

SETD1A augments sorafenib primary resistance via activating YAP in hepatocellular carcinoma

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Abstract

Background: Sorafenib, the approved first-line chemotherapy drug for HCC, remains the key treatment agent which can effectively improve the survival rate of advanced HCC patients. However, the sorafenib primary resistance limits the application of sorafenib for HCC treatment. The aims of current study are to explore the role and mechanism of SETD1A (Histone Lysine Methyltransferase SET Domain Containing 1A) in sorafenib primary resistance.

Methods: The expression of SETD1A in HCC was analyzed by Gene Expression Profiling Interactive Analysis. The survival of HCC patients was analyzed by KM plotter: Kaplan-Meier Plotter. Western Blot and Real-time qPCR were performed to measure the protein and mRNA levels, respectively. Cell counting kit-8 assay and colony formation assay were performed to determine cell viability and proliferation. Propidium Iodide and Trypan Blue staining assays were performed to investigate cell death.

Results: Here, we showed that the expression of SETD1A was markedly upregulated in both HCC cell lines and tumor tissues compared to normal hepatocytes and corresponding non-tumor liver tissues, respectively. The patients who had higher level of SETD1A underwent lower survival rate of overall and sorafenib treated HCC patients, respectively. In addition, SETD1A expression was positively correlated with the IC_{50} of sorafenib treated HCC cell lines. Furthermore, we indicated that knockdown of SETD1A augmented proliferation inhibition and cell death induced by sorafenib. SETD1A deficiency impaired YAP phosphorylation and activation. YAP activation contributed to SETD1A mediated sorafenib primary resistance.

Conclusions: Taken together, the current study demonstrated that SETD1A enhanced YAP activation to induce sorafenib primary resistance in HCC.

Introduction

Hepatocellular carcinoma (HCC) is a malignant tumor with poor prognosis and the five-year relative survival rate of HCC patients is no more than 20% [1]. In past decades, HCC was often diagnosed at advanced stage, there were no reliable and effective treatment options, and the first-line systemic palliative drug approved by the Food and Drug Administration (FDA) was sorafenib [2-4].

Sorafenib, a multi-kinase inhibitor, reduces cancer cell proliferation and represents anti-angiogenic activity by blocking Raf/MEK/ERK (extracellular signaling-regulated kinase), VEGFR (vascular endothelial growth factor receptor), c-Kit (Mast/stem cell growth factor receptor), PDGFR (platelet-derived growth factor receptor) and FLT (FMS-like tyrosine kinase) [4]. Therefore, sorafenib can delay HCC development with prolongation of the survival for nearly 3 months [5]. However, the efficacy of sorafenib is transient owing to primary resistant. Although primary resistance is partially due to HCC heterogeneity, the resistance is mainly caused by long-term exposure to sorafenib which rapidly induced activation of PI3K/AKT (phosphatidylinositol-3-kinase/protein kinase B, EMT (epithelial–mesenchymal transition) and JAK/STAT (janus tyrosine kinase/signal transducer and activator of transcription) pathways [6-10].

SETD1A (Histone Lysine Methyltransferase SET Domain Containing 1A) belongs to the histone methyltransferase family who adds mono-, di-, and trimethyl on histone H3K4[11]. The abnormal activation of SETD1A which promotes oncogenes transcription plays key role in tumorigenesis [12-14]. Previous reports showed that SETD1A was upregulated and cooperated with CUDR to augment liver cancer cell growth and hepatocyte-like stem cell malignant transformation, knockdown of SETD1A suppressed liver cancer cell proliferation, migration and invasion [11, 15]. However, the role of SETD1A in sorafenib primary resistance of HCC was still largely unclear. The aim of this study was to explore the function and potential mechanism of SETD1A in sorafenib primary resistance of HCC.

In the current study, we indicated that SETD1A was upregulated in HCC, and high SETD1A contributed to sorafenib resistance. Inhibition of SETD1A augmented sorafenib-induced proliferation inhibition and cell death by reducing YAP activation.

Materials And Methods

Reagents and Antibodies

DMSO and crystal violet were purchased from Sigma-Aldrich (St. Louis, MO, USA), Sorafenib was purchased from Selleck (Shanghai, P.R. China). Protease Inhibitor Cocktail (100X) was obtained from Cell Signaling (Danvers, MA, USA). Propidium Iodide (PI) was purchased from Meilunbio (Dalian, P.R. China). The antibodies were used as following: anti-SETD1A (Bethyl, Cat#A300-289A, 1:1000 for WB), anti-Cyclin D1 (Abcam, Cat#ab134175, 1:1000 for WB), anti-PCNA (Cell Signaling, Cat#2586, 1:1000 for WB), anti-Cleaved-PARP (Cell Signaling, Cat#5625, 1:1,000 for WB), anti-Cleaved Caspase-3 (Cell Signaling, Cat#9664 1:1,000 for WB), anti-Phospho-YAP (Ser127) (Cell Signaling, Cat#13008, 1:1,000 for WB), anti-YAP (Cell Signaling, Cat#14074, 1:1,000 for WB), anti-CYR61 (Cell Signaling, Cat#14479, 1:1,000 for WB), anti-CTGF (Cell Signaling, Cat#86641, 1:1,000 for WB) and anti- β -actin (Sigma, Cat#A5316, 1:2000 for WB).

