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Supplemental Information

Mutant p53 Gains Its Function via c-Myc Activation upon CDK4 Phosphorylation at Serine 249 and Consequent PIN1 Binding

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Supplementary Information

Figure Legends for Supplementary Figures

Figure S1 (related to Figure 1)

A) Summary of frequency or percentage of R249S mutations of p53 in HCC cell lines or in HCC patients from different geographical regions in China, Africa and Europe.

B) CDK4 interacts with p53-RS, not wt p53. HEK293 cells were co-transfected with CDK4-HA and p53WT or p53-RS, and then harvested for IP-WB analysis using antibodies as indicated.
C) CDK4 forms more complexes with p534MRS in comparison of other mutant p53s. CDK4-HA was co-transfected with p53, p53K280, p53K280-4M, p53K280-4MRS or p53H273 in H1299 cells, and cells were harvested for co-IP-WB analysis with indicated antibodies.
D) The specificity of phosphorylation antibody of p53-S249. The doc blot loaded with non-phos-S249 (right column) and phos-S249 peptides (left column) were pre-incubated with non-phos-S249 or phos-S249 peptides as competitors (as indicated on left of the panel), and then probed with anti-phosphorylated p53-RS antibodies and detected by ECL.
E) Endogenous CDK4 forms complexes with p53-RS, but much less with mutant p53-C220, and none with wt p53. PLC5 (PLC/PRF/5) cells with p53-RS, Huh7 cells with p53-C220, and HepG2 cells with wt p53 were harvested for co-IP with anti-CDK4 antibodies followed by WB with indicated antibodies.
F) The S249/P250 motif is key for p53-RS phosphorylation by the CDK4/Cyclin D1 complex. His-p53SP (S249P250) or His-p53SA (S249A250) was purified from *E. coli* for an *in vitro* kinase assay. Phosphorylated proteins were detected by the anti-phosphor-S249 antibody. Coomassie-stained SDS-PAGE shows the equivalent amount of proteins used for the kinase assay.
G) CDK4 phosphorylates p53-RS or Rb *in vitro*. His-p53-RS or GST-Rb (379-928) was purified from *E. coli* for an *in vitro* kinase assay, and phosphorylated proteins were detected by autoradiography. Coomassie-stained SDS-PAGE shows equivalent amounts of proteins used for the kinase assay.
H) CDK4/Cyclin D1, but not CDK2/Cyclin A1, or CDK6/Cyclin D1, specifically phosphorylates p53-RS *in vitro*. His-p53-RS was incubated with CDK2/Cyclin A1, CDK4/Cyclin D1 and CDK6/Cyclin D1 complexes, individually. Phosphorylated proteins were detected by the anti-phosphor-S249 antibody. Coomassie-stained SDS-PAGE shows equivalent amounts of proteins used for the kinase assay.
I) CDK4/Cyclin D1, but not CDK4/Cyclin D3, specifically phosphorylates p53-RS *in vitro*. His-p53-RS was incubated with CDK4/Cyclin D1 or CDK4/Cyclin D3 complexes individually. Phosphorylated proteins were detected by the anti-phosphor-S249 antibody. Coomassie-stained SDS-PAGE shows equivalent amounts of proteins used for the kinase assay.
J) Knockdown of Cyclin D1, but not Cyclin D3, reduces phosphorylation of p53-RS in cells. Knockdown of Cyclin D1 or Cyclin D3 in PLC/PRF/5 cells with two different of specific siRNAs, and cells were harvested for IP with IgG or the anti-p53 antibody followed by WB analysis with antibodies as indicated.
K) S249 Phosphorylation of p53-RS occurs in the early G1 phase during the cell cycle. PLC/PRF/5 cells were synchronized by Nocodazole, released and harvested at different time points as

indicated for IP with IgG or the anti-p53 antibody followed by WB analysis with antibodies as indicated.

L) More phosphorylation mimic mutant p53-4M-RD molecules with R249D than that of p53-4M-RS are detected in the nuclear fraction. H1299 cells were transfected with p53-RS or p53-RD, and harvested for preparation of nuclear and cytoplasmic fractions 36 hr after transfection. Subcellular fractions were analyzed by WB analysis with antibodies as indicated.

M) Ectopic CDK4 doesn't interact with PIN1 in H1299 cells. CDK4-HA and Flag-PIN1 were co-introduced into H1299 cells that were then harvested for co-IP-WB analysis with antibodies as indicated 36 hr after transfection.

N) Ectopic p53RS doesn't be phosphorylated in WI38 cells. Knockdown of Cyclin D1 or Cyclin D3 in WI38 cells with two different of specific siRNAs, after 24 hr, Flag-p53RS were introduced into WI38 cells that were then harvested for co-IP-WB analysis with antibodies as indicated 48 hr after transfection.

Figure S2 (related to Figure 3)

A) and B) Stable GFP-p53-RS H1299 are treated by CDK4 inhibitor (CDK4i) PD, Pin1 inhibitor (Pin1i) ATRA or not, and are analyzed for subcellular localization by immunofluorescence microscopy and counterstained for DNA with DAPI. Scale bar corresponds to 20 μm (A). Quantification of cytoplasm localization of GFP-p53-RS is shown (B).

C) Pin1 increases the nuclear level of p53-RS. p53-RS was co-introduced with or without Pin1 into H1299 cells. Transfected cells were used for subcellular fractionation 36 hrs later, and subcellular fractions were analyzed by WB analysis with antibodies as indicated.

D) The majority of p53-4MRS-Pin1 complexes are detected in the nucleus. p53-4MRS and Flag-PIN1 were co-transfected in H1299 cells and then harvested for nuclear and cytoplasmic fractionation followed by co-IP-WB analysis with antibodies as indicated.

E) and F) p53-RS was trans-located to the nucleus by Pin1. p53-RS was transfected with Flag-PIN1 or a control vector in H1299 cells. Cells (1600 cells with p53-RS only and 450 cells with both p53-RS and PIN1) were subjected to the analysis by using the ImageStream Imaging Flow Cytometer for the subcellular localization of PIN1 and p53-RS.

G) A graphic presentation of the results from E and F, showing that ~14% of 1600 cells transfected with p53-RS have nuclear p53-RS, but 25% of 450 cells co-transfected with p53-RS and PIN1 have nuclear p53-RS.

