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ORIGINAL ARTICLE

TRIM21 is critical in regulating hepatocellular carcinoma growth and response to therapy by altering the MST1/YAP pathway

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Abstract

Liver cancer is the sixth most common cancer and the third leading cause of cancer-related death globally. Despite efforts being made in last two decades in cancer diagnosis and treatment, the 5-year survival rate of liver cancer remains extremely low. TRIM21 participates in cancer metabolism, glycolysis, immunity, chemosensitivity and metastasis by targeting various substrates for ubiquitination. TRIM21 serves as a prognosis marker for human hepatocellular carcinoma (HCC), but the mechanism by which TRIM21 regulates HCC tumorigenesis and progression remains elusive. In this study, we demonstrated that TRIM21 protein levels were elevated in human HCC. Elevated TRIM21 expression was associated with HCC progression and poor survival. Knockdown of TRIM21 in HCC cell lines significantly impaired cell growth and metastasis and enhanced sorafenib-induced toxicity. Mechanistically, we found that knockdown of TRIM21 resulted in cytosolic translocation and inactivation of YAP. At the molecular level, we further identified that TRIM21 interacted and induced ubiquitination of MST1, which resulted in MST1 degradation and YAP activation. Knockdown of MST1 or overexpression of YAP reversed TRIM21 knockdown-induced impairment of HCC growth and chemosensitivity. Taken together, the current study demonstrates a novel mechanism that regulates the Hippo pathway and reveals TRM21 as a critical factor that promotes growth and chemoresistance in human HCC.

KEYWORDS
chemosensitivity, E3 ubiquitin ligase, sorafenib, therapeutic target

1 | BACKGROUND

Liver cancer is the sixth most common cancer and is the third leading cause of cancer-related mortality globally, resulting in nearly 800,000 deaths annually.[1] More importantly, the incidence of liver cancer is still increasing, and age-specified research has indicated that it is increasingly affecting younger adults.[2,3] In the clinic, hepatocellular carcinoma (HCC) is the most common pathological type of liver cancer and accounts for more than 85% of liver cancer.[4,5] It is well documented that the risk factors for the

development of liver cancer are hepatitis viral infection, hepatic cirrhosis, and alcohol intake.[6] Early diagnosis is crucial for curative therapy for liver cancer, but challenges are faced mainly due to the lack of symptoms of this disease in the early stage, and most liver cancer patients are diagnosed at a relatively advanced stage.[4] For treatment of advanced liver cancer, sorafenib is currently the only first-line chemotherapeutic drug. However, sorafenib administration is frequently associated with a variety of adverse effects and contributes to resistance of chemotherapy in patients.[7] In addition, liver cancer is highly heterogenic in terms of its phenotypes and responsiveness to therapies, including chemotherapy, radiation, and immunotherapy. More importantly, patients with liver cancer frequently develop multidrug resistance, distance metastasis, and recurrence.[8,9] For all the reasons described above, the average 5-year survival for liver cancer is less than 15%.[4] There is an urgent need to improve our understanding of liver cancer pathophysiology, the cellular networks responsible for liver cancer initiation, chemoresistance, and distance metastasis, all of which will potentiate the development of mechanism based on early diagnosis and therapeutic options for liver cancer.[5,10,11]

The Hippo pathway is an evolutionary conserved pathway that plays a vital role in regulating development, and tissue homeostasis and its dysregulation contribute to cancer.[12] Multiple upstream kinases and downstream factors are responsible for the functionality of the Hippo system, including mammalian STE20-like protein kinase (MST1/2), large tumor suppressor kinase 1/2 (LATS1/2), and primary downstream factor Yes-associated protein (YAP) and its paralog TAZ.[12] When the Hippo pathway is activated, MST1/2 induces phosphorylation of LATS1/2, which in turn phosphorylates YAP and retains it in the cytoplasm and thus inhibits YAP/TAZ activation. In contrast, the Hippo pathway inactivation results in dephosphorylation of YAP and its translocation into nucleus and induces target genes expression.[13] Mutations and altered expression of Hippo components are shown to be involved in the development and malignancy of human cancer. In terms of liver cancer, inactivation of Hippo signaling pathway is associated with poor prognosis.[14] In addition, MST1/2 deficiency increases YAP phosphorylation and thus promotes cell proliferation and HCC development.[15] Silencing YAP can restore hepatocyte differentiation and leads to cancer regression in advanced HCC.[16,17] Consistent with these observations, we have previously reported that activation of YAP is an early event of HCC and acts as an independent prognostic factor.[18,19]

Tripartite motif containing (TRIM) protein 21 belongs to the TRIM family, which is structurally characterized by the RING domain for E3 ubiquitin ligase.[20] TRIM21 was initially discovered to regulate the immune system in response to viral infection and autoimmune disease, in which interferons and toll-like receptor ligation enhance its expression.[21–23] Recent evidence suggests that TRIM21 participates in cancer development and progression.[24,25] Multiple key elements of cancer metabolism, glycolysis, immunity, chemosensitivity, and metastasis have been identified as substrates of TRIM21 for ubiquitination.[26–29] Notably, TRIM21 is shown to promote liver cancer development, and knockout TRIM21 prevents DEN-induced hepatocarcinogenesis.[30] Overexpression of TRIM21 serves as a prognosis marker for human HCC.[31] Nevertheless, the mechanism by which TRIM21 regulates HCC tumorigenesis and progression remains poorly understood.

In the present study, we demonstrated that elevated expression of TRIM21 was associated with HCC progression and poor survival. We further revealed that TRIM21 was a critical regulator in determining HCC growth and chemosensitivity, and knocked-down TRIM21 significantly impaired HCC growth and enhanced sorafenib toxicity. Mechanistically, we identified that TRIM21 interacted and induced ubiquitination of MST1, which resulted in MST1 degradation and YAP activation. Knockdown of MST1 or overexpression of YAP reversed TRIM21 knockdown and induced impairment of HCC growth and chemosensitivity. The current study thus demonstrates a novel mechanism that regulates the Hippo pathway and reveals TRM21 as a critical factor that promotes growth and chemoresistance in human HCC.

2 | MATERIALS AND METHODS

2.1 | Cell culture and transfection

Huh7, SK-Hep1, and Hep3B cells were purchased from Procell (Wuhan, China), and HEK 293 FT cells were provided by Drs. Xiyun Deng (Hunan Normal University, Changsha, China). All cells were maintained in DMEM (Invitrogen, USA) supplied with 10% FBS, 50 U/mL penicillin, and 50 mg/mL streptomycin. Cells were cultured in an incubator (Thermo Fisher, USA) under 37°C with 5% CO_2.

2.2 | Plasmids and siRNA

pJ3H-MST1, 8×GTIIC-luciferase, pSMPP-mCherry-hTRIM21, Flag-YAP plasmids were provided by Drs. Jonathan Chernoff, Stefano Piccolo, Leo James, and Yutaka Hata via Addgene (Cambridge, USA). Myc-TRIM21 and catalytically inactive TRIM21CA (C16A/C31A/H33W) plasmid were kindly provide by Dr. Xingzhi Xu (Shenzhen University, China). siRNAs were synthesized by JTS Scientific (Wuhan, China), and sequences were as presented in Table 1. Cells were transfected with Lipofectamine 6000 (Beyotime, China) in serum-free medium (Opti-MEM, Invitrogen).

2.3 | Reagents and antibodies

Anti-YAP (D8H1X), anti-MST1 (D8B9Q), anti-MST2 (3952), anti-p-YAP (Ser 127) (D9W2I), anti-LAST1 (9153), anti-p-LATS1 (9157), anti-TAZ (E8E9G), anti-p53 (DO-7), and anti-β-catenin (D10A8) were purchased from Cell Signaling Technology (MA, USA). Anti-GAPDH (10494-1-AP), anti-TRIM21 (12108-1-AP), and anti-MST1 (22245-1-AP) were purchased from Proteintech (Wuhan, China).

TABLE 1 Sequences of siRNAs and primers used in this study.

