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Reduction of p53 by knockdown of the *UGT1* locus in colon epithelial cells causes an increase in tumorigenesis

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Abstract

BACKGROUND & AIMS—The UDP-glucuronosyltransferases (UGTs) are part of the cells machinery that protects the tissues from a toxicant insult by environmental and host cell metabolites. We have investigated the mechanism behind tumor growth and UGT repression.

METHODS—We initially silenced the *Ugt1* locus in human colon cell lines and investigated markers and responses linked to p53 activation. To examine the role of the *Ugt1* locus in p53-directed apoptosis and tumorigenesis, experiments were conducted to induce acute colon inflammation and chemical induced colon cancer in mice where we have selectively deleted the *Ugt1* locus in the intestinal epithelial cells (*Ugt1*$^{\Delta IEC}$ mice).

RESULTS—Knockdown of the UGT1A proteins by RNAi in human colon cancer cells and knockout of the *Ugt1* locus in intestinal crypt stem cells reduces phosphorylated p53 activation and compromises the ability of p53 to control apoptosis. Targeted deletion of intestinal *Ugt1* expression in *Ugt1*$^{\Delta IEC}$ mice represses colon inflammation-induced p53 production and pro-apoptotic protein activation. When we induced colon cancer, the size and number of the tumors were significantly greater in the *Ugt1*$^{\Delta IEC}$ mice when compared to wild type mice. Furthermore, analysis of endoplasmic reticulum (ER) stress-related markers indicated that lack of UGT1A expression causes higher ER stress in intestinal epithelial cells and tissue, which may account for the lower expression of p53.

CONCLUSIONS—Our results demonstrate that UGT1A expression is required to maintain and sustain p53 activation in stress-induced colon epithelial cells and has a significant impact on p53-

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mediated apoptosis and tumor suppression, thus protecting the colon tissue from neoplastic transformation.

Keywords

ER stress; apoptosis; UGT1A; colon cancer

Introduction

Colorectal cancer (CRC) ranks as the third most common cancer worldwide and the second leading cause of cancer-related deaths in western society [1, 2]. The majority of colorectal tumors are epithelial tumors, whereas lymphomas, endocrine tumors, and mesenchymal tumors are quite uncommon [3]. As an important extrahepatic tissue of xenobiotic metabolism, the colorectum is in direct contact with xenobiotic substances, including potentially toxic or carcinogenic agents, presumably leading to the high incidence rate of CRC [4, 5]. By contrast, cancers of the small intestine are rarely seen, even though the small intestine has a larger mucosal surface area than the colorectum [6]. One plausible explanation is that expression of biotransformation enzymes, including glutathione S-transferases, UDP-glucuronosyltransferases (UGT), and cytochrome P450s, are lower in colorectum than in small intestine. These enzymes are responsible for the detoxification of ingested toxins, carcinogens, or tumor promoting compounds, and their lower expression levels in colorectum are considered to be a contributing factor to the high rate of CRC [7, 8].

As an important part of the detoxification process, glucuronidation provides an effective metabolic process leading toward the biological inactivation of potential toxicants and carcinogens. Previous studies have demonstrated that gastrointestinal UGT activity decreases sharply from the small intestines towards the colon tissue [7]. This decrease in UGT activity contributes to a higher colonic DNA damage caused by carcinogens, such as heterocyclic amines and polycyclic aromatic hydrocarbons, which are usually detoxified through UGT glucuronidation [9–11]. It is speculated that glucuronidation provides a genoprotective defense against the mutagenic actions of chemical carcinogens. Studies found that UGT expression in colorectal tumor tissues is significantly reduced in comparison to surrounding healthy tissues [12–14]. Indeed, the pattern of UGT down-regulation is also identified in other types of cancer, including liver and biliary cancer [15], breast cancer [16], and bladder cancer [17]. These findings indicate that UGT expression is reversely correlated with tissue neoplastic transformation. However, there is no evidence that the UGTs impact the outcome of tumorigenesis, and the underlying mechanism regarding the role of the UGTs in cancer development is largely unexplored.

Recent studies have shown a link between the UGTs and p53, an important regulator of cell cycle, apoptosis, and tumorigenesis. Ariyoshi et al. observed increased constitutive UGT1A activity in *p53*$^{+/-}$ mice [18], and Hu et al. verified that epirubicin up-regulates UGT2B7 expression via a p53 pathway [19]. In contrast to these studies, our initial discovery uncovered an opposite causal relationship between UGT1A and p53 when challenging cells with chemical stress. Cell apoptotic death is a well-defined mechanism that is associated with cancer suppression when the body encounters tumor-promoting challenges. P53 and its

signaling network are known to play a critical role in the regulation of the cell cycle and apoptosis to conserve gene stability, thus suppressing tumor development [20, 21]. Upon occurrence of cellular stress, such as oncogene activation or DNA damage, p53 is activated. When cell damage is minimal, p53 evokes cell cycle arrest by inducing p21 to promote DNA repair and cell survival, whereas sustained p53 activation in response to high damage levels triggers cellular apoptosis, thus preventing the expansion of damaged cells and protecting normal tissue from neoplastic transformation [22, 23]. This is evidenced by the fact that the development of certain tumors in p53 null mice has been associated with decreased apoptosis, implying the important role of p53 in promoting cell death during tumor suppression [24]. In the present study, we explore the role of the UGT1A proteins in CRC by using colon cancer cell lines and an intestinal conditional knockout animal model deficient in *Ugt1* locus expression. By documenting molecular and cellular events that are associated with p53-dependent signaling, this study shed a light on the importance of UGT1A expression on p53-dependent stress responses and tumor suppression.

Materials and methods

Chemicals and reagents

Actinomycin D, etoposide, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), and azoxymethane (AOM) were purchased from Sigma (St. Louis, MO, USA), and dextran sulfate sodium (DSS, molecular weight 36,000–50,000) was obtained from MP Biomedicals (Santa Ana, CA, USA). The Quantitative-real time PCR (Q-PCR) primers were commercially synthesized from Integrated DNA Technologies, Inc (San Diego, CA, USA). Antibodies against UGT1A (Abcam, USA), p21 (Chemicon, USA), p53 and Bax (Santa Cruze, USA), and caspase-9 and caspase-3 (Cell Signaling, USA) were used in Western blot analyses.

Cell culture and UGT1A silencing

Human colon epithelial cell lines, HT29 and LS180, were obtained from the American Type Culture Collection (ATCC, USA). For *UGT1A* gene silencing, two pairs of *UGT1A*-specific siRNAs designed along with non-specific siRNA as a negative control were used. The siRNA (Invitrogen, USA) was mixed with the Lipofectamine RNAiMAX Reagent (Invitrogen, USA) in Opti-MEM I medium (Gibco, USA) at a finial concentration of 30 nM and was incubated at room temperature for 10 minutes according to the manufacturer's instructions. The mixture was added to culture plates, and exponentially growing cells were then seeded in these plates. Cells were incubated for 24–72 hours until further analyses.

