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SIRT7 promotes Hippo/YAP activation and cancer cell proliferation in hepatocellular carcinoma via suppressing MST1

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Abstract

Abnormal activation of the oncogene *YAP* in the Hippo pathway is a major feature in liver cancer and inactivation of MST1/2 has been shown to be responsible for the overactivation of YAP that led to tumorigenesis. However, mechanisms underlying MST1/2 dysregulation remain poorly understood. RNA-seq analysis and genome (KEGG) pathway enrichment analysis were used to identify genes and pathways that were regulated by SIRT7. qRT-PCR, ChIP, and luciferase assay were used to investigate transcriptional regulation. Mass spectrometry, co-immunoprecipitation and immunoprecipitation were used to exam protein–protein interaction and post-transcriptional modification. A xenograft mouse model was used to confirm the effect of SIRT7 and SIRT7 inhibitors on hepatocellular carcinoma (HCC) proliferation in vivo. We found that SIRT7 suppresses MST1 by both transcriptional regulation and post-transcriptional modification, which in turn promotes YAP nuclear localization and transcriptional activation in liver cancer. Mechanistically, we revealed that SIRT7 suppresses MST1 transcription by binding to the MST1 promoter and inducing H3K18 deacetylation in its promoter region. In addition, SIRT7 directly binds to and deacetylates MST1, which primes acetylation-dependent MST1 ubiquitination and protein degradation. In clinical samples, we confirmed a negative correlation between SIRT7 and MST1 protein levels, and high SIRT7 expression correlated with elevated YAP expression and nuclear localization. In addition, SIRT7 specific inhibitor 2800Z sufficiently inhibited HCC growth by disrupting the SIRT7/MST1/YAP axis. Our data thus revealed the previously undescribed function of SIRT7 in regulating the Hippo pathway in HCC and further proved that targeting SIRT7 might provide novel therapeutic options for the treatment of liver cancer.

KEYWORDS
epigenetics, histone deacetylase, liver cancer, oncogenes, post-transcriptional modification

Abbreviations: KEGG, Kyoto Encyclopedia of Genes and Genomes; LATS1/2, large tumor suppressor kinase 1/2; MOD, mean optical density; MST1/2, mammalian STE20-like kinase 1/2; PTM, post-translational modification; YAP, yes-associated protein.

1 | INTRODUCTION

The Hippo signaling pathway is evolutionarily conserved from *Drosophila* to mammals, which is critical for organ size control as well as tumorigenesis by modulating cell proliferation and apoptosis, embryogenesis and development, tissue regeneration and wound healing.[1] The core pathway kinases of the Hippo pathway consist of MST1/2 and LATS1/2, a transcriptional activator and also a Hippo pathway effector, YAP and a WW domain containing transcription regulator protein 1 (WWTR1; TAZ).[2] YAP and TAZ promote tissue growth and regeneration by transcriptional activation of target genes and direct binding to the promoter region.[3,4] Activation of the Hippo pathway leads to the binding of SAV1 to MST1/2, which facilitates MST1/2 phosphorylation and activation. Activated MST1/2 induces LATS1/2 phosphorylation and inactivates YAP/TAZ by inducing phosphorylation and sequestration to the cytosol for proteasome-dependent degradation.[5,6] Activation of YAP/TAZ, mostly resulting from inactivation of MST1/2 alters the cellular responses to nutrient and hormone signals and allows the cells to exit quiescence and enter the cell cycle, which is sufficient to drive aberrant hepatomegaly and neoplastic transformation.[7–9] Maintaining the appropriate kinase activity of MST1/2 is thus critical for the regulation of appropriate YAP/TAZ activity and suppression of tumorigenesis.

Ablation of MST1/2 in mouse liver results in hepatomegaly and eventually liver cancer showing features of both hepatocellular carcinoma and mixed hepatocellular and cholangiocarcinoma.[10] Inactivation of MST1/2 in the liver is associated with a decrease in YAP phosphorylation and an increase in the nuclear localization and protein expression of YAP.[11] Knocking down YAP reversed the transformed phenotype of liver cancer cells and completely prevented these MST1/2 knockout animals from developing hepatocellular carcinoma (HCC).[12] Consistently, YAP activation is a major feature observed in more than 60% of human liver cancer patients and is associated with greatly decreased MST1/2 activation.[13] In addition, emerging evidence suggest that the loss of regulation upstream of MST1/2 is a common abnormality that accounts for YAP activation and tumorigenesis in human cancer.[14] But mechanisms underlying dysregulation of MST1/2 in human cancer remain elusive.

SIRT7 belongs to class III histone deacetylases and plays critical roles in various biological processes, including RNA transcription,[15] DNA damage response,[16] lipid metabolism,[17] bone formation,[18] as well as immune regulation.[19] In addition to histones, SIRT7 is also involved in the deacetylation of non-histone proteins, including p53,[20] CDK9,[21] FOXO3[22] and SMAD4[23] and is associated with tumor differentiation, chemoresistance and metastasis. SIRT7 is highly selective for H3K18 deacetylation, which silences tumor suppressor genes and maintains cancer transformation.[24–26] Aberrant expression of SIRT7 is associated with cancer progression in human liver, breast and pancreatic cancer.[27,28] More importantly, inactive SIRT7 has been shown to reverse malignancy and increase the sensitivity of cancer to therapeutics.[29–31] For HCC, we have previously reported that SIRT7 is elevated in liver cancer and high SIRT7 expression is associated with a poor prognosis.[20] Inactive SIRT7 via small molecular compounds 40569Z and 2800Z significantly increased sensitivity to sorafenib.[32,33] Knocking down of SIRT7 impairs liver cancer growth[27] but mechanisms by which SIRT7 promotes liver cancer proliferation remain elusive. In the present study, we demonstrated that SIRT7 is critical in regulating the YAP nuclear localization and activation via MST1 suppression by both transcriptional regulation and post-transcriptional modification. Reconstitution of MST1 in SIRT7 overexpressed cells or knocking down MST1 in SIRT7 knockdown cells completely abolished SIRT7-mediated liver cancer growth. SIRT7 inhibitor 2800Z[32] sufficiently inhibited HCC growth both in vivo and in vitro by modulating the MST1/YAP axis. Our data thus uncovered a previously undescribed role of SIRT7 in regulating the Hippo pathway and further proved that targeting SIRT7 potentiates novel therapeutic options in human HCC.

2 | MATERIALS AND METHODS

All methods and key reagents can be found in Appendix S1 and Table S1.

3 | RESULTS

3.1 | SIRT7 is essential for HCC growth both in vitro and in vivo

To confirm that SIRT7 regulates HCC proliferation in vitro, we knocked down SIRT7 in HCC cells and measured cell growth (Figure 1A). The knockdown of SIRT7 resulted in significant suppression of cancer cell growth and approximately 75% reduction of colony-forming ability when compared with control cells (Figure 1B–D). Similarly, in vivo results showed that the knockdown of SIRT7 resulted in a significant decrease in tumor size (Figure 1E, F) and tumor weight (Figure 1G) when compared with control cells. We explored the *SIRT7* gene alterations in human cancers using the cBioportal database and the results indicated that SIRT7 was also altered in many human cancers in addition to liver cancer including melanoma, sarcoma, non–small-cell lung cancer, and glioma (Figure S1). Consistent with a previous report,[28] we found that SIRT7 expressions were positively associated with disease progression (Figure S1) and SIRT7 alteration implied a poor prognosis in liver cancer (Figure S1).

