Materials and Methods

Cell proliferation assays and toxicity: The proliferation of multiple cell lines in response to various concentrations of the indicated drugs were determined using live cell imaging in Cytation 5 and IncuCyte imaging systems. The cell counts were determined based on the number of GFP-labelled nuclei and the data were exported to GraphPad Prism for statistical analysis and graph generation. The cell-cycle progression in response to drug treatments were determined using a chemiluminescent ELISA BrdU incorporation assay (Sigma; 11669915001) following the manufacturer's protocol. Luminescence was read on a BioTEK Synergy 2 plate reader. The toxic effects in response to drugs treatments were determined using CTG assay. Each drug treatment was performed in triplicates and confirmed in two independent experiments.

Drug Screen: Cytation 5 live cell imaging system was used to perform drug screen analysis in the PDAC cell line, 1222.that were labelled with H2B-GFP. Cells were seeded in 384-well dish and pretreated with DMSO and palbociclib (200 nM) for 24 H. Following 24 H pretreatment, drugs from the library were added at a dose 500 nM and cell growth was monitored every 12 hours up to 5 days.

Immunoblot analysis: Cells were lysed using RIPA lysis buffer (10 mM Tris HCl, pH 8.0, 1mM EDTA, 150 mM NaCl, 1% Triton-X-100, 0.1% sodium deoxycholate, 0.1% SDS) in the presence of 1X Halt protease inhibitor (Thermo Fisher) and 1 mM PMSF (Sigma, St Louis, MA). The resulting proteins were resolved on an SDS-PAGE gel and the proteins were then transferred to PVDF membrane for immune blotting. The PVDF membranes were incubated with protein-specific primary antibodies overnight at 4°C followed by incubation with HRP-tagged anti-mouse or anti-rabbit secondary antibodies for 1 hour at room temperature. An enhanced chemiluminescence kit (Thermo Fisher, Waltham, MA) was used to detect the immuno-reactive bands. The primary antibodies purchased from Cell-Signaling Technology (Danvers, MA) include pRB S807/811 (8516S), pRB S780 (9307L), pCDK2 T160 (2561S), CDK2 (2546S), CDK4

(12790S), CDK6 (3136S), P27KIP1 (3686S), P21 (2947S), SKP2 (2652S) and cyclin B1 (12231S). Cyclin D1 (SC20044), cyclin A (SC271682), CDK1 (SC-54), kRAS (SC-30), β Actin (SC47778) and GAPDH (SC-47724) were purchased from Santacruz Biotech, Dallas, TX. Mouse-IgGκ-HRP (Santacruz; SC516102) and Goat-anti-rabbit-HRP (Thermo Fisher; 31460) were used as secondary antibodies. An enhanced chemiluminescent substrate (National Diagnostics; CL-300) was used to detect the immunoreactive bands.

I**mmunoprecipitation:** Whole cell extracts were prepared by lysing the cells in the IP-lysis buffer (20 mM Tris-HCl, pH 8.0, 2mM EDTA, 137 mM NaCl, 1% NP-40) in the presence of 1X Halt protease inhibitor (Thermo Fisher) and 1 mM PMSF (Sigma, St Louis, MA). In total, 0.5-0.8 mg of protein from the lysates were incubated with 5 µg of anti-P27KIP1 antibody (Cell Signaling; 3686S) and anti-cyclin D1 antibody (5 µg) (Invitrogen: MA5-12707) overnight at 4°C. Mouse (Cell Signaling, 5415S) or rabbit (Cell Signaling, 3900S) IgG1 isotype control was used. Protein immunocomplexes were then incubated with Protein G-agarose or protein A-agarose (Thermo Fisher) at 4°C up to 4H and were then washed 3 times with IP wash buffer (20 mM Tris-HCl, pH 8.0, 100 mM NaCl, 0,5% NP-40). Complex bound to the protein beads were eluted using 2X SDS buffer and were subjected to western blotting.

***In vitro* kinase reactions:** The cyclin D1 associated CDK4 kinase activity was determined by immunoprecipitating cyclin D1 complexes from the whole lysates. Cells were lysed with kinase - lysis buffer containing 50 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.1% NP-40, 10 mM DTT, 10% glycerol in the presence of 1x Halt protease inhibitor (Thermo Fisher) and 1 mM PMSF. Active cyclin D1 complex was immunoprecipitated by incubating the whole cell lysate (300 µg) with anti-cyclin D1 antibody (5 µg) (Invitrogen: MA5-12707) overnight at 4°C. Normal mouse IgG (Cell signaling Technology, 5415S) was used an isotype control. Protein G-agarose beads were added to each of the IP samples and incubated up to 4 hours at 4°C to immunoprecipitated the cyclin D1 complex. Following immunoprecipitation, the complexes were washed 3 times with the kinase-

lysis buffer and 2 times with the kinase-reaction buffer (50 mM HEPES-KOH, pH 7.5 20 mM $MgCl_2$, 1mM DTT). Kinase reactions were carried out in the presence of 2mM ATP and 0.5 µg of recombinant RB C-terminal as substrate [1]. The resulting RB phosphorylation was detected by western blotting using anti-pRB (S780) antibody (Cell Signaling, 9307L).

The enzymatic activities of CDK2 and CDK1 were determined *in vitro* by following the procedure as described in our previous study [2]. CDK1 was pulled down using the mouse CDK1 antibody from Santa Cruz (SC-54) The RB phosphorylation was detected by western blotting using anti-PRB-antibody (S807/811) (Cell signaling, 8516S).

Immunofluorescence: Cells were seeded on glass coverslips and allowed to grow for 48 hours. Cells were then washed with 1X PBS, fixed with methanol for 5 minutes and permeabilized in 0.5% Triton X-100. To determine the RB expression, the permeabilized cells were blocked using IF buffer (1X PBS, 5% BSA, 0.4% NP-40) and then incubated with the primary antibody, RB(4H1) (Cell Signaling; 9309L) at 37 °C for 1 hour. Following primary antibody incubation, the cover slips were washed and incubated with goat anti-mouse IgG-Alexa Fluor 488 (Invitrogen; A32723) in the presence of DAPI in IF buffer for 30 minutes. For Phalloidin staining, cells on the cover slips were fixed using 4% Formaldehyde for 15 minutes and permeabilized. Cells were incubated with Phalloidin conjugated with Alexa Fluor 488 (Thermo Fisher A12379) in the presence of DAPI for 1 hour. Cover slips were washed again in PBS after secondary antibody incubation and mounted on glass slides. Pictures were taken using EVOS fluorescence microscope at 40X magnification.

