBR Green Supermix (Bio-Rad, Los Angeles, CA). The relative mRNA expression was normalized to the corresponding *actin* mRNA levels. The sequences of real-time PCR primers were described in supplementary material.

Western Blot Analysis and Immunoprecipitation

Western blotting was performed per our previous publication [31]. All commercial antibodies are listed in supplementary material. For immunoprecipitation, 5 μ l p53 antibody (#GTx70214, GeneTex) per ml was added to cell lysate and was incubated overnight at 4 °C. Protein G PLUS-Agarose beads (#sc-2002, Santa Cruz Biotechnology) were then added and incubated for another 2 h. Then, the beads were extensively washed with lysis buffer and eluted with SDS loading buffer by boiling for 5 min, followed by Western blot analysis.

Chromatin Immunoprecipitation (ChIP)

ChIP assays were performed using a SimpleChIP Plus Enzymatic Chromatin IP Kit (Magnetic Beads) (#9005, Cell signaling technology, Danvers, MA). After being transfected with NS or PCAF siRNA for 24 h, cells were treated with 5-FU. DNA-p53 complexes or DNA-Acetyl-

H3 complexes were immunoprecipitated overnight using their respective antibodies, p53 or acetyl-H3 antibodies. The purified DNA was subjected to real-time quantitative PCR with iTaq Universal SYBR Green Supermix (Bio-Rad, Los Angeles, CA).

Animal Studies

The female nu/nu mice (6 weeks old) were purchased from Jackson Laboratory and all animal experiments were maintained in animal facility at the Medical College of Wisconsin. Mice were randomly divided into 2 different groups. HCT116 cells stably expressing Flag-PCAF or empty control vector (5×10^6 in 100 μ l PBS) were inoculated subcutaneously into the oxters of the nude mice, respectively. When the tumor size reached 100 mm³ at Day 10, 5-FU at the dose of 30 mg/kg was i.p. administrated three times per week. Tumors were measured with a caliper every 4 day, and the tumor volume was calculated using the formula $V = 1/2 (\text{width}^2 \times \text{length})$. At Day 26, all mice were sacrificed and the total weight of the tumors in each mouse was measured. Tumor specimens were harvested for IHC staining and western blot analysis. All of the animal experiments were approved by the Institutional Animal Care Use Committee of the Medical College of Wisconsin. Animal care was in accordance with institution guidelines.

Statistical Analysis

Data were analyzed by s SPSS 19.0 statistical software. The statistical significance of quantitative assays was analyzed using either two-tailed Student t-test or ANOVA analysis for more than two groups. A *P*-value <0.05 was considered statistically significant. Data are presented as mean \pm SD.

Results

PCAF is Down-Regulated in 5-FU Resistant CRC Cell Lines

To determine the underlying mechanism by which the acetylation of histone and non-histone proteins affects the efficacy of 5-FU has not been elucidated, we examined the alteration of acetyltransferases and deacetylases in 5-FU resistant CRC cell lines. Following Dr. Liu's protocol for establishing the 5-FU resistant SW620 cell line (SW620/5-FU) [32], we established 5-FU resistant HCT116 and SW48 cell lines by a step-wise increased dose of 5-FU in the culture media. Like SW620/5-FU resistant cells from Dr. Liu's laboratory, both HCT116/5-FU and SW48/5-FU cells can survive better at the treatment of 10 μ g/mL 5-FU than their respective parental cells (Figure S1A). The 5-FU resistant capability of HCT116/5-Fu cells was further determined by colony formation assay as shown in Figure 1A. The HCT116/5-FU can survive well under 10 μ g/mL 5-FU. First, we used real-time PCR array to examine the expression of well-characterized acetyltransferases and deacetylases in HCT116 and HCT116/5-FU cells. Interestingly, our data showed that only one acetyltransferase, PCAF, and two deacetylases, HDAC9 and Sirt 3, mRNA levels decreased in 5-FU resistant HCT116/5-FU cells (Figure 1B). The protein levels of PCAF, HDAC9 and Sirt 3 in HCT116 and HCT116/5-FU cells were determined by Western blot analysis. As shown in Figure 1C, PCAF protein levels also decreased in 5-FU resistant HCT116/5-FU cells as compared to the parental HCT116 cells. Consistently, we appreciate the mRNA and protein levels of PCAF also decreased in other two 5-FU resistant CRC cell lines (SW48/5-FU and SW620/5-FU) as compared to their respective parental CRC cells (SW48 and SW620) (Figure S1B/S1C/S1D). However, we did not