Mouse crypt cell preparations and culture

Crypt cell isolation and culture were carried out as previously described [25]. Intestines from adult $Ugt1^{F/F}$ and $Ugt1^{\Delta IEC}$ mice were dissected, opened longitudinally and gently washed with ice cold PBS buffer. Intestinal tissue was then incubated in PBS buffer containing 2 mM EDTA at 4°C for thirty minutes. The buffer was removed and the tissue was shaken vigorously and then filtered through a 70-μm cell strainer. The filtrate was centrifuged at 1000g for 10 minutes to precipitate the crypt cells, followed by a wash with Advanced DMEM/F12 medium (Life Technologies). Cells were counted and approximately 1000

crypts were suspended into 50 μl of Matrigel (BDBiosience), and the cells plated into 24-well plates. Ten min later, 500 μl of crypt culture medium Advanced DMEM/F12 supplemented with B27 (Life Technologies), N2 (Life Technologies), 1 μM N-acetyl cysteine (Sigma), 100 ng/ml mNoggin and R-spondin 1 (conditioned medium, R-spondin 1 expression 293-HA-Rspol-Fc cell line was a generous gift from Dr. Calvin Kuo, University of Stanford) were added. Growth factors were added every other day and the medium was changed every 4 days. Cells were passaged every 1–2 weeks.

Cytotoxicity assay

Cells were seeded at 7,000 cells per well to a 96-well plate and incubated overnight. The cells were subsequently exposed to the indicated concentrations of actinomycin D or etoposide. After 36 hours, the MTT solution was added to each well at a final concentration of 0.5 mg/ml, and the plate was incubated at 37°C for another 4 hours. The MTT solution was then removed and 150 μL of DMSO per well was added. The absorbance at 595 nm was measured by a microplate reader.

Apoptosis assay

HT29 cells were exposed to the indicated concentration of actinomycin D or etoposide for 36 hours and then harvested by 0.25% trypsin without EDTA. The FITC Annexin V Apoptosis Detection Kit (BioLegend, CA, USA) was used to stain the cells. Samples were analyzed using a flow cytometer (BD FACSCalibur, USA).

Ugt1 conditional knockout mouse lines

Mice carrying *loxP* recombinase sites positioned in *Ugt1a1* intron 2 and intron 4 ($Ugt1^{F/F}$ mice) were previously generated [14]. When the mice were bred with *villin-Cre* mice, Cre-mediated recombination resulted in the deletion of exons 3 and 4 in the common region of the *Ugt1a* locus specifically in intestinal epithelial cells ($Ugt1^{\Delta IEC}$ mice), leading to knockout of the entire *Ugt1* locus.

Animal treatment with DSS or AOM plus DSS

For the acute colitis experiment, 8–10 week-old $Ugt1^{F/F}$ or $Ugt1^{\Delta IEC}$ mice were treated with 3% DSS in drinking water for 5 days, followed by 5 days of regular water. Mice were sacrificed and colons were removed, rinsed with PBS, and cut lengthwise into two segments. One segment was put into liquid nitrogen immediately and then stored at −80°C for subsequent analysis; the other segment was fixed as “Swiss-rolls” in 10% formalin at 4°C overnight and stored in 75% alcohol for paraffin-embedding. For the tumorigenesis study, 8–10 week-old $Ugt1^{F/F}$ or $Ugt1^{\Delta IEC}$ mice were intraperitoneally (i.p.) injected with 10 mg/kg AOM. After 5 days, 2% DSS was given in drinking water for 5 days, followed by 16 days of regular water. The cycle of DSS water followed by regular water was repeated twice. Mice were sacrificed 7 days after the last cycle; their colons were removed, analyzed for the presence of tumors (number counted and size measured), and prepared according to the description above.

TUNEL analysis

Paraffin embedded tissues were sectioned (5 μm) and analyzed with terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining, a common method for detecting DNA fragmentation resulting from apoptosis, by using the in situ cell death detection kit (Roche). DAPI counterstaining was performed before slides were mounted, and they were observed under a Leica fluorescence microscope.

Western blotting

All Western blots were performed by using NuPAGE BisTris-polyacrylamide gels (Invitrogen) with the protocols described by the manufacturer. Membranes were blocked with 5% skim milk and incubated with specific primary antibodies at 4°C overnight. Subsequently, the membranes were incubated with horseradish peroxidase-conjugated secondary antibody for 1 hour at room temperature. Blots were developed by a Western Lightning Plus-ECL agent (PerkinElmer) and were visualized under the BioRad gel documentation system.

Total RNA preparation and gene expression analysis by Q-PCR

The colon sample was homogenized in 1 mL of TRIzol (Invitrogen) and total RNA was extracted and used to generate cDNA with the iScript cDNA Synthesis Kit (BioRad). Following cDNA synthesis, Q-PCR was performed with the Soadvanced SYBR Green Supermix (BioRad) using a CFX96 Touch Real-Time PCR detection system (BioRad). The sequences of the primers used are listed in Supplement Table 1.

Statistical analysis

All data were obtained by at least three independent experiments and are presented as means ± SD. Statistical differences between two treatment groups were evaluated using the Student's t-test. When comparing induction responses between two groups, two-way Anova was performed followed by Bonferroni posttests. P values <0.05 were considered statistically significant, and statistically significant differences are indicated with *, P<0.05; **, P<0.01; ***, P<0.001.

Results

UGT1A knockdown by siRNA silencing and p53 expression

To explore the role of the UGT1A proteins in p53-dependent signaling, the *UGT1A* genes were silenced by UGT1A siRNA transfections in HT29 and LS180 colon cancer cell lines (Figure 1A and Suppl 1). When the cells were treated with anticancer drugs actinomycin D and etoposide, which are known to induce p53 [26, 27], both actinomycin D and etoposide induced p53 total protein expression in a dose-dependent fashion (Figures 1B and 1C, Suppl 1). This treatment led to spontaneous increases in p21 and Bax, both of which are well-defined p53 downstream target genes, along with increases in apoptosis markers caspase-9 and caspase-3 (Figures 1B and 1C). Loss of *UGT1A* gene expression led to a statistically significant decrease in actinomycin D- and etoposide-induced p53 expression in both HT29 and LS180 colon epithelial cells. Consequently, actinomycin D- and etoposide-mediated

induction of p21 and Bax and apoptosis biomarkers caspase-9 and caspase-3 was also inhibited by *UGT1A* silencing, indicating the requirement of UGT1A protein expression for p53 elevation and p53-dependent target gene induction.

The MTT cell proliferation assay measures total mitochondrial activity, which is a measurement of viable cells to assess anti-proliferative effects. The assay detects cell viability but cannot distinguish between apoptosis and proliferation, although proliferation is a common reference for this assay. We observed that the treatment of actinomycin D and etoposide blocked mitochondrial dehydrogenase activity (Relative Absorbance) in a dose-dependent manner in both HT29 and LS180 cells, indicating these agents are apoptotic or anti-proliferative. By comparison, knockdown of UGT1A proteins in these cell lines reduced anti-proliferative/apoptotic effects elicited by actinomycin D and etoposide (Figure 2A). We further detected the impact of chemical treatment on apoptosis by using Annexin V-FITC/PI staining, and the results show significantly fewer apoptotic cells in HT29 cells lacking UGT1A protein expression (Figure 2B). Collectively, these results demonstrate that interruption of UGT1A protein expression influences the regulation of p53 and p53-dependent cell cycle arrest and cell death signaling.

