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Targeting Histone Chaperone FACT Complex Overcomes 5-Fluorouracil Resistance in Colon Cancer

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Abstract

Fluorouracil (5-FU) remains a first-line chemotherapeutic agent for colorectal cancer (CRC). However, a subset of CRC patients who have defective mismatch repair (dMMR) pathway show resistance to 5-FU. Here, we demonstrate that the efficacy of 5-FU in dMMR CRC cells is largely dependent on the DNA base excision repair (BER) pathway. Downregulation of APE1, a key enzyme in BER pathway, decreases IC₅₀ of 5-FU in dMMR CRC cells by 10-fold. Furthermore, we discover that Facilitates Chromatin Transcription (FACT) complex facilitates 5-FU repair in DNA via promoting the recruitment and acetylation of APE1 (AcAPE1) to damage sites in chromatin. Downregulation of FACT affects 5-FU damage repair in DNA and sensitizes dMMR CRC cells to 5-FU. Targeting FACT complex with curaxins, a class of small molecules, significantly improves 5-FU efficacy in dMMR CRC *in vitro* (~50-fold decrease in IC₅₀) and *in vivo* xenograft models. We show that primary tumor tissues of CRC patients have higher FACT and AcAPE1 levels compared to adjacent non-tumor tissues. Additionally, there is a strong clinical correlation of FACT and AcAPE1 levels with CRC patients' response to chemotherapy. Together, our study demonstrates that targeting FACT with curaxins is a promising strategy to overcome 5-FU resistance in dMMR CRC patients.

Keywords

APE1; colon cancer; mismatch repair deficiency; FACT complex; DNA repair; 5-fluorouracil

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Introduction

Colorectal cancer (CRC) is the second leading cause of cancer related deaths in the US. According to the American Cancer Society, more than 50% of new cases are diagnosed at advanced stages and require adjuvant chemotherapy. The pyrimidine analog 5-fluorouracil (5-FU) forms the backbone for almost all chemotherapeutic regimens for CRC (1). However, a subset of CRC patients who develop cancer with microsatellite instability or defective mismatch repair (dMMR) show resistance to 5-FU (2,3). Studies have shown that dMMR CRC patients with stage III tumors do not benefit from 5-FU-based adjuvant (FOLFOX) therapy (2,4). In accordance with clinical observations, *in vitro* studies have shown that dMMR CRC cells are resistant to the cytotoxic effects of 5-FU (5). Therefore, elucidating the mechanisms of 5-FU resistance in dMMR CRC and identifying novel therapeutic targets to increase the efficacy of 5-FU in dMMR CRC represents an unmet need.

Though the mechanism of actions of 5-FU is not completely understood, its cytotoxicity has been ascribed to the inhibition of thymidylate synthase (TS), the key enzyme of *de novo* pyrimidine biosynthesis (6). However, numerous studies have established that 5-FU metabolites can induce cytotoxicity through incorporation into RNA and genomic DNA (7,8), and that both DNA Mismatch Repair (MMR) and Base Excision Repair (BER) pathways are primarily involved in the repair of the resultant DNA lesions (8,9). In the case of FU incorporation opposite dG, the resulting FU:dG mispair would be efficiently processed by the MMR pathway, resulting in single-stranded breaks (SSBs) (9,10). However, repeated incorporation of FU:dG leads to futile attempts by the MMR system and persistent SSBs will result in double-strand breaks that in turn induce apoptosis (11). On the other hand, the BER pathway is able to directly remove FU from newly synthesized DNA in the case of FU:dA or FU:dG (8,12), resulting in apurinic/apyrimidinic (AP) sites that are further processed by AP-endonuclease (APE1) (13). APE1 plays a central role in the BER pathway by cleaving the DNA backbone immediately 5' to lesions (14). The resulting strand breaks are repaired via the highly coordinated BER pathway (15). We have recently shown that APE1 is acetylated (AcAPE1) at AP sites in chromatin by p300 and that acetylation enhances its AP-endonuclease activity (16). We hypothesize that dMMR CRC cells have an increased requirement of BER pathway for efficient repair of 5-FU-induced DNA damages, and that targeting APE1-dependent BER pathway will sensitize dMMR CRC to 5-FU.

In this study, we sought to examine the role of BER pathway in promoting 5-FU resistance in CRC cells with deficient MMR system. We found that downregulation of APE1 sensitizes dMMR CRC cells to 5-FU *in vitro*. Furthermore, we identified FACT (Facilitates Chromatin Transcription) complex as an interacting partner of APE1 in chromatin and characterized the role of FACT complex in BER pathway. Curaxins, a class of small molecules that inhibit FACT complex, were tested extensively in combination with 5-FU using multiple dMMR CRC cell lines *in vitro* and *in vivo* as a means of improving 5-FU therapeutic response. To provide further support of potential applicability of this novel therapeutic strategy, we examined the expression of APE1 and FACT in CRC patient specimens and correlated with the treatment response. Together, our study unveils a novel role of FACT complex in

promoting 5-FU resistance, and demonstrate that targeting FACT with curaxins is a promising strategy to overcome 5-FU resistance in dMMR CRC patients.

Materials and methods

Cell culture, plasmids, siRNAs, transfection and treatments

HCT116 cells (ATCC# CCL-247) were grown in McCoy's 5A medium (Gibco) supplemented with 10% fetal calf serum (FCS; Sigma) and antibiotic mixture of 100 U/ml penicillin and 100 µg/ml streptomycin (Gibco). HCT116 cell line stably expressing APE1-shRNA was a kind gift from Dr. Sheila Crowe (University of California, San Diego) and was cultured in McCoy's 5A supplemented with 0.01% puromycin (Gibco). HEK-293T cells (ATCC # CRL-3216) were cultured in DMEM-high glucose medium (Gibco) with 10% fetal calf serum (FCS; Sigma) and antibiotic mixture of 100 U/ml penicillin and 100 µg/ml streptomycin (Gibco). RKO cell line was obtained from Dr. Jing Wang (Eppley Institute, UNMC). RKO and DLD-1 cells (ATCC# CCL-221) were grown in EMEM medium (ATCC). All cell lines were authenticated using STR DNA profiling by Genetica DNA laboratories (Burlington, NC) two years ago before being used in this study. These cells were routinely assayed for mycoplasma. Mutation of Lys residue (K6, 7, 27, 31 and 32) to arginine or to glutamine in APE1-FLAG-tagged pCMV5.1 plasmid were generated using a site-directed mutagenesis kit (Agilent-Stratagene, Santa Clara, CA) as described previously (16). Exponentially growing HEK293T cells were transfected with wild type (WT) APE1, K6,7,27,31,32 to arginine (K5R) or to glutamine (K5Q), N-terminal 33 amino acid deleted (N 33) mutants expression plasmids. siRNAs targeting SSRP1 (Sigma, EHU015991) and SPT16 (Sigma, EHU039881; Dharmacon, J-009517), as well as control siRNA (Dharmacon, D-001810) were transfected into RKO, HCT116, and HCT116^{APE1shRNA}. APE1 siRNAs were obtained from Sigma-Aldrich (WD04424567) and Dharmacon (J-010237). Cells were transfected using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) and harvested after 48 hrs. Methyl Methanesulfonate (MMS), quinacrine (QC), and 5-FU were obtained from Sigma-Aldrich. CBL0137 was obtained from Cayman Chemical for *in vitro* study, and from Incuron Inc (Buffalo, NY) for *in vivo* study.