Name	Sequence (5′–3′)
Human YAP-forward	5′-CGCTCTTCAACGCCGTCA-3′
Human YAP-reverse	5′-AGTACTGGCCTGTCGGGAGT-3′
Human CTGF-forward	5′-AATGCTGCGAGGAGTGGGT-3′
Human CTGF-reverse	5′-CGGCTCTAATC ATAGTTGGGTCT-3′
Human Cyr61 forward	5′-GAGTGGGTCTGTGACGAGGAT-3′
Human Cyr61-reverse	5′-GGTTGTATAGGATGCGAGGCT-3′
Human MST1-forward	5′-GTAGCCAGCACCATGACTGA-3′
Human MST1-reverse	5′-GCTAACAAACATGAGCAAGCCA-3′
Human MST2-forward	5′-CCCATTCTGGATGCGATGGA-3′
Human MST2-reverse	5′-ACCTAAGGGGACAATAAATAGCC-3′
siTRIM21#1	5′-GGAATGCATCTCTCAGGTT-3′
siTRIM21#2	5′-GGACAATTTGGTTGTGGAA-3′
siMST1	5′-GGGCACTGTCCGAGTAGCAGC-3′

Cycloheximide (CHX) and MG132 were purchased from Sigma Aldrich (MO, USA).

2.4 | Animal procedure

BALB/c nude mice (6 weeks old) were purchased from Gempharmatech (Nanjing, China). All animal experiments were approved by the Institutional Animal Care and Use Committee (IACUC) of Second Xiangya Hospital. Mice randomly received a single tail vein injection of 1×10^6 cells suspended in 100 μL DMEM, and tumor formation was determined in lungs 1 week after injection using H&E staining.

2.5 | Immunohistochemistry

De-identified human liver specimens were obtained from Second Xiangya Hospital. Written informed consent was obtained from all patients, and all studies using human tissue samples were approved by the Human Subjects Committee of Second Xiangya Hospital. After deparaffinization and rehydration, antigen retrieval was achieved by heating in a pressure cooker in 10 mM of sodium citrate. After rinsing three times in PBST and blocking in 4% FBS for 60 min, slides were incubated with primary antibody in humidity chamber box overnight. After washing with PBST, slides were incubated with secondary antibody and then developed with DAB color developer (Biosharp, China), before counterstaining with hematoxylin and mounting. Images were obtained using a Zeiss Axiolab Microscope (Germany).

2.6 | Quantitative PCR

cDNA was synthesized using a high-capacity RNA reverse transcription kit (Thermo Fisher). The CFX96 Real-Time System (Bio-Rad, CA) was used to perform quantitative PCR. Primer sequences are presented in Table 1.

2.7 | Western blotting

Cell lysates were separated on a 10% SDS-PAGE gel and transferred to PVDF membranes. After blocking with 5% skim milk, membrane was incubated with primary antibodies and then HRP-conjugated secondary antibodies. Immunoblots were visualized using the ECL Western Blotting Detection System (Thermo Fisher). Quantifications of western blots were performed by densitometry analysis using the ImageJ (NIH), and relative band intensities were normalized against GAPDH.

2.8 | Immunofluorescence

Cells grown on coverslips were fixed with 4% Paraformaldehyde at room temperature for 5 min and permeabilized by 0.2% Triton X-100. Cells were incubated with primary antibodies followed by Alexa Fluor-conjugated secondary antibodies (Thermo Fisher). After count staining with DAPI, images were acquired using fluorescence microscopy (Leica, Germany).

2.9 | Wound healing and migration assay

For the wound healing assay, cells were grown in 24 well plates, and when cells reached 100% confluence, wounds were scratched using sterile pipette tips. Twenty-four hours later, movement of cells towards the scratched wound was measured as cell motility. Transwell plates (0.4 μm pore size) were used to measure the migration ability of the cells; 2×10^3 cells of FBS-free media were added into the upper chamber, and 600 μL of complete media was added to the bottom chambers. Cells were fixed and cells that

migrated through the membrane were stained with crystal violet, and the number of migratory cells was counted under a microscope.

2.10 | Kaplan–Meier survival curve analysis

The preprocessed RNA-seq data and corresponding clinical information of cancer patients was downloaded from The Cancer Genome Atlas (TCGA) database. The optimal cut-off value was calculated according to the Youden index [(sensitivity + specificity) − 1], and the patient samples were divided into two cohorts according to the median expression of the gene (high vs. low expression). Log-rank *p*-values and hazard ratios with 95% confidence intervals were determined using the survival R package.

2.11 | Statistical analysis

For in vitro experiments, each experiment was repeated at least three times unless indicated. Statistical analyses were calculated using unpaired two-tailed Student's *t*-test to compare mean differences between the two groups and two-way analysis of variance (ANOVA) to compare more than two groups. All quantitative data were presented as the mean ± SEM. *p*-values are indicated by asterisks in the figures: $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$.

3 | RESULTS

3.1 | Elevated TRIM21 expression associated with liver cancer progression and poor survival

We analyzed TRIM21 expression in human HCC using RNA sequencing data collected from The Cancer Genome Atlas (TCGA) database. Consistent with previous studies,[31,32] TRIM21 expression was increased in HCC tissues compared with adjacent non-tumor tissue (Figure 1A). TRIM21 level was positively correlated with disease progression at all stages except stage IV (Figure 1B). To confirm this finding, we examined TRIM21 protein levels from clinical samples and we found that 14 out of 18 patients showed at least 1.3-fold elevation of TRIM21 expression in HCC compared with adjacent non-tumor tissue (Figure 1C,D). Immunohistochemistry (IHC) results indicated elevated TRIM21 in tumor tissue compared with non-tumor tissue and that most TRIM21 was localized in cytoplasm (Figure 1E). The Kaplan–Meier method also suggested that lower TRIM21 expression was associated with increased overall survival (OS) (Figure 1F) as well as progression-free survival (PFS) (Figure 1G) compared with patients with higher levels of TRIM21.

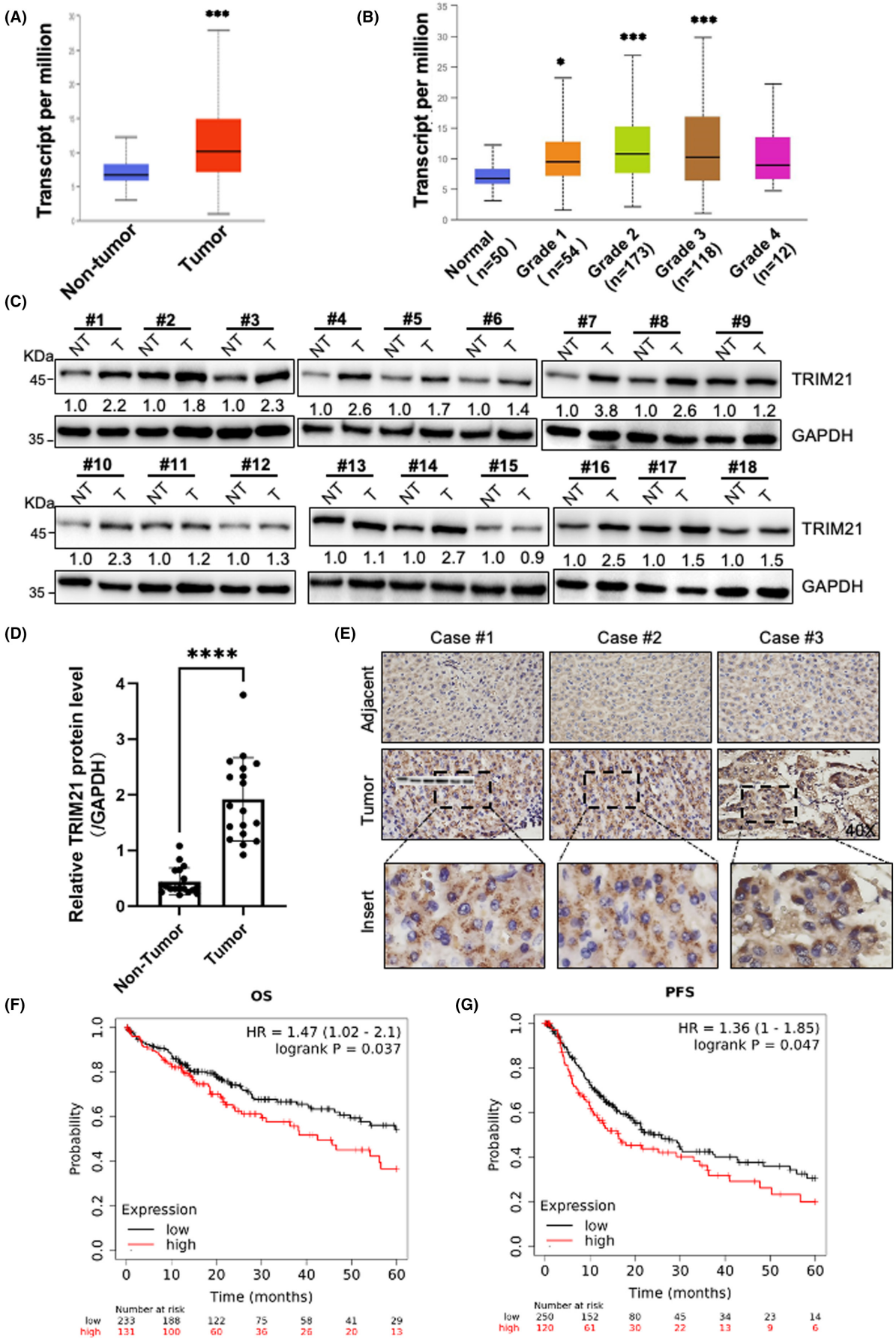
3.2 | TRIM21 is critical for hepatocellular carcinoma growth and sensitivity to sorafenib