Bioinformatic Analysis of Clinical Data

HCC data were obtained from The Cancer Genome Atlas (TCGA). The expression of SETD1A was analyzed by Gene Expression Profiling Interactive Analysis (GEPIA) (<http://gepia.cancer-pku.cn/>)[16]. The survival of HCC patients was analyzed by KM plotter: Kaplan-Meier Plotter (<http://kmplot.com/analysis/>) [17].

Cell Culture

Normal human hepatic cell line LO2, human hepatocellular carcinoma cell lines SMMC-7721, SK-HEP1-1, HLE, HepG2 and Hep3B, and HEK293T were purchased from the Cell Bank of Type Culture Collection of Chinese Academy of Sciences (Shanghai, China). All cells were cultured in DMEM (Invitrogen, Carlsbad, CA, USA) medium containing 10% fetal bovine serum (FBS, Gibco, Cat#10099141) and 1% penicillin-streptomycin (Gibco, Cat#15140122) at 37°C with 5% CO₂.

Nucleus and cytoplasm protein isolation

Nucleus and cytoplasm protein were isolated by a Nuclear and Cytoplasmic Protein Extraction Kit (Beyotime, Shanghai, China) according to the manufacturer's instructions. Briefly, cells were washed with cold PBS, added Cytoplasmic Protein Extraction buffer A on ice for 10 mins, and then add Cytoplasmic Protein Extraction buffer B. After Vortex, the cells were centrifuged for 5 min at 4⁰C and 12,000 rpm. The supernatant was used as Cytoplasmic protein. The pellet was then dissociated with Nuclear and Cytoplasmic Protein Extraction buffer. After 30 min of hard vortex, the homogenates were centrifuged for 10 min at 4⁰C and 12,000 rpm. The supernatants were used as nuclear protein.

Western Blot

Western blot assay was used to measure protein levels. Briefly, after treatment, cells were lysed in RIPA buffer with Protease Inhibitor Cocktail. The concentration of total proteins was measured by the BCA assay (Pierce, Rockford, IL, USA), and 20 µg proteins of each sample were separated by SDS-PAGE gel. Proteins were transferred to PVDF membranes (Millipore, Bedford, MA, USA) and probed with primary antibodies followed by incubation with an HRP conjugated secondary antibody. The primary and second antibody complexes were determined with the ECL Western blot kit (Pierce).

Real Time Quantitative polymerase chain reaction (RT-qPCR)

Total RNAs were isolated by using TRIzol (Invitrogen) and Reverse transcription was performed with 2 µg of total RNA using by gDNA Erase and PrimeScript RT reagent kits (TAKARA Biotechnology, Dalian, China) following with the manufacturer's instructions, respectively. SYBR Green PCR master mix was employed for mRNA quantification. GAPDH was used as a control gene. The primer sequences are as follows: SETD1A: forward, 5'-TTGCCATGTCAGGTCCAAAAA-3', reverse, 5'-CGTACTTACGGCACATATCCTTC-3'; CYR61: forward, 5'-GATCTGCAGAGCTCAGTCAG-3', reverse, 5'-GCACTGCCCGGTAACCTTTGA-3'; CTGF: forward, 5'-TGCCCTCGCGGCTTACCGAC-3', reverse, 5'-TGCAGGAGGCGTTGTCATTG-3'; β-actin: forward, 5'-AGCGAGCATCCCCAAAGTT-3', reverse, 5'-GGGCACGAAGGCTCATCATT-3'.

Establishment of stable SETD1A-knockdown cell lines

Two special SETD1A short hairpin RNAs (shRNAs) were generated by inserting human SETD1A specific targeting sequences shSETD1A-1, 5'-GGAAAGAGCCATCGGAAATTT-3'; shSETD1A-2, 5'-GACAACAACGAATGAAATATT-3' into pU6-puro vector plasmid. HEK293T cells were transfected with lentivirus constructs using PolyJet transfection reagent (SigmaGen Laboratories, Ljamsville, MD, USA) following the manufacturer's instructions. Lentiviral supernatants were harvested during the 48–72-h after transfection and centrifuged at 2,500 rpm for 30 min to remove contaminating cells. SMMC-7721 and HLE were transfected with the viral supernatant in the presence of 10 µg/ml of polybrene (Sigma-Aldrich). Puromycin (1 µg/ml) was used to select stable SETD1A-knockdown cell lines. The knockdown efficiency was confirmed by Western blot.

CCK-8 (Cell Counting Kit-8) assay

CCK-8 assays were used to detect cell growth. A total of 2000 cells of each well were plated into 96-well plates. After treatment, CCK-8 (10 μ l) was added into each well. The plates were incubated at 37°C for 1 h. OD values at 450 nm wavelength of 450 nm were measured.

Clone formation assay

Control and SETD1A knockdown SMMC-7721 and HLE cells (500 cells per well) were plated onto 6-well plates overnight and then treated with sorafenib (10 μ M) for another 2 weeks. The culture medium was changed every 3 days and sorafenib treatment was maintained. Cells were fixed and stained with 0.1% crystal violet.

