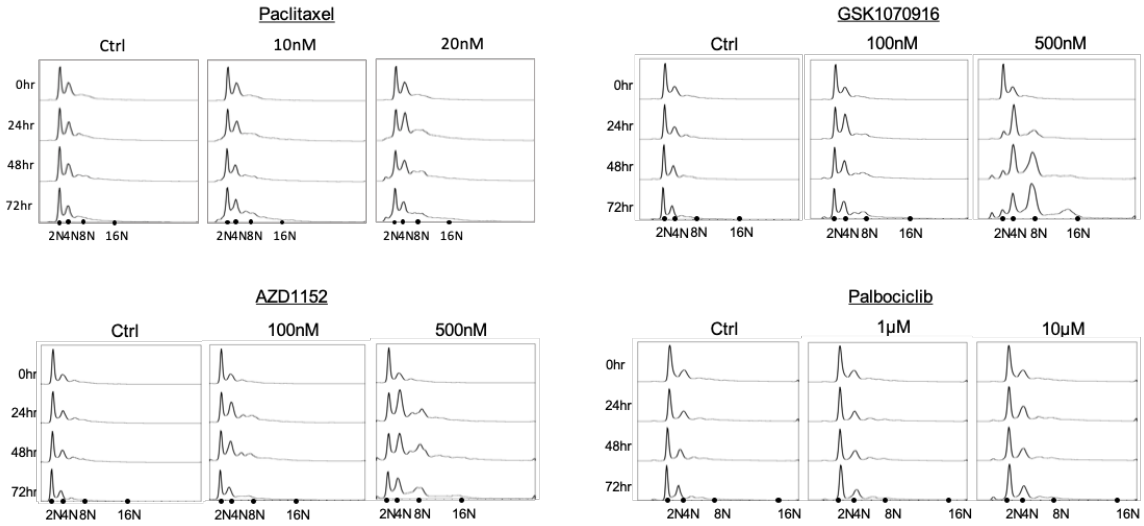
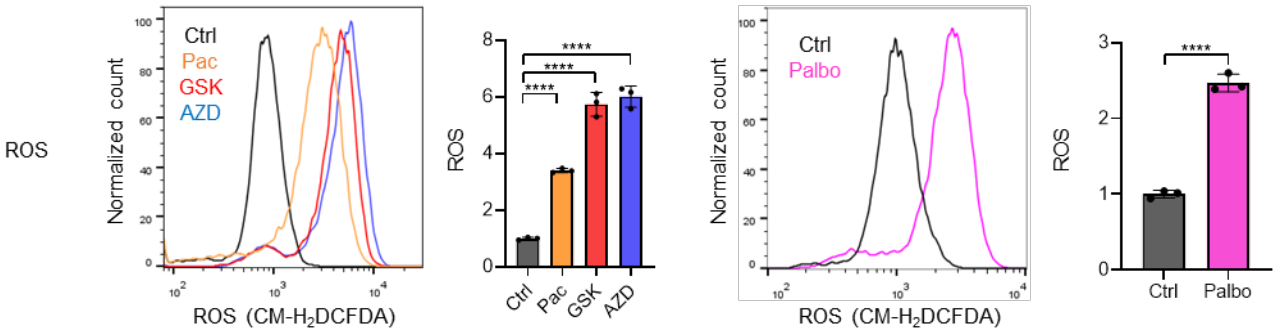
Supplemental material



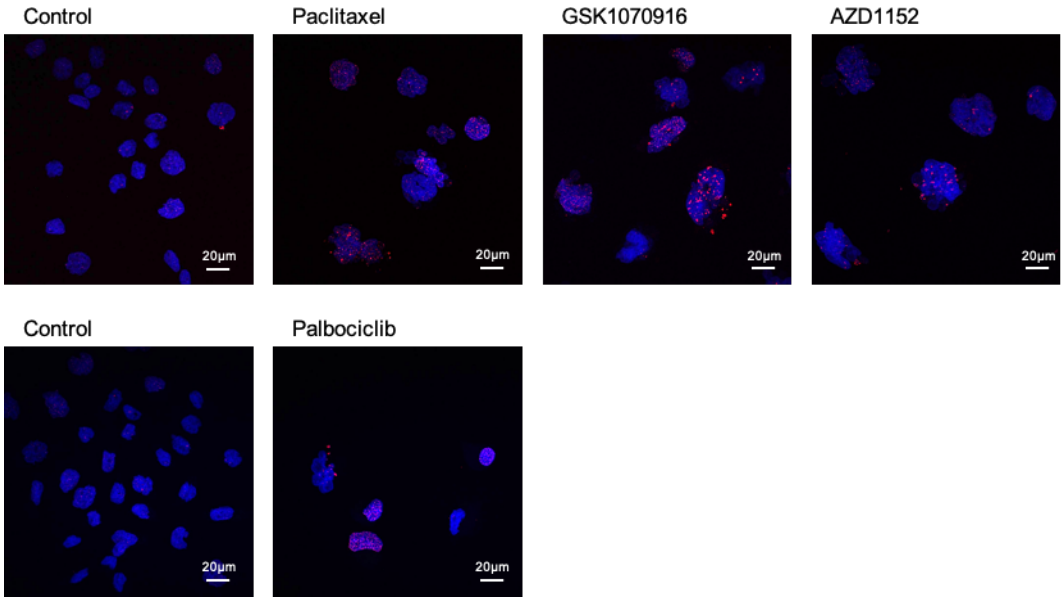
Supplementary figure 1. Cell cycle inhibitors led to genome instability in HCC cells

HCC cell line CLC4 were first synchronized at G1/S boundary with double thymidine block. Upon release from the second thymidine block at 0 hour time point, synchronized HCC cells were then treated with indicated concentration of the cell cycle inhibitors. Cell population were harvested at a 24-hour interval, up to 72 hours. Number of cells analyzed in each treatment (N) ≥ 10,000.



