# Accepted Manuscript


Targeting KDM1A attenuates Wnt/β-catenin signaling pathway to eliminate sorafenib-resistant stem-like cells in hepatocellular carcinoma

Mengxi Huang, Cheng Chen, Jian Geng, Dong Han, Tao Wang, Tao Xie, Liya Wang, Ye Wang, Chun hua Wang, Zengjie Lei, M.D, Ph.D, Xiaoyuan Chu, M.D., Ph.D

PII: S0304-3835(17)30220-3

DOI: 10.1016/j.canlet.2017.03.038

Reference: CAN 13302

To appear in: *Cancer Letters*

Received Date: 15 January 2017

Revised Date: 22 March 2017

Accepted Date: 28 March 2017

Please cite this article as: M. Huang, C. Chen, J. Geng, D. Han, T. Wang, T. Xie, L. Wang, Y. Wang, C.h. Wang, Z. Lei, X. Chu, Targeting KDM1A attenuates Wnt/β-catenin signaling pathway to eliminate sorafenib-resistant stem-like cells in hepatocellular carcinoma, *Cancer Letters* (2017), doi: 10.1016/j.canlet.2017.03.038.

# Targeting KDM1A attenuates Wnt/β-catenin signaling pathway to eliminate sorafenib-resistant stem-like cells in hepatocellular carcinoma

Mengxi Huang [1a], Cheng Chen [1a], Jian Geng[1], Dong Han[3],Tao Wang [2], Tao Xie [3], Liya Wang [1], Ye Wang [3], Chun hua Wang[4], Zengjie Lei [1]*, Xiaoyuan Chu [1]*

**Author affiliations:**

[1] Department of Medical Oncology, Jinling Hospital, School of Medicine, Nanjing University, Nanjing, Jiangsu Province, People's Republic of China

[2]Department of Gastroenterology, Institute of Surgery Research, Daping Hospital, Third Military Medical University, Chongqing, People's Republic of China

[3]Department of Medical Oncology, Jinling Hospital, Nanjing Clinical School of Southern Medical University, Nanjing, Jiangsu Province, People's Republic of China

[4]Gastroenterology Department, Bethune International Peace Hospital (Of The People's Liberation Army), Shijiazhuang City, Hebei Province, People's Republic of China.

***Correspondence: Zengjie Lei**, M.D., Ph.D. or **Xiaoyuan Chu**, M.D., Ph.D., Department of Medical Oncology, Jinling Hospital, School of Medicine, Nanjing University, Nanjing, Jiangsu Province,

China. Tel: +86-25-80860131; Fax: +86-25-80860131; E-mail:leizengjie@163.com

or chuxiaoyuan000@163.com

[a] These authors contributed equally to this study.

**Keywords:** sorafenib resistance, histone demethylation, KDM1A inhibitor, cancer stem cell, hepatocellular carcinoma

**Word count**: 3502 words (excluding highlights, abstract, references and figure legends). 6 Figures

**ABSTRACT**

Use of the tyrosine kinase inhibitor sorafenib in patients with advanced hepatocellular carcinoma (HCC) is often hindered by the development of resistance, which has been recently shown to be associated with the emergence of a cancer stem cell (CSC) subpopulation. However, it remains largely unknown whether epigenetic mechanisms, especially histone posttranslational modifications, are causally linked to the maintenance of stem-like properties in sorafenib-resistant HCC. In this study, we report that the activity of lysine-specific histone demethylase 1A (KDM1A or LSD1) is required for the emergence of cancer stem cells following prolonged sorafenib treatment. As such, KDM1A inhibitors, such as pargyline and GSK2879552, dramatically suppress stem-like properties of sorafenib-resistant HCC cells. Mechanistically, KDM1A inhibitors derepress the expression of multiple upstream negative regulators of the Wnt signaling pathway to downregulate the β-catenin pathway. More importantly, KDM1A inhibition resensitizes sorafenib-resistant HCC cells to sorafenib *in vivo*, at least in part through reducing a CSC pool, suggesting a promising opportunity for this therapeutic combination. Together, these findings suggest that KDM1A inhibitors may be utilized to alleviate acquired resistance to sorafenib, thus increasing the therapeutic efficacy of sorafenib in HCC patients.

## 1. Introduction

As a multiple tyrosine kinase inhibitor, sorafenib is a mainstream molecular targeted drug approved for the treatment of hepatocellular carcinoma (HCC), one of the most common and lethal malignancies worldwide [1-4]. However, the curative effect is hindered due to the frequent occurrence of drug resistance, especially in patients with advanced HCC [5]. Thus, elucidating the potential mechanisms underlying resistance to sorafenib is important for identifying novel agents to use in combination with sorafenib to reduce development of resistance and increase efficacy. Recently, multiple studies have revealed that cancer stem cells (CSCs) are responsible for tumor relapse and primary resistance to molecular targeted drugs[6]. Moreover, acquired resistance to molecular targeted therapy such as sorafenib could also be induced by the enrichment of self-renewing CSCs that leads to tumor progression and dissemination[2, 7-9]. Therefore, further understanding of the molecular mechanisms underlying the enrichment of stem-like properties in sorafenib treated HCC cells may be an important step in reducing and reversing sorafenib resistance.

Previous studies have identified multiple mechanisms of reduced sensitivity to sorafenib in HCC [2], including various molecular and signaling pathway changes, such as activation of the EGFR pathway [10], epithelial-mesenchymal transition (EMT) [11] and induction of autophagy [12, 13]. Although epigenetic abnormalities, which regulate gene expression without alterating DNA sequence [14], has been shown to be an important molecular mechanisms of drug resistance in molecular targeted therapy [15], it remains unclear how epigenetic aberrations are involved in sorafenib resistance.

Epigenetic modifications include posttranslational modifications to histones, DNA methylation and RNA editing [16]. There is accumulating evidence that histone modifications play an important role in many biological processes due to regulating expression of relevant genes. Aberrancies in histone modifications such as histone methylation and demethylation, is tightly associated with hepatocellular carcinoma tumorigenesis [17]. In this regard, we have recently identified that lysine-specific histone demethylase 1A (KDM1A), also known as lysine (K)-specific demethylase 1A (LSD1) [18], is a key histone demethylase responsible for the maintenance of self-renewal in liver cancer stem cells [19]. KDM1A demethylates H3K4me1/2 or H3K9me1/2 at target loci to maintain self-renewal of leukemic stem cells in acute myeloid leukemia (AML) [20, 21] and pluripotency of embryonic stem cells [22]. Here, we further investigate whether aberrancies in KDM1A activity are causally linked to the enrichment of CSCs in sorafenib-resistant HCC cells and its potential translational implications.

## 3. RESULTS

### 3.1 Sorafenib-resistant hepatoma cells acquire stem-like properties

To investigate the molecular mechanism of sorafenib resistance, we obtained sorafenib-resistant subclones from parental PLC and Huh7 HCC cell lines following a stepwise dose incremental protocol (Figure 1A). The resistant subclones were dramatically resistant to sorafenib compared to parental cells as measured by cell viability of parental and resistant cells treated with sorafenib for 24 hours, (Supplementary Figure 1A).

To assess if CSCs have a role in the development of sorafenib resistance in our model, we found that the relative mRNA and protein levels of several liver CSC markers, including Lgr5, Sox9, Nanog and CD90, were elevated in sorafenib-resistant subclones, compared to parental cells (Figure 1B and Supplementary Figure 1B). Moreover, compared to parental cells, the resistant subclones exhibited enhanced sphere formation capacity in stem cell medium, forming significantly more spheres with larger diameters (Figure 1C, D). Furthermore, the percentage of cells expressing Lgr5, a putative marker for liver CSCs, was increased in sorafenib-resistant subclones compared to parental cells (Figure 1E). Together, resistant cells were relatively insensitive to sorafenib and exhibited enhanced stem-like properties compared to parental cells, suggesting that stem-like properties are acquired during the development of sorafenib resistance in hepatocellular carcinoma.

### 3.2 Histone demethylase activity of KDM1A is critical for the induction of a stem-like population in sorafenib-resistant HCC cells.

Histone modifications play a significant role in the development of resistance to targeted therapy [23], we therefore examined whether changes in histone modifications are associated with acquired stem-like traits and sorafenib resistance. To this end, we assessed several histone methylation marks and the

expression levels of KDM1A and its homology KDM1B (LSD2) [24]. We found that H3K4me1 and H3K4me2, but not other histone methylation marks examined, were down-regulated in both PLC and Huh7 sorafenib-resistant cell lines compared to parental control cells (Figure 1F). Although both KDM1A and KDM1B have been reported to target H3K4me1 and H3K4me2 [23], the expression levels of KDM1A were increased in sorafenib-resistant cell lines with minimal change in the abundance of KDM1B (Figure 1F). Moreover, over-expression of KDM1A increased several liver CSC markers, including Lgr5, Sox9, Nanog and CD90 and reduced the sensitivity of sorafenib in HCC cell lines (Figure 1G, H). In contrast, depletion of KDM1A expression attenuated several liver CSC markers, including Lgr5, Sox9, Nanog and CD90 in sorafenib-resistant cell lines (Figure 2A), and shKDM1A cells from both sorafenib-resistant cell lines were more sensitive to sorafenib *in vitro* (Figure 2B). However, depletion of KDM1B expression did not affect sensitivity to sorafenib (Supplementary Figure 2A, B). These results suggest that KDM1A hyper-activation and resulting reduction in H3K4me1/2 levels may contribute to induction of sorafenib resistance in these stem-like cells.

