Co-targeting WIP1 and PARP induces synthetic lethality in hepatocellular carcinoma

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Research

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Abstract

Background

Hepatocellular carcinoma (HCC) is one of the most fatal cancers. Due to limited strategies for effective treatments, patients with advanced HCC have a very poor prognosis. This study aims to identify novel druggable candidate genes for patients with HCC.

Methods

The role of WIP1 (wild type p53 induced protein phosphatase1) in HCC was analyzed in HCC cells, nude mice assay, WIP1 knockout mice, and TCGA database. DNA damage was evaluated by Gene Set Enrichment Analysis, Western blotting, comet assay, and Immunofluorescence.

Results

High expression of WIP1 is associated with poor prognosis of patients with HCC. Genetically and chemically suppression of WIP1 drastically reduced HCC cell proliferation *in vitro* and *in vivo* via inducing DNA damage. WIP1 knockout retarded DEN (Diethylnitrosamine) induced mice hepato-carcinogenesis. In addition, suppression of WIP1 together with PARP inhibition induced synthetic lethality in HCC cells by disrupting DNA damage repair.

Conclusion

WIP1 plays an oncogenic effect in HCC development, and targeting WIP1-dependent DNA damage repair might be a novel strategy for HCC management.

Background

Liver cancer is one of the most common malignance worldwide[1]. Hepatocellular carcinoma (HCC) accounts for 85%-90% of liver cancer. HCC is an aggressive cancer associated with poor survival, frequent recurrence, and high incidence of metastases after surgical resection or chemotherapy[2]. Hence, understanding the molecular mechanisms of HCC pathogenesis is urgently needed to develop novel clinical strategies.

Wild type p53 induced protein phosphatase 1 (WIP1), also known as protein phosphatase magnesium-dependent 1δ (PPM1D), is a member of the PP2C family of Ser/Thr protein phosphatases[3]. It was found to dephosphorylate many proteins and thus implicated in various physio-pathology processes such as DNA damage, immunity, autophagy and so on[4–6]. The expression of WIP1 can be induced by a variety of stresses through p53, p38 MAPK[7], c-Jun[8] and NF-κB[9] pathways. For example, the expression of WIP1 can be up-regulated in a p53-dependent manner in response to ionizing radiation[3]. Interestingly, WIP1 overexpression would inactivate p53 to promote tumorigenesis via abrogating apoptosis and cell-cycle arrest. In addition, it can directly dephosphorylate many other proteins critical for cancer development

such as p53[10], mTOR[11], [12], H2AX[13], p38,[14,15], chk1[16], chk2[17], and UNG2[18]. Moreover, *PPM1D* amplification is found in several solid tumors including medulloblastoma[19], neuroblastoma[20], pancreatic adenocarcinoma[21], ovarian clear cell carcinoma[22] and breast cancer[11,23]. Several studies have found that WIP1 is significantly up-regulated in HCC[24,25]. Wang et al. reported that down-regulated microRNA-29c up-regulates its target gene *PPM1D* expression in HCC, and overexpression of microRNA-29c could decrease WIP1 expression and inhibit HCC cell proliferation[24]. However, the mechanism of WIP1 in HCC development is unclear.

In the current study, we identified that WIP1 depletion inhibited HCC development via increasing DNA damage. In addition, WIP1 inhibition in combination with PARP inhibitors induces HCC synergy lethal *in vitro* and *in vivo*. Therefore, targeting WIP1 dependent DNA damage repair might be a novel strategy for the clinical management of HCC.

Methods

Antibodies, Plasmids and Chemicals

Liver cancer cell lines were obtained from the Cell Bank of the Chinese Academy of Science (Shanghai, China). Cells were cultured in DMEM medium (Thermo Fisher Scientific, Shanghai, China) supplemented with 10% fetal bovine serum at 37℃ with 5% CO_2 and 95% humidity. The following antibodies were used for Western blotting: WIP1 (sc-376257) to detect human samples from Santa Cruz (Shanghai, China); WIP1 (A6204) to detect mouse samples from ABcolonal (Wuhan, China) ; cleaved PARP1(9541), cleaved-caspase3 (9661) and beta-Actin (4970) from Cell Signaling Technology (Shanghai, China); phospho-Histone H2ax (s139) (ab81299) from Abcam (Shanghai, China); ki67 (ER1802-31) from Huabio (Hangzhou, China). WIP1 plasmid was kindly provided by Prof. Zhenyu Ju at Hangzhou Normal University. GSK2830371, Diethylnitrosamine (DEN) and TCPOBOP were purchased from Sigma-Aldrich (Shanghai, China). Olaparib (HY-10162) and Veliparib (HY-10129) were purchased from MedChemExpress (Shanghai, China). Other reagents and chemicals don't list here are commonly commercial available.

siRNAs and Plasmids Transfection

siRNAs mentioned in this article were synthesized by Gene Pharma Company (Shanghai, China), and transfected into cells with Lipofectamine™ RNAiMAX transfection reagent (Thermo Fisher Scientific) at a final concentration of 20-50nM. All siRNAs sequences used were listed in Supplemental Table 1.

For plasmid transfection, cells were seeded overnight in 6 well plates, 2 μg of plasmids were transfected with X-tremeGENE HP DNA Transfection Reagent (Roche Applied Science, Shanghai, China). The mock vector was used as the negative control. Cells were harvested for indicated analysis after 48-72 hours later.

Lentivirus infection

To consistently knock down WIP1, cells were seeded overnight in 6-well plates and infected with lentivirus contain pLKO.1-scramble (shNC) or pLKO.1-shWIP1 (shWIP1). Stable cells were screened by puromycin. Knockdown of WIP1 was verified by Western blotting, and the constructed stable cells were sent out for cell proliferation *in vitro* and *in vivo* respectively. Sequences used were listed in Supplemental Table 2.

Cell growth assay

Cell growth assay was applied with the CellTiter 96® AQueous Non-Radioactive Cell Proliferation Assay kit (Promega, Beijing, China). Briefly, the cells were seeded into a 96-well plate overnight and treated as indicated, and the MTS reagents were added to each well. Cell viability was measured following the manufacturer's instruction. Samples were prepared in triplicates, and the cell viability was determined as the mean ± s.d.

Plate colony-formation assay

Stable knockdown cells were screened by puromycin before colony-formation assays with monolayer cultures. PLC/PRF/5 cells were seeded at 300 cells/well in 6 well plates. Hepa1-6 cells were seeded at 150 cells/well in 6 well plates. After 14 days of culture, cell colonies were counted after staining with 0.5% crystal violet.

Western blotting

HCC cells treated as indicated were harvested with Radio immunoprecitation assay (RIPA) buffer and then were quantitated by BCA protein assay kit (**Bio-Rad Laboratories,** Hercules, CA, USA). Lysates were resolved by SDS-PAGE, transferred to PVDF membrane and incubated with the primary antibodies at 4℃ overnight, then washed with TBS-T (TBS with 0.1% of Tween-20) and incubated with HRP-conjugated second antibodies (Jackson ImmunoResearvh Laboratories) at RT for 2 hours, after that, the membranes were test with FDbio- Femto ECL (FD8030, Fudebio, Hangzhou, China), and pictures were processed with Amersham Imager 600 system (GE Healthcare Life Sciences, Shanghai, China).

Immunohistochemistry (IHC) staining

Formalin-fixed and paraffin-embedded human liver tissue sections were first sent for hematoxylin-eosin (HE) staining and subsequently immunostained with anti-ki-67 antibody using microwave antigen retrieval in 0.01M pH6.0 citrate buffer. After washing, signal was detected using suitable HRP labeled second antibody with DAB as the chromagen (Dako, Denmark).