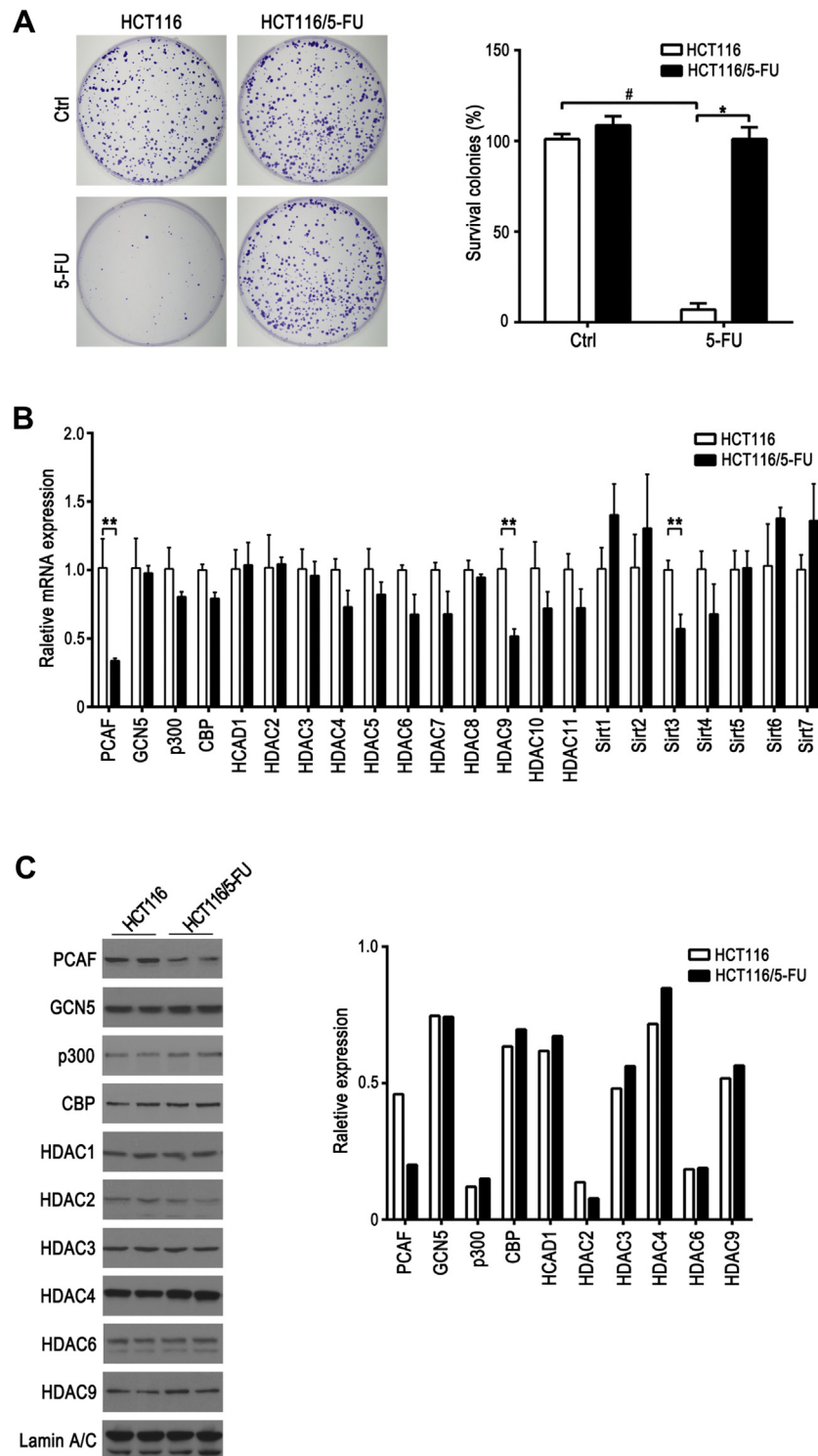


Figure 1. PCAF expression is down-regulated in 5-FU resistant cell lines. **(A)** The 5-FU resistant phenotype of HCT116/5-FU cells was determined by clonogenic survival assay. HCT116 and HCT116/5-FU cells were seeded at 1000 cells/per well and treated with 5-FU (10 μ g/mL) for 48 h. After a 2-week culture, the cell colonies were visualized by Crystal Violet staining (left panel). The quantitative results show the average percentage of surviving colonies (right panel), *: $P < .05$ vs. HCT116, $n = 3$. **(B)** mRNA levels of HATs, HDACs and Sirtuin family in HCT116 and HCT116/5-FU cells were detected by RT-qPCR. The data are means \pm SD of three independent assays, *: $P < .05$ vs. HCT116, $n = 3$. **(C)** PCAF protein level decreased in 5-FU resistant HCT116/5-FU cells (left panel). Nuclear proteins extracted from HCT116 and HCT116/5-FU cells were determined by Western blot analysis. Quantitative analysis of protein level changes in HCT116 and HCT116/5-FU cells by measuring the intensity of western blot band (right panel, $n = 2$).

appreciate the apparently decreased protein levels of HDAC9 and Sirt3 in HCT116/5-FU (Figure 1C, Figure S2) as well as in SW48/5-FU and SW620/5-FU cells (Figure S1C/1D and Figure S2). Also, we did not

observe the consistent alteration of other acetyltransferases (GCN5, p300, CBP) and deacetylases in these three 5-FU resistant cell lines (Figure 1C, Figure S1C, Figure S1D, Figure S2). These findings suggest that PCAF is

a potential acetyltransferase responsible to the resistance of CRC cell line to 5-FU.

Down-regulation of PCAF Transcription in 5-FU Resistant Cells is Dependent on Trimethylation of Histone 3

In contrast, we observed the increase of PCAF in CRC cell lines transiently treated with 5-FU for 24 hours (Figure S3). To further determine the different response of CRC cell lines to the transient and prolonged treatment of 5-FU, we examined the changes of PCAF

protein levels in a time-course treatment of 5-FU. As shown in Figure S4A, PCAF protein level increased at 24 hr. and decreased after 48 hr. treatment with 5-FU. Like other previous studies showing that 5-FU treatment affects the methylation of histone proteins in tumor cells [33,34], the levels of H3K27me3 also increased after 48 hr. treatment with 5-FU (Figure S4A). Consistently, the levels of H3K27me3 increased in the 5-FU-resistant HCT116/5-FU cells as compared to the parental HCT116 (Figure S4B). The binding of H3K27me3 at the promoter region of targeting genes attenuates their transcription