Inhibition of p53 expression in *Ugt1*$^{\Delta IEC}$ intestinal crypt stem cells

To examine if UGT1 protein expression has an impact on sustaining p53 activation through phosphorylation and the apoptosis markers in normal epithelial cells, intestinal crypt cells from *Ugt1*$^{\Delta IEC}$ and *Ugt1*$^{F/F}$ mice were cultured and treated with different concentrations of etoposide (Figure 3). Targeted knockout of the *Ugt1* locus in *Ugt1*$^{\Delta IEC}$ crypt cells led to a reduction in etoposide activated phosph-p53 when compared to the same treatments with *Ugt1*$^{F/F}$ crypt cells. We observed a dose dependent induction with a maximal response at 1 μM etoposide in *Ugt1*$^{F/F}$ mice. A similar profile was observed in *Ugt1*$^{\Delta IEC}$ mice with a statistically significant reduction at 1 μM. Since induction of p53 in both *Ugt1*$^{F/F}$ and *Ugt1*$^{\Delta IEC}$ mice plateaued at 1 μM etoposide, statistical significance was lost at 10 μM. Similar reduction patterns in apoptosis markers caspase 3 and p21 were also evident in *Ugt1*$^{\Delta IEC}$ mice, but there was a lack of statistical significance with caspase 3. The phospho-p53 measured in these experiments is wild type and not mutated p53, indicating that the impact of DNA damage and phosphorylation of p53 activation is linked to expression of the UGT1A proteins in normal epithelial cells. In addition, since induction of p53 protein expression does not result from transcriptional activation of p53 following etoposide treatment (Suppl 5), expression of the UGT1A proteins may interfere with post-translational modulation of the phosphorylation of p53.

Apoptosis is differentially induced in colons of *Ugt1*$^{\Delta IEC}$ and *Ugt1*$^{F/F}$ mice that developed acute colitis with dextran sodium sulfate treatment

As a well-established acute colitis model in mice, dextran sodium sulfate (DSS) through oral administration induces inflammation and a high level of intestinal epithelial cell (IEC) apoptosis in colorectum [28]. It is also appreciated that DSS-induced colonic apoptosis is dependent upon p53 [29]. With recent development of a mouse model targeting deletion of the *Ugt1* locus in the IECs (*Ugt1*$^{\Delta IEC}$ mice) [14], we treated control (*Ugt1*$^{F/F}$) and *Ugt1*$^{\Delta IEC}$ mice with 3% DSS for 5 days. The induction of inflammation leads to a decrease in UGT1A

expression (Figure 4A), indicating that chemically induced p53 activation is independent of UGT1A1 expression. Compared with mice not given DSS, DSS-treated mice exhibited significant increases in the levels of p53, p21, Bax, caspase-3 and caspase-9, accompanied by abundant apoptotic cells in colon tissues detected by the TUNEL assay (Figure 4B). When comparison was made between *Ugt1*$^{F/F}$ and *Ugt1*$^{\Delta IEC}$ mice, DSS-induced p53 and p53-dependent target gene products along with apoptotic biomarkers were significantly reduced in colons of *Ugt1*$^{\Delta IEC}$ mice (Suppl 2). By counting apoptotic epithelial cells in colon, the TUNEL assay showed that apoptosis occurred widely in both differentiated cells on the surface plateau and cells in the crypt proliferative zones in *Ugt1*$^{F/F}$ mice. However, DSS-treated *Ugt1*$^{\Delta IEC}$ mice displayed a 38% decrease in the number of apoptotic cells (Figures 4B and 4C), indicating the reduction of p53-dependent apoptosis in the absence of *Ugt1a* gene expression.

It is interesting to note that although DSS-induced acute colitis is believed to be through inflammatory responses by exposing innate immune cells in the lamina propria to bacteria [30], we found that the extent of colonic inflammation was not correlated to the level of IEC apoptosis between DSS-treated *Ugt1*$^{\Delta IEC}$ and *Ugt1*$^{F/F}$ mice. When *Ugt1*$^{F/F}$ mice were treated with DSS, apoptosis in the IEC was accompanied by severe inflammation and immune activation as the colitis scores and the levels of pro-inflammatory markers, such as tumor necrosis factor-α (TNF-α), interleukin (IL)-1β, IL-6, and IL-8, were increased in colon tissues (Suppl 3). Yet DSS-treated *Ugt1*$^{\Delta IEC}$ mice, exhibiting lower levels of colonic apoptosis, had similar colitis scores and expression levels of pro-inflammatory cytokines TNFα, IL-1β, IL-6, and IL-8 in colon tissues. Colon sections also showed similar morphologies and ulcer numbers in response to DSS treatment as evidenced by H&E staining (Suppl 3C and 3D), indicating no significant differences in inflammatory responses between *Ugt1*$^{\Delta IEC}$ and *Ugt1*$^{F/F}$ mice. These data suggest that *Ugt1* knockout in the IEC did not influence DSS-induced colon inflammation, and the alteration of apoptosis levels may be largely mediated through p53-dependent signaling.

Increased colorectal tumorigenesis in *Ugt1*$^{\Delta IEC}$ *mice with* azoxymethane plus DSS treatment

P53 inactivation and decreased levels of apoptosis have been associated with a higher risk of tissue neoplastic transformation. In particular, loss of p53 has been linked to enhancing the incidence and multiplicity of colitis-associated neoplasia [31, 32]. Since DSS-induced p53 was diminished in colons of *Ugt1*$^{\Delta IEC}$ mice, we further examined the impact of *Ugt1* deletion on CRC formation. To initiate colon tumorigenesis, *Ugt1*$^{F/F}$ and *Ugt1*$^{\Delta IEC}$ mice were injected with a single dose of azoxymethane (AOM) (10 mg/kg), a procarcinogen that induces DNA adduct formation following its metabolic activation by CYP 2E1 [33]. Subsequently, mice were treated with three cycles of 2% DSS administration in drinking water to induce colitis (Figure 5A) [34]. After 9 weeks of treatment, both groups of mice, regardless of genotype, developed colorectal tumors, which were mostly located in the middle to distal colon sections (Figure 5B). Strikingly, *Ugt1*$^{\Delta IEC}$ mice had a greater tumor number and larger tumor size than *Ugt1*$^{F/F}$ mice in response to AOM and DSS treatment. The number of detectable tumors was 62% higher in *Ugt1*$^{\Delta IEC}$ mice than in control *Ugt1*$^{F/F}$ mice (Figures 5B and 5D). In addition, approximately 15% of tumors in the *Ugt1*$^{\Delta IEC}$ mice were >3 mm in

diameter whereas none of the *Ugt1*$^{F/F}$ mice developed tumors >3 mm (Figure 3E). When we performed histological examinations, we found that tumors were adenomas with high-grade dysplasia and inflammatory cell infiltration (Figure 5C).

Western blot analysis showed that AOM plus DSS treatment led to a reduction in UGT1A expression in colonic tumor tissues of Ugt1$^{F/F}$ mice, a phenomenon previously observed in tumor tissue. Treatment with DSS following AOM initiation led to induced p53 and p21 in the control *Ugt1*$^{F/F}$ mice yet was abolished in the *Ugt1*$^{\Delta IEC}$ mice (Figure 6A). These results were similar to what we observed in mice treated with one cycle of DSS alone, although there was no difference in the expression levels of Bax and cleaved caspase-3 between *Ugt1*$^{F/F}$ and *Ugt1*$^{\Delta IEC}$ mice (Figure 6A). Quantitation of these Western blots is presented in Suppl 4. The TUNEL assay showed that *Ugt1*$^{F/F}$ and *Ugt1*$^{\Delta IEC}$ mice appeared to exhibit a similar level of apoptosis (Figures 6B and 6C). These data suggest that UGT1A proteins are an important factor in determining p53 levels following initial activation and protecting IECs from tumorigenesis. However, the p53-dependent signaling may play a lesser role in controlling apoptosis during the late stage of tumor development.