3.2 | SIRT7 regulates YAP nuclear localization and activation

To explore mechanisms by which SIRT7 regulates HCC proliferation we performed RNA sequencing in SIRT7 knockdown Hep3B cells to identify differentially expressed genes (Figure 2A). KEGG pathway enrichment analysis indicated that SIRT7 is involved in the regulation of the

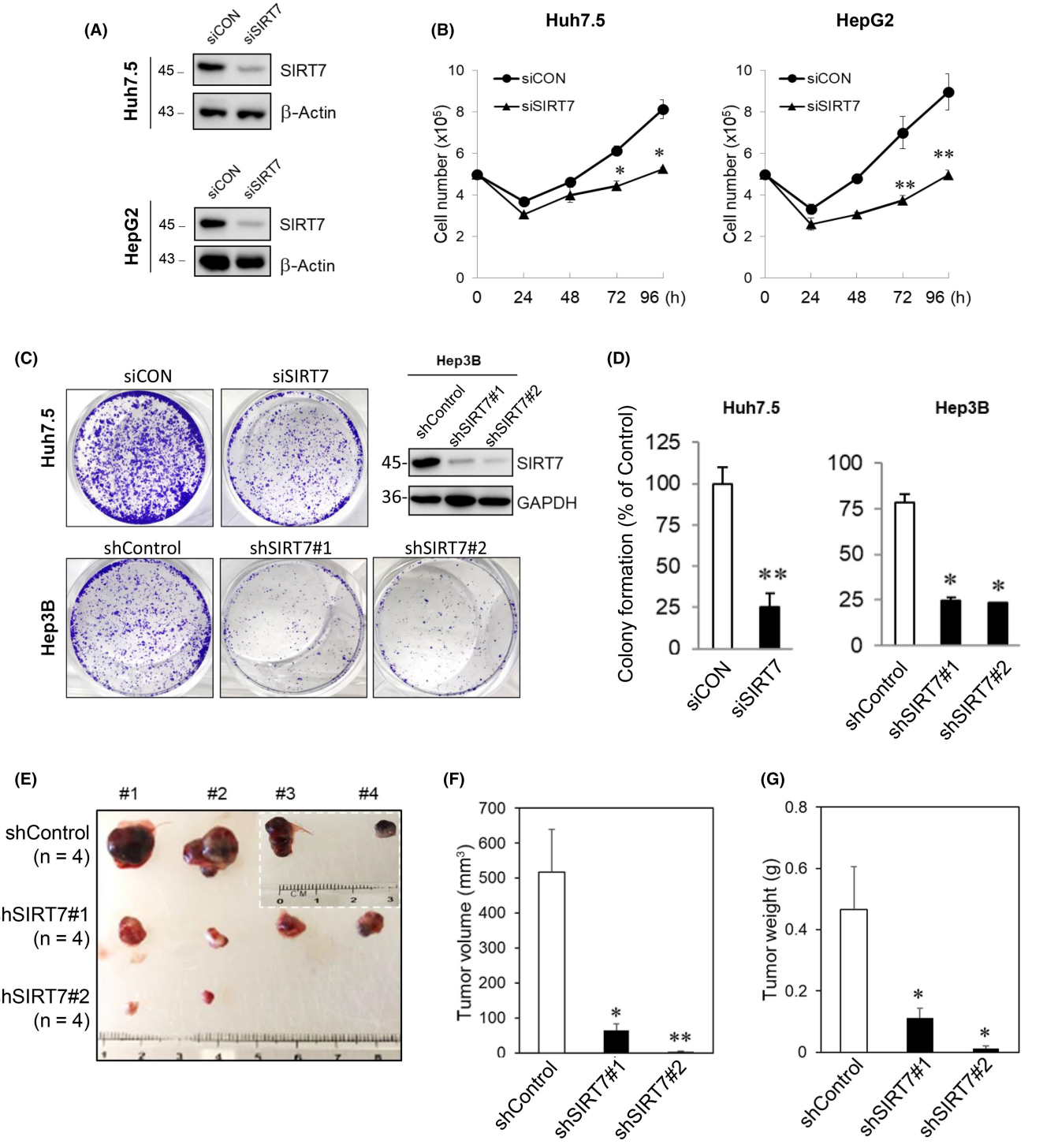
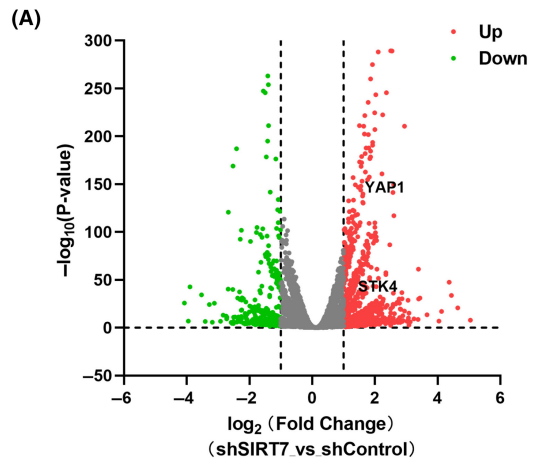
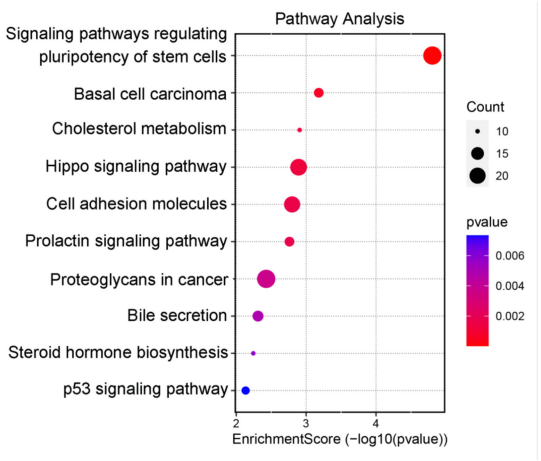
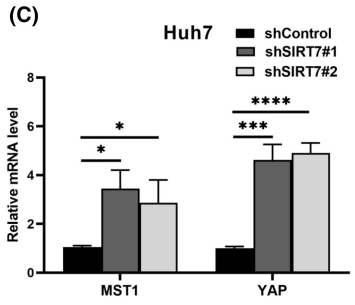


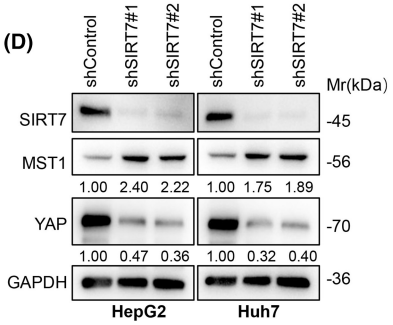
FIGURE 1 SIRT7 is essential for HCC growth both in vitro and in vivo. (A–D) Huh7.5, HepG2 and Hep3B cells transfected with siSIRT7 or transduced with scramble (shControl) or shSIRT7 for 72 h, SIRT7 expression was evaluated by western blot (A), cell proliferation was measured by growth curve (B) and colony-formation assay (C, D). (E–G) Hep3B cells transduced with shControl or shSIRT7, cells were then transplanted into NSG mice, and grass tumor images (E), tumor volume (F) and tumor weight (G) were obtained 14 days after injection. Graphs are presented as means ± SD of at least three replicates. $^{*}p < 0.05$, $^{**}p < 0.01$.

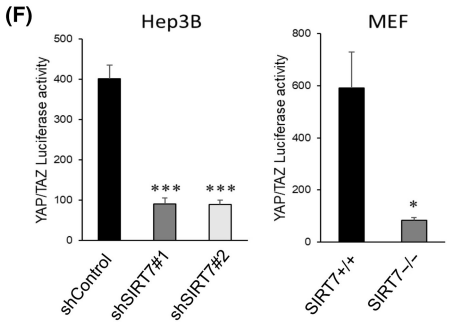
Hippo pathway, cell adhesion and proteoglycans in cancer (Figure 2B). Among those differentially expressed genes, key components of the Hippo pathway including YAP and STK4 (MST1), emerged as responsive genes sensitive to SIRT7 knockdown (Figure 2A). As the Hippo pathway is critical in regulating cell proliferation, we thus focused on whether SIRT7 regulates the Hippo pathway. qRT-PCR confirmed that knocking down SIRT7 resulted in a significant increase in *MST1* and *YAP* mRNA levels (Figure 2C). However, western blot results indicated that

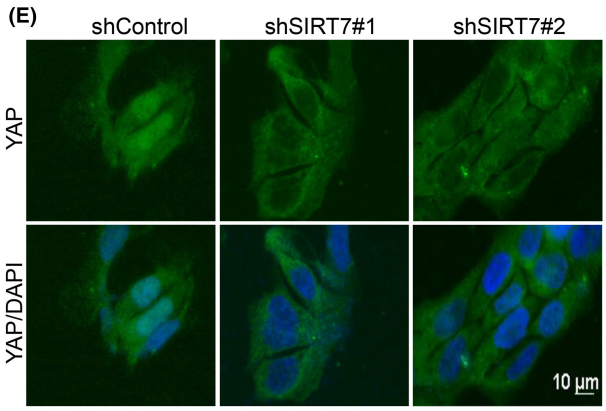

(A)
Up
Down
YAP1
STK4
−log10(P-value)
log2 (Fold Change)
(shSIRT7_vs_shControl)