Cell death assay

Control and SETD1A knockdown SMMC-7721 and HLE cells were plated onto 6-well plates overnight and then treated with sorafenib (10 μ M) for 24 h. Cell death was determined by PI and Trypan Blue staining. For PI staining, cells were harvested and resuspended in PBS with PI at 1 μ g/ml. Cell death was performed by flow cytometry. For Trypan Blue staining, 0.9 ml of cells were mixed together with 0.1 ml of 0.4% Trypan Blue and maintained for 5 min at room temperature. Cells were counted.

Statistical analysis

Data were shown as mean \pm SD of three or more independent experiments, and analyzed using GraphPad Prism 8 (GraphPad Software, San Diego, CA, USA). Statistical differences were analyzed by student t-test, two-way ANOVA and Pearson r, $p < 0.05$ was considered statistically significant.

Results

SETD1A expression is upregulated and associated with poor prognosis in HCC

To determine the function of SETD1A in HCC, we firstly detected the expression of SETD1A in HCC cell lines. The Western Blot and RT-qPCR results showed that SETD1A was significantly upregulated in HCC cell lines compared to normal human hepatocyte (Fig. 1A and B). Then, we analyzed the expression of SETD1A in HCC tumor and corresponding non-tumorous liver tissues obtained from TCGA and found the expression of SETD1A in 369 HCC specimens to be much higher than the 50 non-tumor liver tissues (Fig. 1C). Further analysis revealed the patients who had higher SETD1A expression were associated with poor prognosis with shorter overall survival (Fig. 1D). Although the survival of HCC patients who treated with sorafenib was not markedly different between the SETD1A high-expression and low-expression groups (Fig. 1E), the survival of HCC patients with low SETD1A expression was longer than those with high SETD1A expression (Fig. 1E). The above results suggested that SETD1A may play an essential role in the development and sorafenib primary resistance of HCC.

SETD1A expression is positively correlated with IC₅₀ of Sorafenib in HCC cell lines

To examine whether SETD1A involved in sorafenib primary resistance, we firstly detected sorafenib IC₅₀ of HCC cell lines including SMMC-7721, SK-HEP-1, HLE, HepG2, and Hep3B by treating them with different concentration of sorafenib (0, 1, 5, 10, 50 and 100 μM) for 24 h, and then the cell growth was detected by CCK-8 assay and the IC₅₀ values of each cells were calculated. The IC₅₀ values varied among these different HCC cell lines (Fig. 2A). Furthermore, we determined the correlation between SETD1A expression and sorafenib IC₅₀ among HCC cell lines via Pearson r correlation analysis. Interestingly, we found that the lower SETD1A expressed, the lower IC₅₀ values of the corresponding HCC cell line. There was a markedly positive correlation between IC₅₀ values and SETD1A expression levels (Fig. 2B). Taken together, it stood a good chance that SETD1A was greatly involved in sorafenib primary resistance.

SETD1A knockdown augments sorafenib-induced proliferation inhibition

In order to determinate the function of SETD1A in sorafenib treated HCC cells, we constructed SETD1A knockdown HCC cell lines and then the cells were treated with sorafenib (100 μM) for 24 h. CCK-8 assay was employed to examine the cell growth. The results revealed that knockdown of SETD1A augmented sorafenib-induced cancer cell proliferation inhibition in SMMC-7721 (Fig. 3A) and HLE (Fig. 3B), with higher inhibitory rates of sorafenib to DMSO treatment in SETD1A knockdown groups compared to control group. Next, the clone formation assay results showed that the clone formation abilities of SETD1A knockdown cells were markedly lower than control cells both with or without sorafenib treatment in SMMC-7721 (Fig. 3C) and HLE (Fig. 3D), with higher inhibitory rates of sorafenib to DMSO treatment in SETD1A knockdown groups compared to control group. Furthermore, knockdown of SETD1A significantly reduced the cell cycle checkpoint protein Cyclin D1 and proliferation marker protein PCNA in SMMC-7721 (Fig. 3E) and HLE (Fig. 3F). These data indicated that knockdown of SETD1A enhanced sorafenib-induced liver cancer cell proliferation inhibition.

SETD1A knockdown augments sorafenib-induced cell death

It had been reported that sorafenib can induce cell death, we detected whether SETD1A suppressed sorafenib-induced HCC cell death [18, 19]. Control and SETD1A knockdown HCC cell lines were treated with sorafenib (100 μM) for 24 h. Trypan blue and PI staining were performed to determine cell death. The Trypan blue staining results revealed that knockdown of SETD1A enhanced sorafenib-induced cancer cell death in SMMC-7721 (Fig. 4A) and HLE (Fig. 4B). Next, these findings were confirmed by the PI staining, SETD1A knockdown markedly increased PI positive cells both with or without sorafenib treatment in SMMC-7721 (Fig. 4C) and HLE (Fig. 4D). Furthermore, knockdown of SETD1A markedly increased the expression of the cleaved caspase 3 and PARP in SMMC-7721 (Fig. 4E) and HLE (Fig. 4F). These results indicated that knockdown of SETD1A enhanced sorafenib-induced liver cancer cell death.

