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RAD51AP1 loss attenuates colorectal cancer stem cell renewal and sensitizes to chemotherapy

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Abstract

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DNA damage, induced by either chemical carcinogens or environmental pollutants, plays an important role in the initiation of colorectal cancer (CRC). DNA repair processes, however, are involved in both protecting against cancer formation, and also contributing to cancer development, by ensuring genomic integrity and promoting the efficient DNA repair in tumor cells, respectively. Although DNA repair pathways have been well exploited in the treatment of breast and ovarian cancers, the role of DNA repair processes and their therapeutic efficacy in CRC is yet to be appreciably explored. To understand the role of DNA repair, especially homologous recombination (HR), in chemical carcinogen-induced CRC growth, we unraveled the role of RAD51AP1 (RAD51-Associated Protein 1), a protein involved in HR, in genotoxic carcinogen (Azoxymethane, AOM)-induced CRC. Although AOM treatment alone significantly increased *RAD51AP1* expression, the combination of AOM and Dextran Sulfate Sodium (DSS) treatment dramatically increased by several folds. RAD51AP1 expression is found in mouse colonic crypt and proliferating cells. *RAD51AP1* expression is significantly increased in majority of human CRC tissues, including BRAF/KRAS mutant CRC, and associated with reduced treatment response and poor prognosis. *Rad51ap1* deficient mice were protected against AOM/DSS-induced CRC. These observations were recapitulated in a genetically engineered mouse model of CRC ($Apc^{Min/+}$). Further, chemotherapy-resistant CRC is associated with increased RAD51AP1 expression. This phenomenon is associated with reduced cell proliferation and CRC stem cell (CRCSC) self-renewal. Overall, our studies provide evidence that RAD51AP1 could be a novel diagnostic marker for CRC and a potential therapeutic target for CRC prevention and treatment.

Implications: This study provides first *in vivo* evidence that RAD51AP1 plays a critical role in CRC growth and drug resistance by regulating CRCSC self-renewal.

Introduction

Colorectal cancer (CRC) is the third most common cancer in both men and women, and the second leading cause of cancer-related death in the United States, with ~150,000 new cases and 53,200 expected deaths in 2020 (1). More than one-half of all these cases and deaths are associated with modifiable risk factors, such as smoking, an unhealthy diet, high alcohol consumption, reduced physical activity, or excess body weight (2). Although the currently available treatment regimens for CRC such as surgery, radiotherapy, chemotherapy, and the combination of these regimens has slightly improved the overall survival, the local recurrence, distant metastasis, and eventual relapse remain common complications for many CRC patients. Notably, the five-year overall survival for patients with metastatic CRC (mCRC) is only 14% (3-5). It is, therefore, imperative to explore novel diagnostic markers and pathways that may be the focus of developing new CRC therapies.

The DNA repair pathway has been considered as a promising target for cancer therapy development. DNA is constantly exposed to a variety of exogenous (e.g., UV rays, ionizing radiation) or endogenous (e. g., reactive oxygen species) genotoxic agents. In normal cells, the integrity of the genome is ensured by a very efficient DNA damage response signaling network, which includes cell-cycle checkpoints and DNA repair pathways. Cancer cells are thought to arise through the accumulation of numerous genetic alterations that confer growth and survival advantages, often circumventing checkpoints that initiate cellular senescence or

apoptosis. Dysregulation of DNA repair pathways can promote the accumulation of DNA errors leading to genomic instability, a major driving force for both cancer initiation and progression. Double strand breaks (DSBs) are the most damaging form of DNA and the genomic stability is dependent on the accurate repair of DSBs. A major mechanism by which DSBs are repaired is homologous recombination (HR), which is essential for the preservation of genome integrity and maintenance of accurate genome duplication (6, 7). In eukaryotic cells, HR is mediated by RAD51 or DMC1 recombinases, both of which are orthologs of bacterial RecA (8-10). Most chemotherapeutic agents and radiotherapy exert their cytotoxic effects by inducing DNA DSBs. The damage incurred by chemotherapeutic agents and radiotherapy are often recognized and repaired by DNA repair proteins. Therefore, aberrant expression of DNA repair proteins can be used as biomarkers to predict possible resistance to chemotherapy or radiotherapy (11).

Recent studies have shown enhanced expression of RAD51AP1 (Rad51-associated protein 1), a HR protein involved in D-loop formation during HR, in human cancers such as cholangiocarcinoma (12), hepatocellular carcinoma (13), acute myeloid leukemia (14), lung cancer (15), and, recently, breast cancer (16, 17). RAD51AP1 is a vertebrate-specific RAD51-interacting protein. RAD51AP1 interaction with either RAD51 or DMC1 recombinases greatly enhances their recombinase activity, stimulating D-loop formation (8-11), a key step in HR-mediated DNA repair (18-20). RAD51AP1 is involved in growth-promoting signaling (16, 17). RAD51AP1 knockdown cells showed increased levels of genomic instability (21, 22) and are sensitized to the cytotoxic effects of chemotherapeutic agents and ionizing radiation. However, the functional role of RAD51AP1 in chemical carcinogen-induced CRC growth and the underlying molecular mechanism(s) by which RAD51AP1 regulates tumorigenesis have not been fully understood. In this study, we provide direct evidence showing that RAD51AP1 may be critical for not only chemical carcinogen-induced CRC, but also in the early steps of neoplasia, where increased cell proliferation and replication stresses occur at higher than normal levels. Using *Rad51ap1* knockout mice, we provide genetic and biochemical evidence that RAD51AP1 is critical for tumor growth and stem cell self-renewal in CRC. We also provide evidence that enhanced *RAD51AP1* expression in human CRC is correlated with a poor prognosis and is associated with chemotherapy resistance. Given the strong correlation between *RAD51AP1* overexpression in CRC and reduced overall survival, RAD51AP1 could be recognized as a novel prognostic and diagnostic marker for CRC and an important target for anti-cancer therapies

Materials and methods

Institutional compliance:

The animal experiments reported in this study were approved by the Augusta University IACUC (Institutional Animal Care and Use Committee) and Biosafety Committee. Similarly, human CRC tissue and the surrounding normal tissues were obtained from the Augusta University tumor bank as per the approval of the Institutional Review Board and Human Assurance Committee as described in our previous manuscript (23).

Collection of normal and CRC tissues:

Paired normal colon and CRC specimens were collected from 18 adult patients with CRC, after obtained a written informed consent from the patients. Therefore, the studies were conducted in accordance with recognized ethical guidelines and that the studies were approval by an institutional review board (IRB) as described in our previous manuscript (23, 24). RNA was extracted from these tissues to determine *RAD51AP1* gene expression.

Cell lines:

The human colon cancer cell lines SW480 and SW620 cell lines were obtained from ATCC. These cell lines were grown in RPMI 1640 medium with 10% FBS. Both of these cell lines have been routinely tested for mycoplasma contamination using the Universal Mycoplasma Detection Kit obtained from ATCC (Manassas, VA), with the last mycoplasma test performed in April 2020. Mycoplasma-free cell lines were used in all of our experiments.

Generation of 5-flurouracil-resistant cell lines:

To generate a 5-flurouracil (5-FU)-resistant cells in the human CRC cell lines (SW480 and SW620), the stepwise method of treatment was used as described previously for cisplatin (25). SW480 and SW620 cells were treated with the predetermined 24h 30% inhibitory concentration (IC_{30}) of 5-FU for 3-4 days. The drug was then removed for 3-4 days before repeating the treatment. This treatment cycle lasted for 2-4 weeks before the drug dose was increased to the IC_{40} concentration. This process was repeated until the cells were growing successfully at IC_{80} concentration of 5-FU. The final concentration of 5-FU in drug-resistant cells was 100 μM. The final 5-FU-resistant cells were maintained at 1 μM 5-FU. Single cell-derived clones were obtained by limiting dilution, in which cells were serially diluted in a 96-well plate, and single clones were expanded.

Clonogenic assays:

Briefly, colonic epithelial cells derived from *Rad51ap1*$^{+/+}$ and *Rad51ap1*$^{-/-}$ mice were seeded in 6-well plates at $1x10^3$ cells/well. Cells were cultured for 2 weeks, changing the medium every 3 days, and then washed with PBS and fixed in 100% methanol for 30 min followed by staining with KaryoMax Giemsa stain for 1 h. The wells were washed with water and dried overnight at room temperature. Finally, cells were lysed with 1% SDS in 0.2 N NaOH for 5 min and the absorbance of the released dye was measured at 630 nm as described previously (26).