ER stress is involved in the down-regulation of p53 caused by UGT1A knockdown

Enzymes localized in the endoplasmic reticulum (ER) play a vital role in maintaining ER homeostasis in response to a variety of challenges. Recent studies have indicated that there may be regulatory links between drug transforming enzymes and ER stress responding to xenobiotic stimuli [35]. It is also known that ER stress plays a role in accelerating p53 degradation and inhibiting cell apoptosis [36, 37]. With the known and important association between p53 and ER stress, we elected to explore the involvement of ER stress in UGT1A-dependent p53 regulation by examining ER stress marker genes, including *GRP78*, *GRP94*, spliced *XBP1*, *ATF4*, by Q-PCR (Figure 7). In both intestinal HT29 and LS180 cell lines and the *Ugt1*$^{\Delta IEC}$ mouse model, we found that actinomycin D, etoposide, and DSS, acting as ER stress-inducing agents, induced gene expression of all of the ER stress markers that we examined in addition to activating p53 induction. The knockdown of the *Ugt1a* genes further potentiated ER stress, resulting in significantly higher expression of ER stress markers (Figure 7). These results indicate that UGT1A expression may have a role in maintaining ER homeostasis, particularly when the cells are under stress, and that increased levels of ER stress resulting from UGT1A knockdown may have a direct impact on expression and function of p53 after p53 activation has taken place.

Discussion

In healthy cells and organs, low p53 levels are maintained by Mdm2 through a negative-feedback loop [38]. When cells sense stress signals, p53 can accumulate and transcriptionally regulate genes to control cell cycle and cell death. For example, p53 accumulation can lead to cell cycle arrest by inducing expression of p21, a cyclin-dependent kinase inhibitor. P53 accumulation is also required for the occurrence of apoptosis by inducing the expression of various pro-apoptotic genes, such as *Bax*, *caspase-3* and *caspase-9* [39]. The maintenance of the growth-inhibitory responses by elevated levels of p53 is essential for cells to adapt to the stress and accounts for p53-dependent anti-tumorigenesis activity. In this study, we found

that when intestinal cells are under stress, induced by actinomycin D or etoposide, UGT1A expression is required for the maintenance of elevated levels of p53, which determines whether the cell undergoes apoptosis and/or cell arrest. The absence of UGT1A expression, through siRNA silencing, disturbs this adaptive mechanism initiated by p53, leading to inhibition of up-regulation of p53-dependent target genes, such as proapoptotic genes *caspase-3* and *caspase-9*, and to subsequent inhibition of apoptosis. When an in vivo study was performed using DSS to induce colitis in colon tissue, we observed p53 accumulation accompanied by a higher incidence of apoptosis. However, when colitis is induced in mice deficient in intestinal *Ugt1a* gene expression, p53 accumulation is reduced and apoptosis is inhibited. More importantly, when challenged with AOM in combination with DSS, $Ugt1^{\Delta IEC}$ mice exhibited a large increase in tumor multiplicity and size compared to control mice, indicating that activated p53 requires UGT1A to maintain and exert its function in protecting cells from tumorigenesis.

Down-regulation of UGT1A proteins occurs in solid tumors when compared to the surrounding healthy tissue [13–17]. UGT down-regulation is also observed in inflammation-related diseased organs (e.g., intestine and liver) [40, 41]. Possible contributing factors that cause a decrease in *UGT1A* gene expression in tumor and/or diseased tissues have been reported to be epigenetic modulation, inflammatory factor-mediated inhibition, and reduction of xenobiotic nuclear receptor (XNR) expression [42]. These studies emphasize that the down regulation of *UGT1A* genes is the result of the disease state in various tissues and its impact on the detoxification capacity of xenobiotics, especially cancer drugs. By comparison, we report that under stress, UGT1A expression is a key factor that determines p53-dependent cell cycle regulation and cell death and has a direct impact on anti-tumorigenesis in colon tissue. Our result is of significance because it indicates that UGT1A proteins play a role in cell protection through both chemical detoxification and - reported here for the first time - maintenance of p53 activation in response to cellular stress. Reduced expression of *Ugt1a* genes in the diseased colon tissue not only leads to lower detoxification capacity for their substrates, it also exacerbates the disease condition by inhibiting p53-dependent pro-apoptosis and/or cell arrest. This finding may explain that the patients with inflammatory diseases often have a higher risk of developing cancer [43] because lower UGT1A expression in the disease condition and subsequent inactivation of the p53-dependent anti-tumorigenesis pathway may be one of the contributing mechanisms that account for the tumorigenic action. We speculate that UGT1A activity may have a global effect on maintaining p53 activation in various tissues when cells encounter stress.

A central mechanism linking UGT1A expression and the sustainment of activated p53 is unclear. We explored the involvement of ER stress since it has been shown to induce p53 cytoplasmic localization and degradation, preventing p53-dependent apoptosis [36, 37, 44]. ER stress is activated by physiological conditions such as nutrient deprivation or by xenobiotic and drug toxicities, leading to the accumulation of unfolded proteins [45]. The generation of unfolded proteins stimulates an adaptive process, the unfolded protein response (UPR) that leads to the activation of cellular machinery needed to repair the unfolded proteins. Interestingly, recent studies indicate that there may be a regulatory link between ER stress and biotransformation enzymes: in vivo and in vitro experiments uncovered that overexpression or induction of CYPs can cause up-regulation of the UPR [35]. Since the

UGTs play a key role in xenobiotic metabolism and cellular protection against toxicants, regulatory links controlling the UGTs, p53 and ER stress may be an important network during tissue neoplastic transformation. We have observed that when colon cells are under stress, p53 elevation is accompanied by perturbation of ER homeostasis reflected by induction of ER-resident proteins. Loss of UGT1A expression, particularly in DSS treated *Ugt1*$^{\Delta IEC}$ mice, potentiates ER stress while dramatically down-regulating the expression of p53. Coinciding with our results, previous studies have shown that ER stress promotes p53 degradation in the cytoplasm by the ER-resident ubiquitin ligase Synoviolin [44]. We can speculate that since the UGT1A proteins and Synoviolin are located in the ER, further investigation as to the novel role of the UGT1A proteins in down-regulating Synoviolin-generated ubiquitin ligase targeting of p53 would shed light on how the absence of UGT1A proteins could potentially lead to ER-associated p53 degradation. The UGTs exist as dimers in the ER and form both momomeric and heterodimer complexes [46]. The UGTs have also been shown to dimerize with other ER based proteins, such as the CYPs [47–50]. Since knockout of the UGT1A proteins during stress in colon tissue leads to decreases in p53 abundance, the possibility exists that the UGTs may interact through protein-protein interactions and play an important role in controlling activation of ER-localized Synoviolin, which controls the levels of phosphorylated cytoplasmic p53. Our working model of this study is illustrated in Figure 8.