Identification of interacting proteins of AcAPE1

Chromatin extracts were immunoprecipitated (IP) with anti-AcAPE1 and control IgG antibodies (16). The IP samples were boiled for 5 min and resolved in 12.5 % SDS-PAGE gel followed by staining with Coomassie blue (PageBlue™, Thermo Scientific). Identification of protein bands was performed by MALDI-TOF-TOF analysis in the Mass Spectrometry and Proteomics Core Facility (University of Nebraska Medical Center, Omaha, NE, USA).

Western Blot Analysis

Cell fractionation was performed as described previously (17). Whole cell lysates or cell fractions were resolved on 10 to 12.5 % SDS-PAGE gel and transferred to nylon membranes for blotting. Whole cell lysates of HCEC, GEO, LoVo and SW620 were provided by Dr. Jing Wang (UNMC). Primary antibodies were used including SPT16 (Abcam, 204343), SSRP1 (Biolegend, 609702), FLAG (Sigma, F1804), TRF1 (Abcam, 10579), α-HSC70 (B6-

Sc7298, Santa Cruz Biotechnology), H2A (Abcam, 26350), APE1 (Novus Biologicals, NB100-116), α -tubulin (Abcam, 52666) and AcAPE1 (14,18). Immunoblot signals were detected using Super Signal West pico chemiluminescent substrate (Thermo Scientific) after treating with HRP-conjugated secondary Ab (GE Healthcare).

MTT assay

Cells were seeded at a density of 5×10^3 in 96-well plates. After 24-hour incubation in the medium to allow for cell attachment, the fresh medium was added and cells were treated with vehicle control (DMSO alone) or indicated doses of 5-FU dissolved in DMSO for 72 hours. The MTT reagent (Sigma-Aldrich, M5655) was added to a final concentration of 0.5 mg/ml to each well. The assay was performed as per manufacture's protocol. Three independent experiments with six replicates were performed for each group.

Patient tissue samples and analysis

Colon cancer samples were obtained from tissue bank at University of Nebraska Medical Center (UNMC) and University of Texas Medical Branch. Tissues were collected in accordance with institution's review board approval and informed consent was waived. The deparaffinized sections were stained per standard IHC protocol. The antibodies used were: AcAPE1 (1:200), Ki67 (1:500, CST, 9027) and SSRP1 (1:100). Staining intensity and percentage of positive cells were analyzed by Definiens Releases Tissue Studio® 4.3. We used a stain deconvolution algorithm to separate the DAB chromogen stain and the hematoxylin counterstain in all tissue cores. We then measured the brown chromogen intensity across all tissues to obtain the range of pixel density. Based on the range, we divided the staining intensity into 3 categories using one third threshold increment in the range. Tissue lysates were prepared and analyzed by Western Blot as described previously (19).

Treatment response is assessed by clinician using modified Ryan Tumor Regression Grading System (20).

Complete response: no viable cancer cells

Moderate response: single cells, or small groups of cancer cells

Minimal response: residual cancer outgrown by fibrosis

No response: minimal or no tumor killed; extensive residual cancer

Immunoprecipitation (IP) and FLAG-IP

Nuclear and chromatin extracts of HCT116 or RKO cells were pre-cleared with protein A/G Plus agarose beads and IP was performed with AcAPE1 antibody or control IgG (Santa Cruz, sc-2003). The chromatin extracts of control and MMS-treated cells were immunoprecipitated with the same antibody. FLAG-IP was done with mouse monoclonal α -FLAG M2 antibody-conjugated agarose beads (Sigma-Aldrich, A2220) in nuclear extracts of HEK293T cells transfected with FLAG tagged constructs as described previously (18). The immunoprecipitated proteins were resolved in SDS-PAGE and identified by Western Blot analysis with the indicated antibodies.

Immunofluorescence

Cells grown on coverslips were fixed with 4% formaldehyde (Sigma-Aldrich) and stained with immunofluorescence as described previously (16). Primary antibodies used were mouse monoclonal anti-APE1 (1:100; Novus Biologicals, NB100-116), anti-AcAPE1 (1:50), SSRP1 (1:100; Biolegend, 609702), SPT16 (1:50; Abcam, 204343). Images were acquired by use of a fluorescence microscope with a 63× oil immersion lens (LSM 510; Zeiss), and structured illumination microscopy (SIM) was done with an Elyra PS.1 microscope (Carl Zeiss) by using a 63× objective with a numerical aperture of 1.4. ImageJ software was used to measure Manders colocalization using the JaCoP plug-in.

Chromatin Immunoprecipitation (ChIP) Assay

ChIP assay was performed after double crosslinking of cells with disuccinimidyl glutarate and formaldehyde, with protein A/G Plus agarose beads (Santa Cruz, sc-2003) using with AcAPE1, SPT16 and control IgG (Santa Cruz) following the procedure as described earlier (16,18). The immunoprecipitated purified DNA was used to amplify the p21 and DTL promoters regions using SYBR GREEN-based (Thermo Scientific) Real Time PCR analysis. The following primers are used: p21 forward 5'-CAGGCTGTGGCTCTGATTGG-3', reverse 5'-TTCAGAGTAACAGGCTAAGG-3'; DTL forward 5'-TCCTGCAAATTTCCCGCAAC-3', reverse 5'-GGCTATGGCGAACAGGAAC-3'. Data were represented as relative enrichment with respect to IgG control based on 2^{-CT} method.

AP site measurement assay

HCT116 cells were transfected with control and siRNA against SSRP1 and SPT16. After 48 hrs, cells were treated with 1 mM MMS for 1 hr and released in fresh media for 6 hrs. Total genomic DNA was isolated by Qiagen DNeasy kit following manufacturer's protocol. AP sites were measured using aldehyde reactive probe (Dojindo Laboratories) as described previously (16).

Fluorescence Recovery after Photobleaching (FRAP)

N-terminal GFP tagged -APE1 (21) was transfected into HCT116 cells. 24 hours after transfection of control and FACT siRNAs, cells were treated with DMSO or MMS. FRAP experiments were performed as described previously (22). All FRAP data were normalized to the average prebleached fluorescence after removal of the background signal. The curve was plotted using GraphPad Prism 7 and each curve represented an average of 10 measurements from different regions of cells.