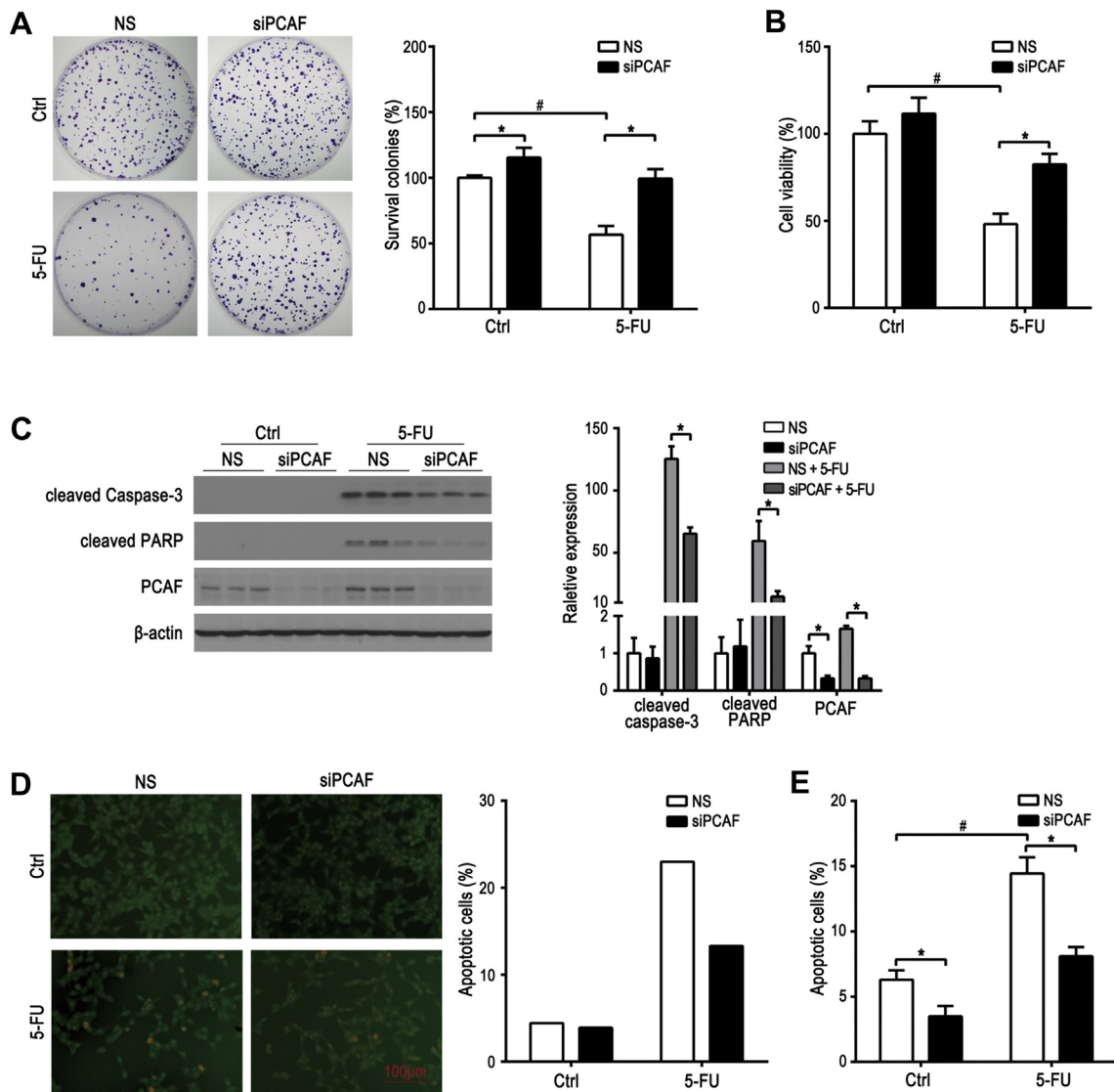


Figure 2. PCAF knockdown enhances the chemoresistance of HCT116 cells to 5-FU. **(A)** Knockdown of PCAF increases the clonogenicity of HCT116 cells. Clonogenic formation assay was used for determining the clonogenicity of HCT116 cells treated with 5-FU (1 $\mu\text{g}/\text{mL}$) (left panel). The quantitative results show the average percentage of surviving colonies (right panel). **(B)** PCAF knockdown increases chemoresistance of HCT116 cells to 5-FU. Cell viability of HCT116 cells treated with 5-FU (2 $\mu\text{g}/\text{mL}$) for 48 h was determined by CCK8 assay. **(C)** PCAF knockdown decreases the amount of cleaved caspase-3 and cleaved PARP induced by 5-FU (5 $\mu\text{g}/\text{mL}$) treatment (left panel). Quantitative analysis of protein level changes in HCT116 cells by measuring the intensity of western blot band (right panel). The data are means \pm SD of three independent assays, *: $P < .05$ vs. NS, #: $P < .05$ vs. Ctrl, n = 3. **(D)** PCAF knockdown reduces apoptosis of HCT116 cells induced by 5-FU. AO/EB staining was used for measuring apoptotic cell population in HCT116 cells treated with 5-FU (5 $\mu\text{g}/\text{mL}$) (left panel). The quantitative results show the average percentage of apoptotic cells from 3 images taken from each group (right panel). **(E)** PCAF knockdown attenuated the 5-FU-induced apoptosis of HCT116 cells. Annexin V-PI dual staining-based flow cytometry assay was used for measuring apoptotic cell population in HCT116 cells treated with 5-FU (5 $\mu\text{g}/\text{mL}$). The total number of cells in the Q2 and Q4 quadrant was regarded as apoptotic cells. Percentages of apoptotic cells are shown in the bar graph. The data are means \pm SEM of three independent assays. *: $P < .05$ vs. NS. #: $P < .05$ vs. Ctrl. (n = 3).

activity [35]. Based on the ENCODE database, we identified several binding sites of H3K27me3 at the promoter region of PCAF (Figure S4C). To test the hypothesis that 5-FU treatment affects the transcription of PCAF due to the alteration of trimethyl H3K27 (H3K27me3), we performed the chromatin immunoprecipitation (ChIP) of H3K27me3 and examined the alteration of their binding to the promoter region of PCAF by quantitative PCR (qPCR). The ChIP-qPCR results showed the significantly increased binding of H3K27me3 to two different promoter regions (-770- -652 and -529- -429) of PCAF in the HCT116/5-FU cells (Figure S4D). It indicates the 5-FU-caused histone trimethylation in CRC cell lines contributes to the alteration of PCAF in response to 5-FU treatment. The mechanism by which 5-FU affects the trimethylation of histone proteins is beyond the scope of this report and needs further investigation.

Knockdown of PCAF Increases the Resistance of HCT116 Cells to 5-FU

To determine the contribution of PCAF to the resistance of CRC to 5-FU, we first knocked down PCAF in HCT116 cells using siRNA targeting PCAF (siPCAF). The clonogenic survival assay was performed to determine the cytotoxicity of 5-FU. Cells were treated with 5-FU for 48 h, and cell colonies were counted 2 weeks later. As shown in Figure 2A, 5-FU treatment reduced the colony size and number of HCT116 cells transfected with non-silencing control siRNA (NS) as compared to the non-treatment group (Ctrl). However, PCAF knockdown increased the colony size and number

of HCT116 cells treated with 5-FU. Similarly, 5-FU treatment reduced the viability of HCT116 cells, but PCAF knockdown increased the viability of 5-FU treated HCT116 cells (Figure 2B). Consistently, 5-FU treatment increased the cleaved caspase-3 and PARP, which are involved in facilitating the apoptosis [36,37]. However, knockdown of PCAF reduced the amount of cleaved caspase-3 and PARP in HCT116 cells (Figure 2C). To determine if PCAF knockdown increases the resistance to 5-FU-induced apoptosis, we first examined the apoptosis of HCT116 cells using AO/EB method, which has validated by others and our previous publications [38,39]. As shown in the representative images of AO/EB staining in Figure 2D, we appreciated that 5-FU induced the apoptosis of HCT116 cells but PCAF knockdown reduced the 5-FU-induced apoptosis in HCT116 cells. We further used the Annexin V-FITC/propidium iodide (PI) staining-based flow cytometry approach to determine cell apoptosis (Figure 2E). Consistently, knockdown of PCAF attenuated the 5-FU-induced apoptosis in HCT116 cells.

Overexpression of PCAF Decreases the Resistance of HCT116 Cells to 5-FU

If loss of PCAF increases the resistance of HCT116 cells to 5-FU, PCAF overexpression should increase the susceptibility of HCT116 cells to 5-FU. To prove this hypothesis, we transfected Flag-tagged PCAF plasmid DNA to HCT116 cells and examined the effects of PCAF overexpression on the capability of colony formation and cell viability. As shown in Figure 3A, PCAF overexpression decreased the capability of colony formation under either no treatment or 5-FU

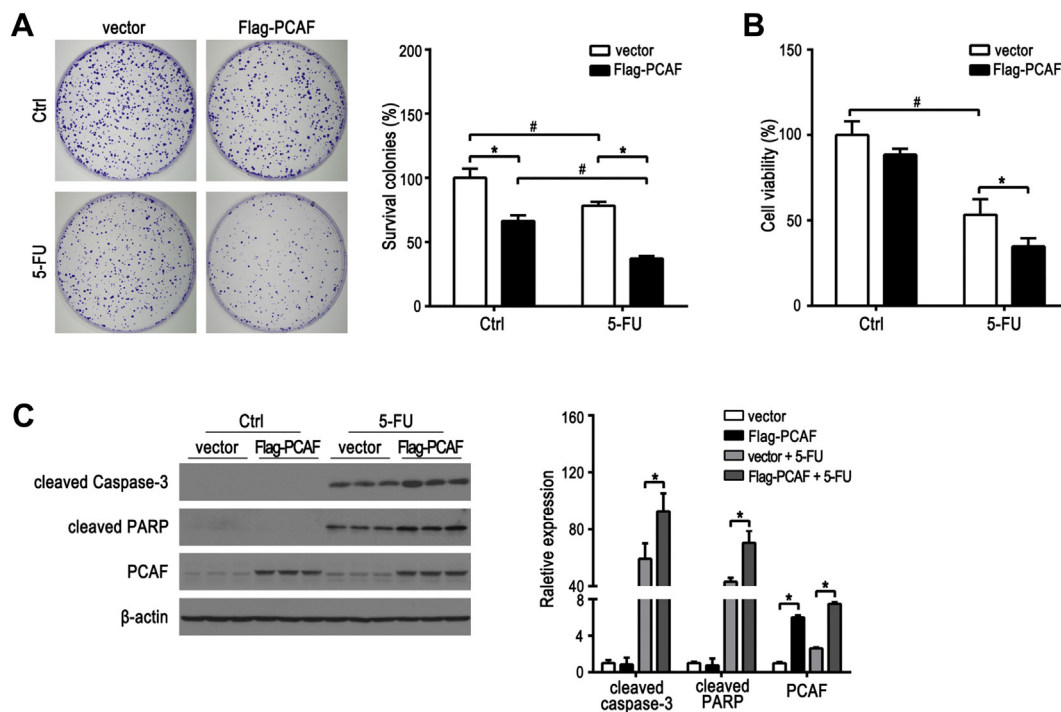


Figure 3. Exogenous overexpression of PCAF increases the susceptibility of HCT116 cells to 5-FU. **(A)** Overexpression of PCAF decreases the clonogenicity of HCT116 cells. Plasmid DNA expressing the Flag tagged PCAF was transfected to HCT116 cells by lipofectamine. Clonogenic formation assay was used for determining the clonogenicity of HCT116 cells treated with 5-FU (1 μ g/mL) (left panel). The quantitative results show the average percentage of surviving colonies (right panel). **(B)** Exogenous overexpression of PCAF attenuates the chemoresistance of HCT116 cells to 5-FU. Cell viability was determined by CCK8 assay in HCT116 cells treated with 5-FU (2 μ g/mL) for 48 h. **(C)** PCAF overexpression increases the amount of cleaved caspase-3 and cleaved PARP induced by 5-FU (5 μ g/mL) treatment (left panel). Quantitative analysis of protein level changes in HCT116 cells by measuring the intensity of western blot band (right panel). The data are means \pm SD of three independent assays, *: $P < .05$ vs. vector, #: $P < .05$ vs. Ctrl, $n = 3$.