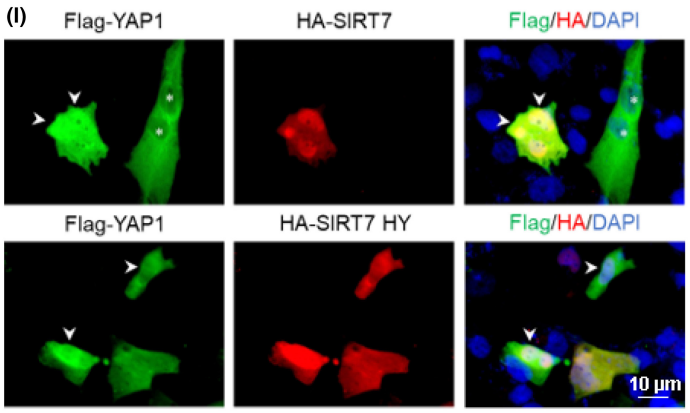

(B)
Pathway Analysis
Signaling pathways regulating pluripotency of stem cells
Basal cell carcinoma
Cholesterol metabolism
Hippo signaling pathway
Cell adhesion molecules
Prolactin signaling pathway
Proteoglycans in cancer
Bile secretion
Steroid hormone biosynthesis
p53 signaling pathway
Count
pvalue
EnrichmentScore (−log10(pvalue))

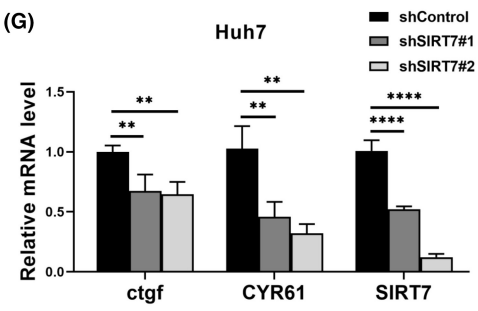

(C)
Huh7
shControl
shSIRT7#1
shSIRT7#2
Relative mRNA level
MST1
YAP

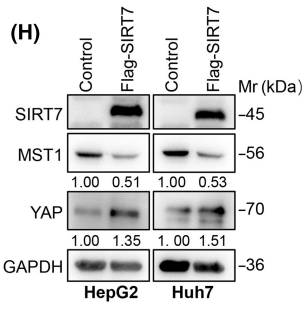

(D)
shControl
shSIRT7#1
shSIRT7#2
Mr(kDa)
SIRT7
MST1
YAP
GAPDH
HepG2
Huh7


(F)
Hep3B
MEF
YAP/TAZ Luciferase activity
shControl
shSIRT7#1
shSIRT7#2
SIRT7+/+
SIRT7−/−


(E)
shControl
shSIRT7#1
shSIRT7#2
YAP
YAP/DAPI
10 μm


(I)
Flag-YAP1
HA-SIRT7
Flag/HA/DAPI
Flag-YAP1
HA-SIRT7 HY
Flag/HA/DAPI
10 μm


(G)
Huh7
shControl
shSIRT7#1
shSIRT7#2
Relative mRNA level
ctgf
CYR61
SIRT7


(H)
Control
Flag-SIRT7
Mr (kDa)
SIRT7
MST1
YAP
GAPDH
HepG2
Huh7

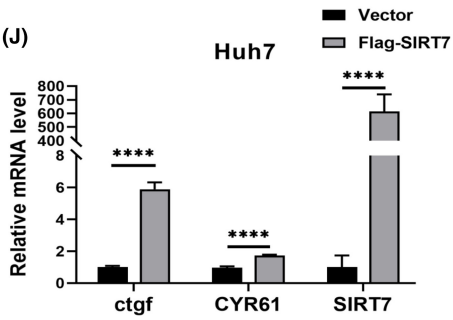

(J)
Huh7
Vector
Flag-SIRT7
Relative mRNA level
ctgf
CYR61
SIRT7

FIGURE 2 SIRT7 regulates YAP nuclear localization and activation. (A) Volcano plot of differentially regulated genes in Hep3B cells transduced with scramble shRNA (shControl) or SIRT7 shRNA (shSIRT7). Green indicates a decrease, and red indicates an increase. (B) Differential gene-enriched signaling pathways were identified using KEGG pathway enrichment analysis. (C, D) qRT-PCR and western blot analysis of MST1 and YAP in SIRT7 knockdown cells. The numbers below the lane indicate relative band intensity normalized to GAPDH. (E) Immunofluorescence staining of YAP (green) in Hep3B cells with or without SIRT7 knockdown. (F) 8xGTIIC-luciferase plasmids were transfected in SIRT7-knockdown Hep3B or SIRT7$^{-/-}$ MEF cells; luciferase assay was performed 24 h after transfection. (G) qRT-PCR analysis of *ctgf* and *CYR61* mRNA expression in Huh7 cells with SIRT7 knockdown. (H) Western blot analysis of YAP and MST1 expression in HepG2 and Huh7 cells with SIRT7 overexpression. The numbers below the lane indicate relative band intensity normalized to GAPDH. (I) Immunofluorescence staining of YAP (green) in Hep3B cells transfected with Flag-SIRT7 or Flag-SIRT7 HY (red). (J) qRT-PCR analysis of *ctgf* and *CYR61* mRNA expression in Huh7 cells with SIRT7 overexpression. Graphs are presented as means ± SD of at least three replicates. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$.

SIRT7 knockdown increased MST1 but decreased YAP protein expression in both cells (Figure 2D). Consistent with western blotting results, we observed that nuclear localization of YAP was greatly hindered in SIRT7 knockdown cells (Figure 2E; Figure S2A, B). More importantly, we found that SIRT7 was essential for YAP/TAZ activation in both SIRT7 knockdown and primary SIRT7 knockout mouse embryonic fibroblasts (MEF) (Figure 2F). As a result, SIRT7 knockdown suppressed YAP downstream target genes *ctgf* and *CYR61* expression (Figure 2G; Figure S2C). Conversely, SIRT7 overexpression downregulated MST1 and increased YAP protein in HCC cells (Figure 2H). WT SIRT7 (HA-SIRT7) but not the enzymatic inactive form SIRT7 H187Y (HA-SIRT7 HY) increased the nuclear localization of YAP (Figure 2I) and increased *ctgf* and *CYR61* mRNA levels in HCC cells (Figure 2J; Figure S2D).

3.3 | SIRT7 suppresses MST1 transcription via H3K18 deacetylation

To further investigate how SIRT7 regulates the Hippo pathway, we detected Hippo components in SIRT7-knockdown cells (Figure 3A; Figure S3A,B). Knockdown of SIRT7 resulted in an increase in MST1, p-MST1, p-LATS1 as well as p-YAP S127 levels. Accordingly, YAP protein was significantly decreased. However, MST2, LATS1, LATS2 and PAN-TEAD protein levels were mildly affected by SIRT7 knockdown (Figure 3A; Figure S3A,B). Overexpression of SIRT7 exhibited opposite effects in both cell lines (Figure 3B, S3C,D). Immunofluorescence (IF) results confirmed that overexpression of wild-type SIRT7 but not SIRT7 HY reduced MST1 expression, while MST2 was not affected in either case (Figure 3C). Knockdown of SIRT7 enhanced *MST1/2* mRNA expression while overexpression SIRT7 but not SIRT7 HY suppressed *MST1/2* mRNA levels (Figure 3D,E). We performed a ChIP assay to assess whether SIRT7 bound to the MST1 promoter and we found direct binding of SIRT7 to the MST1 promoter region (Figure 3F,G). To further assess whether SIRT7 induced histone H3K18 deacetylation in the MST1 promoter region, we knocked down SIRT7 and evaluated H3K18 acetylation using a ChIP assay. Knockdown of SIRT7 resulted in an increase in H3K18 acetylation in the MST1 promoter region and SIRT7 overexpression showed the opposite effects (Figure 3H,I). Meanwhile, H3K56 acetylation levels, a known substrate of SIRT6, remain unchanged in either case (Figure 3J).