We measured protein levels in HCC cell lines, and the results indicated that increased TRIM21 protein level in HCC cell lines compared to primary mouse hepatocyte (PMH) and LO2 cell (Figure 2A). To further confirm the role of TRIM21 in HCC, we transiently knocked down TRIM21 in Huh7 and SK-Hep1 cells, which showed high expression of TRIM21 by using siRNA (Figure 2B,C), and determined cell growth using a growth curve (Figure 2D,E) and a colony formation assay (Figure 2F). Knockdown of TRIM21 resulted in a significant decrease in cell growth and impaired the colony formation capability in Huh7 and SK-Hep1 cells compared with those of control cells (Figure 2D–F). To further confirm the effect of TRIM21 in HCC and rule out off-target effects of siRNAs, we used siRNA targeting TRIM21 untranscriptional region (UTR) and performed rescue experiments via ectopic TRIM21 expression (Figure S1A). Knockdown TRIM21 impaired cell growth in both cells but ectopic expression of TRIM21 reversed these effects (Figure S1B,C). We further evaluated whether TRIM21 regulates HCC sensitivity to sorafenib in vitro. The results indicated that knockdown TRIM21 itself slightly but not significantly increased TUNEL positivity in HCC cells. Sorafenib treatment induced a significant increase in TUNEL positivity in control cells, and knockdown TRIM21 further enhanced sorafenib-induced TUNEL positivity in both Huh7 and SK-Hep1 cells (Figure 2G,H; Figure S2A). Conversely, overexpression of TRIM21 in Hep3B and HepG2 cells, which showed relatively low TRIM21 protein levels, resulted in elevated cell growth (Figure 2I,J), colony formation capability (Figure 2K), and reduced sorafenib-induced TUNEL positivity in both Hep3B and HepG2 cells (Figure 2L; Figure S2B).

3.3 | TRIM21 is required for hepatocellular carcinoma metastasis both in vitro and in vivo

To further investigate whether TRIM21 regulates liver cancer metastasis, we transiently knocked down TRIM21 in HCC cells using two independent siRNAs (siTRIM21#1, siTRIM21#2) and

FIGURE 1 Elevated TRIM21 expression in human HCC associated with poor prognosis. (A) Analysis of TRIM21 mRNA expression in normal tissue ($n = 50$) and HCC (347) from the TCGA database. (B) Analysis of TRIM21 mRNA expression in differential stages based on TNM classification of HCC as in (A). (C) Western blot (WB) analysis of SIRT7 protein levels in human HCC and paired adjacent non-tumor tissue ($n = 18$). The number below the lane indicates the relative band intensity normalized to GAPDH. (D) Quantifications of WB results as in (C) by measuring relative band intensity normalized to GAPDH. Graphs show mean ± SEM, $^{****}p < 0.0001$, Student's *t* test. (E) Representative immunohistochemistry (IHC) staining of TRIM21 in human HCC and paired adjacent non-tumor tissue. (F, G) Kaplan–Meier analysis of overall survival (OS, $n = 364$) and progression-free survival (PFS, $n = 370$) in human HCC patients based on TRIM21 expression. HCC, hepatocellular carcinoma; TCGA, The Cancer Genome Atlas Program; TNM, Tumour, node and metastasis staging.


(A)
Transcript per million
***
Non-tumor
Tumor
(B)
Transcript per million
*
***
***
Normal (n=50)
Grade 1 (n=54)
Grade 2 (n=173)
Grade 3 (n=118)
Grade 4 (n=12)
(C)
#1 #2 #3 #4 #5 #6 #7 #8 #9
NT T
KDa
45
1.0 2.2 1.0 1.8 1.0 2.3
1.0 2.6 1.0 1.7 1.0 1.4
1.0 3.8 1.0 2.6 1.0 1.2
TRIM21
35
GAPDH
#10 #11 #12 #13 #14 #15 #16 #17 #18
1.0 2.3 1.0 1.2 1.0 1.3
1.0 1.1 1.0 2.7 1.0 0.9
1.0 2.5 1.0 1.5 1.0 1.5
(D)
Relative TRIM21 protein level (/GAPDH)
****
Non-Tumor
Tumor
(E)
Case #1
Case #2
Case #3
Adjacent
Tumor
Insert
40X
(F)
OS
HR = 1.47 (1.02 - 2.1)
logrank P = 0.037
Probability
Expression
low
high
Time (months)
Number at risk
low 233 188 122 75 58 41 29
high 131 100 60 36 26 20 13
(G)
PFS
HR = 1.36 (1 - 1.85)
logrank P = 0.047
Probability
Expression
low
high
Time (months)
Number at risk
low 250 152 80 45 34 23 14
high 120 61 30 22 13 9 6

evaluated cell migration. The results indicated that knockdown of TRIM21 significantly impaired the cell migration in Huh7 and SK-Hep1 cells compared with those of controls (siCtrl, Figure 3A–C) in addition to the repressed cell growth in both cells. Conversely, overexpression of TRIM21 resulted in significant enhancement of cell migration in both Hep3B and HepG2 cells (Figure 3D,E). We further evaluated whether TRIM21 regulates HCC metastasis in vivo (Figure 3F,G). Similar to in vitro experiments, we found that TRIM21 suppression markedly decreased both the number and size of lung metastatic foci compared with control cells (siCtrl, Figure 3F,G; Figure S3).

3.4 | Knockdown TRIM21 impairs YAP nuclear localization and activation

To investigate mechanisms underlying TRM21 mediated HCC growth and metastasis, we measured multiple well-known factors that are pivotal for cancer growth and metastasis, including β-catenin, FOXO3, p53, and YAP in TRIM21 knockdown cell lines (Figure 4A). Knockdown of TRIM21 significantly decreased YAP protein compared with those of controls in both Huh7 and SK-Hep1 cells, while mildly affected other protein expressions (Figure 4A,B). We further performed immunofluorescences to examine cellular localization of YAP in TRIM21 knockdown SK-Hep1 cells (Figure 4C). The results indicated that YAP predominantly localized in nuclear in control cells but after TRIM21 knockdown YAP was exclusively localized in cytosol (Figure 4C). Consistent with these observations, we found that knockdown of TRIM21 significantly impaired YAP/TAZ luciferase activities (Figure 4D) and decreased mRNA expression of YAP target genes CTGF and Cyr61 but not YAP mRNA itself (Figure 4E).