Animals:

C57BL/6 (Stock # 000664), *Rad51ap1*-knockout [B6N(Cg)-*Rad51ap1*$^{tm1.1(KOMP)Vlcg/J}$, Stock# 025176), and *Apc*$^{Min/+}$ (Stock # 002020) mice were obtained from the Jackson laboratories. All these mice were bred, maintained, and euthanized in Augusta University Animal Facility in accordance with the guidelines of the IACUC and Laboratory Animal Services (LAS) of Augusta University.

RNA isolation and PCR analysis:

Total RNA was prepared using Trizol according to manufacturer's protocol (Invitrogen). The quality and concentration of RNA were determined by Agilent Bioanalyzer/NanoDrop Spectrometer. Isolated and purified total RNA was reverse-transcribed by a cDNA synthesis kit (Invitrogen). Taqman and CT kit was used for real time expression from the sorted cells. Real-time PCR primers were designed using either Primer-BLAST (NCBI) or PrimerBank (https://pga.mgh.harvard.edu/primerbank/index.html).

Reverse transcriptase PCR:

Expression of RAD51AP1 mRNA was determined by semi-quantitative reverse-transcriptase PCR (RT-PCR). Total RNA, isolated from human and mouse tumor tissues, pLKO.1 and RAD51AP1shRNA transfected cells, was reverse-transcribed using the GeneAmp RNA PCR kit (Applied Biosystems). PCR was done on Veriti Thermocycler (Applied Biosystems) using human- and mouse-specific primers. Representative images of triplicate experiments are shown.

Immunoblot analysis:

For immunoblot analysis, cell lysates were prepared from cell lines and colon tissues as described previously (27, 28). Protein samples were fractionated on SDS-PAGE and transferred to Protran nitrocellulose membrane (Whatman GmbH). Membranes were blocked with 5% non-fat dry milk and exposed to primary antibody at 4 °C overnight followed by treatment with appropriate horseradish peroxidase-conjugated secondary antibody at room temperature for 1h, and developed by Enhanced Chemiluminescence Super Signal Western System and the signals captured on x-ray film.

Methods for: (1) Generation of *Rad51ap1*$^{-/-}$-*Apc*$^{Min/+}$ mice; (2) mouse genotyping; (3) AOM/DSS administration and analysis; (4) Analysis of *Rad51ap1-Apc*$^{Min/+}$ mice; (5) Colon single cell preparation, RNA isolation, and PCR analysis; (6) Analysis of stem/progenitor and colorectal cancer stem cells (CRCSCs); (7) Generation of colon organoids; (8) immunohistochemical analysis; and (9) MTT assay are provided in the Supplementary Methods.

Statistical analysis:

Statistical analysis was done using one-way ANOVA followed by Bonferroni multiple comparison test and also using Student's t-test with two-tail distribution. The software used was Graph Pad Prism, version 8.0. A value of $p < 0.05$ was considered statistically significant. Kaplan-Meier analyses (http://kmplot.com/analysis/) were used to assess group differences in tumor-free survival. TCGA database, UCGC genome, DAVID (http://david.abcc.ncifcrf.gov) and cBioportal (http://www.cbioportal.org) were also used to analyze *RAD51AP1* expression. GraphPad, Sigma Plot and Excel programs were used to draw figures.

Results

Rad51ap1 knockout mice are normal, fertile, and have normal colonic homeostasis:

To understand the role of DNA damage repair proteins in cancer growth and development, several knockout mice have been generated, especially for *Rad51*, *Brca1*, *Brca2*, and *Atm*. However, most of these knockouts were embryonically lethal and proved to be not useful for studying the functional role of these proteins in cancer growth. *Rad51ap1* knockout mice, however, are normal, fertile, and do not show any discernable abnormal phenotype. Therefore, *Rad51ap1* knockout mice offer a useful tool to study the role of DNA damage repair signaling in cancer growth and development. We examined the role of Rad51ap1 in colon tissue by comparing the morphology of colon between wild type ($Rad51ap1^{+/+}$) and *Rad51ap1* knockout ($Rad51ap1^{-/-}$) mice. Rad51ap1 is uniformly expressed in colon and small intestine (duodenum, jejunum, and ileum) (Fig. 1A-B and S1A). Homeostasis in the colon is maintained by a delicate balance between proliferation driven by stem cells at the base of the colonic crypts and apoptotic death in fully differentiated cells close to the luminal surface (29). This balance helps to regulate turnover in the colon and serves to prevent conditions favorable for carcinogenesis to occur. Changes in signaling mechanisms can tilt this balance one way or the other, thereby altering susceptibility for tumor formation (30). To determine if the loss of *Rad51ap1* has a significant effect on colon homeostasis, colon sections were stained with H&E to study morphometric changes, Ki67 to analyze cell proliferation, TUNEL and cleaved caspase 3 (CC3) to analyze apoptotic cell death. Morphometric analysis did not show any significant differences between wild type and *Rad51ap1* knockout mice (Fig. 1C and S1B). Similarly, the number of Ki67-, TUNEL-, and CC3-positive cells in the crypt was not significantly changed between these two genotypes (Fig. 1C). These results show that *Rad51ap1* knockout does not affect the balance of colonic homeostasis.

In the colonic crypt, proliferation and subsequent self-renewal of existing cells rely on its stem cell population, which is replenished roughly every 3.5 days (30). Therefore, self-renewal plays an important role in colonic epithelial cell formation and maintenance. To determine the self-renewal potential of the colonic stem cell populations, we collected colonic tissues from $Rad51ap1^{+/+}$ and $Rad51ap1^{-/-}$ mice, and prepared single cell suspensions as described previously (25, 31). We stained these cells with anti-Lgr5 antibody, a well-established colon stem cell marker (32), and CD24. We also generated colonospheres from single cells isolated from $Rad51ap1^{+/+}$ and $Rad51ap1^{-/-}$ mice. *Rad51ap1* deficiency significantly reduced colon stem cell populations ($Lgr5^{hi}CD24^{hi}$) and colonosphere size and number compared to wild type mice (Fig. 1D, F and G). To confirm this observation, we stained the colonic sections derived from $Rad51ap1^{+/+}$ and $Rad51ap1^{-/-}$ mice with anti-Lgr5 antibody. We found that Lgr5 mainly expressed in the basal crypt of the colon in both wild type and *Rad51ap1* knockout mice. However, the number of Lgr5 positive cells are significantly reduced in *Rad51ap1* deficient mice when compared with wild type mice (Fig. 1E and S1C). We further examined the expression of genes involved in stem cell self-renewal and found that *Rad5ap1* deletion significantly reduced Nanog (Fig. 1H), one of the stem cell master regulatory genes that has been linked to tumor initiation and progression

(33, 34). Taken together, these data provide evidence that *Rad51ap1* deletion does not affect the normal colonic epithelial homeostasis but does affect self-renewal signaling.

Increased RAD51AP1 expression in human CRC tissues is associated with poor prognosis:

cBioPortal database analyses uncovered evidence that *RAD51AP1* expression is not only amplified in CRC but also mutated in this cancer subtype (Fig. S1D). It is not clear, however, whether this mutation is associated with a loss of function, in which case RAD51AP1 functions as a tumor suppressor, or a gain of function wherein RAD51AP1 functions as a tumor promoter. We analyzed the levels of *RAD51AP1* gene transcript in paired samples of normal and CRC patients (n=18) and found that *RAD51AP1* expression is significantly increased in majority of CRC patients when compared to normal (Fig. 2A and B). This observation was further confirmed by the Oncomine database (TCGA) analyses which showed an increased expression of *RAD51AP1* in CRC tissues compared to their respective controls (Fig. 2C and D). This increased *RAD51AP1* expression in CRC patients predicted reduced overall survival (Fig. 2E). We also examined the correlation between BRAF/KRAS mutation and RAD51AP1 expression and its relevance to overall survival in CRC patients. We found that increased RAD51AP1 expression in BRAF/KRAS mutant CRC patients and is associated with reduced overall survival (Fig. 2F-G & S2A-B). We then stratified the overall survival of CRC patients who has low RAD51AP1 expression with KRAS wild type (Group 1) and low RAD51AP1 expression with KRAS mutation (Group 2) as well as high RAD51AP1 expression with KRAS wild type (Group 3) and high RAD51AP1 expression with KRAS mutation (Group 4). We found that the Group 1 patients (low RAD51AP1/ KRAS WT) have better survival and Group 4 patients (high RAD51AP1/KRAS MUT) have worst survival (Fig. 2H) among the four groups suggesting that highly proliferative cancer cells are more dependent on DNA repair proteins like RAD51AP1. We also analyzed the correlation between *RAD51AP1* expression and treatment response in CRC patients. We found that treatment responses are reciprocally associated with *RAD51AP1* expression, high *RAD51AP1* expression is correlated with unresponsive to the therapy and leading to stable disease (Fig. S2C-D). Given the strong correlation between *RAD51AP1* overexpression in CRC and the reduced overall survival, RAD51AP1 could be recognized as a novel prognostic and diagnostic marker for CRC and an important target for anti-cancer therapies.