Protein Stability assays: The stability of cyclin D1 was determined using cycloheximide (CHX) (Santacruz Biotechnology; SC-3508). Cells were cultured in the appropriate growth media up to 48 hours and were exposed to CHX (50 µg/ml) for different time points. Following CHX treatment cells were harvested and lysed for western blotting.

Flow Cytometry Cell-cycle analysis: To determine the cell-cycle profile based on DNA content, cells were trypsinized and fixed in ice cold 70% ethanol overnight at -20°C. Fixed cells were pelleted and washed with PBS. Prior to analysis using BD LSR FORTESSA flow cytometer, cells were stained with propidium iodide (40 µg/ml) in the presence of RNaseA (200 µg/ml).

Senescence Associated β- Galactosidase staining: MCF7-WT, MCF7 D1/K4 and MCF7-RB-del cell lines were treated with PF06873600 (200 nM) up to 6 days. The cells were stained for β-galactosidase to determine the senescence phenotype by using the commercially available kit (Cell Signaling; 9860) according to the manufacturer's protocol. Cell images were taken using phase-contrast microscope at 20X magnification.

Knockdown experiments: Cells were reverse transfected with siRNA using Dharmacon Human On-target plus siRNA: CDK4 (L-003238-00-0005), CDK6 (L-003240-00-0005), Cyclin E1 (M-003213-02-0005), Cyclin A (L-003205-00-0005), kRAS (L-005069-00-0005), CDKN1B (L-003472-00-0005), SKP2 (L-003324-00-0005) and non-targeting siRNA (D-001810-10-05). CDK2 siRNA (ID# 103569) was purchased from Thermo Fisher. Transfection was performed using Lipofectamine RNAiMax Transfection Reagent (Invitrogen, 13778150). Following 24 H transfection cells were treated with palbociclib or DMSO for another 48 H and 72 H for Western blotting and BrdU incorporation assay respectively as described in supplementary information.

Organoid Cell culture: The H2B-GFP labelled T47D and 1222 cell lines were seeded in 96 well dish (4000 cells/well) that were precoated with 75% Matrigel (Corning; 354234). Cells were allowed to form organoids up to 24 H and were treated with different concentrations of palbociclib. Immediately after drug treatment, the growth of organoids was monitored every day using live cell imaging (Cytation 5 cell imager). Based on the organoid area, the relative growth was calculated to determine the drug effect. Data were exported to GraphPad Prism for statistical analysis and

graph generation. Cells from each condition were seeded in triplicates and the experiment was performed at two independent times.

Mice and xenografts: Mice bearing MCF7 xenografts and PDAC PDX were treated for 3 weeks by gastric gavage with vehicle control, palbociclib (100 mg/kg) diluted in 50 mM lactate buffer, pH 4.0 was administered orally for 5 days during the 1st week followed by a 2-day break and then administered every other day during the 2nd and 3rd week. For the combination arm in PDAC PDX, trametinib (0.5 mg/kg) diluted in 50 mM lactate buffer, pH 4.0 was administered orally for 5 days during the 1st week followed by a 2-day break and then administered every other day during the 2nd and 3rd week. For HCC1806 xenografts mice were treated with palbociclib (75 mg/kg) diluted in 50 mM lactate buffer, pH 4.0 was administered orally for 5 days during the 1st week followed by a 2-day break and then administered every other day during the 2nd week. PF06873600 (30 mg/kg) diluted in 40% PEG300, 5% Tween-80, 45% saline and 10% DMSO was administered orally every day. Tumor size was measured every other day using digital calipers and the tumor volume was calculated based on the following equation: (greatest diameter*(shortest diameter^2))/2. Mice were sacrificed, and the tumors were harvested at the end of treatment or when the tumor size reached 2000 mm^3.