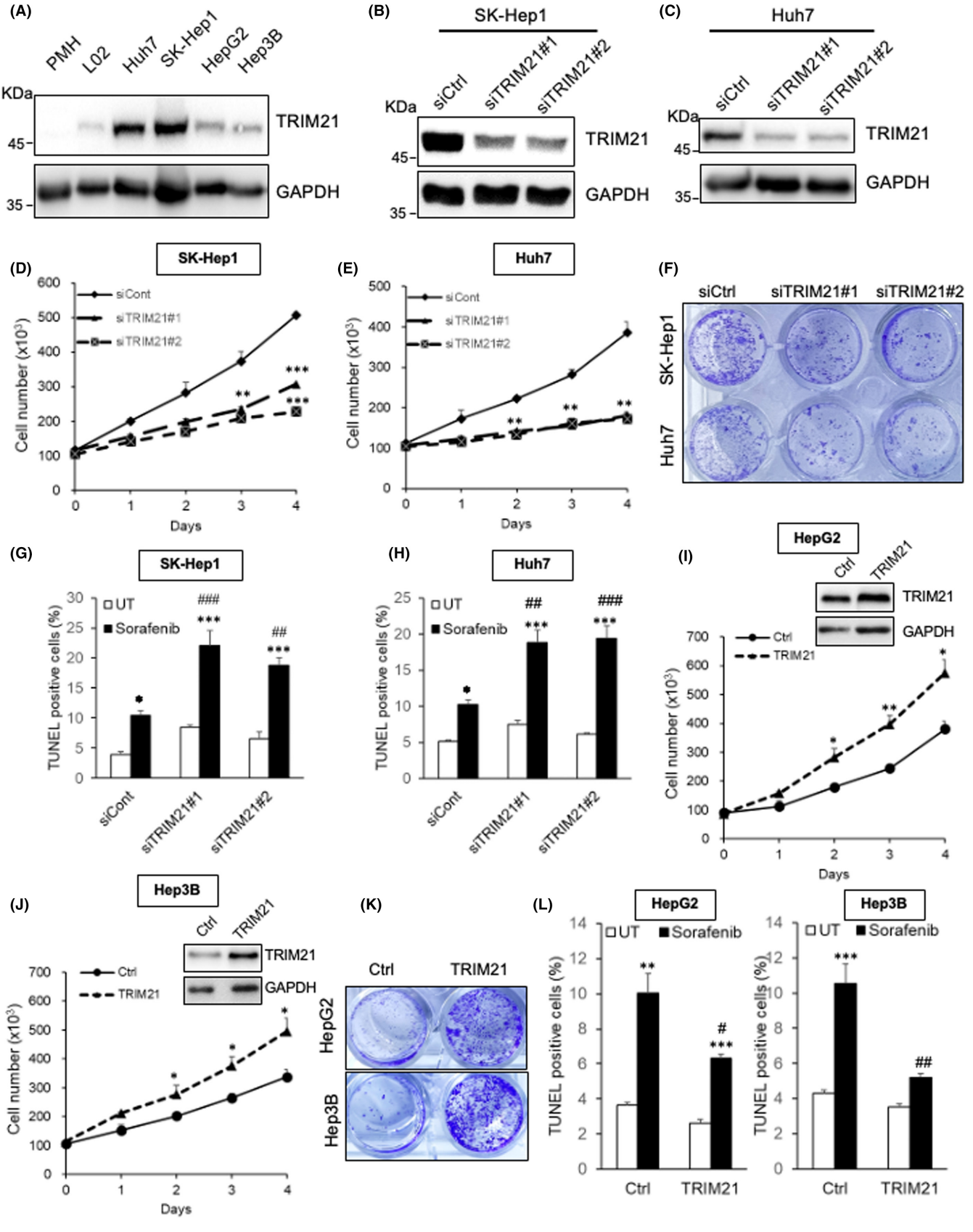
3.5 | TRIM21 regulates MST1 protein levels in human hepatocellular carcinoma

To further explore the mechanisms underlying TRIM21-mediated YAP cytoplasmic sequestration and inactivation, we measured Hippo upstream kinases that are responsible for YAP regulation (Figure 5A). We found that knockdown of TRIM21 significantly increased MST1, serine 909 phosphorylation of LAST1, and serine 127 phosphorylation of YAP, but the expression of LATS1, MST2, and TAZ was not changed (Figure 5A). The mRNA levels of MST1 and MST2 were not affected by TRIM21 knockdown (Figure 5B). To evaluate whether TRIM21 regulates MST1 through post-translational modification, we measured MST1 half-life in HCC cells after TRIM21 knockdown by treating the cells with cycloheximide,[33,34] and the results indicated that knockdown of TRIM21 prolonged MST1 half-life (Figure 5C). To confirm whether TRIM21 was sufficient to induce MST1 degradation, we overexpressed TRIM21 and measured MST1 protein levels in both Huh7 and SK-Hep1 cells. In both cases, overexpression of TRIM21 was sufficient to decrease MST1 protein expression (Figure 5D). Similar results were observed where TRIM21-transfected cells decreased MST1 but not MST2 fluorescence intensity (Figure 5E). We further examined TRIM21 and MST1 protein levels in patients, and the results indicated a trend towards a negative correlation ($\rho = -0.3243$, $p = 0.092$) between TRIM21 and MST1 protein levels in human HCC (Figure 5F,G).

3.6 | TRIM21 interacts with and induces MST1 polyubiquitination

To examine how TRIM21 regulates MST1, we first tested whether TRIM21 interacted with MST1 by overexpressing MST1 or TRIM21 and performing immunoprecipitation (IP) experiments, and the results indicated direct interaction between MST1 and TRIM21 (Figure 6A). In addition, we further confirmed that endogenous TRIM21 was also associated with MST1 in HCC cells (Figure 6B). Because TRIM21 belongs to the E3 ubiquitin ligase, we examined whether TRIM21 induces MST1 ubiquitination by comparing the MST1 ubiquitination level in TRIM21 knockdown cells, and the results indicated that knockdown TRIM21 significantly decreased MST1 ubiquitination (Figure 6C). In contrast, overexpression of TRIM21 significantly increased MST1 ubiquitination, but the enzymatic inactive form of TRIM21 (TRIM21 CA) failed to induce these effects (Figure 6D). To examine whether TRIM21 promotes

FIGURE 2 TRIM21 is critical for hepatocellular carcinoma growth and sensitivity to sorafenib. (A) Western blot (WB) analysis of TRIM21 protein levels in primary mouse hepatocyte (PMH), L02, Huh, HepG2, Hep3B, and SK-Hep1 cells. (B, C) Huh7 and SK-Hep1 cells were treated with non-target siRNA (siCtrl) or siRNA targeting TRIM21 (siTRIM21#1 and siTRIM21#2) for 72 h; protein levels of TRIM21 were evaluated by WB analysis. (D–F) Huh7 and SK-Hep1 cells were treated with siRNA targeting TRIM21 for 72 h; cell proliferation was evaluated using growth curve (D, E) and colony formation assay (F). Graphs show mean ± SEM. $^{**}p < 0.01$, $^{***}p < 0.001$, one-way ANOVA. (G, H) Huh7 and SK-Hep1 cells were treated with siRNA targeting TRIM21 for 72 h; cells were untreated (UT) or treated with sorafenib for an additional 24 h, and cell death was evaluated by TUNEL assay. (I) Graphs show mean ± SEM. $^{*}p < 0.05$, $^{***}p < 0.001$ versus UT, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ versus siCont/Sorafenib, one-way ANOVA. (I–K) Hep3B and HepG2 cells were transfected with empty vector (Ctrl) or mCherry TRIM21 (TRIM21) for 24 h; cell proliferation was evaluated using a growth curve (I, J) and a colony formation assay (K). Graphs show mean ± SEM of at least three independent experiments. Inserts indicate TRIM21 protein levels after transfection. $^{*}p < 0.05$, $^{**}p < 0.01$ versus Ctrl, Student's *t*-test. (L) Hep3B and HepG2 cells were transfected with empty vector (Ctrl) or mCherry TRIM21 (TRIM21) for 24 h; cells were untreated (UT) or treated with sorafenib for an additional 24 h, and cell death was evaluated by TUNEL assay. Graphs show mean ± SEM. $^{**}p < 0.01$, $^{***}p < 0.001$ versus UT, $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ versus Ctrl/Sorafenib, one way ANOVA.



proteasome-dependent degradation of MST1, we compared proteins levels in cells with overexpressed TRIM21 in the absence or presence of proteasome inhibitor MG132 (Figure 6E). Overexpression of TRIM21 decreased the MST1 level compared with control cells, but MG132 completely prevented this effect (Figure 6E).