Rad51ap1 deficiency protects against chemical carcinogen-induced colorectal tumorigenesis:

To understand the precise role of RAD51AP1 in the colon and its potential contribution to CRC, we used two different mouse models of CRC in the presence and absence of functional *Rad51ap1*: the inflammation-induced CRC model AOM/DSS [Azoxymethane (AOM)/Dextran Sodium Sulfate (DSS)], which is a chemically induced model of colitis-associated CRC (35) and the genetic model of CRC $Apc^{Min/+}$ (36). Intraperitoneal injection of AOM was performed in 12-week-old $Rad51ap1^{+/+}$ and $Rad51ap1^{-/-}$ mice. Seven days following AOM injection, the first course of DSS treatment (3% in drinking water) was begun for 7 days. Mice were then allowed to recover for two weeks before the subsequent round of DSS (1.5%) was begun. In total, mice received three rounds of DSS treatment (Fig. S2E). Mice were sacrificed 70 days after AOM injection and colons were examined for

polyps. Bodyweight of *Rad51ap1*$^{+/+}$ and *Rad51ap1*$^{-/-}$ mice were monitored and percent changes were calculated. There was no significant difference in weight loss between *Rad51ap1*$^{+/+}$ and *Rad51ap1*$^{-/-}$ mice (Fig. S2F). Analysis of *Rad51ap1* expression in colon tissues of wild type mice treated with AOM, DSS, and AOM+DSS revealed a significantly higher *Rad51ap1* expression in treated mice when compared to untreated control mice (Fig. 3A). We also analyzed the expressions of HR-associated genes that are directly associated with RAD51AP1, in AOM/DSS-induced CRC tissues. Expression of *Rad51*, *Atm*, *Brca1*, and *Brca2* have significantly increased in *Rad51ap1*$^{-/-}$ mice compared to *Rad51ap1*$^{+/+}$ mice (Fig. S2G). We next asked whether *Rad51ap1* deficiency protected or promoted CRC development. Interestingly, *Rad51ap1* deficiency protects AOM/DSS induced colorectal tumorigenesis (Fig. 3B & C). Colonic sections from AOM/DSS treatment group revealed a significant damage to the mucosa with epithelial erosion, inflammation with infiltrating leukocytes, loss of normal epithelial architecture, frequent ulceration, and loss of crypt structure in *Rad51ap1*$^{+/+}$ mice (indicated by arrows). This damage was significantly rescued by *Rad51ap1* deficiency (Fig. 3D and S2H). TUNEL staining also confirmed that increased epithelial erosion and loss of crypt structure by increased apoptotic cell death in AOM/DSS treated colon of *Rad51ap1*$^{+/+}$ mice and Rad51ap1 deficiency significantly rescued by reducing apoptosis (Fig. 3F-G and S2I). Ki67 staining further revealed that *Rad51ap1* deficiency is associated with significantly reduced cell proliferation (Fig. 3D & E). We also tested whether the increased epithelial damage observed in *Rad51ap1*$^{+/+}$ mice will lead to increased tumor burden by increasing CRC stem cells, analyzed by the expression of CRC stem cell marker Lgr5. We stained the tumor tissue sections derived from the AOM/DSS treated *Rad51ap1*$^{+/+}$ and *Rad51ap1*$^{-/-}$ mice with anti-Lgr5 antibody and found that higher expression of Lgr5 in the tumor tissues of *Rad51ap1*$^{+/+}$ mice when compared to *Rad51ap1*$^{-/-}$ mice (Fig. 3H). Taken together these results provide evidence that Rad51ap1 could play a critical role in inflammation-driven CRC and *Rad51ap1* deficiency provide barrier against inflammation-driven colorectal carcinogenesis.

Rad51ap1 deficiency reduces Apc$^{Min/+}$-driven CRC:

To better understand the role of RAD51AP1 in CRC further, we used the genetically engineered mouse model of CRC, *Apc*$^{Min/+}$ mice. Analysis of *Rad51ap1* expression in control and tumor tissues obtained from the *Apc*$^{Min/+}$ mice shows significantly increased expression of *Rad51ap1* in tumor tissues of *Apc*$^{Min/+}$ mice (Fig. 4A). This observation was further confirmed by the immunohistochemical (IHC) analysis of colon tissues from the control and *Apc*$^{Min/+}$ mice using anti-RAD51AP1 antibody (Fig. 4B). We then crossed *Rad51ap1*$^{-/-}$ mice with *Apc*$^{Min/+}$ mice and generated *Rad51ap1*$^{+/+}$-*Apc*$^{Min/+}$ and *Rad51ap1*$^{-/-}$-*Apc*$^{Min/+}$ offspring. Mice were monitored for 5-6 months for cancer burden, and then both small intestine and colons were assessed for polyps. *Rad51ap1*$^{-/-}$-*Apc*$^{Min/+}$ mice exhibited smaller and fewer tumors in the small intestine and colon, compared to their age-matched *Rad51ap1*$^{+/+}$-*Apc*$^{Min/+}$ littermates (Fig. 4C). H&E and Ki67 staining showed that *Rad51ap1* deficiency significantly protected the mice from *Apc*$^{Min/+}$-driven colorectal tumorigenesis (Fig. 4D-G and S3A & B). TUNEL staining provide further evidence that *Rad51ap1* deficiency significantly reduced *Apc*$^{Min/+}$-driven tumor growth by activating programmed cell death (Fig. 4H & I). Altogether, these results provide evidence that RAD51AP1 plays a critical role in CRC, and that *Rad51ap1* deficiency reduces tumor

growth in inflammation-driven as well as in a genetically engineered mouse (GEM) model of CRC.

Rad51ap1 deficiency reduces cell growth in mouse models of CRC by inhibiting stem cell self-renewal:

To dissect out the underling molecular mechanism by which *Rad51ap1* deficiency reduces tumor growth, we isolated colonic epithelial cells from *Rad51ap1*$^{+/+}$ and *Rad51ap1*$^{-/-}$ mice as described previously (16, 31). *Rad51ap1* deficient CRC cells grow significantly slower than the wild type (Fig. 5A & B). Colony formation assay confirmed this observation (Fig. 5C). Analysis of genes that involved in cell cycle regulation and apoptosis showed reduced expression of growth-promoting signaling, like *Cyclin D1* and *C-Myc*, and increased expression of apoptotic genes, like *Caspase 8* and Bax, in *Rad51ap1* deficient colon epithelial cells compared to wild type colon cells (Fig. 5D). We also generated colonic organoids (colonosphere) using the single-cell suspension prepared from the colonic tissue of AOM/DSS treated *Rad51ap1*$^{+/+}$ and *Rad51ap1*$^{-/-}$ mice, and found that *Rad51ap1* deficiency significantly reduced the organoid size and number (Fig. 5E & F). We also analyzed stem cell self-renewal genes in AOM/DSS-induced CRC and found that *Rad51ap1* deletion significantly reduced the expression of *Nanog*, *Kif4*, and *Epcam* (Fig. 5G). A similar observation was also recapitulated in *Rad51ap1*$^{-/-}$-*Apc*$^{Min/+}$ mice (Fig. 5H). Taken together, these results demonstrate the importance of RAD51AP1 in stem cell self-renewal signaling.