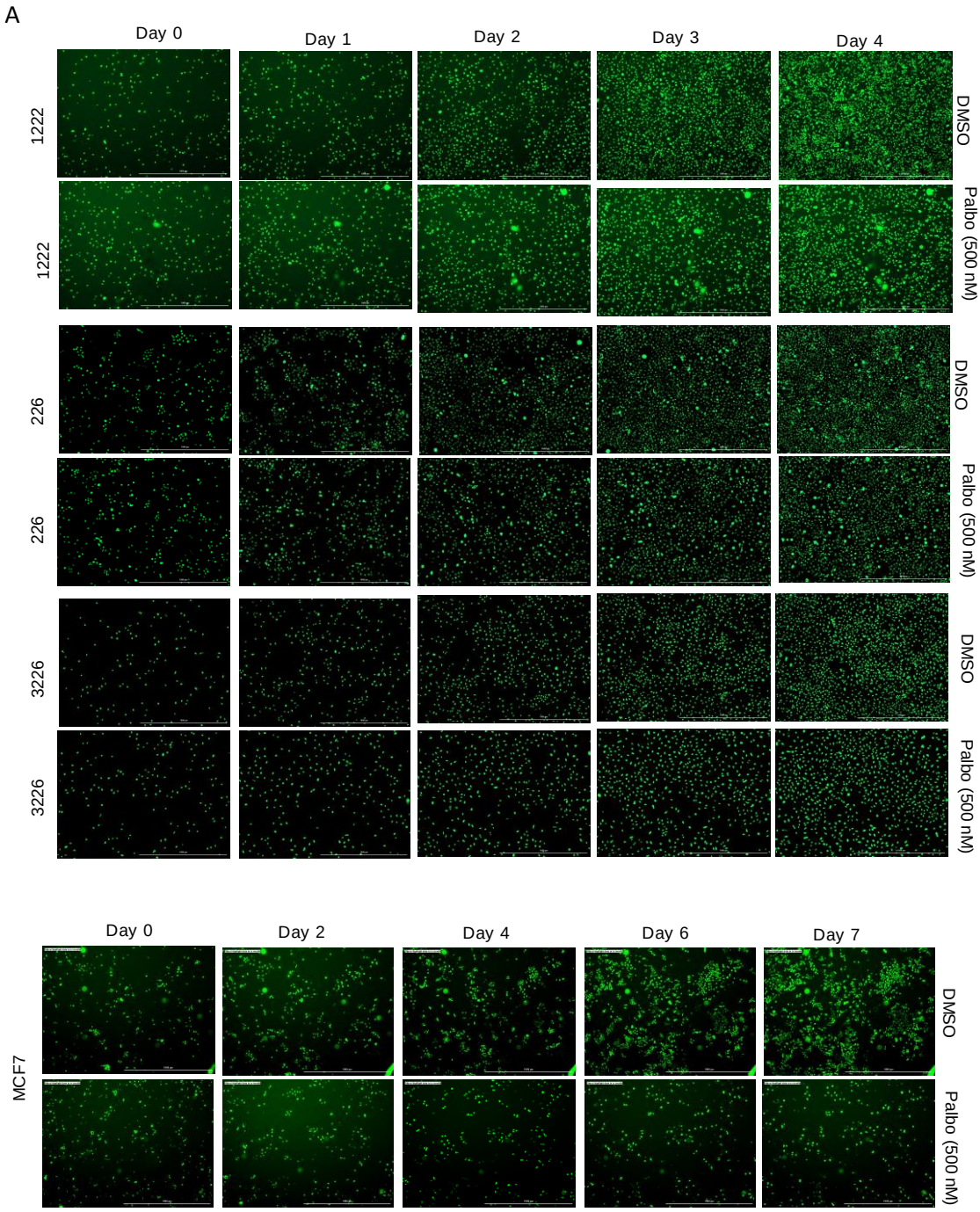
Gene expression analysis: RNA was isolated from MCF7 and 226 PDAC cell lines that were treated with palbociclib (250 nM) and PF06873600 (200 nM) up to 48 hours using RNeasy Plus kit (Quiagen). Extracted RNA was used for 50 bp paired-end RNA sequencing as described in previous studies [2, 3]. The downregulated genes were generated based on the log fold change and Student's two-sided t test p-value for each treatment conditions relative to the control from the normalized reads. Genes that were significantly downregulated in the palbociclib and PF06873600 treated samples were used for gene ontology analysis using ENRICHR as described previously [4]. All log-fold change and statistical information is provided in the supplemental data tables S1 and S2.

Immunohistochemical and multi-spectral imaging: Hematoxylin and eosin (H&E) staining and immunohistochemistry with KI67 antibody (RM-9106S1-1:150) on tumor tissues were performed following formalin fixation using the standard procedures. Staining was done on a Leica auto-stainer and image processing was performed using Aperio (Leica Biosystems). The panel for multi-spectral staining include primary antibodies include; pRB S807/811 (Cells Signaling; 8516S), P27 (Santacruz; SC-1641) and pHH3 (S10) (Millipore; #06-570). Images were captured on a Vectra Polaris Instrument (Perkin Elmer) and phenotype counts were determined using InFORM software (Perkin Elmer).