YAP activation contributes to SETD1A mediated sorafenib primary resistance

Previous reports had demonstrated that YAP aberrant activation contributed to sorafenib resistance [20-22]. The phosphorylation of YAP at S127 was reported to prevent YAP translocating to nuclear and suppress YAP transactivation [23, 24]. So, we hypothesized that SETD1A augmented sorafenib resistance by decreasing YAP phosphorylation and downstream target genes expression. Compared with control cells, the phosphorylation of YAP in the cytoplasm was significantly increased in SETD1A knockdown SMMC-7721 (Fig. 5A) and HLE (Fig. 5B) cells, but the activated YAP in the nucleus was not changed in SETD1A knockdown SMMC-7721 (Fig. 5A) and HLE (Fig. 5B) cells. In addition, knockdown of SETD1A decreased the protein (Fig. 5A and B) and mRNA (Fig. 5C and D) levels of YAP downstream target genes CYR61 and CTGF in SMMC-7721 (Fig. 5A and C) and HLE (Fig. 5B and D) cells. Furthermore, we tested whether SETD1A induced sorafenib primary resistance dependent on YAP activation. Overexpression of YAP in SETD1A knockdown cells, and then treated with sorafenib (10 μ M) for 24 h. Cell proliferation and cell death were detected by CCK-8 and Trypan blue staining. As expected, overexpression of YAP reversed sorafenib sensitive induced by knockdown of SETD1A in SMMC-7721 (Fig. 5E and G) and HLE (Fig. 5F and H) cells. In addition, knockdown YAP in SETD1A overexpression cells, and then detected the effects of YAP blocked in SETD1A mediated sorafenib primary resistance. Agreed with the former results, knockdown of YAP reversed sorafenib resistance induced by overexpression of SETD1A in HepG2 (Fig. 5I and K) and Hep3B (Fig. 5J and L) cells. Taken together, these data suggested that knockdown of SETD1A increased the phosphorylation of YAP to inhibit YAP activation and YAP activation contributed to SETD1A mediated sorafenib primary resistance.

Discussion

Epigenetic alterations were employed as pivotal mediators of cancer development and drug resistance [25-29]. Histone lysine methylation, maintains by lysine methyltransferases and lysine demethylase, has been involved in both transcriptional repression (H3K9, H3K27 and H4K20) and activation (H3K4, H3K36 and H3K79) [13, 30]. Aberrant methylation of H3K4, which induces oncogene expression, contributes to numerous tumorigenesis [31-35]. As we all know, SETD1A belongs to SET Domain containing histone lysine methyltransferase that adds methyl on H3K4, which plays an important role in cancer development [36]. Previous reports showed that SETD1A was upregulated and promoted HCC development [11, 15]. However, the role of SETD1A in sorafenib primary resistance of HCC was still largely unclear. Here, we uncovered the function of SETD1A in sorafenib primary resistance to HCC. Our results showed that the SETD1A expression was highly positively correlated with IC₅₀ of sorafenib in HCC cells and knockdown of SETD1A augmented sorafenib-induced cell proliferation inhibition and cell death (Fig. 2, 3 and 4). It should be noted that the current study just demonstrated that inhibition of SETD1A augmented sorafenib-induced HCC cell proliferation inhibition and cell death in vitro. Future studies in vivo are needed to confirm our findings.

YAP, the core transcriptional coactivator of Hippo signaling pathway, induces the transcription of downstream target genes, such as CYR61, CTGF and so on, and plays an important part in cell proliferation, apoptosis, invasion and metastasis [37, 38]. Previous studies have also reported that YAP

signaling links to resistance to targeted, chemo-, and immunotherapies in numerous solid tumors [20, 39]. Tian-yi Zhou et al reported that YAP aberrant activation contributed to sorafenib resistance [21]. Agreed with this previous study, we uncovered that overexpression of YAP suppressed sorafenib-induced cell proliferation inhibition and cell death in SETD1A knockdown cells (Fig. 5E and F). A previous study had indicated SET1A mediated YAP K342 mono-methylation and block its nuclear translocation disrupting the binding of YAP to CRM1, resulting in lung and colorectal cancer cell proliferation and tumorigenesis [40]. Similarly, in the current study we also found that knockdown of SETD1A induced the S127 phosphorylation of YAP in the cytoplasm and reduced the expression of CYR61 and CTGF in HCC cells (Fig. 5A-D).

Conclusion

In the current study, we demonstrated that SETD1A acted as a novel initiator of sorafenib primary resistance with decreasing sorafenib-induced cell proliferation inhibition and cell death. Furthermore, YAP activity was enhanced by SETD1A and contributed to SETD1A-initiated sorafenib resistance. All these findings suggested that SETD1A was a potential target for overcoming sorafenib primary resistance in HCC.

Declarations

Acknowledgments

Not applicable.

Author contributions

Y Gu and H chai contributed to the study concepts, study design and manuscript edit and review. JG Wu and HJ Chai carried out the study design, most experiments, data acquisition and analysis, manuscript preparation. F Li and Y Xia performed some cellular experiments. All authors read and approved the final manuscript.

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Availability of data and materials

The datasets generated and analyzed during the current study are available from the corresponding author on reasonable request.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Conflict of interest

The authors declared no conflict of interest.

