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ORIGINAL ARTICLE

c-Rel–dependent Chk2 signaling regulates the DNA damage response limiting hepatocarcinogenesis

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Abstract

Background and Aims: Hepatocellular carcinoma (HCC) is a leading cause of cancer-related death. The NF-κB transcription factor family subunit c-Rel is typically protumorigenic; however, it has recently been reported as a tumor suppressor. Here, we investigated the role of c-Rel in HCC.

Approach and Results: Histological and transcriptional studies confirmed expression of c-Rel in human patients with HCC, but low c-Rel expression correlated with increased tumor cell proliferation and mutational burden and was associated with advanced disease. *In vivo*, global ($Rel^{-/-}$) and epithelial specific (Rel^{Alb}) c-Rel knockout mice develop more tumors, with a higher proliferative rate and increased DNA damage, than wild-type (WT) controls 30 weeks after N-diethylnitrosamine injury. However, tumor burden was comparable when c-Rel was deleted in hepatocytes once tumors were established, suggesting c-Rel signaling is important for preventing HCC initiation after genotoxic injury, rather than for HCC progression. *In vitro*, $Rel^{-/-}$ hepatocytes were more susceptible to genotoxic injury than WT controls. ATM-CHK2 DNA damage response pathway proteins were

Abbreviations: Adeno-associated virus (AAV), ATM, ataxia-telangiectasia mutated; ATR, ataxia-telangiectasia and RAD3-related; CCl_4, carbon tetrachloride; Chk2, checkpoint kinase 2; Chk2i, inhibitor of Chk2; DEN, N-nitrosodiethylamine; HCC, hepatocellular carcinoma; LDH, lactate dehydrogenase; ROS, reactive oxygen species; WT, wild-type.

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suppressed in *Rel*$^{-/-}$ hepatocytes following genotoxic injury, suggesting that c-Rel is required for effective DNA repair. To determine if c-Rel inhibition sensitizes cancer cells to chemotherapy, by preventing repair of chemotherapy-induced DNA damage, thus increasing tumor cell death, we administered single or combination doxorubicin and IT-603 (c-Rel inhibitor) therapy in an orthotopic HCC model. Indeed, combination therapy was more efficacious than doxorubicin alone.

Conclusion: Hepatocyte c-Rel signaling limits genotoxic injury and subsequent HCC burden. Inhibiting c-Rel as an adjuvant therapy increased the effectiveness of DNA damaging agents and reduced HCC growth.

INTRODUCTION

Hepatocellular carcinoma (HCC) is the most common primary liver malignancy and a leading cause of cancer-related deaths worldwide, affecting ~840,000 new patients in 2018, and is predicted to rise to 1,361,836 in 2040.[1,2] HCC is an inflammation associated cancer, typically developing in patients with chronic liver disease or cirrhosis, and age, gender, and genetics, as well as lifestyle and environmental factors, can all increase the risk of developing HCC.[3] In the Western world, NAFLD and alcoholic-related liver disease are the primary drivers of liver carcinoma, accounting for 15%–20% and 15%–30% of HCC cases, respectively, whereas in Asia, HCC arises typically with the background of viral infection, either chronic hepatitis B or hepatitis C.[4–6]

Current therapeutic options to treat HCC are limited, and most patients are not suitable for resection or transplant because of either the size or position of the tumor or the underlying liver dysfunction.[7] For advanced, unresectable disease, medical treatments are restricted to targeted systemic therapies, with combination atezolizumab plus bevacizumab therapy, which offers a median progression free survival of ~6.8 months, now being the first-line standard of care.[8] The multikinase inhibitors sorafenib and lenvatinib (second-line therapies) and regorafenib, cabozantinib, and ramucirumab (third-line therapies) typically extend median survival by approximately 12 weeks but often have poor tolerability. However, not all patients respond to systemic therapies. The combination of a lack of effective therapeutic options for those diagnosed with advanced disease and the increasing numbers of patients with chronic liver disease mean that HCC mortality rates are still rising. Therefore, there is a need to gain deeper insights into the disease biology to reveal new therapeutic avenues and potentially stratify HCC therapies.

The NF-κB family of transcription factors regulates a variety of cellular functions important for tissue homeostasis, but when dysregulated, NF-κB signaling can cause disease pathology. Each of the five NF-κB subunits; RelA, c-Rel, RelB, p50, and p52 can form either homo- or heterodimers, and it is the dimer composition and injury stimulus that direct discrete transcriptional and biological responses.[9] Activation of NF-κB signaling can stimulate inflammation, cellular proliferation, differentiation, and migration, as well as cell death, which are processes that are implicated in the pathogenesis of chronic disease and cancer.[10–13] Because of the complexity of NF-κB signaling, the cell-specific role of the individual NF-κB subunits in the initiation and progression of cancer has not been fully elucidated. Typically, the c-Rel subunit has been characterized as having an oncogenic role in cancer progression. c-Rel is the cellular homologue of the avian Rev-T retroviral oncoprotein v-Rel, which causes lymphoma in birds,[14,15] and subsequent work revealed that *REL* gene amplifications are common in human B cell lymphomas,[16,17] whereas in pancreatic ductal adenocarcinoma, mutant Kirsten rat sarcoma virus-RAS-like proto-oncogene B-TANK-binding kinase 1 signaling axis promotes the c-Rel–dependent activation of antiapoptotic and stemness genes conveying resistance to therapy.[18] However, this dogma has recently been challenged in studies using *Rel*$^{-/-}$ mice, which describe c-Rel as a tumor suppressor in a model of B cell lymphoma,[19] as playing a protective role in *Helicobacter*-associated models of gastric and colon cancer[20,21] and limiting oral squamous cell carcinoma development in a carcinogen model.[22]

Further to the intrinsic role of c-Rel in the tumor cell, in the immune system, cell-specific roles of c-Rel that either promote or limit tumor development have been reported. c-Rel is a critical regulator of T-cell activation and plays a central role in immune surveillance through activation of a Th1 response.[23,24] Paradoxically, c-Rel has been described as a cancer immunotherapy checkpoint, where c-Rel–dependent activation of regulatory T cells and myeloid cells limits CD8^{+} T-cell–

mediated tumor killing, thereby promoting tumor survival.[25]

Here, we have gained insights into the role of c-Rel signaling in HCC and discovered that low tumor c-Rel expression in patients with HCC is associated with a more advanced disease stage and poor outcome. whereas in mice, c-Rel in hepatocytes protects against genotoxic DNA damage and suppresses tumorigenesis independent of the immune response.

METHODS

Mice

All animal experiments were approved by the Newcastle Ethical Review Committee and performed under a UK Home Office license and recieved humane care in accordance with the Animal Research: Reporting of In Vivo Experiments guidelines. Mice were housed in pathogen-free conditions and kept under standard conditions with a 12-h day/night cycle and free access to food and water. Power calculations were not routinely performed; however, animal numbers were chosen to reflect the expected magnitude of response considering the variability observed in previous experiments. *In vivo* and *in vitro* experiments were performed on either C57BL/6 WT control mice or c-Rel knockout mice ($Rel^{-/-}$). $Rel^{fl/fl}$ mice were crossed with Alb-$cre^{+/-}$ to generate Alb-$cre^{+/-}$ $Rel^{fl/fl}$ ($Rel^{\Delta Alb}$) mice or received a single intravenous tail vein injection of 1×10^{11} plaque-forming unit of AAV8-TBG-Cre to generate c-Rel–deficient hepatocyte $Rel^{fl/fl}$ ($Rel^{\Delta AAV}$) mice.

Orthotopic liver cancer models

Liver cancer was induced by intrahepatic injection of 1×10^6 Hep53.4 cells (CLS) into 8- to 10-week-old male WT or $Rel^{-/-}$ mice as described previously.[39] Fourteen days postinjection, mice received either IT-603 (3 times weekly byintraperitonal injection, 24 mg/kg), doxorubicin (1 times weekly by intravenous injection, 2 mg/kg), or dual IT-603 + doxorubicin therapy for 2 weeks. Mice were humanely killed 28 days postsurgery.