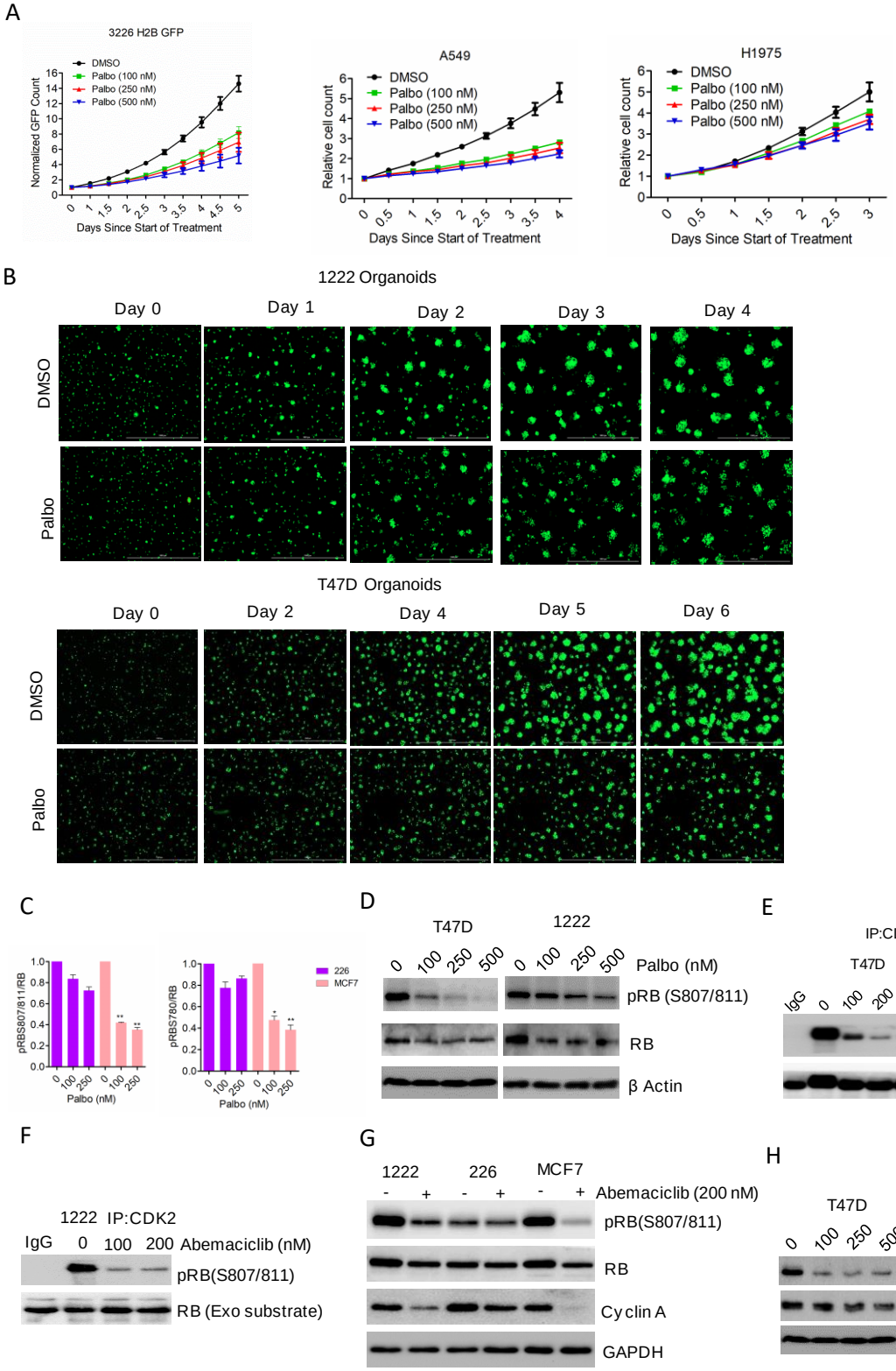
References

1. Knudsen, E.S. and J.Y. Wang, *Differential regulation of retinoblastoma protein function by specific Cdk phosphorylation sites.* J Biol Chem, 1996. **271**(14): p. 8313-20.
2. Knudsen, E.S., et al., *Cell cycle plasticity driven by MTOR signaling: integral resistance to CDK4/6 inhibition in patient-derived models of pancreatic cancer.* Oncogene, 2019.
3. Knudsen, E.S., et al., *Pancreatic cancer cell lines as patient-derived avatars: genetic characterisation and functional utility.* Gut, 2018. **67**(3): p. 508-520.
4. Kumarasamy, V., et al., *Chemotherapy impacts on the cellular response to CDK4/6 inhibition: distinct mechanisms of interaction and efficacy in models of pancreatic cancer.* Oncogene, 2020. **39**(9): p. 1831-1845.

Fig S1

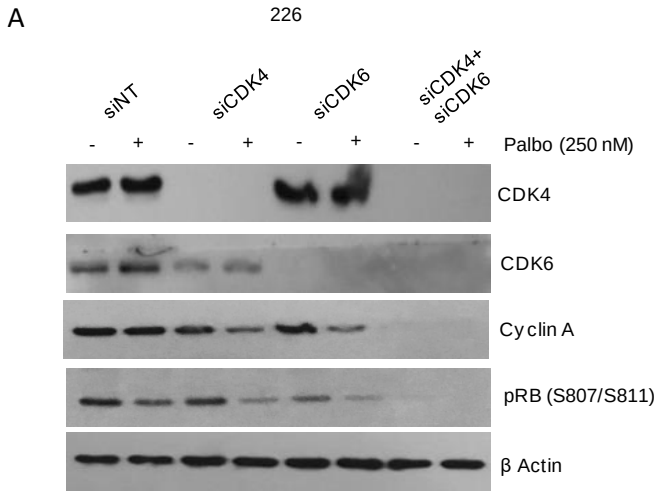


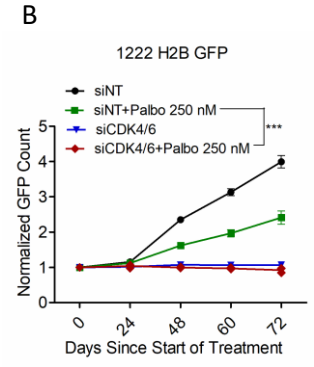
Supplementary Figure 1: (A) Representative images indicating the proliferation of different PDAC models (1222, 226 and 3226) and MCF7 cells in the absence and presence of palbociclib (500 nM) at different time points.

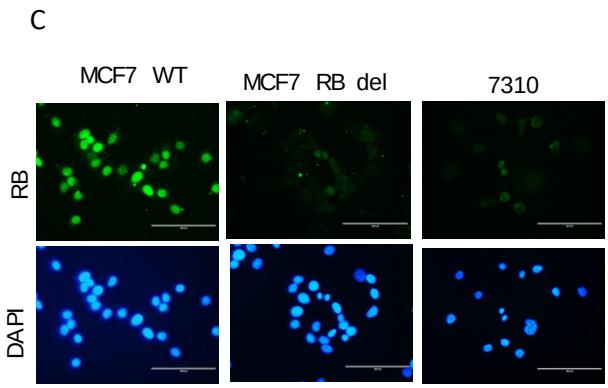


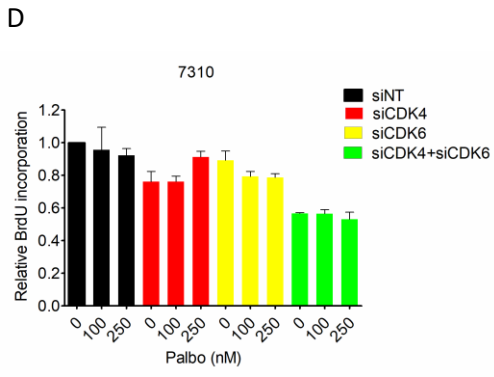
Supplementary Figure 2: (A) Growth of 3226, A549 and H1975 following the exposure to palbociclib. Graphs represent mean and SD (B) Representative images of organoids derived from 1222 and T47D cells at different days following treatment with DMSO and palbociclib (500 nM). (C) Densitometry analysis on RB phosphorylation in MCF7 and 226 cells in the presence of different concentrations of palbociclib. Colom represents mean and SEM (t-test). (*p<0.05, **p<0.01). (D) Western blot analysis in 1222 and T47D cells after 48 H exposure with palbociclib. (E) *In vitro* CDK2 kinase assays were performed using lysates from T47D and 226 cells treated with palbociclib up to 48 H. Representative blot images were shown. (F) *In vitro* CDK2 kinase assay in 1222 cells following 48 H exposure with abemaciclib. (G) Western blot on 1222, 226 and MCF7 cells in the presence of abemaciclib. (H) Western blot analysis on the indicated proteins from ER+ breast cancer models and PDAC models following 48 H exposure with palbociclib.