Figure S3 (related to Figure 4)

A) Go analysis of the ChIP-on-chip data after p53-RS knockdown. ChIP-on-chip analysis was carried out using anti-p53 antibodies in PLC/PRF/5 cells after being transfected with control siRNA or p53-RS siRNA.

B) c-Myc interacts with p53-RS, but not p53WT. HA-c-Myc was co-transfected with p53WT or p53-

RS in H1299 cells. Cells 36 hours after transfection were harvested for IP-WB analysis with antibodies as indicated.

C) Knockdown of CDK4 alleviates the interaction of p53-RS with c-Myc. H1299 cells transfected with CDK4 or control SiRNA for 24 h were transfected with combination of plasmids encoding HA-c-Myc, Flag-p53RS, and harvested for co-IP-WB or straight WB analysis with antibodies as indicated.

D) p53-RS reduces FBW7a-mediated c-Myc ubiquitination. H1299 cells transfected with His-Ub, HA-c-Myc, 4XFlag-FBW7a and p53-RS, and harvested for His-Ub experiment or straight WB analysis with antibodies as indicated.

E) Knockdown of p53-RS reduces c-Myc protein level in the BT-549 cells. BT-549 cells transfected with p53 or control SiRNA for 72 h were treated with or without MG132 and harvested for analysis of protein levels by WB with indicated antibodies.

F) The CDK4 inhibitor doesn't affect the half-life of p53-RS. PLC/PRF/5 or HepG2 cells were treated with a CDK4 inhibitor (PD) at indicated concentrations overnight, then treated by CHX, and harvested at the time points as indicated. Protein levels of p53-RS or wt p53 were detected by WB with antibodies as indicated.

G) More c-Myc molecules interact with phosphorylation mimic mutant p53-RD than with p53-RS. H1299 cells were transfected with combination of plasmids encoding HA-c-Myc, p53-4M-RS or p53-4M-RD for co-IP-WB or straight WB analysis with antibodies as indicated.

Figure S4 (related to Figure 5)

A) p53-RS was introduced into Hep3B cells, which were harvested for WB analysis with indicated antibodies.

B) p53-RS was introduced into Hep3B cells, and cells were harvested for RNA analysis by using Q-PCR.

C) Knockdown of p53-RS or c-Myc in PLC/PRF/5 cells. PLC/PRF/5 cells were transfected with scramble, anti-p53-RS, anti-c-Myc siRNA and then harvested for RNA analysis by using Q-PCR.

D) Knockdown of p53WT or c-Myc in HepG2 cells. HepG2 cells were transfected with scramble, anti-wtp53 or anti-c-Myc siRNA and harvested for RNA analysis by using Q-PCR.

Figure S5 (related to Figure 6)

A) The Correlation of p53-RS with CCND1, CCND2, MYC and CDK4 amplification and expression in patients from the cBioPortal for Cancer Genomics (http://www.cbioportal.org/).

B) The mRNA expression of CDK4 in normal liver or hepatocellular carcinoma from the ONCOMINE (https://www.oncomine.org/).

C) Knockdown of wt p53 does not affect the sensitivity of HepG2 cells to a CDK4 inhibitor (PD). SiNC or Sip53 was introduced into HepG2 cells via transfection, and then treated with different concentrations of PD for a colony formation assay (left panel), and the quantification of colonies is shown in the graph (right panel).

Figure S6 (related to Figure 7)

A) Summary of the HBV status of HCC patient samples used in Figures 7A-7D.

Figure S1 is related to Fig.1

A

Regions/ Cells	HepG2 cells	Guangxi (China)	Qidong (China)	Sub-Saharan Africa	Shanghai (China)	USA Europe	Anhui (China)
AFB exposure	Yes	Yes	High	High	Moderate	Low	Unknown
S249 * (percentage)	8.4/10^7 Frequency	31.0%	96.7%	92.3%	68.4%	5.7%	10.5%
HCC prevalent area		Yes	Yes	Yes	Yes	No	No
reference	Aguilar et al 1993	Qi et al 2015	Gouas et al 2009	Gouas et al 2009	Gouas et al 2009	Gouas et al 2009	Liu et al 2002

