The Ubiquitin E3 Ligase TRIM21 Promotes Hepatocarcinogenesis by Suppressing the p62-Keap1-Nrf2 Antioxidant Pathway

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SUMMARY
The ubiquitin E3 ligase Tripartite motif-containing protein 21 (TRIM21) is highly expressed in hepatocellular carcinoma (HCC). Genetic ablation of TRIM21 suppresses diethylnitrosamine (DEN)-induced hepatocarcinogenesis by activating the p62-Keap1-Nrf2 antioxidative pathway.

BACKGROUND AND AIM: TRIM21 is a ubiquitin E3 ligase that is implicated in numerous biological processes including immune response, cell metabolism, redox homeostasis, and cancer development. We recently reported that TRIM21 can negatively regulate the p62-Keap1-Nrf2 antioxidative pathway by ubiquitylating p62 and preventing its oligomerization and protein sequestration function. As redox homeostasis plays a pivotal role in many cancers including liver cancer, we sought to determine the role of TRIM21 in hepatocarcinogenesis.

METHODS: We examined the correlation between TRIM21 expression and the disease using publicly available data sets and 49 cases of HCC clinical samples. We used TRIM21 genetic knockout mice to determine how TRIM21 ablation impact HCC induced by the carcinogen DEN plus phenobarbital (PB). We explored the mechanism that loss of TRIM21 protects cells from DEN-induced oxidative damage and cell death.

RESULTS: There is a positive correlation between TRIM21 expression and HCC. Consistently, TRIM21 knockout mice are resistant to DEN-induced hepatocarcinogenesis. This is accompanied by decreased cell death and tissue damage upon DEN treatment, hence reduced hepatic tissue repair response and compensatory proliferation. Cells deficient in TRIM21 display enhanced p62 sequestration of Keap1 and are protected from DEN-induced ROS induction and cell death. Reconstitution of wild-type but not the E3 ligase-dead and the p62 binding-deficient mutant TRIM21 impedes the protection from DEN-induced oxidative damage and cell death in TRIM21-deficient cells.

CONCLUSIONS: Increased TRIM21 expression is associated with human HCC. Genetic ablation of TRIM21 leads to protection against oxidative hepatic damage and decreased hepatocarcinogenesis, suggesting TRIM21 as a preventive and therapeutic target. (Cell Mol Gastroenterol Hepatol 2021;11:1369–1385; https://doi.org/10.1016/j.jcmgh.2021.01.007)

Keywords: TRIM21; Hepatocellular Carcinoma; p62; Nrf2; Diethylnitrosamine.

A major risk factor in hepatocellular carcinoma (HCC) is dysregulated redox homeostasis that is associated with various oncogenic events such as hepatitis B and C viral infection, obesity-induced nonalcoholic fatty liver disease, and alcohol-associated fatty liver diseases.1-3 Increased reactive oxygen species (ROS) can lead to oncogenic events such as elevated mitogenic signaling, genomic instability, increased metastatic potential, and altered tumor microenvironment. On the one hand, it is generally thought that reducing oxidative stress can be cancer preventive by lowering the chance of accumulating oncogenic mutations. On the other hand, it has been increasingly recognized that chronic and excessive antioxidant response can be oncogenic by promoting cancer cell survival and reducing

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Abbreviations used in this paper: 8-oxo-dG, 8-oxo-2-deoxyguanosine; α-SMA, α-smooth muscle actin; AFP, α-fetoprotein; cDNA, complementary DNA; DEN, diethylnitrosamine; DMEM, Dulbecco’s modified Eagle’s medium; FBS, fetal bovine serum; HCC, hepatocellular carcinoma; IHC, immunohistochemistry; Keap1, Kelch-like ECH-associated protein 1; KO, knockout; LD, lipase dead; MEF, mouse embryonic fibroblast; mRNA, messenger RNA; Nrf2, nuclear factor E2-related factor 2; PB, phenobarbital; PBS, phosphate-buffered saline; PBST, phosphate-buffered saline with 0.02% Tween 20; PCNA, proliferation cell nuclear antigen; PCR, polymerase chain reaction; PI, propidium iodide; RNA-seq, RNA sequencing; ROS, reactive oxygen species; SDS, sodium dodecyl sulfate; TCGA, The Cancer Genome Atlas; TRIM21, tripartite motif-containing protein 21; WB, western blotting; WT, wild-type.