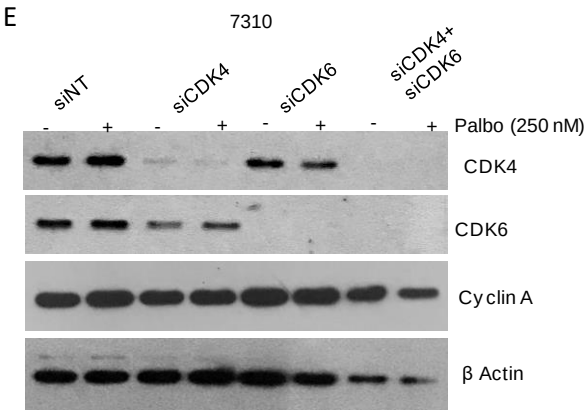
Fig S3





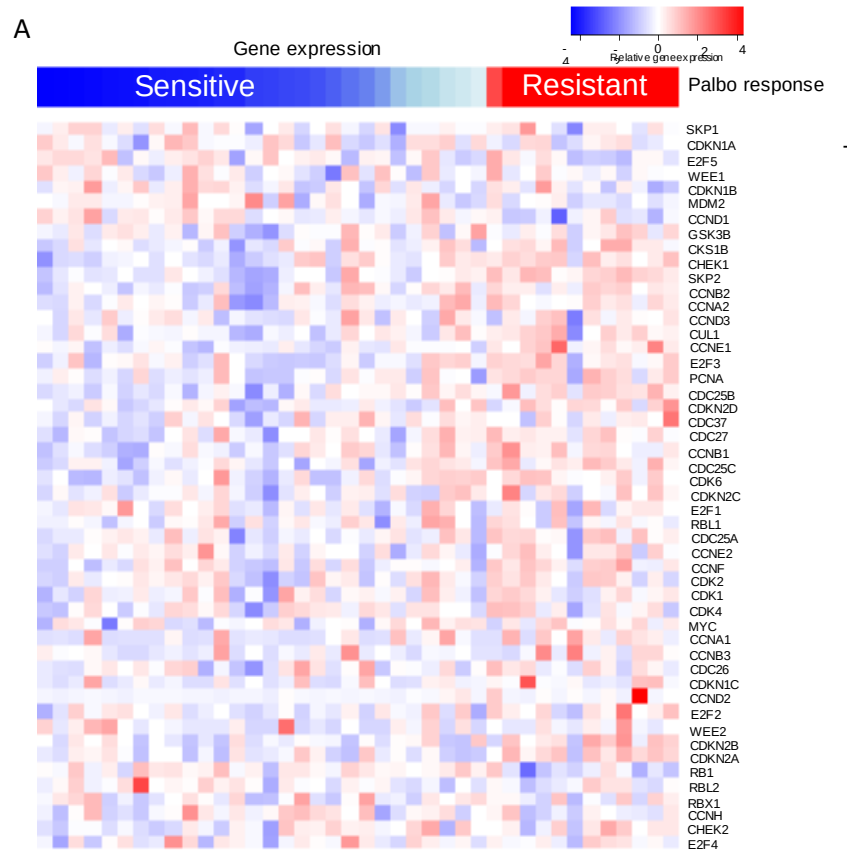






Supplementary Figure 3: (A) Western blot analysis on the indicated proteins in 226 cells following the knockdowns of CDK4 and CDK6. (B) Growth of 1222 cells following the knockdown of CDK4/6 in the presence of palbociclib. Data shows mean and SD (n=4). (*** $p<0.001$ as determined by 2-Way ANOVA) (C) Immunofluorescence staining of RB in MCF7-WT, MCF7-RB-del and 7310 cells. (D) BrdU incorporation in 7310 cell line following the knockdowns of CDK4 and CDK6. Mean and SD are shown from two independent experiments. (E) Western blot analysis on the indicated proteins in 7310 cells following the knockdowns of CDK4 and CDK6.

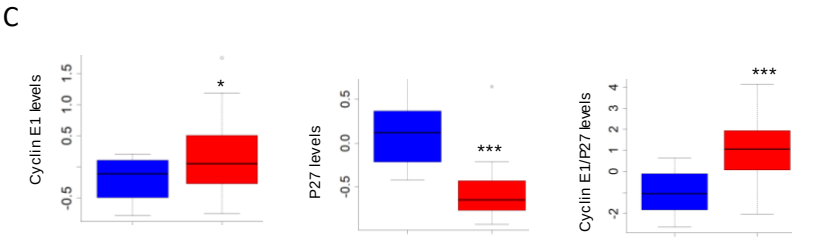
Fig S4

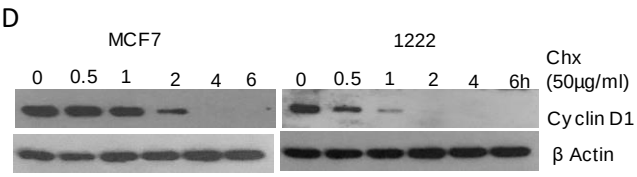


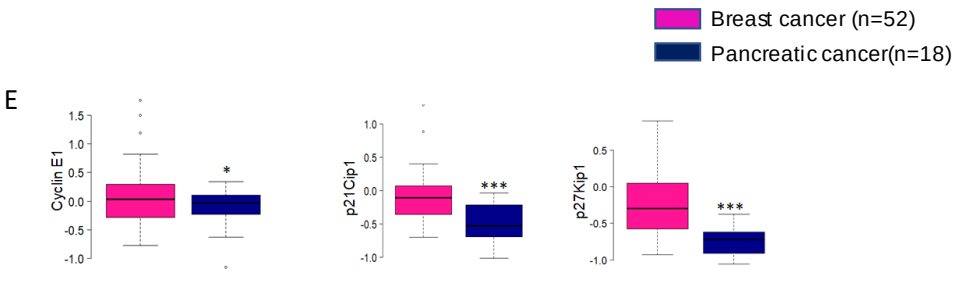
B

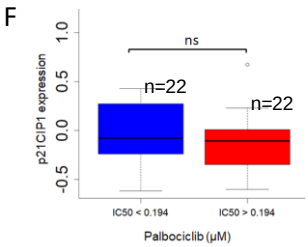
Top-10 significantly correlated genes

Genes	Q val
CDC25B	5.044416467
CHEK1	3.701057313
SKP2	3.687483645
CDK6	3.684473321
CCNE1	2.49440071
CDC25C	2.440171296
CCNB1	2.398065363
CCNB2	2.394187691
CCNA2	1.961079005
CUL1	1.94358537