The KDM1A protein contains a SWIRM domain and an amine oxidase domain with catalytic activity [24]. To further ascertain the role of KDM1A in sorafenib resistance, we introduced a lysine-to-alanine mutation at lysine 661 (K661A) in KDM1A (KDM1A-K661A) to disrupt its demethylase activity [24] and ectopically expressed KDM1A-WT or KDM1A-K661A in *KDM1A*-deplted HCC cells (Figure 2C). Depletion of endogenous KDM1A in the sorafenib-resistant cell lines led to reduced sphere formation in stem cell medium, forming significantly less spheres with smaller diameters, which could be efficiently rescued by re-introducing KDM1A-WT but not KDM1A-K661A (Figure 2D and Supplementary Figure 2C). Furthermore, depletion of endogenous KDM1A in the resultant sorafenib-resistant cell lines reduced the $Lgr5^+$ liver CSC population, whereas

ectopic expression of KDM1A-WT, but not KDM1A-K661A, maintained the $Lgr5^+$ liver CSCs subset (Figure 2E and Supplementary Figure 2E). In normal serum-containing medium, sorafenib-resistant cell lines expressing endogenous KDM1A or ectopic KDM1A-WT exhibited enhanced proliferative capacity, compared to *KDM1A*-depleted cells or cells expressing KDM1A-K661A (Figure 2F and Supplementary Figure 2D). These results demonstrate that the histone demethylase activity of KDM1A is required for the induction of a stem-like population during the development of sorafenib resistance in HCC cells.

### 3.3 Inhibiting histone demethylase activity of KDM1A attenuates the stemness of sorafenib-resistant cells.

To confirm a role for KDM1A in the maintenance of stemness in sorafenib-resistant cells, we used GSK2879552 [18] and pargyline [21, 25], two potent KDM1A inhibitors, to inhibit the histone demethylase activity of KDM1A. To this end, sorafenib-resistant cells treated with pargyline or GSK2879552 displayed reduced mRNA expression levels of stem cell markers, such as Lgr5, Sox9, Nanog and CD90, and elevated mRNA expression levels of differentiation markers Alb and Hnf4, while the hepatic stem/progenitor cell marker CK19 [26, 27] remained unchanged (Figure 3A, B). Consistently, the protein levels of these biomarkers exhibited similar expression patterns (Figure 3C, D). Remarkably, treatment with increasing concentrations of KDM1A inhibitors in sorafenib-resistant cells led to reduced sphere formation in stem cell medium, resulting in significantly less spheres with smaller diameters (Figure 3E-G and Supplementary Figure 3A). Moreover, treatment with KDM1A inhibitors in the sorafenib-resistant cell lines significantly reduced the $Lgr5^+$ liver CSCs population (Figure 3H; Supplementary Figure 3B, C, D). Thus, inhibition of KDM1A histone demethylase activity in sorafenib-resistant cells attenuates their stem-like properties.

### 3.4 KDM1A inhibitors derepress the transcription of Wnt antagonists and downregulate β-catenin signaling activity in sorafenib-resistant cells.

Previous studies have demonstrated that KDM1A demethylates histone H3K4Me1/2 at the promoters of multiple Wnt antagonists to promote β-catenin activity, thus it is conceivable that KDM1A inhibitors might exploit a similar molecular mechanism to inhibit stemness of sorafenib-resistant HCC cells [6]. To this end, we observed that the mRNA expression levels of three antagonists of the β-catenin signaling pathway, including Prickle1 [19, 28], APC [29, 30] and Sfrp5 [31] were upregulated in resistant cells treated with KDM1A inhibitors (Figure 4A, B and Supplementary Figure 4A, B). Moreover, we performed ChIP in PLC resistant cells treated with pargyline using antibodies against KDM1A, H3K4me1, H3K4me2 and H3K4me3. We found that pargyline treatment enriched H3K4me1 and H3K4me2 on the promoter of Prickle1, APC and Sfrp5 in sorafenib-resistant PLC cells (Figure 4C and Supplementary Figure 4C, D), suggesting that KDM1A inhibitors may enhance activating histone marks on the promoters of Wnt antagonists to promote their transcriptional activity.

Consistently, treatment with KDM1A inhibitors elevated protein expression levels of three antagonists of the β-catenin signaling, including Prickle1, APC and Sfrp5, thereby inactivating downstream β-catenin signaling in sorafenib-resistant cells (Figure 4D, E). Moreover, sorafenib-resistant PLC cells treated with pargyline exhibited relatively reduced TOP/FOP activity, compared to control cells treated DMSO (Figure 4F). The KDM1A inhibitors also reduced mRNA levels of β-catenin signaling pathway target genes c-Myc and Cyclin D1 in sorafenib-resistant PLC cells (Supplementary Figure 4E), suggesting attenuated activity of the β-catenin signaling pathway upon KDM1A inhibition. These results indicate that KDM1A inhibitors activate the expression of

multiple Wnt antagonists, Prickle1, APC and Sfrp5, and downregulate β-catenin signaling activity in sorafenib-resistant cells.

**3.5 Targeting KDM1A sensitizes resistant stem-like cells to sorafenib by suppressing Wnt/β-catenin signaling.**

Next, we assessed whether reduced β-catenin signaling activity underlies the physiological function of KDM1A inhibitors in suppressing stem-like properties of sorafenib-resistant cells. Sorafenib-resistant cells treated with KDM1A inhibitors were further incubated with CT99021[32], a GSK-3 inhibitor that stabilized the expression of β-catenin by suppressing its proteasomal degradation. By performing sphere formation assay, we found that similar to depleting *KDM1A*, KDM1A inhibitors exhibited inhibitory effect on sphere formation, reducing average sphere diameter and numbers of spheres (Figure 5A, B; Supplementary Figure 5A, B), while this effect of KDM1A inhibitors could be rescued by CT99021.

We next utilized CCK8 assays to assess if these inhibitor combinations could resensitize sorafenib-resistant cells to sorafenib treatment. To this end, KDM1A inhibitors treated in combination with sorafenib inhibited cell proliferation compared to cells treated with KDM1A inhibitor or sorafenib alone (Figure 5C, D and Supplementary Figure 5C, D). However, reactivating β-catenin signaling using the GSK-3 inhibitor CT99021 efficiently restored cell growth in the presence of both KDM1A inhibitor and sorafenib. Consistently, reduced colony formation of sorafenib-resistant cells due to combined treatment with KDM1A inhibitor and sorafenib was also rescued by addition of CT99021 (Figure 5E, F and Supplementary Figure 5E, F). These results demonstrate that KDM1A inhibition resensitizes resistant HCC cells to sorafenib which is, at least in part, through suppressing the Wnt/β-catenin signaling pathway.

### 3.6 Targeting KDM1A attenuates resistance to sorafenib *in vivo*.

To further validate the increased sensitivity to sorafenib by KDM1A inhibition, we examined the therapeutic efficacy of these agents on xenografts formed by sorafenib-resistant HCC cells. To this end, sorafenib-resistant HCC cells were injected subcutaneously into nude mice. After xenograft establishment, nude mice were treated with either pargyline or sorafenib alone, or with both pargyline and sorafenib in combination. We found that treatment with either pargyline or sorafenib alone could only minimally reduce the growth of subcutaneous xenografts in nude mice (Figure 6A, B). However, combined treatment with pargyline and sorafenib efficiently suppressed growth of sorafenib-resistant HCC cells (Figure 6A, B), suggesting that KDM1A inhibition increases the therapeutic efficacy of sorafenib on resistant cells *in vivo*.

Moreover, the $Lgr5^+$ liver CSC population was enriched in xenografts treated with sorafenib, whereas combined treatment with KDM1A inhibitor pargyline significantly reduced $Lgr5^+$ liver CSCs (Figure 6C) and sphere formation capacity (Supplementary Figure 6A). Consistently, analysis of tissue lysates revealed that protein expression levels of Wnt antagonists Prickle1, APC and Sfrp5 were unregulated, while β-catenin was reduced in xenografts treated with pargyline and sorafenib in combination, compared to sorafenib monotherapy (Figure 6D). Therefore, our results suggest that KDM1A inhibitors can resensitizes sorafenib-resistant HCC xenografts to sorafenib in part by suppressing Wnt/β-catenin signaling and eliminating stem-like cells *in vivo*.

## 4. Discussion

Sorafenib has been shown to provide longer survival in HCC patients, but most patients develop uncontrollable disease progression after treatment due to development of resistance to sorafenib [33]. In the present study, we demonstrates that the histone demethylase KDM1A is critical for the development of sorafenib resistance, suggesting it as a potential therapeutic target to increase sorafenib efficacy. Furthermore, we have identified a potential mechanism by which KDM1A inhibition suppresses Wnt/β-catenin signaling and attenuates the stemness of sorafenib-resistant HCC cells *in vitro* and *in vivo*.

The potential mechanisms of sorafenib resistance have been deeply investigated over several decades [34-36], including the impacts of CSCs that have been identified in many cancers, such as brain, lung, breast and liver [37, 38]. Self-renewal capacity, differentiating into heterogeneous tumor cells and drug resistance are all characteristics of CSCs. With the successful establishment of sorafenib-resistant cells, we provide evidence that sorafenib resistant cells express enhanced levels of Lgr5, Sox9, Nanog and CD90, which are key markers of liver CSCs. Furthermore, we identified that sorafenib-resistant cells had greater self-renewal capacity. We previously found that KDM1A was crucial for the stemness of liver CSCs by epigenetic modification, therefore we tested if KDM1A was involved in the maintenance of stemness in sorafenib-resistant cells through regulating histone methylation. We assessed key histone methylation marks along with the expression levels of KDM1A and KDM1B and observed that only H3K4me1/2, which are targets of KDM1A, were altered in sorafenib-resistant cell lines.