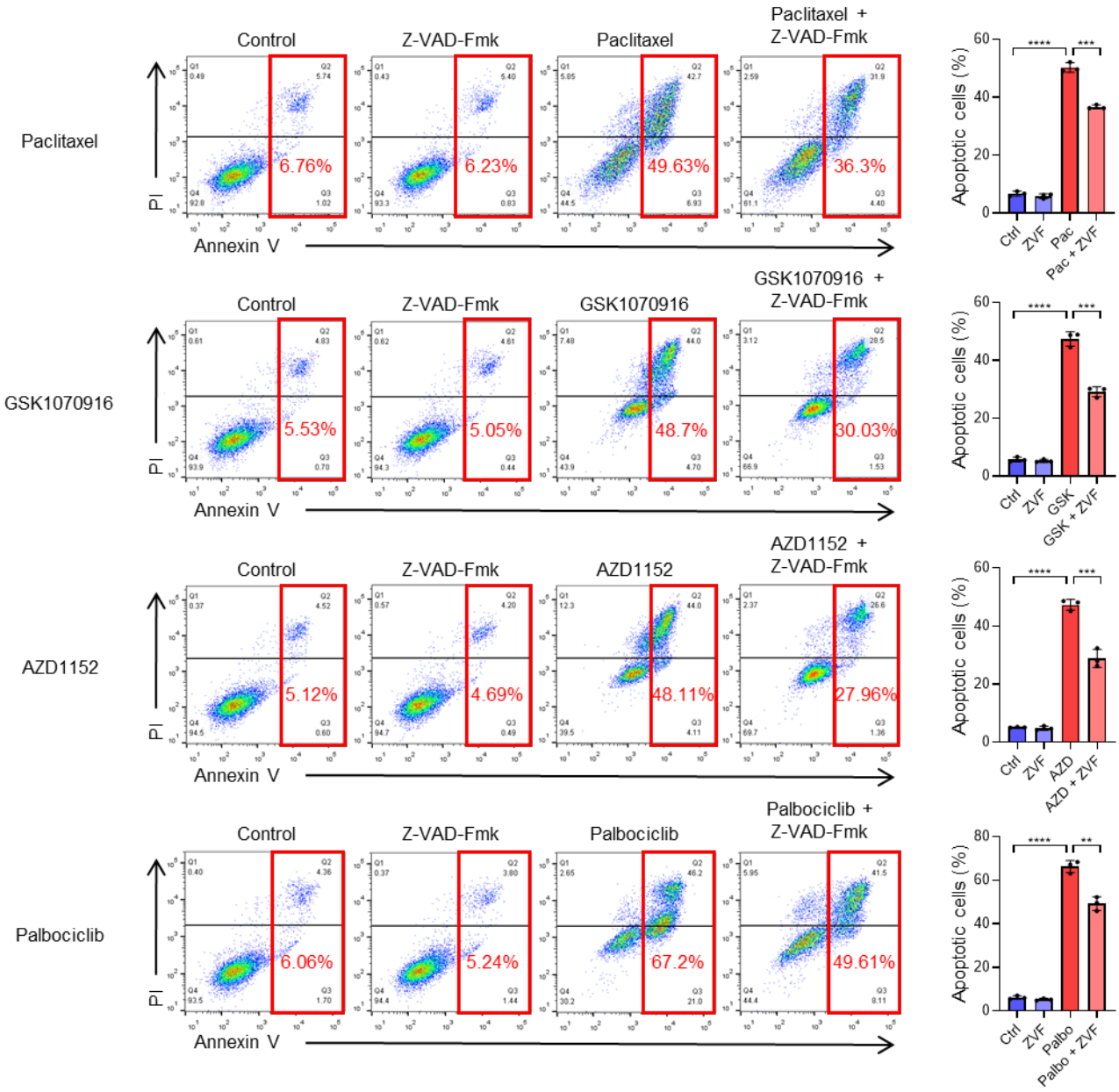
Supplementary figure 2. Cell cycle inhibitors induced cellular oxidative stress in HCC cells

MHCC97L cells were treated with cell cycle inhibitors for 48 hours. The intracellular ROS level was detected using CM-H_2DCFDA. The ROS level in inhibitor-treated cells was normalized to that in control cells (n=3/group). Scatter dot plot: mean with SD. Student's t test. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$.



Supplementary figure 3. Representative images showing DNA damage in cell cycle inhibitors-treated HCC cells