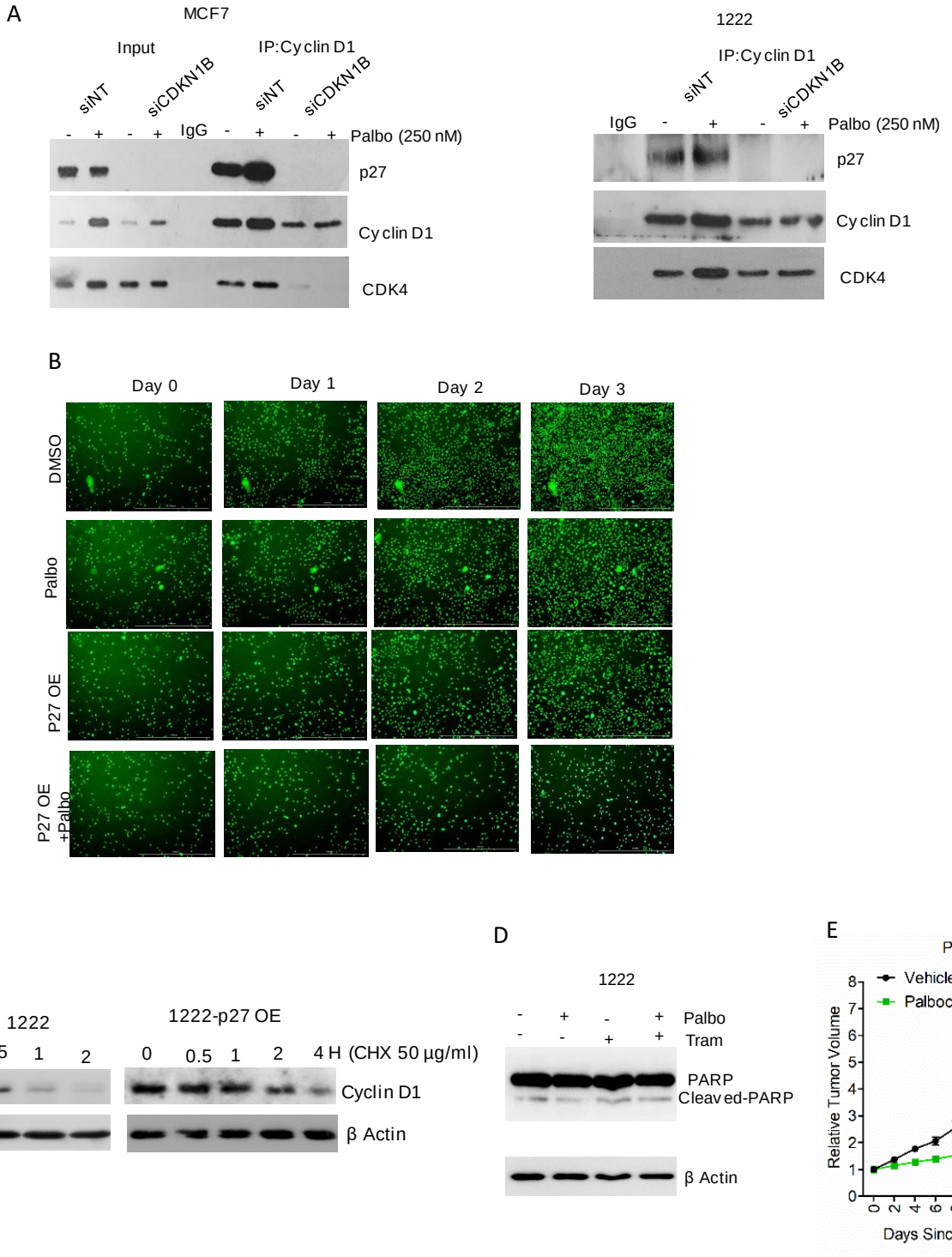




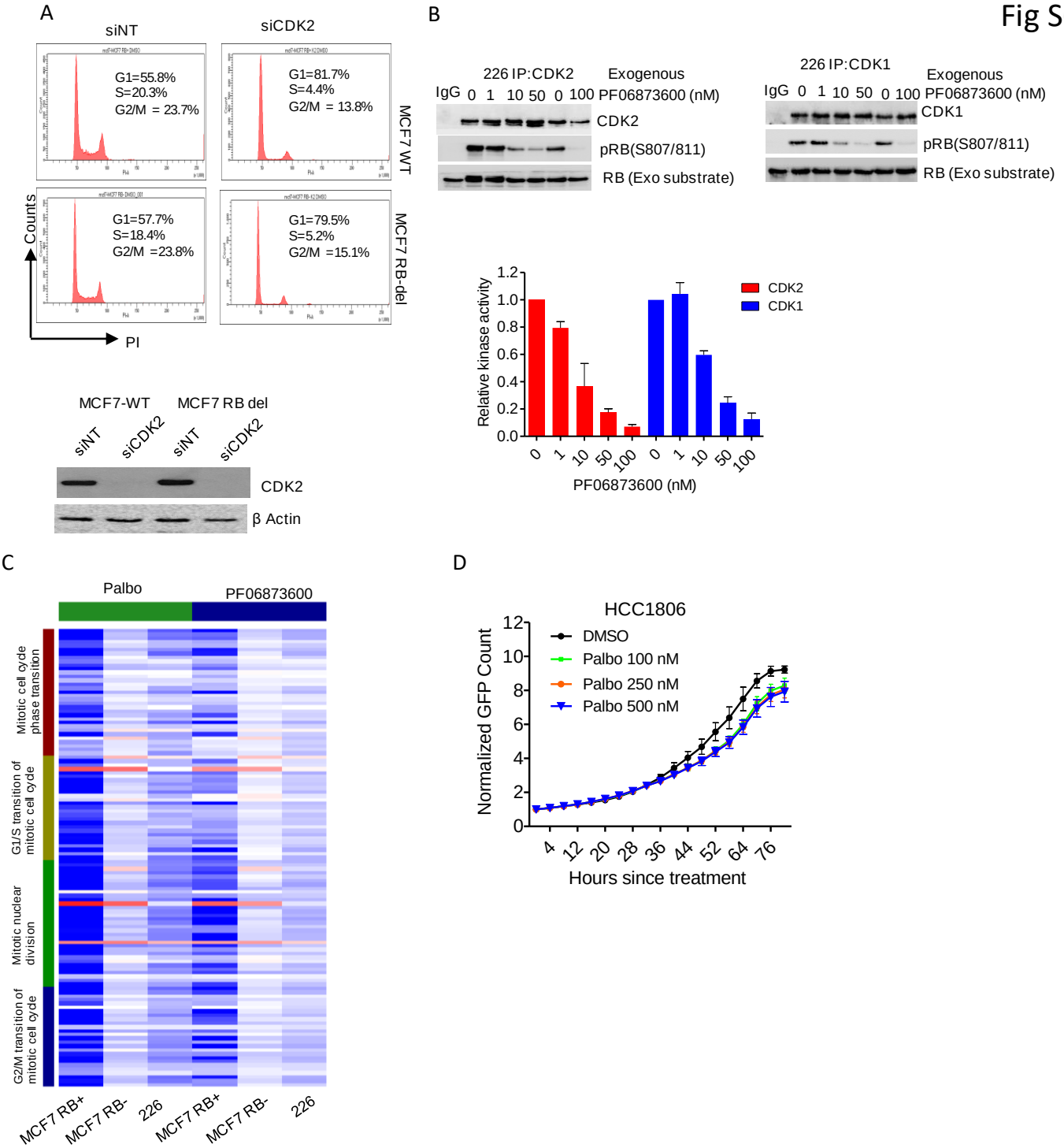


Supplementary Figure 4: (A) Heat map representing the expression of different cell-cycle genes in the breast cancer cells that were sorted based on their IC50 values of palbociclib. (B) Table indicating the top 10 genes that showed significant correlation with the response to palbociclib. (C) Box plot representing the distribution of Cyclin E1, P27KIP1 protein expression and the ratio of cyclin E1/P27KIP1 based on the IC50 values of abemaciclib in different breast cancer cells (*p<0.05; ***p<0.001 as determined t-test). (D) Stability of cyclin D1 in MCF7 and 1222 cells at the indicated time points following the treatment with cycloheximide (Chx) (50 µg/ml). (E) Box plot representing the distribution of fold change of cyclin E1, p21CIP1 and P27KIP1 expressions in different breast and pancreatic cancer cell lines (*p<0.1, ***p<0.001 as determined by t-test.). (F) Box plot representing the distribution of fold change of p21CIP1 expression based on the IC50 values of palbociclib in different breast cancer cells.