3.4 | SIRT7 interacts with MST1 and alters its stability

To further evaluate whether SIRT7 also regulated MST1 through PTM, we first examined protein–protein interactions using immunoprecipitation-mass spectrometry (IP-MS) analysis. The results indicated the abundant presence of SIRT7 peptide in the immunoprecipitated MST1 protein complex (Figure S4A). Using IP, we confirmed direct SIRT7/MST1 interaction in HCC cells co-transfected with MST1 and SIRT7 (Figure 4A,B). Moreover, we observed interactions between endogenous MST1 and SIRT7 in all three HCC cell lines as well as 293FT cells (Figure 4C). We further examined whether SIRT7 regulates MST1 stability by measuring protein half-life and the results indicated that overexpression of SIRT7 led to a significant decrease in MST1 protein half-life (Figure 4D; Figure S4B,C). In contrast, the knockdown of SIRT7 prolonged the half-life MST1 and shortened the half-life of YAP in both cells (Figure 4E–H). To evaluate how SIRT7 regulated MST1 stability, we measured MST1 ubiquitination in the presence or absence of SIRT7. SIRT7 knockdown significantly decreased the ubiquitination of MST1 (Figure 4I). Similarly, the ubiquitination of endogenous MST1 was markedly decreased in SIRT7 knockdown cells (Figure 4J,K). By contrast, SIRT7 overexpression increased ubiquitination of endogenous MST1 (Figure S4D).

3.5 | SIRT7 reduces MST1 stability via deacetylation-dependent ubiquitination

SIRT7 is known to deacetylate multiple non-histone proteins, we thus examined whether SIRT7 catalyzes MST1 deacetylation. The results indicated that SIRT7 knockdown increased the acetylation of both exogenous and endogenous MST1 (Figure 5A–D). To further evaluate the relationship between acetylation and protein stability of MST1, we transfected SIRT7 HY mutant and measured the MST1 protein half-life (Figure 5E–H). Interestingly, SIRT7 HY did not change the MST1 half-life in HCC cells (Figure 5E–H). We further examined whether deacetylation of MST1 was essential for its ubiquitination by comparing ubiquitination levels of MST1 in SIRT7 or SIRT7 HY

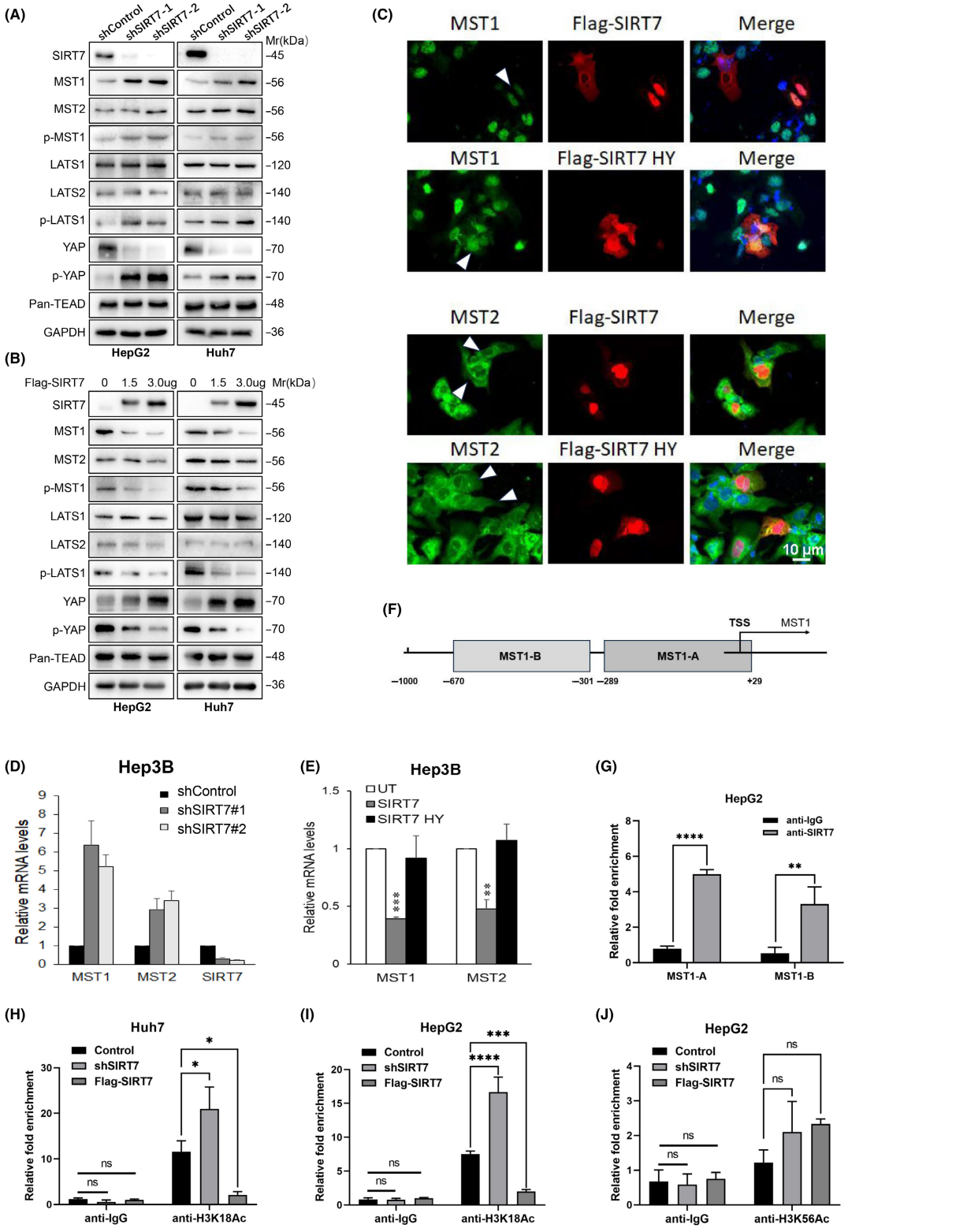


FIGURE 3 SIRT7 suppresses MST1 transcription via H3K18 deacetylation. (A, B) HepG2 and Huh7 cells were transduced with scrambled or SIRT7 shRNA (shSIRT7#1, shSIRT7#2) for 72 h, or transfected with Flag-SIRT7 for 24 h, and protein levels of the Hippo pathway were analyzed using western blot. (C) Immunofluorescence staining of transfected Flag-SIRT7 or Flag-SIRT7 HY (red) and endogenous MST1 and MST2 (green). (D, E) mRNA expression levels of *MST1/2* after SIRT7 knockdown (D) and Flag-SIRT7 or Flag-SIRT7 HY overexpression (E). (F) Illustration of the MST1 promoter regions (MST1-A, MST1-B) accessed using ChIP analysis. (G) Promoter binding of SIRT7 to the MST1 promoter regions (MST1-A, MST1-B) was evaluated using ChIP assay in HepG2 cells using antibodies as indicated. (H–J) Cells were transduced with shSIRT7 or transfected with Flag-SIRT7; acetylation levels within the promoter regions of MST1 (MST1-A) were measured by ChIP assay using antibodies as indicated. Graphs are presented as means ± SD of at least three replicates. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$.