* p53S249 mutant percentage in all mutant codons of p53

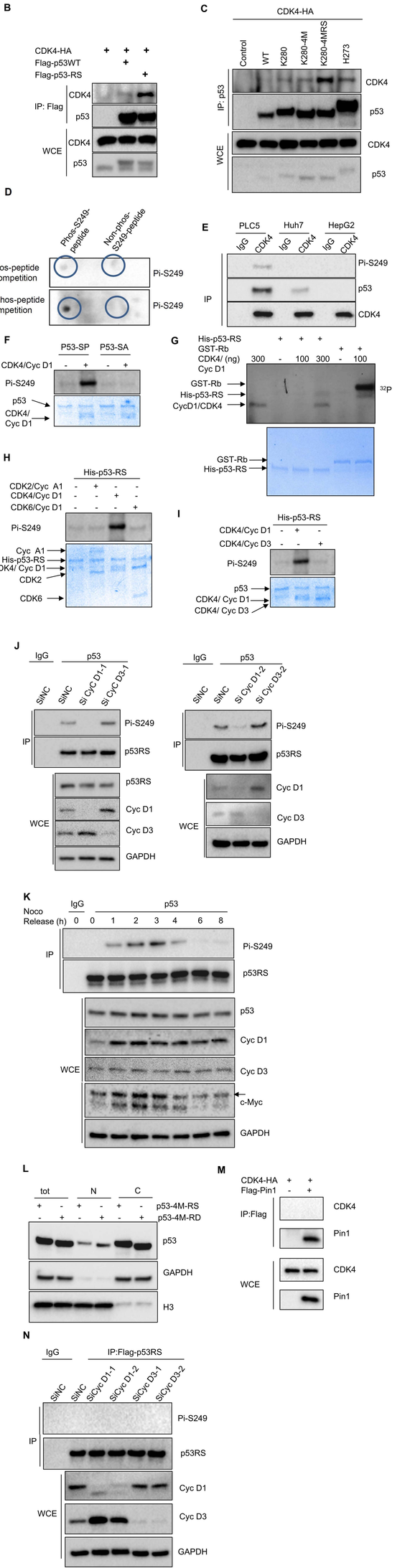
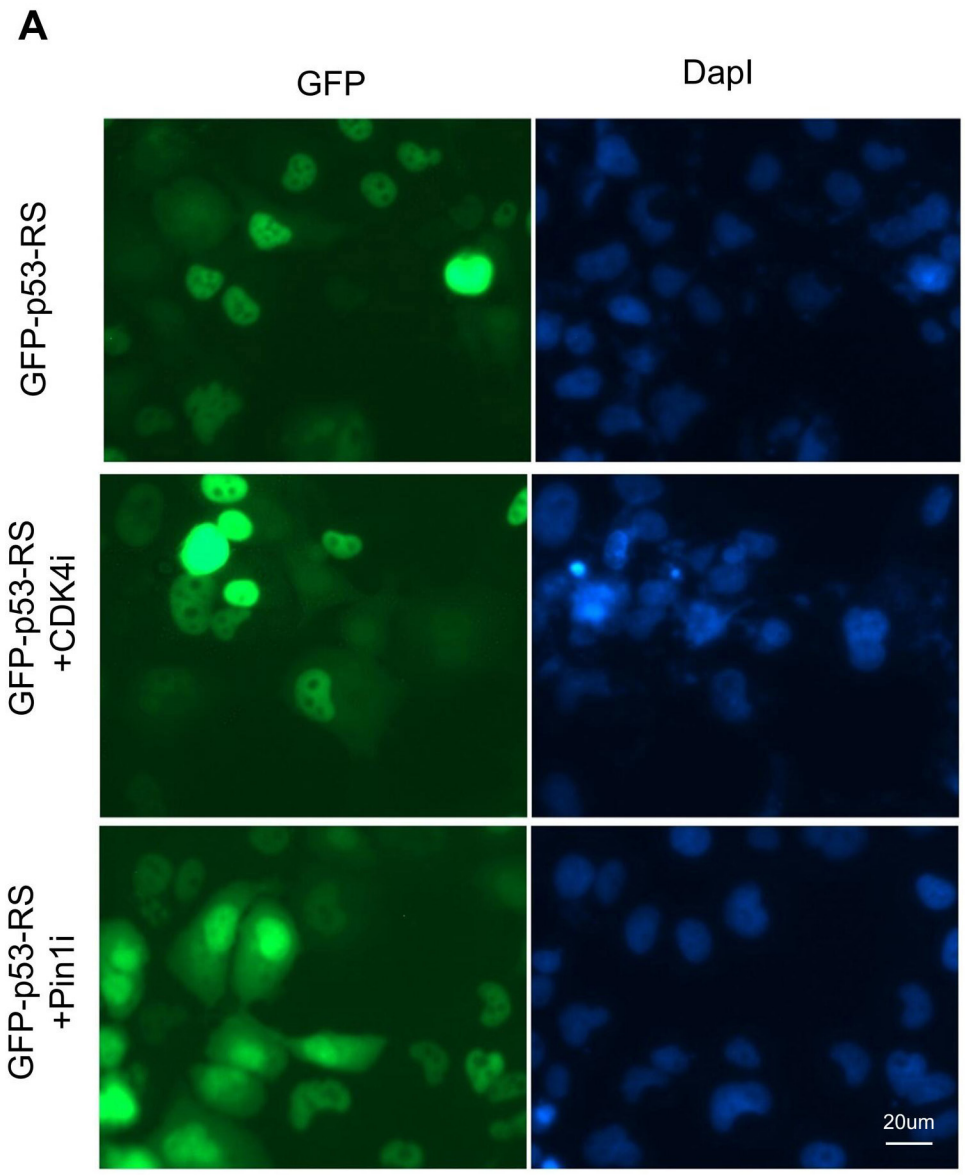