Importantly, the increased stemness and proliferation of sorafenib-resistant cells were dependent on the demethylase activity of KDM1A. Previous studies have shown that KDM1A is overexpressed in many human cancers [18, 39-45], and depletion of KDM1A may inhibit growth and metastasis of different tumors, such as acute myeloid leukemia (AML), small cell lung carcinoma (SCLC) and colon cancer. As a result, many KDM1A inhibitors have been developed clinically for AML and small cell lung carcinoma, such as GSK2879552[46]. It has been reported that treatment of SCLC cells with a KDM1A inhibitor led to increased expression of genes important for differentiation and development [18]. In another study, KDM1A inhibitor treatment reduced proliferation, migration, and invasion of NSCLC cells [46]. Additionally, the effects of KDM1A inhibitors on differentiation had also been discovered in adipogenic differentiation of hESCs [47]. However, the mechanisms by which KDM1A inhibitors function has remained elusive. Using the two established inhibitors of KDM1A, GSK2879552 and pargyline, we confirmed our observation that inhibition of KDM1A attenuates the stem-like properties of sorafenib resistant cells. This effect was similarly shown in glioblastoma, where KDM1A inhibitors reduced the stemness of glioma stem cells (GSCs) resulting in their differentiation and apoptosis [48].

In this study, our results suggests the potential mechanism by which KDM1A leads to sorafenib resistance through the regulation of key antagonists of the β-catenin signaling pathway and observed that KDM1A inhibitors upregulated their expression levels to downregulate Wnt/β-catenin signaling pathway, which is consistent with previous findings [49]. Furthermore, our results suggest that

KDM1A inhibitors may promote H3K4me1/2 methylation on the promoters of Prickle1, APC and Sfrp5 to upregulate their expression. And moreover, KDM1A inhibitors weakened stemness of sorafenib-resistant cells by suppressing β-catenin signaling.

Finally, our study indicates that KDM1A inhibitors may act to resensitize sorafenib-resistant tumors as treatment of sorafenib-resistant cells with KDM1A inhibitors had an inhibitory effect on sphere formation and cell proliferation *in vitro* and tumor formation *in vivo* when administered together with sorafenib. Moreover, we concluded that KDM1A inhibitors resensitizes sorafenib-resistant cells at least partially by suppressing β-catenin signaling and reducing their self-renewing capability. Our findings provide a more complete understanding of the mechanisms of sorafenib resistance and help to elucidate more effective drug therapeutic combinations for HCC patients. Taken together, we have demonstrated that KDM1A is required for stemness maintenance in sorafenib-resistant cells, and KDM1A inhibitors increase sensitivity to sorafenib by suppressing Wnt/β-catenin signaling pathway.

## 2. Materials and methods

### 2.1.Cell culture and cell lines

HCC cell lines (PLC/PRF/5 and Huh7) were purchased from the Shanghai Cell Collection (Shanghai, China) were maintained in DMEM medium (HyClone, USA) containing high glucose, 10% fetal bovine serum (FBS, HyClone, USA) and 1% antibiotic/antimycotic solution (Sigma) at 37°C in a humidified atmosphere containing 5% $CO_2$. And cells were cultured under different conditions in some experiments, including treated with sorafenib (LC laboratories, Boston, MA), GSK2879552, pargyline or CT99021 (Selleckchem, Houston, TX).

### 2.2.Development of sorafenib resistant cell lines

PLC and Huh7 sorafenib-resistant cell lines were selected based on constant exposure of the parental cells to sorafenib in a stepwise dose incremental strategy. Through increasing selection pressure in liquid culture, the surviving cells which became resistant to sorafenib were picked and transferred to DMEM medium containing increasing concentrations of sorafenib. After establishment, these resistant cell lines were continuously cultured in the presence of sorafenib.

### 2.3.Cell viability assay

Viable cells were measured in Cell Counting Kit-8 (CCK8) assay (Dojindo, Japan). Briefly, cells were cultured in a 96-well plate overnight at a density of $5\times 10^3$ cells per well and treated with the indicated concentrations of sorafenib (0 uM, 40 uM or 80 uM) for 24 hours. Subsequently, the cells were incubated with 10 μl CCK8 for 60 mins at 37°C, 5% $CO_2$. The absorbance of optical density at 450 nm (A450) was determined with Varioskan Flash (Thermo Scientific, USA).

2.4.Tumorosphere formation assay

For tumorosphere cultures, the isolated cells at 200 cells/well were cultured in the 24-well plates in the DMEM/F12 medium (GIBCO) containing 20 ng/ml of EGF, FGF, 10 ng/mL of HGF (PeproTech), B27 supplement (Invitrogen, Grand Island, USA), and 4 μg/mL of insulin (Sigma-Aldrich) as well as 1% methyl cellulose (Sigma-Aldrich) for two weeks. The cells were exposed to fresh medium every 4 days. At least 20 tumorospheres were examined with a microscope to calculate the average diameters.

2.5.Quantitative reverse transcription PCR (qRT-PCR)

According to the manufacturer's protocol, total RNA was extracted with TRIzol reagent (Invitrogen, USA) and then treated with DNase. Using primeScriptTM RT kit (Takara, Japan), RNA was reverse transcribed into cDNA and the relative levels of mRNA transcripts were detected by real-time PCR (BioRad, Hercules, CA, USA). The fold change between target gene mRNA transcripts and control

β-actin were calculated and shown in the histogram. . Primer sequences can be found in Supplementary Table 2. All the experiments were performed in triplicate.

2.6.Flow cytometric analysis of Lgr5+ HCC cells

Cells were suspended in blocking solution (PBS – BSA 1% - FBS 10%) for 5 min at 4°C after trypsin digestion, and subsequently incubated with antibody after centrifugation for 20 min at 4°C. $Lgr5^{+}$ cells were indicated by FCA.

2.7.Western blotting

Total protein was extracted from treated cells using M-PER Mammalian Protein Extraction Reagent (Thermo Scientific, Waltham, MA). Samples were separated by sodium dodecyl sulfate/polyacrylamide gel electrophoresis (SDS-PAGE) and electro transferred onto a nitrocellulose membrane (Bio-Rad, Hercules, CA, USA). Membranes were blocked with 5% skim milk, and incubated with primary specific antibodies for 24 hours (see supplementary Table 1) and subsequently with HRP conjugated secondary antibodies, and detected with Immobilon Western Chemiluminescent HRP Substrate detection reagents (Millipore, Billerica, USA).

2.8.Chromatin immunoprecipitation (ChIP)

ChIP-qPCR assays were carried out on $2 \times 10^6$ cells per well which were cross-linked with 1% formaldehyde. Cells were lysed by SDS lysis buffer and sonicated to DNA fragments of lengths between 200 and 1,000 bp [19]. The DNA fragments were immunoprecipitated with antibodies against KDM1A, H3K4me2, H3K4me1 and H3K4me3. Next, the samples were analyzed using PCR assays with primers specific for the promoter of Prickle1. Input was collected for further analysis. Fold changes relative to input DNA were quantified. Primer set the KDM1A_peak_5254 of Prickle1 locus (forward: 5'-TGGGCTTGCTTTGAGGAT-3', reverse: 5'- CAGGTCACGCGATGTACTAAC -3'). The results were expressed as fold changes relative to input DNA.

2.9.Lentivirus production and cell infection

For KDM1A knockdown experiments, PLC and Huh7 sorafenib-resistant cells were transfected with doxycycline (Dox) inducible lentiviruses pLVT-shRNA-KDM1A and pLVT-scramble (SBO Medical Biotechnology, Shanghai, China). Sequences for shKDM1A: CCACCTGACAGTAAGGAAT. According to the manufacturer's instructions, the KDM1A-specific shRNA-expressing or control lentiviruses were incubated together with lentiviral transfection enhancer (Polybrene 5 μg/ml, Sigma-Aldrich). Cells were induced to express shRNAs with Dox (10 ng/mL, Sigma-Aldrich) after selection with puromycin for 3 days. We ectopically expressed KDM1A cells resistant to shRNA by a

similar strategy. In addition, KDM1A with mutation at K661A (lysine-to-alanine mutation) in the demethylase domain was generated by mutagenesis polymerase chain reaction [50].

### 2.10.TCF/LEF reporter assay

The activation of the Wnt/β-catenin was detected by TCF reporter luciferase assay. The cells were transfected with a TCF reporter vector (TOPflash) (Millipore, Billercia, MA, USA) or the renilla luciferase reporter vector (pRL-TK) (40:1), using Lipofectamine 2000 (Invitrogen). With a dual-luciferase reporter assay kit (BMG Labtech GmbH, Germany), TCF and renilla luciferase activities were measured 48 hours after transfection in the cell suspension. The relative TOP/FOP activity (%) was calculated to show the changes in activation of the Wnt/β-catenin.

### 2.11.Tumorigenicity in vivo

4-weeks-old male nude mice were injected with stable clones of cells subcutaneously. Tumor weights were measured weekly over a period of 6 weeks. Finally tumors were harvested and photographed.

### 2.12.Statistical analysis

Differences among experimental groups were evaluated by an ANOVA and Student's t test using SPSS software (version 17.0). A *p* value less than 0.05 was considered statistically significant.

## Conflict of Interest

The authors declare no conflict interest.

**Acknowledgments**

This work was supported by the grants from the National Natural Science Foundation of China (NSFC Nos.81572457, 81272394).