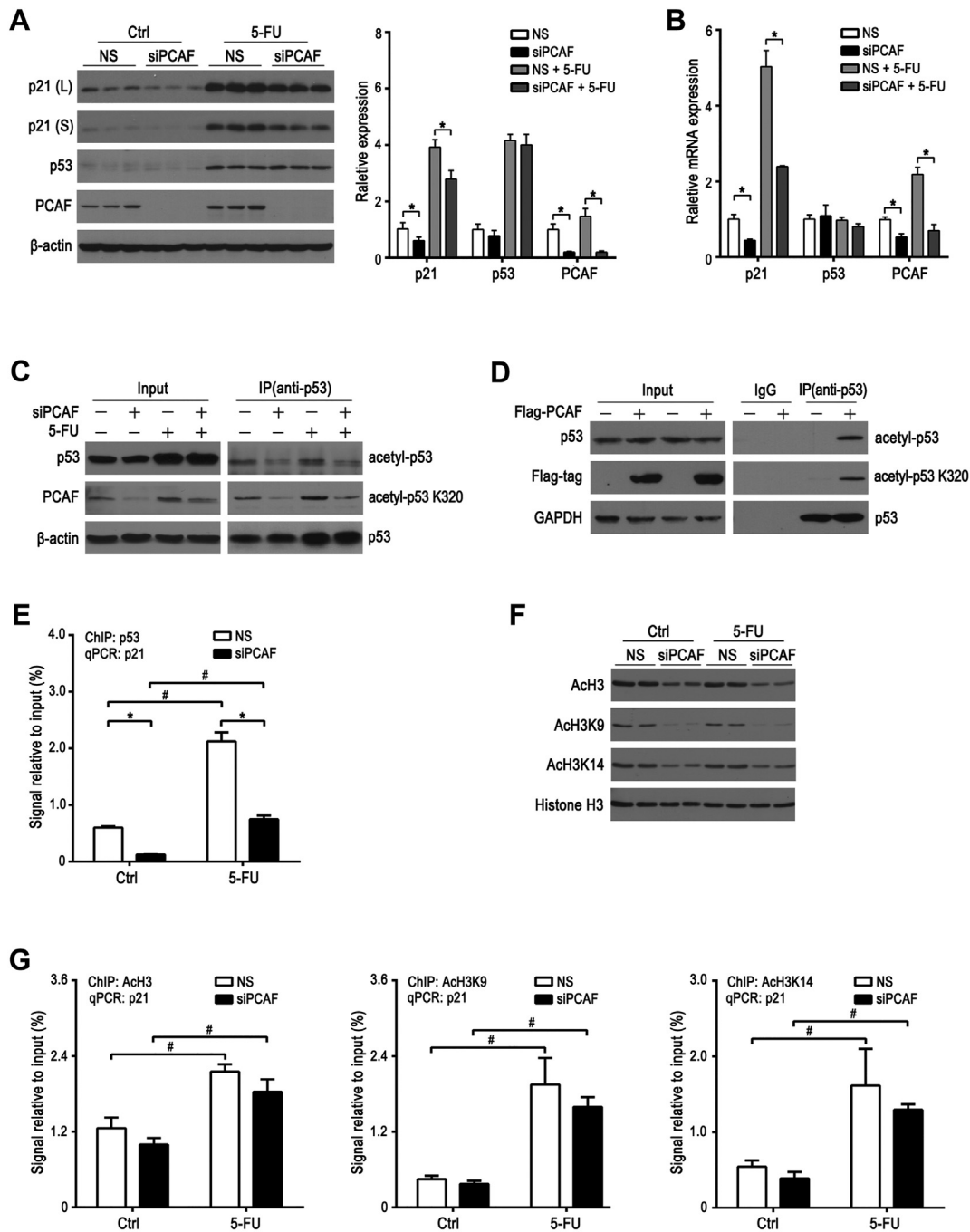


Figure 4. PCAF regulates the acetylation of p53 and p53-dependent expression of p21. **(A)** The protein level of p21 is down-regulated in PCAF knockdown HCT116 cells with or without 5-FU treatment (left panel). (L): long time exposure, (S): short time exposure. Quantitative analysis by measuring the intensity of western blot band (right panel). **(B)** PCAF knockdown reduces the transcription of p21 in HCT116 cells. The alteration of mRNA levels was determined by RT-qPCR. **(C)** The acetylation of p53 is induced by 5-FU treatment and diminished by PCAF knockdown in HCT116 p53^{-/-} cells overexpressing Flag-p53. The acetylation levels were detected by immunoprecipitation of p53 and immunoblot using the anti-acetylated-Lysine antibody (Cell Signaling #9441) and anti-K320Ac-p53 antibody (Sigma #06-1283), respectively. **(D)** Overexpression of Flag-PCAF increased p53 acetylation and K320Ac-p53 in HCT116/5-FU cells. **(E)** ChIP-qPCR assay reveals that p53 occupancy at the region of p21 promoter. PCAF knockdown reduces the binding of p53 to the p21 promoter region in HCT116 cells with or without 5-FU treatment. **(F)** PCAF knockdown reduces the acetylation of total Histone H3 (Ach3), Histone H3 (Lys9) (Ach3K9) and Histone H3 (Lys14) (Ach3K14). 5-FU treatment does not affect the acetylation of Histone H3. The acetylation levels were detected by using the anti-acetylated-H3 antibodies from Cell Signaling. **(G)** ChIP-qPCR assay reveals that PCAF knockdown does not affect the binding of the Ach3, Ach3K9 or Ach3K14 at the p53 occupancy region of p21 promoter. The data are means \pm SD of three independent assays, *: $P < .05$ vs. NS, #: $P < .05$ vs. Ctrl, $n = 3$.

treatment condition. Similarly, cell viability data demonstrated that PCAF overexpression in HCT116 cells increased the cytotoxicity of 5-FU (Figure 3B). Consistently, PCAF overexpression increased the

amount of cleaved caspase-3 and PARP in HCT116 cells treated with 5-FU as compared to 5-FU treated HCT116 cells transfected with control vector (Figure 3C).

PCAF Regulates the Acetylation of p53 and p53-Dependent Expression of p21

To elucidate the underlying mechanism by which PCAF regulates the resistance of CRC cell lines to 5-FU, we first examined the change of p21 protein levels because p21 plays an essential role in developing the 5-FU resistance [40,41]. As shown in Figure 4A, 5-FU transient treatments not only increased PCAF but also increased the protein levels of p21 and p53 as compared to no-treatment controls. Knockdown of PCAF decreased the 5-FU-induced expression of p21 in HCT116 cells. However, loss of PCAF does not affect the protein level of p53 in HCT116 cells. Real-time PCR results further demonstrated that PCAF regulated the transcription of p21 (Figure 4B). 5-FU treatment increases the transcript levels of p21, and PCAF knockdown with siRNA decreased both basal and 5-FU-induced transcription of p21 in HCT116 cells. However, either 5-FU treatment or PCAF knockdown does not affect the transcription of p53 (Figure 4B). Previous reports from other groups revealed that p53 is one of the non-histone proteins acetylated by PCAF [19]. Western blot data shown in Figure 4C demonstrated that PCAF knockdown in HCT116 cells impaired the acetylation of p53. However, overexpression of PCAF in HCT116 cells increased the acetylation of p53 (Figure 4D). Based on previous reports that p53 binds the promoter region of p21 and regulates the transcription of p21, we hypothesized that impaired acetylation of p53 in PCAF knockdown cells decreased the binding of p53 as to attenuate the transcription of p21. As a proof of concept, we used CHIP-qPCR approach to demonstrate that 5-FU treatments increased the binding of p53 to the promoter region of p21 and PCAF knockdown attenuated the binding of p53 (Figure 4E). As the known substrates of PCAF, acetylation of a pan-H3 histone protein, H3K9, and H3K14 also decreased in HCT116 cells transfected with PCAF siRNA as compared to NS group (Figure 4F). However, the CHIP-qPCR results did not show any significant down-regulation of AcH3, AcH3K9, and AcH3K14 binding to the promoter region of p21 (Figure 4G). These data suggest that PCAF regulates the transcription of p21 through PCAF-mediated acetylation of p53 instead of H3 histone acetylation.