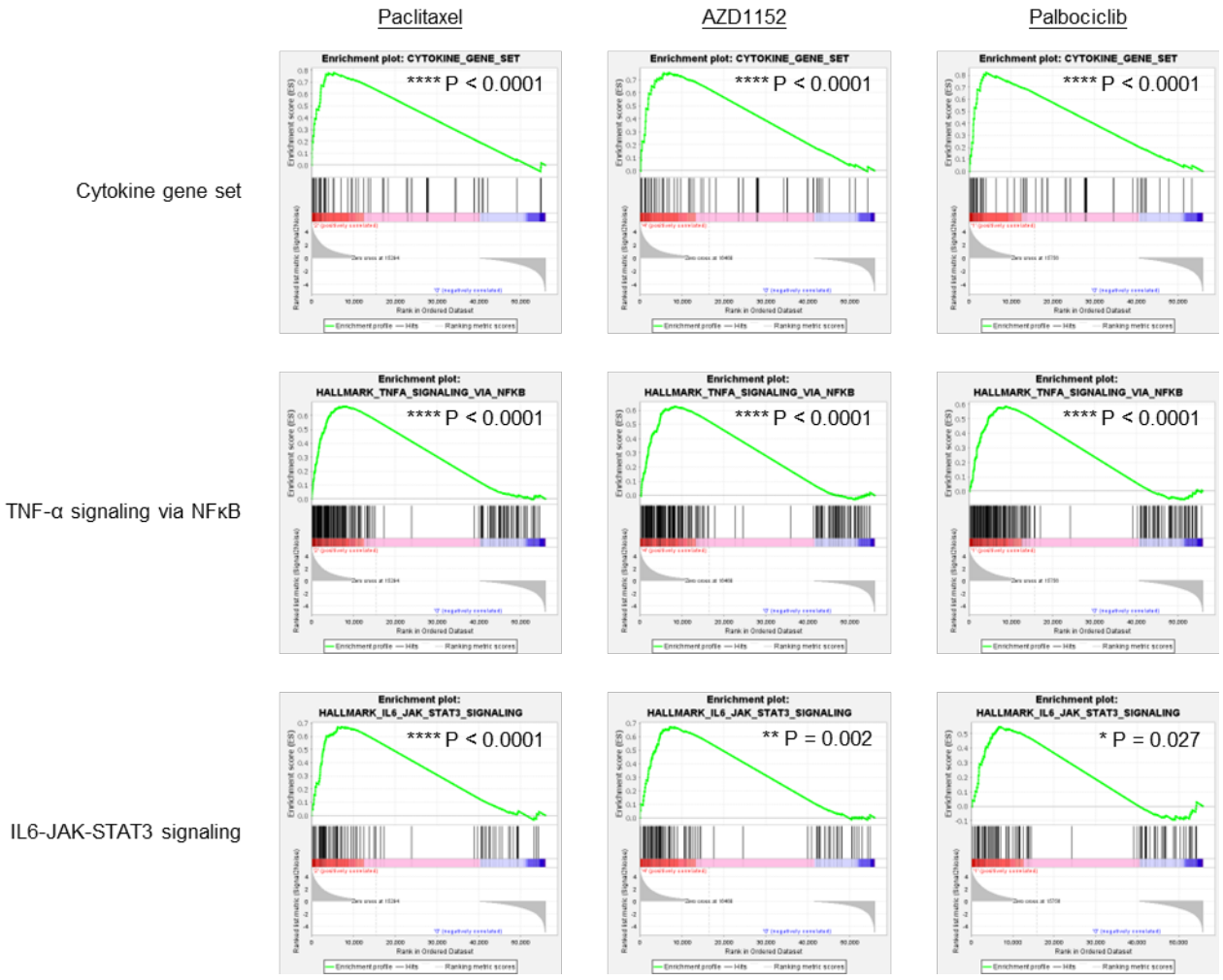
MHCC97L cells were treated with cell cycle inhibitors for 48 hours and subjected to IF staining at 40x magnification. DNA damage was detected using γ-H2A.X. Blue: DAPI; Red: γ-H2A.X. Number of cells analyzed in each treatment (N) ≥ 85. Scale: 20 μm.



Supplementary figure 4. Cell cycle inhibitors induced apoptosis

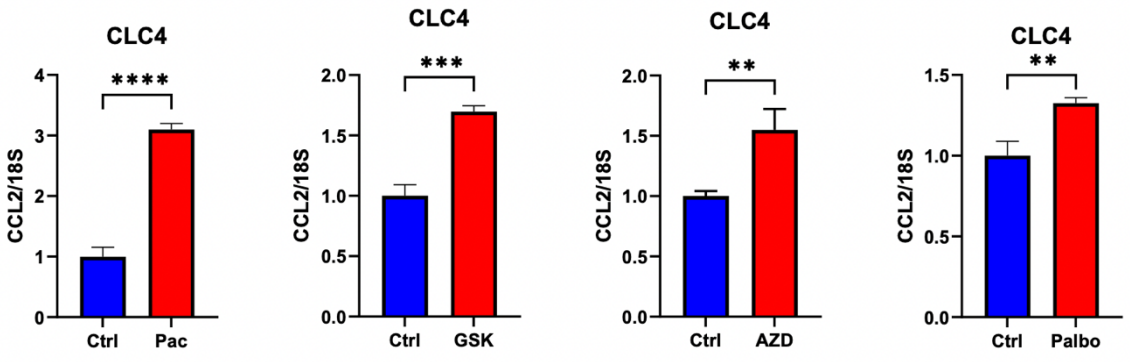
MHCC97L cells were treated with cell cycle inhibitors alone, ZVF alone or co-treated with both cell cycle inhibitors and ZVF for 72 hours. Apoptosis was detected using Annexin V and PI staining and analyzed using flow cytometry. The percentage of apoptotic cells was determined by both Annexin V single positive staining cells and PI-Annexin V double

positive staining cells (n=3/group). Scatter dot plot: mean with SD. Student's t test. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$.



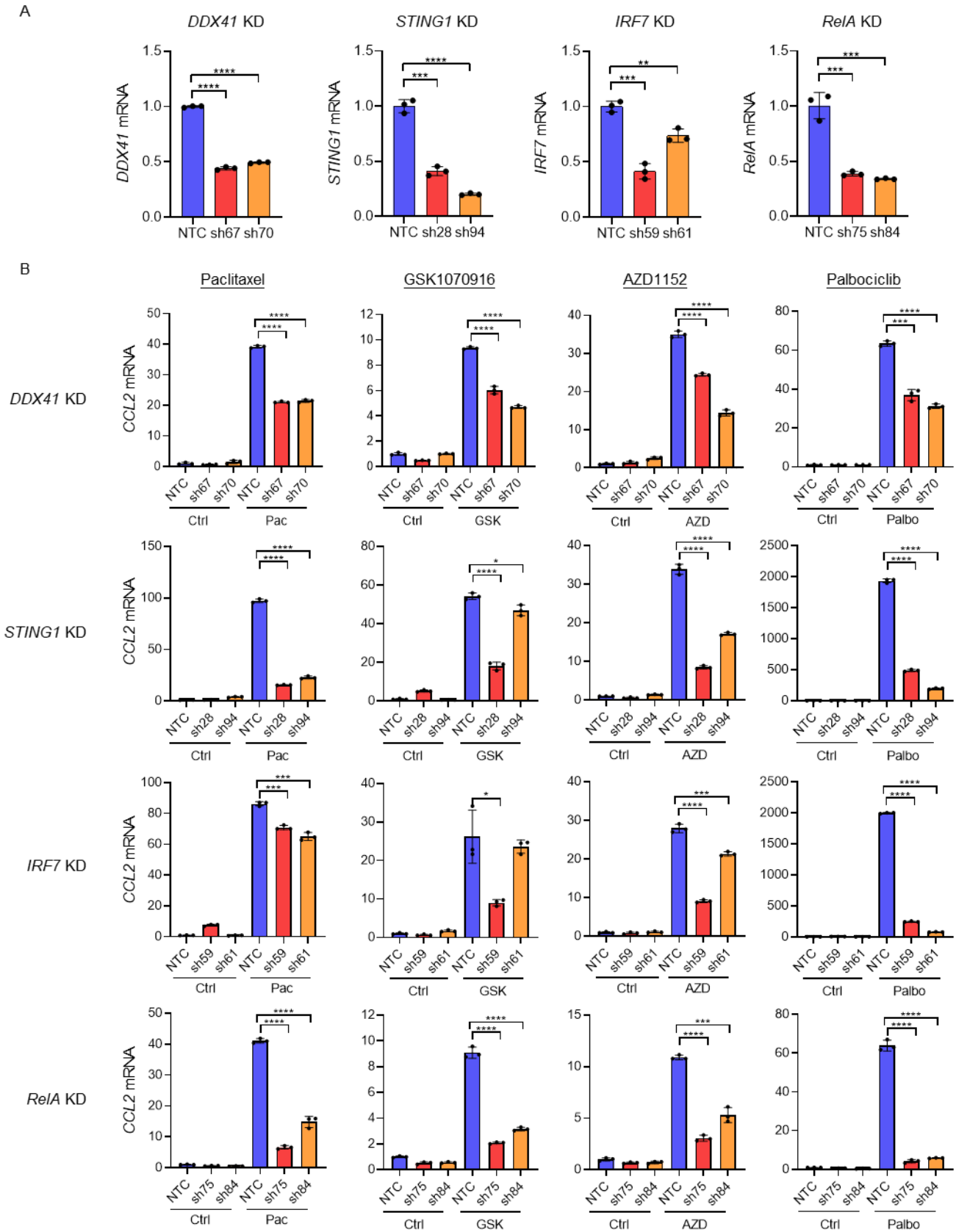
Supplementary figure 5. Pathways enriched in cell cycle inhibitors-treated HCC cells