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Results

TRIM21 Expression Is Elevated in Human HCC

We first examined the expression of TRIM21 in human HCC using publicly available datasets. In the Gene Expression Omnibus Series,19 6 of 18 studies showed elevated TRIM21 expression in HCC compared with normal liver tissues (Figure 1A). The Cancer Genome Atlas (TCGA) also showed that TRIM21 transcript level is elevated in tumor tissues and positively correlates with disease progression (Figure 1B), and that the expression level of TRIM21 correlates with a worse overall survival although not with disease-free survival (Figure 1C). These observations are opposite to that was reported in GSE14520, in which increased TRIM21 correlates with better survival20 and in other studies in the TCGA. To closely examine the role of TRIM21 in HCC, we performed TRIM21 immunohistochemistry (IHC) using cohorts of 49 HCC samples, 29 paracancerous tissues, and 20 hepatic hemangioma tissues as normal liver sample controls. TRIM21 protein level was markedly higher in HCC tumor tissues than that in nonmalignant liver tissues from peripheral hepatic hemangioma or in pericancreatic tissues (Figure 1D and E). Among the 49 patients, 35 (71.43%) were infected with hepatitis B virus and 36 (73.47%) patients were infected with cirrhosis, suggesting a potential etiological connection between TRIM21 expression and these HCC risk factors. We also compared the diagnostic capacity of TRIM21 with the commonly used HCC biomarker α-fetoprotein (AFP). TRIM21 showed higher area under the receiver-operating characteristic curve (= 0.798) than AFP (= 0.756) (Figure 1F). The diagnostic performance was calculated and showed the sensitivity (79.6% vs 61.2%), specificity (80.0% vs 90.0%), positive predictive value (90.7% vs 93.8%), negative predictive value (61.5% vs 48.6%), and accuracy (79.7% vs 69.6%) for TRIM21 (TRIM21 high expression) vs AFP (AFP ≥15 ng/mL), respectively. Taken together, these analyses indicate that TRIM21 expression is elevated in HCC and correlates with a poorer prognosis.

These results are opposite to a previous study that showed high level of TRIM21 in normal liver tissue but decreased TRIM21 in HCC tumor tissues.20 To reconcile the seemingly drastically different results between ours and the previous report, we acquired the same TRIM21 antibody as described in Ding et al.20 To our surprise, this antibody failed to recognize both endogenous and ectopically expressed TRIM21 by immunoblotting (Figure 1G) and did not detect high signal in IHC of both peri-cancerous and HCC tissues (Figure 1H). Therefore, while we cannot fully explain why the results in Ding et al.20 are drastically different from ours, we suspect that the discrepancy may be caused, at least in part, by the different resources and validity of reagents. Taken together, our previous analyses indicate that TRIM21 expression is elevated in HCC and correlates with a poorer prognosis.

TRIM21-Deficiency Inhibits HCC Development in the Diethylnitrosamine/Phenobarbital Model

We next determined the role of TRIM21 in HCC development. We chose to use the chemical carcinogen diethylnitrosamine (DEN)/phenobarbital (PB)-induced HCC model, which has been characterized to have histology and gene expression similar to human HCC.21–23 DEN is bio-activated in the liver by cytochrome P450 to cause oxidative damage and genotoxic lesions and subsequent tissue repair response. PB promotes the progression from DEN-induced early dysplastic lesions to fully malignant tumors. TRIM21 wild-type (WT) and knockout (KO) male mice were given a
single intraperitoneal injection of DEN at day 14 after birth. All WT mice developed HCC 10 months later. Strikingly, TRIM21−/− mice showed markedly less HCC development than the WT mice, while the heterozygote TRIM21+/− mice showed an intermediate phenotype (Figure 2A–D). Close examination of the tumor tissues showed that solid type...
HCC that was poorly differentiated (including tumor giant cell) in WT, moderately differentiated in TRIM21+/−, and well differentiated in TRIM21/− mice, respectively (Figure 2E). While both WT and KO tumors expressed high levels of a HCC marker HSP70, the WT tumor cells proliferated much faster than the KO tumor cells (Figure 2F). Tissue fibrotic response was also markedly decreased in TRIM21/− mice, as indicated by the expression of α-smooth muscle actin (α-SMA) and Sirius Red staining (Figure 2G). These data indicate that TRIM21 deficiency confers resistance to DEN/PB-induced HCC development in mice.

TRIM21+/− Mice Are Protected From DEN-Induced Liver Damage

DEN is a genotoxic agent that induces oxidative DNA damage, cell death, and dysplastic lesions. The resulting chromatin instability, activating oncogenic mutations, and compensatory proliferation of the surviving hepatocytes contribute to DEN-induced carcinogenesis. Therefore, we examined whether TRIM21+/− mice were protected from DEN-induced acute liver damage. WT and TRIM21 KO mice were treated with 1 intraperitoneal injection of DEN for 12, 24, and 48 hours. TRIM21+/− mice were protected from acute liver damage caused by DEN treatment as indicated by the serological levels of alanine aminotransferase (Figure 3A) and aspartate aminotransferase (Figure 3B). Both the untreated WT and TRIM21+/− livers showed similar levels of the DEN-metabolizing enzymes, suggesting that the decreased acute liver damage in TRIM21+/− mice was not due to decreased expression of the DEN-metabolizing enzymes (Figure 3C). Hematoxylin and eosin staining showed that in WT mice, DEN treatment for 12 and 24 hours induced perivenular necrosis, with hepatocytes showing microvesicular change and inclusions of scattered neutrophils, lymphocytes, and monocytes (Figure 3D and E), with cells around the portal tracts spared. These changes were resolving at 48 hours (Figure 3D and E). The extent of liver damage was drastically less severe in TRIM21 KO mice at all time points (Figure 3D). Consistently, DEN treatment led to more profound infiltration of F4/80-positive monocytes in WT than in KO livers (Figure 3E). Moreover, acute DEN treatment is known to induce accumulation of lipid droplets via oxidative inhibition of β-oxidation enzymes. This DEN-induced accumulation of lipid droplets was significantly suppressed in TRIM21/− livers at 12- and 24-hour time points, which was further resolved at 48 hours (Figure 3F and G). These results indicate that TRIM21+/− mice are protected from acute oxidative hepatocyte damage induced by DEN treatment.