Human biopsies

HCC tumor and nontumor biopsy tissue and liver tissue from surgical resections were obtained with informed patient consent, in wirting and under full ethical approval from the Newcastle and North Tyneside Regional Ethics Committee, the Newcastle Academic Health Partners Bioresource (NAHPB), the Newcastle-upon-Tyne NHS Foundation Trust Research and Development department, and the CEPA biobank. (reference numbers: 10/H0906/41; NAHPB Project 48; REC 12/NE/0395; R&D 6579; Human Tissue Act license 12534; 17/NE/0070). All research was conducted in accordance and conformed to the study protocol and ethical guidelines of the 1975 Declarations of Helsinki and Istanbul.

Statistical analysis

Results are presented as means ± SEM. GraphPad Prism 8 was used to perform either a two-tailed unpaired Student *t*-test, two-tailed paired Student *t*-test, or two-way analysis of variance with a Tukey post hoc test $p < 0.05$, $p < 0.01$, or $p < 0.001$ was considered statistically significant.

Other methods are in the Supporting Material.

RESULTS

Low c-Rel/*REL* expression in HCC is associated with more advanced disease and poorer prognosis.

To investigate the role of c-Rel in HCC, we histologically assessed c-Rel expression in matched HCC tumor and nontumor biopsy tissues from patients. Hepatocyte c-Rel levels were variable but significantly increased in HCC tumor tissue compared with the matched nontumor tissue (Figure 1A,B).[26–31] When tumor biopsy samples were stratified by tumor grade and clinical features, c-Rel expression was higher in well-differentiated tumors, which are typically less advanced, compared with moderate/poorly differentiated tumors but was not different when categorized by tumor stage, Barcelona Clinic Liver Cancer score, cirrhosis, type 2 diabetes, gender, or etiology (Figures 1C,D and S1A–F).[26–31] We have previously shown in the partial hepatectomy model of liver regeneration that loss of hepatocyte c-Rel correlates with increased hepatic proliferation.[32] Similarly, in our patient biopsies, low tumor c-Rel expression was negatively correlated with a higher rate of cellular proliferation (Figure S1G,H). This association and reduction in c-Rel in less differentiated, more advanced tumors led us to hypothesize that patients with lower tumor *REL* expression may have a more progressive disease. Kaplan-Meier survival curves generated through a KM plotter that stratify patients with HCC based on NF-κB subunit mRNA expression revealed that lower *REL* expression correlates with reduced length of survival (Figure 1E). Survival was also reduced in patients with high *RELB* gene expression but not with other NF-κB subunits (Figure S1I–L).

Global c-Rel knockout mice develop more liver cancer

To test our hypothesis that c-Rel signaling limits liver cancer growth, we performed the N-diethylnitrosamine

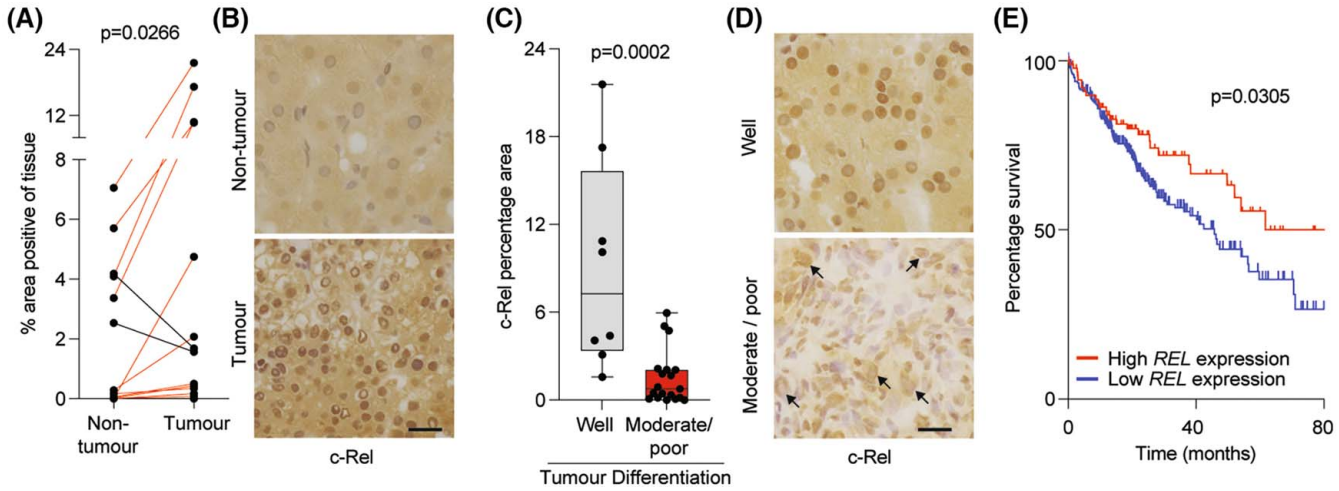

FIGURE 1 Low *REL* expression in human liver cancer is associated with increased HCC proliferation and poorer prognosis. (A,B) Graph and representative images showing c-Rel expression in matched nontumor and HCC tumor tissue expressed as percentage of area positive of tissue, *p* value calculated using a paired *t*-test. (C,D) Graph and representative images showing c-Rel expression in HCC tumor tissue characterized, as well or moderately/poorly differentiated HCC expressed as percentage of area positive of tissue. The *p* value was calculated using an unpaired *t*-test. (E) Kaplan-Meier plots showing patient survival in patients with high versus low *REL* mRNA levels plotted against time in months. Data generated from publicly available datasets available on Kaplan-Meier plotter; *p* value calculated using the log-rank Mantel-Cox test. HCC, hepatocellular carcinoma.

(DEN) model of liver cancer in wild-type (WT) and global c-Rel knockout mice ($Rel^{-/-}$) (Figure 2A).[26–31] Confirming our hypothesis, the liver to body weight ratio and small (< 0.5 mm) and large (> 0.5 mm) macroscopic tumor counts were greater in $Rel^{-/-}$ mice than WT controls (Figures 2B–D and S2A). Histological typing and grading confirmed that the tumors in $Rel^{-/-}$ mice were more advanced, with a significant increase in both the number of hepatocellular adenomas (HCAs) and HCCs (Figure 2E), as well as a greater proliferative capacity (Figure S2B). Hepatic expression of tumorigenic growth factors and angiogenic gene expression was elevated in the $Rel^{-/-}$ mice, consistent with more advanced tumors (Figure S2C). Given the key role c-Rel plays in the adaptive immune response, we next assessed tumoral $CD8^+$ T-cell recruitment. Infiltration of intratumoral $CD8^+$ T cells was significantly decreased in $Rel^{-/-}$ mice compared with WT controls, suggesting a suppression in immune surveillance (Figures 2F and S2D). Innate immune cell recruitment was also assessed. Infiltration of $F4/80^+$ macrophages and $Ly6G^+$ neutrophils into the tumor and nontumor tissue of $Rel^{-/-}$ mice was also significantly reduced compared with WT controls (Figure S2E,F), suggesting an impairment of total inflammation, even in the context of larger tumors.

To determine if c-Rel signaling in the background liver and/or inflammatory cells contributes to HCC growth, we implanted the Hep53.4 HCC cell line, which expresses c-Rel and the other canonical NF-κB subunits RelA and p50, into the livers of WT and $Rel^{-/-}$ mice (Figures 2G and S2G). Tumor burden was significantly increased in $Rel^{-/-}$ mice in this orthotopic model, and consistent with the $Rel^{-/-}$ mice in the DEN model, there was also impaired immune surveillance, shown by a significant reduction in infiltration of $CD8^+$ T cells into to the tumor (Figure 2H,I). These data suggest that expression of c-Rel in nontumor cells is required for effective immune surveillance and to limit tumorigenesis.