## References

[1] E.A. Kuczynski, C.R. Lee, S. Man, E. Chen, R.S. Kerbel, Effects of Sorafenib Dose on Acquired Reversible Resistance and Toxicity in Hepatocellular Carcinoma, Cancer research, 75 (2015) 2510-2519.

[2] J. Chen, R. Jin, J. Zhao, J. Liu, H. Ying, H. Yan, S. Zhou, Y. Liang, D. Huang, X. Liang, H. Yu, H. Lin, X. Cai, Potential molecular, cellular and microenvironmental mechanism of sorafenib resistance in hepatocellular carcinoma, Cancer letters, 367 (2015) 1-11.

[3] J. Lo, E.Y. Lau, R.H. Ching, B.Y. Cheng, M.K. Ma, I.O. Ng, T.K. Lee, Nuclear factor kappa B-mediated CD47 up-regulation promotes sorafenib resistance and its blockade synergizes the effect of sorafenib in hepatocellular carcinoma in mice, Hepatology, 62 (2015) 534-545.

[4] J. Ferlay, I. Soerjomataram, R. Dikshit, S. Eser, C. Mathers, M. Rebelo, D.M. Parkin, D. Forman, F. Bray, Cancer incidence and mortality worldwide: sources, methods and major patterns in GLOBOCAN 2012, International journal of cancer. Journal international du cancer, 136 (2015) E359-386.

[5] Q. Wu, Z. Yang, Y. Nie, Y. Shi, D. Fan, Multi-drug resistance in cancer chemotherapeutics: mechanisms and lab approaches, Cancer letters, 347 (2014) 159-166.

[6] Z.J. Lei, J. Wang, H.L. Xiao, Y. Guo, T. Wang, Q. Li, L. Liu, X. Luo, L.L. Fan, L. Lin, C.Y. Mao, S.N. Wang, Y.L. Wei, C.H. Lan, J. Jiang, X.J. Yang, P.D. Liu, D.F. Chen, B. Wang, Lysine-specific demethylase 1 promotes the stemness and chemoresistance of Lgr5(+) liver cancer initiating cells by suppressing negative regulators of beta-catenin signaling, Oncogene, 34 (2015) 3188-3198.

[7] J. Wang, Z.J. Lei, Y. Guo, T. Wang, Z.Y. Qin, H.L. Xiao, L.L. Fan, D.F. Chen, X.W. Bian, J. Liu, B. Wang, miRNA-regulated delivery of lincRNA-p21 suppresses beta-catenin signaling and tumorigenicity of colorectal cancer stem cells, Oncotarget, 6 (2015) 37852-37870.

[8] B. Wang, Q. Wang, Z. Wang, J. Jiang, S.C. Yu, Y.F. Ping, J. Yang, S.L. Xu, X.Z. Ye, C. Xu, L. Yang, C. Qian, J.M. Wang, Y.H. Cui, X. Zhang, X.W. Bian, Metastatic consequences of immune escape from NK cell cytotoxicity by human breast cancer stem cells, Cancer research, 74 (2014) 5746-5757.

[9] B. Wang, J. Liu, L.N. Ma, H.L. Xiao, Y.Z. Wang, Y. Li, Z. Wang, L. Fan, C. Lan, M. Yang, L. Hu, Y. Wei, X.W. Bian, D. Chen, J. Wang, Chimeric 5/35 adenovirus-mediated Dickkopf-1 overexpression suppressed tumorigenicity of CD44(+) gastric cancer cells via attenuating Wnt signaling, Journal of gastroenterology, 48 (2013) 798-808.

[10] A.X. Zhu, D.G. Duda, D.V. Sahani, R.K. Jain, HCC and angiogenesis: possible targets and future directions, Nature reviews. Clinical oncology, 8 (2011) 292-301.

[11] Y.D. Shaul, E. Freinkman, W.C. Comb, J.R. Cantor, W.L. Tam, P. Thiru, D. Kim, N. Kanarek, M.E. Pacold, W.W. Chen, B. Bierie, R. Possemato, F. Reinhardt, R.A. Weinberg, M.B. Yaffe, D.M. Sabatini, Dihydropyrimidine accumulation is required for the epithelial-mesenchymal transition, Cell, 158 (2014) 1094-1109.

[12] N. Prieto-Dominguez, R. Ordonez, A. Fernandez, A. Garcia-Palomo, J. Muntane, J. Gonzalez-Gallego, J.L. Mauriz, Modulation of Autophagy by Sorafenib: Effects on Treatment Response, Frontiers in pharmacology, 7 (2016) 151.

[13] S. Shimizu, T. Takehara, H. Hikita, T. Kodama, H. Tsunematsu, T. Miyagi, A. Hosui, H. Ishida, T. Tatsumi, T. Kanto, N. Hiramatsu, N. Fujita, T. Yoshimori, N. Hayashi, Inhibition of autophagy potentiates the antitumor effect of the multikinase inhibitor sorafenib in hepatocellular carcinoma, International journal of cancer. Journal international du cancer, 131 (2012) 548-557.
[14] J. Borley, R. Brown, Epigenetic mechanisms and therapeutic targets of chemotherapy resistance in epithelial ovarian cancer, Annals of medicine, 47 (2015) 359-369.
[15] M.A. Dawson, T. Kouzarides, Cancer epigenetics: from mechanism to therapy, Cell, 150 (2012) 12-27.
[16] E.L. Greer, Y. Shi, Histone methylation: a dynamic mark in health, disease and inheritance, Nature reviews. Genetics, 13 (2012) 343-357.
[17] H. Alam, B. Gu, M.G. Lee, Histone methylation modifiers in cellular signaling pathways, Cellular and molecular life sciences : CMLS, 72 (2015) 4577-4592.
[18] H.P. Mohammad, K.N. Smitheman, C.D. Kamat, D. Soong, K.E. Federowicz, G.S. Van Aller, J.L. Schneck, J.D. Carson, Y. Liu, M. Butticello, W.G. Bonnette, S.A. Gorman, Y. Degenhardt, Y. Bai, M.T. McCabe, M.B. Pappalardi, J. Kasparec, X. Tian, K.C. McNulty, M. Rouse, P. McDevitt, T. Ho, M. Crouthamel, T.K. Hart, N.O. Concha, C.F. McHugh, W.H. Miller, D. Dhanak, P.J. Tummino, C.L. Carpenter, N.W. Johnson, C.L. Hann, R.G. Kruger, A DNA Hypomethylation Signature Predicts Antitumor Activity of LSD1 Inhibitors in SCLC, Cancer cell, 28 (2015) 57-69.
[19] Z.J. Lei, J. Wang, H.L. Xiao, Y. Guo, T. Wang, Q. Li, L. Liu, X. Luo, L.L. Fan, L. Lin, C.Y. Mao, S.N. Wang, Y.L. Wei, C.H. Lan, J. Jiang, X.J. Yang, P.D. Liu, D.F. Chen, B. Wang, Lysine-specific demethylase 1 promotes the stemness and chemoresistance of Lgr5+ liver cancer initiating cells by suppressing negative regulators of β-catenin signaling, Oncogene, (2015).
[20] D.P. Mould, A.E. McGonagle, D.H. Wiseman, E.L. Williams, A.M. Jordan, Reversible inhibitors of LSD1 as therapeutic agents in acute myeloid leukemia: clinical significance and progress to date, Medicinal research reviews, 35 (2015) 586-618.
[21] W. Fiskus, S. Sharma, B. Shah, B.P. Portier, S.G. Devaraj, K. Liu, S.P. Iyer, D. Bearss, K.N. Bhalla, Highly effective combination of LSD1 (KDM1A) antagonist and pan-histone deacetylase inhibitor against human AML cells, Leukemia, 28 (2014) 2155-2164.
[22] N.A. Kearns, H. Pham, B. Tabak, R.M. Genga, N.J. Silverstein, M. Garber, R. Maehr, Functional annotation of native enhancers with a Cas9-histone demethylase fusion, Nature methods, 12 (2015) 401-403.
[23] T.A. Katz, S.N. Vasilatos, E. Harrington, S. Oesterreich, N.E. Davidson, Y. Huang, Inhibition of histone demethylase, LSD2 (KDM1B), attenuates DNA methylation and increases sensitivity to DNMT inhibitor-induced apoptosis in breast cancer cells, Breast cancer research and treatment, 146 (2014) 99-108.
[24] S.M. Kooistra, K. Helin, Molecular mechanisms and potential functions of histone demethylases, Nature reviews. Molecular cell biology, 13 (2012) 297-311.