Immunofluorescence and microscopy

Cells were seeded on coverslips overnight and treated as indicated. Briefly, the cells were fixed with cold methanol for 10 min, permeabilized in 0.25% Triton X-100 for 10 min and blotted with 3% BSA (bovine serum albumin; diluted in PBS) for 30 min. The appropriate primary antibodies were diluted with 3% BSA and incubated with the cells at 4 °C overnight. Then, the coverslips were washed three times with 0.5%

PBS-T (PBS with 0.5% Tween-20), incubated with the appropriate secondary antibodies for 1 h at room temperature, washed and sealed with mounting medium including DAPI. Images were captured on microscope (Olympus, Japan).

Animal Studies

Den induced mice liver cancer model

Wip1 KO mice were kindly provided by Prof. Lawrence A. Donehower[26]. The mice were maintained and treated under specific pathogen-free conditions. To induce hepatocellular carcinogenesis, 100 mg/kg DEN was i.p. injected into 4-weeks-old male mice, and after 2 weeks, 3 mg/kg TCPOBOP (Sigma) was i.p. injected into the mice every other weeks for 8 times. Ten months after the DEN injection, mice were euthanized. The liver tissues were collected and divided, one half was immediately frozen in liquid nitrogen and stored at -80°C until sent for quantitative real-time RT-PCR and Western blot analysis, another half was fixed with 4% formaldehyde immediately and sent for HE staining. The numbers of liver tumor (diameter>2mm) of each mice was counted and student's t test was performed for statistical analysis.

Tumor-formation assay in C57BL/6J mice

Male C57BL/6J mice (6-8 weeks of age) were obtained from Shanghai Laboratory Animal Center and housed in the laboratory-animal research center of Zhejiang University. Cultures of Hepa1-6-shNC or Hepa1-6-ShWIP1 cells were resuspended with PBS. Mice were randomized divided into two groups (n=5/group), and 1×10^6 of cells were subcutaneous injected into each mouse. After 7 days, growth of implanted tumors was monitored using Vernier calipers every 2 days. Tumor volume (cm^3) = 0.5 × Tumor length× Tumor $width^2$. All mice were sacrificed after 17 days.

Tumor-formation assay in nude mice

Male BALB/c nude mice (n=56, 6-8 weeks of age) were obtained from Shanghai Laboratory Animal Center and housed in the laboratory-animal research center of Zhejiang University. PLC/PRF/5 cells were resuspended with PBS, and 3×10^6 cells were subcutaneous injected in to each mouse. After 7 days, mice were randomized divided into 4 groups (n=7) and oral treated with Blank/GSK2830371 (100mg/kg) /Olaparib (50mg/kg)/ GSK2830371（50mg/kg）+ Olaparib(25mg/kg) three times a week. For another experiment, 3×10^6 PLC/PRF/5 cells were subcutaneous injected in to each mouse. After 7 days, mice were randomized divided into 4 groups (n=7) and oral treated with Blank/GSK2830371 (100mg/kg) /Veliparib (100mg/kg)/ GSK2830371（50mg/kg）+ Veliparib(50mg/kg) three times a week. For each experiment, the growth of implanted tumors was monitored using Vernier calipers three times a week: Tumor volume (cm^3) = 0.5 × Tumor length × Tumor $width^2$. All mice were sacrificed after 23 days.

Bioinformatics analysis

To identify the association of WIP1 expression with DNA damage response, global gene expression profiles in paired human HCC tissues was obtained from GEO database (GSE57957), and was analyzed with Gene Set Enrichment Analysis(GSEA) using GSEA 3.0 software (http://www.broadinstitute.org/gsea/), the Gene Set of DNA double strand break response and Mismatch Repair from MsigDB was employed for GSEA[27]. And the survival analysis and correlation analysis were performed via GEPIA2.0 (http://gepia2.cancer-pku.cn/). The TMB data(Tumor mutation burden) of HCC patients was download from TCGA database, and was calculated via maftools R packge[28].

Statistical analysis

An independent Student's t test was performed to analyze the assay results. Pearson analysis was performed to analyze the correlation. *p* value < 0.05 was considered statistically significant. Results are expressed as mean ± SD as indicated. All experiments were repeated at least three times.

Results

High expression of WIP1 correlates with a poor prognosis in HCC

To determine whether WIP1 is associated with HCC development, we first compared WIP1 mRNA expression level in normal liver and hepatocellular carcinoma tissues. Analysis of multiple microarray data sets in the Oncomine (www.oncomine.org) confirmed that WIP1 mRNA was significantly increased in HCC tissues compared to normal liver tissues (Fig. 1A, $p < 0.01$). In addition, WIP1 mRNA expression in HCC tissues was significantly increased compared to corresponding paired noncancerous tissues (Fig. 1B, $p < 0.01$). Furthermore, both mRNA array analysis and RNAseq analysis from Cancer Cell Line Encyclopedia database[29] revealed high expression of WIP1 in liver cancer cell lines (Fig. 1C). Consistently, up-regulation of WIP1 protein was confirmed in most of human HCC tissues (Fig. 1D). Moreover, higher level of WIP1 mRNA was detected in patients with higher tumor degree (www.cbioprotal.org) (TCGA database, Fig. 1E). And up-regulated WIP1 was associated with shortened patient overall survival (OS) (Fig. 1F, HR = 1.5, Log rank p = 0.018). Collectively, these data suggested that WIP1 is up-regulated, and high expression of WIP1 correlates with a poor prognosis in human HCC.

Suppression of WIP1 inhibits proliferation of HCC cells in vitro

Next, we want to investigate the role of WIP1 in hepatocellular carcinogenesis. Firstly, knockdown of WIP1 could significantly inhibit HCC cell growth (Fig. 2A, and supplemental Fig. 1A). On the other hand, ectopic expression of WIP1 predominantly increased HCC cell growth (Fig. 2B, and supplemental Fig. 1B). As an allosteric inhibitor of WIP1, GSK2830371 interacts with a 'flap' subdomain located near the Wip1 catalytic site and thereby confers selectivity over other phosphatases[30]. Indeed, GSK2830371 inhibited cell proliferation as well (Fig. 2C). Similarly, WIP1 knockdown or treatment with GSK2830371 attenuated clone formation of HCC cells (Fig. 2D-E, supplemental Fig. 1C). In addition, suppression of WIP1 expression or activity could induce apoptosis in HCC cells (Fig. 2F-G, and supplemental Fig. 1D-F). In summary, suppression of WIP1 reduces cell proliferation and induces apoptosis in HCC cells.

WIP1 suppression inhibits HCC development*in vivo*

To further explore the relevance of WIP1 to HCC development *in vivo*, xenograft mice model was applied. Compared to stable PLC/PRF/5-shNC cells, the growth of shWIP1 cells-formed tumors were significantly impaired (Fig. 3A-3C). In addition, a widely used Diethylnitrosamine (DEN) induced hepatocellular carcinogenesis mice model was adopted to evaluate the role of WIP in HCC[31,32]. Firstly, we confirmed that WIP1 protein expression was upregulated in mouse liver cancer tissues compared with paired normal tissues (Fig. 3D). Furthermore, in consistence with *in vitro* results, WIP1 deficient (+/-) mice showed a significant decrease in the number of liver tumors compared to wild type mice (Fig. 3E-G, supplemental Fig. 2A-B). And WIP1 deficient decreased expression of Ki67 compared with controls (Fig. 3H). These data confirmed that suppression of WIP1 inhibits HCC development.