We showed previously that TRIM21 is a negative regulator of the p62-Keap1-Nrf2 antioxidant pathway, and that TRIM21-deficient mice are protected from oxidative damage in arsenic-induced liver damage and aortic transverse operation–induced heart failure. Our previous results suggest that TRIM21 deficiency confers protection from DEN-induced oxidative hepatocyte damage. We then looked at whether the p62-Keap1-Nrf2 pathway is involved in DEN-induced liver damage. Indeed, consistent with our previous finding that TRIM21 negatively regulates p62 oligomerization and protein aggregation, TRIM21 KO livers showed increased p62 and Keap1 aggregation upon DEN treatment, as indicated by increased integrated optical density of both proteins (Figure 4A), as well as increased NQO1 expression indicative of elevated Nrf2 activity (Figure 4B). These were accompanied by decreased H2AX phosphorylation (a DNA damaging marker), 8-oxo-2-deoxyguanosine (8-oxo-dG) (an oxidative derivative of deoxyguanosine), and cleaved caspase-3 (Figure 4A). Interestingly, Akt activation as indicated by phosphorylated Akt was higher in DEN-treated TRIM21 KO livers (Figure 4B), suggesting that Akt may play a cytoprotective role in the hepatocytes exposed to DEN, although this increased phospho-Akt may also lead to predisposition of increased tumorigenesis. Collectively, these results indicate that TRIM21 KO livers have increased p62-Keap1-Nrf2 antioxidative activity and are protected from DEN-induced oxidative damage.
TRIM21 ablation leads to decreased HCC induced by DEN/PB. (A) TRIM21+/+(n = 8), TRIM21-/- (n = 15), and TRIM21-/- (n = 14) 14-day-old male mice were intraperitoneal injected with DEN (5 mg/kg), then fed with 0.05% PB in drinking water 7 days later. Livers from DEN/PB-treated mice were collected after 10 months. Representative images are shown. (B) Liver weight and body weight were measured 10 months after DEN/PB treatment. Ratio of liver weight to body weight was plotted. (C) Total tumor number was counted and plotted. Each dot represents 1 mouse. (D) Tumors with >0.3 cm diameter was counted and plotted. (E) Liver tissue sections were processed for hematoxylin and eosin staining. TRIM21+/+(left panel), TRIM21+/+(middle panel), and TRIM21-/- (right panel) liver tissue showed solid type hepatocellular carcinoma, with poorly differentiated (including tumor giant cell), moderately differentiated, and well differentiated, respectively. (F) Liver tissue sections were stained for HSP70 and PCNA. PCNA-positive cells were counted and the means of 3 randomly selected fields are shown. (G) Liver tissue sections were processed for Sirius Red staining and α-SMA/DAPI staining by immunofluorescence. Representative images are shown. *P < .05. **P < .01. ***P < .001. IOD, integrated optical density.

TRIM21 KO Mouse Livers Show Enhanced Antioxidant Capacity and Decreased Compensatory Proliferation

To further determine the effect of TRIM21 ablation on the liver tissue response to DEN treatment, we performed RNA sequencing (RNA-seq) analysis of liver tissues of TRIM21 WT or KO mice treated with DEN for 48 hours. Differentially expressed genes were identified by a cutoff of 4-fold and P < .05 (Figure 5A). Upon DEN treatment, markedly more genes in TRIM21 WT (n = 843) were altered than in TRIM21 KO (n = 339), with 266 genes in common (Figure 5B; Supplementary Table S1). Gene Ontology analysis revealed that the 577 genes uniquely altered in TRIM21 WT livers in response to acute DEN treatment were associated with redox regulation and chemical carcinogenesis (Figure 5C). In contrast, no significant enrichment was found in the 73 genes specifically altered in TRIM21 KO livers. The enrichment associated with chemical carcinogenesis was not significant in the 266 commonly altered genes. While DEN treatment did not significantly alter hepatocyte and cholangiocyte markers in WT and KO livers, it significantly induced the expression of markers of hepatic stellate cells and Kupffer cells (Figure 5D), consistent with our observations that DEN-induced fibrosis was lower in the KO livers than in the WT livers (Figure 2G). In addition, no significant difference in cytochrome P450 (Cyp2e1 and Cyp2f2) expression was observed between TRIM21 WT vs KO livers, before and after DEN treatment (Figure 5D), consistent with our observation by immunoblotting (Figure 3C). Quantitative reverse-transcriptase polymerase chain reaction (qRT-PCR) analysis confirmed the significantly decreased expression of antioxidant genes GSTM1 and GSTM2 and increased expression of proliferation-related proteoglycans c-FOS, c-JUN, and JUNB in TRIM21 WT but not KO livers.
Consistently, IHC analysis demonstrated that the proliferation cell nuclear antigen (PCNA) was highly induced upon DEN treatment in TRIM21 WT but not in KO livers (Figure 5F). Taken together, these results indicate that TRIM21 KO livers have better antioxidant capacity and decreased compensatory hepatic proliferation compared with TRIM21 WT livers.