PCAF Regulates the 5-FU-Induced Apoptosis of HCT116 Cells in a p21-Dependent Manner

As the cyclin-dependent kinase (CDK)-inhibitory protein, p21 is involved in developing 5-FU resistance [40,41]. As shown in Figure 5A, knockdown of p21 using siRNA increased the resistance of HCT116 cells to 5-FU. To determine if PCAF regulates the 5-FU-induced apoptosis of HCT116 cells in a p21-dependent manner, we first used Western blot to examine the effects of PCAF overexpression on the alterations of p21 and p21-related cell cycle regulators in 5-FU resistant HCT116 cells (HCT116/5-FU). The results of Western blot (Figure 5B) demonstrated that both PCAF and p21 decreased in HCT116/5-FU cells (lane 2) as compared to the parent HCT116 cells (lane 1). However, cyclin D1 and phosphorylation of Retinoblastoma 1 (phos-Rb) increased in HCT116/5-FU cells (lane 2) as compared to the parent HCT116 cells (lane 1). Retinoblastoma 1 (Rb), as a tumor suppressor gene, inhibits the G1-S phase transition. However, the CDK-mediated phosphorylation of Rb (Ser807/811) results in the loss of Rb function and triggers the p53 apoptotic pathway [42,43]. When transfecting the plasmid DNA overexpressing Flag-PCAF to HCT116/5-FU cells (lane 3), we appreciated increased p21 and decreased cyclin D1/phos-Rb. If knocking down p21 in HCT116/5-FU cells overexpressing Flag-

PCAF (lane 4) using siRNA targeting p21 (si-p21), p21 knockdown does not change PCAF protein levels but increases cyclin D1 and phos-Rb. The Western blot data shown in Figure 5B suggests that PCAF regulates p21-related cell cycle regulators. As shown in representative images of AO/EB staining (Figure 5C), 5-FU induced the apoptosis of parent HCT116 cells (left upper panel) but not in 5-FU resistant HCT116/5-FU cells (right upper panel). Moreover, overexpression of Flag-PCAF increased the efficacy of 5-FU in inducing the apoptosis of 5-FU resistant HCT116 cells (HCT116/5-FU) (Figure 5C, left bottom panel). However, p21 knockdown abolished the effects of Flag-PCAF overexpression (Figure 5C, right bottom panel). It suggests that PCAF is dependent on p21 to promote the sensitivity of HCT116/5-FU cells to 5-FU.

We further examined effects of PCAF on changing cell cycle because cyclin D1 and phos-Rb regulates the cell cycle progression [43], and the cell cycle arrest is one of the well-known factors attributing to chemotherapy resistance [44]. We used propidium iodide staining-based flow cytometry method to determine the change of the cell cycle. As shown in Figure S5A, knockdown of PCAF in HCT116 cells decreased the G1 phase and increased S phase. We further examined the protein levels of cell cycle regulators. The western blot analysis data (Figure S5B) showed that knockdown of PCAF in HCT116 cells increases the protein levels of cyclin D1 and phosphorylation of Retinoblastoma 1 (phos-Rb), which drives the G1/S phase transition [43]. Our data indicate that the loss of PCAF increases the S phase of the cell cycle, which may be beneficial for DNA repair and preventing the 5-FU-induced apoptosis of HCT116 cells. In contrast, PCAF overexpression increased G1 phase and decreased S-phase of HCT116 cells (Figure S6A). PCAF overexpression decreases the amount of cyclin D1 and phos-Rb (Figure S6B). As shown in Figure 5B, the levels of cyclin D1 and phos-Rb increased in HCT116/5-FU resistant cells as compared to HCT116 cells. The overexpression of Flag-PCAF in HCT116/5-FU resistant cells decreased the levels of cyclin D1 and phos-Rb. Consequently, overexpression of Flag-PCAF also increases the sensitivity of HCT116/5FU resistant cells to 5-FU determined by cell viability assay (Figure 5D) and colony formation assay (Figure 5E), respectively.

Overexpression of PCAF Decreases the Resistance of HCT116 cells to 5-FU In Vivo

The further determine if and the extent to which PCAF overexpression decreases the resistance of CRC to 5-FU, we established HCT116 cells stably expressing Flag-PCAF by a drug selection approach described in our previous publications [45]. After being subcutaneously transplanted into nude mice, the size of tumor xenografts was measured by a caliper as described in our previous publication [46]. When the tumor size reached 100 mm³ at Day 10, 5-FU at the dose of 30 mg/kg was intraperitoneally administrated three times per week. As shown in Figure 6, PCAF overexpression significantly decreased the tumor growth determined by tumor size (Figure 6A) and tumor weight (Figure 6B). The images of tumors isolated from nude mice were presented in Figure 6C. As shown in Figure 6D, the number of tumor cells showing positive immunostaining of p21 increased significantly in the tumors overexpressing Flag-PCAF. The increased expression of PCAF in HCT116 tumor xenografts carrying Flag-PCAF plasmid DNA was confirmed by Western blot analysis (Figure 6E). Consistently, the protein levels of