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Figures

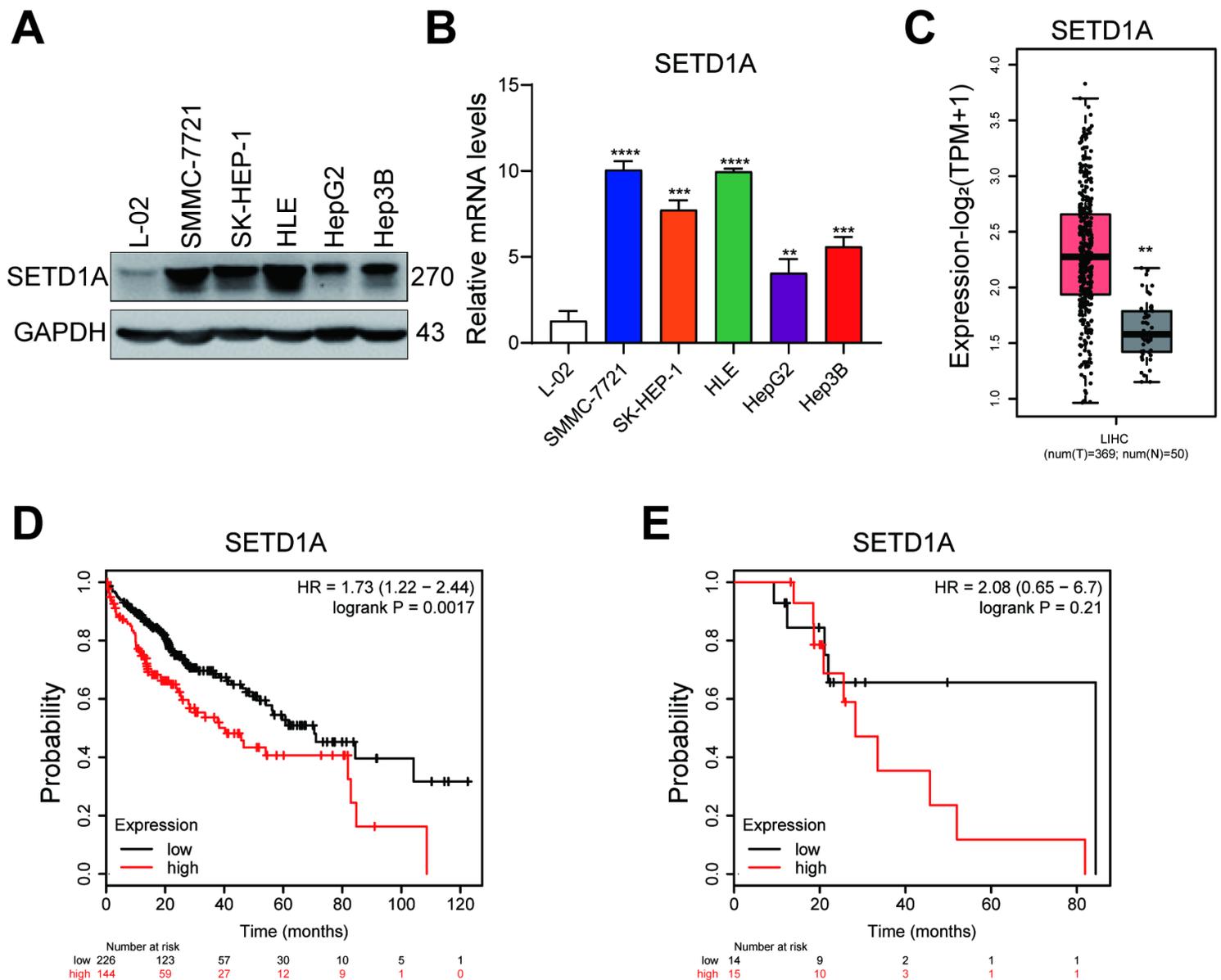
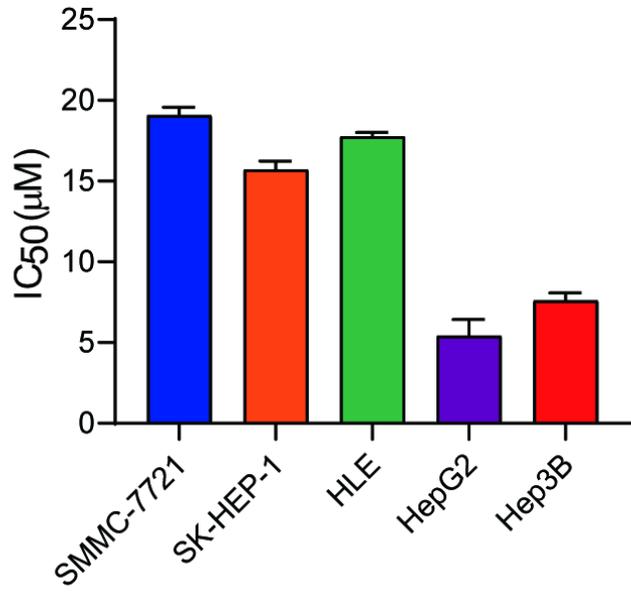
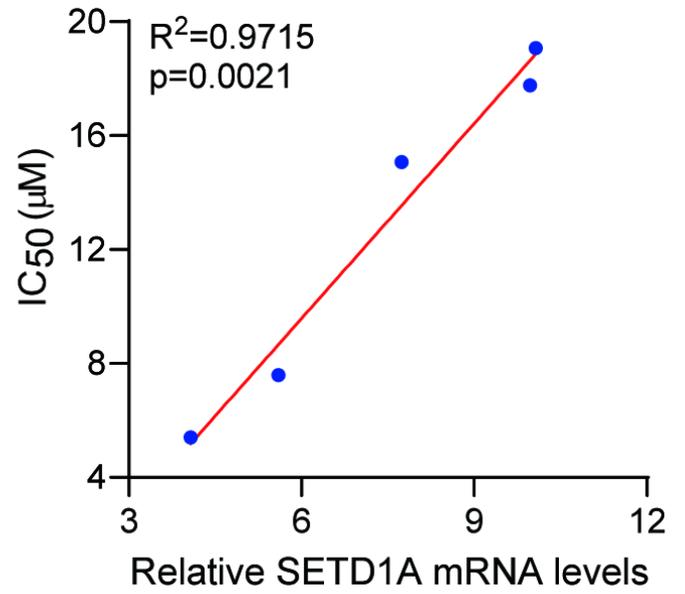