Chemotherapy resistance is associated with increased RAD51AP1 expression in human CRC cells:

Cancer stem cells (CSCs) have been implicated in resistance to conventional chemotherapy. CSCs are a small population of cells (1-2%) with unique characteristics, such as self-renewal, high proliferation rate, and the ability to generate heterogenic lineages of cancer cells. A tumor is a heterogenous entity with several CSCs, each with different mutational profiles and in different stages of the cell cycle. Within a tumor, however, there is usually one dominant population of CSC with a particular genetic profile driving tumor progression while other CSCs with varying other genetic profile wait for an opportunity to expand. This opportunity often comes when the dominant CSC population is destroyed during chemotherapy. The minor CSC population that was either in a quiescent state during treatment, or has acquired resistance to therapeutics, can emerge and promote new tumor growth. The newly emerged tumor is often more aggressive and resistant to the previous therapeutics, increasing the risk for metastasis and requiring a new treatment strategy to be devised.

As shown in Figs. 1, 3, and 5, RAD51AP1 appears to play an important role in CSC maintenance and self-renewal. Based on these data, we asked whether chemotherapy (5-Fluorouracil, 5-FU) resistance is correlated with an increased RAD51AP1 expression which in turn increases stem cell self-renewal. We generated 5-FU-resistant (5-FU-R) colon cancer cell lines in SW480 and SW620. Analysis of RAD51AP1 expression in 5-FU-sensitive (5-FU-S) and 5-FU-R cell lines revealed an increased RAD51AP1 expression in 5-FU-R cell line when compared to 5-FU-S cell line (Fig. 6A). We generated colonospheres using

SW480-5-FU-S and SW480-5-FU-R as well as SW620-5-FU-S and SW620-5-FU-R cell lines and found that 5-FU resistance is associated with increased colonosphere size and number (Fig. 6B & C). Then, we analyzed stem cell self-renewal genes and found a functional correlation among 5-FU resistance, *RAD51AP1* expression, and self-renewal gene expression (Fig. 6D & E). Taken together, these data provide evidence showing that RAD51AP1 plays a critical role in chemotherapy resistance and that *RAD51AP1* deficiency would not only potentiate chemotherapy response, but also resensitize the resistant cancer cells to chemotherapy. Overall, our results provide evidence that RAD51AP1 could be a novel diagnostic marker for CRC detection and a potential target for CRC treatment, especially for chemotherapy-resistant CRC.

Discussion

Although the CRC death rates are slowly declining in the United States and Europe (37, 38), the five-year overall survival for patients with metastatic CRC (mCRC) is only 14% (5). The standard chemotherapeutic regimen for mCRC is 5-FU in combination with either oxaliplatin (FOLFOX) or irinotecan (FOLFIRI) (39). Although these chemotherapeutic agents induce genotoxic damage in tumor cells, DNA repair pathways recognize and repair the DNA damage induced by these chemotherapeutic agents and recover the tumor cells from cell death (40). With this in mind, DNA-repair-targeting therapies needs to be used in combination with current chemotherapeutic drugs in CRC. In this study, we identified RAD51AP1 is overexpressed in CRC and also associated with chemotherapy resistance. We provided evidence showing that RAD51AP1 plays a critical role in tumor growth by regulating cancer stem cell (CSC) self-renewal. Although RAD51AP1 plays a central role in HR and genome integrity, it also plays an important role in growth by promoting signaling in early stages of tumor growth where replication stress occurs at higher than normal levels. Further, we also provided direct evidence that RAD51AP1 plays a critical role in chemotherapy resistance by targeting CSC self-renewal and that *Rad51ap1* deficiency could resensitize cancer cells for chemotherapy by inhibiting CSC self-renewal.

Despite recent studies showing increased expression of RAD51AP1 in human cancers (12-17), there was no evidence of RAD51AP1's involvement in tumor growth and CSC self-renewal in CRC. Our investigation shows that RAD51AP1 expression is increased in CRC and is inversely associated with chemotherapy response and overall survival. Furthermore, RAD51AP1 depletion in mice abrogated tumor growth in genetic mouse models of CRC. Importantly, however, Rad51ap1 depletion did not affect the normal epithelial homeostasis in colonic epithelium, suggesting that Rad51ap1 is dispensable for normal growth. Therefore, functional inactivation of RAD51AP1 would have far-reaching implications in cancer prevention and treatment without affecting normal cells. Additionally, given the overexpression of RAD51AP1 in CRC patients, it could be explored as a potential biomarker for CRC.

The colon and small intestines of the gastrointestinal tract are very unique and each contain their own unique populations of stem cells at the bases of their respective functional crypts (41). These stem cells are responsible for proliferation and subsequent self-renewal of existing cells that make up the organ, driving the complete turnover of luminal surface

every 3-4 days (29). These intestinal stem cells are therefore reliant on myriad of pathways to regulate their functions and maintenance of their population. This is how balance is effectively maintained in the colonic environment. Therefore, their capability for self-renewal is an important quality that is expected to have a profound effect on colon function if altered. Further, self-renewal plays an important role in the initiation of CRC. Cancer progression usually begins with the aberrant activation of one or more proliferative or self-renewal signaling pathways (30). Most of the CRCs are sporadic with tumor development occurring over the course of several years, rarely manifesting as cancerous polyps until patients are in their 50-60 years of age. This type of CRC is usually identified in patients in its early stages during preventative screenings and is therefore feasibly treatable by conventional therapies. However, there are two other types of CRC that manifest themselves before the screening age and are often only recognized in late stages or after the cancer has already metastasized to the liver. First, inflammation and high ROS levels can drive the development of tumorigenesis along a faster timeline (30). Therefore, inflammation-driven CRC often occurs in patients as young as in their twenties. Almost all patients with this form of cancer polyps have a pre-existing gastrointestinal disorder, such as inflammatory bowel disease (IBD). Second, CRC can also be brought on by genetic mutations present from birth - in particular, patients who have a condition known as familial adenosis polyposis (FAP) (1). These patients have one mutant copy of the *APC* gene. Later in life, they either develop or lose expression of the other copy, leading to the excessive formation of polyps. Some of these polyps may start out as benign, but eventually they will be driven towards carcinogenesis. This often occurs in patients much younger than 50 years of age and is therefore more difficult to detect before symptoms start to manifest. Typically, at that point, the tumor has reached its later stages and will, therefore, be harder to treat. In our mouse models of CRC, we utilized one of each of these forms of CRC. The AOM/DSS model mimics inflammation-driven CRC while $Apc^{Min/+}$ mimics FAP-driven CRC. However, in the latter model, polyps develop in the small intestine much more abundantly than in the colon, thus underlining an important distinction between man and mouse in terms of APC function. It is interesting to note that the loss of *Rad51ap1* decreased tumor size and polyp number in both models. This demonstrates that Rad51ap1's contribution to tumorigenesis is not bound by inflammatory or genetic origins, instead, self-renewal plays a much more significant role in driving tumor development. Several studies have demonstrated that mutations associated with changes in the proliferative capacity of colon stem cells are not sufficient enough to drive tumorigenesis. For example, hyperproliferation due to the loss of *Apc* can be induced in the intestinal compartment (42). However, without the aberrant expression of the nuclear factor-κB (NF-κB) due to either inflammation-driven pathways or subsequent mutation of KRAS, malignant tumors will not develop. Therefore, it is clear that CRC is quite a multi-faceted disease and loss of *Rad51ap1*, which is involved in DNA repair and stem cell self-renewal, leads to a significant reduction in tumor size and number, making it a promising candidate for anti-cancer therapeutics. If the function of Rad51ap1 is blocked in the colon, tumor progression may be inhibited, allowing for conventional therapeutics to destroy cancerous cells, and limiting its capacity to regenerate. It is feasible, therefore, that this two-hit treatment strategy would target not only colon CSCs but also prevent tumor recurrence. Though promising, more studies need to be conducted to investigate the

feasibility of long-term Rad51ap1 inhibition and whether or not CSCs can work around this obstacle to their self-renewal mechanisms.