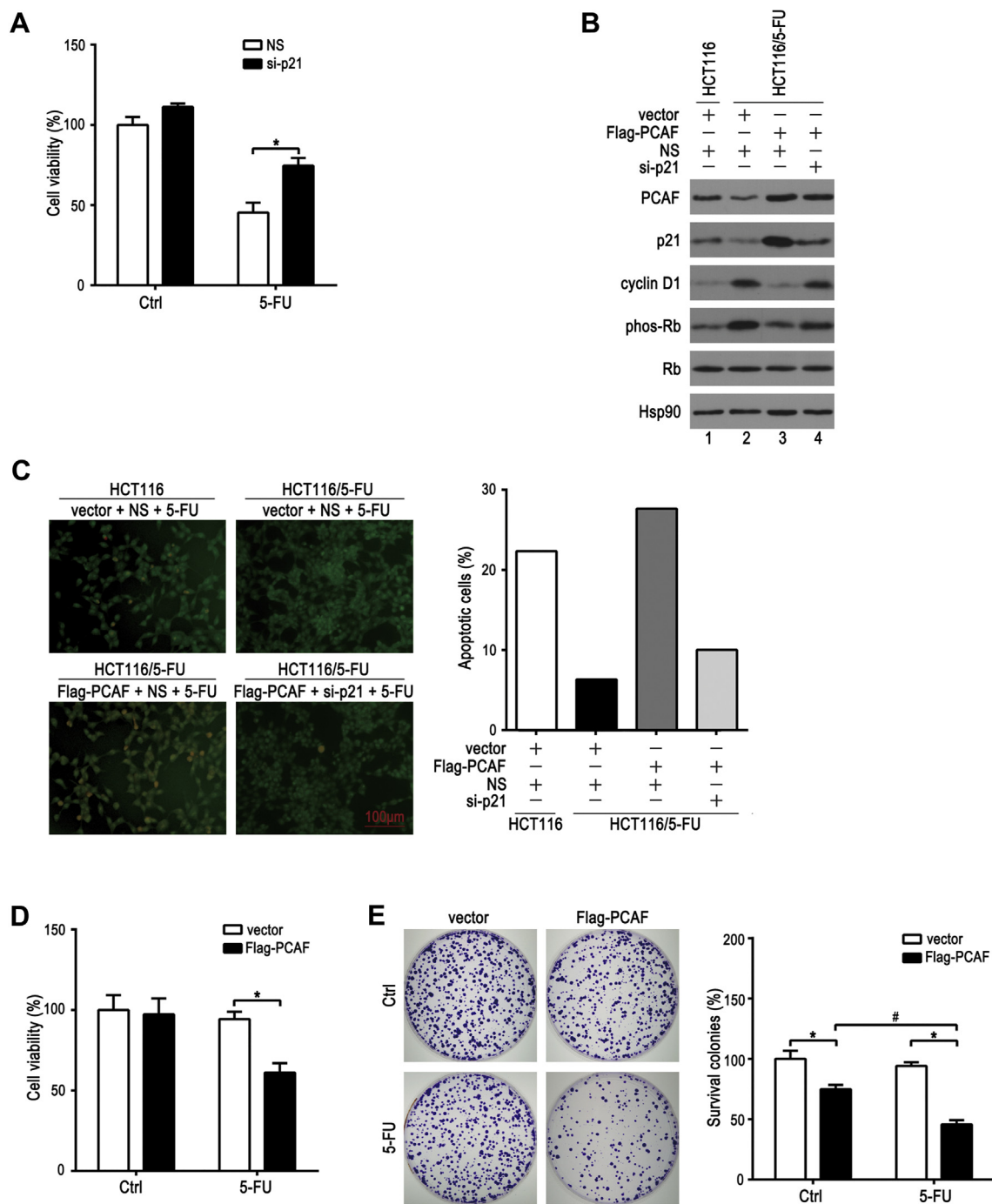


Figure 5. PACF regulates the 5-FU-induced apoptosis of HCT116 cells in a p21-dependent manner. **(A)** Knockdown of p21 increases chemoresistance of HCT116 cells to 5-FU. Cell viability was analyzed by CCK-8 assay in HCT116 cells treated with 5-FU (2 μ g/mL) for 48 h. The data are means \pm SD, *: $P < .05$, $n = 4$. **(B)** PACF regulates the expression of p21 and p21-related cell cycle regulators. The overexpression of Flag tagged PACF in HCT116/5-FU cells was carried out by transfecting the plasmid DNA of Flag-PCAF. The knockdown of p21 was achieved by transfecting siRNA targeting p21. The changes of proteins and phosphorylated protein were determined by Western blot analysis using respective antibodies. **(C)** Exogenous PACF overexpression promotes the 5-FU-induced apoptosis of HCT116/5-FU cells in a p21-dependent manner. AO/EB staining was used for measuring apoptotic cell population in HCT116 and HCT116/5-FU cells treated with 5-FU (5 μ g/mL). The quantitative results show the average percentage of apoptotic cells from 3 images taken from each group (right panel). **(D)** Exogenous PACF overexpression restores chemo-sensitivity of HCT116/5-FU cells to 5-FU. Cell viability was analyzed by CCK-8 assay in HCT116/5-FU cells treated with 5-FU (10 μ g/mL) for 48 h. The data are means \pm SD, *: $P < .05$, $n = 4$. **(E)** Overexpression of PACF decreases the clonogenicity of HCT116/5-FU cells. Clonogenic formation assay was used for measuring clonogenicity of HCT116/5-FU cells treated with 5-FU (10 μ g/mL) (left panel). The quantitative results show the average percentage of surviving colonies (right panel). The data are means \pm SD, *: $P < .05$ vs. vector, #: $P < .05$ vs. Ctrl, $n = 3$.

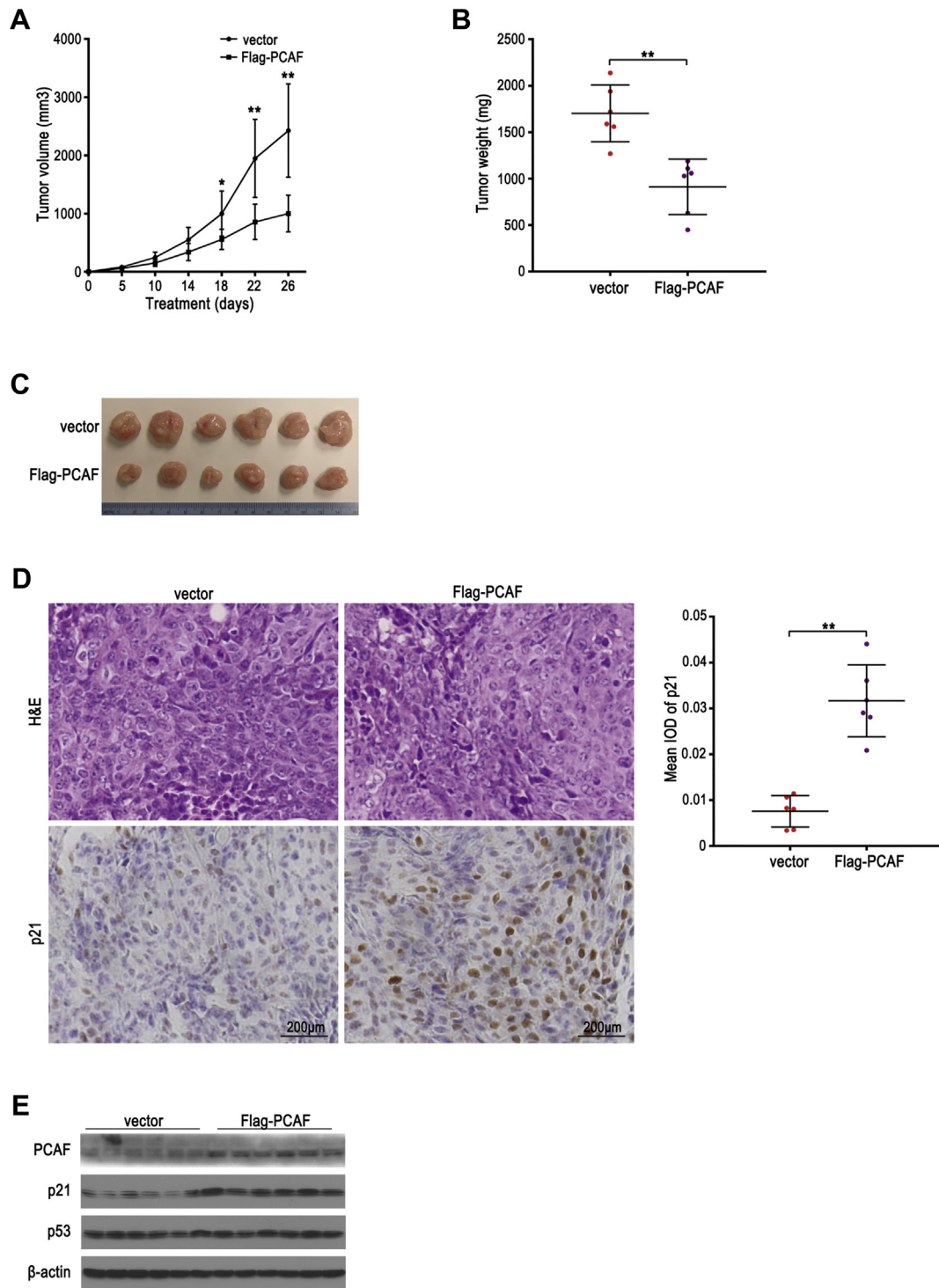


Figure 6. Overexpression of PCAF decreases the resistance of HCT116 cells to 5-FU *in vivo*. **(A)** Overexpression of PCAF decreases the tumor size. HCT116 cells expressing either empty vector or Flag-PCAF were inoculated subcutaneously into the oexter of the nude mice, respectively. When the tumor size reached 100 mm³ at Day 10, 5-FU at the dose of 30 mg/kg was i.p. administrated three times per week. Tumor volumes of different tumor and treatment groups were calculated as described in methods. **(B)** Overexpression of PCAF decreases the tumor weights. **(C)** The representative images of tumor xenografts of implanted HCT116 cells expressing either empty vector or Flag-PCAF. **(D)** Immunohistochemical (IHC) staining of p21 revealed a higher p21 expression in tumors overexpressing PCAF group than in control group (left panel). The quantitative results show the mean integrated optical density (IOD) of p21 (right panel). **(E)** Overexpression of PCAF increases p21 protein level and does not affect p53 protein level *in vivo*. The data are means \pm SD, *: $P < .05$, **: $P < .01$, $n = 6$.