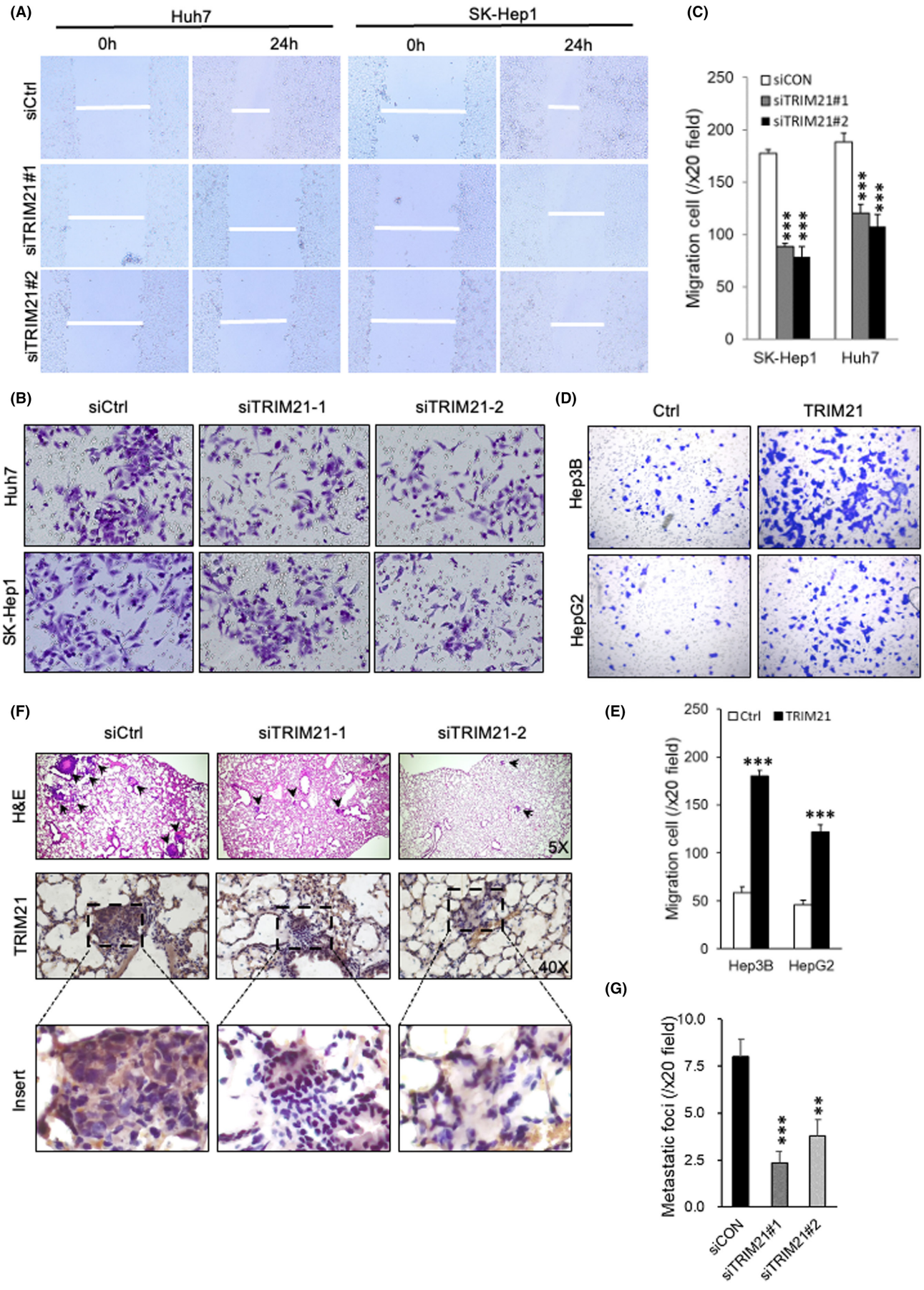

(A)
Huh7
SK-Hep1
0h
24h
0h
24h
siCtrl
siTRIM21#1
siTRIM21#2
(C)
siCON
siTRIM21#1
siTRIM21#2
Migration cell (/x20 field)
250
200
150
100
50
0
SK-Hep1
Huh7
(B)
siCtrl
siTRIM21-1
siTRIM21-2
Huh7
SK-Hep1
(D)
Ctrl
TRIM21
Hep3B
HepG2
(F)
siCtrl
siTRIM21-1
siTRIM21-2
H&E
TRIM21
Insert
5X
40X
(E)
Ctrl
TRIM21
Migration cell (/x20 field)
250
200
150
100
50
0
Hep3B
HepG2
(G)
Metastatic foci (/x20 field)
10.0
7.5
5.0
2.5
0.0
siCON
siTRIM21#1
siTRIM21#2

FIGURE 3 TRIM21 is required for hepatocellular carcinoma metastasis both in vitro and in vivo. (A–C) Huh7 and SK-Hep1 cells were treated with siRNA targeting TRIM21 for 72 h. Cell migration was evaluated by using wound healing (A) and a migration assay (B). Quantification of the migration assay was evaluated by counting migrated cells of five independent microscope fields (C). (D, E) Hep3B and HepG2 cells were transfected with empty vector (Ctrl) or mCherry TRIM21 (TRIM21) for 24 h; cell migration was evaluated using a migration assay (D). Quantification of the migration assay was evaluated by counting migrated cells of five independent microscope fields (E). (F, G) SK-Hep1 cells were treated with siRNA targeting TRIM21 (5 nM) overnight; 1×10^6 cells were randomly injected into nude mice via tail vein injection ($n=5$ each group); tumor formation was determined in lungs 1 week after injection (F); and quantified data are shown in (G). The graph shows mean ± SEM. $^{**}p<0.01$, $^{***}p<0.001$, one-way ANOVA. Arrow heads indicate metastatic foci in the lung. Scale bar indicates 50 μm.

3.7 | TRIM21 promotes hepatocellular carcinoma growth and resistance to sorafenib via the Hippo pathway

To examine whether TRIM21 regulates HCC growth, chemosensitivity, and metastasis through the MST1/YAP axis, we first confirmed whether TRIM21 regulate YAP through MST1. We generated MST1 knockout cells using the CRIPR Cas9 system (Figure S4A) and knocked down or overexpressed TRIM21 in those cells and monitored YAP expression. The result indicated that knocked down TRIM21 enhanced MST1 and p-YAP and decreased YAP expression in control cells; MST1 knockout completely prevented these effects. Reversely, TRIM21 overexpression barely affected MST1 and p-YAP expression in MST1 knockout cells (Figure S4B). We further knocked down MST1 or overexpressed YAP in TRIM21 knockdown HCC cells and measured cell growth, chemosensitivity, and metastasis. Because both MST1 and YAP play important roles in mediating cell growth, chemosensitivity, and migration, we titrated MST1 and YAP protein expression in rescue experiments similar as control cells to make clear comparison. As a result, downregulation of MST1 via siRNA reversed the TRIM21 knockdown-mediated upregulation of MST1 and downregulation of YAP expression and activation (Figure 7A; Figure S5A), the impairment of HCC growth in both Huh7 and SK-Hep1 cells (Figure 7B,C), and the increase of sorafenib sensitivity (Figure 7D; Figure S6A). However, cell migration impairment caused by TRIM21 knockdown was not rescued by MST1 knockdown (Figure S7A,B). In contrast, ectopic expression of YAP reversed TRIM21 knockdown-mediated downregulation of YAP protein expression and activation (Figure 7E; Figure S5B), the impairment of HCC growth in both Huh7 and SK-Hep1 cells (Figure 7F,G), and the increase of sorafenib sensitivity (Figure 7H; Figure S6B). Again, cell migration impairment caused by TRIM21 knockdown was not reversed by YAP overexpression (Figure S7A,B).

4 | DISCUSSION

In the present study, we demonstrated that TRIM21 expression was frequently upregulated and high TRIM21 expression was associated with aggressive pathological features in human HCC. By using in vitro and in vivo models, we revealed that knockdown of TRIM21 in HCC cell lines not only impaired cell growth and metastasis but also increased sensitivity to sorafenib. At the molecular level, we found that knockdown of TRIM21 resulted in impairment of nuclear localization and activation of YAP. Mechanistically, we identified MST1 as TRIM21 substrate for ubiquitination, which resulted in MST1 degradation and YAP activation. MST1 knockdown or overexpression of YAP reversed TRIM21 knockdown-induced impairment of HCC growth and chemosensitivity. Our data thus revealed that TRIM21 is involved in Hippo regulation, and the newly identified TRIM21–MST1–YAP axis partially illustrates the molecular mechanism of HCC growth and chemosensitivity, which highlights a potential therapeutic target for treatment of human HCC.

Sorafenib is currently a first-line chemotherapeutic drug for treatment of advanced HCC and metastatic colon cancer.[35] However, oral administration of sorafenib is associated with a variety of adverse effects, including diarrhea and fatigue, which can lead to discontinuation of chemotherapy and cardiovascular events, including hypertension.[36] More importantly, patients frequently develop multidrug resistance (MDR) shortly after sorafenib exposure.[37] While efforts have been made to understand the mechanisms underlying MDR, there is still a need to increase sorafenib sensitivity and decrease its side effects.[38,39] We showed that knockdown of TRIM21 increased sorafenib toxicity in HCC and clearly indicated that TRIM21 plays important roles in regulating the response to sorafenib and could serve as a therapeutic target in HCC. Whether TRIM21 regulates chemosensitivities in other cancer types and studies focused on development of specific TRIM21 inhibitors and evaluating its effects in HCC would be of great interest. We further revealed that YAP is primarily responsible for TRIM21-mediated drug sensitivity. YAP has been reported to be associated with chemoresistance in varies types of human cancer,[40–42] but the downstream target genes responsible for this regulation remain to be explored.