Hepatocyte c-Rel is important for chemical-induced liver cancer initiation but not progression

To explore the role of c-Rel in the liver parenchyma in HCC, we generated $Rel^{\Delta Alb}$ mice in which c-Rel is selectively deleted in the hepatocytes and then subjected the mice to the 30-week DEN HCC model (Figure 3A).[26–31] These mice retain c-Rel expression in all immune cells,[32] and therefore, immune surveillance following DEN administration should not be impaired. Like the global $Rel^{-/-}$ mice, $Rel^{\Delta Alb}$ mice have an increased liver to body weight ratio and greater tumor burden, develop larger tumors, and have significantly more HCAs and HCCs than $Rel^{fl/fl}$ controls (Figures 3B–D and S3A). The tumors of $Rel^{\Delta Alb}$ mice also displayed a significant increase in the number of proliferating cell nuclear antigen positive tumor cells (Figure SF3B) and an increased expression of protumorigenic growth factors and angiogenic genes (Figure S3C). Given that albumin-driven expression of cre-recombinase also leads to the deletion of c-Rel in cholangiocytes, it was important to assess the biliary status of tumor-bearing mice. Cytokeratin 19 immunohistochemistry revealed no difference in biliary injury or ductular reaction between $Rel^{\Delta Alb}$ mice and $Rel^{fl/fl}$ controls (Figure S3D).

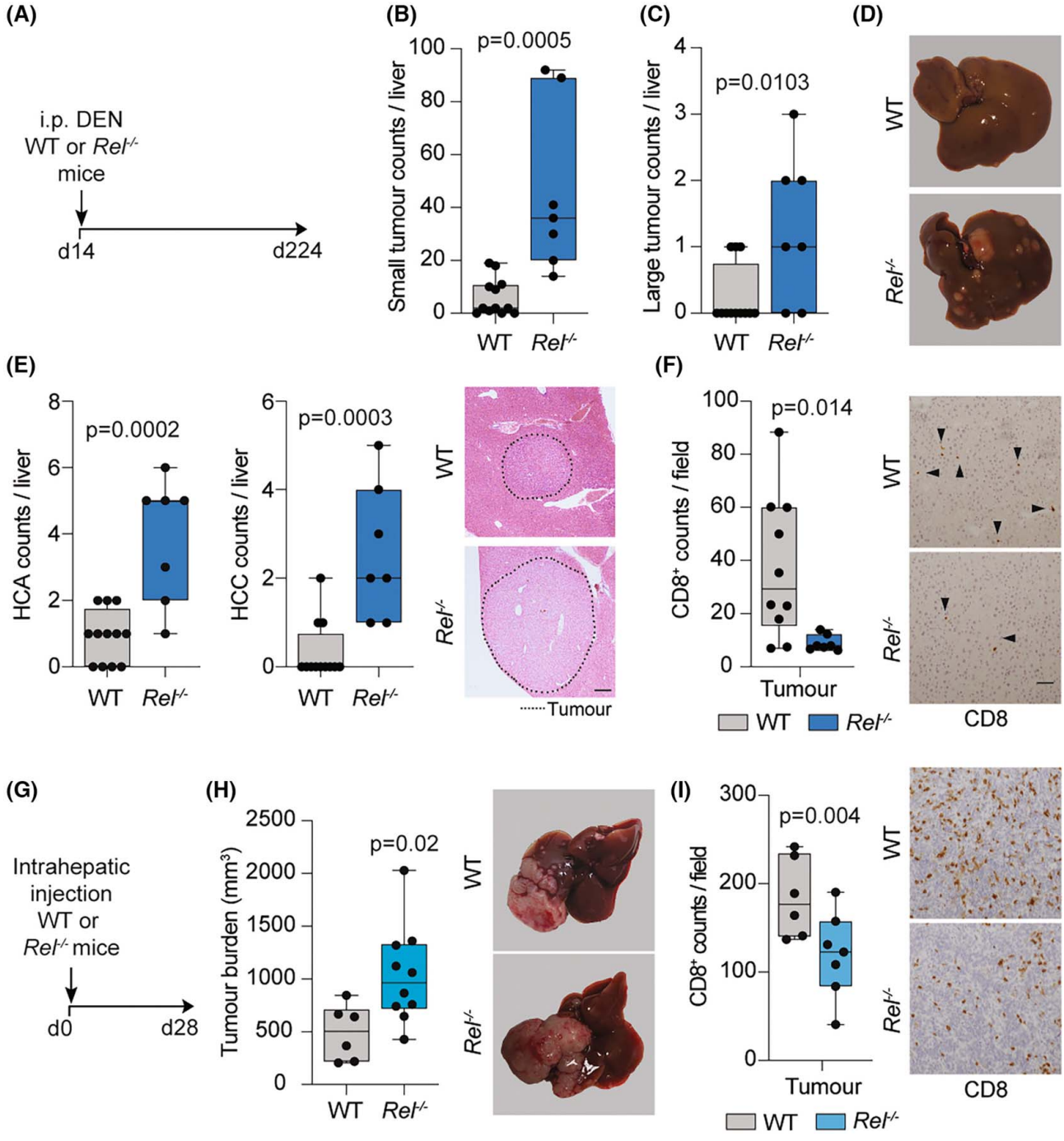

FIGURE 2 Global c-Rel knockout mice develop more liver cancer. (A) Experimental timeline of chronic DEN model, mice recieve an intraperiotneal injection of DEN at day 14 (d14), tumours are then allowed to develop until day 224 (d224). (B–D) Graphs showing small (B) or large (C) tumor counts and accompanying representative liver pictures (D) *ex vivo* from chronic DEN-injured WT or $Rel^{-/-}$ mice. (E) Graphs showing frequency of histologically graded hepatocellular adenomas (HCAs) and hepatocellular carcinomas (HCCs) with representative images of H&E-stained livers in chronic DEN-injured WT or $Rel^{-/-}$ mice. Dotted line denotes tumor region. (F) Graph and representative images showing recruitment of $CD8^+$ T cells to the tumor regions of chronic DEN-injured WT or $Rel^{-/-}$ mice. (G) Experimental timeline of orthotopic model. (H) Graph showing tumor burden and representative pictures of livers *ex vivo* orthotopic model tumors in WT or $Rel^{-/-}$ mice. (I) Graph and representative images showing recruitment of $CD8^+$ T cells to tumors of orthotopic HCC WT or $Rel^{-/-}$ mice. Data are mean ± SEM in up to eight mice/group; each data point represents a different donor. The *p* values were calculated using an unpaired *t*-test. DEN, N-diethylnitrosamine; H&E, haematoxylin and eosin; WT, wild-type.

We next assessed tumor and nontumor immune cell infiltration. Unlike global $Rel^{-/-}$ mice, there was no difference in $CD8^+$ T-cell, $F4/80^+$ macrophage, and $Ly6G^+$ neutrophil recruitment in $Rel^{\Delta Alb}$ and $Rel^{fl/fl}$ mice (Figures 3E and S3E–G), suggesting active immune surveillance mechanisms in $Rel^{\Delta Alb}$ mice.