WIP1 inhibition disrupts DNA damage repair by increasing H2AX phosphorylation

To investigate how WIP1 promotes HCC development, we firstly analyzed the gene expression files from GSE57957 by gene sets enrichment analysis (GSEA). And we found that up-regulated WIP1 was associated with activated DNA double strand break response and mismatch repair signature (Fig. 4A). In fact, the high WIP1 mRNA level was positively correlated with the expression of DNA double strand break response and mismatch repair signature in TCGA LIHC database (supplemental Fig. 3A and 3B). Meanwhile, we confirmed that DNA double strand break response and mismatch repair signature were up-regulated in HCC tumor tissues compared to paired non-tumor tissues (supplemental Fig. 2C and 2D). And the up-regulated signature was associated with shortened overall survival (OS) of HCC patients (supplemental Fig. 3E and 3F). In addition, the up-regulated WIP1 mRNA expression was correlated with lower tumor mutation burden (TMB) in TCGA LIHC database (Fig. 4C). These results indicated that up-regulated WIP1 expression could enhance DNA damage repair to promote HCC development.

Previous studies had found that WIP1 play critical roles in the regulation of DNA damage repair through directly dephosphorylation of several DNA damage repair associated proteins including p53, H2AX[13], p38,[14,15], and chk1. As a result, the phosphorylation of H2AX was regulated by WIP1 in HCC cells. The level of phosphorylated H2AX (gamma-H2AX, γ-H2AX) was up-regulated in HCC cells with WIP1 knockdown or inhibition (Fig. 4C-D). And increasing DNA damage in HCC cells with WIP1 knockdown or inhibition was found via comet assay (Fig. 4E-F), which was further confirmed via immunofluorescence staining ofγ-H2AX foci in HCC cells with WIP1 knockdown or inhibition (Fig. 4G-H, and supplemental Fig. 4A-B). In summary, suppression of WIP1 could abrogate DNA damage repair in HCC cells.

WIP1 and PARP inhibition pronounced DNA damage

Inhibition of poly-(ADP-ribose) polymerase (PARP), a key enzyme in base excision repair, efficiently kills cancer cells with defective Homologous recombination (HR), which turned as synthetic lethal due to enhanced DNA damage[33]. Upon these findings, PARP inhibitors including olaparib are now clinically used for the treatment of BRCA1/2-deficient breast and ovary cancers [33]. Sinceγ-H2AX plays an important role

in HR, we want to know whether suppression of WIP1 together with PARP inhibition could be synthetic lethal in HCC cells. Indeed, combined treatment with PARP inhibitors (olaparib and veliparib) and WIP1 knockdown or inhibition increased the level ofγ-H2AX in HCC cells (Fig. 5A -D). And the increasing DNA damage was also confirmed via immunofluorescence staining ofγ-H2AX foci in HCC cells with WIP1 knockdown or inhibition combined with PARP inhibitors (Fig. 5E and 5F, and supplemental Fig. 5A and 5B). The above findings suggested that suppression of WIP1 synergy with PARP inhibition to enhance DNA damage.

WIP1 and PARP inhibition induce synthetic lethality in HCC both *in vitro* and *in vivo*.

According to the above findings, we explored the synthetic lethal effect of WIP1 and PARP inhibition in HCC. As expected, WIP1 knockdown increased the sensitivity of PARP inhibitors and promoted apoptosis in HCC cells (Fig. 6A–D, supplemental Fig. 6A-D). Consistently, WIP1 inhibition by GSK2830371 also increased the sensitivity of PARP inhibitors and promoted apoptosis in HCC cells (Fig. 6E–H, supplemental Fig. 6E). Furthermore, in line with *in vitro* findings, the combination of GSK2830371 with PARP inhibitors significantly retarded tumor growth in nude mice xenograft model (Fig. 7A-F). Compared to either single agent, more DNA damage, apoptosis, and proliferation attenuation were found after the combined treatment of GSK2830371 and PARP inhibitors (Fig. 7G-J). In summary, the combinational inhibition of WIP1 and PARP could induce synthetic lethality in HCC.

Discussion

Hepatocellular carcinoma (HCC) is one of the leading causes of cancer deaths worldwide. Understanding the molecular mechanisms of HCC pathogenesis is urgently needed to develop novel clinical strategies. Previous studies have found that WIP1 is up-regulated in HCC, and down-regulated microRNA-29c contributes to its target gene WIP1 high expression[24]. Consistently, we confirmed high expression of WIP1 in HCC, and further found that WIP1 high expression correlates with a poor prognosis in HCC patients (Fig. 1). Additionally, we found that suppression of WIP1 could remarkably inhibit HCC cell proliferation both *in vitro* and *in vivo* via increasing DNA damage(Fig. 2–4). And WIP1 deficiency significantly retarded DEN-induced hepato-carcinogenesis (Fig. 3). Thus, WIP1 might work as an oncoprotein in HCC.

As a phosphatase, WIP1 is implicated in DNA damage repair pathways by directly dephosphorylating several proteins including p53[7], H2AX[13], p38[14,15], chk1[16], and chk2[34]. Herein, we found that γ-H2AX, but not other DNA damage associated proteins, was increased in HCC cells after WIP1 inhibition (Fig. 4).

Genomic instability is one of the hallmarks of cancer cells, which is associated with a greater propensity to accumulate DNA damage[35]. Hence, DNA damage repair (DDR) signaling is usually pronounced to control the genome integrity in cancer cells. In the process of DNA damage repair, phosphorylated H2AX (γ-H2AX) play a crucial role in recruiting DNA damage repair factors such as BRCA1, MRE11/RAD50/NBS1 complex, and 53BP1 to repair damaged DNA[36]. Upon the completion of DNA damage repair, γ-H2AX need to be dephosphorylated and removed for checkpoint recovery[37]. Thus the

turnover ofγ-H2AX need to be precisely controlled during DNA damage repair process. Indeed, WIP1 acts as a checkpoint regulator that could dephosphorylate γ-H2AX directly and remove γ-H2AX from chromatin to disassociate the DNA damage repair complex, which promotes repaired cells to re-enter cell cycle arrest [38]. Recent studies revealed that clonal hematopoiesis with the gain of function mutations in WIP1 outcompeted their wild-type counterparts in *vivo* after exposure to DNA damage stress[39,40]. Therefore, WIP1 could function as a homeostatic regulator during DNA damage[41]. In line with these findings, our results indicated the expression of WIP1 had positive correlation with DNA damage repair signature in HCC. And suppression of WIP1 induced DNA damage and apoptosis in HCC cells via increasing γH2AX (Fig. 4).

The PARP family of enzymes covalently add Poly(ADP- ribose) (PAR) chains on to target proteins, termed PARylation, which has been found to be involved in chromatin modification, DNA damage repair, maintenance of telomeres and so on[42]. PARP1/2 are the best-studied PARP enzyme, PARP1/2 and BRCA inhibition induce cancer cell synthetic lethal via disturbing DNA damage repair[43]. And PARP inhibitors (PARPi) are now used for clinically treatment of BRCA1/2-deficient breast and ovary cancer[44,45]. Combinations of PARPi with other drugs are now being intensively investigated to prevent the development of resistance to PARPi and to extend their use beyond BRCA1/2-deficient tumors including HCC[46–48]. Interestingly, WIP1 suppression together with PARP inhibition induced synthetic lethality in HCC via enhancing DNA damage, and combination of WIP1 and PARP inhibition suppressed HCC cell proliferation significantly both *in vitro* and *in vivo* (Fig. 5–7). These results suggested that in WIP1 high expressed HCC, WIP1 inhibition might extend the PAPRi indication in future.