p21 were also increased in HCT116 tumor xenografts carrying Flag-PCAF plasmid DNA (Figure 6E). These data suggest that PCAF overexpression can reduce the resistance of CRC to 5-FU through increasing p21.

The Down-Regulation of PCAF is Associated With the Poor Outcome of CRC

To determine if and the extent to which PCAF expression is related to clinical outcome of CRC, we first analyzed the published data from

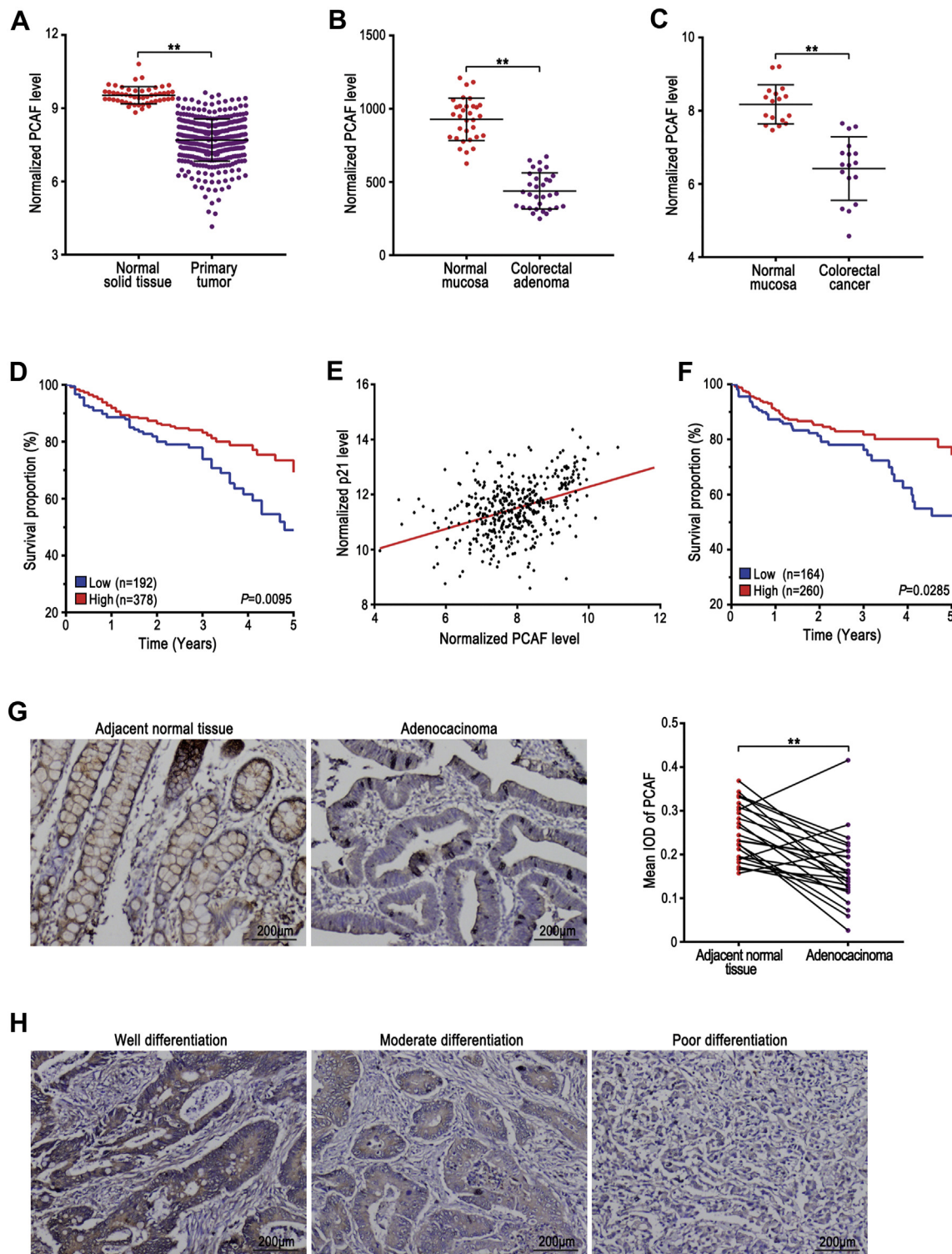


Figure 7. The down-regulation of PCAF is associated with the poor outcome of CRC. **(A)** PCAF mRNA levels in normal solid tissues ($n = 51$) and primary tumor tissues ($n = 380$) ($P < .0001$, Student's t-test). The data were retrieved from the Cancer Genome Atlas (TCGA). **(B, C)** The expression of PCAF compared between normal mucosa and colorectal adenoma. The microarray data were retrieved from Gene Expression Omnibus (GEO) via the accession numbers GDS2947 (normal mucosa ($n = 32$) and colorectal adenoma ($n = 32$)) and GDS4382 (normal mucosa ($n = 17$) and colorectal adenoma ($n = 17$)), respectively. ($P < .0001$, Student's t-test). **(D)** The expression levels of PCAF are associated with the survival outcome of CRC patients. Kaplan-Meier plot of 570 patients with survival data from TCGA revealed a reduced survival rate for patients with low PCAF expression in CRC tumors ($P < .01$). **(E)** Correlation of gene expression between p21 and PCAF in 424 patients with CRC tumors ($\rho = 0.4001$, $P < .0001$). **(F)** Kaplan-Meier plot of 424 patients with survival data from TCGA revealed a reduced survival rate for patients with low p21 expression in CRC tumors ($P < .05$). **(G)** Immunohistochemical (IHC) staining of PCAF revealed a lower expression of in adenocarcinoma than in adjacent normal tissue from 24 patients (left panel). The quantitative results show the mean integrated optical density (IOD) of PCAF (right panel) ($P < .0001$). **(H)** IHC staining shows a trend that higher PCAF expression in well differentiated than in poorly differentiated adenocarcinoma.

the Cancer Genome Atlas (TCGA) and appreciated a significant decrease of PCAF (KAT2B) RNA levels in primary tumors as compared to normal tissues (Figure 7A). However, there is no significant difference among the different stages of primary CRC tumors (Figure S7). The microarray data from Gene Expression Omnibus (GEO) also revealed remarkable down-regulation of PCAF in colorectal adenoma, a kind of precancerous lesion, and CRC, respectively (Figure 7, B and C). In addition, PCAF microarray data were retrieved from a gene-expression profiling dataset of 570 colorectal cancer cases. Kaplan–Meier analysis revealed a reduced 5-year survival probability of 192 patients with low PCAF-expression in tumors as compared to 378 patients with high PCAF-expression in tumors (Figure 7D). Interestingly, the expression of p21 in these patients is positively correlated with the expression of PCAF (Figure 7E). Kaplan–Meier analysis revealed a reduced 5-year survival probability of 164 patients with low p21-expression in tumors as compared to 260 patients with high p21-expression in tumors (Figure 7F). Consistently, the immunohistochemical (IHC) staining of PCAF in paired CRC tissue array from the BioChain showed that the PCAF staining intensity in CRC tissues is weaker than in adjacent mucosa tissues (Figure 7G). The quantitative IHC analysis of the paired samples of adjacent normal tissues and adenocarcinoma from 16 patients was presented in Figure 7G left panel. The 13 from 16 patients showed decreased IHC staining of PCAF in adenocarcinoma as compared to their adjacent normal tissues. In addition, we appreciated a trend that IHC staining intensity of PCAF in well-differentiated colorectal tumors is higher than in poorly differentiated colorectal tumor (Figure 7H). However, the sample number is not sufficient for statistical analysis among these three groups. These results collectively suggest that the down-regulation of PCAF is associated with the poor outcome of CRC.