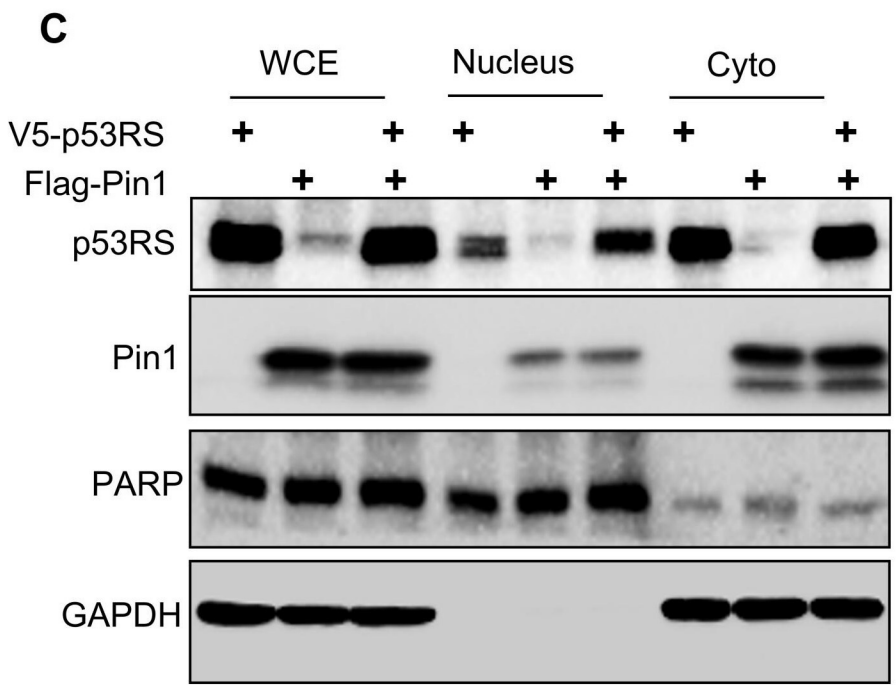
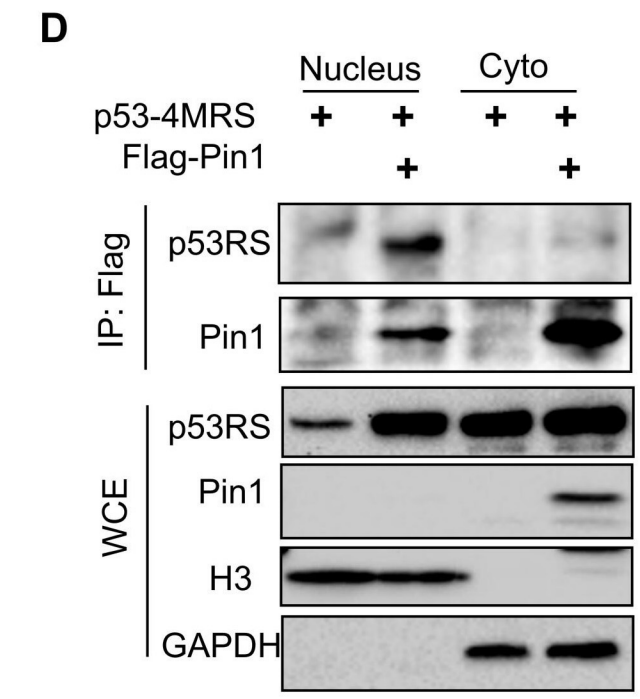
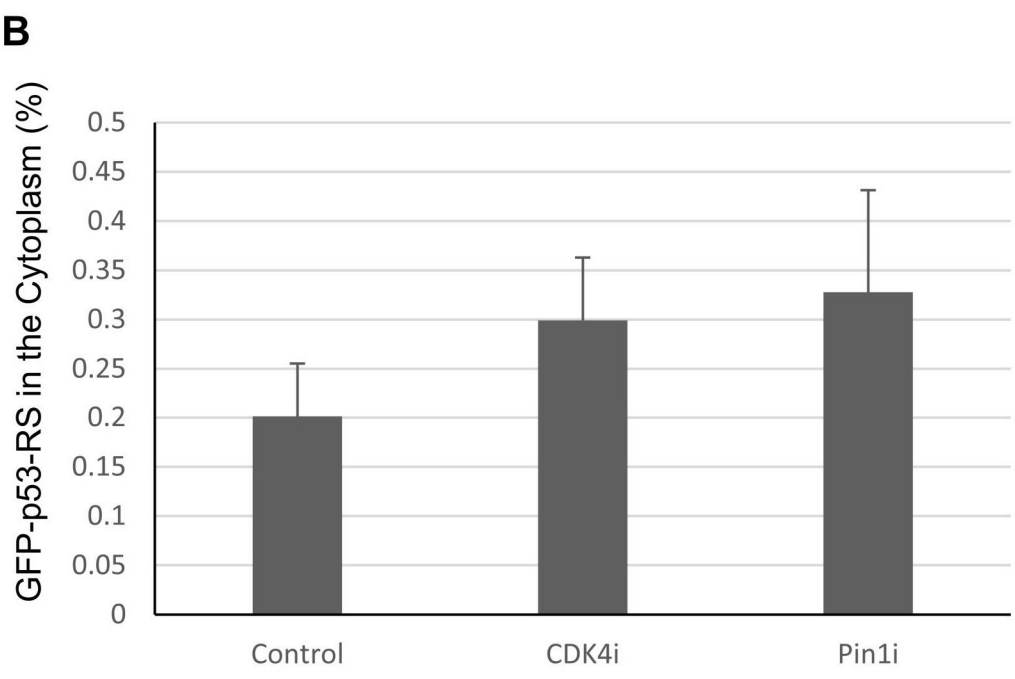