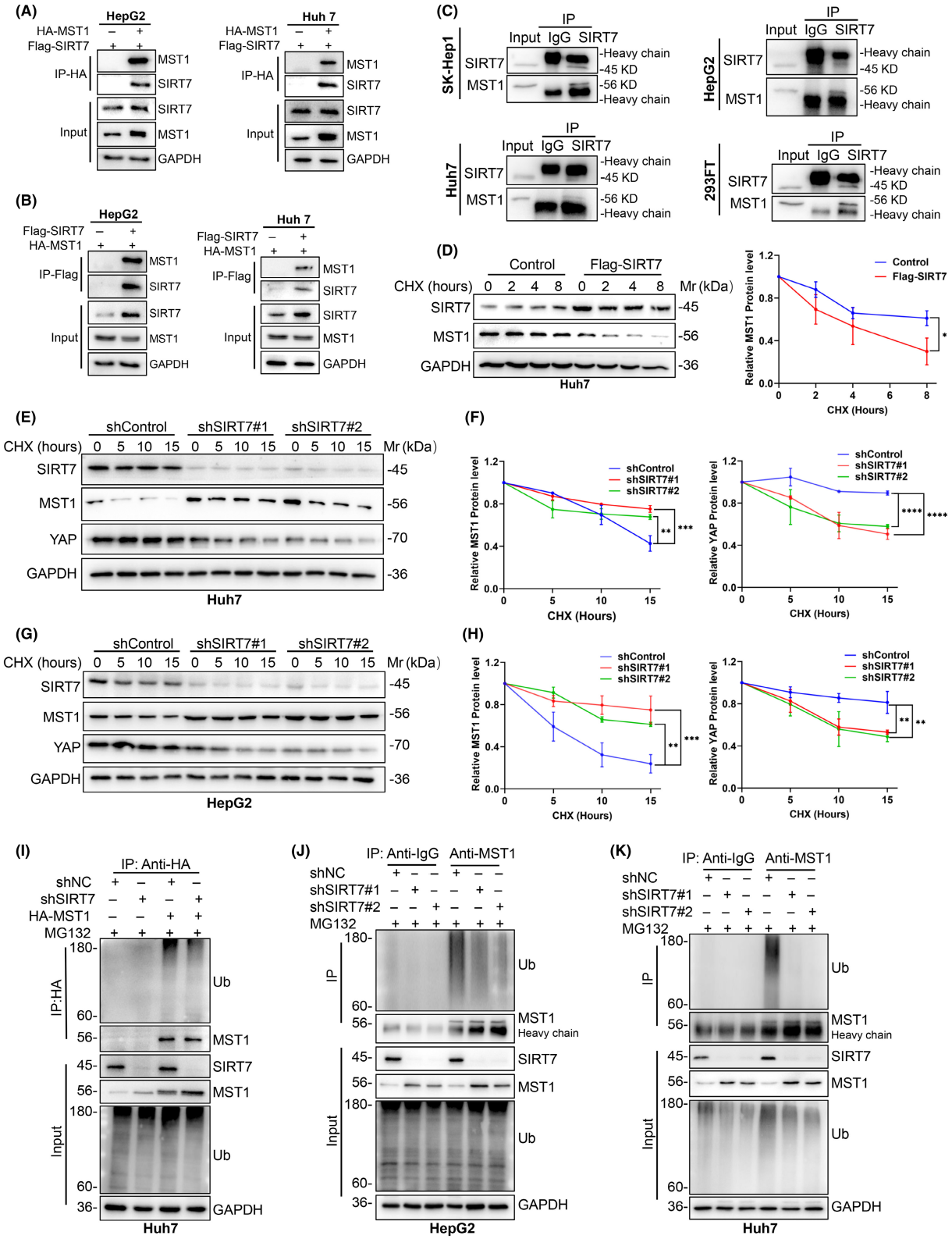


FIGURE 4 SIRT7 interacts with MST1 and alters its stability. (A, B) Cells were transfected with either HA-MST1 or Flag-SIRT7 alone or in combination. Proteins were immunoprecipitated by HA or Flag magnetic beads; immunocomplexes were analyzed using western blotting. (C) Cell lysates were immunoprecipitated with SIRT7 antibody-conjugated magnetic beads, and immunocomplexes were analyzed by western blot. (D–H) Protein half-life analysis of MST1 protein in HCC cells with SIRT7 overexpression (D) or knockdown (E–H). (I) Huh7 cells were transduced with scrambled or shSIRT7 for 72 h, and then transfected with HA-MST1 for another 24 h. Cells were treated with MG132 (20 μM) for 4 h, exogenous MST1 protein was immunoprecipitated; ubiquitination was analyzed by western blot. (J, K) Cells were treated with MG132 for 4 h, endogenous MST1 protein was immunoprecipitated, and its ubiquitination was analyzed by western blot. Graphs are presented as the means ± SD of at least three replicates. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$.

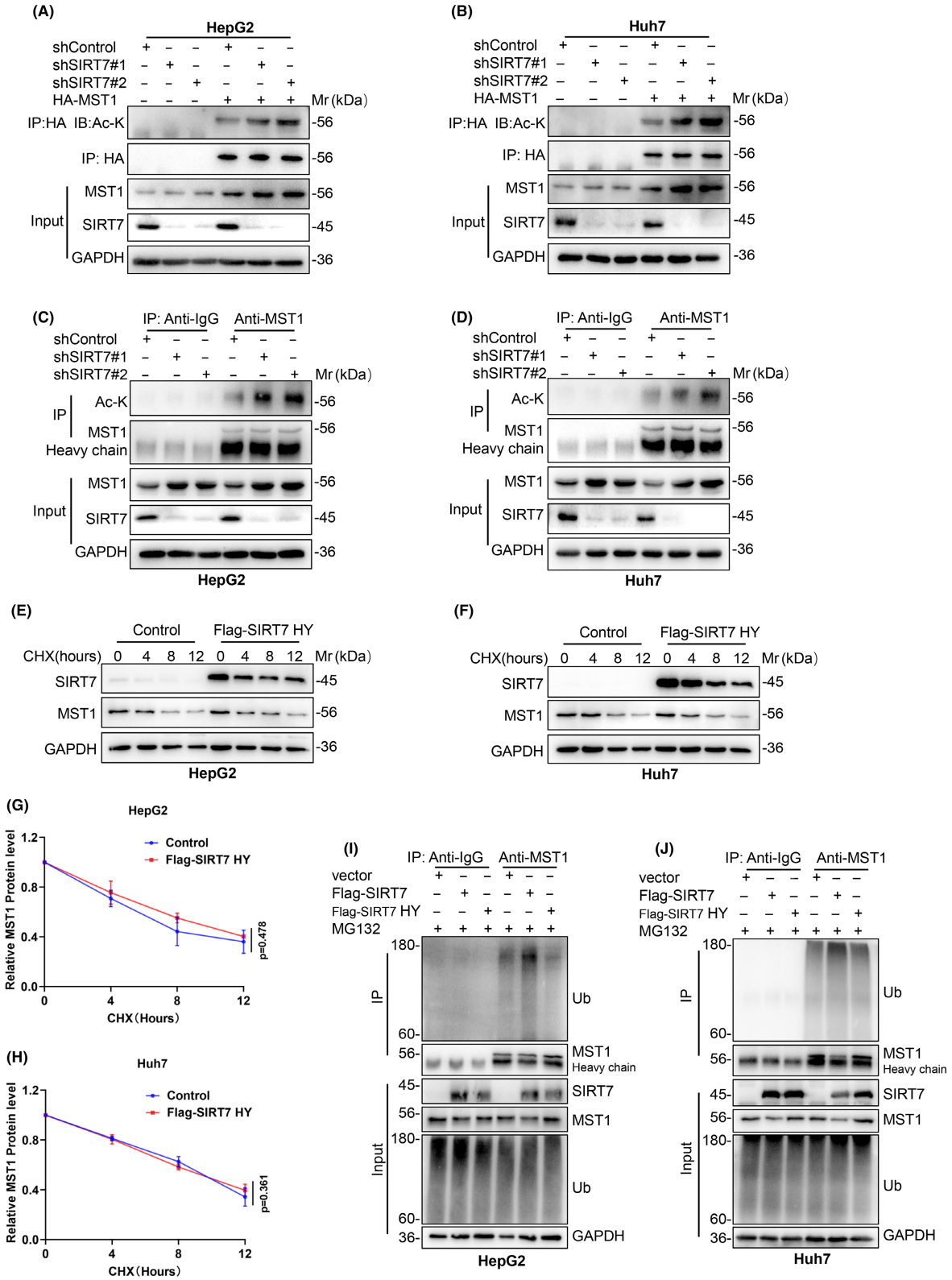


FIGURE 5 SIRT7 reduces MST1 stability via deacetylation-dependent ubiquitination. (A, B) Cells were transduced with scrambled or SIRT7 shRNA for 72 h, and then transfected with HA-MST1 for another 24 h. Cells were treated with TSA (2 μM) for 4 h, exogenous MST1 protein was immunoprecipitated and its acetylation was analyzed using western blotting with acetyl K (Ac-K-103) antibody. (C, D) Cells were treated with TSA (2 μM) for 4 h, endogenous MST1 protein was immunoprecipitated, and its acetylation was analyzed using western blotting with acetyl K (Ac-K-103) antibody. (E, F) Cells were transfected with Flag-SIRT7 HY for 24 h and then treated with cycloheximide (CHX) for the indicated times; the expression of MST1 protein was examined by western blot. (G, H) Protein half-life of MST1 with or without Flag-SIRT7 HY transfection ($n = 3$). (I, J) After 24 h of Flag-SIRT7 or Flag-SIRT7 HY transfection, cells were treated with MG132 for 4 h, endogenous MST1 protein was immunoprecipitated, and its ubiquitination was detected using western blotting.

transfected cells and the results indicated SIRT7 dramatically increased MST1 ubiquitination, but the mutant showed nearly no effects (Figure 5I,J).