In terms of the molecular mechanism(s) by which RAD51AP1 is involved in tumor growth and progression, our study provides evidence that RAD51AP1 plays a critical role in CSC self-renewal. CSCs are capable of driving tumor progression in its early stages, hence the alternate name of tumor-initiating cells for this population of cells (43). Normal stem cells are capable of driving excessive proliferation, a role they promote during early embryogenesis. These pathways, however, are strictly regulated in the adult stem cell to keep proliferation and self-renewal in check. When these pathways become deregulated, they have the capability to promote tumorigenesis. Even though they make up a small fraction of the tumor, the proliferative influence of CSCs is quite powerful and can have profound, abnormal, growth-promoting effects on non-CSCs (44). Therefore, targeting CSCs will aim to destroy cancer cells at its origin. In our study, *Rad51ap1* deficiency not only reduced the CSCs population but also their self-renewal capacity. This later effect is associated with a reduced stem cell population, sphere regeneration, and tumorigenesis. Self-renewal is the most efficient way to expand cancer cells quickly over a short period of time; however, proliferation only confers a slight growth advantage and takes longer to initiate (45). This notion is consistent with the heterogeneous populations among CSCs, with each population conferring different types of growth advantage. As these clones compete with one another to drive tumor growth and progression, a new set of clones always is ready to take its place when the current set is eradicated. This inherent ability of the cell to make more of itself ensures that a set of clones always survives. In summary, self-renewal is key for tumor maintenance and the loss of self-renewal would have disastrous consequences for the tumor. Our studies support this notion that inhibition of self-renewal of CSC has a profound impact on tumor growth and progression in CRC.

The loss of Rad51ap1 leads to loss of stem cell self-renewal capability through the reduction of NANOG. Self-renewal is maintained in stem cells by Notch, Wnt, and Hedgehog (Hh) signaling pathways (46). These signaling pathways promote regeneration through activation of NANOG, KLF4, OCT4, and SOX2 expression, making their stemness, and serving as a functional readout for self-renewal capacity. As a result, expression of one or a combination of these genes has been demonstrated to correlate with cancer development and progression. It has been shown that reduced self-renewal drivers, such as Notch, Hh, and Wnt, lead to decreased polyp number (46). Wnt is one of the chief drivers of aberrant proliferation in the colon, as Apc is one of its chief regulators. When Apc function is lost, Wnt becomes overexpressed and its signaling cascade drives self-renewal and stemness, whereas low Wnt signaling drives differentiation (47). A significant reduction in NANOG expression following the loss of RAD51AP1 is in part responsible for this reduction in self-renewal. There are four key genes involved in pluripotency - SOX2, OCT4, KLF4, and NANOG - of which NANOG is the master regulator. NANOG is a transcription factor involved in the maintenance of stem cell self-renewal. Many studies have demonstrated the role of NANOG in both normal and cancer stem cell function. High expression of NANOG is associated with several types of cancers from melanoma to glioma and from colon to hepatocellular carcinoma and also with the promotion of excessive proliferation, chemoresistance, inhibition of apoptosis, and activation of EMT - leading to metastasis (33).

NANOG expression exhibits a high capacity for self-renewal, which can easily promote initiation and growth of tumors. High levels of proliferation have been linked to excessive NANOG expression in cancer phenotypes (33). NANOG expression also promotes EMT and metastasis through positively regulating the TWIST/BMI1/SNAIL1/SNAIL2 signaling pathway, which has been well-documented to drive EMT (48). Knockdown of NANOG has been demonstrated to decrease this metastatic potential. Another perceived role of NANOG is in the regulation of E-cadherin expression. NANOG blocks E-cadherin, whose expression is essential in preventing EMT. Knockdown of NANOG has been shown to allow E-cadherin expression to renew (49). Therefore, decreasing the expression of NANOG would not only serve to reduce stem cell self-renewal but also promote apoptosis. In our study, we did not observe any drastic change in apoptosis following RAD51AP1 loss and associated decrease in NANOG expression. This may be due to cells entering cell cycle arrest instead, a hypothesis fully supported by the fact that compromised DNA repair also forces cells into cell cycle arrest (50). This is definitely an area for future studies to explore.

Accumulating evidences suggest that CSCs are responsible for the chemoresistance and cancer relapse, made possible by its ability to self-renew and also to differentiate into the heterogeneous lineages of cancer cells in response to chemotherapeutic agents (51, 52). Chemotherapeutic drugs target only the bulk of the cancer cells, but not CSC, and survival of CSCs reinitiate tumor formation in about 20-45% of patients within years or decades after treatment. Thus, targeting CSCs is an attractive strategy for cancer prevention and treatment. CSCs retain higher levels of DNA damage repair ability than normal healthy cells. Due to their high frequency of replication, understanding the mechanisms that promote successful repair and clearance of DNA strand breaks will allow us to determine the best way to undermine the system to either make cells more sensitive to conventional therapies or force them into apoptosis on their own. In this study, we have demonstrated the importance of RAD51AP1 in stem cell maintenance and also provided sufficient evidence showing that RAD51AP1 deficiency has a profound impact on stem cell self-renewal by inhibiting genes involved in pluripotency (Nanog and Klf4). Thus, reduced expression of Nanog in Rad51ap1 deficient mice could provide possible explanations for the reduced self-renewal and tumor growth in Rad51ap1 deficient mouse models of colon cancer. Therefore, RAD51AP1 should be considered as a novel target for cancer treatment that is applicable to a wide variety of cancer subtypes. Altogether, our study provides direct evidence that RAD51AP1 has great potential as a novel drug target for CRC prevention and treatment and for overcoming drug resistance to conventional chemotherapies.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments.

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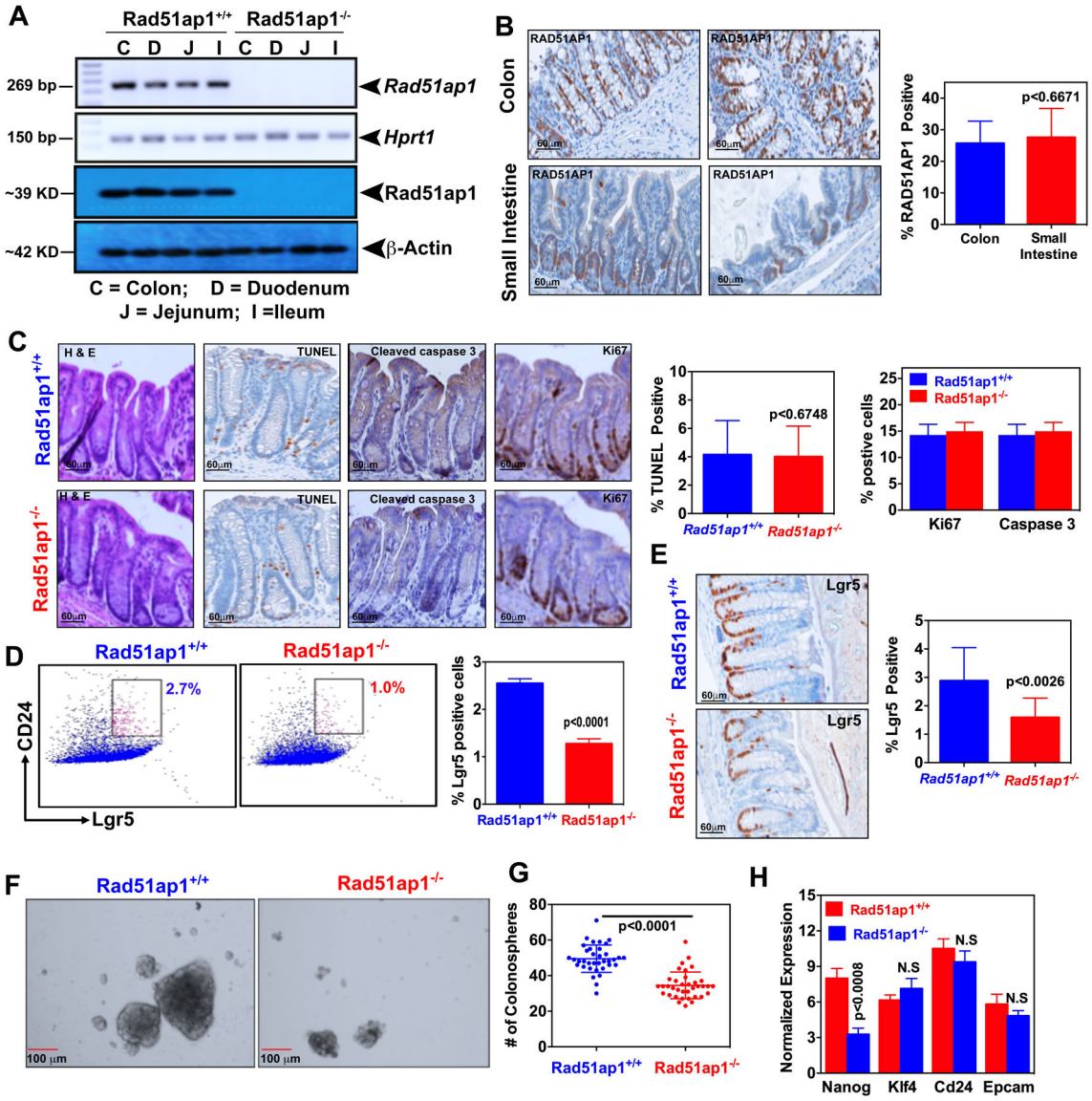

Figure 1.