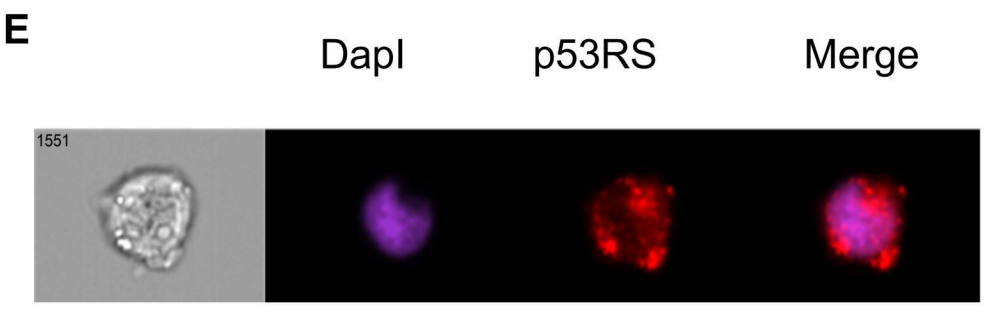
Figure S2 is related to Fig.3

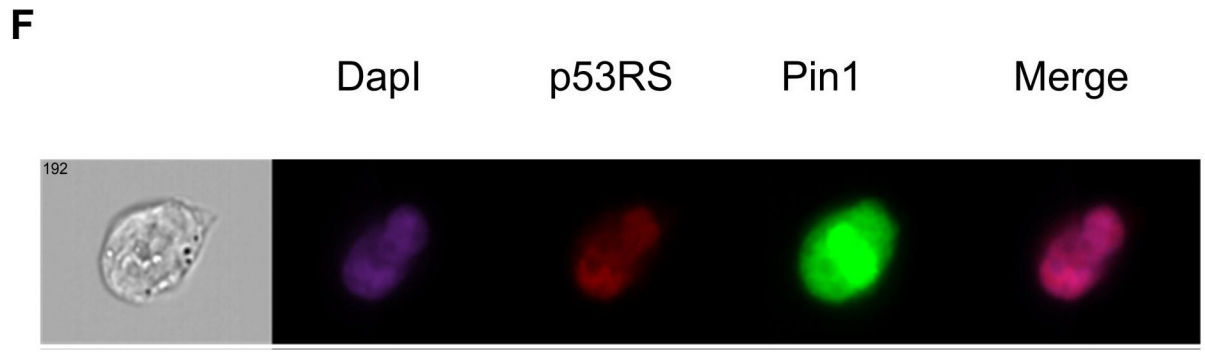












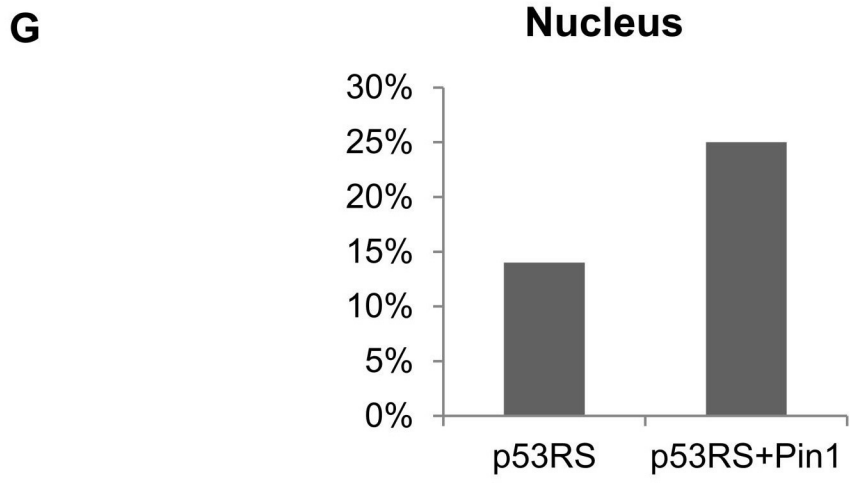