Xenograft studies

All animal experiments were performed following the approval of Institutional Animal care and use committee (IACUC). The experiments and reports are adhered to the Animal Research: Reporting of In Vivo Experiments (ARRIVE) guidelines. HCT116 and DLD-1 cells (2×10⁶ in 100 µl medium) were injected subcutaneously over the left and right flanks in 6-week old male athymic nude mice (Charles Rivers, Wilmington, MA). The average weight was 27 ± 3.6 grams. Subcutaneous tumors were allowed to grow for 1-2 weeks before treatments. The mice were divided into four treatment groups (each groups n=5 mice) and

received treatments every other day for four weeks. The following drugs: 5-FU 20 mg/kg, QC 50 mg/kg, CBL0137 30 mg/kg were injected intraperitoneally. Combination group received both 5-FU and QC or CBL0137. Normal saline 100 μ L was given to control group. Body weight and tumors volume were measured before each treatment. The mice were euthanized in gas canister with gradual fill carbon dioxide after the end of treatment cycles. Xenograft tumor was fixed in formalin and paraffin-embedded tissue sections were used to perform IHC staining Ki67 and TUNEL assay. The percentage of positive staining was quantified with 10 random high-power field images using TMARKER (23). Additive or synergistic effect was examined using online tool SynergyFinder (<https://synergyfinder.fimm.fi>) (24).

Statistical analysis

Results are shown as the mean $\pm$ SEM of three independent experiments. Paired data were evaluated by Student's t-test, and one-way analysis of variance (ANOVA) was used for multiple comparisons. Friedman test was used for non-parametric test. A p-value of less than 0.05 is considered statistically significant. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

Results

Downregulation of APE1 sensitizes dMMR CRC cells to 5-FU *in vitro*

To examine the role of APE1 in promoting 5-FU resistance in CRC, we used highly 5-FU resistant dMMR colon adenocarcinoma HCT116 (5) and isogenic HCT116 cells expressing APE1-specific shRNA (16). We found that downregulation of APE1 sensitized HCT116 to 5-FU (Fig. 1A). This phenomenon was confirmed in DLD-1 and HCT116 by siRNA interference (Fig. 1B), as evidenced by a decrease in the IC_{50} by approximately 10-fold (Fig. S1).

APE1 interacts with nucleosome remodeling histone chaperone FACT complex in chromatin

In the absence of highly selective and nontoxic small molecule inhibitors of DNA repair function of APE1 (25), we set out to identify targets that regulate APE1 function in cells. To identify the interacting partners of AcAPE1, we immunoprecipitated (IP) endogenous AcAPE1 from the chromatin fraction using our AcAPE1-specific antibody. After separation in SDS-PAGE followed by identification of protein bands by MALDI-TOF-TOF analysis, we identified a large number of proteins involved in the repair of damaged DNA as the prominent AcAPE1 interacting partners (Fig. 1C). We found DNA Ligase III, PARP1, both subunits (SPT16 & SSRP1) of FACT complex, nucleolin, chromatin assembly factor 1a (CHAF1a), and all four core nucleosome histones H2A, H2B, H3 and H4 in the AcAPE1 IP complex. We focused on the FACT complex because increasing evidence suggests that FACT complex plays a role at sites of UV damage and single-strand breaks (SSBs) in cells (26,27). FACT complex, a heterodimer of Structure-Specific Recognition Protein1 (SSRP1) and Suppressor of Ty (SPT16), was originally identified as a histone chaperone complex that facilitates the removal and deposition of histone H2A/H2B in nucleosome during transcription initiation and elongation (28,29). We confirmed the interaction of AcAPE1

with FACT complex by immunoprecipitating AcAPE1 from nuclear and chromatin extracts followed by Western blot analysis. We found both subunits of FACT in AcAPE1 IPs, in both chromatin and nuclear fractions (Fig. 1D & 1E). Confocal microscopy revealed colocalization of AcAPE1 with SPT16 and SSRP1 in the nucleus (Fig. 1F). To examine whether acetylation of APE1 is required for its interaction with FACT complex, we immunoprecipitated FLAG-tagged WT-APE1 and nonacetylatable K5R mutant from nuclear fractions. No significant differences were observed in the amount of SPT16 bound with WT and non-acetylatable K5R APE1 IPs, indicating that acetylation of APE1 is not essential for its interaction with FACT complex (Fig. 1G and 1H). We also used the FLAG-tagged N-terminal 33 amino acids deleted N 33 mutant and K5Q APE1 mutant which cannot enter the nucleus and stably bind to chromatin in cells (16,21). Our data showed that inhibition of either nuclear localization or chromatin binding of APE1 significantly reduced the amount of SPT16 or SSRP1 in APE1 IP (Fig. 1H). Together, these data indicate that APE1 forms complex with FACT in chromatin, and acetylation is not essential for this interaction.

Induction of AP sites enhances colocalization of AcAPE1 and FACT in chromatin

To determine whether induction of DNA damage promotes interaction of APE1 and FACT at damage sites, we generated AP sites in the genome by 5-FU or MMS treatment (a widely used alkylating agent that induces AP sites in the genome) (30). Structured Illumination Microscopy (SIM) revealed enhanced colocalization of both subunits of FACT complex with APE1 or AcAPE1 upon treatment (Fig. 2A, 2B & S2). The Pearson correlation coefficient (PCC) was used to quantify the degree (+1 perfect correlation to -1 perfect but negative correlation) of colocalization between fluorophores (Fig. 2C & 2D). There was significant increase of colocalization of APE1 or AcAPE1 with FACT complex upon induction of DNA damages, raising the possibility that recruitment of FACT to the damage sites may promote binding and acetylation of APE1 during the DNA repair process. We examined the levels of FACT and AcAPE1 in chromatin fraction at several time points following MMS treatment. Treatment of MMS resulted in increasing levels of AcAPE1 and both subunits of FACT complex in chromatin fraction in a time-dependent manner (Fig. 2E). Our Co-IP data showed that there was an increasing association of AcAPE1 and FACT complex upon induction of DNA damage (Fig. 2F & 2G). Previously, we showed that APE1 regulates *p21* expression via binding to the *p21* and *DTL* proximal promoter regions and functions as a coactivator or corepressor depending on the p53 status of the cells (31). To understand if FACT facilitates the recruitment and/or binding of APE1 to damage sites in *p21* and *DTL* promoters, we treated the cells with MMS and cross-linked the chromatin. We performed ChIP assays with AcAPE1 and SPT16 antibodies. We found that induction of DNA damage significantly increased the binding/occupancy of AcAPE1 and SPT16 to the *p21* and *DTL* gene promoter regions (Fig. 2H & 2I).

FACT complex facilitates the binding and acetylation of APE1 to damage site in chromatin.

To examine if FACT is required for facilitating the binding and acetylation of APE1 at damage sites in chromatin, we used siRNA to downregulate SPT16 and SSRP1 individually and both together (Fig. 3A). Consistent with prior report, we found that downregulation of either subunit SPT16 or SSRP1 affected the level of the other subunit in cells (32). We found a significant decrease of AcAPE1 level when both subunits of FACT were downregulated

(Fig. 3A). SIM demonstrated that FACT knockdown reduced the AcAPE1 level but did not alter total APE1 level in cells (Fig. 3B & S3A). As acetylation of APE1 occurs after binding to AP site in chromatin, these data indicate that the absence of FACT complex significantly reduced the access or binding of APE1 to damage sites and its subsequent acetylation in chromatin. We examined the binding or occupancy of unmodified APE1 and AcAPE1 to *p21* and *DTL* promoter in FACT downregulated cells by ChIP assays. ChIP assays revealed that FACT downregulation significantly abrogated the occupancy of APE1 and AcAPE1 to *p21* and *DTL* promoters upon DNA damages (Fig. 3C–3D and Fig. S3B–3C). Together, these data provide evidence that the FACT complex promotes the binding and subsequent acetylation of APE1 to damage sites in chromatin.