[25] Y. Liang, J.L. Vogel, A. Narayanan, H. Peng, T.M. Kristie, Inhibition of the histone demethylase LSD1 blocks alpha-herpesvirus lytic replication and reactivation from latency, Nature medicine, 15 (2009) 1312-1317.
[26] X.R. Yang, Y. Xu, B. Yu, J. Zhou, S.J. Qiu, G.M. Shi, B.H. Zhang, W.Z. Wu, Y.H. Shi, B. Wu, G.H. Yang, Y. Ji, J. Fan, High expression levels of putative hepatic stem/progenitor cell biomarkers related to tumour angiogenesis and poor prognosis of hepatocellular carcinoma, Gut, 59 (2010) 953-962.
[27] H. Kim, G.H. Choi, D.C. Na, E.Y. Ahn, G.I. Kim, J.E. Lee, J.Y. Cho, J.E. Yoo, J.S. Choi, Y.N. Park, Human hepatocellular carcinomas with "Stemness"-related marker expression: keratin 19 expression and a poor prognosis, Hepatology, 54 (2011) 1707-1717.
[28] D. Landeira, H. Bagci, A.R. Malinowski, K.E. Brown, J. Soza-Ried, A. Feytout, Z. Webster, E. Ndjetehe, I. Cantone, H.G. Asenjo, N. Brockdorff, T. Carroll, M. Merkenschlager, A.G. Fisher, Jarid2 Coordinates Nanog Expression and PCP/Wnt Signaling Required for Efficient ESC Differentiation and Early Embryo Development, Cell reports, 12 (2015) 573-586.
[29] M. Masuda, Y. Uno, N. Ohbayashi, H. Ohata, A. Mimata, M. Kukimoto-Niino, H. Moriyama, S. Kashimoto, T. Inoue, N. Goto, K. Okamoto, M. Shirouzu, M. Sawa, T. Yamada, TNIK inhibition abrogates colorectal cancer stemness, Nature communications, 7 (2016) 12586.
[30] S.H. Choi, C. Estaras, J.J. Moresco, J.R. Yates, 3rd, K.A. Jones, alpha-Catenin interacts with APC to regulate beta-catenin proteolysis and transcriptional repression of Wnt target genes, Genes & development, 27 (2013) 2473-2488.
[31] H. Mori, T.C. Prestwich, M.A. Reid, K.A. Longo, I. Gerin, W.P. Cawthorn, V.S. Susulic, V. Krishnan, A. Greenfield, O.A. Macdougald, Secreted frizzled-related protein 5 suppresses adipocyte mitochondrial metabolism through WNT inhibition, The Journal of clinical investigation, 122 (2012) 2405-2416.
[32] Y. Fu, C. Huang, X. Xu, H. Gu, Y. Ye, C. Jiang, Z. Qiu, X. Xie, Direct reprogramming of mouse fibroblasts into cardiomyocytes with chemical cocktails, Cell research, 25 (2015) 1013-1024.
[33] F. Fornari, D. Pollutri, C. Patrizi, T. La Bella, S. Marinelli, A. Casadei Gardini, G. Marisi, M. Baron Toaldo, M. Baglioni, V. Salvatore, E. Callegari, M. Baldassarre, M. Galassi, C. Giovannini, M. Cescon, M. Ravaioli, M. Negrini, L. Bolondi, L. Gramantieri, In hepatocellular carcinoma miR-221 modulates Sorafenib resistance through inhibition of caspase-3 mediated apoptosis, Clinical cancer research : an official journal of the American Association for Cancer Research, (2017).
[34] X. Sun, X. Niu, R. Chen, W. He, D. Chen, R. Kang, D. Tang, Metallothionein-1G facilitates sorafenib resistance through inhibition of ferroptosis, Hepatology, 64 (2016) 488-500.
[35] F.Q. Wu, T. Fang, L.X. Yu, G.S. Lv, H.W. Lv, D. Liang, T. Li, C.Z. Wang, Y.X. Tan, J. Ding, Y. Chen, L. Tang, L.N. Guo, S.H. Tang, W. Yang, H.Y. Wang, ADRB2 signaling promotes HCC progression and sorafenib resistance by inhibiting autophagic degradation of HIF1alpha, Journal of hepatology, 65 (2016) 314-324.

[36] H.A. Chen, T.C. Kuo, C.F. Tseng, J.T. Ma, S.T. Yang, C.J. Yen, C.Y. Yang, S.Y. Sung, J.L. Su, ANGPTL1 Antagonizes MET Receptor Activity to Repress Sorafenib Resistance and Cancer Stemness in Hepatocellular Carcinoma Cells, Hepatology, (2016).
[37] J.E. Visvader, G.J. Lindeman, Cancer stem cells: current status and evolving complexities, Cell stem cell, 10 (2012) 717-728.
[38] S. Sekiya, A. Suzuki, Direct conversion of mouse fibroblasts to hepatocyte-like cells by defined factors, Nature, 475 (2011) 390-393.
[39] X. Zhang, F. Lu, J. Wang, F. Yin, Z. Xu, D. Qi, X. Wu, Y. Cao, W. Liang, Y. Liu, H. Sun, T. Ye, H. Zhang, Pluripotent stem cell protein Sox2 confers sensitivity to LSD1 inhibition in cancer cells, Cell reports, 5 (2013) 445-457.
[40] Y. Qin, W. Zhu, W. Xu, B. Zhang, S. Shi, S. Ji, J. Liu, J. Long, C. Liu, L. Liu, J. Xu, X. Yu, LSD1 sustains pancreatic cancer growth via maintaining HIF1alpha-dependent glycolytic process, Cancer letters, 347 (2014) 225-232.
[41] A. Sprussel, J.H. Schulte, S. Weber, M. Necke, K. Handschke, T. Thor, K.W. Pajtler, A. Schramm, K. Konig, L. Diehl, P. Mestdagh, J. Vandesompele, F. Speleman, H. Jastrow, L.C. Heukamp, R. Schule, U. Duhrsen, R. Buettner, A. Eggert, J.R. Gothert, Lysine-specific demethylase 1 restricts hematopoietic progenitor proliferation and is essential for terminal differentiation, Leukemia, 26 (2012) 2039-2051.
[42] T. Schenk, W.C. Chen, S. Gollner, L. Howell, L. Jin, K. Hebestreit, H.U. Klein, A.C. Popescu, A. Burnett, K. Mills, R.A. Casero, Jr., L. Marton, P. Woster, M.D. Minden, M. Dugas, J.C. Wang, J.E. Dick, C. Muller-Tidow, K. Petrie, A. Zelent, Inhibition of the LSD1 (KDM1A) demethylase reactivates the all-trans-retinoic acid differentiation pathway in acute myeloid leukemia, Nature medicine, 18 (2012) 605-611.
[43] D.P. Mould, A.E. McGonagle, D.H. Wiseman, E.L. Williams, A.M. Jordan, Reversible Inhibitors of LSD1 as Therapeutic Agents in Acute Myeloid Leukemia: Clinical Significance and Progress to Date, Medicinal research reviews, (2014).
[44] Y. Huang, E. Greene, T. Murray Stewart, A.C. Goodwin, S.B. Baylin, P.M. Woster, R.A. Casero, Jr., Inhibition of lysine-specific demethylase 1 by polyamine analogues results in reexpression of aberrantly silenced genes, Proceedings of the National Academy of Sciences of the United States of America, 104 (2007) 8023-8028.
[45] Y. Lu, N. Wajapeyee, M.S. Turker, P.M. Glazer, Silencing of the DNA mismatch repair gene MLH1 induced by hypoxic stress in a pathway dependent on the histone demethylase LSD1, Cell reports, 8 (2014) 501-513.
[46] L. Kong, P. Zhang, W. Li, Y. Yang, Y. Tian, X. Wang, S. Chen, Y. Yang, T. Huang, T. Zhao, L. Tang, B. Su, F. Li, X.S. Liu, F. Zhang, KDM1A promotes tumor cell invasion by silencing TIMP3 in non-small cell lung cancer cells, Oncotarget, (2016).
[47] Y. Xiong, E. Wang, Y. Huang, X. Guo, Y. Yu, Q. Du, X. Ding, Y. Sun, Inhibition of Lysine-Specific Demethylase-1 (LSD1/KDM1A) Promotes the Adipogenic Differentiation of hESCs Through H3K4 Methylation, Stem Cell Reviews and Reports, 12 (2016) 298-304.

[48] G.R. Sareddy, S. Viswanadhapalli, P. Surapaneni, T. Suzuki, A. Brenner, R.K. Vadlamudi, Novel KDM1A inhibitors induce differentiation and apoptosis of glioma stem cells via unfolded protein response pathway, Oncogene, (2016).
[49] X. Liu, Z. Li, Y. Song, R. Wang, L. Han, Q. Wang, K. Jiang, C. Kang, Q. Zhang, AURKA induces EMT by regulating histone modification through Wnt/beta-catenin and PI3K/Akt signaling pathway in gastric cancer, Oncotarget, (2016).
[50] M.G. Lee, C. Wynder, N. Cooch, R. Shiekhattar, An essential role for CoREST in nucleosomal histone 3 lysine 4 demethylation, Nature, 437 (2005) 432-435.

**Figure Legends**

**Figure 1. KDM1A demethylase activity is required for induction of a stem-like population in sorafenib-resistant cells.**

A. A schematic model depicting experimental protocol to establish sorafenib-resistant PLC and Huh7 cell lines in the present study.

B. Immunoblot analysis of stem cell markers in control or sorafenib-resistant cells. Representative data derived from parental and sorafenib-resistant PLC and Huh7 cell lines is shown.

C. Representative floating tumorspheres generated by parental or sorafenib-resistant cells in stem cell medium. Quantification of average sphere diameter is shown.

D. Quantification of spheres generated by parental or sorafenib-resistant PLC and Huh7 cells shown as numbers of spheres formed per 2,000 cells.

E. Flow cytometric analysis of the percentage of $Lgr5^+$ cells in parental and sorafenib-resistant PLC and Huh7 cell lines.

F. The levels of multiple histone methyl marks were examined by western blot analysis using whole cell lysates from parental and sorafenib-resistant cells.

G. The expression of stem cell markers examined by western blot analysis from lysates derived from control or ectopically expressing KMD1A sorafenib-resistant cells.

H. Cell viability of control or ectopically expressing KMD1A sorafenib-resistant cells.incubated with sorafenib for 24 hrs.

**Figure 2. KDM1A is critical for the induction of a stem-like population in sorafenib-resistant HCC cells.**

A. Western blot analysis of stem cell markers in control or shKDM1A expressing sorafenib resistant cells. Representative data derived from sorafenib-resistant PLC and Huh7 cell line are shown.