TRIM21-Deficient Cells Are Protected From DEN-Induced Oxidative Damage via p62-Mediated Sequestration of Keap1

We showed previously that TRIM21 interacts with and ubiquitylates p62 and suppresses p62 oligomerization and Keap1 sequestration, which negatively regulates cellular antioxidant response. To determine whether the
protection of TRIM21 KO livers from DEN treatment is via this mechanism, we utilized cultured TRIM21 KO mouse embryonic fibroblast (MEFs) and the human cancer cell line SMMC-7721 for acute DEN treatment. Although DEN is commonly used for in vivo studies, it has also been shown to possess cytotoxicity in cultured cells via similar mechanisms as in vivo.29–31 TRIM21-KO MEFs were reconstituted with HA-tagged WT, the ubiquitin E3 ligase dead (LD), and p62 binding-deficient mutant (W381/383A) mutants of TRIM21 (Figure 6A). DEN treatment induced DNA damage as indicated by H2AX phosphorylation (γH2AX) in TRIM21 WT MEFs, which was largely suppressed in TRIM21 KO MEFs.
Reconstitution of WT, but not the E3 LD and the p62 interaction–deficient (W381/382A) mutants, resumed the DEN-induced DNA damage (Figure 6B). Consistently, WT TRIM21 reconstituted cells displayed increased ROS indicated by H2DCFDA staining, while the LD and W381/383A mutants showed similar level of ROS as the KO MEFs (Figure 6C). Immunofluorescence analysis showed that DEN induced the formation of protein aggregates that were
positive for p62 and Keap1 in TRIM21 KO MEFs and those reconstituted with the LD and W381/383A mutants but not with WT TRIM21 (Figure 6D). Cellular fractionation of detergent (1% Triton X-100) soluble and insoluble fractions also showed that DEN treatment led to increased insoluble p62 and Keap1 in TRIM21 KO MEFs, which was reduced by the reconstitution of WT but not the LD and W381/383 mutants of TRIM21 (Figure 6E). These results indicate that TRIM21 negatively regulates DEN-induced p62 aggregation and Keap1 sequestration. Ablation of TRIM21 leads to decreased oxidative stress.

Similarly, in the human SMMC-7721 cancer cell line, acute DEN treatment led to significant amount of cell death, which was alleviated by the ROS scavenger N-acetylcysteine (Figure 7A). This indicates that DEN-induced cell death is mediated by increased oxidative stress (Figure 7A). We then used 2 independent short hairpin RNA of TRIM21 to silence TRIM21 (Figure 7B). Upon DEN treatment in vitro, the knockeddown cells increased cell viability (Figure 7C). Consistent with increased p62 sequestration of Keap1 in TRIM21-deficient cells upon other oxidative stress,18 DEN treatment led to increased p62 and Keap1 in the detergent-insoluble fraction in the TRIM21-silenced cells (Figure 7D). This was correlated with increased Nrf2 in the nuclear fraction (Figure 7E), and decreased DNA damage as indicated by H2AX phosphorylation (γH2AX) (Figure 7F). Taken together, the previous results demonstrate that ablation of TRIM21 leads to increased p62 oligomerization and Keap1 sequestration, which protects hepatocytes from DEN-induced genotoxic damage and cell death.

Discussion
In this study, we report that TRIM21 expression is elevated in human HCC by examining clinical samples and publicly available datasets. We also show that TRIM21 KO mice are resistant to hepatocarcinogenesis induced by carcinogen DEN. The decreased HCC development is correlated with decreased ROS production, DNA damage, and cell death in TRIM21-deficient mice and cells upon DEN treatment. As DEN is known to induce HCC by causing genomic instability, cellular damage, and subsequent compensatory proliferation of the remaining survived cells, our data indicate that ablation of TRIM21 plays a protective role in maintaining hepatocyte homeostasis and survival and hence reduces carcinogenesis.