To further examine the role of FACT in regulating APE1 binding dynamics to damage sites, we used Fluorescence Recovery After Photobleaching (FRAP) (33) to quantify the mobility of GFP-tagged APE1 in the presence or absence of FACT complex. Fluorescence was bleached using an excitation laser, and the recovery of fluorescence in that region due to binding of new GFP-tagged APE1 into chromatin was monitored (33). The mobile fraction represents the fraction of recovered fluorescence and the half-life ($T_{1/2}$) is the time it takes for fluorescence intensity to reach half the maximum of the plateau level. In the presence of MMS, the mobile fraction of APE1 in FACT downregulated cells was significantly lower compared to control cells (Fig. 3E), suggesting that FACT regulates the mobility and binding dynamics of APE1 to damage sites in chromatin.

FACT is required for efficient repair of AP site damages in cells and downregulation of FACT sensitizes CRC cells to 5-FU

As FACT promotes binding and subsequent acetylation of APE1, we deduced that cells would accumulate AP sites in the absence of FACT. We depleted FACT complex by siRNA and quantitated AP sites in the genome. As expected, depleting FACT complex significantly increased the number of AP sites in the genome compared to control (Fig. 3F). We also treated these cells with MMS to induce AP site damages. As shown in Fig. 3F, AP sites accumulated significantly in the genome after MMS treatment in both control and FACT downregulated cells. However, after 6 hours of release, FACT knockdown cells retained significantly more AP sites, indicating that efficient AP site repair depends on the function of FACT complex. To provide further evidence for the role of FACT in facilitating the AP site or SSBs repair in cells, we used single cell alkaline comet assay which detects the SSBs and DSBs damages in the genome in cells. Knockdown of FACT significantly delayed the repair of MMS-induced DNA damages in the genome compared to control cells (Fig. 3G & 3H). Consistently, we found that downregulation of FACT sensitizes HCT116 and RKO cell lines to 5-FU (Fig. 3I). Together, these data indicate that FACT complex plays a crucial role in AP site or SSBs repair in cells.

Targeting FACT with curaxins enhances the efficacy of 5-FU in dMMR CRC cells *in vitro*

Several studies have shown that curaxins, a class of small molecule drugs (Fig. S4A), have broad anticancer activity and function as an inhibitor of FACT complex (34–36). FACT binds to unfolded nucleosomes and curaxins trap FACT in chromatin (34). Consistent with previous studies, our data show that Quinacrine (QC) (37), a first generation curaxin,

reduced SSRP1 and SPT16 levels from soluble nuclear fraction but had no effect on the chromatin-bound fraction (Fig. 4A). CBL0137, a second generation curaxin (38), exhibited a similar effect on FACT complex and decreased the level of AcAPE1 while total APE1 level remained unchanged (Fig. 4B – 4D & S4B). Of note, we found that HCT116 cells were unable to repair 5-FU- or MMS induced damage in the presence of FACT inhibitor CBL0137 (Fig. 4E–4F, S4C–S4D). Since our studies and others show that FACT is involved in AP site or SSBs repair in cells, we examined whether targeting FACT with CBL0137 enhanced the efficacy of 5-FU in dMMR CRC *in vitro*. To eliminate the possibility that QC or CBL0137 show cytotoxic effect *per se* by inducing DNA damages, cells were treated with different doses of QC or CBL0137 alone. We found minimal DNA damage with treatment of increasing doses of CBL0137 treatment on comet assay (Fig. S4E). Moreover, 4 μ M CBL0137 or 10 μ M QC alone had a minimal effect (20% cell death) on cell viability (Fig. S4F & S4G). However, combination of 2 μ M CBL0137 or 5 μ M QC with 5-FU significantly enhanced the sensitivity (~50-fold decrease in IC₅₀) of 5-FU resistant dMMR HCT116 and RKO cells to 5-FU (Fig. 4G & 4H), suggesting that targeting FACT complex with curaxins could be a promising strategy to overcome 5-FU resistance in dMMR CRC cells *in vivo*.

FACT inhibitor Curaxin sensitizes dMMR-CRC tumor to 5-FU *in vivo*

To examine whether the combination of curaxins and 5-FU inhibits dMMR-CRC tumor growth *in vivo*, we utilized tumor xenograft models. The effects of QC and CBL0137 were tested alone and in combination of 5-FU. Tumor growth curve showed that single agent treatment with 5-FU, QC or CBL0137 alone had very little or moderate effect on tumor growth compared to vehicle group (Fig. 5A–5D, S5A–5D), while combination group significantly inhibited tumor growth, demonstrating a synergistic effect. The combination of QC with 5-FU was well tolerated at the scheduled doses. All mice were weighted at each time point of treatment during the study, and there was 10-15% total weight loss at the end of study period. No cachectic appearance was noted (Fig. S5E). Moreover, no major histological abnormality was identified in vital organs including lung, liver and kidney (Fig. S5F). Further analysis showed that combination group suppressed the proliferation and induced apoptosis in these tumors (Fig. 5E–H). Additionally, long-term QC treatment resulted in decreased SSRP1 level in nucleus and the residual SSRP1 was trapped in chromatin (Fig. 5I), suggesting that QC alters FACT expression and localization *in vivo*. These data together demonstrate that inhibition of FACT function with curaxins can overcome 5-FU resistance and inhibits dMMR CRC growth both *in vitro* and *in vivo*.

FACT is overexpressed in colon cancer tissue and cell lines

Previously, we showed that primary tumor tissues of CRC and other types of cancer patients have higher AcAPE1 levels compared to adjacent-non-tumor tissues (19,39). Recent reports demonstrate that FACT expression is strongly associated with poorly differentiated cancers and low overall survival (32,35,36,38). Here we examined the levels of SSRP1 and AcAPE1 in CRC patients' tumor tissues. Both subunits of FACT complex and AcAPE1 were overexpressed in tumor but not in adjacent normal tissues (Fig. 6A & 6B). This finding was confirmed in various colon cancer cell lines using HCEC cells for comparison (Fig. 6C & 6D). These data indicate a potential role of overexpression of FACT and AcAPE1 in inducing chemoresistance.

FACT (SSRP1) expression and AcAPE1 levels positively correlate with chemoresistance in CRC patients

To determine the clinical significance of elevated levels of FACT and AcAPE1 in CRC, we extended our analyses by assessing SSRP1 and AcAPE1 levels in 39 CRC patients at different T stages. Among them, 19 patients had a moderate response and the other 20 had no response or minimal response to chemotherapy. Four (10.3%) were characterized as microsatellite instable or MMR deficient. This is consistent with prior reports that sporadic, noninherited dMMR CRC constitutes 10-15% of all CRCs (40). The percentages of positive cells and the staining intensity of SSRP1 and AcAPE1 level were significantly higher in CRC tumor tissue compared to control (Fig. 6E). Although SSRP1 or AcAPE1 staining varied among the samples within a particular stage of CRC, we found a significant increase in percentage of positive SSRP1 and AcAPE1 staining cells from stage T2 to T4, indicating that SSRP1 and AcAPE1 level increases with tumor depth invasion (Fig. 6F & 6G). Analysis of staining intensity of AcAPE1 and SSRP1 in tumor samples (characterized as low, medium and high intensity) revealed higher numbers of positive cells exhibiting high intensity staining with increasing tumor stage (Fig. 6H & 6I).