B. Cell viability of control or shKDM1A expressing sorafenib-resistant cells following treatment with sorafenib for 24 hrs.

C. A schematic diagram of domain structure of wild-type (WT) KDM1A and mutant KDM1A used in this study, highlighting the KDM1A-K661A (lysine-to-alanine) mutation deficient in histone demethylase activity. The expression levels of stably reintroduced KDM1A-WT or KDM1A-K661A in *KDM1A*-depleted sorafenib-resistant HCC cells were validated by immunoblot analysis.

D .Quantification of sphere formation of *KDM1A*-depleted sorafenib-resistant HCC cells stably expressing KDM1A-WT or KDM1A-K661A cultured in stem cell medium .

E. Flow cytometric analysis of the percentage of $Lgr5^{+}$ cells generated in *KDM1A*-depleted sorafenib-resistant HCC cells stably expressing KDM1A-WT or KDM1A-K661A cultured in stem cell medium.

F. Cell proliferation curves of *KDM1A*-depleted sorafenib-resistant HCC cells stably expressing KDM1A-WT or KDM1A-K661A cultured in serum-containing medium.

**Figure 3. Inhibition of KDM1A histone demethylase activity induces differentiation of sorafenib-resistant cells and attenuates stemness properties.**

A, B. qRT-PCR analysis of the relative mRNA levels of stem cell and differentiation markers in sorafenib-resistant cells treated with KDM1A inhibitors pargyline or GSK2879552 for indicated time.

C, D. Immunoblot analysis of the expression of stem cell and differentiation markers in sorafenib-resistant cells treated with KDM1A inhibitors pargyline or GSK2879552 for indicated time.

E, F. Quantitative analysis of the average sphere diameter of sorafenib-resistant cells treated the indicated concentration of KDM1A inhibitors pargyline or GSK2879552.

G. Quantification of spheres generated by sorafenib-resistant cells treated with indicated concentration of KDM1A inhibitor GSK2879552.

H. Flow cytometric analysis of the percentage of $Lgr5^+$ cells from sorafenib-resistant cell line treated with indicated concentration of KDM1A inhibitor GSK2879552.

**Figure 4. KDM1A inhibitors reactivate transcription of multiple Wnt repressors and downregulate β-catenin signaling pathway in sorafenib-resistant cells.**

A, B. qRT-PCR analysis of mRNA levels of Prickle1, APC, and Sfrp5 in sorafenib-resistant PLC cells incubated with or without KDM1A inhibitors, pargyline or GSK2879552.

C. The abundance of KDM1A and H3K4me1/2 on the promoter of Prickle1 in sorafenib-resistant PLC cells treated with or without pargyline determined by ChIP assay.

D, E. Immunoblot analysis of Prickle1, APC and Sfrp5 as well as H3K4me1, H3K4me2, a-β-catenin and t-β-catenin in sorafenib-resistant PLC cells treated with or without pargyline or GSK2879552.

F. Relative TOP/FOP activity (%) following KDM1A inhibition by paragyline reveals reduction in the activity of β-catenin signaling pathway in treated sorafenib-resistant PLC cells.

**Figure 5. Targeting KDM1A resensitizes resistant stem-like cells to sorafenib in part by suppressing Wnt/β-catenin signaling.**

A, B. PLC sorafenib-resistant cells incubated with GSK2879552, in the presence or absence of the Wnt activator CT99021, were assessed for sphere size and number.

C, D. Cell Counting Kit-8 assays to measure cell proliferation of PLC and Huh7 sorafenib-resistant cells treated with GSK2879552 and/or sorafenib, in the presence or absence of the Wnt activator CT99021.

E, F. Numbers of colonies formed by sorafenib-resistant stem-like PLC and Huh7 cells incubated with GSK2879552 and/or sorafenib, in the presence or absence of the Wnt activator CT99021.

**Figure 6. KDM1A inhibitor sensitizes resistant stem-like cells to sorafenib *in vivo*.**

A. The representative images of subcutaneous xenograft tumors formed by sorafenib-resistant PLC cells in nude mice treated with pargyline with or wthout sorafenib. Six weeks after implantation, the subcutaneous xenografts were dissected and shown.

B. Growth curves of subcutaneous xenografts formed by sorafenib-resistant PLC cells in nude mice treated with GSK2879552 with or without sorafenib.

C. Flow cytometric analysis of the percentage of $Lgr5^+$ cells in subcutaneous xenografts generated by sorafenib-resistant HCC cells in mice treated with a combination of KDM1A inhibitor pargyline and/or sorafenib.

D. Immunoblot analysis of the expression of Wnt/β-catenin signaling in xenografts formed by sorafenib-resistant PLC cells in nude mice treated with pargyline and/or sorafenib.

**Supplementary Figure 1**.

A. Cell viability of parental and sorafenib-resistant cells incubated with indicated concentrations of sorafenib for 24 hrs.

B. qRT-PCR analysis of the stem cell markers in control and sorafenib-resistant cells.

**Supplementary Figure 2**.

A. qRT-PCR and western blot analysis of *KDM1B* expression in sorafenib-resistant cells either uninfected, or the cells infected with shGFP, shKDM1B-a or shKDM1B-b.

B. Cell viability of control or shKDM1B expressing sorafenib-resistant cells treated with sorafenib for 24 hrs.

C. Average sphere diameter of sorafenib-resistant cells stably expressing exogenous KDM1A-WT or KDM1A-K661A.

D. Cell proliferation of sorafenib-resistant Huh7 cells using CCK8 assay.

E. Flow cytometric analysis of percentage of $Lgr5^{+}$ cells in sorafenib-resistant cells.

**Supplementary Figure 3.**

A. Quantification of spheres generated from sorafenib-resistant cells treated with indicated concentrations of KDM1A inhibitor pargyline.

B. Flow cytometric analysis of the percentage of $Lgr5^+$ cells from sorafenib-resistant cell line treated with indicated concentrations of KDM1A inhibitor pargyline.

C, D. Flow cytometric analysis of the percentage of $Lgr5^+$ cells from sorafenib-resistant cells treated with or without KDM1A inhibitors, pargyline or GSK2879552.

**Supplementary Figure 4.**

A, B. qRT-PCR analysis of mRNA levels of Prickle1, APC, and Sfrp5 in sorafenib-resistant Huh7 cells treated with or without KDM1A inhibitors, pargyline or GSK2879552.

C,D. The abundance of KDM1A and H3K4me1/2 on the promoter of APC and Sfrp5 in sorafenib-resistant Huh7 cells treated with or without pargyline determined by ChIP assay

E. qRT-PCR analysis of c-Myc and CyclinD1 in sorafenib-resistant PLC cells incubated with or without KDM1A inhibitors, pargyline or GSK2879552.

**Supplementary Figure 5.**

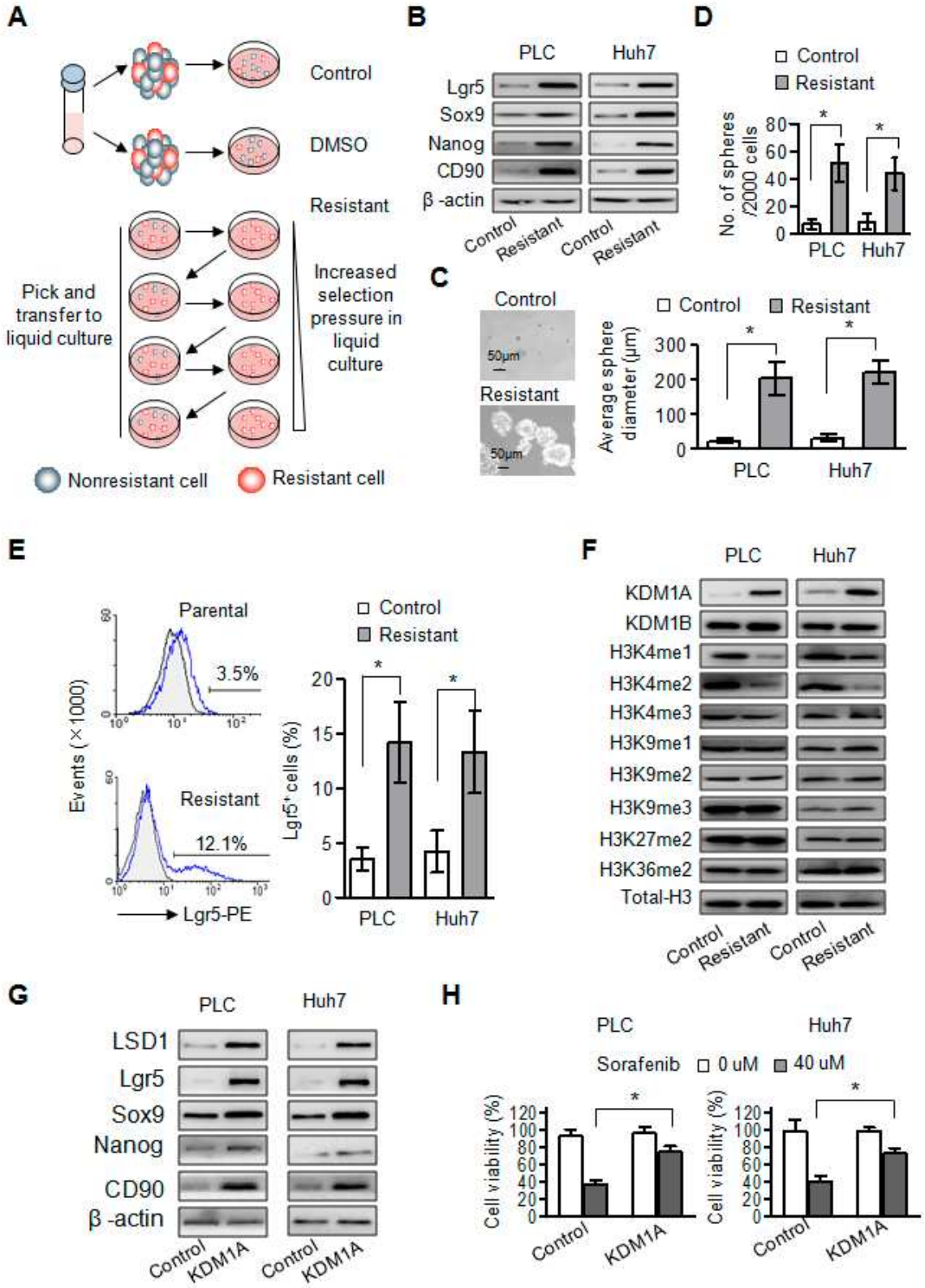
A, B. Huh7 sorafenib-resistant cells incubated with pargyline, in the presence or absence of CT99021, were assessed for sphere size and number.