Conclusion

WIP1 plays an oncogenic role in HCC development via regulating DNA damage repair. Targeting WIP1 regulating DNA damage repair provide a novel strategy for HCC precise management (Fig. 7K).

Abbreviations

HCC: hepatocellular carcinoma; WIP1: wild-type p53-induced phosphatase 1; DEN: Diethylnitrosamine; RIPA: radio immunoprecitation assay; IHC: Immunohistochemistry; HE: hematoxylin-eosin; GSEA: Gene Set Enrichment Analysis; TMB: Tumor mutation burden; OS :overall survival; CCLE: Cancer Cell Line Encyclopedia; PI: propidium iodide; C-PARP1:Cleave-PARP1; C-Caspase3: Cleave-Caspase3; WT: WIP1 wild-type; LW/BW: liver weight/body weight ratio; γ-H2AX: phosphorylated H2AX at Ser139; PARP: poly-(ADP-ribose) polymerase; HR: Homologous recombination; DDR: DNA damage repair; PARPi: PARP inhibitors.

Declarations

Ethics approval and consent to participate

This study was approved by the ethics committee of Sir Run Run Shaw Hospital and written consents were obtained from all patients involved.

Consent for publication

All the authors have read the manuscript and agreed with the evidence and conclusions in it. We confirm that this paper has not been published previously and if acceptable, will not be published elsewhere.

Availability of data and materials

Available upon request.

Competing interest

There is no conflict of interest to declare.

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Authors' contributions

Hongchuan Jin and Lifeng Feng designed the study; Lifeng Feng, Hongchuan Jin and Miaoqin Chen analyzed the data and wrote the manuscript; Miaoqin Chen, Weikai Wang, Shiman Hu, Yifan Tong, Yiling Li, Qi Wei, Lei Yu, Liyuan Zhu, Leiming Liu, Zhenyu Ju and Xian Wang performed the experiments.

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Figures

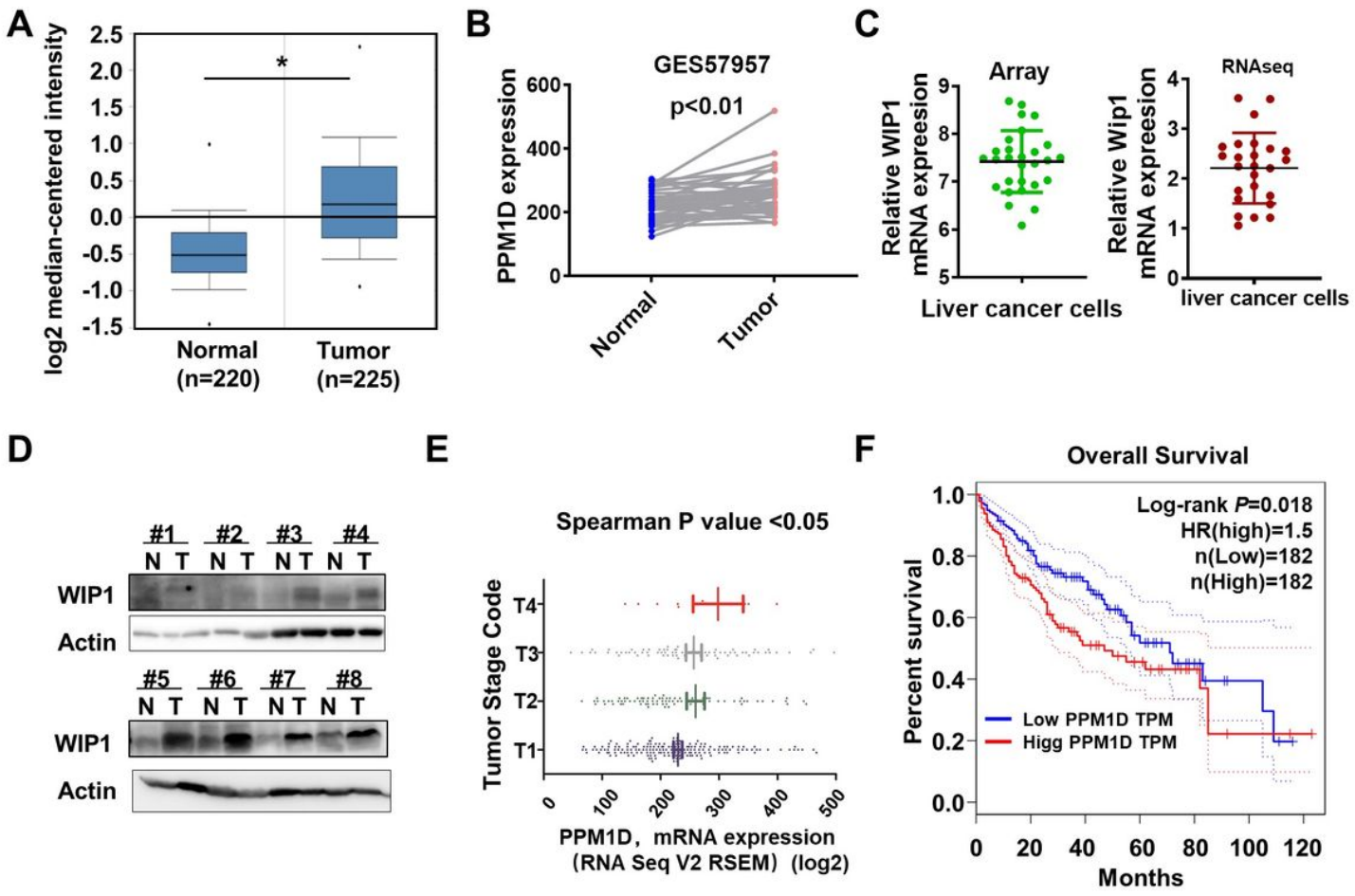


Figure 1

High expression of WIP1 correlates with a poor prognosis in HCC A. WIP1 mRNA expression of normal liver tissues and hepatocellular carcinoma of Roessler Liver 2 cohort in Oncomine database (T-test; p<0.001). B. Expression of WIP1 mRNA in 37 pairs of liver cancer tissues and adjacent non-tumor tissues from GEO data sets GES57957 (Paired t test; p<0.001) C. The mRNA expression levels (array data in left and RNAseq data in right) of WIP1 in liver cancer cell lines in the Cancer Cell Line Encyclopedia (CCLE) database. (T-test; p<0.05). D. Expression of WIP1 protein in 8 pairs of liver cancer tissues and adjacent non-tumor tissues was analyzed by Western blotting. E. The expression of WIP1 mRNA levels in different American Joint Committee on Cancer Tumor Stage Code of TCGA. (Spearman p value <0.05). F. The impact of WIP1 mRNA expression on overall survival (OS) was analyzed by Kaplan-Meier survival curve (patients were grouped based on median WIP1 mRNA expression).