Figure 1

SETD1A expression is upregulated and associated with poor prognosis in HCC. (A) Western Blot and (B) RT-qPCR analysis showed SETD1A was upregulated in HCC cell lines compared with normal hepatocytes (n=3). (C) Data from the TCGA database showed a marked upregulation of SETD1A mRNA in HCC tissues compared with nontumorous liver tissues. (D-E) KM plotter analysis showed high SETD1A expression suffered shorter overall survival (D) and sorafenib treated survival (E). **p<0.01, ***p<0.001, ****p<0.0001.

A**B****Figure 2**

SETD1A expression was positively associated with sorafenib IC50 values of HCC cell lines. (A) IC50 values of sorafenib in different HCC cell lines (n=3). (B) Positive correlation between SETD1A expression and IC50 in HCC cell lines.

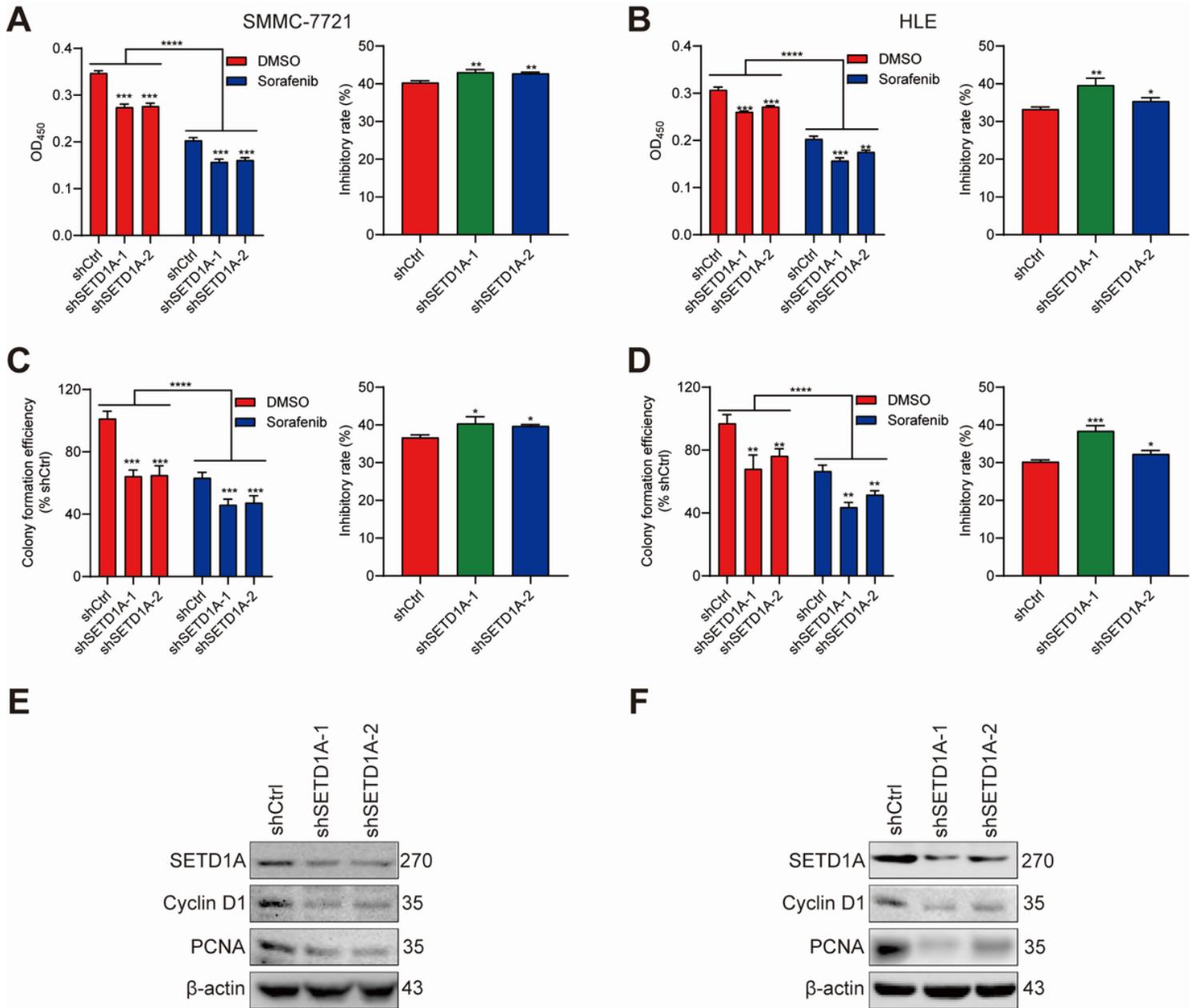


Figure 3