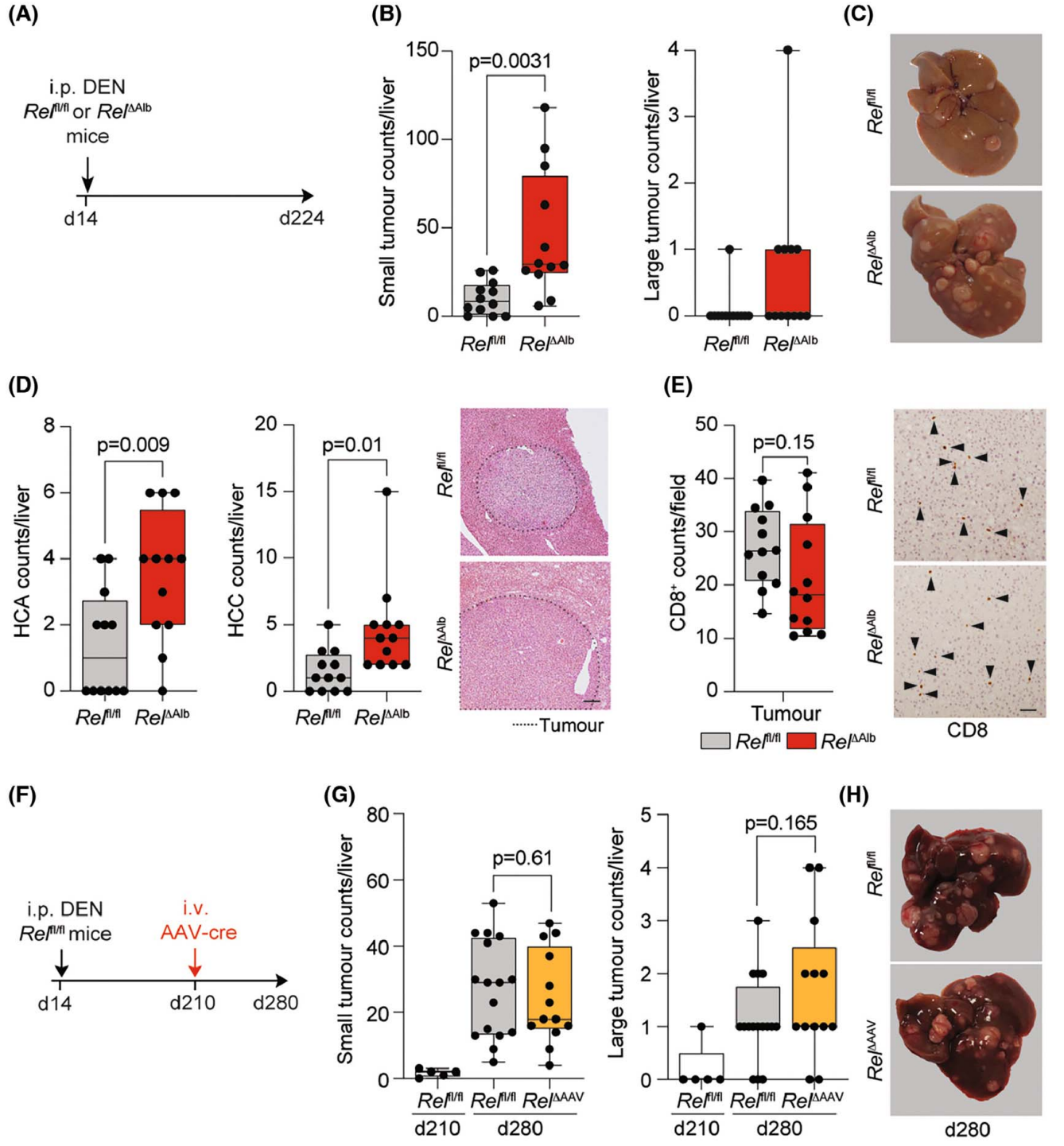

FIGURE 3 Hepatocyte c-Rel is important for chemical-induced liver cancer initiation but not progression. (A) Experimental timeline of chronic DEN model. (B,C) Graphs showing small (left) or large (right) tumor counts (B) and accompanying representative pictures of livers (C) *ex vivo* from chronic DEN-injured $Rel^{fl/fl}$ and $Rel^{\Delta Alb}$ mice. (D) Graphs showing frequency of histologically graded HCA and HCC, with accompanying representative images of H&E-stained livers from chronic DEN-injured $Rel^{fl/fl}$ and $Rel^{\Delta Alb}$ mice. Dotted line denotes tumor region. (E) Graph showing recruitment of $CD8^+$ T lymphocytes to the tumor regions of chronic DEN-injured $Rel^{fl/fl}$ and $Rel^{\Delta Alb}$ mice. Data are mean ± SEM in 12 mice/group; each data point represents a different donor. (F) Experimental timeline depicting temporal deletion of c-Rel via AAV-Cre administration via intravenous injection (i.v.) to $Rel^{fl/fl}$ mice in chronic DEN model. (G,H) Graphs showing small (left) or large (right) tumor burden (g) and accompanying representative pictures of livers (H) *ex vivo* from chronic DEN-injured $Rel^{fl/fl}$ mice at Day 210 prior to AAV-TBG-Cre and $Rel^{fl/fl}$ and $Rel^{\Delta AAV}$ mice at Day 280. Data are mean ± SEM in up to 11 mice/group; each data point represents a different donor. The *p* values were calculated using an unpaired *t*-test or one-way ANOVA with Tukey's post hoc test. AAV, Adeno-associated virus; DEN, N-diethylnitrosamine; H&E, haematoxylin and eosin; HCA, hepatocellular adenoma; HCC, hepatocellular carcinoma.

To determine if c-Rel signaling in hepatocytes is important for HCC initiation and/or progression, we chronically injured $Rel^{fl/fl}$ mice with DEN; then, after 196 days (mouse age Day 210), once macroscopic tumors had developed in this model,[33] we administered an AAV8-TBG-Cre to the livers of $Rel^{fl/fl}$ mice to selectively deplete c-Rel in hepatocytes ($Rel^{\Delta AAV}$) and then allowed tumors to continue growing for a further 70 days (total of 280 days post-DEN injury) (Figure 3F). Similar to $Rel^{\Delta Alb}$ mice, no difference in $CD8^+$ T-cell, F4/80^+ macrophage, or $Ly6G^+$ neutrophil recruitment was observed between $Rel^{\Delta AAV}$ and $Rel^{fl/fl}$ controls (Figure S3H–J). There was no difference in tumor burden between the two genotypes at Day 280 (Figure 3G,H). suggesting that c-Rel signaling, specifically in hepatocytes, may play a role in limiting genotoxic injury or early events in liver cancer development.

$Rel^{-/-}$ mice are more susceptible to genotoxic injury

A role for c-Rel in maintaining genomic stability has been suggested via regulation of claspin, an adaptor protein facilitating ataxia-telangiectasia and RAD3-related (ATR)–dependent DNA damage responses.[34] To determine if c-Rel is an important regulator of DEN-induced hepatocyte DNA damage, we performed an acute DEN injury in $Rel^{fl/fl}$ and $Rel^{\Delta Alb}$ mice in which hepatocyte death peaks at 24 h (Figure 4A). Acute DEN challenge resulted in a significant increase in hepatocyte cell death in $Rel^{\Delta Alb}$ mice compared with controls, shown by an increase in necrotic area (Figure 4B), an elevation in the liver damage markers alanine aminotransferase and aspartate aminotransferase (AST), and a marked reduction in liver to body weight ratio (Figure S4A–C). The increased hepatocellular injury in the $Rel^{\Delta Alb}$ mice was associated with a significant increase in the number of hepatocytes with the DNA damage marker γH2AX and DEN-induced promutagenic O6-ethyl-2-deoxyguanosine adducts (Figure 4C,D). These data suggest that, in the absence of c-Rel, there is a failure to effectively resolve DNA damage following genotoxic injury.

Genomic instability, because of persistent DNA damage in the absence of c-Rel, could cause accumulation of genetic mutations in the liver and development of HCC. To investigate this, we interrogated the cBioPortal (The Cancer Genome Atlas (TCGA), provisional) database[35] to ascertain if there is a correlation between tumor *REL* mRNA expression and aberrant genetic alteration counts (tumor mutational burden). Consistent with our hypothesis, hepatic *REL* expression negatively correlated with mutational burden in patients with HCC (Figure 4E). Of note, *NFKB1* expression also negatively correlated with mutational burden, but there was no correlation with the other three NF-κB subunits (Figure S5A–D).