To achieve proper function, p53 is tightly regulated by means of post-translational modifications, cofactor binding, and subcellular localization. For example, a classic p53 regulator Mdm2, an E3 ubiquitin ligase, inactivates p53 by accelerating its nuclear export and degradation when cells are under a basal condition [38]. Our findings suggest that *UGT1A* genes act as an important p53 regulator by maintaining p53 elevation following cellular stress. Studies have shown that when p53 is activated, post-transcriptional modifications such as p53 phosphorylation and acetylation can protect p53 from degradation [38]. This statement is consistent with our finding that p53 protein phosphorylation expression was down-regulated with unchanged transcriptional activity (Suppl 5), as we observed a loss of UGT1A expression following actinomycin D, etoposide, and DSS treatment. As a tumor suppressor gene, p53 is involved in tumorigenesis through one of three mechanisms: 1. Complete loss of wild type p53 leading to dysregulation of cell cycle and cell death; 2. Suppression of normal function of the wild type p53 by mutant p53; and 3. Pro-oncogenic properties exerted by the mutant p53 [51]. In our study, the tumorigenic action elicited by loss of UGT1A expression is most likely through inhibition of wild type p53. Our results imply that, at least in certain tissues and conditions, UGT1A levels may be a reliable clinical marker that can predict the tumorigenic potential of these tissues. In summary, this study provides compelling evidence that UGT1A expression is essential to maintain p53 accumulation, promoting apoptosis and preventing cells from undergoing tumorigenesis in the disease state.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

AOM	Azoxymethane
CRC	Colorectal cancer
DSS	Dextran sodium sulfate
ER	Endoplasmic reticulum
IEC	Intestinal epithelial cells
TUNEL	Terminal deoxynucleotidyl transferase dUTP nick end labeling
UGT	UDP-glucuronosyltransferase
UGT1	Human *UGT1* locus
Ugt1	Murine *Ugt1* locus

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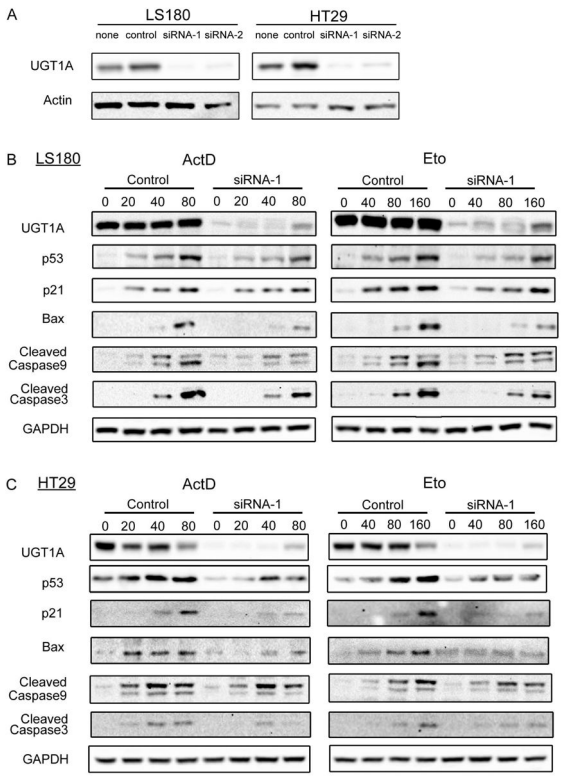

Figure 1. *UGT1A* silencing inhibits actinomycin D and etoposide-induced p53 protein expression and the P53-dependent apoptosis pathway in LS180 and HT29 colon epithelial cells

(A) Assessment of gene-silencing efficiency for the two pairs of *UGT1A* siRNAs (siRNA1, siRNA2). Cells were treated with UGT1A-specific siRNA or non-specific siRNA for 48 hours, and protein expression levels were examined by Western blot analysis. The cell sample without any treatment was indicated as “none” and treated with non-specific siRNA was indicated as “control.” (B) Protein expression of p53 and genes regulated by p53. LS180 cells and HT29 cells (C) were pretreated with UGT1A-specific siRNA or non-specific siRNA for 48 hours and then incubated with actinomycin D (ActD, 20, 40, 80 nM) or etoposide (Eto, 40, 80 160 μM) for 36 hours. Whole cell lysates were prepared and expression of UGT1A, p53, p21, Bax, cleaved caspases-9, and cleaved caspases-3 were examined by Western blots. These represent an example taken from 3 independent experiments, where combined expression levels are quantitated in Supplemental Figures 1A and 1B.

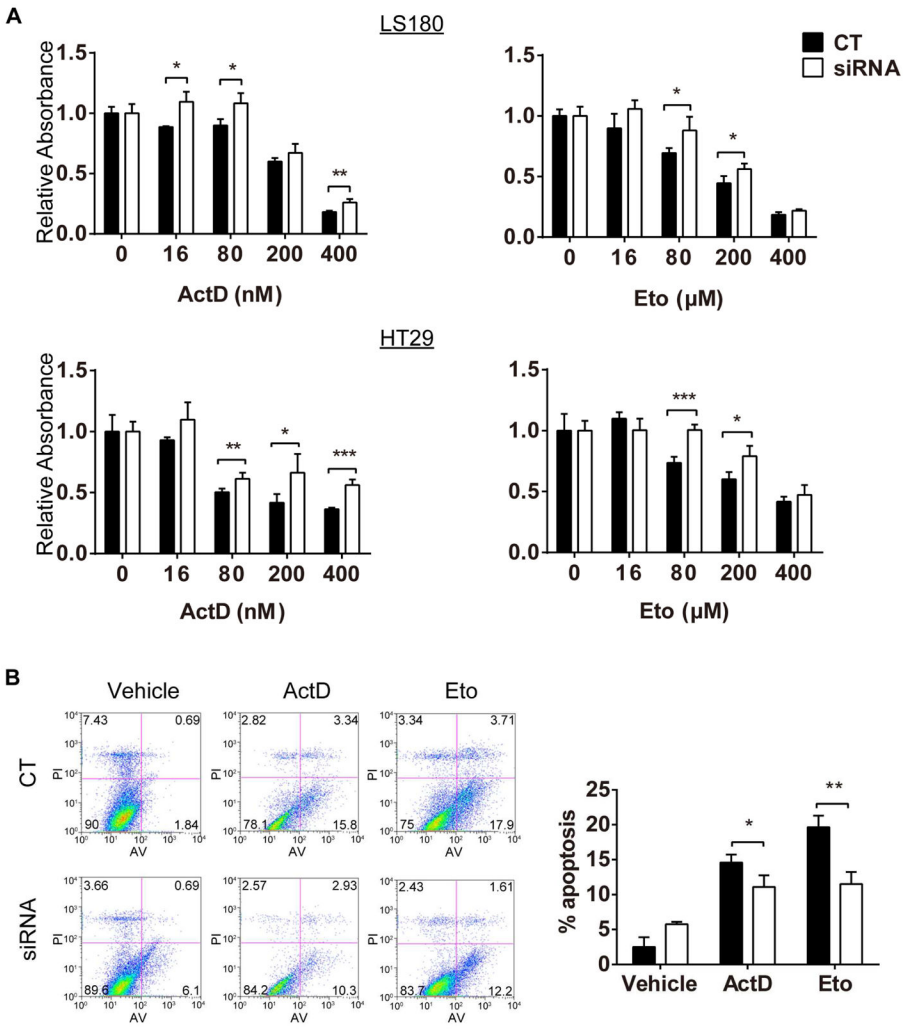

Figure 2. *UGT1A* silencing lowers the anti-proliferative activity of actinomycin D and etoposide in both HT29 and LS180 cells

Cells were pretreated with UGT1A siRNA1 or non-specific siRNA for 24 hours and then exposed to gradient concentrations of actinomycin D or etoposide for 36 hours. (A) Cell proliferation was measured by MTT assay in actinomycin- or etoposide-treated LS180 and HT29 cells. The proliferation rate of cells is expressed as Relative Absorbance (mitochondrial dehydrogenase activity) with non-specific siRNA is expressed as 1. (B) Annexin V FITC/PI staining of HT29 cells was examined by a flow cytometer. Results are presented as mean ± SD of at least four independent experiments (* P<0.05, ** P<0.01, *** P<0.001).