SETD1A knockdown augments sorafenib-induced proliferation inhibition. (A-B) Knockdown of SETD1A inhibited cell growth both with or without sorafenib treatment in SMMC-7721 (A) and HLE (B) cells (n=4). (C-D) Knockdown of SETD1A inhibited clone formation both with or without sorafenib treatment in SMMC-7721 (C) and HLE (D) cells (n=3). (E-F) Knockdown of SETD1A suppressed Cyclin D1 and PCNA expression in SMMC-7721 (E) and HLE (F) cells. **p<0.01, ***p<0.001, ****p<0.0001.

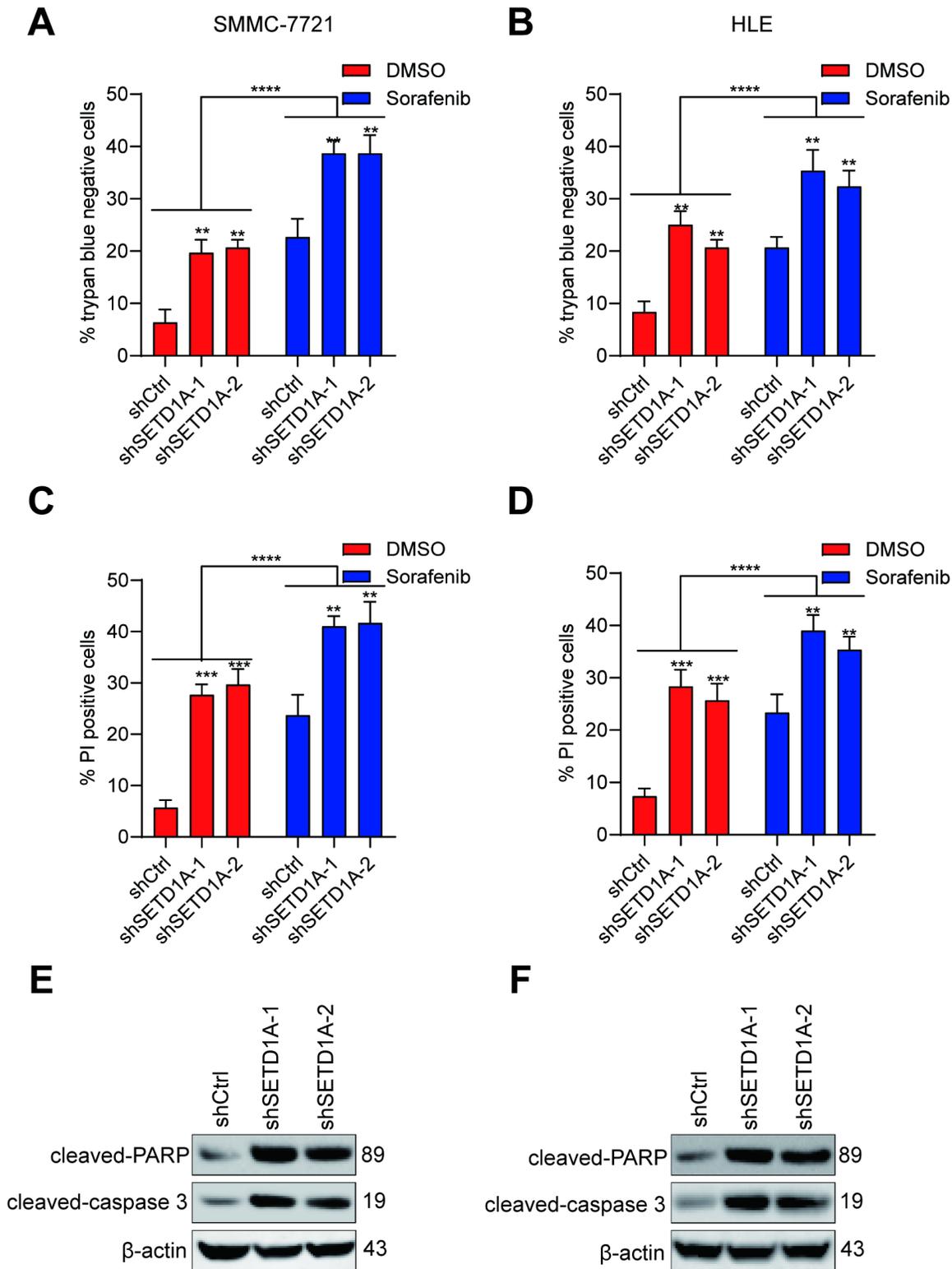


Figure 4

SETD1A knockdown augments sorafenib-induced cell death. (A-B) Knockdown of SETD1A increased trypan blue negative cell ratio both with or without sorafenib treatment in SMMC-7721 (A) and HLE (B) cells (n=4). (C-D) Knockdown of SETD1A increased PI positive cell ratio both with or without sorafenib treatment in SMMC-7721 (C) and HLE (D) cells (n=3). (E-F) Knockdown of SETD1A induced cleaved-PARP

and cleaved-caspase 3 expression in SMMC-7721 (E) and HLE (F) cells. ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