Next, we determined the relationship between FACT expression and acetylation of APE1 across all patient's samples. We revealed a moderate but significantly positive correlation between SSRP1 and AcAPE1 levels in CRC samples (Fig. 7A). We found that patients exhibiting no or minimal response to 5-FU had distinct staining patterns compared to patients exhibiting moderate responses (Fig. 7B). Quantitation of the percentage of positive staining cells showed that the percentage of positive SSRP1 or AcAPE1 was two-fold higher in non- or minimal responders compared to moderate responders (Fig. 7C & 7D). Additionally, three out of four patients who have loss of MSH2 and MSH6 were found to have minimal or no response and they had high levels of AcAPE1 and FACT (Fig. S6). Overall, these data indicate that the expression levels of SSRP1 and AcAPE1 positively correlates with 5-FU resistance in CRC patients.

Discussion

Resistance to 5-FU remains a major challenge in the treatment of dMMR CRC. Several mechanisms are believed to contribute to 5-FU resistance, including overexpression of TS enzyme due to gene amplification, deficient MMR pathway activity, and enhanced DNA damage repair resulting in reduced apoptosis (2,41). However, TS levels do not explain the observed therapeutic resistance to 5-FU in dMMR CRC (42), and several clinical studies have shown that defective mismatch repair is a strong predictor for lack of response of 5-FU based adjuvant therapy in dMMR CRC (2). Here, we demonstrate that loss of APE1 significantly sensitizes dMMR CRC to 5-FU, indicating the significant role of BER pathway in dMMR CRC.

Overexpression of APE1 in different cancer types including CRC and its association with chemotherapeutic resistance as well as poor prognosis are well documented (43). AP sites or SSB are common intermediates in the BER pathway that are generated after removal of damages bases induced by many chemotherapeutic drugs, including 5-FU and alkylating agents (13). The repair of AP sites/SSBs by APE1 on naked DNA or nucleosomal DNA

substrate has been extensively investigated *in vitro* (44). However, to date, how APE1 repairs AP sites in the context of the nucleosome in chromatin remains largely unknown. Earlier, we discovered that human APE1 could be acetylated (AcAPE1) at multiple lysine (Lys 6, 7, 27, 31, 32 & 35) residues in the N-terminal domain by p300 (16,45). Acetylation of these Lys residues modulates both DNA damage repair function of APE1 and the expression of multiple genes (16,18,45). Furthermore, we demonstrated that tumor tissues of diverse origins have higher levels of acetylated APE1 and the absence of APE1 acetylation sensitizes cells to many chemotherapeutic agents (19). It is likely that at the initiating steps of repair, DNA glycosylase responsible for removing the incorporated 5-FU facilitates the recruitment of FACT to sites of damage through physical interaction. Consistent with this, SSRP1 was shown to interact with OGG1 DNA glycosylase (46). We predict that FACT remains at damage sites and might cooperate to facilitate complete repair by promoting chromatin relaxation and subsequent recruitment of downstream repair proteins APE1 and XRCC1 through physical interaction (27). This may also facilitate the recruitment of histone acetyltransferase p300 to acetylate APE1 and acetylation in turn enhances the endonuclease activity of APE1 and promotes faster repair. Consistent with this hypothesis, a recent study shows that SSRP1 cooperates with PARP1 and XRCC1 to facilitate SSBs repair by chromatin priming (27).

The second generation curaxin CBL0137 is in the phase I multicenter clinical trial for metastatic or unresectable advanced solid malignancies (). This small molecule modulates several important signaling pathways through inhibition of FACT function (35,36). Increasing evidence suggests that CBL0137 itself has low direct cytotoxic effects (34,38). Our data also suggest that combination of curaxins with 5-FU has no major toxicity in vital organs in mice. Studies have shown that curaxins do not cause DNA damage or affect general transcription and are therefore expected to be well tolerated (35). We propose that combination of CBL0137 has several advantages, including high efficiency in reaching nuclear DNA (as they are not substrate for multidrug transporters), and high DNA affinity that facilitates altering nucleosome without causing DNA damage (34). Although our data show that combination of CBL0137 with 5-FU inhibits DNA damage repair and provides better cell killing *in vivo*, we cannot eliminate the possibility that curaxins also affect the expression of other genes involved in modulating the tumor growth or sensitivity to 5-FU.

In conclusion, our study identifies FACT complex as the interacting partner of APE1 and discovers a novel role of FACT in inducing 5-FU resistance via modulating APE1-dependent BER pathway. The FACT complex facilitates the recruitment and acetylation of APE1 which in turn promotes 5-FU resistance in dMMR CRC. In this preclinical study, we have shown that targeting FACT complex with small molecules CBL0137/curaxin significantly improves the efficacy of 5-FU in dMMR CRC *in vitro* and *in vivo*. The readily available small molecule, CBL0137, warrants further testing to determine its biodistribution, efficacy and toxicity profile *in vivo*. The combination therapy represents a highly translatable and targeted therapeutic approach that can be used clinically to overcome 5-FU resistance in dMMR CRC patients after further validation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

APE1	Apurinic/Apyrimidinic Endonuclease-1
BER	Base excision repair
CRC	Colorectal cancer
dMMR	Defective mismatch repair
FACT	Facilitates chromatin transcription
5-FU	5-Fluorouracil
SPT16	Suppressor of Ty 16
SSRP1	Structure Specific Recognition Protein 1

References

1. Andre T, Boni C, Mounedji-Boudiaf L, Navarro M, Tabernero J, Hickish T, et al. Oxaliplatin, fluorouracil, and leucovorin as adjuvant treatment for colon cancer. *N Engl J Med* 2004;350(23):2343–51 doi 10.1056/NEJMoa032709. [PubMed: 15175436]
2. Ribic CM, Sargent DJ, Moore MJ, Thibodeau SN, French AJ, Goldberg RM, et al. Tumor microsatellite-instability status as a predictor of benefit from fluorouracil-based adjuvant chemotherapy for colon cancer. *N Engl J Med* 2003;349(3):247–57 doi 10.1056/NEJMoa022289. [PubMed: 12867608]
3. Carethers JM, Smith EJ, Behling CA, Nguyen L, Tajima A, Doctolero RT, et al. Use of 5-fluorouracil and survival in patients with microsatellite-unstable colorectal cancer. *Gastroenterology* 2004;126(2):394–401. [PubMed: 14762775]
4. Sinicrope FA, Mahoney MR, Smyrk TC, Thibodeau SN, Warren RS, Bertagnolli MM, et al. Prognostic impact of deficient DNA mismatch repair in patients with stage III colon cancer from a randomized trial of FOLFOX-based adjuvant chemotherapy. *J Clin Oncol* 2013;31(29):3664–72 doi 10.1200/JCO.2013.48.9591. [PubMed: 24019539]
5. Bracht K, Nicholls AM, Liu Y, Bodmer WF. 5-Fluorouracil response in a large panel of colorectal cancer cell lines is associated with mismatch repair deficiency. *Br J Cancer* 2010;103(3):340–6 doi 10.1038/sj.bjc.6605780. [PubMed: 20606684]