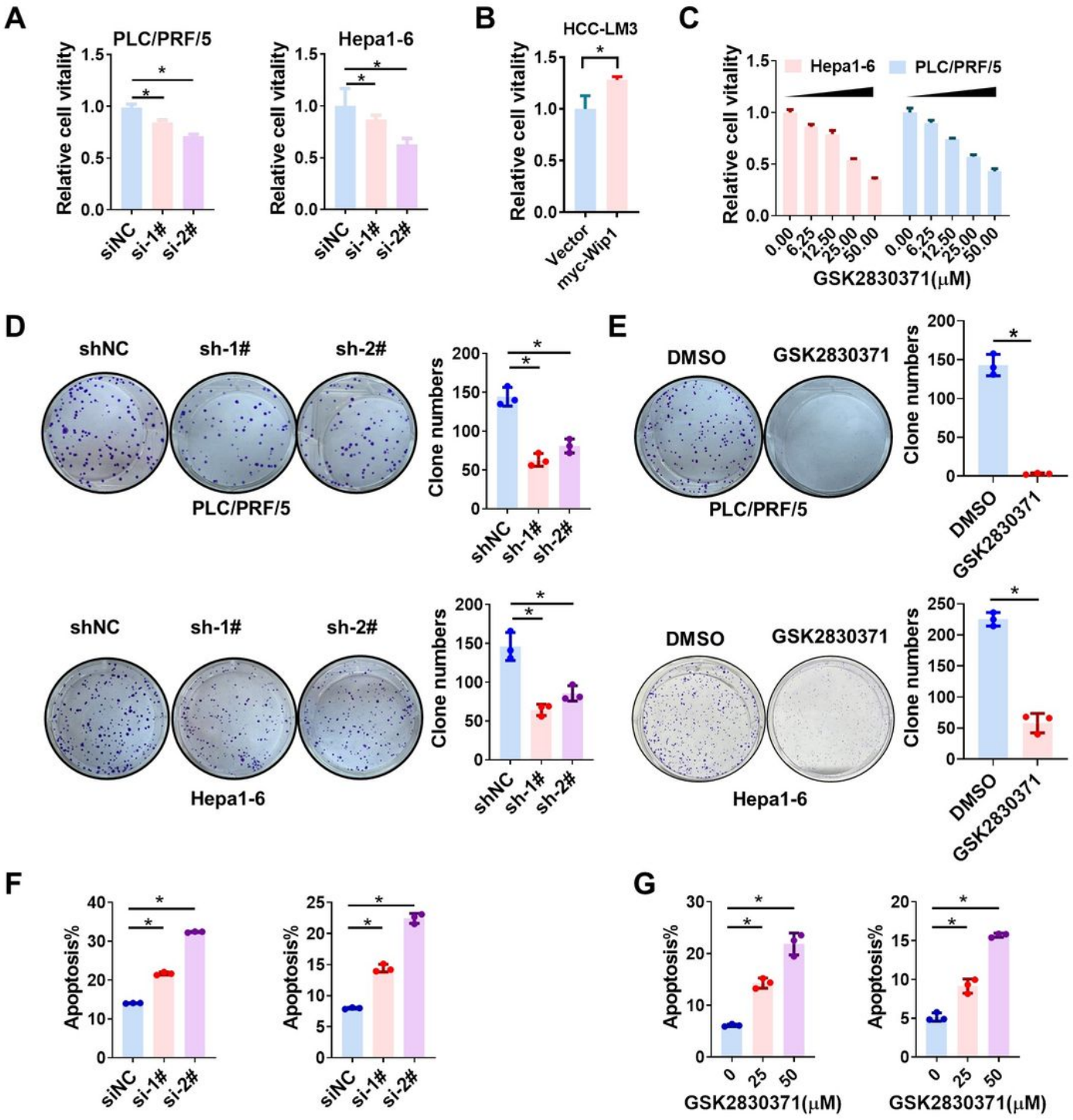
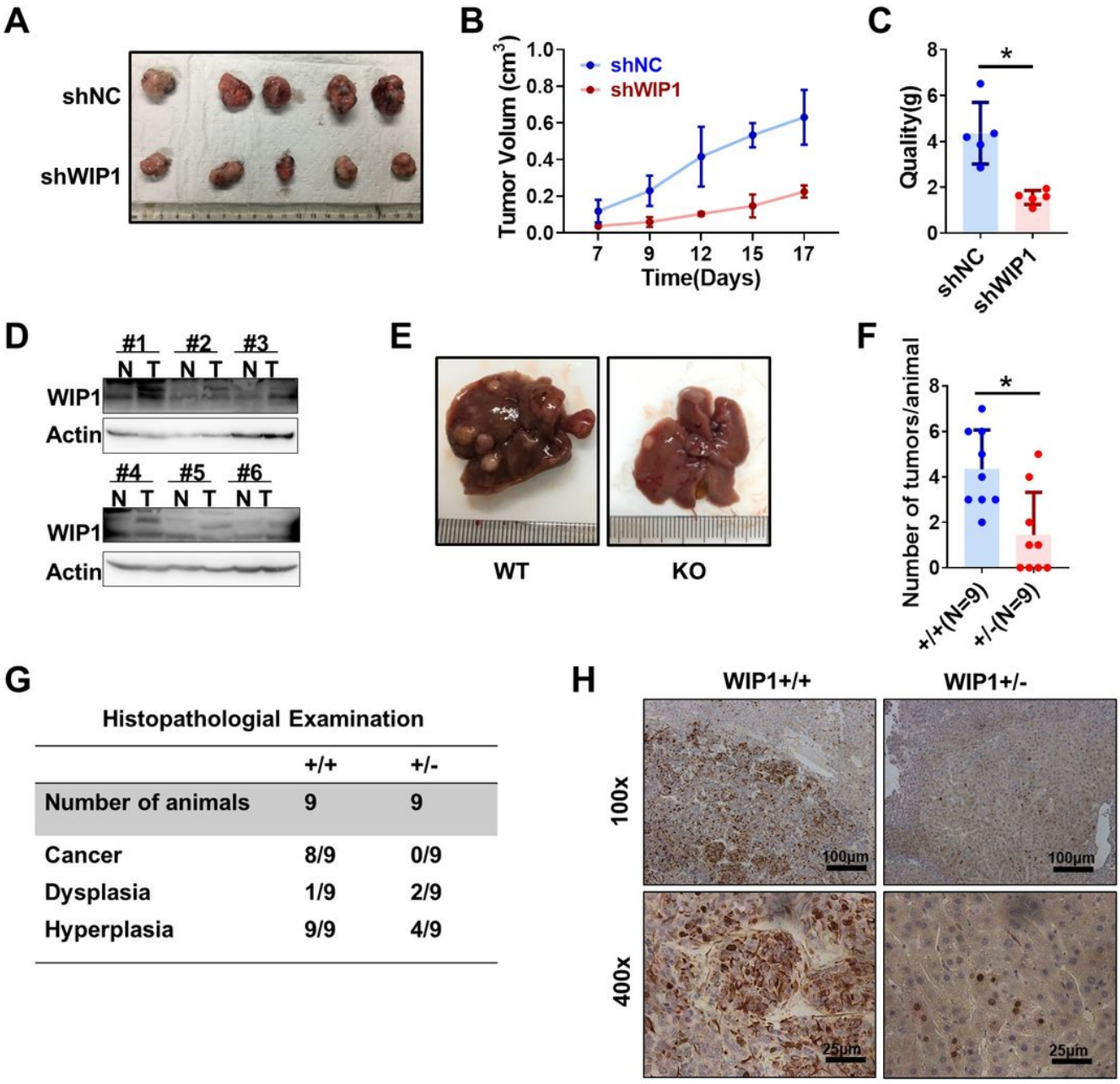


Figure 2

Suppression of WIP1 inhibits proliferation of HCC cells in vitro A. Cell viability of PLC/PRF/5 and Hepa1-6 cells with or without WIP1 knockdown by siRNA was measured with MTS. B. Cell viability of HCC-LM3 cells with ectopic overexpression of WIP1 was measured with MTS. C. Cell viability of PLC/PRF/5 and Hepa1-6 cells treated with WIP1 inhibitor GSK2830371 with indicated concentrations for 72 hours was measured with MTS. D. Representative image of colony formation and quantitative analysis of colony

numbers of PLC/PRF/5 and Hepa1-6 cells with WIP1 stable knockdown with shRNA. E. Representative image of colony formation and quantitative analysis of colony numbers of PLC/PRF/5 and Hepa1-6 cells with GSK2830371(12.5μM, 24 hours). F. The apoptosis of HCC cells with or without WIP1 knockdown with siRNA was assessed via flow cytometry with PI and annexin V–FITC staining. G. The apoptosis of HCC cells with or without GSK2830371 treatment for 72 hours was assessed via flow cytometry with PI and annexin V–FITC staining.