Discussion

In this study, we found PCAF was remarkably decreased in 5-FU resistant cell lines (Figure 1 and Figure S1), which is mediated by 5-FU caused histone protein trimethylation (Figure S4). PCAF knockdown attenuated 5-FU-induced apoptosis of HCT116 cells (Figure 2). Conversely, PCAF overexpression increases the susceptibility of HCT116 cells to 5-FU (Figure 3). Mechanistically, we demonstrated that PCAF regulates the transcription of p21 through PCAF-mediated acetylation of p53 (Figure 4). PCAF is dependent on p21 induction to increase the sensitivity of HCT116/5-FU resistant cells to 5-FU (Figure 5). Furthermore, we used CRC tumor xenograft to demonstrate PCAF overexpression can increase p21 and reduce the resistance of CRC to 5-FU *in vivo* (Figure 6). The clinical data also revealed that significant down-regulation of PCAF in colorectal adenoma and CRC was associated with the poor outcome of CRC patients (Figure 7).

Although 5-fluorouracil (5-FU) is widely used in the chemotherapy of CRC, drug resistance still is a major challenge for achieving effective chemotherapy [9]. 5-FU is one type of DNA-damaging drugs, leading to the incorporation of fluoronucleotides into RNA and DNA as to inhibit the activity of nucleotide synthesis enzyme [47]. DNA-damaging drugs have been shown to inhibit cell growth and DNA repair by altering regular cell cycle [48]. Numerous previous studies have revealed that the cell-cycle perturbation might be attributed to acquired DNA-damaging drugs resistance [49,50]. Alterations of cell cycle regulators, such as cycle-dependent kinase inhibitor p21^{Cip1/WAF1}, play an essential role in regulating drug

sensitivity [51]. Previous studies from other laboratories have demonstrated that p21 may be required for 5-FU-induced apoptosis in colon cancer cells [40,41]. Consistently, in our study, knockdown of p21 increased the resistance of HCT116 cells to 5-FU (Figure 5A) and attenuated the effects of PCAF overexpression on promoting 5-FU-induced apoptosis of HCT116/5-FU cells (Figure 5C).

However, the transcription of p21 is regulated by p53, which is one of the non-histone proteins acetylated by PCAF [19–23,52]. The acetylation significantly modulates the function of p53 since p53 deacetylation by histone deacetylase-associated proteins compromises p53-induced cell cycle arrest and apoptosis [53]. Previous reports from other laboratories also demonstrated that HADC2 regulates the expression of p53-targeted genes through the deacetylation of p53 [22,23,54]. PCAF directly acetylates p53 and thereby increases the capability of p53 in binding to DNA [19]. The transcriptional activation of p21 regulated by p53 is one of the critical events leading to cell cycle arrest [55,56]. Acetylation of p53 has previously been linked to p21 regulation *via* two separate mechanisms. Firstly, p53 acetylation facilitates the recruitment of coactivator and HATs onto the promoters of p21 to promote p21 transcription [57]. Second, acetylated p53 blocks the recruitment of Mdm2 and Mdmx to the p21 promoter in a ubiquitination-independent manner so as to reduce p21 degradation [58]. In this study, we show that PCAF knockdown did not affect p53 protein levels but decreased p21 expression at transcriptional and protein levels (Figure 4, A or B). Mechanistically, our data demonstrated that PCAF knockdown attenuated the acetylation of p53 and resulted in decreased binding of p53 to the promoter region of p21 (Figure 4, C–E). The previous report by Love et al. reveals that PCAF acts as a true HAT to acetylate H3K9 and H3K14, which share the p53 binding site in the p21 promoter region *via* acetyl-p53-independent manner [59]. In our study, PCAF knockdown indeed decreased the acetylation of a pan-H3 histone protein, H3K9, and H3K14, but did not significantly attenuate the binding of AcH3, AcH3K9, and AcH3K14 to the promoter region of p21 (Figure 4, F or G). Therefore, our data suggest that PCAF-mediated acetylation of the p53 attribute to the transcription of p21 in 5-FU resistant CRC cell lines.

Although the beneficial effect of histone deacetylase inhibitors treatment combined with 5-FU has been fully identified in CRC chemotherapy [9], it is essential to find a biomarker can indicate the alteration of acetylation for early diagnosis and prognosis evaluation. As shown in Figure 7, the levels of PCAF and p21 expression in CRC patients are significantly associated with 5-year survival probability. Together with our result that down-regulated expression of PCAF and p21 in 5-FU resistant CRC cell lines, PCAF and p21 fulfill the credential for being potential tumor biomarkers or therapeutic targets for CRC. The underlying molecular mechanism by which PCAF is decreased in CRC, especially in 5-FU resistant CRC, needs further investigation.

Data in Figure S4 suggests that histone methylation is one of the causal factors in regulating the expression of PCAF. Methylation, another type of epigenetic modification, can regulate gene expression by inhibiting the binding of transcription factors or by recruiting repressive proteins involved in gene expression [60]. Histone methylation modulates chromatin structure and thereby regulates DNA-based nuclear processes such as transcription, replication, and repair [61,62]. The function of histone methylation, either transcriptional activation or repression, depends on the different lysine residues. While H3K4me2/3 and H3K79me3 are stimulatory

for transcription, H3K9me2/3, H3K27me2/3 and H4K20me3 are signals for transcriptional silencing [63,64]. Several studies provide evidence to suggest that mutations in or altered expression of histone methylation may play a role in a variety of different cancers [33,34]. In this study, we demonstrated that 5-FU treatment increases the level of H3K27me3 and increased binding of H3K27me3 in the promoter region of PCAF in the 5-FU resistant HCT116 cells (Figure S4). These data indicate that the 5-FU-caused histone trimethylation in CRC cell lines contributes to the alteration of PCAF in response to 5-FU treatment.

In summary, our results demonstrate that PCAF-mediated p53 acetylation is an essential regulatory mechanism that contributes to the sensitivity of CRC to 5-FU both *in vitro* and *in vivo*. This study provides compelling evidence to support the feasibility of targeting PCAF as an alternative approach for improving the treatment efficacy for 5-FU resistant CRC.

Conflict of Interest

The authors declare that they do not have any conflicts of interest related to this study.

Author Contributions

T.L. performed tumor xenograft experiments. T.L., X.W., W.H., Z. F, Y.J. performed cell function assays and cell signaling analysis. T.L. contributed to the manuscript writing and figure preparation. Q.R.M. contributed to the editing of the manuscript. Q.R.M., T.L. X.W. and X.F. were responsible for concept development and preparation of the manuscript; Q.R.M. was responsible for overall integration and execution of the scientific approaches.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.neo.2019.03.011>.

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