MHCC97L cells were treated with cell cycle inhibitors for 96 hours. RNA was extracted and prepared for RNA sequencing. RNA sequencing data was used to analyze the enrichment of cytokine gene set, TNF-α signaling via NFκB and IL6-JAK-STAT3 signaling using GSEA. Number of cells analyzed in each treatment (N) ≥ 1e5. Student's t test. * P < 0.05, ** P < 0.01, *** P < 0.001, **** P < 0.0001.



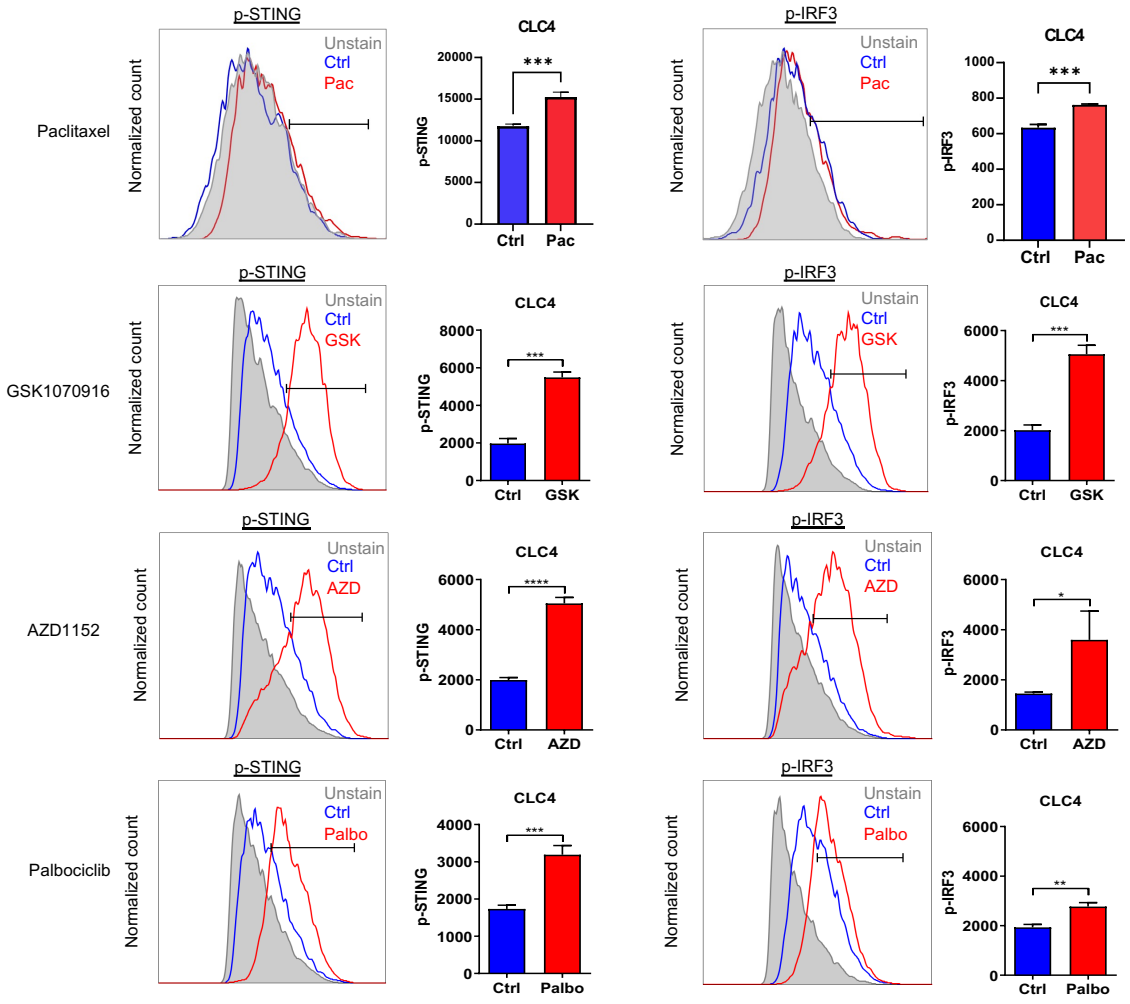
Supplementary figure 6. Cell cycle inhibitors induced SASP expression in HCC cell line

After treatment with cell cycle inhibitors for 192 hours, RNA was extracted HCC cell line CLC4 and mRNA expression of the target gene *CCL2* was determined using RT-qPCR and normalized to housekeeping gene *18S* (n=3/group). Column bar graph: mean with SD. Student's t test. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$.