6. Longley DB, Harkin DP, Johnston PG. 5-fluorouracil: mechanisms of action and clinical strategies. *Nat Rev Cancer* 2003;3(5):330–8 doi 10.1038/nrc1074. [PubMed: 12724731]
7. Sawyer RC, Stolfi RL, Martin DS, Spiegelman S. Incorporation of 5-fluorouracil into murine bone marrow DNA in vivo. *Cancer Res* 1984;44(5):1847–51. [PubMed: 6713387]
8. Kunz C, Focke F, Saito Y, Schuermann D, Lettieri T, Selfridge J, et al. Base excision by thymine DNA glycosylase mediates DNA-directed cytotoxicity of 5-fluorouracil. *PLoS Biol* 2009;7(4):e91 doi 10.1371/journal.pbio.1000091. [PubMed: 19402749]
9. Fischer F, Baerenfaller K, Jiricny J. 5-Fluorouracil is efficiently removed from DNA by the base excision and mismatch repair systems. *Gastroenterology* 2007;133(6):1858–68 doi 10.1053/j.gastro.2007.09.003. [PubMed: 18054558]
10. Tajima A, Hess MT, Cabrera BL, Kolodner RD, Carethers JM. The mismatch repair complex hMutS alpha recognizes 5-fluorouracil-modified DNA: implications for chemosensitivity and resistance. *Gastroenterology* 2004;127(6):1678–84. [PubMed: 15578504]
11. Meyers M, Wagner MW, Hwang H-S, Kinsella TJ, Boothman DA. Role of the hMLH1 DNA Mismatch Repair Protein in Fluoropyrimidine-mediated Cell Death and Cell Cycle Responses. *Cancer Research* 2001;61(13):5193. [PubMed: 11431359]
12. Mauro DJ, De Riel JK, Tallarida RJ, Sirover MA. Mechanisms of excision of 5-fluorouracil by uracil DNA glycosylase in normal human cells. *Mol Pharmacol* 1993;43(6):854–7. [PubMed: 8316218]
13. Li M, Wilson DM 3rd. Human apurinic/apyrimidinic endonuclease 1. *Antioxid Redox Signal* 2014;20(4):678–707 doi 10.1089/ars.2013.5492. [PubMed: 23834463]
14. Chattopadhyay R, Das S, Maiti AK, Boldogh I, Xie J, Hazra TK, et al. Regulatory role of human AP-endonuclease (APE1/Ref-1) in YB-1-mediated activation of the multidrug resistance gene MDR1. *Mol Cell Biol* 2008;28(23):7066–80. [PubMed: 18809583]
15. Mitra S, Izumi T, Boldogh I, Bhakat KK, Hill JW, Hazra TK. Choreography of oxidative damage repair in mammalian genomes. *Free Radic Biol Med* 2002;33(1):15–28. [PubMed: 12086678]
16. Roychoudhury S, Nath S, Song H, Hegde ML, Bellot LJ, Mantha AK, et al. Human Apurinic/Apyrimidinic Endonuclease (APE1) Is Acetylated at DNA Damage Sites in Chromatin, and Acetylation Modulates Its DNA Repair Activity. *Mol Cell Biol* 2017;37(6) doi 10.1128/MCB.00401-16.
17. Nath S, Roychoudhury S, Kling MJ, Song H, Biswas P, Shukla A, et al. The extracellular role of DNA damage repair protein APE1 in regulation of IL-6 expression. *Cell Signal* 2017;39:18–31 doi 10.1016/j.cellsig.2017.07.019. [PubMed: 28751279]
18. Sengupta S, Mantha AK, Mitra S, Bhakat KK. Human AP endonuclease (APE1/Ref-1) and its acetylation regulate YB-1-p300 recruitment and RNA polymerase II loading in the drug-induced activation of multidrug resistance gene MDR1. *Oncogene* 2011;30(4):482–93 doi 10.1038/onc.2010.435. [PubMed: 20856196]
19. Sengupta S, Mantha AK, Song H, Roychoudhury S, Nath S, Ray S, et al. Elevated level of acetylation of APE1 in tumor cells modulates DNA damage repair. *Oncotarget* 2016;7(46):75197–209 doi 10.18632/oncotarget.12113. [PubMed: 27655688]
20. Kim SH, Chang HJ, Kim DY, Park JW, Baek JY, Kim SY, et al. What Is the Ideal Tumor Regression Grading System in Rectal Cancer Patients after Preoperative Chemoradiotherapy? *Cancer Res Treat* 2016;48(3):998–1009 doi 10.4143/crt.2015.254. [PubMed: 26511803]
21. Jackson EB, Theriot CA, Chattopadhyay R, Mitra S, Izumi T. Analysis of nuclear transport signals in the human apurinic/apyrimidinic endonuclease (APE1/Ref1). *Nucleic Acids Res* 2005;33(10):3303–12. [PubMed: 15942031]
22. Hoogstraten D, Nigg AL, Heath H, Mullenders LH, van Driel R, Hoeijmakers JH, et al. Rapid switching of TFIIH between RNA polymerase I and II transcription and DNA repair in vivo. *Mol Cell* 2002;10(5):1163–74. [PubMed: 12453423]
23. Schuffler PJ, Fuchs TJ, Ong CS, Wild PJ, Rupp NJ, Buhmann JM. TMARKER: A free software toolkit for histopathological cell counting and staining estimation. *J Pathol Inform* 2013;4(Suppl):S2 doi 10.4103/2153-3539.109804. [PubMed: 23766938]