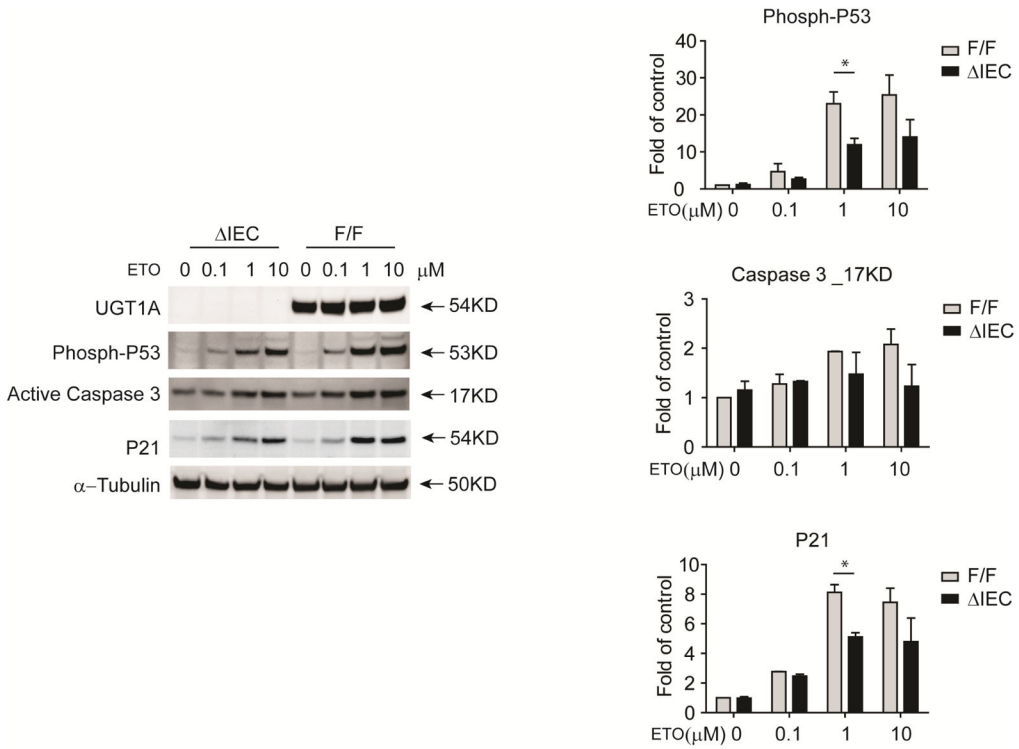

Figure 3. *Ugt1* knockout in intestinal crypt stem cells reduces p53 activation by etoposide

Intestinal crypt stem cells were cultured from $Ugt1^{F/F}$ and $Ugt1^{\Delta IEC}$ adult mice. After passage, approximately 500–1000 cells per 50 μl of Matrigel per well were cultured in 24-well plates. Four days later, cells were exposed to fresh medium containing vehicle control or different concentrations of etoposide (ETO). Twenty four hours after ETO exposure, cells were collected in RIPA buffer. Whole cell extracts were prepared and protein concentrations were quantitated. Thirty μg of protein were used for gel electrophoresis and Western blot analysis. Primary antibodies directed towards UGT1A proteins (Santa Cruz), phosphorylated P53 (Ser 15, Cell Signaling), p21 (Cell Signaling), and active Caspase 3 (Cell Signaling) were used. Shown is a representation of multiple Western blots. Quantitation of multiple Western blots was performed by density analysis visualized by a ChemiDoc Touch Imaging System (Bio-Rad). Each band was normalized to α-tubulin and expressed as "Fold of control". Since induction patterns were being compared between two groups, the $Ugt1^{F/F}$ and $Ugt1^{\Delta IEC}$ mice, statistical comparisons were performed using two-way Anova followed by the Benferroni correction using Graphpad Prism. *$P < 0.05$.

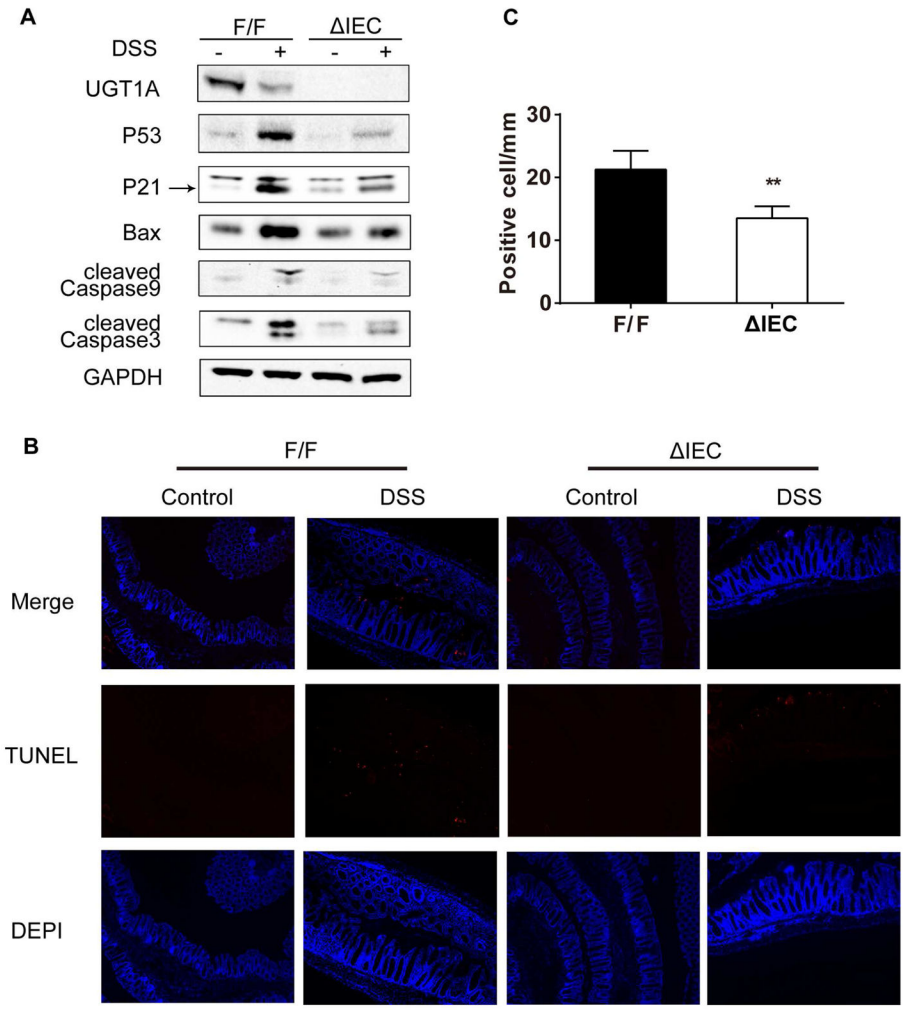

Figure 4. The impact of DSS induced colitis on p53 and apoptotic cells in *Ugt1*$^{\Delta IEC}$ mice