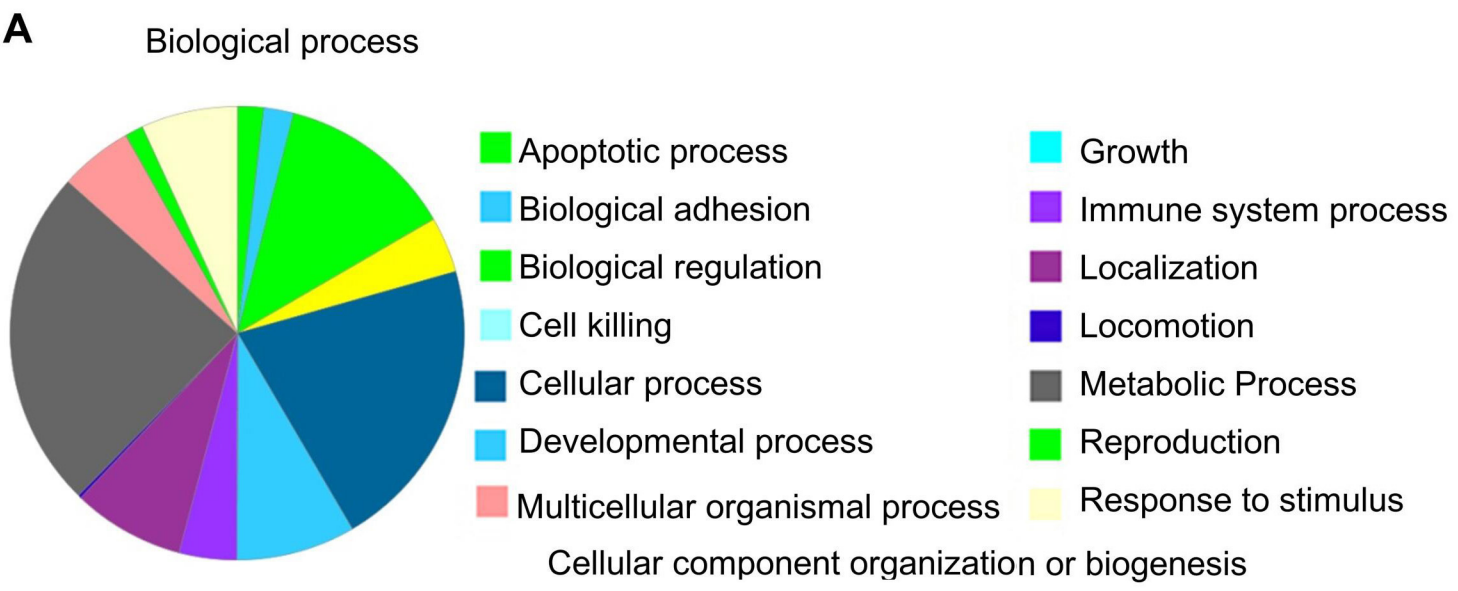
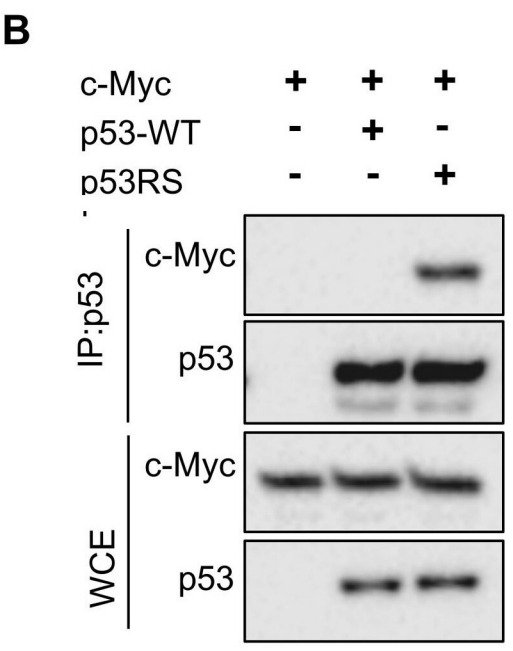
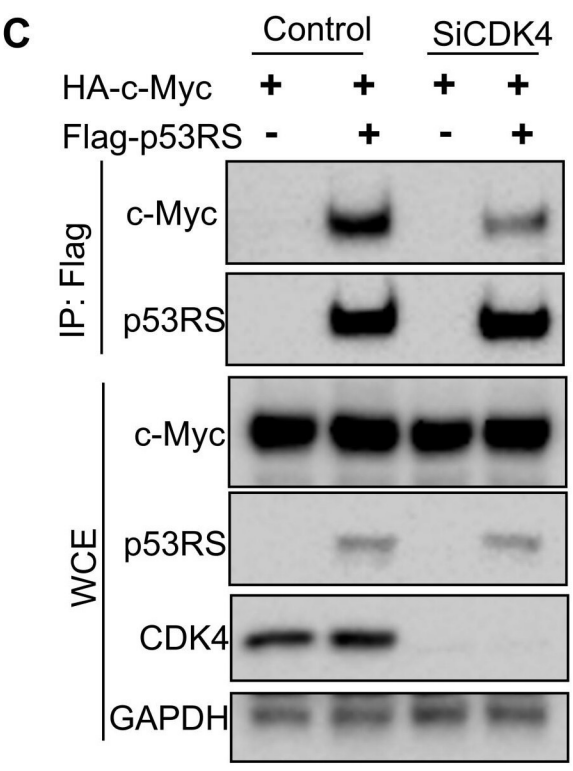
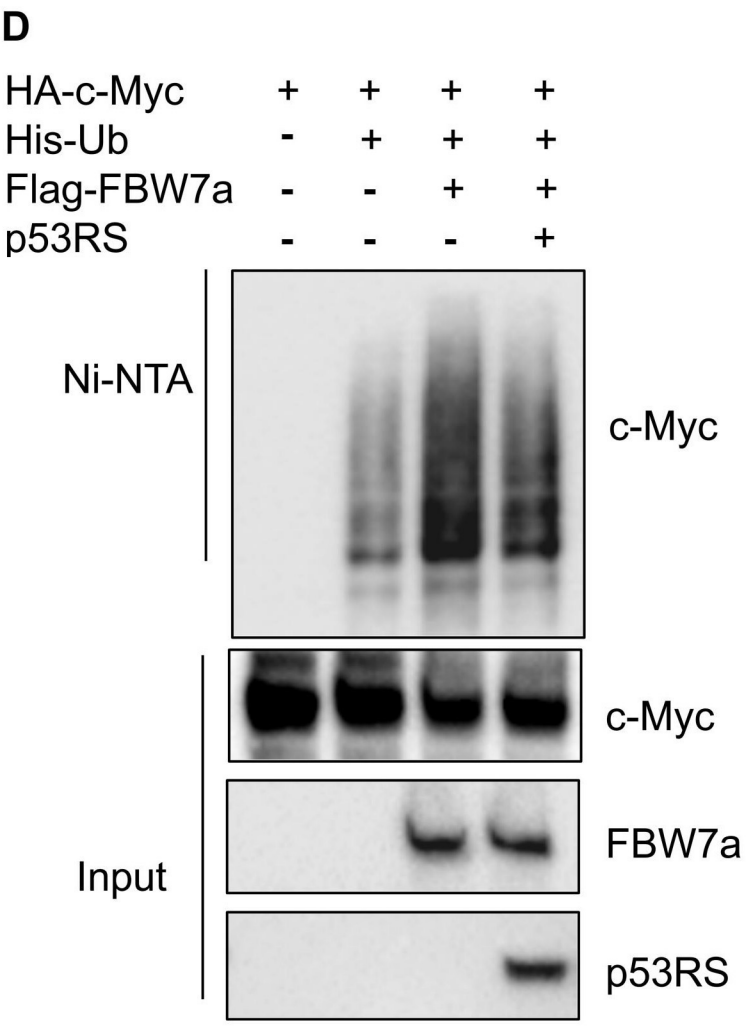