To determine if sensitivity to DNA damage in the livers of $Rel^{-/-}$ mice is due to an intrinsic hepatocyte defect, we challenged *in vitro* cultured WT and $Rel^{-/-}$ hepatocytes with DEN (Figure 4F). Baseline metabolic output, a proxy for the number of viable cells and lactate dehydrogenase (LDH) release was comparable in unchallenged WT and $Rel^{-/-}$ hepatocytes. However, upon DEN challenge, the metabolic output of $Rel^{-/-}$ hepatocytes was significantly reduced (Figure S6A); LDH release and the number of apoptotic cells significantly increased compared with DEN-challenged WT hepatocytes, suggestive of more hepatocyte death (Figure 4G–I).

We next asked if $Rel^{-/-}$ hepatocytes were susceptible to other DNA damaging agents or if this observation was specific to DEN-induced genotoxic injury. WT and $Rel^{-/-}$ hepatocytes were treated with the DNA damaging agents doxorubicin, γ-irradiation, etoposide, and cisplatin. Hepatocytes were also treated with hydrogen peroxide to simulate reactive oxygen species (ROS) release caused by DEN-induced genotoxic injury. A significant increase in cell death and decline in metabolic function were observed in $Rel^{-/-}$ hepatocytes compared with WT controls, irrespective of the DNA damaging agent used (Figure S6B–E). However, unlike genotoxic challenge, ROS-induced cellular damage caused by hydrogen peroxide resulted in similar changes in metabolic function and cell death in the WT and $Rel^{-/-}$ hepatocytes (Figure S6F). To explore this *in vivo*, we performed the acute carbon tetrachloride (CCl_4) liver injury model in WT and $Rel^{-/-}$ mice (Figure S6G). In this model, hepatic metabolism of CCl_4 causes oxidative stress–induced hepatocyte necrosis and apoptosis and hepatic inflammation. We observed no difference in hepatocellular injury or DNA damage in $Rel^{-/-}$ mice injured by CCl_4 (Figure S6H,I). We propose that c-Rel plays an important role in limiting hepatocellular damage caused by DNA damaging agents and that this is independent of cellular production of ROS following injury.

c-Rel regulates the ATM-CHK2-P53 DNA damage response axis

Having established a role for c-Rel in the regulation of the hepatocyte response to genotoxic injury, we next wished to discern the cellular mechanisms underpinning this observation. The DNA damage response is predominantly coordinated by DNA-dependent protein kinase catalytic subunit (DNA-PKcs), RAD3-related (ATR), and ataxia-telangiectasia mutated (ATM), each of which assemble distinct cellular machinery to repair damaged DNA.[36] Expression of these kinases and the DNA damage repair machinery was assessed in the

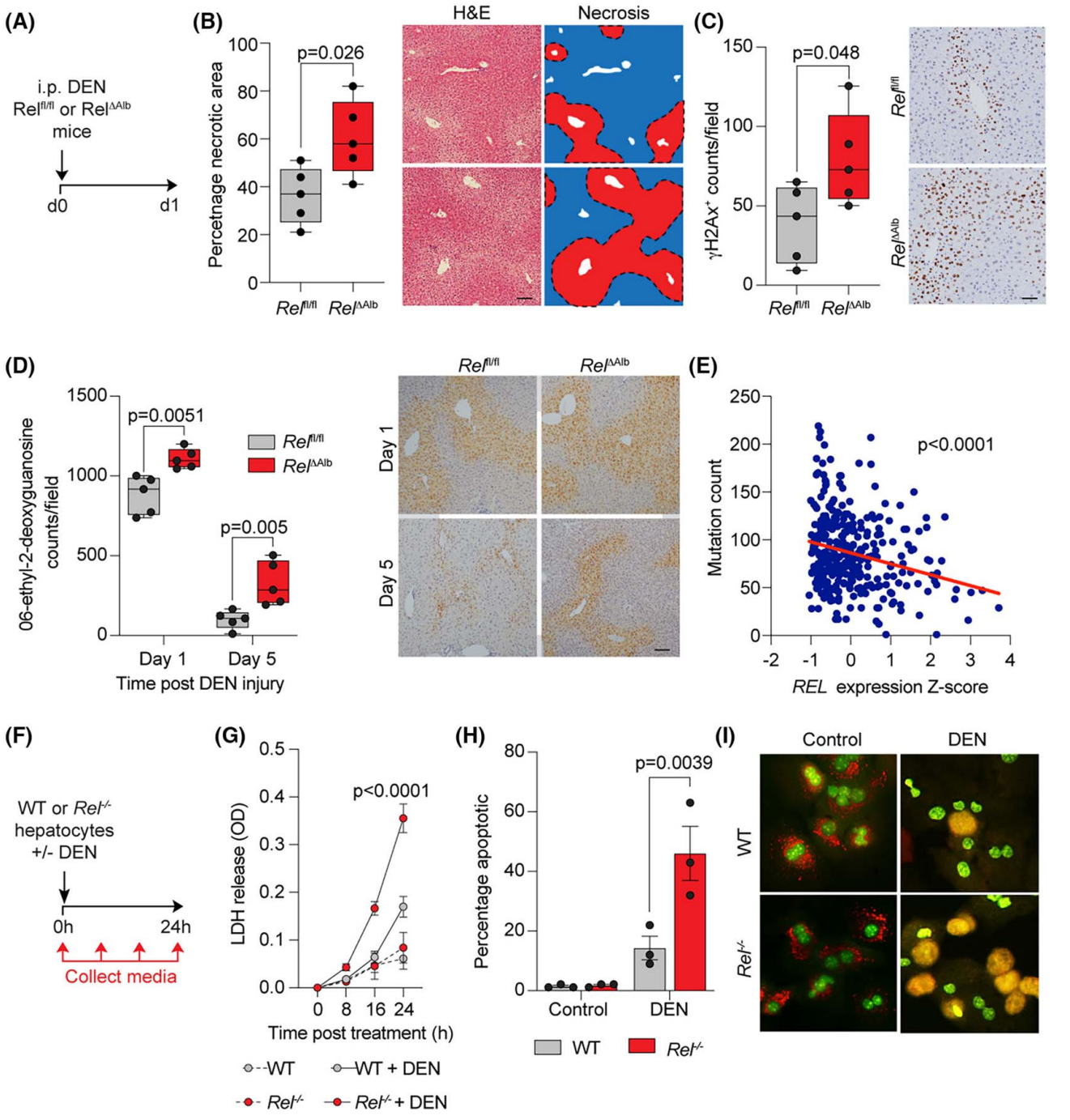

FIGURE 4 *Rel*$^{-/-}$ mice are more susceptible to genotoxic injury. (A) Experimental timeline of acute DEN injury model. (B) Graph shows the percentage of area of necrotic liver tissue in acute DEN-injured *Rel*$^{fl/fl}$ and *Rel*$^{\Delta Alb}$ mice. Representative images show H&E-stained liver sections (left panel) and masking of necrotic tissue (right panel) highlighted in red, blue denotes healthy liver tissue, and vessels are colored white. (C,D) Graphs showing mean γH2AX positive hepatocytes (C) and mean O6-ethyl-2-deoxyguanosine (d), with accompanying histology images in acute DEN-injured *Rel*$^{fl/fl}$ and *Rel*$^{\Delta Alb}$ mice. (E) Correlation of mutational burden with *REL* mRNA expression in patients with HCC, generated using data from the cBioPortal The Cancer Genome Atlas (TCGA) database. (F) Experimental timeline of *in vitro* hepatocyte DEN injury. (G–I) Graphs showing cell death (LDH release, expressed as optical density (OD)) and apoptosis (acridine orange staining) with representative fluorescent images in untreated (control) or DEN-injured WT or *Rel*$^{-/-}$ hepatocytes. Data are mean ± SEM in five mice/group or cells isolated from three independent donors. The *p* values were calculated using either an unpaired *t*-test or repeated measure ANOVA with Tukey's post hoc test. HCC, hepatocellular carcinoma; LDH, lactate dehydrogenase; WT, wild-type.