Ugt1$^{\Delta IEC}$ and *Ugt1*$^{F/F}$ mice were treated with 3% DSS in drinking water for 5 days to induce acute colitis and IEC apoptosis. After another 5 days recovery with regular water, colon tissues were collected. (A) Expression of UGT1A, p53, p21, Bax, caspase-9, and caspase-3 detected by Western blot anaylsis. GAPDH was used as a loading control. Quantitation of each Western blot is presented in Supplemental Figure 2. (B) TUNEL staining of colons from untreated mice or DSS-treated mice (100× Magnification). At least four mice were analyzed in each group and the experiment was repeated twice. (C) Quantitation of the number of TUNEL positive cells in DSS-treated mice.

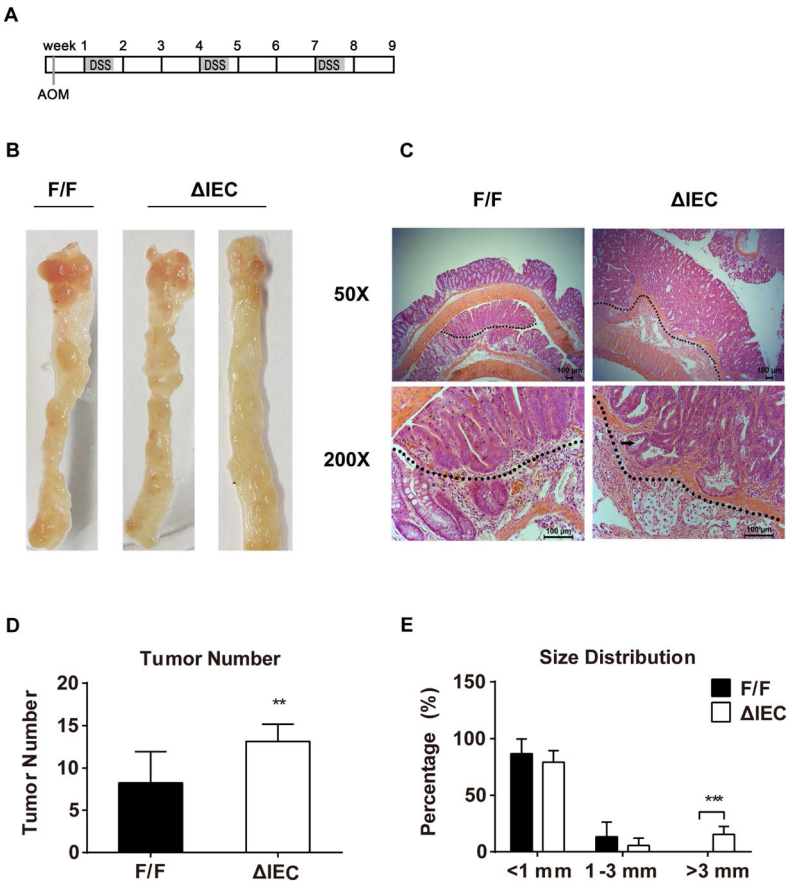

Figure 5. AOM plus DSS treatment causes an increased incidence and increased size of tumors in the colorectum of *Ugt1*$^{\Delta IEC}$ mice compared to *Ugt1*$^{F/F}$ mice

(A) A schematic schedule is shown as the AOM plus DSS feeding protocol. Each rectangle represents one week. (B) Photos of colon tumors in *Ugt1*$^{F/F}$ and *Ugt1*$^{\Delta IEC}$ mice. (C) Representative tumor morphologies in colonic sections by H&E staining (50× and 200× magnification). The black arrow indicates the penetrating adenoma. (D) Tumor incidence and (E) size distribution in *Ugt1*$^{F/F}$ and *Ugt1*$^{\Delta IEC}$ mice. At least seven mice were analyzed in each group, and results are presented as mean ± SD (* P<0.05, ** P<0.01, *** P<0.001).

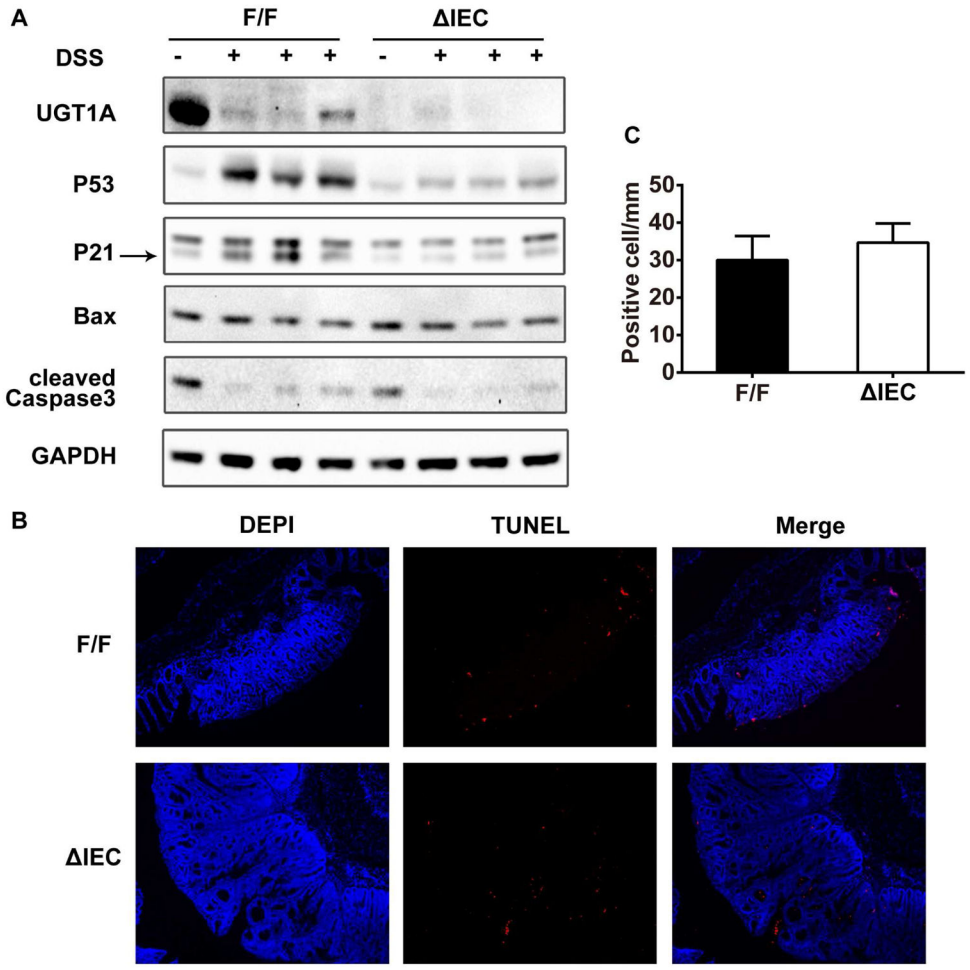

Figure 6. Protein expression of p53 and p53-dependent genes and detection of apoptosis in the colorectum of *Ugt1*$^{F/F}$ and *Ugt1*$^{\Delta IEC}$ mice with colorectal neoplasia following AOM and DSS treatment

Colorectum tissues from AOM plus DSS-treated *Ugt1*$^{\Delta IEC}$ and *Ugt1*$^{F/F}$ mice were collected and analyzed. (A) Expression levels of UGT1A, p53, p21, Bax, and cleaved caspase-3 were examined by Western blot analysis with GAPDH as a loading control. Quantitation of the Western blots is shown in Supplemental Figure 4 (B) TUNEL staining of colon sections (100× Magnification). (C) Quantification of the number of TUNEL positive cells.