Rad51ap1 deficiency did not affect the normal colonic epithelium but reduced colon stem cell self-renewal signaling. **A,** Representative semi-quantitative RT-PCR and western blot analyses show the *Rad51ap1* transcript and protein expression in colon, duodenum, jejunum, and ileum in *Rad51ap1*$^{+/+}$ and *Rad51ap1*$^{-/-}$ mice. (n=3 mice in each genotype). **B,** Rad51ap1 immunohistochemical (IHC) analysis using anti-RAD51AP1 antibody in mouse colon and small intestine shows that Rad51ap1 is expressed in basal crypt as well proliferating colonic epithelial cells in both colon and small intestine (n=3 mice). RAD51AP1 positive cells were counted and represented as a bar diagram (mean ± SD). **C,** Representative images of H&E, TUNEL, Ki67, and cleaved caspase 3 expression in colon tissues of *Rad51ap1*$^{+/+}$ and *Rad51ap1*$^{-/-}$ mice. (n=3 mice in each genotype). TUNEL, Ki67 and cleaved caspase 3 positive cells in *Rad51ap1*$^{+/+}$ and *Rad51ap1*$^{-/-}$ mice were counted and represented as a bar diagram (mean ± SD of n=3-5 mice in

each genotype). **D,** Representative FACS dot plots and quantification of colon stem like populations ($Lin^{-}Lgr5^{+}CD24^{+}$) in *Rad51ap1*$^{+/+}$ and *Rad51ap1*$^{-/-}$ mice. (n=3-5 mice in each genotype). **E,** Representative IHC image of anti-Lgr5 antibody staining in colon tissues of Rad*51ap1*$^{+/+}$ and *Rad51ap1*$^{-/-}$ mice. (n=3-5 mice in each genotype). Lgr5 positive cells in *Rad51ap1*$^{+/+}$ and *Rad51ap1*$^{-/-}$ mice were counted and represented as a bar diagram (mean ± SD, n=3 mice each genotype). **F and G,** Representative image of colonosphere and quantification of total colonosphere number derived from the single-cell populations of the colon tissues of *Rad51ap1*$^{+/+}$ and *Rad51ap1*$^{-/-}$ mice. (n=3-5 mice in each genotype). H, Normalized expression of stem cell self-renewal genes in the colonic tissues of *Rad51ap1*$^{+/+}$ and *Rad51ap1*$^{-/-}$ mice. (n=3-5 mice in each genotype).

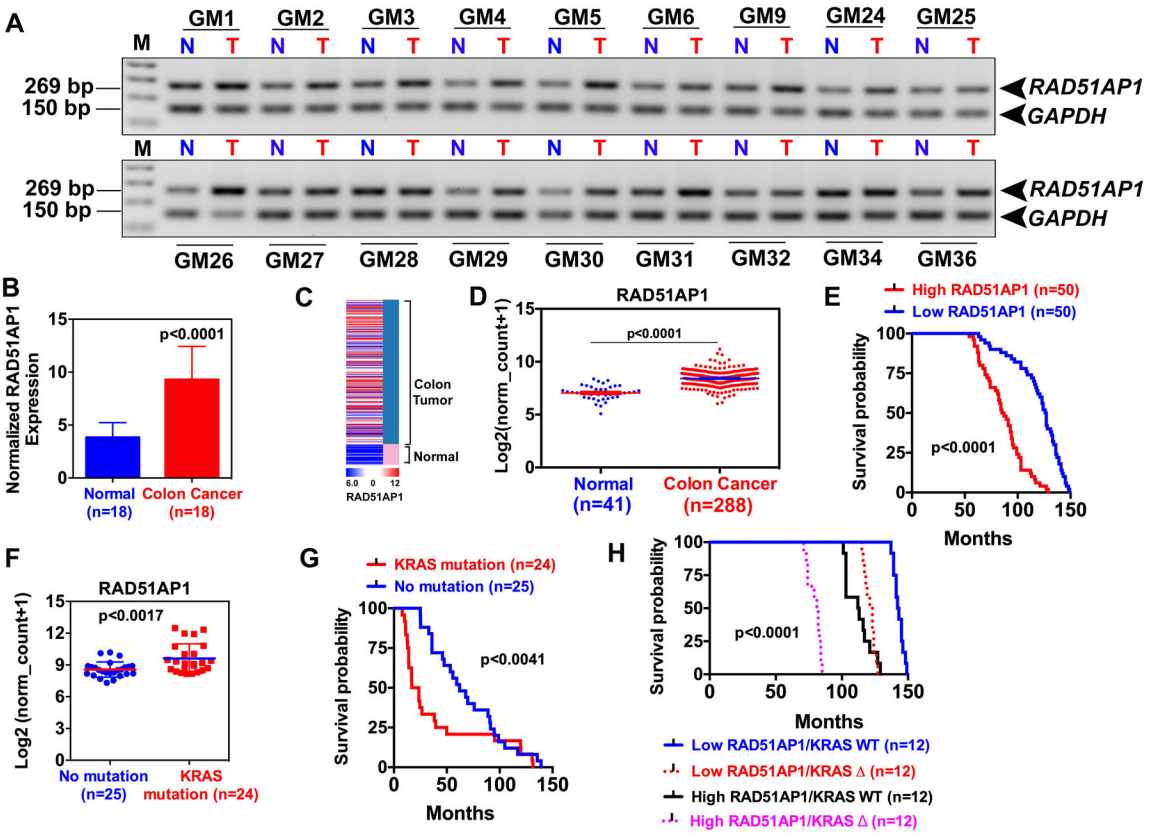

Figure 2.

Increased RAD51AP1 expression in human CRC tissues is associated with reduced overall survival. **A and B,** Representative images of semi-quantitative RT-PCR and qRT-PCR analyses show that high *RAD51AP1* mRNA expression in human CRC tissues (T) compared with their respective normal (N) tissues. **C and D,** Heat map and normalized *RAD51AP1* mRNA expression in log scale data generated from TCGA database analysis provide evidence that *RAD51AP1* expression is increased in human CRC tissues (n=288) compared to their respective control normal (n=41) tissues. **E,** Kaplan-Meier Plotter analysis show that low *RAD51AP1* expression (n=50) is associated with better overall survival when compared with high *RAD51AP1* expression (n=50). **F,** *RAD51AP1* expression was analyzed in KRAS mutant (n=24) CRC patients and compared with CRC patients without KRAS mutation (n=25). G, Overall survival probability between *RAD51AP1* expression and KRAS mutation was stratified from the TCGA database (n=24 with KRAS mutation and n=25 without KRAS mutation). **H,** Overall survival probability was stratified in CRC patients who had low *RAD51AP1* expression with and without KRAS mutation and high *RAD51AP1* expression with and without KRAS mutation from the TCGA database (n=12 patients in each).