liver tissue of acute DEN-injured WT and *Rel*$^{-/-}$ mice. c-Rel was largely dispensable for expression of ATR and DNA-PKcs mediated DNA repair pathways proteins; however, the transcriptional regulation of the Atm-Chk2-p53 axis was blunted in *Rel*$^{-/-}$ mice (Figure 5A). Failure to maintain expression of key components of the ATM-

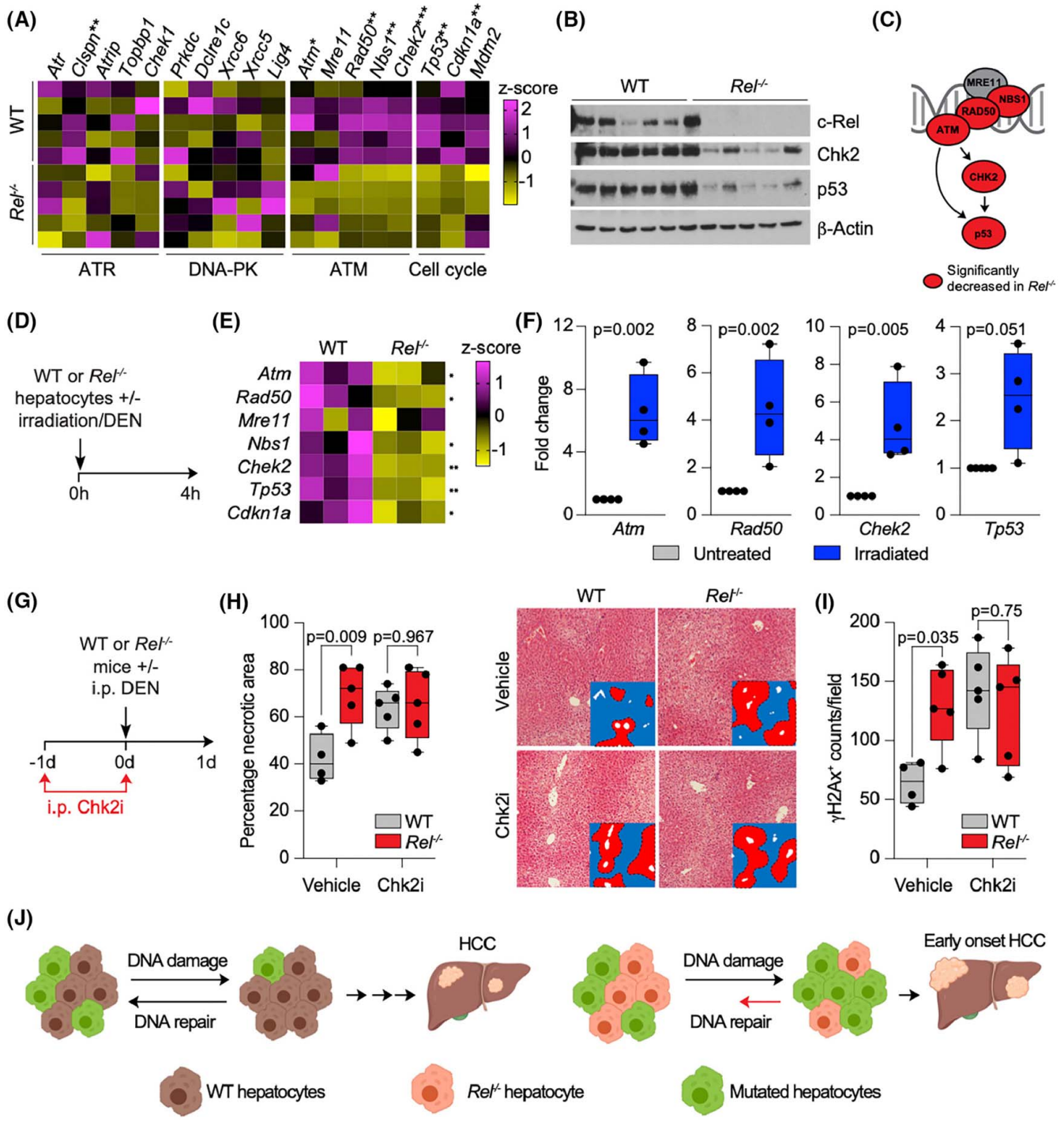

FIGURE 5 c-Rel regulates the ATM-CHK2-P53 DNA damage response axis. (A) Heatmap showing hepatic expression of DNA damage repair machinery measured by qPCR in acute DEN-injured WT or $Rel^{-/-}$ mice. (B) Western blot showing Chk2, p53, and c-Rel expression compared with β-actin loading control in DEN-injured WT or $Rel^{-/-}$ mice. (C) Schematic showing the ATM-mediated DNA damage response pathway; proteins colored in red are differentially expressed in $Rel^{-/-}$ versus WT hepatocytes. (D) Experimental timeline of *in vitro* hepatocyte γ-irradiation/DEN injury. (E) Heatmap showing expression of *Atm, Rad50, MRE11, NBS1, Chek2, Tp53*, and *Cdkn1a* in γ-irradiated WT or $Rel^{-/-}$ hepatocytes. (F) ChIP analysis of c-Rel enrichment at the *Atm, Rad50, Chek2*, and *Tp53* promoters of untreated or γ-irradiated WT hepatocytes. (G) Experimental timeline of Chk2 inhibitor (Chk2i) therapy in the acute DEN injury model. (H) Average percentage area of necrotic liver tissue and representative images of H&E-stained liver sections in acute DEN-injured WT or $Rel^{-/-}$ mice treated ± Chk2i. Masking of necrotic tissue (lower right corner) is highlighted in red, blue denotes healthy liver tissue, and vessels are colored white. (I) Graph showing mean γH2AX positive hepatocytes in WT or $Rel^{-/-}$ mice treated ± Chk2i. (J) Schematic showing that loss of c-Rel sensitizes hepatocytes to DNA damaging agents. Failure to induce key genes in the DNA damage response pathway results in genomic instability and earlier onset of HCC development. The *p* values are calculated using a two-way ANOVA with a Sidak post hoc test or paired *t*-test. ATM, ataxia-telangiectasia mutated; ATR, ataxia-telangiectasia, RAD3-related; Chk2i, Chk2 inhibitor; ChIP, chromatin immunoprecipitation; HCC, hepatocellular carcinoma; WT, wild-type.

mediated signaling pathway was validated at the protein level, with *Rel*$^{-/-}$ mice displaying a reduction in both checkpoint kinase 2 (Chk2) and p53 (Figure 5B). The schematic shows c-Rel–regulated Atm-Chk2-p53 pathway genes highlighted in red (Figure 5C).

To investigate this further, cultured WT and *Rel*$^{-/-}$ hepatocytes were γ-irradiated or challenged with DEN to induce DNA damage (Figure 5D). Expression of the Atm-Chk2-p53 pathway genes, *Atm*, *Rad50*, *Chek2*, and *p53*, were significantly reduced in *Rel*$^{-/-}$ hepatocytes compared with WT hepatocytes in response to γ-irradiation or DEN (Figures 5E and S7A,B). Chromatin immunoprecipitation assays confirmed that γ-irradiation promotes recruitment of c-Rel to promoter regions of the *Atm, Rad50, Chek2*, and *p53* genes (Figure 5F), indicating that c-Rel is a potential upstream transcriptional regulator of these genes in hepatocytes following genotoxic injury.