Redox regulation plays a critical role in organismal homeostasis. While certain level of ROS is beneficial by serving as signaling molecules and by promoting antipathogen responses, elevated ROS can cause oxidative damage to macromolecules thus genomic instability, cellular damage, and ultimately cell death. The role of ROS in cancer has been complex and is dependent on tissue types and temporal stages of tumor initiation and development. Antioxidant response on one hand can prevent tumor initiation by maintaining cellular redox homeostasis and suppressing oxidative damage, on the other hand can enhance survival and growth of established tumor cells hence facilitate tumor progression and therapy-resistance.12-34 We have previously shown that TRIM21, a RING finger-containing E3 ubiquitin ligase, negatively regulates the p62-Keap1-Nrf2 antioxidant pathway.18 Therefore, it is foreseeable that loss of TRIM21 should be equivalent to gain of function of p62 and Nrf2 and of loss of function of Keap1, in their capacity in regulating redox balance and cellular homeostasis. Our previous study showed that TRIM21 KO hearts are protected from cardiac failure induced by pressure overload and livers protected from arsenic-induced liver damage,19 and the current study shows that TRIM21-deficient cells and liver are protected from DEN-induced oxidative damage. Indeed, numerous studies on the p62-Keap1-Nrf2 axis have demonstrated consistent results in protection against cellular and tissue damages. Activation of Nrf2 attenuated myocardial ischemia and reperfusion injury35 and oxidative liver damage.36-40

In cancer, the p62-Keap1-Nrf2 axis plays a paradoxical role.13,41 While some studies showed that activation of Nrf2 promotes tumorigenesis,12-44 others showed it functions as a tumor-suppressor by maintaining redox homeostasis.14,45,46 KEAP1-inactivating mutations and Nrf2-activating mutations are prevalent in a wide spectrum of cancers, suggesting that NRF2 functions as an oncoprotein. Increased p62 in hepatocytes promotes HCC,44 whereas increased p62 in hepatic stellate cells suppresses liver inflammation, fibrosis, and HCC.16 Therefore, the role of the p62-Keap1-Nrf2 axis in cancer is highly context dependent and may vary depending on different stages of tumor initiation and progression. NRF2 activation may inhibit chemically
induced tumor initiation but accelerates tumor progression at tumor progression stages by increasing the antioxidant capacity for cancer cells against the harsh tumor microenvironment. Similarly, the role of TRIM21 in cancer is complex. While genetic deletion of TRIM21 or reduced TRIM21 expression has been found to associate with the development and poor prognosis in non-small cell lung cancer and diffuse large B cell lymphoma, [47–49](#)

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**Figure A**

- **HA**: TRIM21
- **TRIM21**: GADPH
- **B**: TRIM21+/+ vs. TRIM21−/−
- **γ-H2A.X (ser139)**
- **GADPH**

**Figure C**

- **Cell counts**
- **H2DCFDA (log)**

**Figure D**

- **p62**
- **keap1**
- **DAPI**
- **Merge**

**Figure E**

- **p62**
- **Keap1**
- **β-tubulin**

(Images and data from Cellular and Molecular Gastroenterology and Hepatology Vol. 11, No. 5)
Figure 7. Silencing of TRIM21 protects cells from DEN-induced genotoxic damage and cell death. (A) SMMC-7721 cells were treated with DEN (50 mM) alone or together with N-acetylcysteine (5 mM) for indicated time. Cell viability was analyzed by PI exclusion. ***$P < .001$. (B) TRIM21 was silenced in SMMC-7721 cells using 2 independent short hairpin RNAs. Cells were lysed in RIPA buffer with 1% SDS and subjected to WB. (C) SMMC-7721 with nontargeted control, shTRIM21 #1 and shTRIM21 #2 were treated with DEN (50 mM) for indicated periods of time. Cell viability was determined by trypan blue exclusion. Shown are the mean plus SD of 3 countings. *$P < .05$. (D) SMMC-7721 with nontargeted control, shTRIM21 #1, and shTRIM21 #2 were treated with DEN (50 mM) for 6 hours. Cells were lysed in immunoprecipitation lysis buffer containing 1% Triton X-100. The insoluble fraction was dissolved in RIPA buffer containing 1% SDS. Both Triton X-100 soluble and insoluble fractions were subjected to WB, and 30-μg soluble proteins and corresponding volume of insoluble proteins were used for WB. The numbers below Western blot panels indicate the ratios of absolute levels of insoluble and soluble proteins, respectively. (E) SMMC-7721 cells were treated with DEN (50 mM) for 6 hours. Cells were separated into cytoplasmic and nuclear fractions, and 20-μg cytosolic proteins and corresponding volume of nuclear proteins were used for WB. (F) SMMC-7721 cells were treated with DEN (50 mM) for 6 hours. Cells were lysed in RIPA buffer (1% SDS) and subjected to WB.