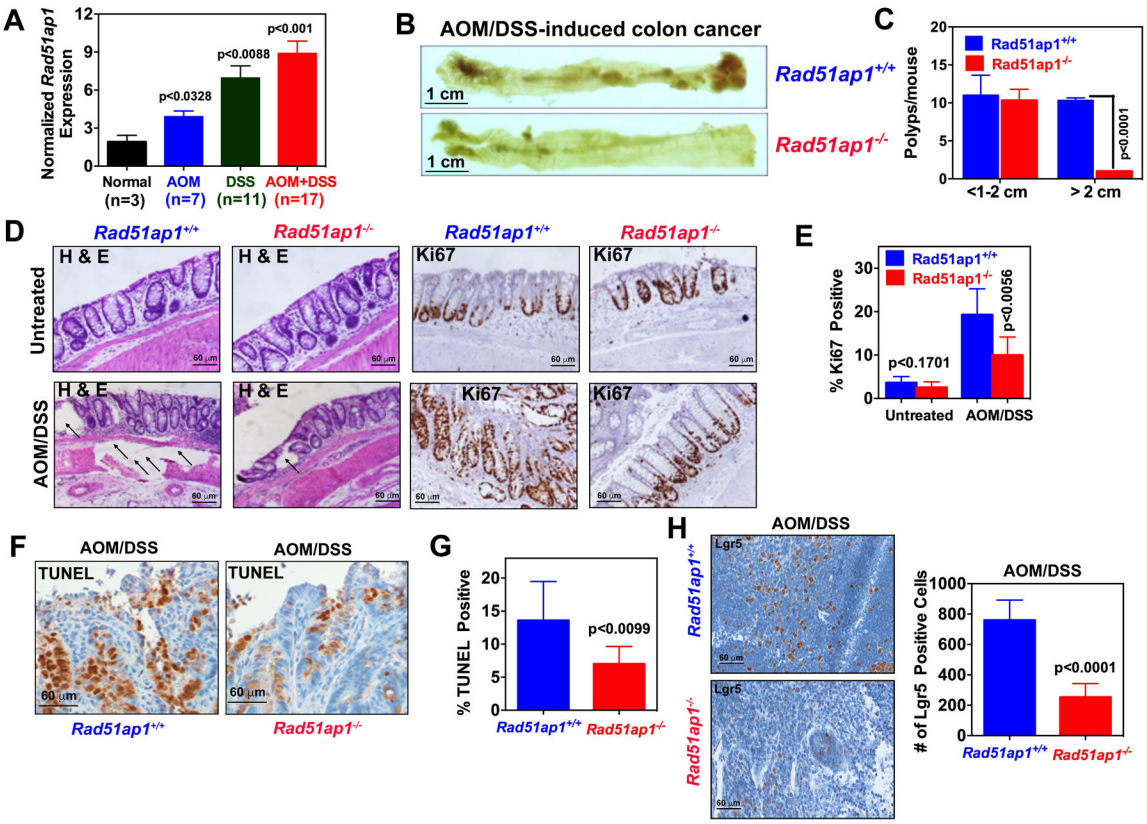

Figure 3.

Rad51ap1 deficiency reduces inflammation-induced (AOM/DSS) colon carcinogenesis. **A,** Real-time RT-PCR (qRT-PCR) analysis shows increased *Rad51ap1* gene transcript in colon tissues obtained from AOM (n=7), DSS- (n=11), and AOM+DSS (n=17) treated mice compared with normal control (n=3) mice. **B,** Representative images of colon polyps induced by AOM/DSS in *Rad51ap1*$^{+/+}$ and *Rad51ap1*$^{-/-}$ mice. (n=5-7 mice). **C,** Average polyp size (mm) separated into small (<1-2 cm) and large (>2 cm) polyps from colons of *Rad51ap1*$^{+/+}$ and *Rad51ap1*$^{-/-}$ mice. (n=5-7 mice in each genotype). **D,** Histological evidences observed from H&E and Ki67 staining reveal that *Rad51ap1* deficiency significantly protects the normal epithelial architecture by reducing the inflammation and infiltrating leukocytes in AOM/DSS-induced carcinogenesis when compared to *Rad51ap1*$^{+/+}$ mice (n=3-5 mice in each). **E,** Ki67 positive cells were counted in *Rad51ap1*$^{+/+}$-AOM/DSS and *Rad51ap1*$^{-/-}$-AOM/DSS mice and represented as a bar diagram (n = 3 mice). **F,** Representative images of TUNEL staining in *Rad51ap1*$^{+/+}$-AOM/DSS and *Rad51ap1*$^{-/-}$-AOM/DSS mice show that increased apoptosis in *Rad51ap1*$^{+/+}$-AOM/DSS mice. **G,** TUNEL positive cells were counted in *Rad51ap1*$^{+/+}$-AOM/DSS and *Rad51ap1*$^{-/-}$-AOM/DSS mice and represented as a bar diagram (n = 3 mice). **H,** Representative images of anti-Lgr5 antibody staining in tumor tissues of *Rad51ap1*$^{+/+}$-AOM/DSS and *Rad51ap1*$^{-/-}$-AOM/DSS mice show that high Lgr5 expression in *Rad51ap1*$^{+/+}$-AOM/DSS mice when compared witn *Rad51ap1*$^{-/-}$-AOM/DSS mice. Lgr5 positive cells were counted in *Rad51ap1*$^{+/+}$-AOM/DSS and *Rad51ap1*$^{-/-}$-AOM/DSS mice and represented as bar diagram (n = 3 mice).

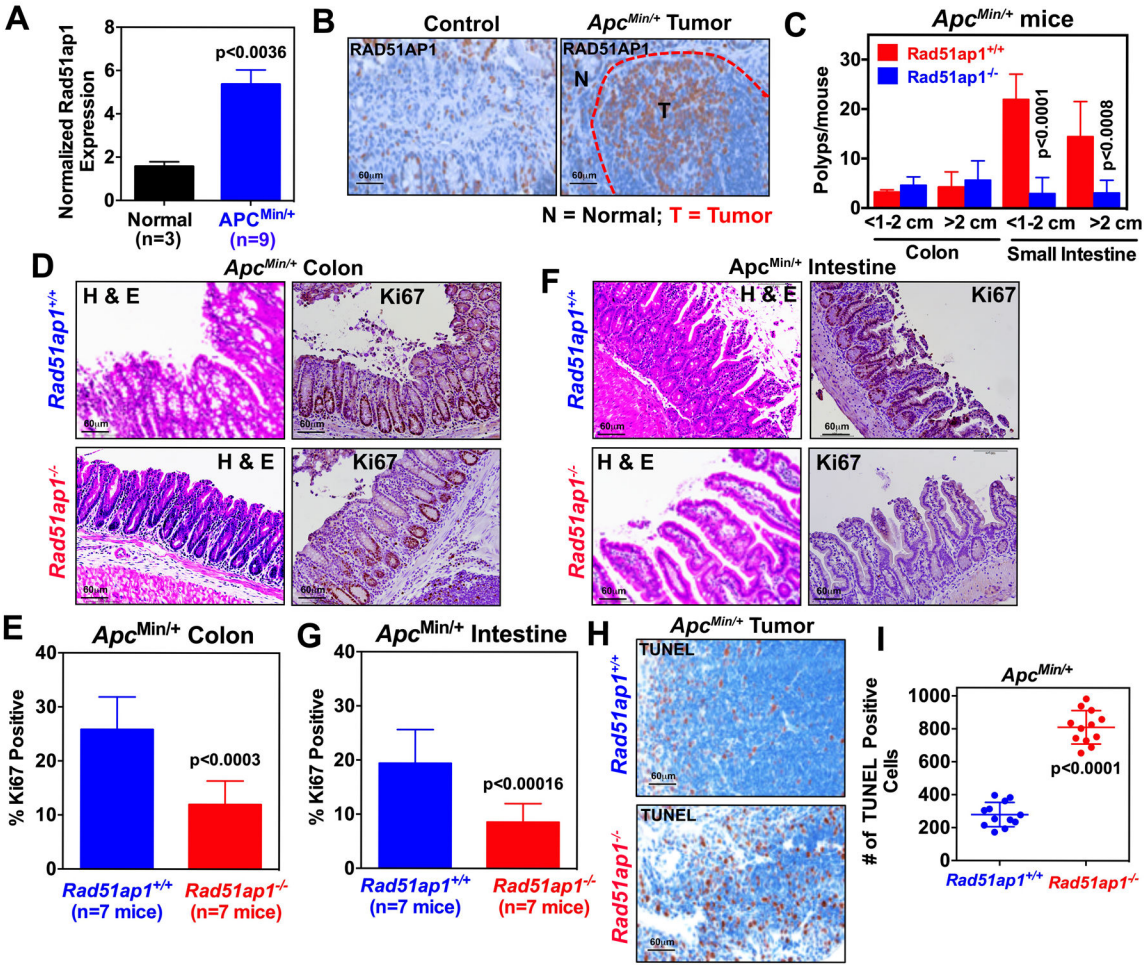

Figure 4.