3.6 | MST1 is responsible for SIRT7-mediated HCC proliferation

We next evaluated the role of MST1 in SIRT7-mediated YAP activation and HCC growth in vitro. SIRT7 overexpression decreased MST1, p-YAP(S127), and increased YAP expression. Consistent with these findings, reconstitution of HA-MST1 in Flag-SIRT7 overexpressed HCC cells partially restored MST1 and p-YAP levels, and downregulated YAP expression (Figure 6A,B). In both cells, SIRT7 overexpression decreased the p-YAP/YAP ratio while reconstitution of MST1 abolished these effects (Figure 6B). In addition, overexpression of SIRT7 enhanced the colony-formation efficiency and proliferation of HCC cells that were abrogated by ectopic expression of MST1 in both cells (Figure 6C,D). Conversely, knockdown SIRT7 increased MST1, p-YAP (S127), decreased YAP, and inhibited colony formation and proliferation in HCC cells (Figure 6E–H). Knockdown of MST1 could reverse the effects caused by SIRT7 deficiency (Figure 6E–H).

3.7 | SIRT7 is negative correlated with MST1 expression in HCC

We further determined correlations between SIRT7, MST1 and YAP in clinical samples ($n=14$; Table S2). The data indicated that the MST1 expression pattern and the MOD were negatively correlated with SIRT7, while YAP expression was positively correlated with SIRT7 (Figure 7A–C). We further divided specimens into groups with low and high SIRT7 expression based on the medium staining score and compared MST1 expression in each group (Figure 7D). The results further confirmed that the MST1 level was signifyingly higher in the SIRT7-low group (Figure 7D). In addition, we measured mRNA expression levels of *SIRT7* and *MST1* in our cohort and found a negative correlation between mRNA levels of *MST1* and *SIRT7* (Figure 7E). More importantly, we observed that the percentage of YAP nuclear-positive staining cells among the patients with high expression of SIRT7 was significantly higher than that of patients with low SIRT7 expression (Figure 7F,G).

3.8 | SIRT7 inhibition suppresses HCC growth via MST1/YAP axis

We finally tested the potency of targeting SIRT7 in HCC using the SIRT7-specific inhibitor 2800Z.[32] Here, 2800Z treatment increased MST1 and p-YAP but decreased YAP in vitro (Figure 8A; Figure S5A,B). In addition, 2800Z administration inhibited SIRT7 binding to the MST1 promoter and increased H3K18 acetylation at the MST1 promoter region (Figure 8B,C; Figure S5C). As a result, the mRNA level of *MST1* was significantly enhanced after 2800 treatment (Figure 8D). At the protein level, 2800Z increased MST1 protein and decreased MST1 ubiquitination in HCC cells (Figure 8E; Figure S5D). In addition, 2800Z treatment induced YAP sequestration to the cytosol and suppressed YAP target genes *ctgf* and *CYR61* transcription (Figure 8F–H; Figure S5E–H). We finally investigated whether 2800Z could reshape the Hippo pathway and inhibit tumor growth in vivo. To this end, mice with transplanted tumors were treated with the same dose of sorafenib or 2800Z (4 mg/kg) for 14 days; 2800Z showed comparable or even profound effects of tumor inhibition when compared with sorafenib (Figure 8I,J). Unlike in vitro data, we found that 2800Z administration in vivo reduced the positive staining for SIRT7 that was associated with increased MST1 protein levels (Figure 8K,L). We found that 2800Z administration enhanced p-YAP expression and decreased YAP nuclei localization in vivo (Figure 8M). In addition, the YAP expression and transcription levels of *ctgf* and *CYR61* were also significantly decreased in the 2800Z-treated tumors (Figure 8N).

4 | DISCUSSION

In the present study, we demonstrated that SIRT7 is critical in regulating YAP activation and liver cancer growth both in vitro and in vivo. Knockdown SIRT7 resulted in significant impairment of liver cancer growth and YAP activation, while SIRT7 overexpression showed the opposite effects. We further revealed that SIRT7 targets MST1 via transcriptional and post-translational regulation. SIRT7 suppresses MST1 transcription by binding to the MST1 promoter and induced its H3K18 deacetylation. In addition, SIRT7 directly binds to and deacetylates MST1 that in turn primes MST1 ubiquitination. Reconstitution of MST1 in SIRT7-overexpressed cells or knocking down MST1 in SIRT7-knockdown cells completely abolished SIRT7-mediated HCC growth in vitro. Using the SIRT7-specific inhibitor 2800Z, we showed that inactive SIRT7 significantly suppressed liver cancer growth both in vitro and in vivo via the reshaped Hippo pathway.

As the most important upstream kinase that restricts YAP activation, the inactivation of MST1/2 is responsible for the overactivation of YAP and tumorigenesis.[34,35] Previous data have demonstrated that Myc and EZH2-mediated histone methylation modulates MST1 transcription.[36] Our data newly identified that SIRT7-dependent transcriptional and post-transcriptional modification also participates in the regulation of the MST1/YAP axis. Similar to MST1, SIRT7 also suppressed MST2 transcription, whereas it did not affect its protein expression, indicating that other factors also participate in the regulation of MST2 protein expression in human HCC. Of note, it is well known that the activity of MST1/2 is essential for the YAP regulation and that the combined deficiency of MST1/2 resulted in the loss of inhibitory Ser127 phosphorylation of YAP and tumorigenicity. However, in mouse liver, re-expression of MST1 itself was sufficient to promote