G

Histopathologial Examination

	+/+	+/-
Number of animals	9	9
Cancer	8/9	0/9
Dysplasia	1/9	2/9
Hyperplasia	9/9	4/9

Figure 3

WIP1 suppression inhibits HCC development in vivo Xenograft model (n=5 per group) was performed with Hepa1-6 cells with or without WIP1 stable knockdown via shWIP1 or shNC lentivirus. And tumor pictures (A), tumor growth curve (B) and tumor weight (C) were shown respectively. D. WIP1 protein levels in 6 pairs of DEN-induced mice liver cancer tissues and adjacent non-tumor tissues was analyzed by Western blotting. E. Representative macroscopic images of WIP1 wild-type (+/+) or knockout (+/-) mice with DEN-induced HCC. F. The average number of tumors per mouse in DEN-induced HCC (size>2mm) in WIP1 +/+ or +/- mice. G. The histopathological examination of +/+ or +/- mice liver tissue. H. The ki-67 immunohistochemical staining of +/+ or +/- mice liver tissue.

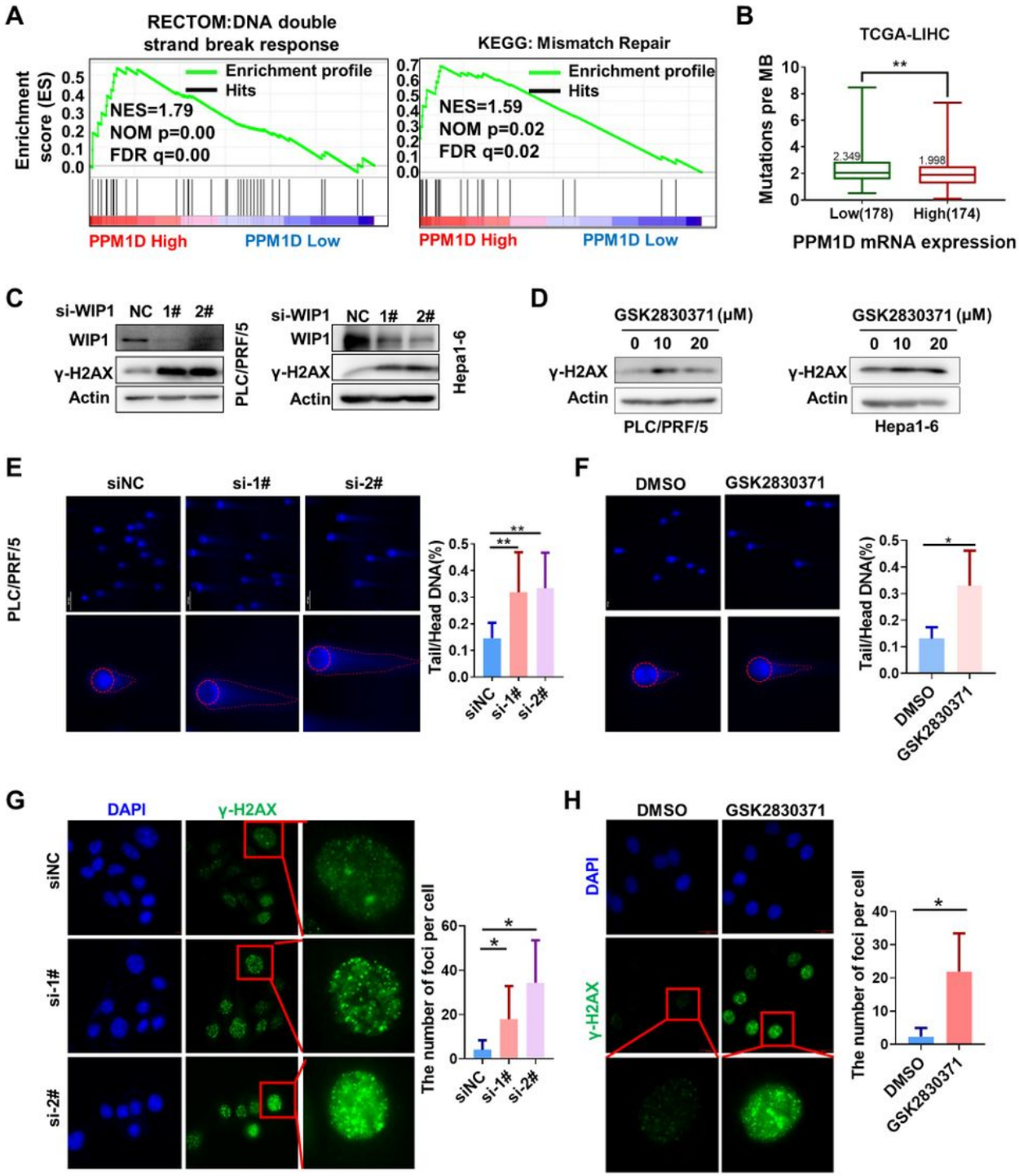


Figure 4

WIP1 inhibition disrupts DNA damage repair by increasing H2AX phosphorylation. A. Gene set enrichment analysis (GSEA) of the gene expression profiles in WIP1 high expression and WIP1 low expression human liver cancer tissues. Red indicates WIP1 high expression; blue indicated WIP1 low expression. B. Tumor mutation burden (TMB) was compared between WIP1 high expression and low expression liver cancer tissues from TCGA. The phosphorylation of H2AX at S139 (γ-H2AX) was measured via Western blotting

in HCC cells after WIP1 knockdown (C) or GSK2830371 inhibition (D). The Comet assay was performed to detect the DNA double strand break of PLC/PRF/5 cells after WIP1 knockdown (E) or GSK2830371 inhibition (25 μM, 48 hours) (F). CASP software was used to calculate Tail/Head DNA percent of every single cell. The foci of phosphorylation of H2AX at S139 (γ-H2AX) was measured via immunofluorescence to evaluated the DNA damage levels of PLC/PRF/5 cells after WIP1 knockdown (G) or GSK2830371 inhibition (25 μM, 48 hours) (H). And the numbers of foci per cell were counted.