24. Ianevski A, He L, Aittokallio T, Tang J. SynergyFinder: a web application for analyzing drug combination dose-response matrix data. *Bioinformatics* 2017;33(15):2413–5 doi 10.1093/bioinformatics/btx162. [PubMed: 28379339]
25. Gordon MS, Rosen LS, Mendelson D, Ramanathan RK, Goldman J, Liu L, et al. A phase 1 study of TRC102, an inhibitor of base excision repair, and pemetrexed in patients with advanced solid tumors. *Invest New Drugs* 2013;31(3):714–23 doi 10.1007/s10637-012-9876-9. [PubMed: 23054206]
26. Dinant C, Ampatzidis-Michailidis G, Lans H, Tresini M, Lagarou A, Grosbart M, et al. Enhanced chromatin dynamics by FACT promotes transcriptional restart after UV-induced DNA damage. *Mol Cell* 2013;51(4):469–79 doi 10.1016/j.molcel.2013.08.007. [PubMed: 23973375]
27. Gao Y, Li C, Wei L, Teng Y, Nakajima S, Chen X, et al. SSRP1 Cooperates with PARP and XRCC1 to Facilitate Single-Strand DNA Break Repair by Chromatin Priming. *Cancer Res* 2017;77(10):2674–85 doi 10.1158/0008-5472.CAN-16-3128. [PubMed: 28416484]
28. Belotserkovskaya R, Oh S, Bondarenko VA, Orphanides G, Studitsky VM, Reinberg D. FACT facilitates transcription-dependent nucleosome alteration. *Science* 2003;301(5636):1090–3 doi 10.1126/science.1085703. [PubMed: 12934006]
29. Winkler DD, Luger K. The histone chaperone FACT: structural insights and mechanisms for nucleosome reorganization. *J Biol Chem* 2011;286(21):18369–74 doi 10.1074/jbc.R110.180778. [PubMed: 21454601]
30. Wyatt MD, Pittman DL. Methylating agents and DNA repair responses: Methylated bases and sources of strand breaks. *Chem Res Toxicol* 2006;19(12):1580–94 doi 10.1021/tx060164e. [PubMed: 17173371]
31. Sengupta S, Mitra S, Bhakat KK. Dual regulatory roles of human AP-endonuclease (APE1/Ref-1) in CDKN1A/p21 expression. *PLoS One* 2013;8(7):e68467 doi 10.1371/journal.pone.0068467. [PubMed: 23874636]
32. Garcia H, Miecznikowski JC, Safina A, Commane M, Ruusulehto A, Kilpinen S, et al. Facilitates chromatin transcription complex is an “accelerator” of tumor transformation and potential marker and target of aggressive cancers. *Cell Rep* 2013;4(1):159–73 doi 10.1016/j.celrep.2013.06.013. [PubMed: 23831030]
33. Reits EA, Neefjes JJ. From fixed to FRAP: measuring protein mobility and activity in living cells. *Nat Cell Biol* 2001;3(6):E145–7 doi 10.1038/35078615. [PubMed: 11389456]
34. Neshier E, Safina A, Aljahdali I, Portwood S, Wang ES, Koman I, et al. Role of chromatin damage and chromatin trapping of FACT in mediating the anticancer cytotoxicity of DNA-binding small molecule drugs. *Cancer Res* 2018 doi 10.1158/0008-5472.CAN-17-2690.
35. Dermawan JK, Hitomi M, Silver DJ, Wu Q, Sandlesh P, Sloan AE, et al. Pharmacological Targeting of the Histone Chaperone Complex FACT Preferentially Eliminates Glioblastoma Stem Cells and Prolongs Survival in Preclinical Models. *Cancer Res* 2016;76(8):2432–42 doi 10.1158/0008-5472.can-15-2162. [PubMed: 26921329]
36. Koman IE, Commane M, Paszkiewicz G, Hoonjan B, Pal S, Safina A, et al. Targeting FACT complex suppresses mammary tumorigenesis in Her2/neu transgenic mice. *Cancer Prev Res (Phila)* 2012;5(8):1025–35 doi 10.1158/1940-6207.capr-11-0529. [PubMed: 22689915]
37. Dermawan JK, Gurova K, Pink J, Dowlati A, De S, Narla G, et al. Quinacrine overcomes resistance to erlotinib by inhibiting FACT, NF-kappaB, and cell-cycle progression in non-small cell lung cancer. *Mol Cancer Ther* 2014;13(9):2203–14 doi 10.1158/1535-7163.mct-14-0013. [PubMed: 25028470]
38. Gasparian AV, Burkhart CA, Purmal AA, Brodsky L, Pal M, Saranadasa M, et al. Curaxins: anticancer compounds that simultaneously suppress NF-kappaB and activate p53 by targeting FACT. *Sci Transl Med* 2011;3(95):95ra74 doi 10.1126/scitranslmed.3002530.
39. Bhakat KK, Sengupta S, Adeniyi VF, Roychoudhury S, Nath S, Bellot LJ, et al. Regulation of limited N-terminal proteolysis of APE1 in tumor via acetylation and its role in cell proliferation. *Oncotarget* 2016;7(16):22590–604 doi 10.18632/oncotarget.8026. [PubMed: 26981776]
40. Walther A, Johnstone E, Swanton C, Midgley R, Tomlinson I, Kerr D. Genetic prognostic and predictive markers in colorectal cancer. *Nature Reviews Cancer* 2009;9:489 doi 10.1038/nrc2645. [PubMed: 19536109]

41. van Triest B, Pinedo HM, van Hensbergen Y, Smid K, Telleman F, Schoenmakers PS, et al. Thymidylate synthase level as the main predictive parameter for sensitivity to 5-fluorouracil, but not for folate-based thymidylate synthase inhibitors, in 13 nonselected colon cancer cell lines. *Clin Cancer Res* 1999;5(3):643–54. [PubMed: 10100718]
42. Sinicrope FA, Rego RL, Halling KC, Foster NR, Sargent DJ, La Plant B, et al. Thymidylate synthase expression in colon carcinomas with microsatellite instability. *Clin Cancer Res* 2006;12(9):2738–44 doi 10.1158/1078-0432.ccr-06-0178. [PubMed: 16675565]
43. Kakolyris S, Kaklamanis L, Engels K, Turley H, Hickson ID, Gatter KC, et al. Human apurinic endonuclease 1 expression in a colorectal adenoma-carcinoma sequence. *Cancer Res* 1997;57(9):1794–7. [PubMed: 9135024]
44. Li M, Volker J, Breslauer KJ, Wilson DM 3rd. APE1 incision activity at abasic sites in tandem repeat sequences. *J Mol Biol* 2014;426(11):2183–98 doi 10.1016/j.jmb.2014.03.014. [PubMed: 24703901]
45. Lirussi L, Antoniali G, Vascotto C, D'Ambrosio C, Poletto M, Romanello M, et al. Nucleolar accumulation of APE1 depends on charged lysine residues that undergo acetylation upon genotoxic stress and modulate its BER activity in cells. *Mol Biol Cell* 2012;23(20):4079–96 doi 10.1091/mbc.E12-04-0299. [PubMed: 22918947]
46. Charles Richard JL, Shukla MS, Menoni H, Ouararhni K, Lone IN, Roulland Y, et al. FACT Assists Base Excision Repair by Boosting the Remodeling Activity of RSC. *PLoS Genet* 2016;12(7):e1006221 doi 10.1371/journal.pgen.1006221. [PubMed: 27467129]

Figure 1. APE1 plays a pivotal role in inducing 5-FU resistance in dMMR-CRC cells and interacts with SSRP1 and SPT16.