The MST1–YAP axis plays a paradoxical role in human cancer, and dysregulated Hippo components coordinate to promote cancer development and disease progression.[43,44] Numerous studies have shown that high YAP and TAZ expression possess higher proliferative capability through transcriptional regulation of genes associated with cell cycle and division.[45–47] Except for YAP/TAZ, upregulation of their target genes, including CTGF, Cyr61, and AREG, has also been shown to promote tumorigenesis in gastric cancer.[48–50] MST1 inhibits YAP activation and acts as a tumor suppressor.[12] Genetic knockout MST1 results in hyperactivation of YAP and tumorigenesis.[15,51] How MST1 exerts its tumor suppressor function in human cancer remains largely unknown. Previous study has shown that MST1 promotes cancer growth by inhibiting AMPK-SIRT3 mediated mitochondrial fission for cell death.[52] Our data thus provides new evidence that TRIM21 interacts with and induces MST1 ubiquitination

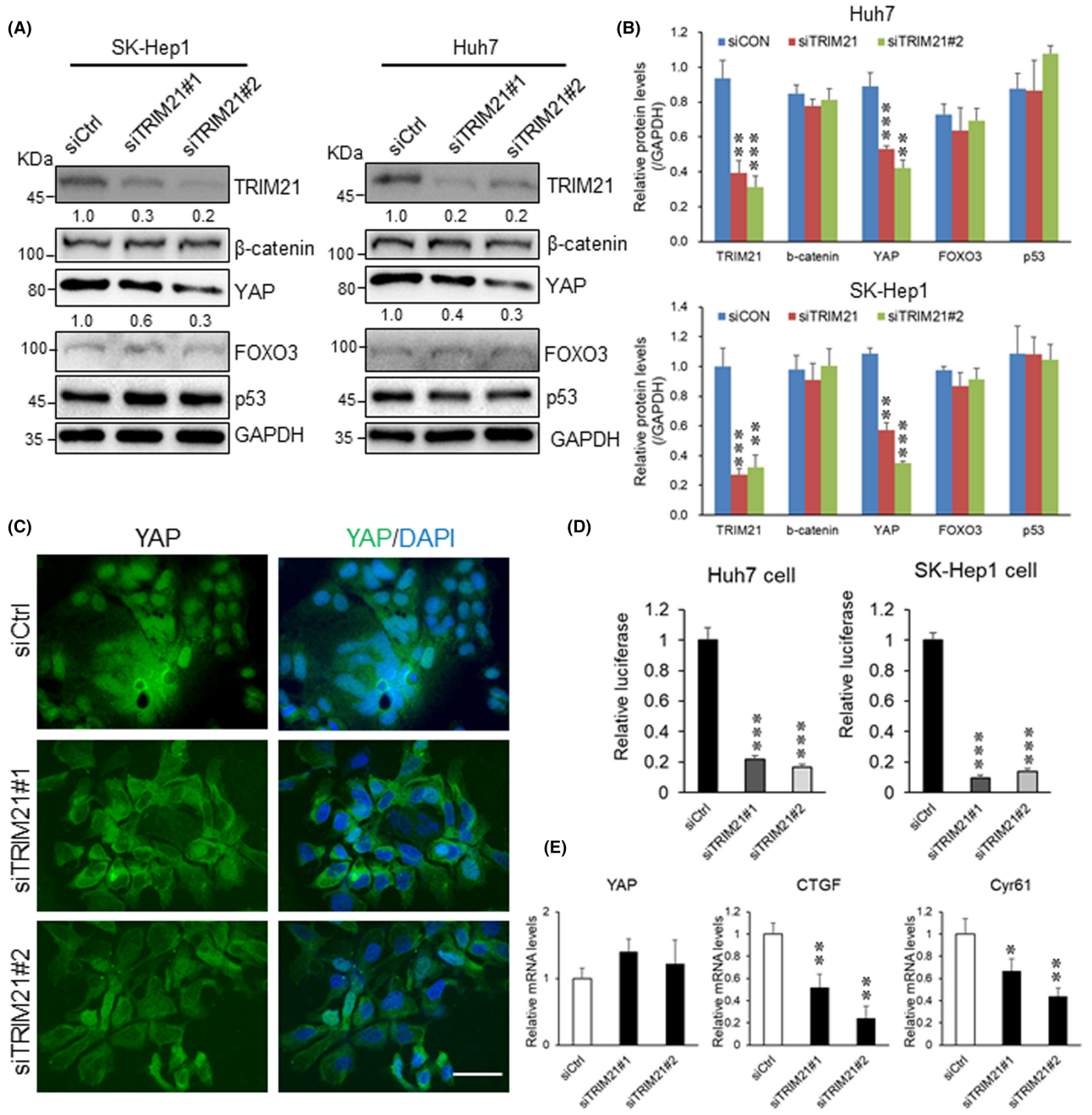
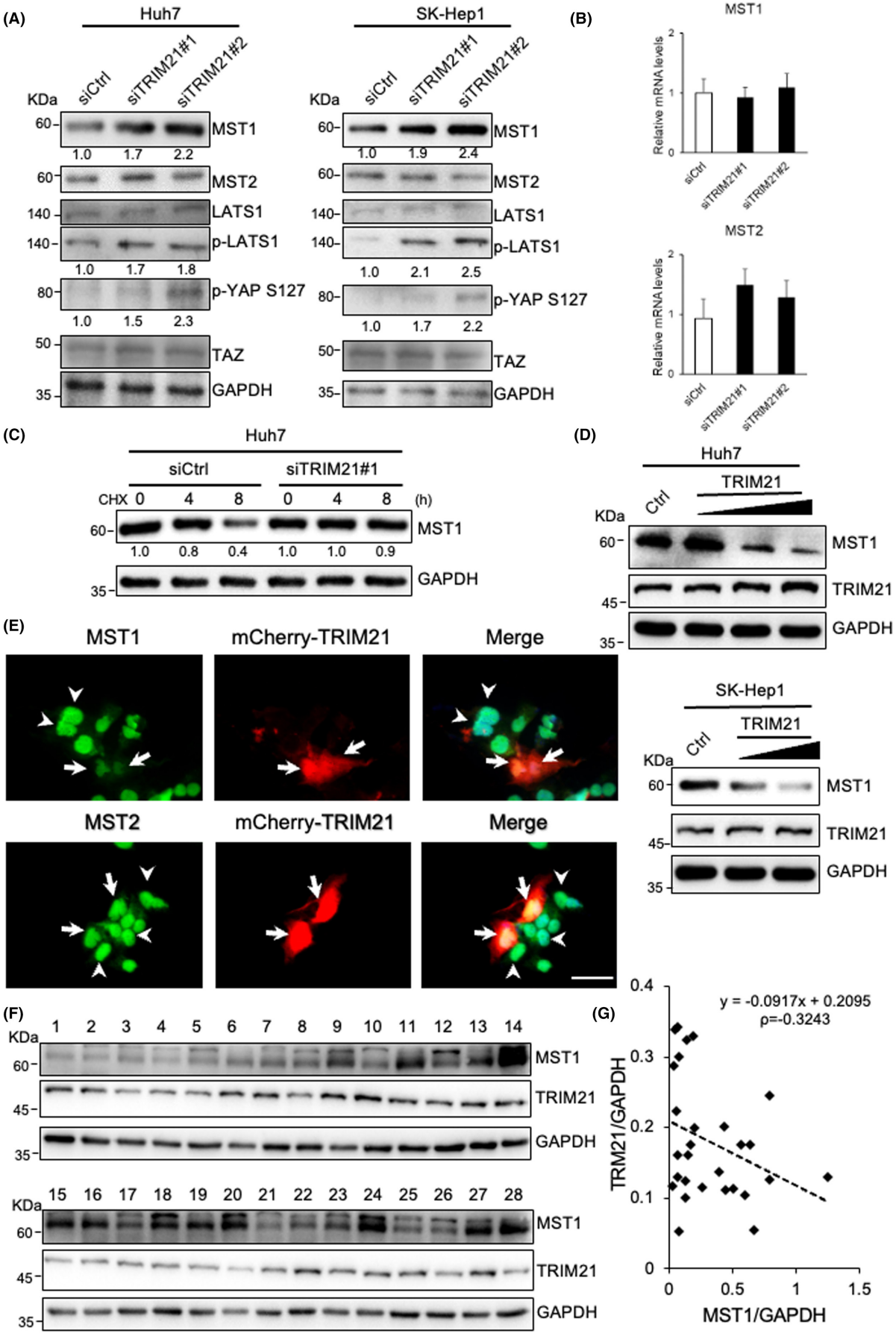


FIGURE 4 Knockdown of TRIM21 impairs YAP nuclear localization and activation. (A, B) Huh7 and SK-Hep1 cells were treated with siRNA targeting TRIM21 for 72 h; protein levels of TRIM21, β-catenin, YAP, FOXO3, and p53 were evaluated by western blot (WB) analysis (A) The number below the lane indicates relative band intensity normalized to GAPDH. (B) Quantifications of WB results as in (C) by measuring relative band intensity normalized to GAPDH. (C) Immunofluorescence for YAP (green) in cells treated with siTRIM21 as in (A). Scale bar indicates 50 μm. (D) Cells in (A) were transfected with YAP/TAZ luciferase plasmids for 24 h, and transcription activity was evaluated by luciferase assay. (E) mRNA levels of YAP and its target genes CTGF and Cyr61 were evaluated by qRT-PCR. Graphs show mean ± SEM. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, one way ANOVA.