Figure 6. (See previous page). TRIM21 silencing protects cells from DEN-induced oxidative damage. TRIM21$^{+/−}$ MEFs reconstituted with vector, HA-TRIM21 WT, HA-TRIM21 LD, and HA-TRIM21 W381/383A mutant. (A) Cells were lysed in RIPA buffer with 1% SDS and subjected to WB. (B–E) Cells were treated with DEN (20 mM) for 6 hours. (B) Cells were harvested and probed for indicated proteins by WB. (C) Cells were stained with H2DCFDA and analyzed by flow cytometry. Quantification of relative H2DCFDA intensity (geometry mean of 3 repeats) is shown on the right. ***$P < .001$. (D) Cells were subjected to IF with Keap1 and p62 antibodies and observed under deconvolution microscope. Cells with p62/Keap1 aggregates were counted blindly. Data shown are the averages plus SD of at least 3 countings with over 200 cells. ***$P < .001$. (E) Cells were separated into Triton X-100 (1%) soluble and insoluble fractions, and 30-μg soluble proteins and corresponding volume of insoluble proteins were used for WB. The numbers below Western blot panels indicate the ratios of absolute levels of insoluble and soluble proteins, respectively.
overexpression of TRIM21 has been reported to promote tumor aggressiveness and therapy resistance in glioma, nasopharyngeal carcinoma, colon and pancreatic cancer. In HCC, while one study shows that TRIM21 is a favorable molecule whose downregulation promotes cancer, several Gene Expression Omnibus Series studies, the TCGA dataset, and our own analysis using clinical samples indicate an opposite effect of TRIM21 (ie, its elevated expression correlates with HCC incidence and poor outcome) (Figure 1). It is possible that TRIM21 plays different and even opposite roles in HCC owing to the multifaceted causes and risk factors of this highly heterologous disease. Moreover, it is important to note that while our current study shows that TRIM21 inhibition can decrease HCC development by protecting hepatocytes against oxidative DNA damage, TRIM21’s other functions such as preventing guanosine 5’-monophosphate synthases (GMPs) from stabilizing p53 hence promoting oncogenesis and its role in immune checkpoint regulation may also contribute to its oncogenic activity. Nonetheless, our study uncovers an important role of TRIM21 in HCC development and suggests that it may serve as a prognostic marker and therapeutic target in HCC as it functions as an important regulator of the redox homeostasis.

Materials and Methods

Mouse Experiments

TRIM21 KO mice with C57BL/6J background were kind gift from Dr Keiko Ozato. To induce HCC, 14-day-old male mice were injected via intraperitoneal with DEN (5 mg/kg; Sigma-Aldrich, St. Louis, MO; N-0756), then fed with 0.05% Sigma-Aldrich, St. Louis, MO; N-0756), then fed with 0.05% DEN-treated mice have been uploaded and are available at Gene Expression Omnibus (GSE164369).

Plasmids

Human TRIM21 complementary DNA (cDNA) was amplified using primers 5’-CGAACCTTACGAGCTGAATACCGGAATTC-3’ and 5’-GGAGTACAGAGTCTTCTGAGGATCGCTTTTACGTA-3’ and were cloned into the retroviral LPC vector. TRIM21 LD and W381/383A mutants were generated following standard site-directed mutagenesis procedure. The primers used for TRIM21 mutagenesis were: for LD C16A, 5’-CGGAATTCACAGCCCTATCTGCAGCTGAACGC-3’ and 5’-GCAGTACAGACTCCTGAGGATCGCCTTACGTA-3’; for W381/383A, 5’-CGGAATTCACAGCCCTATCTGCAGCTGAACGC-3’ and 5’-TTTTTGGTGTTCGCAACGC-3’.

Cell Death Assay

Cell viability was quantified by either PI exclusion or Trypan blue staining. For PI exclusion, cells were collected and resuspended in culture medium with PI (1 μg/mL). Cell viability was determined by flow cytometry (BD Biosciences, Franklin Lakes, NJ; FACScanto II) and analyzed using FlowJo 7.6.1 software (FlowJo, Ashland, OR). For Trypan blue staining, 0.5 mL of cells (1 × 10⁵ cells per mL) were mixed with 0.1 mL of 0.4% Trypan blue and incubated at room temperature for 3 minutes. Cells were counted under a phase-contrast light microscope.

ROS Detection

A total of 5 × 10⁵ cells were plated into 6-cm petri dish. After overnight recovery, cells were untreated or treated with 20-mM DEN. six hours after treatment, cells were incubated with H2DCFDA (MCE HY-D0940, 5 μM) in 5% CO₂ at 37°C. After a 30-min incubation, cells were harvested and analyzed by flow cytometry (FACScanto II) using FlowJo 7.6.1 software.

Cell Culture, Transfection, and Retroviral Infection

HEK293T cells and MEFs were cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% FBS, 100 units/mL penicillin, and 100-μg/mL streptomycin (Invitrogen, Carlsbad, CA). Lipofectamine 2000 (Invitrogen) was used for transfection. TRIM21 KO MEFs were infected with the retroviral linear predictive coding vector to generate reconstruction with HA-TRIM21 WT, HA-TRIM21 LD, and HA-TRIM21 W381/383A, respectively. One day after infection, the virus-containing medium was replaced with fresh medium. Repeat viral infection every 12 hours for 3 times. Cells were collected for at least 5 days to reach sufficient protein expression.
leak that was confirmed by immunoblotting with HA-Tag or TRIM21 antibodies.