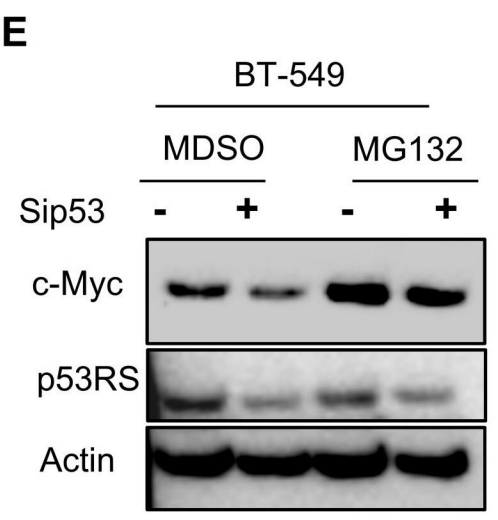
Figure S3 is related to Fig.4

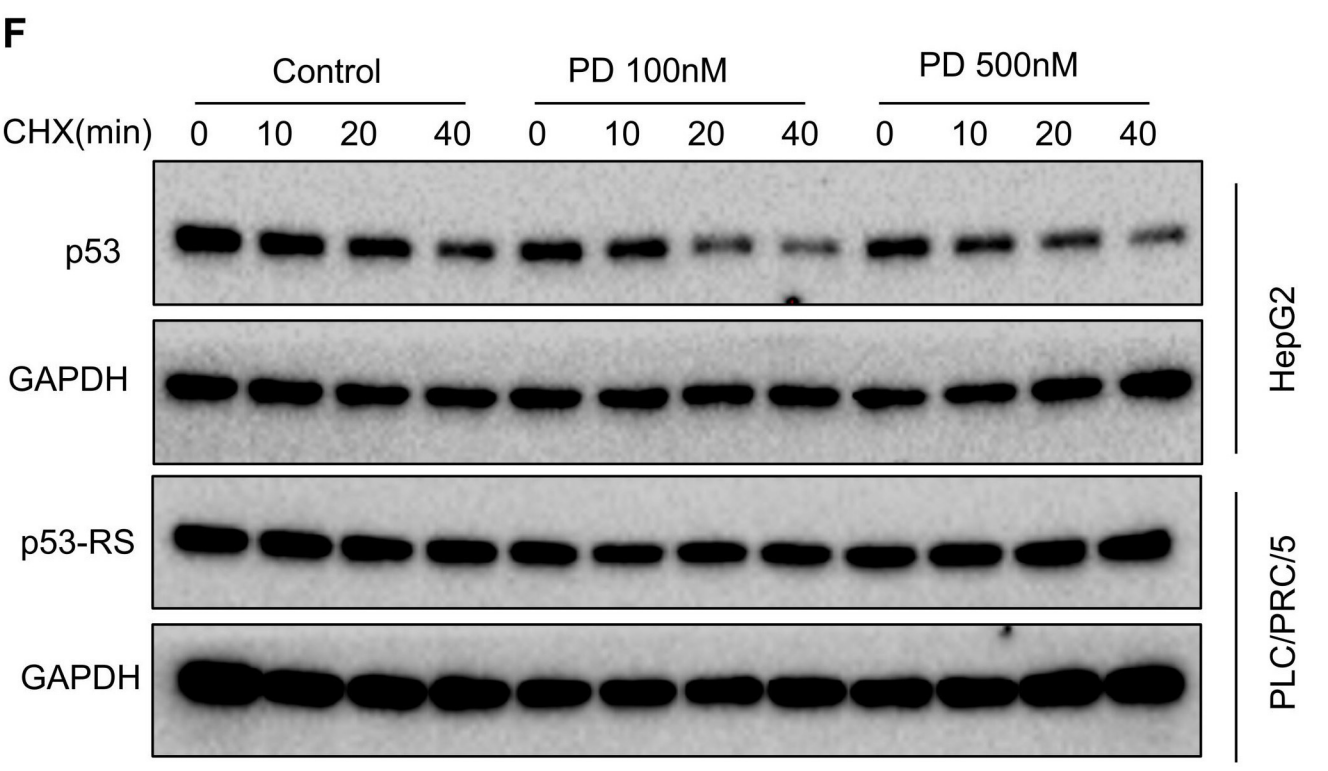












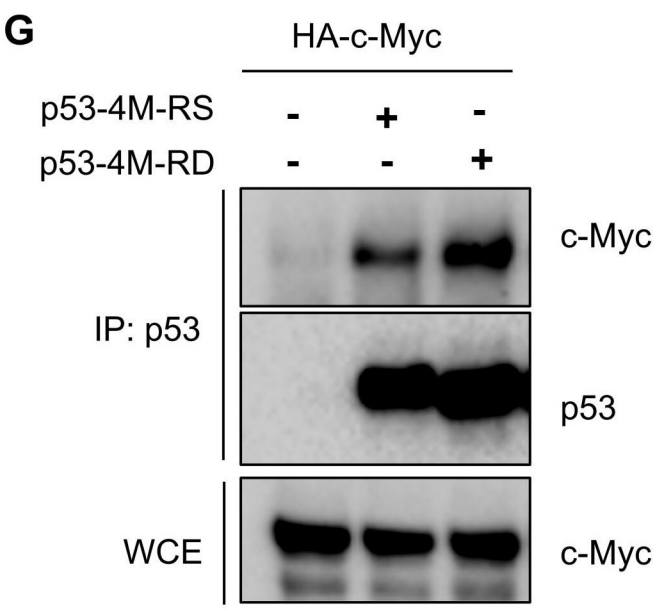


Figure S4 is related to Fig.5

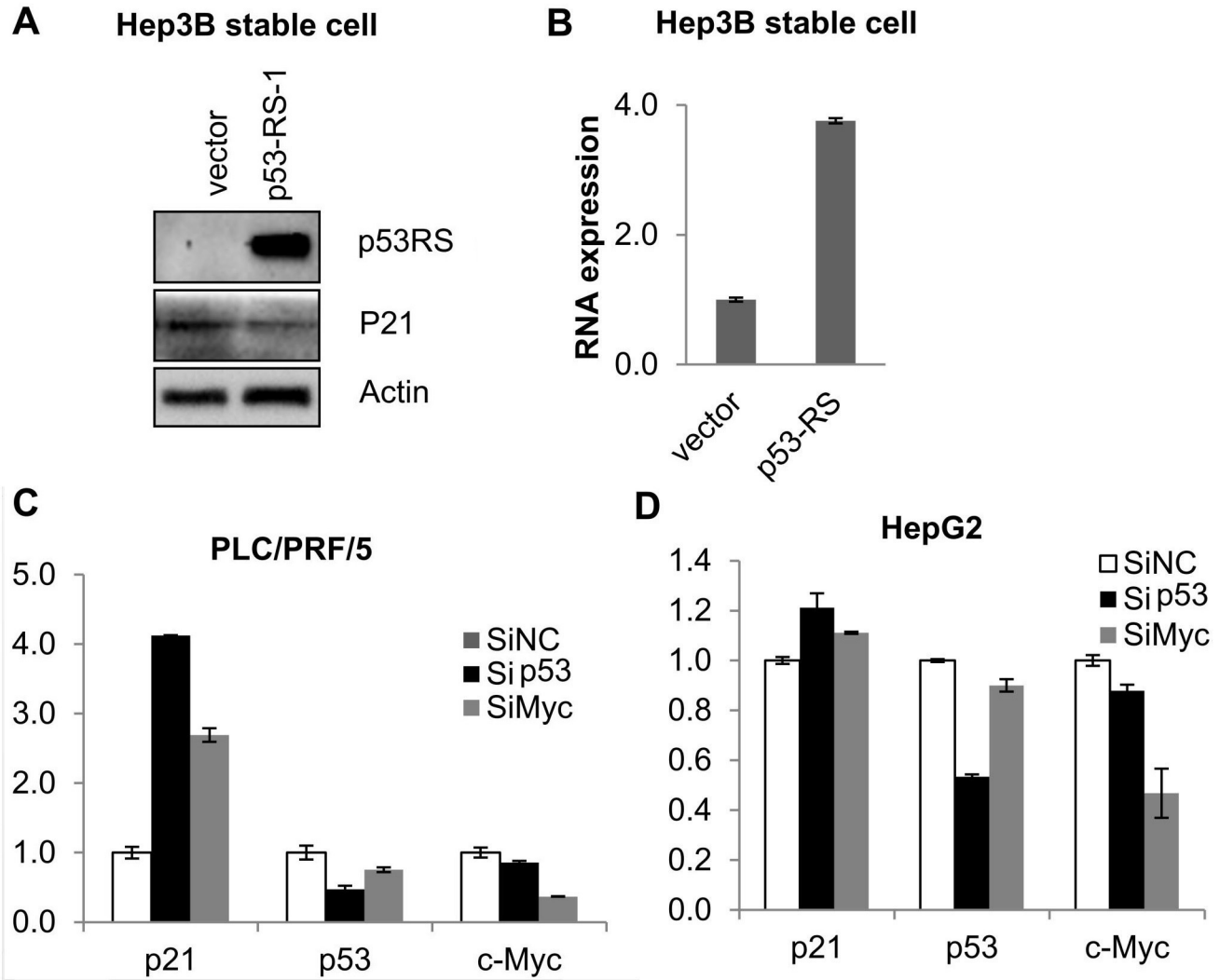


Figure S5 is related to Fig.6

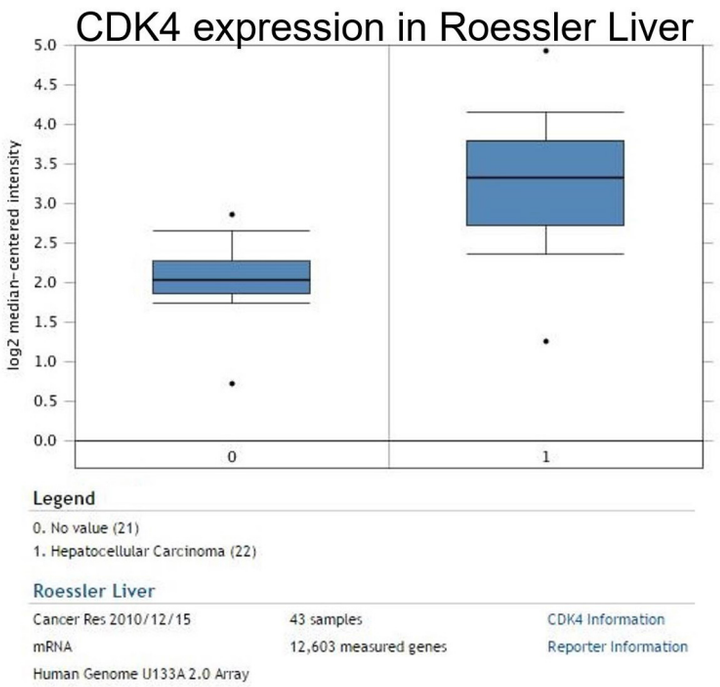
A

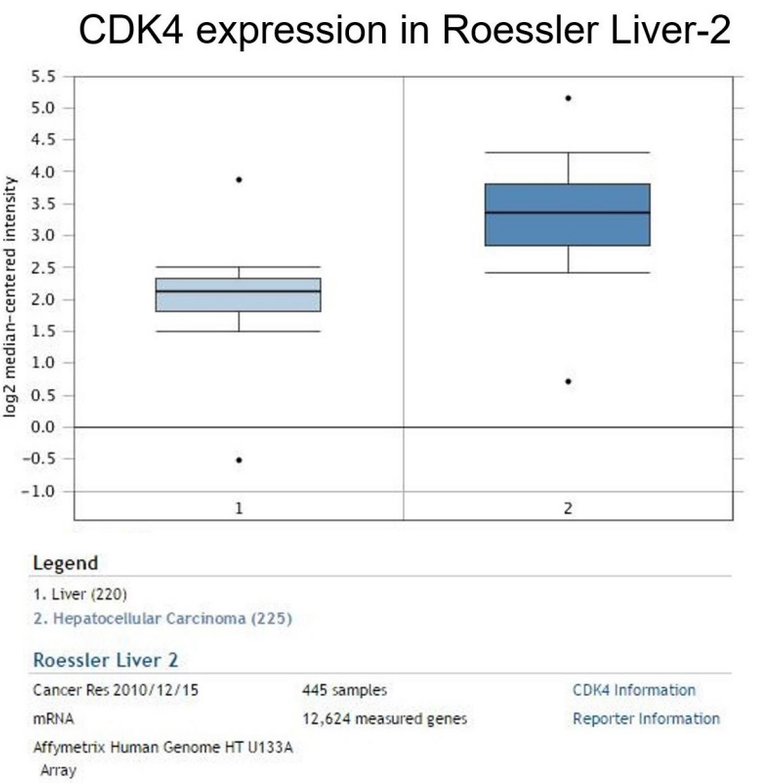
patient sample	R249S	CCND1	CCND2	MYC	CDK4
TCGA-90-7766-01	+	+			
MB-0609	+	+		+	
TCGA-2Y-A9GS-01	+	+			
TCGA-CC-A3M9-01	+	+			
TCGA-CC-A3MB-01	+	+			
TCGA-CQ-5326-01	+	+			
TCGA-CV-7418-01	+	+			
TCGA-HU-A4GY-01	+	+			
WA15	+	+			+
LEXANDERCELLS_LIVE	+				
H092610	+				
ICGC_0228	+				
ICGC_0279	+				
ISTMES2_PLEURA	+			+	
LUAD-S01467	+				
LUAD-S01467-Tumor	+				
MB-6228	+				
MD-279 X	+				
TCGA-34-8455-01	+				
TCGA-55-8089-01	+				
TCGA-66-2734-01	+				