FIGURE 5 TRIM21 regulates MST1 protein levels in human HCC. (A, B) Huh7 and SK-Hep1 cells were treated with siRNA targeting TRIM21 for 72 h. Protein levels of MST1, MST2, LATS1, p-LATS1 (S909), and p-YAP (S127) were evaluated by western blot (WB) analysis (A), and the number below the lane indicates relative band intensity normalized to GAPDH. (B) mRNA levels of MST1 and MST2 were evaluated by qRT-PCR. Graphs show mean ± SEM. (C) Cells in (A) were treated with cycloheximide (CHX, 100 μM) for various time points as indicated, and protein levels of MST1 were evaluated by WB analysis. (D) Huh7 and SK-Hep1 cells were transfected with Myc-TRIM21, and protein levels of TRIM21 and MST1 were evaluated by WB analysis. (E) Huh7 cells were transfected with plasmid express mCherry-tagged TRIM21 (red) for 24 h; the cellular localization of MST1 and MST2 (green) was evaluated by IF. Arrows indicate transfected cells and arrowheads indicate untransfected cells. Scale bar indicates 50 μm. (F) WB analysis for TRIM21 and MST1 in human HCC ($n = 28$). (G) Spearman correlation between TRIM21 and MST1 expression as in (D) ($\rho = -0.3243$, $p = 0.092$). HCC, hepatocellular carcinoma.


(A)
Huh7
SK-Hep1
siCtrl
siTRIM21#1
siTRIM21#2
KDa
MST1
MST2
LATS1
p-LATS1
p-YAP S127
TAZ
GAPDH
(B)
MST1
MST2
Relative mRNA levels
(C)
Huh7
siCtrl
siTRIM21#1
CHX 0 4 8 0 4 8 (h)
(D)
Huh7
Ctrl
TRIM21
SK-Hep1
(E)
MST1
mCherry-TRIM21
Merge
MST2
(F)
TRIM21
(G)
y = -0.0917x + 0.2095
ρ=-0.3243
TRM21/GAPDH
MST1/GAPDH

and degradation, which in turn activates YAP and promotes liver cancer proliferation.

Unlike the Hippo pathway, the role of TRIM21 in human cancer seems more complex and highly cancer type dependent. Elevated expression of TRIM21 has been observed as associated with aggressive phenotypes of human glioma and colon and pancreatic cancer.[26,53] In contrast, reduced TRIM21 expression has been reported to be associated with tumorigenesis and poor survival in lung cancer and lymphoma patients.[54,55] In HCC, while one study shows that TRIM21 is a favorable molecule and its downregulation promotes HCC,[56] multiple groups show that TRIM21 is upregulated in human HCC, and high TRIM21 expression correlates with HCC incidence and poor survival.[31,32] Using a mice model, Wang et al. showed that genetic deletion of TRIM21 protects mice from hepatocarcinogenesis by reducing

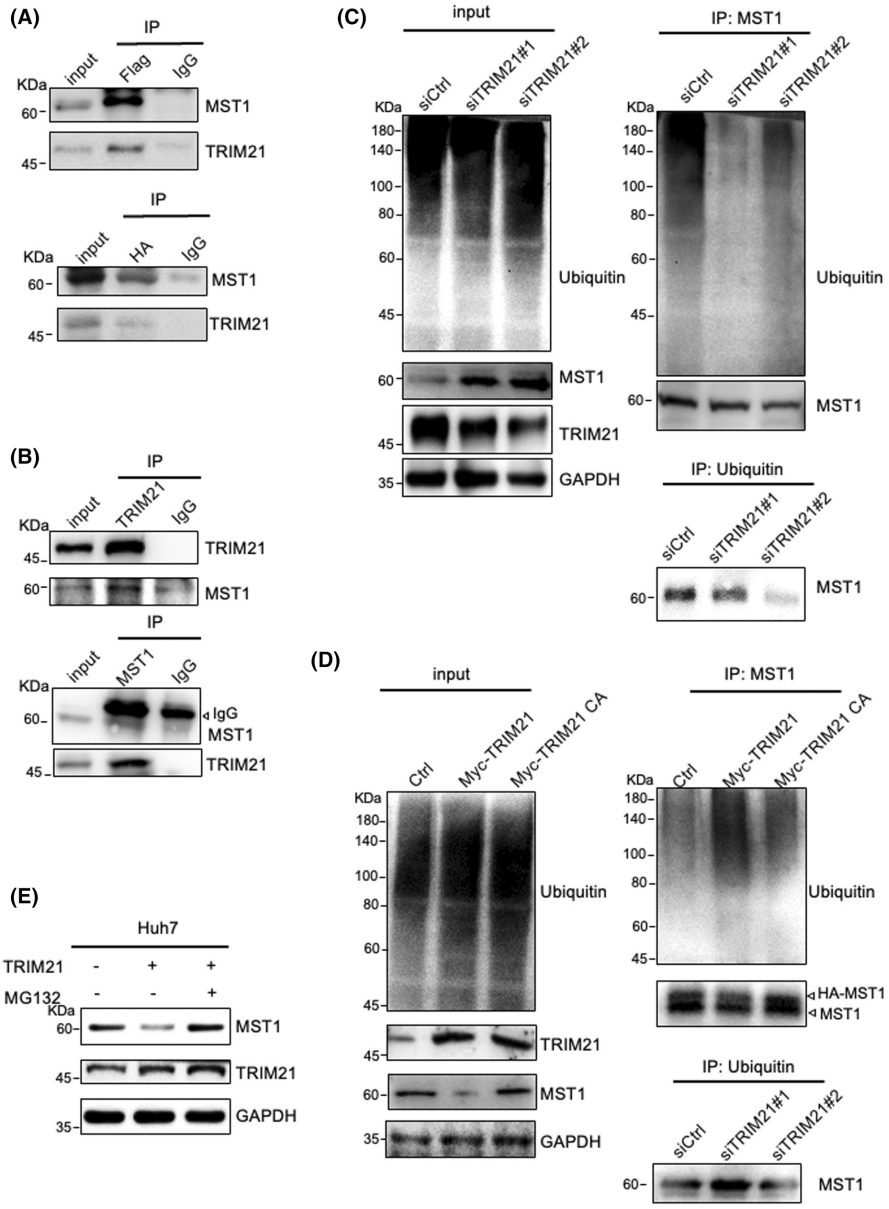
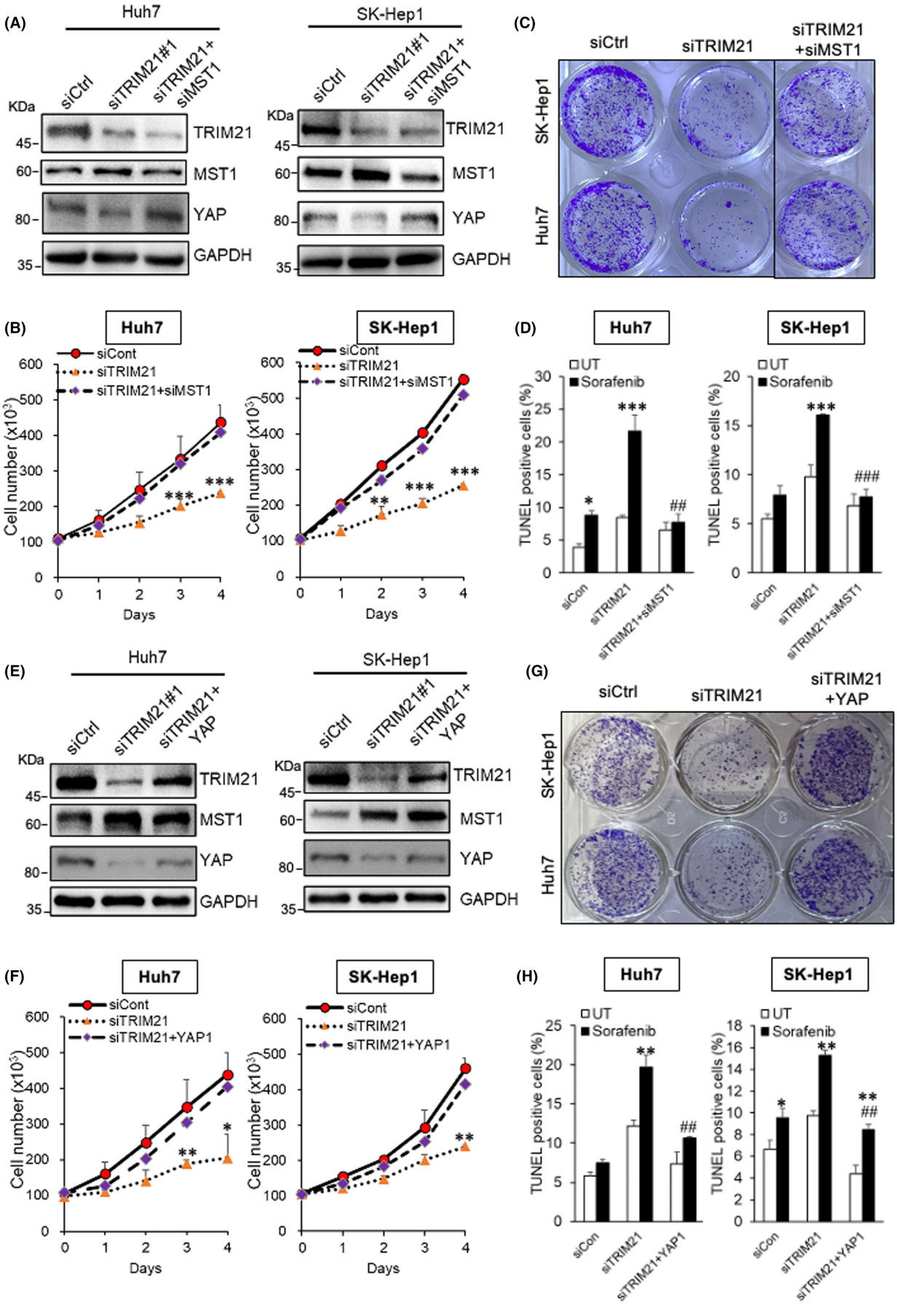