SMCC-7721 and MHCC-97L cells were cultured in RPMI 1640 medium supplemented with 10% FBS, 100 units/mL penicillin, and 100-μg/mL streptomycin (Invitrogen). Lipofectamine 2000 (Invitrogen) was used for transfection. Cells were infected with the retroviral nontargeted control, shTRIM21#1, and shTRIM21#2, respectively. One day after infection, the virus-containing medium was replaced with fresh medium. Viral infection was repeated every 12 hours for 3 times. Cells were cultured for at least 5 days before verifying TRIM21 silencing by immunoblotting.

Antibodies

The following antibodies were used: AKT (Cell Signaling, Danvers, MA; #4965; 1:1000 for western blotting [WB]), Phospho-AKT308 (Cell Signaling; #2965; 1:1000 for WB), Phospho-AKT473 (Cell Signaling; #4508; 1:1000 for WB), Cytochrome p450 2E1 (Abcam, Cambridge, United Kingdom; ab28146; 1:2000 for WB), Cytochrome p450 2B10 (Santa Cruz, Dallas, TX; sc-53242; 1:2000 for WB), Cytochrome p450 2D1 (1:2000 for WB).[56] Cytochrome p450 3A (1:2000 for WB),[56] NQO1 (Abcam; ab28947; 1:1000 for WB), Nrf2 (Abcam; ab62352; 1:1000 for WB), GADPH (Cell Signaling; #97166; 1:5000 for WB), β-actin (Cell Signaling; #4970; 1:1000 for WB), β-tubulin (Cell Signaling; #86298; 1:1000 for WB), Lamin B1 (Cell Signaling; #12586; 1:1000 for WB), p62 (Novus Biologicals, Littleton, CO; C11, H00008878-MOL; 1:1000 for WB and IF), Keap1 (Proteintech, Rosemont, IL; 10503-2-AP; 1:1000 for WB and IF), Phospho-histone H2AX (Ser139) (Cell Signaling; #9718; 1:1000 for WB and 1:100 for IHC), Ki-67 (Cell Signaling; #12202; 1:100 for IHC), PCNA (Cell Signaling; #13110; 1:100 WB and 1:100 for IHC), 8-oxo-dG (R&D Systems, Minneapolis, MN; 4354-MC-050; 1:100 for IHC), cleaved caspase-3 (Cell Signaling; #9661; 1:300 for IHC), HA-Tag (Cell Signaling; #3742; 1:1000 for WB), TRIM21 (Abcam; ab207728; 1:1000 for WB; Proteintech; 12108-1-AP; 1:1000 for WB and 1:100 for IHC), F-4/80 (Cell Signaling; #70076; 1:200 WB and 1:200 for IHC), HSP70 (Abcam; ab2787; 1:200 for IHC).

Immunofluorescence

Cells were fixed in 4% paraformaldehyde in PBS for 15 minutes at room temperature. After 3 washes with PBS, cells were permeabilized with 0.2% Triton X-100 in PBS for 15 minutes. After a brief wash with PBS with 0.02% Tween 20 (PBST) and 1.5% FBS, cells were incubated with primary antibodies in PBST with 1.5% FBS for 1 hour in dark at room temperature. Cells were then washed 3 times with PBST. After 2 washes with dH2O, the cells were mounted with ProLong Gold antifade reagent with DAPI (Invitrogen; 2047396). Slides were observed and imaged using inverted fluorescence microscope (Olympus, Tokyo, Japan; IX71) and analyzed using Image-Pro Plus software (version 5.1.0.20; Media Cybernetics, Rockville, MD).

RNA Extraction, Complementary DNA Synthesis, and PCR

After treatment, total RNA of the cells was isolated with RNeasy kit (Qiagen, Hilden, Germany). Reverse transcription was carried out with 2 μg of total RNA using the Superscript III First Strand Synthesis system (Invitrogen). The synthesized cDNA was used for real-time quantitative PCR with the PerfeCTa SYBR Green Super mix (Quanta Bioscience, Beverly, MA; 95055) on the StepOnePlus (Applied Biosystems, Foster City, CA). The following primers were used for quantitative reverse-transcription PCR: PCNA: forward 5′-TTTGAGGACGCCCTGATCC-3′; reverse 5′-GGAGACGCTGA- GAGGAGTCCAT-3′; c-Jun: forward 5′-CCTTCTACGAGATGCCCTC-3′; reverse 5′-GGTCTAGCAGTCTGTTTTT-3′; TRIM21 forward 5′-ATCATCCGGAAGAAGTG-3′ and reverse 5′-GGTCTT GGTAGCTCGTGGGC-3′; GSTM1: forward 5′-TGTTTTAGGCGGAGCACGCTG-3′ and reverse 5′-AGAGCAATGCATCTCG-3′; GSTM2: forward 5′-AGAGCAATGCGAGCTGC-3′ and reverse 5′-GGACTCATGCGAGCAGC-3′; FOS: forward 5′-GGAATGGTGTAAGAAGCT-3′ and reverse 5′-ACTGCT- TGAAGTTGTTGAGC-3′.