TCGA-77-7142-01	+		+		
TCGA-85-8580-01	+				
TCGA-CC-A5UE-01	+			+	
TCGA-CC-A7II-01	+			+	
TCGA-CC-A8HU-01	+				
TCGA-CG-4300-01	+				
TCGA-DD-A114-01	+				
TCGA-E6-A1LZ-01	+				
TCGA-ED-A7XP-01	+				
TCGA-G3-A25U-01	+				
TCGA-NA-A4R1-01	+			+	
TCGA-QA-A7B7-01	+				
TCGA-QK-A8Z9-01	+			+	
WA50	+				

+: amplification

Valid R249S samples: 35
Amplification samples of CCND1/CCND2 or MYC: 15

B





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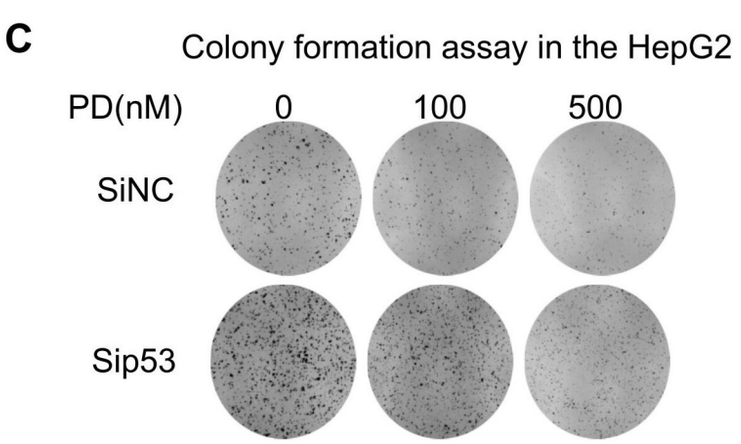

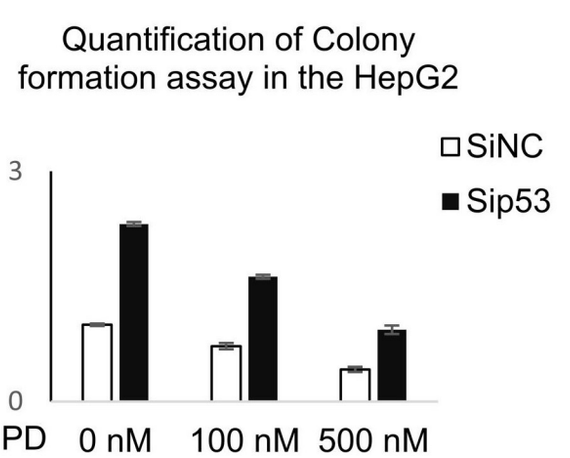

Figure S6 is related to Fig.7

	Patient No.	HBV
P53-S249 patients (mutant)	D0518931	+
	D0559051	+
	D0578322	+
	D0591271	+
	D0473903	+
	D0456940	+
	D0728546	+
P53-R249 patients (WT)	D0167324	-
	D0517844	-
	D0360182	-
	D0439581	+
	D0341468	+
	+: positive; -: negative	