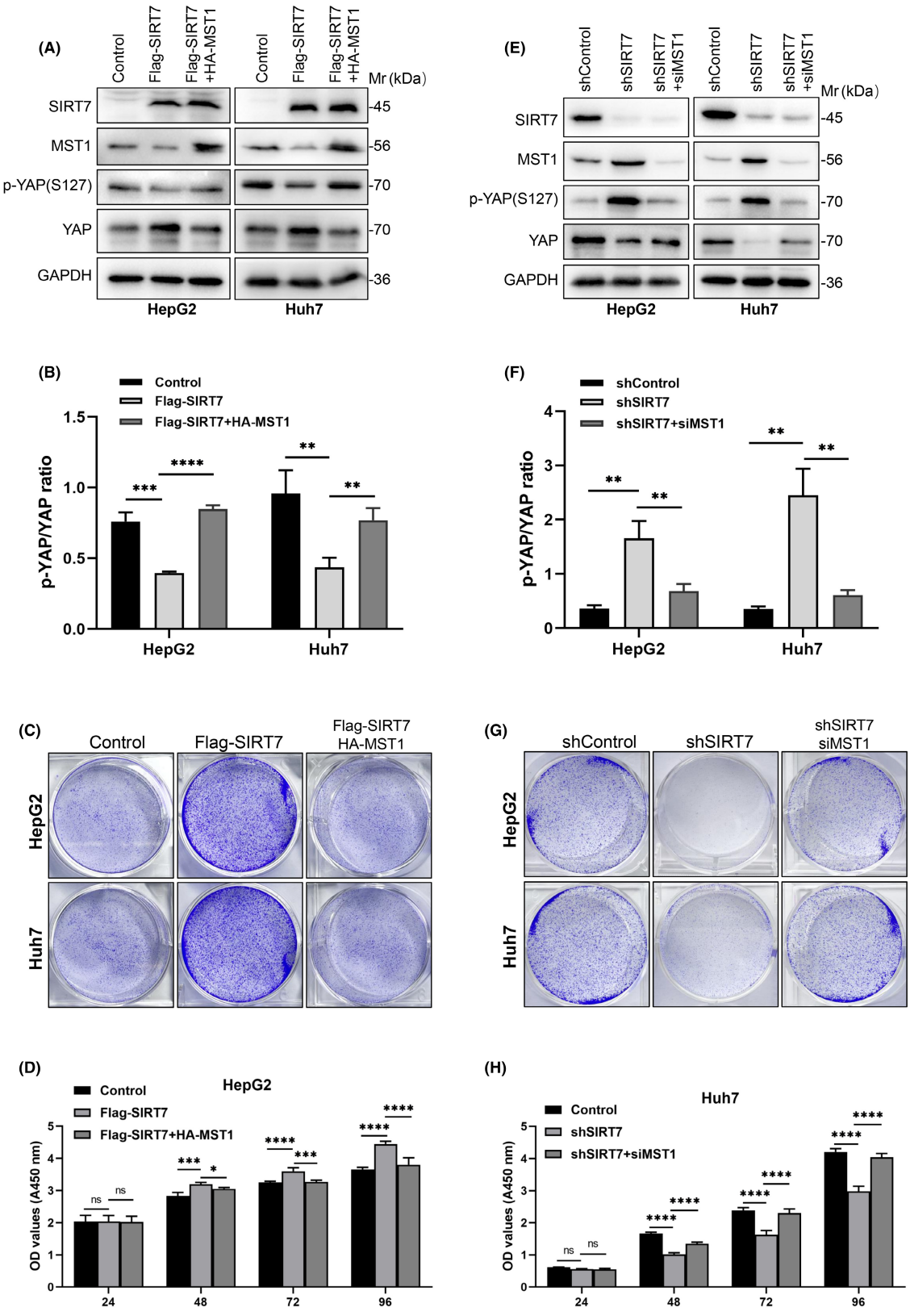


FIGURE 6 MST1 is responsible for SIRT7-mediated HCC proliferation. (A, B) Cells were untransfected or transfected with Flag-SIRT7 in the presence or absence of HA-MST1, (A) protein expression was evaluated using western blotting, (B) ratios of p-YAP to YAP were quantified by measuring the relative expression normalized to GAPDH. (C) Colony-formation assay and (D) CCK-8 assay of the cells as in (A). (E, F) Cells were transduced with scrambled or SIRT7 shRNA in the presence or absence of siMST1, (E) protein expression was evaluated using western blotting and (F) ratios of p-YAP to YAP were quantified by measuring the relative expression normalized to GAPDH. (G) Colony-formation assay and (H) CCK-8 assay of the cells as in (E). Graphs are presented as means ±SD of at least three replicates. $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$, $^{****}p<0.0001$.

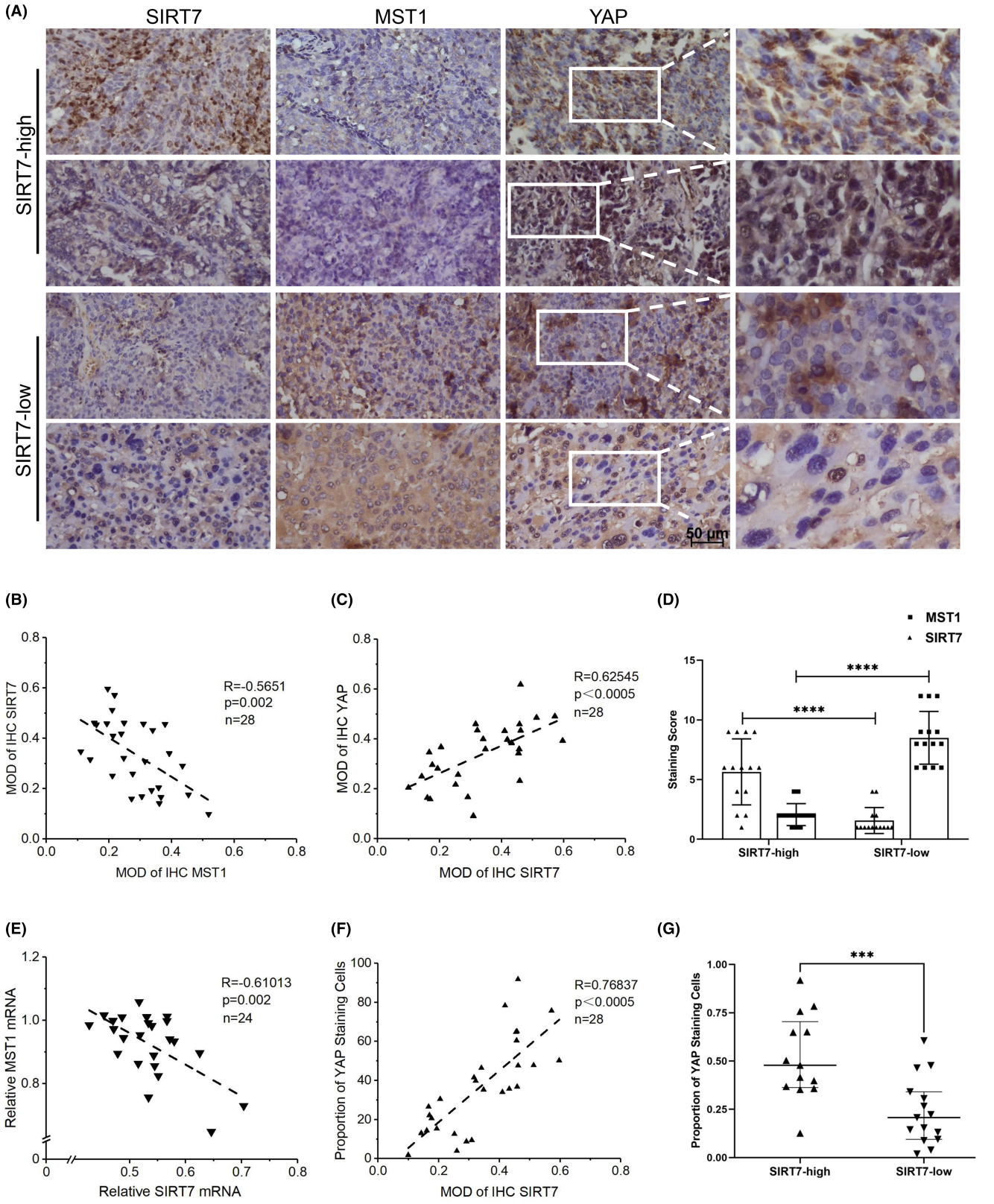


FIGURE 7 SIRT7 is negatively correlated with MST1 expression in human HCC. (A) Representative immunohistochemistry (IHC) staining of SIRT7, MST1 and YAP proteins in human HCC ($n=14$). The mean optical density (MOD) values of each protein in each sample were calculated using ImageJ software, and the linear correlation scatter plots of (B) SIRT7 and MST1, and (C) SIRT7 and YAP were plotted. Tumor and adjacent nontumor tissue ($n=28$) were divided into SIRT7-high and SIRT7-low groups according to SIRT7 expression; the median value was used as the cut-off, and then its correlation with (D) MST1 staining score and (G) YAP-positive cell proportion was analyzed. (E) Correlation analysis between *SIRT7* and *MST1* mRNA levels in human HCC. (F) Correlation between the proportion of YAP-positive cells and the SIRT7 MOD value was analyzed based on the results of immunohistochemistry. Correlation analysis was assessed using Pearson's correlation test (two-sided).