Rad51ap1 deficiency reduces *Apc*$^{Min/+}$-driven colon carcinogenesis. **A & B,** Rad51ap1 mRNA and protein expression was analyzed by qRT-PCR and IHC analysis, respectively, in colon tissues harvested from control (n=3) and *Apc*$^{Min/+}$ (n=9) mice. **C,** Average polyp size (mm) separated into small (<1-2 cm) and large (>2 cm) polyps from colons and small intestine of *Rad51ap1*$^{+/+}$-*Apc*$^{Min/+}$ and *Rad51ap1*$^{-/-}$-*Apc*$^{Min/+}$ mice (n=5-7 mice). **D - G,** Morphometric analysis (H&E) and Ki67 staining reveal that *Rad51ap1* deficiency significantly protects mice from the *Apc*$^{Min/+}$-induced colon cancer (n=3-5 mice in each). **H and I,** Representative images of TUNEL staining in tumor tissues collected from *Rad51ap1*$^{+/+}$-*Apc*$^{Min/+}$ and *Rad51ap1*$^{-/-}$-*Apc*$^{Min/+}$ mice (n=5-7 mice). TUNEL positive cells were counted and represented as a bar diagram (mean ± SD, n=5 mice each genotype) .

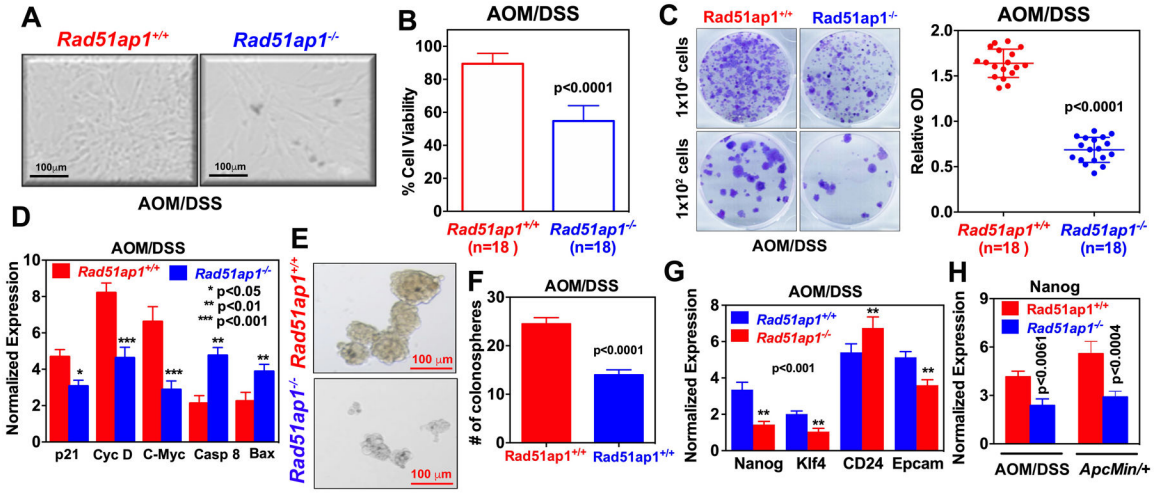

Figure 5.

Rad51ap1 deficiency reduces cell growth and self-renewal signaling. **A,** Representative images of colon epithelial cells isolated from AOM/DSS treated *Rad51ap1*$^{+/+}$ and *Rad51ap1*$^{-/-}$ mice and cultured *in vitro* in the 2D model. **B,** Cell viability assay was performed in the colonic epithelial cells as described in A by MTT assay and plotted as mean ± SD (n= 3 independent experiments with 6 replicates in each). **C,** Representative images of colony-formation assay using Giemsa staining show reduced colony number in colonic epithelial cells derived from *Rad51ap1*$^{-/-}$ mice than *Rad51ap1*$^{+/+}$ mice. Giemsa positive cells were lysed with lysis buffer and the released dye was measured and plotted (n=18). **D,** Normalized mRNA expression of genes involved in cell cycle regulation (p21, CycD, and C-Myc), and apoptosis (Casp8 and Bax) in AOM/DSS treated *Rad51ap1*$^{+/+}$ and *Rad51ap1*$^{-/-}$ mice. **E & F,** Representative images and quantitative count of *in vitro* 3D colonospheres derived from AOM/DSS treated *Rad51ap1*$^{+/+}$ and *Rad51ap1*$^{-/-}$ mice. **G,** Normalized mRNA expression of self-renewal genes *Nanog*, *Klf4*, *CD24*, and *Epcam* from AOM/DSS treated *Rad51ap1*$^{+/+}$ and *Rad51ap1*$^{-/-}$ mice. **H,** Normalized mRNA expression of *Nanog* from AOM/DSS treated *Rad51ap1*$^{+/+}$ and *Rad51ap1*$^{-/-}$ mice and *Apc*$^{Min/+}$-*Rad51ap1*$^{+/+}$ and *Apc*$^{Min/+}$-*Rad51ap1*$^{-/-}$ mice.

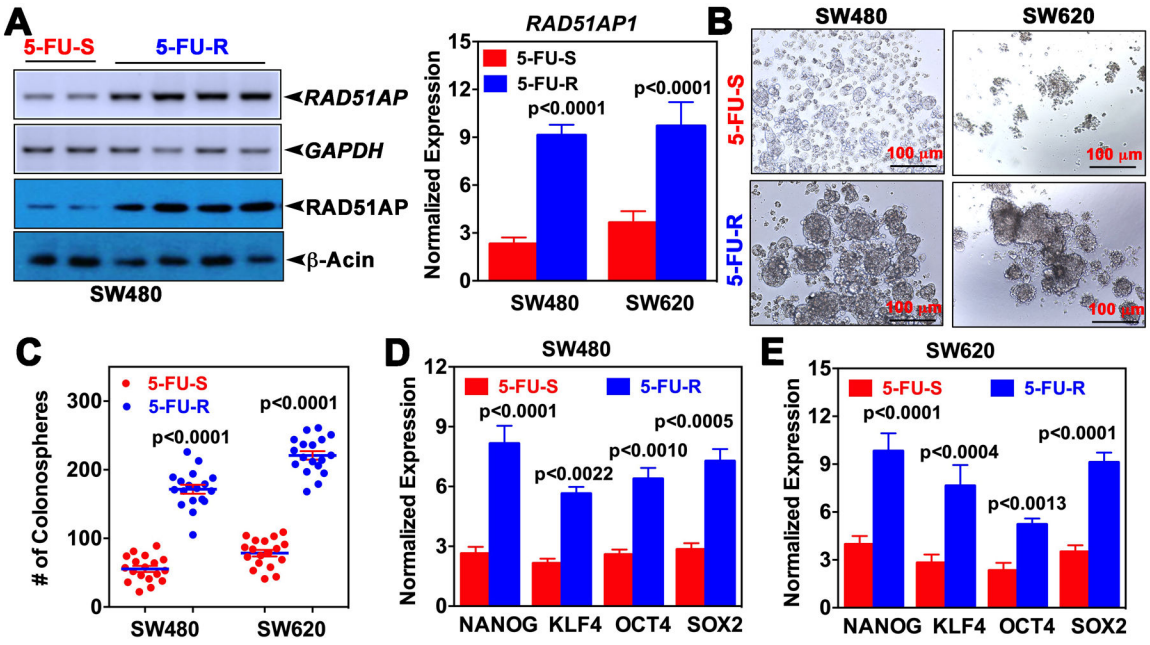

Figure 6.

Chemotherapy resistance is associated with enhanced RAD51AP1 expression and stem cell self-renewal signaling. **A,** Representative images of semi-quantitative RT-PCR, qRT-PCR, and western blot show increased RAD51AP1 expression in 5-flurouracil-resistant (5-FU-R) CRC cell lines (SW480 and SW620) than in the 5-flurouracil-sensitive (5-FU-S) CRC cell lines. (n=4 5-FU-R clones). **B and C,** Representative images of colonospheres and also colonosphere size and number derived from 5-FU-S and 5-FU-R SW480 and SW620 cell lines. **D and E,** Normalized expression of stem cell self-renewal genes in 5-FU-S and 5-FU-R SW480 and SW620 cell lines, respectively.