A. HCT116 cells stably expressing control (ctrl) shRNA or APE1-shRNA were treated with various doses of 5-FU and viable cells were quantitated by MTT assay. APE1 levels in these cells were measured by Immunoblot analysis. **B.** APE1 level in HCT116 and DLD-1 was downregulated by siRNA transfection and cells were treated with 5-FU. Cell viability was measured by MTT. Immunoblot image showing the levels of APE1 after siRNAs transfection. Yellow asterisks mark the comparison between HCT116-ctrl siRNA and HCT116-APE1 siRNA. Red asterisks mark the comparison between DLD1-ctrl siRNA and DLD1-APE1 siRNA. **C.** Endogenous AcAPE1 in chromatin extracts was

immunoprecipitated (IP) and resolved in SDS-PAGE gel followed by MALDI-TOF-TOF analysis. **D & E.** Co-IP followed by Western blot analysis showed the presence of SSRP1 and SPT16 in AcAPE1 IP complex from nuclear and chromatin extracts. **F.** Colocalization of AcAPE1 with SPT16 and SSRP1 in nuclei was visualized using confocal microscope. Bar = 50 μ m. **G.** Schematic diagram showing the mutation (red) and deletion sites in the N-terminus of APE1. **H.** Cells were transfected with FLAG-tagged WT-APE1 or mutant APE1 expression plasmids and cell extracts were immunoprecipitated with FLAG antibody followed by Western blot analysis with SSRP1 and SPT16 antibodies.

Figure 2. Interaction of AcAPE1 with FACT complex enhances upon induction of DNA damages.

A & B. Colocalization of SSRP1 or SPT16 with AcAPE1 in HCT116 cells before and after treatment with MMS or 5-FU was examined by Structured-Illumination Microscopy (SIM). Representative images are shown. Bar = 5 μ m. **C & D.** Pearson correlation coefficient was used to quantify the colocalization of AcAPE1 with SSRP1 and SPT16. * $p=0.035$; *** $p<0.001$. **E.** Cells were treated with 1 mM MMS for various time periods as indicated. AcAPE1, SSRP1 and SPT16 proteins levels were examined in chromatin extracts. **F.** AcAPE1 was immunoprecipitated after MMS treatment and immunoblotted with SSRP1 or

SPT16 antibodies. **G.** Quantification SPT16 and SSRP1 in IP from **F** showing the fold change of SSRP1 and SPT16 levels before and after MMS treatment. **H & I.** Occupancy of AcAPE1 and SPT16 to *p21* and *DTL* promoter regions was examined before and after treatment with MMS by ChIP analysis.

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Figure 3. FACT complex facilitates AP site and SSB repair by facilitating APE1 access and acetylation in chromatin.

A. SPT16 or SSRP1 level individually or both together (FACT) was downregulated by siRNA for 24 hours and the levels of AcAPE1 and APE1 were measured by Immunoblot analysis. Note that FACT siRNA means siRNAs of SSRP1 plus SPT16. **B.** AcAPE1 level was examined in SPT16 and SSRP1 downregulated cells by SIM. **C & D.** Occupancy of AcAPE1 to *p21* and *DTL* promoters was examined before and after treatment with MMS by ChIP analysis. Yellow asterisks mark the comparisons between ctrl siRNA and FACT siRNA

without MMS treatment. Red asterisks mark the comparisons between ctrl siRNA and FACT siRNA at the presence of MMS. **E.** GFP-tagged APE1 expression plasmid was transfected in control or FACT downregulated cells and specific regions were bleached with laser and the recovery of GFP fluorescence was examined. **F.** Control and FACT downregulated cells were treated with MMS and then release for 6 hours. The number of AP sites in the genomic DNA was quantitated using aldehyde reactive probe. **G.** Control and FACT downregulated cells were treated with MMS and release for 6 hours. DNA damage was examined by single cell alkaline comet assay **H.** Average tail moment before and after MMS treatment was shown. **I.** Cell viability were examined after FACT KD in HCT116 and RKO cells. *** $p < 0.001$, **** $p < 0.0001$ (t test).

Figure 4. FACT inhibitor Curaxin inhibits efficient repair of 5-FU induced DNA damages and sensitizes dMMR CRC cells to 5-FU in vitro.

A - C. HCT116 cells were treated with indicated doses of QC or CBL0137 for 1 hour and whole cell extract (WCE), soluble nuclear and chromatin-bound fractions were prepared. SSRP1 and SPT16 levels in these extracts were examined by immunoblot analysis. **D.** Cells were treated with indicated doses of CBL0137 for 1 hour and the AcAPE1 level in cells was examined by SIM. **E.** HCT116 cells, pretreated with or without CBL0137 for 1 hour, were exposed to 5-FU for 6 hours and then allow to recover for 26 hours. DNA damage was examined by alkaline comet assay. **F.** Average tail moment before and after 5-FU treatment was shown. **G & H.** HCT116 and RKO were treated with and without QC or CBL0137 for 1 hour then exposed to various doses of 5-FU. Cell viability was measure by MTT assay. Yellow asterisks mark the comparisons between HCT116 and HCT116/CBL0137 (G) or

HCT116 and HCT116/QC (H). Red asterisks mark the comparisons between RKO and RKO/CBL0137 (G) or RKO and RKO/QC (H).

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Figure 5. FACT inhibitor Curaxin sensitizes dMMR-CRC tumor growth in vivo.

A & C. Vehicle, 5-FU, QC and combination of 5-FU and QC were administered to mice intraperitoneally for 3 weeks (**A**). In a separate experiment, 5-FU, CBL0137 and combination of 5-FU and CBL0137 were used (**C**). Resected xenograft tumor after completion of treatment were shown. **B & D.** Tumor volume was measured at indicated days and tumor growth curve was plotted. **E.** Paraformaldehyde-fixed xenograft tumor section from each treatment groups were stained for Ki67 to examine cell proliferation. **F.** TUNEL assay was performed in tumor sections and the representative images are shown. **G&H.** Box

chart depicting the Ki67 or TUNEL positive cell percentage among groups. Data report the median, 25th and 75th percentiles of percentages of positive cells. **I.** Tumor sections from each treatment groups were stained with SSRP1 antibody. Zoomed images of portions of the IHC staining indicate the chromatin trapping of FACT due to QC treatment.

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Figure 6. AcAPE1 and SSRP1 levels are elevated in human CRC samples and CRC cell lines. **A.** Levels of APE1, AcAPE1, SSRP1 and SPT16 in paired adjacent normal (N) and tumor (T) tissue extracts of CRC patients. **B.** The expression level of each protein in tumor was presented in fold change compared to normal adjacent tissues. Data were expressed as mean $\pm$ SEM of three independent experiments. **C.** The levels of AcAPE1, SSRP1, and SPT16 were analyzed in various colon cancer cell lines as compared to normal colon cell line HCEC. **D.** Bar graph showing elevated levels of SPT16, SSRP1 and AcAPE1 in multiple CRC cell lines compared to normal HCEC cells. Expression levels were presented in fold change with respect to normal HCEC cells. **E.** IHC staining of AcAPE1 and SSRP1 from a total of 39 CRC patients with different T stages were performed. Representative images are shown. **F & G.** The percentage of cells positive for AcAPE1 or SSRP1 from ten random high field in each sample was pooled. **H & I.** The percentage of cells with low, medium and high staining intensity in each group was analyzed and plotted.

Figure 7. Elevated level of AcAPE1 or SSRP1 in CRC patients is associated with poor treatment response to chemotherapy.

A. The expression of AcAPE1 and SSRP1 was analyzed using linear regression. **B.** Representative images of AcAPE1 and SSRP1 staining in patients with moderate and minimal/none response to chemotherapy are shown. **C & D.** The percentages of cells with positive AcAPE1 and SSRP1 staining were compared between moderate and minimal/none response groups.