A
DDX41 KD
STING1 KD
IRF7 KD
RelA KD
DDX41 mRNA
STING1 mRNA
IRF7 mRNA
RelA mRNA
NTC sh67 sh70
NTC sh28 sh94
NTC sh59 sh61
NTC sh75 sh84
B
Paclitaxel
GSK1070916
AZD1152
Palbociclib
DDX41 KD
STING1 KD
IRF7 KD
RelA KD
CCL2 mRNA
Ctrl
Pac
GSK
AZD
Palbo

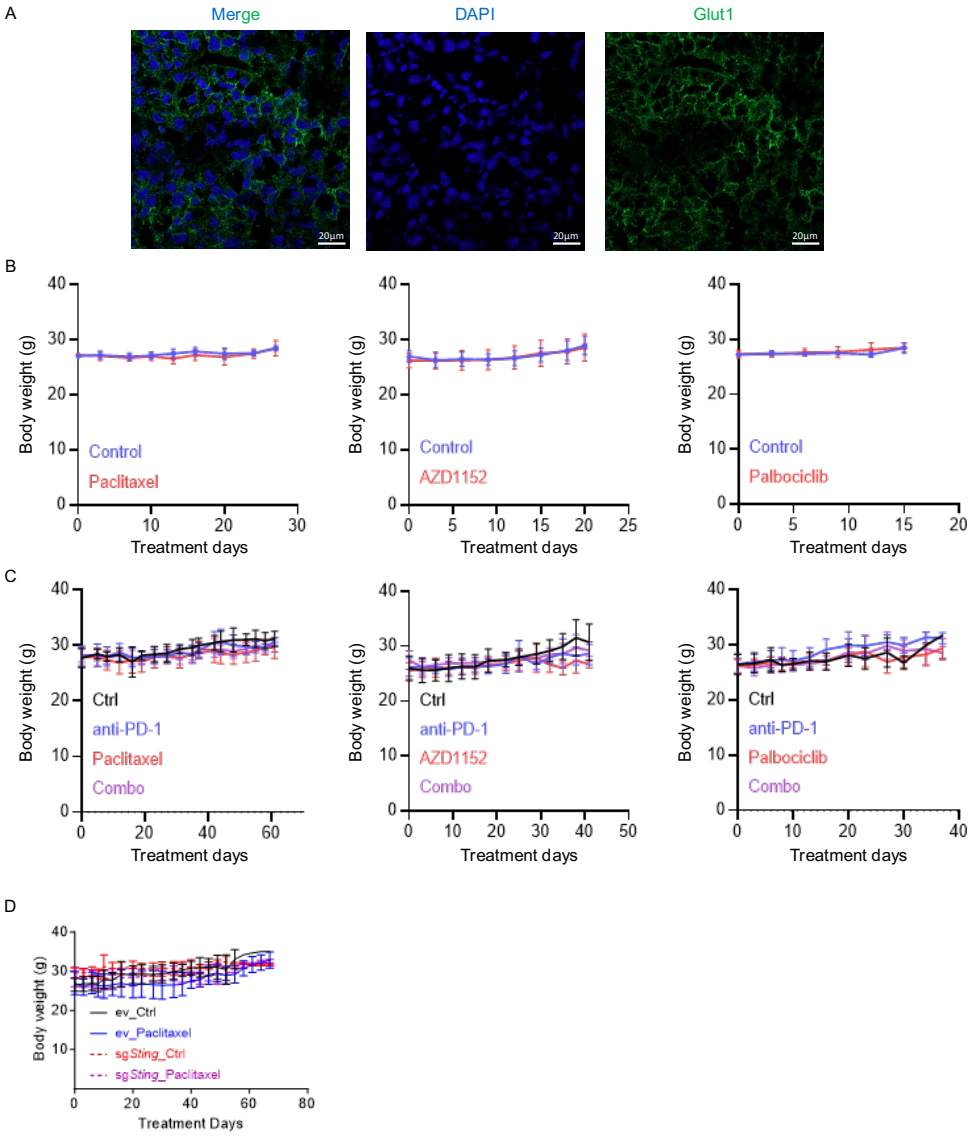
Supplementary figure 7. Cell cycle inhibitors induced SASP expression via DDX41-STING pathway

A. Efficiency of KD of genes. *DDX41*, *STING 1*, *IRF7* and *RelA* KD clones were generated in MHCC97L cells using shRNA. The cells from each KD clones were collected and RNA was extracted. The relative mRNA expression of the target gene was determined using RT-qPCR and normalized to housekeeping gene *18S*. The KD efficiency was determined by normalizing the target gene expression in KD clone to that in non-targeting control (NTC) clone (n=3/group). **B.** MHCC97L stable KD clones were treated with cell cycle inhibitors for 96 hours. The RNA was extracted and mRNA expression of *CCL2* was determined using RT-qPCR (n=3/group). Scatter dot plot: mean with SD. Student's t test. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$.



Supplementary figure 8. Cell cycle inhibitors led to genome instability in HCC cells