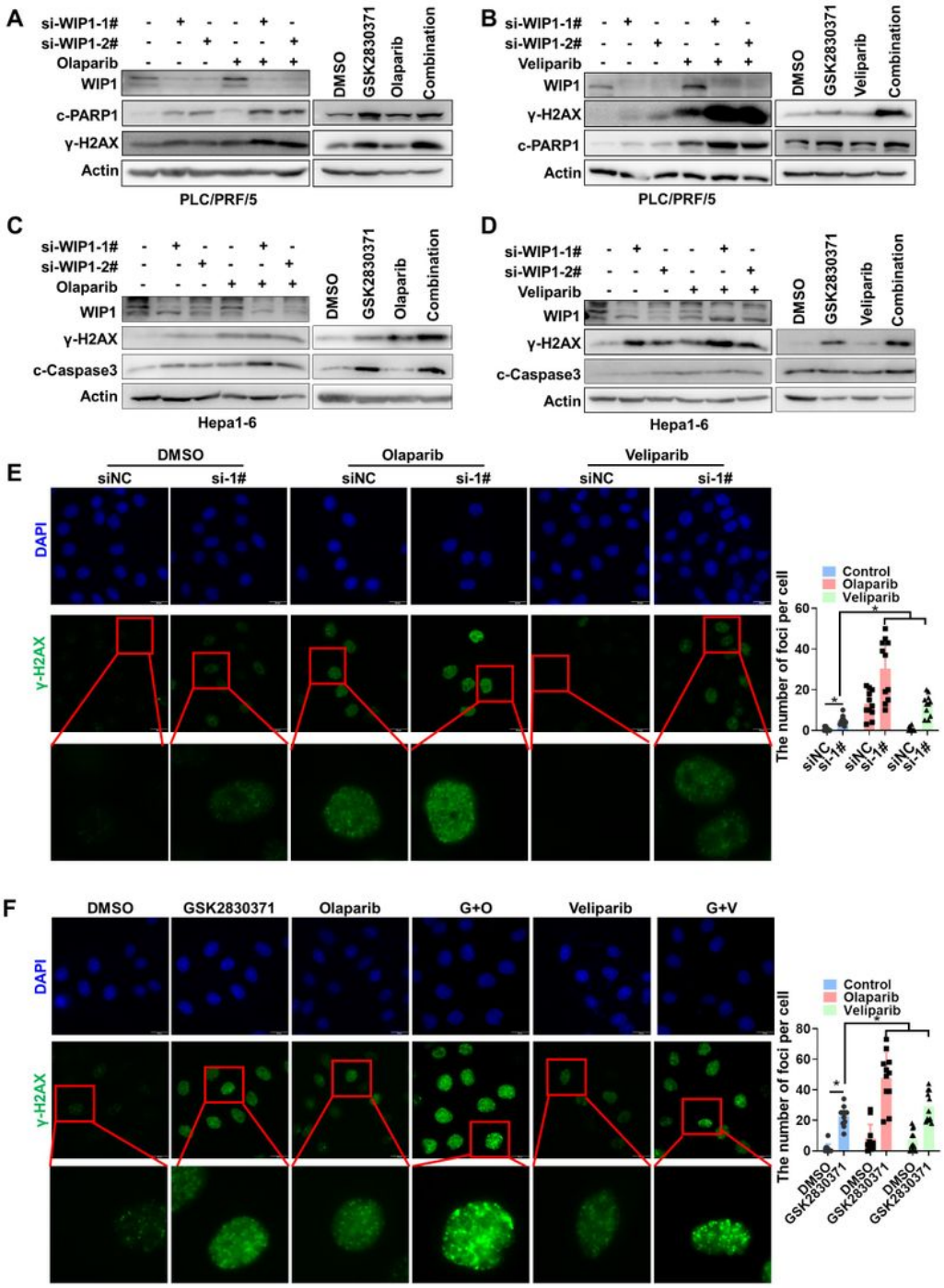


Figure 5

WIP1 and PARP inhibition pronounced DNA damage A. The phosphorylation of H2AX at S139 and cleaved-PARP1 (C-PARP1) were measured via Western blotting in PLC/PRF/5 cells after WIP1 knockdown or inhibition combined with Olaparib (50μM, 24 hours) treatment. B. The phosphorylation of H2AX at S139 (γ-H2AX) and C-PARP1 was measured via Western blotting to evaluated the DNA damage levels and apoptosis of PLC/PRF/5 cells after WIP1 knockdown or inhibition combined with Veliparib (50μM, 24 hours) treatment. C. The phosphorylation of gama-H2AX at S139(γ-H2AX) and C-PARP1 was measured via Western blotting in Hepa1-6 cells after WIP1 inhibition combined with Olaparib (50μM, 24 hours) treatment. D. The phosphorylation of H2AX at S139 (γ-H2AX) and c-PARP was measured via Western blotting to evaluated the DNA damage levels and apoptosis of Hepa1-6 cells after WIP1 inhibition combined with Veliparib (50μM, 24 hours) treatment. E. The foci of phosphorylation of H2AX at S139 (γ-H2AX) was measured via immunofluorescence in PLC/PRF/5 cells after WIP1 knockdown combined with Olaparib (50μM) or Veliparib (50μM) treatment for 24 hours. And the numbers of foci were counted. F. The foci of phosphorylation of H2AX at S139(γ-H2AX) was measured via immunofluorescence to evaluated the DNA damage levels of PLC/PRF/5 cells after GSK2830371(25μM) combined with Olaparib (50μM) or Veliparib (50μM) treatment for 24 hours. And the numbers of foci were counted.