To test if Chk2 activation was a key step in limiting genotoxic hepatocellular damage, we prophylactically treated acute DEN-injured WT and *Rel*$^{-/-}$ mice with CCT-241533, a potent and highly selective small molecule inhibitor of Chk2 (Chk2i)[37] (Figures 5G and S7C). Consistent with previous data (Figure S4), there was a significant increase in necrotic area, serum ALT and AST, and γH2AX positive hepatocytes in *Rel*$^{-/-}$ mice compared with vehicle-treated WT mice (Figures 5H,I and S7D–F). Prophylactic treatment with a Chk2i sensitized WT mice to DEN-mediated injury, with serum aminotransferases, necrotic area, and γH2AX positive hepatocytes at levels comparable to *Rel*$^{-/-}$ mice (Figure 5H,I). As expected, the Chk2i did not further exacerbate liver injury in *Rel*$^{-/-}$ mice, suggesting that the induction of Chk2 by c-Rel following injury is critical in this model. We propose that c-Rel acts as a putative tumor suppressor, where loss of c-Rel and subsequent transcriptional regulation of DNA repair machinery lead to genomic instability and acquisition of genetic mutations, ultimately promoting earlier onset of HCC (Figure 5J).

c-Rel inhibition sensitizes tumors to chemotherapy and limits HCC growth

Given that c-Rel limits genotoxic injury, we asked if this could be exploited therapeutically and predicted that pharmacological inhibition of c-Rel could increase the effectiveness of chemotherapy on promoting HCC cell death and reducing HCC growth when given in combination. To test this *in vitro*, we created spheroids from the human HuH7 cell line and treated them with either the c-Rel inhibitor IT-603,[38] doxorubicin, or a combination of IT-603 and doxorubicin (Figure 6A). As anticipated, doxorubicin reduced spheroid growth, but this was further suppressed by dual IT-603/doxorubicin therapy, whereas IT-603 alone modestly increased spheroid growth (Figure 6B,C). Given the effectiveness of the dual IT-603/doxorubicin therapy, we asked if IT-603 could enhance the efficacy of the tyrosine kinase inhibitor, sorafenib (Figure S8A). Sorafenib significantly reduced spheroid growth compared with both vehicle- and IT-603–treated groups alone; however, combining it with IT-603 did not result in a further suppression in spheroid growth (Figure S8B,C). These data suggest that IT-603 may only sensitize cells to specific types of anticancer agents, likely those that result in DNA damage.

To determine whether these results could be recapitulated *in vivo*, we performed a syngeneic orthotopic HCC model,[39] where Hep53.4 cells, a murine HCC cell line, were intrahepatically injected into WT mice (Figure 6D). Tumor burden was significantly reduced by administration of both mono IT-603 and doxorubicin treatment, but as predicted, these therapies synergize to suppress growth further (Figure 6E,F). Given that we have previously demonstrated a key role for c-Rel in the immune system (Figure 2), we next wanted to assess whether IT-603 inhibition of c-Rel alters the tumor immune microenvironment to promote tumor clearance. Paradoxically, infiltration of intratumoral CD8$^+$ T cells was significantly increased in IT-603/doxorubicin–treated mice compared with vehicle-treated controls and both monotherapies (Figure 6G). Similarly, IT-603/doxorubicin dual therapy also resulted in an increase in F4/80$^+$ macrophages (Figure 6H). Macrophage secretion of the chemokine Chemokine (C-X-C motif) ligand 19 (*Cxcl9*) has been shown to be important for the recruitment of antitumor CD8$^+$ T cells.[40] Tumor gene expression analysis revealed that IT-603/doxorubicin dual therapy results in an increase in *Cxcl9* expression (Figure 6I). These data suggest that IT-603 not only sensitizes tumor cells to genotoxic injury but also by indirect mechanisms may contribute to reprogramming of tumor immune microenvironment, increasing immune-mediated antitumor activity. These data highlight the complexity and challenges when pharmacologically targeting the NF-κB pathway in cancer.

DISCUSSION

Persistent, uncontrolled NF-κB signaling is associated with chronic inflammation, disease, and cancer. In tumor cells, NF-κB can regulate a plethora of genes controlling cancer-related cellular processes, including apoptosis, cellular proliferation, angiogenesis, tumor-promoting inflammation, and metastasis and therefore mechanistically underpins many of the "hallmarks of cancer."[41] However, NF-κB signaling is complex, and its role in cancer initiation and progression can be both subunit and cancer-type specific. In the liver, both tumor promotor and suppressor roles of canonical NF-κB activation have been described. RelA signaling in