C, D. Cell Counting Kit-8 assays to measure cell proliferation of PLC and Huh7 sorafenib-resistant cells treated with pargyline and/or sorafenib, in the presence or absence of CT99021.

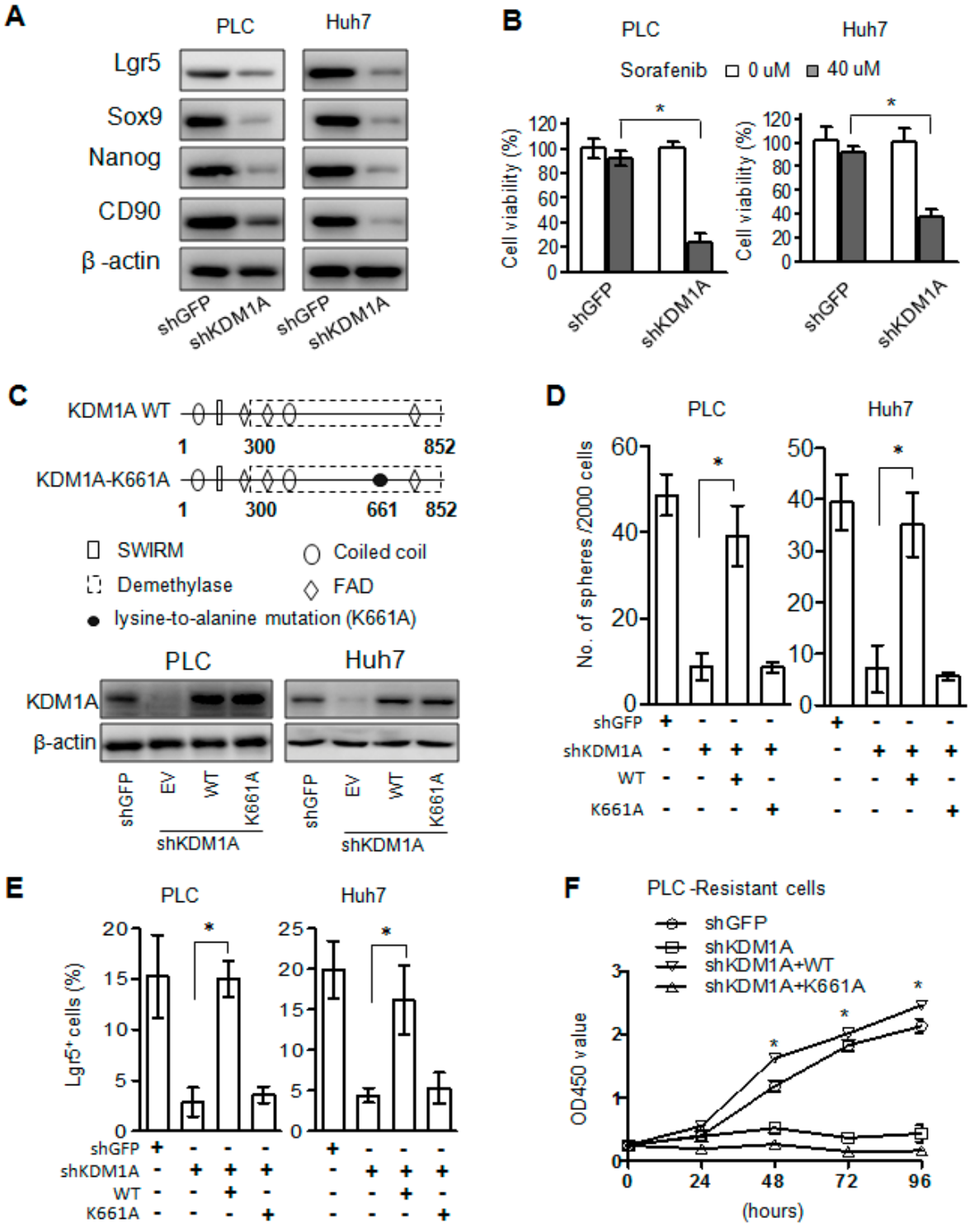
E. F. Representative images of colonies formed by sorafenib-resistant stem-like cells incubated with GSK2879552 and/or sorafenib, in the presence or absence of the Wnt activator CT99021.

**Supplementary Figure 6**

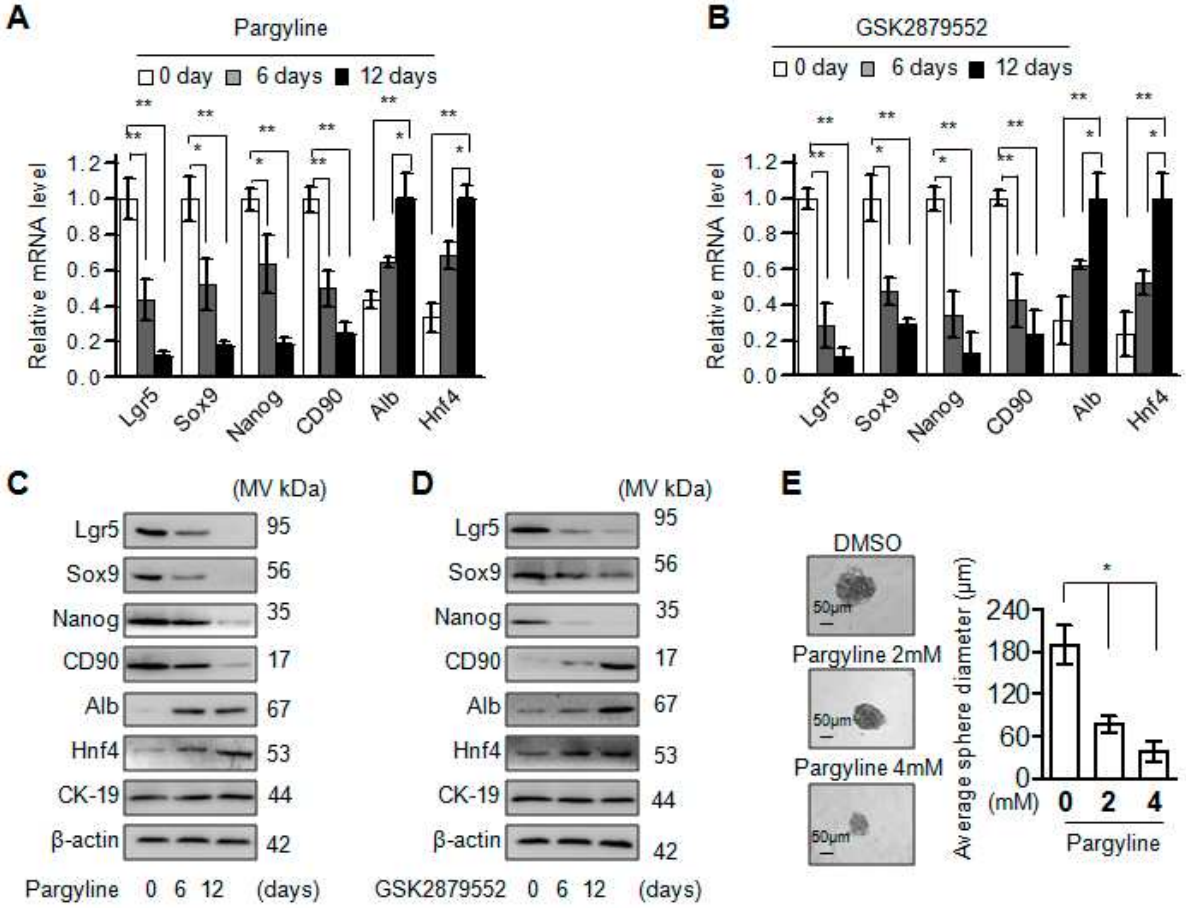
A. Quantification of spheres generated by sorafenib-resistant cells in subcutaneous xenografts generated by sorafenib-resistant HCC cells in mice treated with pargyline and/or sorafenib.