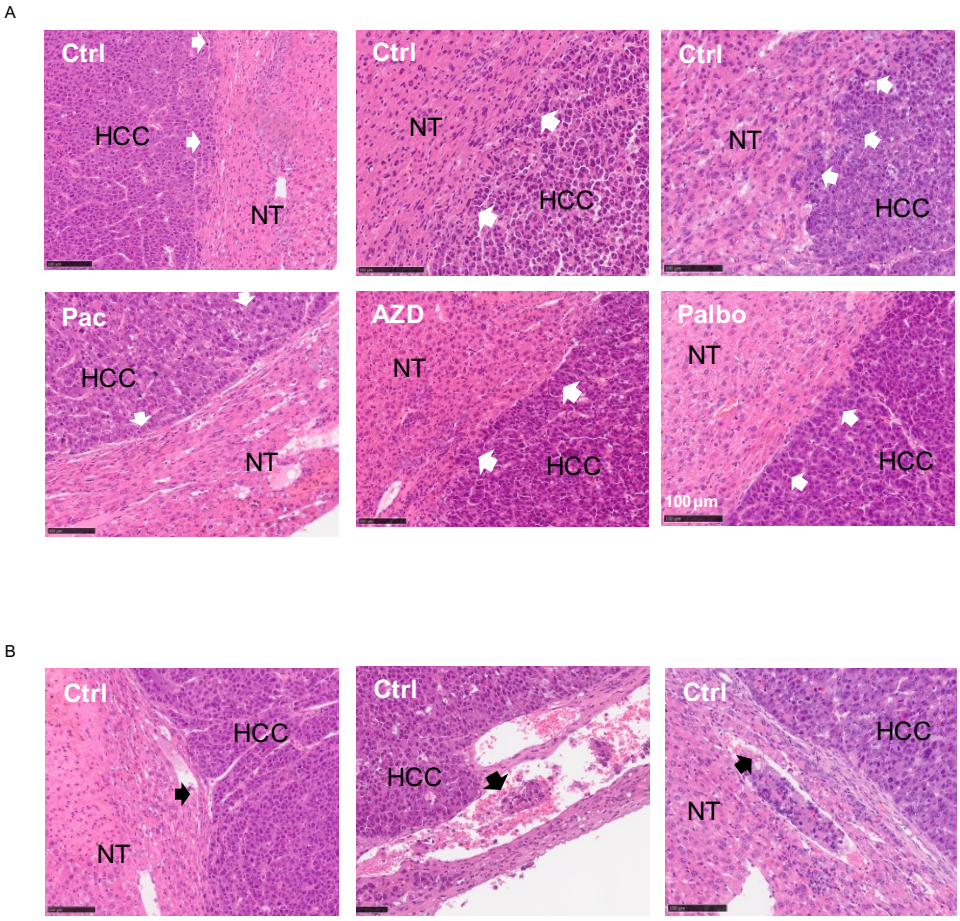
HCC cell line CLC4 were treated with 10 nM Paclitaxel for 192 hours, 100 nM GSK1070916, 100 nM AZD1152 or 10 μM Palbociclib for 120 hours. Upon collection, cells were stained for the phospho-STING (p-STING) and phospho-IRF3 (p-IRF3) with specific antibodies, hence, detected and analyzed using flow cytometry (n=3/group). Column bar graph: mean with SD. Student's t test. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$.



Supplementary figure 9. Cell cycle inhibitors treatment did not cause weight loss in mice with hypoxic HCC tumors

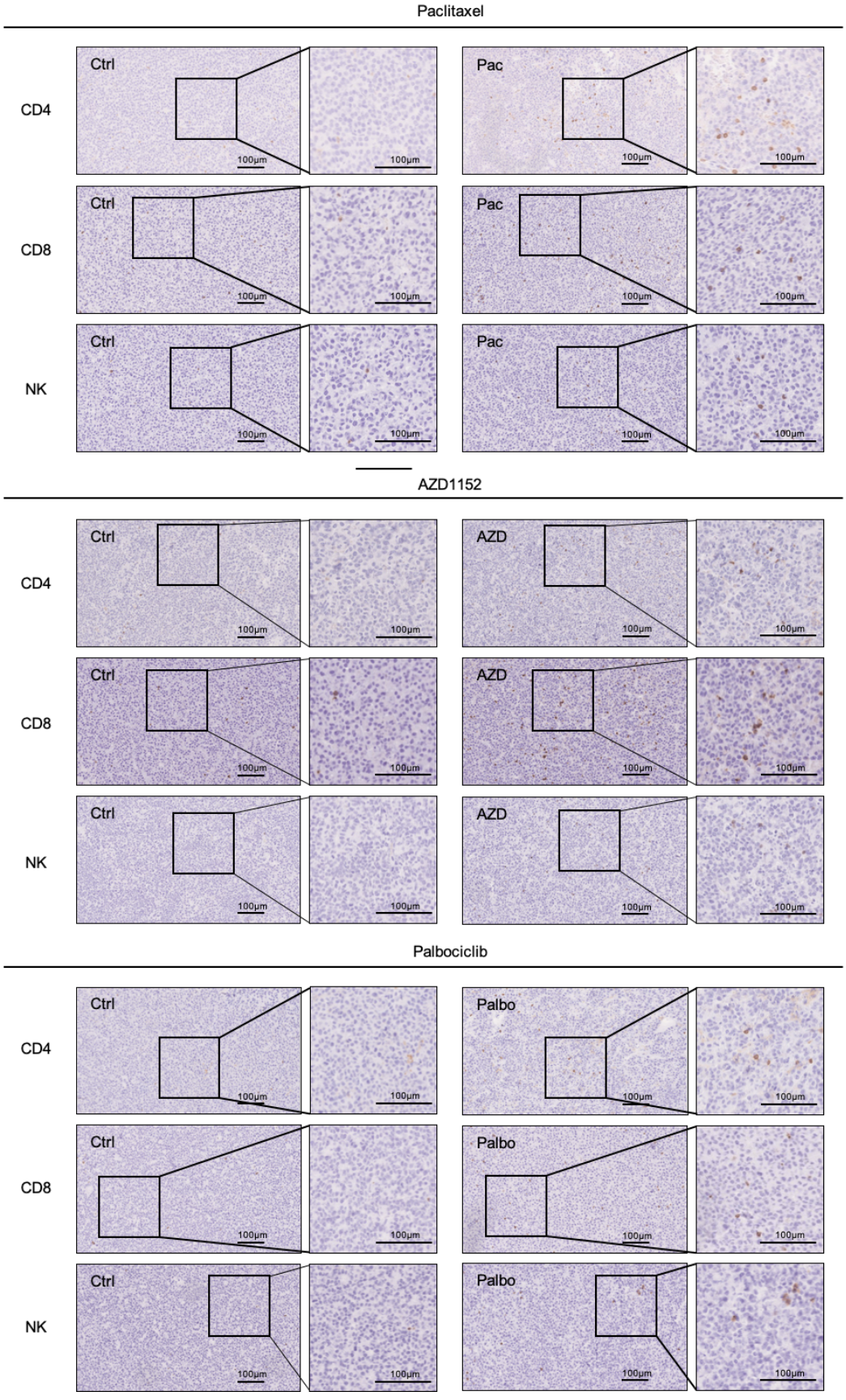
A. Hypoxia in *Trp53*^{KO}/*c-Myc*^{OE} HCC tumor-bearing C57BL/6N mice was detected using glut1 in IF staining. Blue: DAPI; Green: Glut1. Scale: 20 μm. **B - C.** The body weight of mice treated with **B.** single cell cycle inhibitor treatment, or **C.** combination treatment of cell cycle inhibitor with anti-PD-1 were measured regularly during the treatment period. **D.**