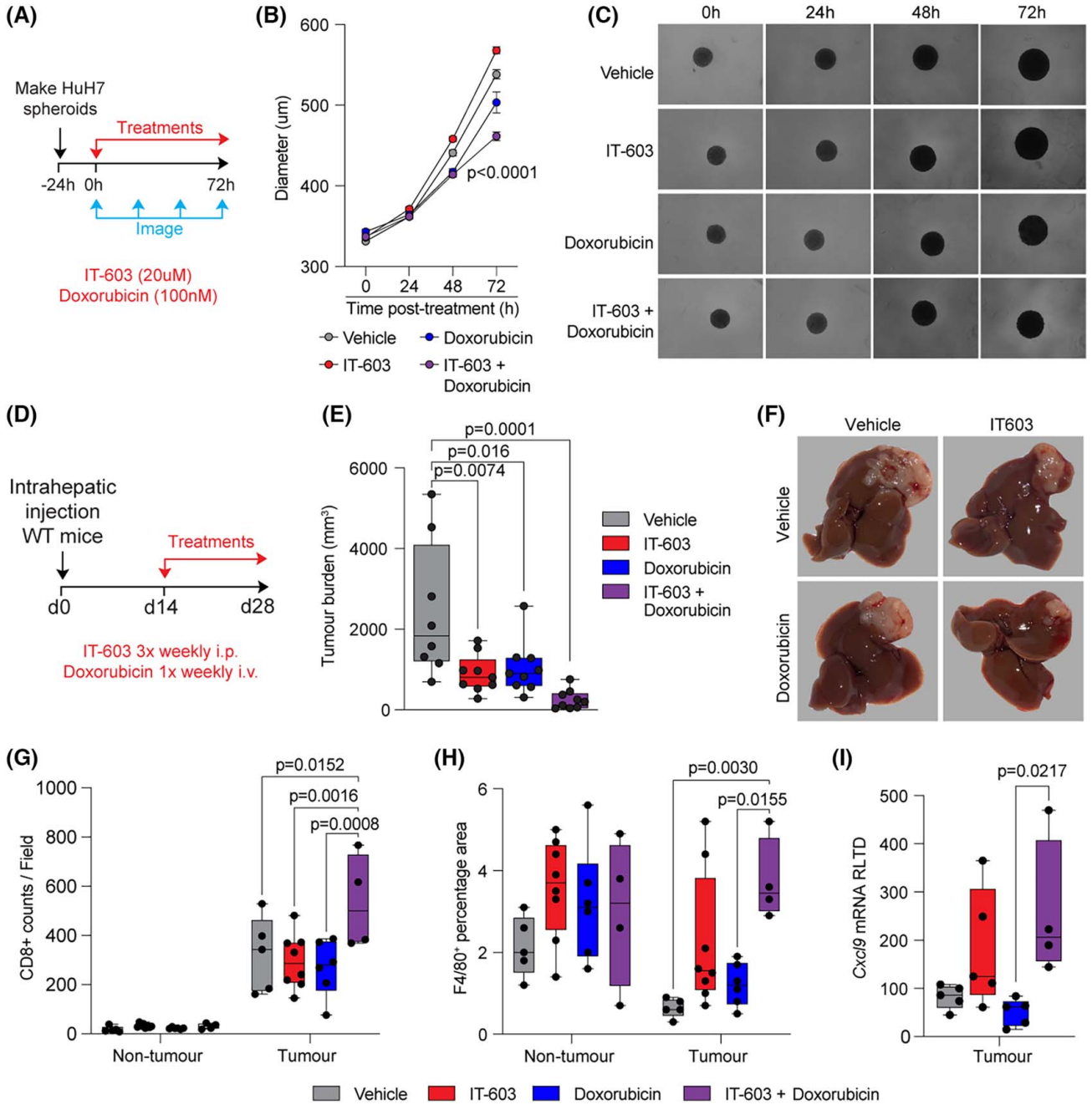

FIGURE 6 Combination c-Rel inhibitor and chemotherapy is more effective than monotherapy in limiting HCC growth. (A) Experimental timeline of IT-603 and doxorubicin mono and dual therapy in the *in vitro* Huh7 spheroid model. (B,C) Graph showing Huh7 spheroid growth over 72 h (B) and representative spheroid pictures (C) after either vehicle or IT-603 and doxorubicin mono or dual therapy. Data are mean ± SEM in eight spheroids/group. (D) Experimental timeline of IT-603 and doxorubicin mono and dual therapy in the orthotopic model. (E,F) Graph showing tumor burden (E) and representative pictures (F) of livers *ex vivo* of orthotopic model tumors in WT mice receiving either IT-603 and doxorubicin mono or dual therapy. (G,H) Graph showing recruitment of $CD8^+$ T cells(g) or $F4/80^+$ macrophages (H) into the tumor and nontumor tissue of orthotopic mice with HCC receiving either IT-603 and doxorubicin mono or dual therapy. (I) Graph showing tumor expression of *Cxcl9* mRNA in orthotopic mice with HCC receiving either IT-603 and doxorubicin mono or dual therapy. Data are mean ± SEM in 10–12 mice/group; each data point represents a different donor. The *p* values were calculated using two-way ANOVA with Sidak's post hoc test.

hepatocytes promotes hepatic inflammation and compensatory proliferation in the damaged liver but was only shown to be important in HCC progression, not initiation.[42] Mice lacking *nfkb1* (p105/p50) spontaneously develop liver cancer with age[43] and have accelerated tumorigenesis in the DEN model[33] because of a loss of p50:p50 homodimers, which suppress hepatic inflammation and neutrophil recruitment. Interestingly, bioinformatic interrogation of the cBioPortal TGCA database revealed that low NFKB1 mRNA expression in tumors of patients with HCC was associated with reduced survival (Figure S5A).

In the liver, whole body or hepatocyte- or myeloid-specific c-Rel deletion limits fibrosis,[32,44] suggesting that c-Rel plays a protective role in chronic liver disease. However, the cell-specific actions of c-Rel in regulating hepatocyte regeneration are more complex. In the partial hepatectomy model, c-Rel deletion in hepatocytes induced expression of cell cycle genes and increased cellular proliferation,[32] whereas hepatocyte proliferation was attenuated in whole body or myeloid-specific c-Rel null mice, suggesting that crosstalk between epithelial and immune cells regulates hepatic regeneration.[32,44] c-Rel also has key roles in the immune system, regulating myeloid cell activation and T-cell development and function.

Here, we investigated a role for c-Rel in liver carcinoma. Unexpectedly, we discovered that low c-Rel expression in tumors of patients with HCC at the protein and mRNA level was indicative of more advanced disease, poorer prognosis, and a greater mutational burden. Synonymous with this observation, tumor burden was increased in $Rel^{-/-}$ and $Rel^{\Delta Alb}$ mice compared with controls in the DEN model of HCC. However, if c-Rel was deleted once precancerous lesions and early HCC had formed ($Rel^{\Delta AAV}$ mice), there was no difference in tumor size and frequency between the $Rel^{fl/fl}$ and $Rel^{\Delta AAV}$ animals, suggesting that c-Rel signaling is important to limit tumor initiation but not required to promote tumor progression. This was confirmed *in vivo* in the acute DEN model, in which $Rel^{\Delta Alb}$ mice develop more DNA damage, and *in vitro*, in which $Rel^{-/-}$ hepatocytes were more sensitive to genotoxic injury but independent of ROS production (Figures 4 and S6). An extensive body of literature describes an association between NF-κB signaling and the DNA damage response; however, this typically focuses on the role of canonical NF-κB (RelA/p50) signaling.[36,41,45,46] Evidence for c-Rel–dependent regulation of the DNA damage response is limited and exclusively confined to the ATR/CHK1 pathway via regulation of CLSPN[34]. In our study, we identified a previously unrealized role for c-Rel in regulating the Atm-Chk2-p53 pathway in response to genotoxic injury, and we propose that disruption of this pathway in $Rel^{-/-}$ mice drives genomic instability and tumorigenesis. Given that c-Rel signaling is required to maintain genomic stability and cell survival after genotoxic injury, we were able to exploit this and use the c-Rel inhibitor IT-603 to enhance the antitumorigenic actions of doxorubicin therapy. This effect was limited to anti-cancer agents that cause direct DNA damage, with IT-603 unable to enhance the efficacy of the tyrosine kinase inhibitor, sorafenib. In future studies, it would be interesting to determine if similar therapeutic benefits could be achieved if IT-603 was administered in combination with either radiotherapy, transcatheter arterial chemoembolization, or selective internal radiotherapy.

It is important not to overlook the contribution of c-Rel signaling in the nontumor liver and immune cells to liver cancer. In the DEN and orthotopic model, larger tumors developed in the c-Rel–deficient animals, and this was associated with a significant reduction in recruitment of $CD8^+$ T cells to the tumor, suggesting a loss of antitumor immunity. To maintain immunological integrity and tumor suppressive function of c-Rel in the immune system, we deleted c-Rel in hepatocytes only ($Rel^{\Delta Alb}$ mice). These animals developed more DEN-induced tumors than control mice, but the recruitment of inflammatory cells was unaffected, identifying a previously unreported tumor suppressive role for c-Rel outside of the immune system.

Pharmacological inhibition of c-Rel using IT-603 monotherapy resulted in an unexpected reduction in orthotopic HCC tumor burden. Similarly, dual therapy with IT-603 and doxorubicin led to a reprogramming of the tumor immune microenvironment and an unexpected increase intratumoral $CD8^+$ T cells. Whether these observations are the result of partial c-Rel inhibition, indirect or off target effects of the compound, needs to be further explored; however, our data do highlight a complex role for c-Rel in the immune system in cancer that can be exploited alongside sensitizing genotoxic agents to improve cancer therapy efficacy.

Here, we identify c-Rel as a regulator of the ATM-Chk2-p53 pathway and limiting DNA damage in hepatocytes caused by genotoxic insults. Loss of c-Rel signaling in hepatocytes in HCC is associated with more advanced stage of disease and worse outcome. Pharmacological inhibition of c-Rel sensitizes HCC cells in culture to chemotherapy, and this can be exploited therapeutically. We propose that chemo-sensitizing adjuvant therapy could be explored therapeutically for HCC.

AUTHOR CONTRIBUTIONS

Jack Leslie performed a majority of the laboratory-based work and analyses presented in the manuscript. Lauren G. Russell, Amy Collins, Amelia Rushton, Erik Ramon-Gil, Maja Laszczewska, Misti McCain, Marco Y. W. Zaki, Amber Knox, Yixin Seow, Laura Sabater, Daniel Geh, and Jill E. Hunter performed a portion of the laboratory experiments and their related analyses. Dina Tiniakos, Derek A. Mann, Helen L. Reeves, and Neil D. Perkins provided advice, samples, and/or contributed to the experimental design and writing. Jack Leslie, Jill E. Hunter, and Fiona Oakley conceived the studies, designed the experiments, and wrote the manuscript. All authors read and commented on the final manuscript.

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CONFLICTS OF INTEREST

Derek A. Mann owns stock in, is employed by, received grants from, and owns intellectual property rights in FibroFind Ltd. Jack Leslie owns stock in Fibrofind Ltd. Fiona Oakley owns stock in, received grants from, and is employed by FibroFind Ltd. She owns stock in FibroFind IP Ltd and FF Estates Ltd.

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