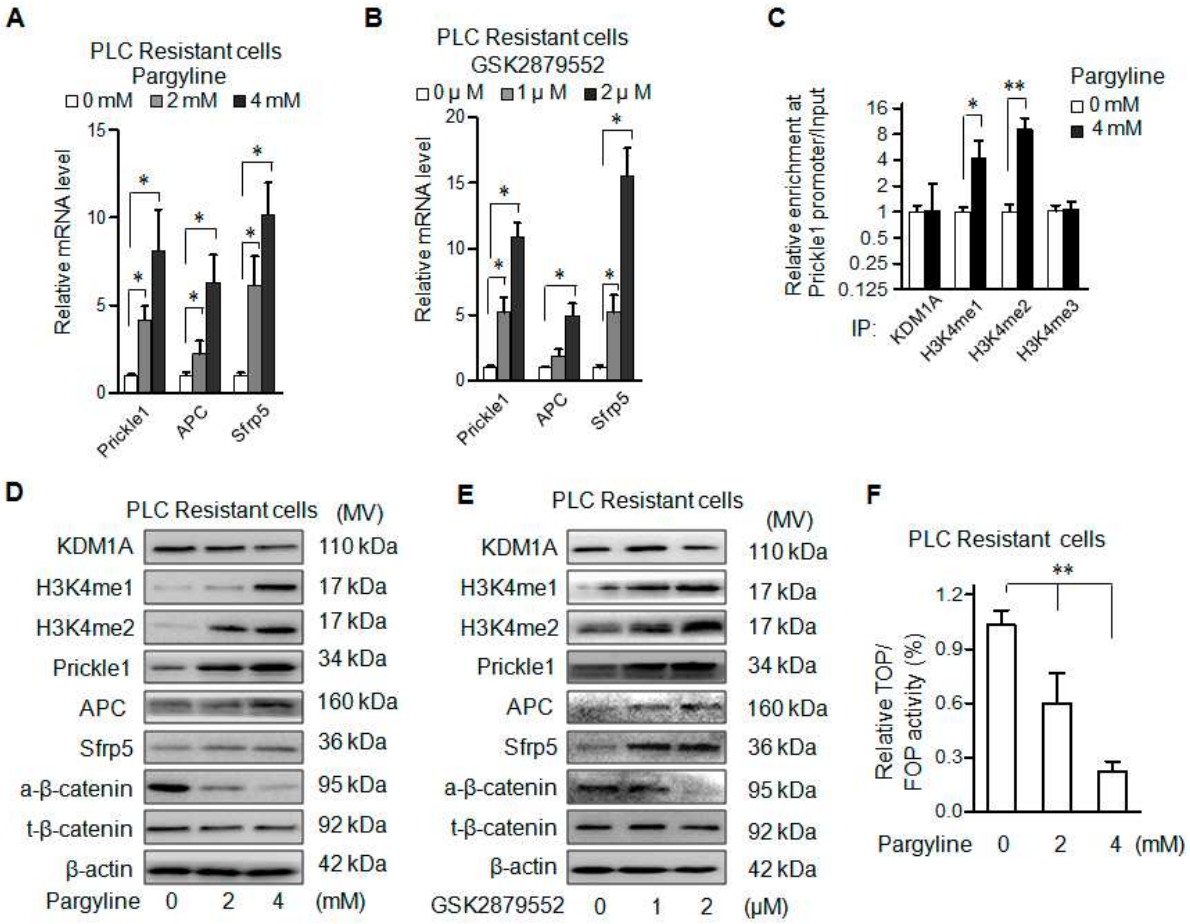

*Huang MX et al. Figure 1*



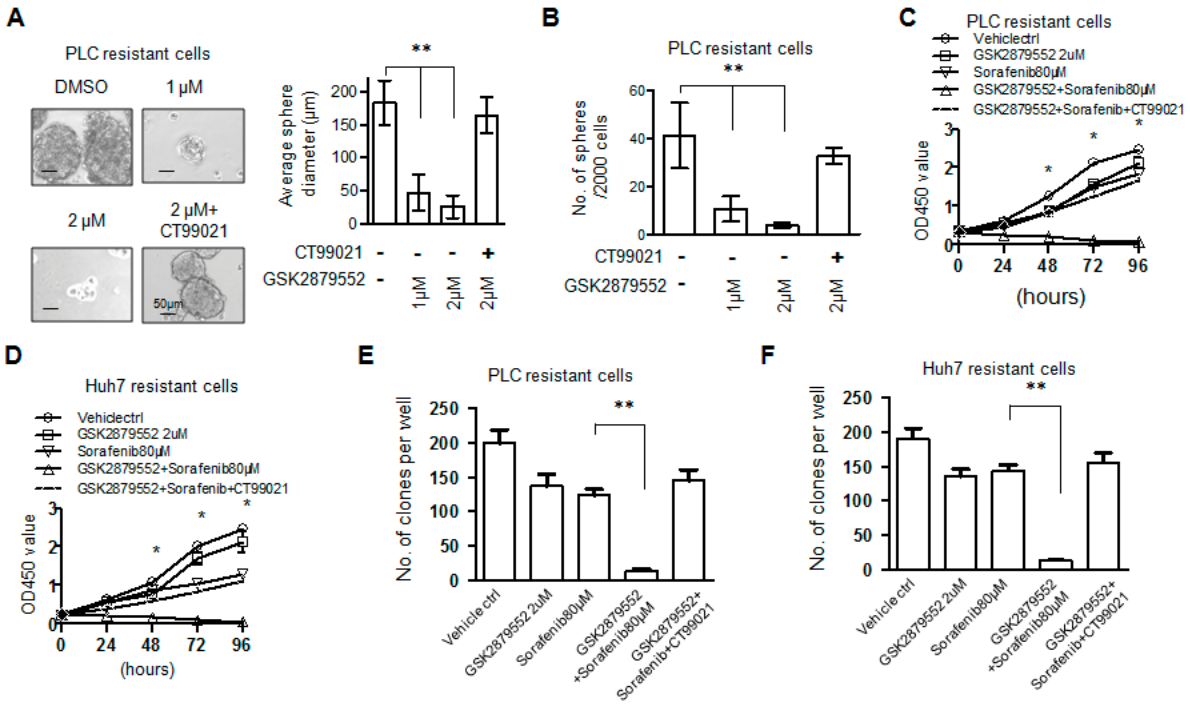

*Huang MX et al. Figure 2*



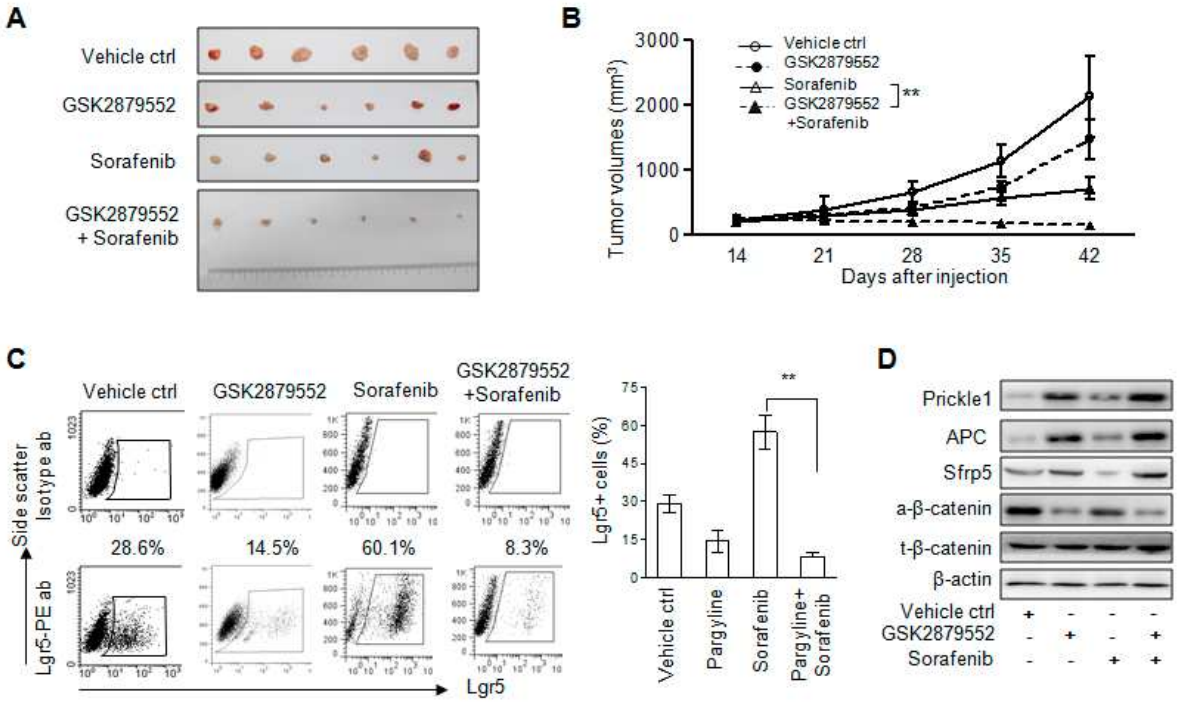

*Huang MX et al. Figure 3*




*Huang MX et al. Figure 4*




*Huang MX et al. Figure 5*




*Huang MX et al. Figure 6*

# Targeting KDM1A attenuates Wnt/β-catenin signaling pathway to eliminate sorafenib-resistant stem-like cells in hepatocellular carcinoma

Mengxi Huang [1a], Cheng Chen [1a], Tao Wang [2], Tao Xie [3], Liya Wang [1], Ye Wang [3],Dong Han[3], Jian Geng[1], Chun hua Wang[4], Zengjie Lei [1]*, Xiaoyuan Chu [1]*

## Highlights

1. The histone demethylase activity of KDM1A is critical for the acquired stem-like properties of sorafenib-resistant HCC cells.

2. KDM1A inhibitors de-repress multiple Wnt antagonists and down-regulate β-catenin signaling activity in sorafenib-resistant HCC cells.

3. Targeting KDM1A eliminates the stem-like cells and attenuates their resistance to sorafenib both *in vitro* and *in vivo*.