The body weight of mice treated with vehicle control (Ctrl) or Paclitaxel treatment. Mean and error: SD**.**



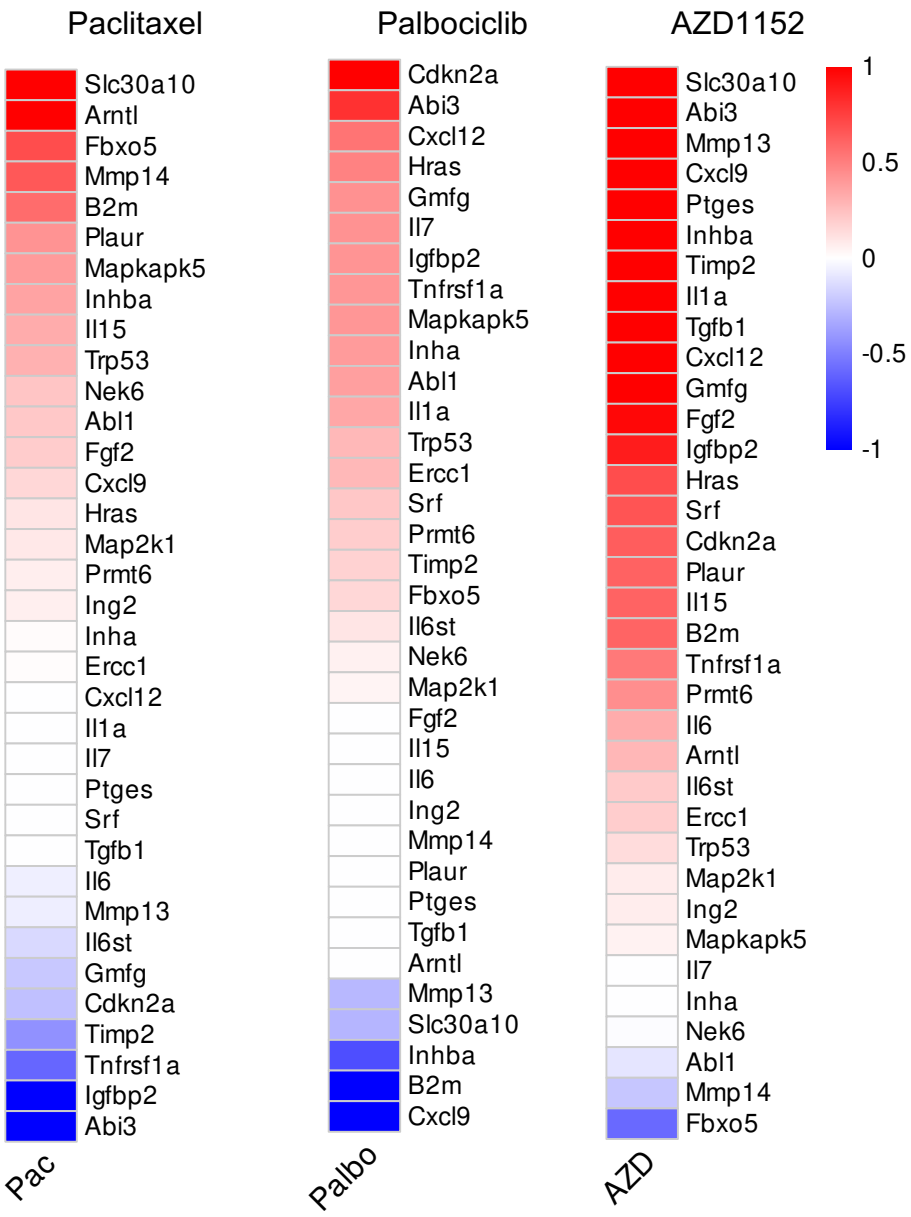
Supplementary figure 10. H&E staining of livers in HCC bearing mice treated with vehicle control (Ctrl) and cell cycle inhibitors (Pac, AZD, Palbo)

Representative images illustrating (A) HCC and non-tumorous (NT) tissues boundary (B) presence of venous invasion (left: Paclitaxel, middle: AZD1152, right: Palbociclib experiments). **A.** White arrows indicate HCC and NT tissues boundary. **B.** Black arrows indicate venous invasion.


Paclitaxel
CD4
CD8
NK
Ctrl
Pac
AZD1152
AZD
Palbociclib
Palbo
100μm

Supplementary figure 11. Representative images showing the IHC staining of CD4 T cells, CD8 T cells and NK cells

Cell cycle inhibitors were administrated to *Trp53*^{KO}/*c-Myc*^{OE} HCC tumor-bearing C57BL/6N mice. The tumors were harvested and prepared for paraffin-embedded slices (The plots were analyzed with 3 sections from each mouse, in total 15 sections from 5 mice). CD4 T cells, CD8 T cells and NK cells were detected using mouse CD4, CD8α and KLrb1c/CD161c antibodies in IHC staining. Scale: 100 μm.



Supplementary figure 12. Senescence markers and SASPs in cell cycle inhibitors-treated mouse HCC.

Heatmap showing results of RNA sequencing performed on cell cycle inhibitors-treated mouse HCC tissues. Drug treated samples were compared with vehicle control treated counterparts. Log2 fold change of gene expressions is shown.