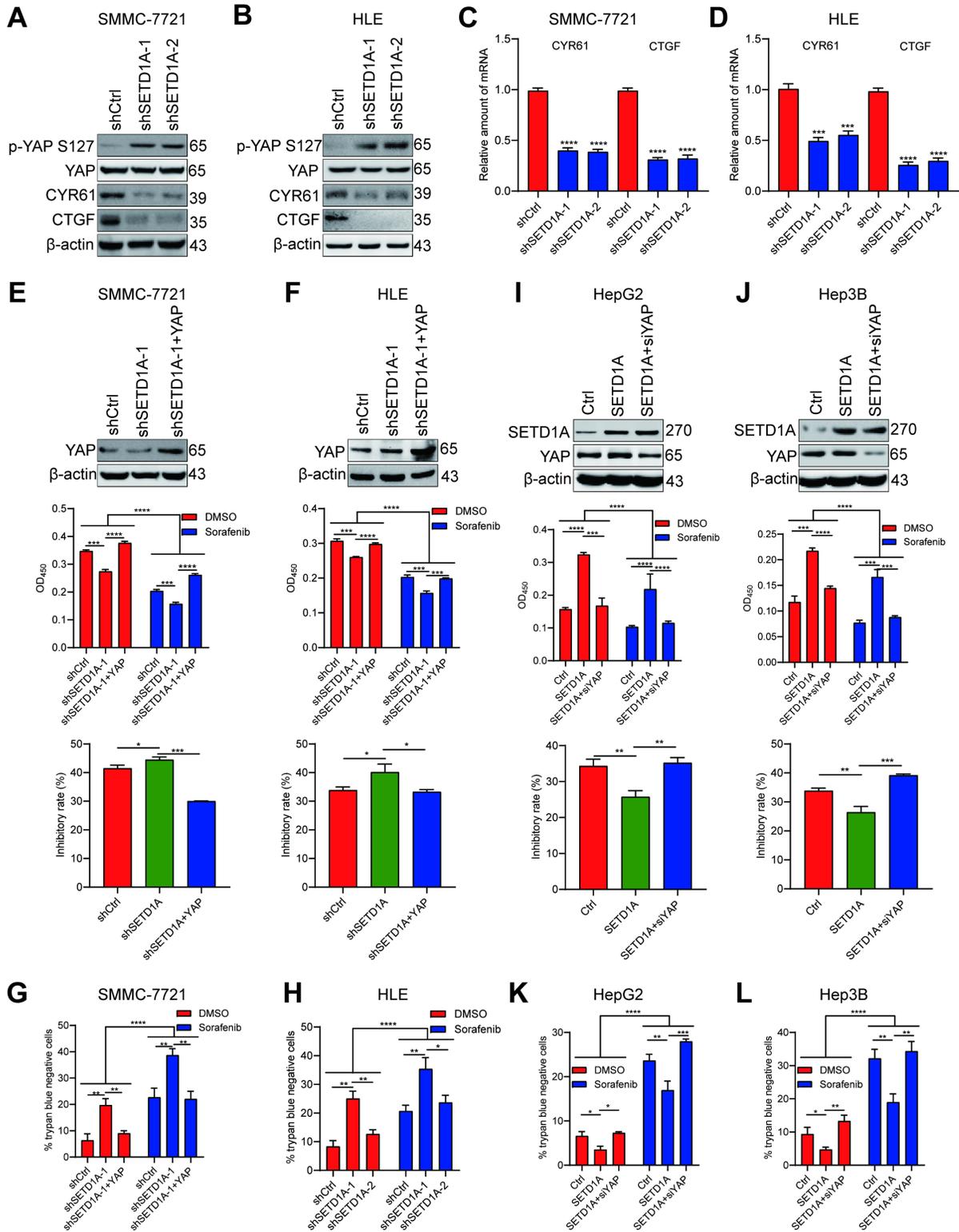


Figure 5

YAP activation contributes to SETD1A mediated sorafenib primary resistance. (A-B) Knockdown of SETD1A increased YAP phosphorylation and decreased the protein levels of CYR61 and CTGF in SMMC-7721 (A) and HLE (B) cells. (C-D) Knockdown of SETD1A decreased the mRNA levels of CYR61 and CTGF

in SMMC-7721 (C) and HLE (D) cells (n=3). (E-F) Overexpression of YAP in SETD1A knockdown cell reversed SETD1A knockdown induced cell proliferation inhibition both with or without sorafenib treatment in SMMC-7721 (E) and HLE (F) cells (n=3). (G-H) Overexpression of YAP in SETD1A knockdown cell reversed SETD1A knockdown induced cell death both with or without sorafenib treatment in SMMC-7721 (G) and HLE (H) cells (n=3). (I-J) Knockdown of YAP in SETD1A overexpression cell reversed SETD1A overexpression arrested cell proliferation inhibition both with or without sorafenib treatment in HepG2 (I) and Hep3B (J) cells (n=3). (K-L) Knockdown of YAP in SETD1A overexpression cell reversed SETD1A overexpression arrested cell death both with or without sorafenib treatment in HepG2 (K) and Hep3B (L) cells (n=3). *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.