RNA-Seq and Data Analysis

RNA-seq was performed by the University of Texas Health San Antonio Genome Sequencing Facility. Approximately 500-ng total RNA was used for RNA-seq library preparation following the Illumina TruSeq stranded messenger RNA (mRNA) sample preparation guide (Illumina, San Diego, CA). The first step in the workflow involves purifying the poly-A-containing mRNA molecules using poly-T oligo-attached magnetic beads. Following purification, mRNA is fragmented into small pieces using dTTP

Rnase A. Strand specificity is achieved by replacing dTTP
with dUTP in the Second Strand Marking Mix. These cDNA fragments then go through an end repair process, the addition of a single “A” base, and then ligation of the adapters. The products are then purified and enriched with PCR to create the final RNA-seq library. After RNA-seq libraries were subjected to quantification process, pooled for cBot amplification and subsequent 50 bp single-read sequencing run with Illumina HiSeq 3000 platform. After the sequencing run, demultiplexing with Bcl2fastq2 was employed to generate the fastq file for each sample. An average of 37 million reads was obtained for this set of samples.

The raw reads were aligned to the reference Mus musculus genome (UCSC mm9) with TopHat2.60 Genes were annotated and quantified by HTSeq,61 and DEseq was used to find differentially expressed genes.59 Significantly differentially expressed genes were identified with Benjamini and Hochberg-adjusted P value <0.05 and fold change ≥2 when the gene has read abundance with reads per kilobase per million reads ≥1 within at least 1 treatment (samples). Gene Ontology analysis was performed using the DAVID online tool (http://david.abcc.ncifcrf.gov/).63 Plots were generated and the related statistics were performed using R version 3.4.1 (R Foundation for Statistical Computing, Vienna, Austria).

Histology, IHC, and Immunofluorescence
Liver tissues were fixed and processed for hematoxylin and eosin and IHC staining. In brief, paraffin-embedded liver sections were deparaffinized, rehydrated, and microwave heated for 10 minutes in 0.01-Mol/L citrate buffer (pH 6.0) for antigen retrieval. A total of 3% hydrogen peroxide was applied to block endogenous peroxidase activity. After 30 minutes of blocking with 5% goat serum, primary antibodies (TRIM21, γH2AX, p62, Keap1, PCNA, cleaved caspase 3, 8-oxo-dG, F-4/80, or control IgG) were applied and incubated overnight at 4°C. After wash, slides were incubated with biotinylated secondary antibody and the streptavidin-biotin complex (Vector Laboratories, Burlingame, CA) were applied, each for 60 minutes at room temperature. A total of 3% hydrogen peroxide was applied to block endogenous peroxidase activity. After 30 minutes of blocking with 5% goat serum, primary antibodies (TRIM21, γH2AX, p62, Keap1, PCNA, cleaved caspase 3, 8-oxo-dG, F-4/80, or control IgG) were applied and incubated overnight at 4°C. After wash, slides were incubated with biotinylated secondary antibody and the streptavidin-biotin complex (Vector Laboratories, Burlingame, CA) were applied, each for 60 minutes at room temperature. After rinsing with PBS, slides were immersed for 5 minutes in the coloring substrate DAB (Vector Labs, Burlingame, CA; cat SK-4100) 0.4 mg/mL with 0.003% hydrogen peroxide, then rinsed with distilled water, counterstained with hematoxylin, dehydrated, and coverslipped.

For immunofluorescence analysis, liver optimum cutting temperature compound (OCT) cryosections were fixed in cold acetone for 15 minutes and immunostained with anti-α-SMA (1:100; Abcam; cat ab124964) overnight at 4°C. After wash with PBS for 3 times, cryosections were incubated with goat anti-rabbit IgG-Alexa488 (1:500; Invitrogen, Grand Island, NY) and donkey anti-guinea pig IgG-Alexa651 (1:500; Millipore, Temecula, CA) for 1 hour at room temperature. Images were visualized and captured using the All-in-One BZ-X700 fluorescence microscope (Keyence, Elmwood Park, NJ).

For Sirius Red staining, paraffin-embedded liver sections were deparaffinized, rehydrated, and stained with hematoxylin for 10 minutes, followed by staining in Picrosiris red (Direct Red 80 [Sigma Aldrich; 365548], Picric acid [Sigma-Aldrich; 197378]) for 1 hour. After washing in 2 changes of 0.5% acidified water, slides were dehydrated and coverslipped.

For Oil Red O staining, liver frozen sections were fixed in cold propylene glycol for 2 minutes, then incubated slide in Oil Red O solution (Oil Red O stain kit; ab150678) for 8 minutes. Slides were differentiated in 85% propylene glycol for 1 minute, rinsed in 2 changes of distilled water, incubated in hematoxylin for 2 minutes, and softly rinsed in tap water and 2 changes of distilled water. A coverslip was mounted using an aqueous mounting medium. Images were visualized and captured using the Virtual/Digital Slice microscope (VS120; Olympus).

Access to Data
All authors had access to all data and have reviewed and approved the final manuscript.

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Conflicts of Interest
The authors disclose no conflicts.

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