FIGURE 6 TRIM21 interacts with and induces MST1 ubiquitination. (A) SK-Hep1 cells were transfected with HA-tagged MST1 or myc-tagged TRIM21 for 24 h, and MST1 were immunoprecipitated with magnetic beads and the presence of TRIM21 or MST1 was evaluated by western blot (WB) analysis. (B) Endogenous TRIM21 or MST1 in SK-Hep1 cells were immunoprecipitated by TRIM21 or MST1 antibodies, and the presence of MST1 or TRIM21 was evaluated by WB analysis. (C) SK-Hep1 cells were transfected with siRNA targeting TRIM21 for 72 h and treated with protease inhibitor MG132 (50 mM) for an additional 4 h; ubiquitination of MST1 was evaluated by immunoprecipitation of MST1 or ubiquitin followed by immunoblotting with ubiquitin or MST1. (D) SK-Hep1 cells were transfected with TRIM21 or enzymatic inactive TRIM21 (TRIM21 CA) for 24 h and treated with protease inhibitor MG132 for an additional 4 h; ubiquitination of MST1 was evaluated by immunoprecipitation of MST1 or ubiquitin followed by immunoblotting with ubiquitin or MST1. (E) SK-Hep1 cells were transfected with TRIM21 for 24 h; cells were untreated or treated with MG132 (50 mM) for an additional 4 h. Protein levels of TRIM21 and MST1 were evaluated by WB analysis.

FIGURE 7 TRIM21 promotes hepatocellular carcinoma growth and resistance to sorafenib via MST1 and YAP. (A–C) Huh7 and SK-Hep1 cells were treated with siRNA targeting TRIM21 or TRIM21 and MST1 for 72 h; protein levels of TRIM21, MST1, and YAP were evaluated by Western blot (WB) analysis (A). Cell proliferation was evaluated using a growth curve (B) and a colony formation assay (C). Graphs show mean ± SEM. $^{**}p < 0.01$, $^{***}p < 0.001$, one-way ANOVA. (D) Cells in (A) were untreated (UT) or treated with sorafenib for an additional 24 h, and cell death was evaluated by TUNEL assay. $^{**}p < 0.01$, $^{***}p < 0.001$ versus UT, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ versus siTRIM21/sorafenib, one-way ANOVA. (E–G) Huh7 and SK-Hep1 cells were treated with siRNA targeting TRIM21 for 72 h and with/without YAP transfection for an additional 24 h; protein levels of TRIM21, MST1, and YAP were evaluated by WB (E). Cell proliferation was evaluated using a growth curve (F) and a colony formation assay (G). Graphs show mean ± SEM of at least three independent experiments. $^{*}p < 0.05$, $^{**}p < 0.01$, one-way ANOVA. (H) Cells in (A) were untreated (UT) or treated with sorafenib for and additional 24 h, and cell death was evaluated by TUNEL assay. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ versus UT, $^{\#\#}p < 0.01$ versus siTRIM21/sorafenib, one-way ANOVA.


(A)
Huh7
SK-Hep1
siCtrl
siTRIM21#1
siTRIM21+siMST1
KDa
TRIM21
MST1
YAP
GAPDH
45
60
80
35
(C)
siCtrl
siTRIM21
siTRIM21+siMST1
SK-Hep1
Huh7
(B)
Huh7
SK-Hep1
siCont
siTRIM21
siTRIM21+siMST1
Cell number (x10³)
Days
(D)
Huh7
SK-Hep1
UT
Sorafenib
TUNEL positive cells (%)
siCon
siTRIM21
siTRIM21+siMST1
(E)
Huh7
SK-Hep1
siCtrl
siTRIM21#1
siTRIM21+YAP
(G)
siCtrl
siTRIM21
siTRIM21+YAP
SK-Hep1
Huh7
(F)
Huh7
SK-Hep1
siCont
siTRIM21
siTRIM21+YAP1
Cell number (x10³)
Days
(H)
Huh7
SK-Hep1
UT
Sorafenib
TUNEL positive cells (%)
siCon
siTRIM21
siTRIM21+YAP1

ROS production and cell death after DEN treatment.[30] In line with these results, we observed that TRIM21 was frequently unregulated in human HCC, and increased TRIM21 expression was associated with HCC progression and poor survival. It is possible that TRIM21 has different and even opposite functions in HCC because of the risk factors and multifaceted causes of this highly heterologous disease. Notably, our current data showed that TRIM21 promotes MST1 degradation, which in turn activates YAP to promote HCC proliferation, but it is still possible that other functions of TRIM21, such as immune checkpoint regulation,[57,58] metabolic regulation,[59,60] and inhibiting pyroptotic cell death,[61] might also contribute to its oncogenic activity. Due to the high heterogeneity of liver cancer and the relatively small number of samples, the correlation between MST1 and TRIM21 from our cohort did not reach statistical significance. Therefore, the clinical relevance of TRIM21-dependent MST1 regulation needs further investigation. In addition, our data demonstrated that TRIM21 is required for HCC metastasis, but this function seems independent of the MST1/YAP axis. The mechanisms underlying TRIM21-mediated HCC metastasis thus need to be further explored, but we cannot exclude the possibility that TRIM21-mediated HCC proliferation contributes to its in vivo metastasis.

In summary, our data reveals the important roles of TRIM21 in human HCC and suggests that it might serve as a prognostic marker and therapeutic target in HCC as it functions as a critical regulator of the Hippo pathway.

AUTHOR CONTRIBUTIONS

Bo Shu: Investigation; writing – original draft. **Yingxia Zhou:** Investigation; methodology. **Guoqiong Lei:** Methodology. **Yu Peng:** Methodology. **Cong Ding:** Methodology. **Zhuan Li:** Conceptualization; project administration. **Chao He:** Conceptualization; funding acquisition; project administration.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

All data are within the manuscript and supporting information. Any additional information or data is available upon request.

ETHICS STATEMENT

Approval of the research protocol by an Institutional Review Board: All studies using human tissue samples were approved the Human Subjects Committee of Second Xiangya Hospital, Central South University.

Informed Consent: Written informed consent was obtained from all patients.

Registry and the Registration No. of the study/trial: N/A.

Animal Studies: All animal handing procedures were approved by the Institutional Animal Care and Use at Second Xiangya Hospital, Central South University.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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