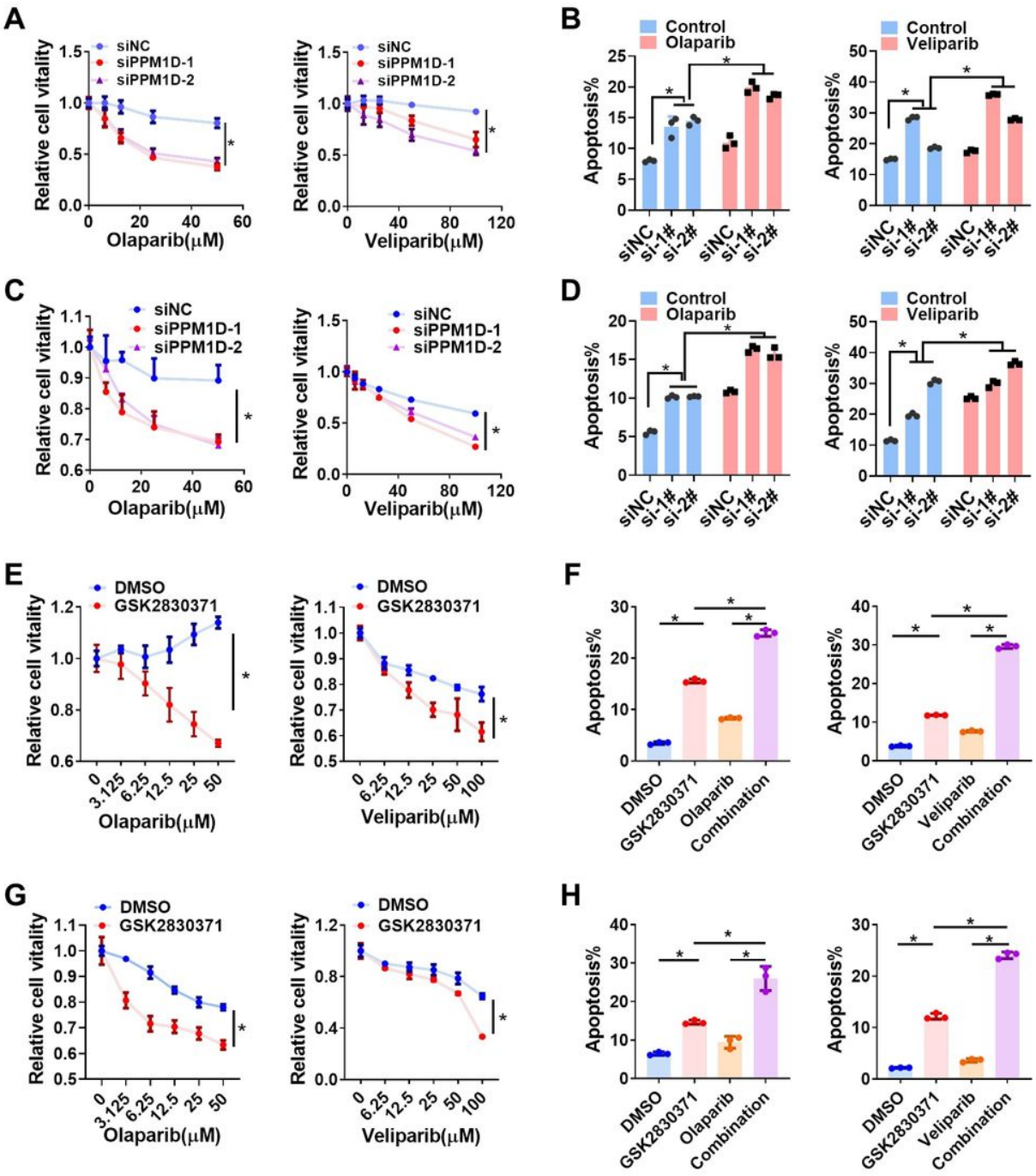


Figure 6

WIP1 and PARP inhibition confers HCC cells synthetic lethal in vitro A. Cell viability of Hepa1-6 cells after Olaparib (48 hours) or Veliparib (48 hours) treatment with or without WIP1 knockdown was measured with MTS. B. The apoptosis of Hepa1-6 cells after Olaparib (50μM, 48 hours) or Veliparib treatments with or without WIP1 knockdown was measured via flow cytometry with PI and annexin V–FITC staining. C. Cell viability of PLC/PRF/5 cells after Olaparib or Veliparib treatment with or without WIP1 inhibitor GSK2830371 was measured with MTS. D. The apoptosis of PLC/PRF/5 cells after Olaparib or Veliparib

treatment with or without WIP1 inhibitor GSK2830371 was measured via flow cytometry. E. Cell viability of Hepa1-6 cells after Olaparib or Veliparib treatments with or without GSK2830371 treatment was measured with MTS. F. The apoptosis of Hepa1-6 cells after Olaparib or Veliparib treatments with or without GSK2830371 treatment was measured via flow cytometry. G. Cell viability of PLC/PRF/5 cells after Olaparib or Veliparib treatments with or without GSK2830371 treatment was measured with MTS. H. The apoptosis of PLC/PRF/5 cells after Olaparib or Veliparib treatments with or without GSK2830371 treatment was measured via flow cytometry.

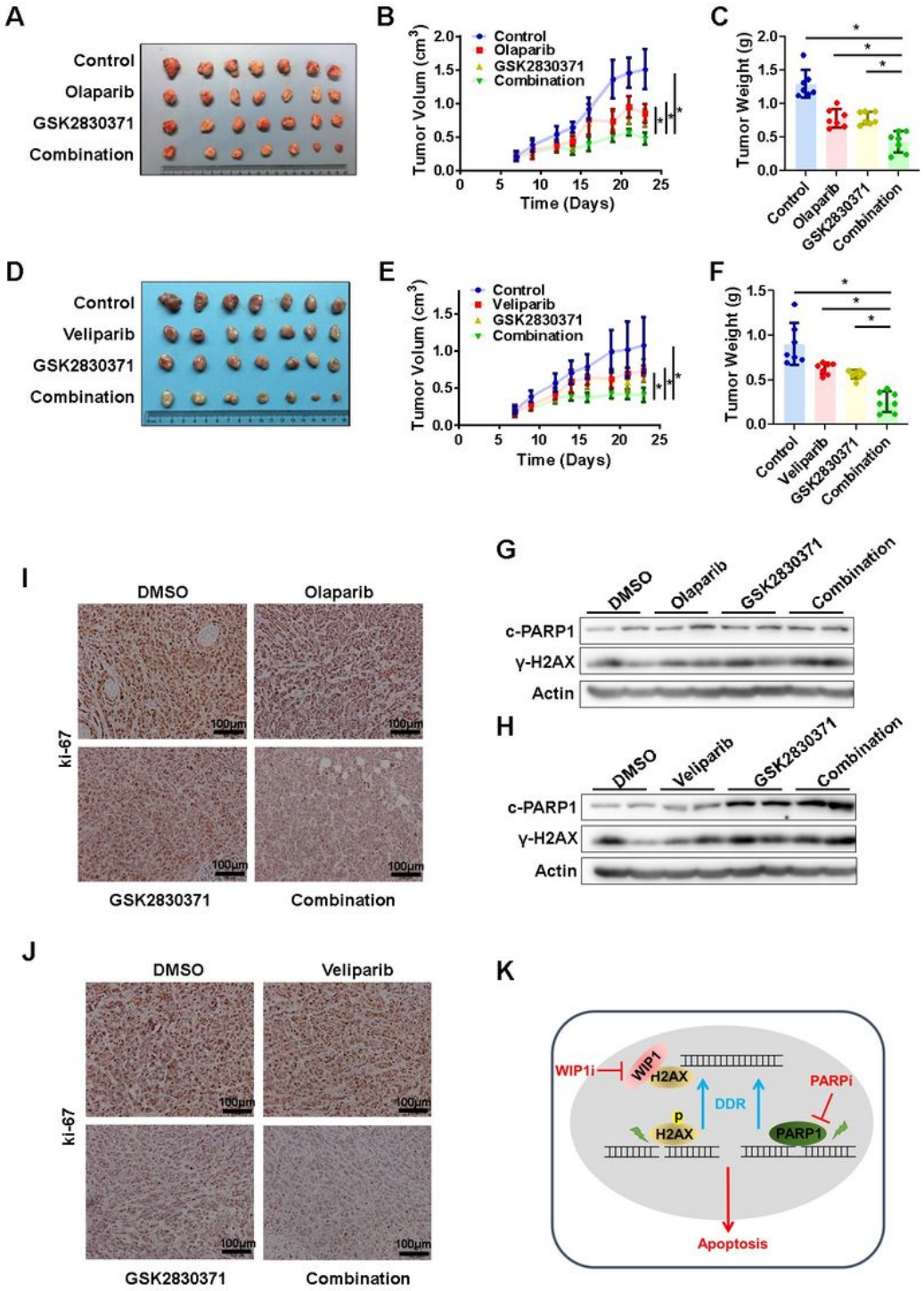


Figure 7

WIP1 and PARP inhibition induce synthetic lethality of HCC cells in vivo. Xenograft model (n=7 per group) was generated by subcutaneous inoculation of PLC/PRF/5 cells. Mice were then treated with Olaparib and GSK2830371 as indicated. Tumor pictures (A), tumor growth curve (B) and tumor weight (C) were shown or summarized respectively. Veliparib and GSK2830371 combination treatment experiment was performed as above, and tumor pictures (D), tumor growth curve (E) and tumor weight (F) were shown or summarized respectively. C-PARP1 and γ-H2AX in tumor tissues were evaluated by Western blotting, Olaparib and GSK2830371 combination in (G), Veliparib and GSK2830371 combination in (H). I-J. The ki-67 immunohistochemical staining of tumor tissues with indicated treatment was shown. K. Working model. WIP1 functions as a homeostatic regulator during DNA double strand break by de-phosphorylatingγ-H2AX at the end of DNA damage repair. Thus, co-targeting WIP1 and PARP could induce HCC synthetic lethality via disrupting DNA damage repair, which likes the PARPi works in BRCA1/2 deficient cancers.

Supplementary Files

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