Tables

Treatment group	Mouse_id	Growth Front	Venous Invasion
Ctrl	C1	mostly irregular	venous invasion
	C2	Partial irregular, partial regular	NA
	C3	No tumor boundary could be seen	
	C4	mostly irregular	NA
	C5	Irregular	Multiple Venous Invasion
	C6	mostly irregular	NA
	C7	Partial irregular, partial regular	NA
	C8	Irregular	NA
Paclitaxel	P1	Irregular	NA
	P2	mostly regular	NA
	P3	mostly irregular	NA
	P4	Partial irregular, partial regular	NA
	P5	Partial irregular, partial regular	NA
	P6	Mostly regular	NA
	P7	Mostly irregular	NA
	P8	mostly irregular	NA

Supplementary Table 1. Analysis of H&E staining in vehicle control (Ctrl) treated and Paclitaxel treated mice.

Treatment group	Mouse_id	Growth Front	Venous Invasion
Ctrl	C1	Partially regular, partially irregular	Multiple Venous Invasion
	C2	Irregular	NA
	C3	Irregular	NA
	C4	Irregular	NA
	C5	Irregular	NA
	C6	Irregular	Venous Invasion
	C7	regular	NA
	C8	regular	NA
AZD	A1	Irregular	NA
	A2	regular	NA
	A3	regular	NA
	A4	regular	NA
	A5	regular	NA
	A6	regular	NA
	A7	regular	NA
	A9	Partially regular, partially irregular	NA

Supplementary Table 2. Analysis of H&E staining in vehicle control (Ctrl) treated and AZD1152 treated mice.

Treatment group	Mouse_id	Growth Front	Venous Invasion
Ctrl	C1	Partially regular, partially irregular	NA
	C2	Partially regular, partially irregular	Venous Invasion
	C3	Irregular	NA
	C4	Partially regular, partially irregular	NA
	C5	Irregular	NA
	C6	Irregular	NA
	C7	Irregular	NA
	C8	Partially regular, partially irregular	NA
Palbo	Palbo1	regular	NA
	Palbo2	Partially irregular	NA
	Palbo3	Irregular	NA
	Palbo4	regular	NA
	Palbo5	regular	NA
	Palbo6	regular	NA
	Palbo7	regular	NA

Supplementary Table 3. Analysis of H&E staining in vehicle control (Ctrl) treated and Palbociclib treated mice.

shRNA	Target sequence
HIF1A	GTTACGTTCCTTCGATCAG
HIF1B-97	GGCTCAAGGAGATCGTTTATT
HIF1B-98	ACTAGGTCCCACAGCTAATTT
DDX41-67	GCCACTACCTTCATCAACAAA
DDX41-70	CGCCACTACCTTCATCAACAA
STING1-28	CCAACATTCGCTTCCTGGATA
STING1-94	GTTTACAGCAACAGCATCTAT
IRF7-59	GCTGGACGTGACCATCATGTA
IRF7-61	CTGTTCGGAGAGTGGCTCCTT
RelA-75	GGAGTACCCTGAGGCTATAAC
RelA-84	CGGATTGAGGAGAAACGTAAA

Supplementary Table 4. List of shRNA target sequences

Target gene	5'-3' sequence
18S Forward	GAGGATGAGGTGGAACGTGT
18S Reverse	AGAAGTGACGCAGCCCTCTA
CCL2 Forward	CAAGTGTCCCAAAGAAGCTGTG
CCL2 Reverse	GGTTTGCTTGTCCAGGTGGT
DDX41 Forward	ATCCCACCACCCATCAAGAG
DDX41 Reverse	TGTCACGGCCAGATAGAATGG
STING1 Forward	CATGGGCTGGCATGGTCATA
STING1 Reverse	CCCCGTAGCAGGTTGTTGTA
IRF7 Forward	GCTCCCCACGCTATACCATCTA
IRF7 Reverse	AGCCAGGGTTCCAGCTTCAC
RelA Forward	GCGAGAGGAGCACAGATACC
RelA Reverse	TCCCCACGCTGCTCTTCTAT
DDX41_HRE_536F	TCAAAACCACTGCCATCCGT
DDX41_HRE_536R	GGGATAAACCGCTCGACACA
DDX41_HRE_422/365F	TGTGTCGAGCGGTTTATCCC
DDX41_HRE_422/365R	CTCCCAGAGCATGGCGTCTT
DDX41_HRE_36/2F	CGTCGTTCGCCTCTTCACA
DDX41_HRE_36/2R	GGTTCCGACTCCTCCATTCTTT

Supplementary Table 5. List of primer sequences

Antibody	**Supplier**	**Amount / Dilution**	**Catalogue no.**	**purpose**
HIF-1α	Abcam	5 µg	ab1	ChIP
HIF-1β	Abcam	5 µg	ab2	ChIP
Mouse IgG	Santa Cruz	5 µg	sc2762	ChIP
Rabbit IgG	Invitrogen	5 µg	10500C	ChIP
DDX41 (D3F1Z)	Cell Signaling Technology	1:1000	15076	WB
Histone H3	Sigma	1:3000	05-928	WB
STING (D2P2F)	Cell Signaling Technology	1:1000	13647	WB
HIF-1α	Cell Signaling Technology	1:1000	3716	WB
Phospho-Histone H2A.X (Ser139)	Sigma	1:1000	05-636-I	WB
β-actin	Sigma	1:2500	A5316	WB
Mouse IgG HRP Linked	Sigma	1:2500	NA931	WB
Rabbit IgG HRP Linked	Sigma	1:2500	NA934	WB
Glut 1	Abcam	1:200	ab15309	IF
Phospho-Histone H2A.X (Ser139)	Sigma	1:200	05-636-I	IF
Goat anti-Rabbit IgG (H+L) Alexa Fluor 488	Invitrogen	1:1000	A27304	IF
Goat anti-Mouse IgG (H+L) Alexa Fluor 594	Invitrogen	1:600	A11005	IF
Mouse CD4	Cell Signaling Technology	1:100	25229	IHC

Mouse CD8α	Cell Signaling Technology	1:200	98941	IHC
Mouse KLrb1c/CD161c (NK)	Cell Signaling Technology	1:200	24991	IHC
Phospho-STING (Ser366)	Cell Signaling Technology	1:50	41622	FC
Phospho-IRF3 (Ser386)	Cell Signaling Technology	1:50	96421	FC

Supplementary Table 6. List of antibodies