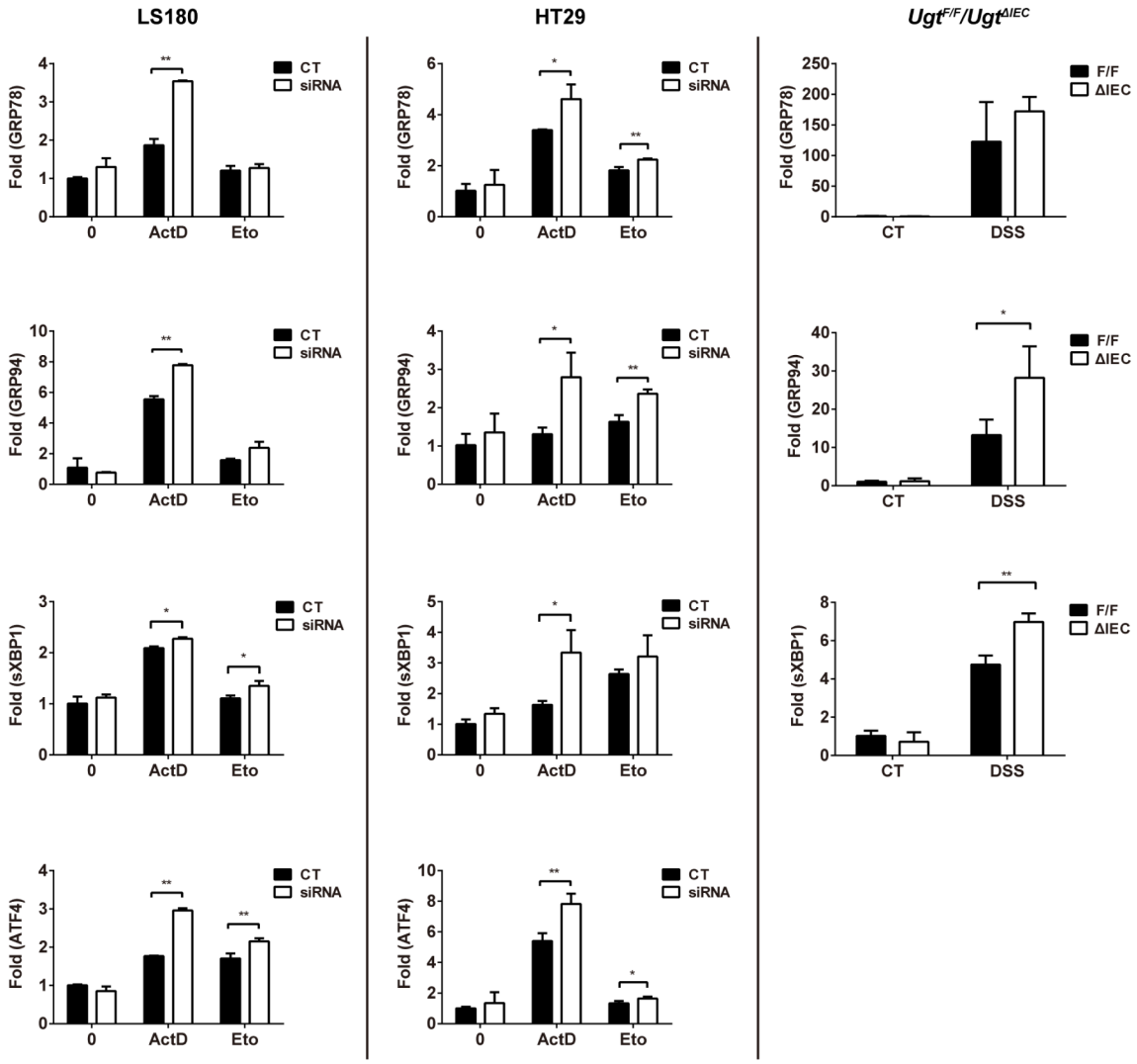

Figure 7. Loss of UGT1A potentiates ER stress induced by actinomycin D, etoposide, or DSS treatment

LS180 and HT29 cells were pretreated with UGT1A siRNA or non-specific siRNA for 48 hours and then incubated with actinomycin D (ActD, 40 nM) or etoposide (Eto, 80 μM) for 36 hours. *Ugt1*$^{\Delta IEC}$ and *Ugt1*$^{F/F}$ mice were treated with 3% DSS in drinking water and colon tissues were collected. Expression of ER stress-responsive genes was quantitated by Q-PCR.

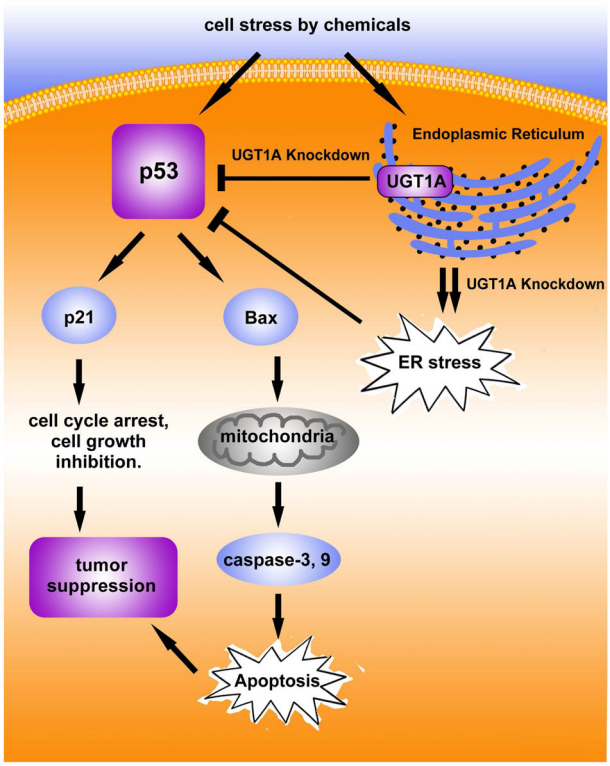

Figure 8. A working model that demonstrates control of apoptosis and tumorigenesis by UGT1A proteins through regulation of p53

In this model, we are showing that p53 when activated can induce apoptosis and repress tumor formation. Following p53 activation by cellular or chemical stress the presence of UGT1A proteins in the endoplasmic reticulum (ER) is required to sustain and maintain p53 levels. Knockdown of the *UGT1* locus and the resulting UGT1A proteins increases ER stress leading to inhibition of p53 activation/elevation and subsequent reduction in apoptosis and tumor suppression.