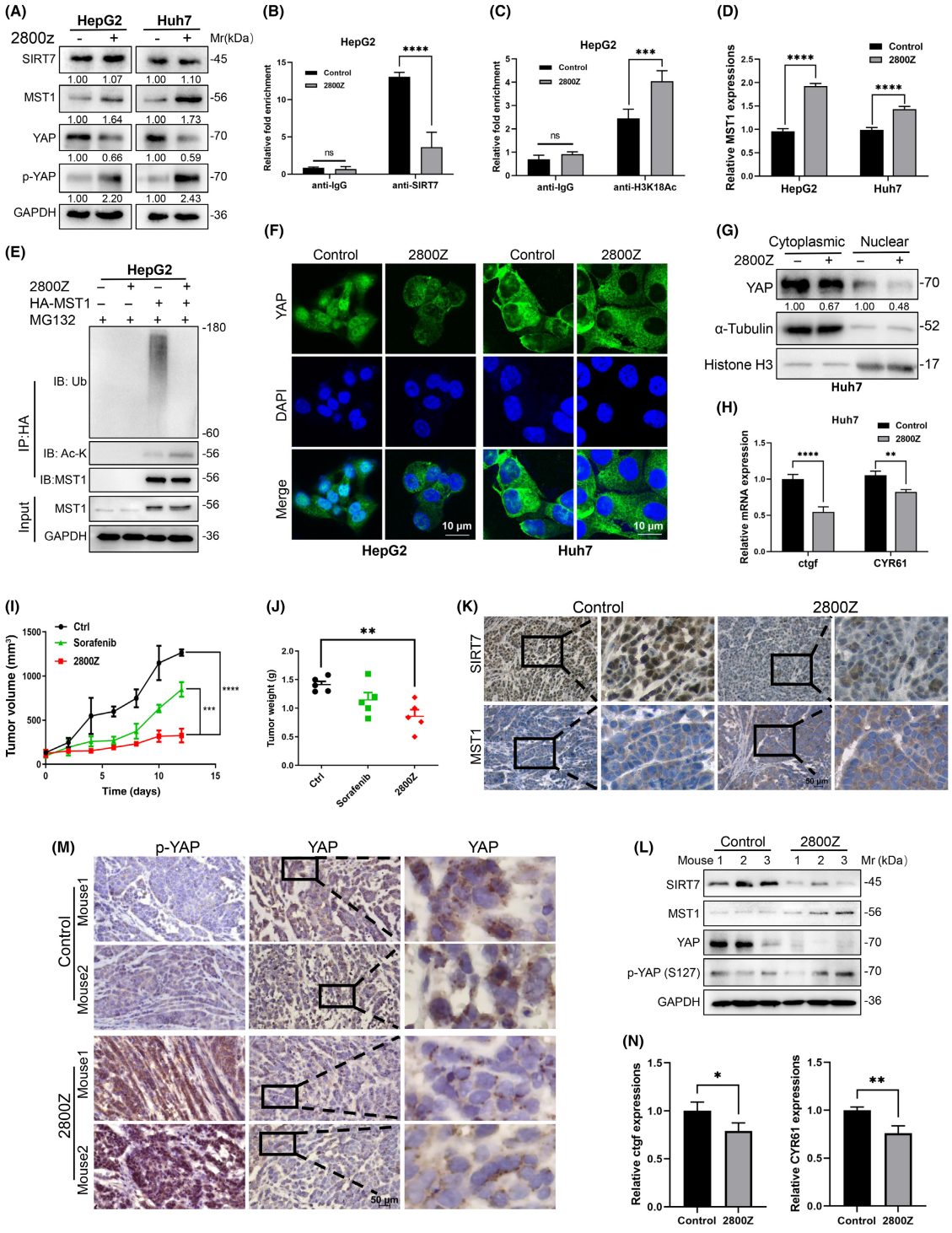


FIGURE 8 SIRT7 inhibition suppresses HCC growth via the MST1/YAP axis. (A) Western blotting analyzed the protein levels of SIRT7, MST1, YAP, and p-YAP in HCC cells after 2800Z (50 μM) treatment for 48 h. The numbers below the lane indicate relative band intensity normalized to GAPDH. (B, C) ChIP assay to evaluate binding of SIRT7 to the MST1 promoter (B) and H3K18 acetylation at the MST1 promoter region (C) after 2800Z treatment. (D) mRNA levels of *MST1* in cells after 2800Z treatment. (E) HA-MST1 plasmid was transfected into the HepG2 cells with or without 2800Z. Cells were treated with MG132 for 4 h, exogenous MST1 protein was immunoprecipitated and its ubiquitination was detected using western blotting. (F) Immunofluorescence staining of YAP in HCC cells after 2800Z treatment. (G) Subcellular fractionation analysis of YAP in Huh7 cells after 2800Z treatment. The numbers below the lane indicate relative band intensity normalized to GAPDH. (H) qRT-PCR analysis of ctgf and CYR61 in cells after 2800Z treatment. (I) Tumor growth curves and (J) tumor weights of tumor-bearing nude mice that received vehicle, sorafenib, or 2800Z (4 mg/kg) treatment for 14 days. IHC staining of SIRT7 and MST1 (K), YAP and p-YAP (M) in tumor tissues. (L) Protein levels of SIRT7, MST1, YAP, and p-YAP in tumors. (N) mRNA levels of *ctgf* and *CYR61* in tumors. Graphs are presented as means ± SD of at least three replicates. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$.

YAP Ser127 phosphorylation and abrogated MST1/2 deficiency-induced HCC formation,[10] which indicated that MST1 had a tumor suppressor function. In addition, overexpression of MST1 promoted YAP inactivation, apoptosis and increased chemosensitivity in the HCC cell line.[37] Consistent with those observations, we observed that SIRT7-mediated YAP activation and HCC cell growth regulation primarily relied on MST1.

SIRT7-specific inhibitor 2800Z was able to increase H3K18 acetylation at the MST1 promotor region and thus activate MST1 transcription. Notably, it is unclear how 2800Z decreased SIRT7 binding to the MST1 promoter, but we speculate that the compound may affect the DNA accessibility of SIRT7. In addition, PTMs precisely regulate the protein stability and activity of MST1.[38–41] Multiple proteins, including HDAC6, USP46 c-Abl, and Cullin-7, have been shown to associate with MST1 and modulate its protein stability through acetylation, ubiquitination and phosphorylation.[42–45] Studies have shown that, whereas K35 residue is critical for MST1 stability, Y433, T387, K59 seems to be critical in regulating its activity.[46,47] The precise sites of SIRT7 deacetylation remain to be determined, but our data clearly indicated that SIRT7-dependent MST1 deacetylation was required for its ubiquitination and degradation. Further investigation focused on the deacetylation site as well as whether acetylation and other PTMs coordinately determine MST1 degradation would be of interest. In addition, given the multiple roles of the Hippo pathway in human cancer, it is still unclear whether the transcriptional and post-transcriptional regulation of MST1 by SIRT7 contribute equally to the overactivation of YAP that led to tumorigenesis and disease progression.

SIRT7 is frequently upregulated in human cancer and high SIRT7 expression is associated with aggressive phenotype and a poor survival.[48–51] The majority of studies has been focused on its functions and therapeutic options by targeting SIRT7[20,23,26,52] but the mechanisms underlying its regulation remain elusive. We have previously indicated that alcohol has the ability to upregulate SIRT7, which requires CYP2E1, the major component of the ethanol oxidizing system. We newly identified that SIRT7 regulates YAP activation by suppressing MST1, thus extending our finding of SIRT7 functions and downstream targets in cancer. It is unclear whether MST1 is the only target responsible for SIRT7-mediated HCC proliferation, but reconstitution of MST1 in SIRT7-overexpressed cells or knock down of MST1 in SIRT7-knockdown cells completely abolished SIRT7-mediated HCC growth. We did not observe a direct interaction between SIRT7 and YAP using LS-MS/MS and immunoprecipitation. Emerging evidence has suggested that inactive SIRT7 shows beneficial effects in various types of cancers, which make it an attractive therapeutic target.[53–57] Using virtual screening, we developed specific SIRT7 inhibitors 2800Z and 40569Z, and have reported that both inhibitors sensitize HCC cells to sorafenib. We further demonstrated that 2800Z significantly inhibited liver cancer growth both in vitro and in vivo by reactivating the Hippo pathway. It is unclear why 2800Z suppressed SIRT7 expression in vivo, but we speculate that high SIRT7 expression tumors are more sensitive to the drug. Nevertheless, our data clearly demonstrated the important role of SIRT7 in regulating HCC proliferation and therapy sensitivity. Therapeutically targeting SIRT7 may thus offer novel options for the treatment of human HCC. In addition, it would be of interest to further explore whether these compounds inhibit the proliferation of other cancer types with high SIRT7 expression.

In summary, our data demonstrated the crucial role of SIRT7 in regulating the Hippo pathway and illustrated the molecular mechanisms responsible for MST1 dysregulation that led to YAP activation and tumorigenesis. Our findings thus provide a useful target for the development of mechanism-based therapeutic strategies for the management of human HCC and further prove that targeting SIRT7 may provide novel therapeutic options for liver cancer treatment.

AUTHOR CONTRIBUTIONS

Yiying Gu: investigation; data curation; writing validation. **Cong Ding:** Data curation; investigation. **Tingzi Yu:** Investigation. **Bohao Liu:** Formal analysis. **Wenbin Tang:** Data curation. **Zhiqiang Wang:** Data curation. **Xiaohui Tang:** Validation. **Gaoshuang Liang:** Validation. **Jinying Peng:** Methodology. **Xiangwen Zhang:** Data curation. **Zhuan Li:** Conceptualization; supervision; writing – review and editing.

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CONFLICT OF INTEREST STATEMENT

The authors have no conflict of interest to disclosure.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

Approval of the research protocol by an Institutional Reviewer Board: This study has been approved by the Ethics Committee of Hunan Normal University (2019083). Informed Consent: De-identified human liver specimens obtained from The First Affiliated Hospital of Hunan Normal University (People's Hospital of Hunan Province) and Second Xiangya Hospital, Central South

University. Written informed consent was obtained from all patients and all studies using human tissue samples were approved by the Human Subjects Committee of Hunan Normal University (2020274). Registry and the Registration No. of the study/trial: N/A. Animal Studies. All procedures involving mice were approved by the Ethics Committee of Hunan Normal University (2019083).

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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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