Fig S5

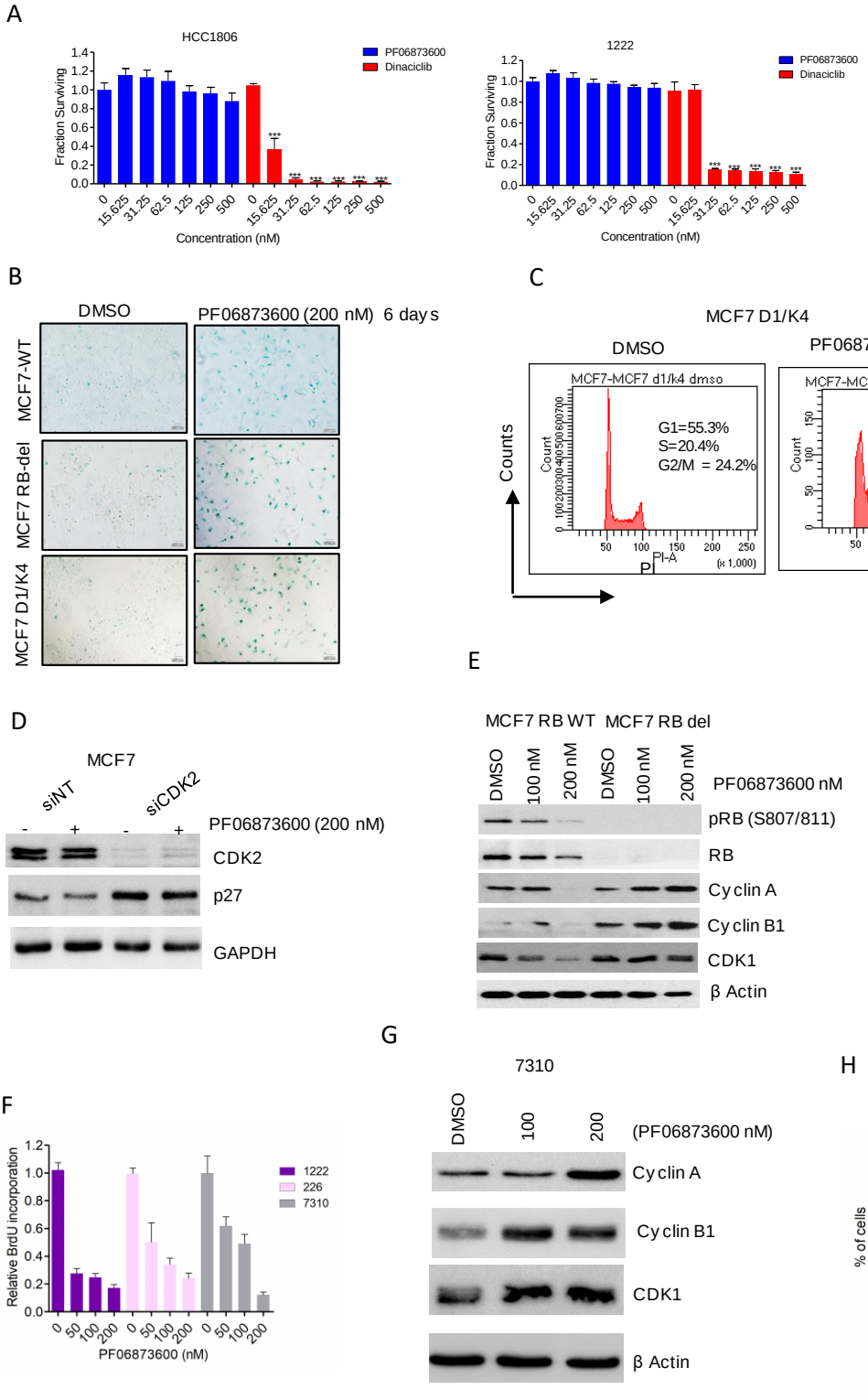


Supplementary Figure 5 (A) Immunoprecipitation of cyclin D1 from MCF7 and 1222 cells following the knockdown of P27KIP1 in the absence and presence of palbociclib (200 nM). Coimmunoprecipitated cyclin D1 and CDK4 were determined by immunoblotting. (B) Representative images indicating the proliferation of 1222-WT and 1222 P27KIP1 OE cells in the absence and presence of palbociclib at different time points. (C) Stability of cyclin D1 was determined in 1222-WT and 1222 P27KIP1 cells at the indicated time points following the treatment with cycloheximide (Chx) (50 µg/ml). (D) Western blot analysis to investigate the effect of palbociclib (200 nM) in combination with trametinib (20 nM) on PARP cleavage. (E) Mice bearing different PDAC models derived PDXs including 1222, 226 and 3226 were randomized for treatment with Vehicle (n=39) and palbociclib (n=52). This is a composite graph being adapted from our previous study (2). Data show the mean and SEM (***p<0.001 as determined 2-way ANOVA).



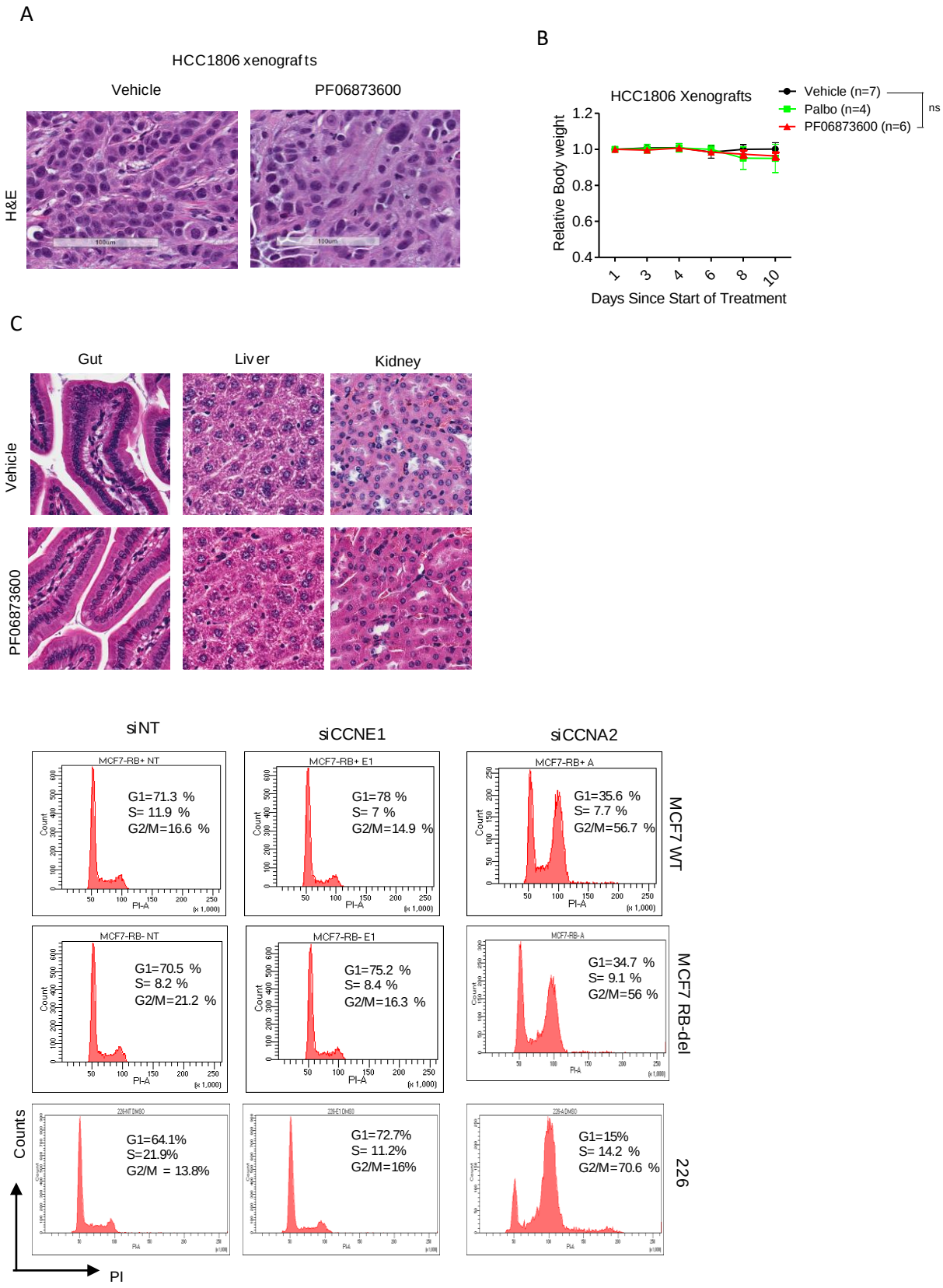
Supplementary Figure 6: (A) Cell-cycle profile based on PI staining in MCF7-WT and MCF7-RB-del cells following CDK2 knockdown. Western blot analysis on CDK2 expression to validate the knockdown efficacy. (B) *In vitro* CDK2 and CDK1 kinase assays from actively proliferating PDAC cell lines. Different concentrations of PF06873600 was exogenously added to the kinase reaction mix. Representative blot images and mean and SEM are shown. (C) Heatmap shows the relative transcriptional repression achieved in the presence of palbociclib (250 nM) and PF06873600 (200 nM) in the indicated cell lines. (D) Growth of HCC1806 cells, treated with palbociclib up to the indicated number of days. Graphs are representative of 2 independent experiments. Bars indicate the mean and standard deviation (SD) from triplicates.

Fig S7



Supplementary Figure 7. (A) CTG assay on 1222 and HCC1806 cells treated with increasing concentrations of PF06873600 and dinaciclib up to 72 H. Graphs are representative of 2 independent experiments. Bars represent mean and SD from triplicates. (***p<0.001 as determined by t-test). (B) Senescence associated β-galactosidase staining from MCF7-WT, MCF7 RB-del and MCF7 D1/K4 cells treated with PF06873600 (200 nM) up to 6 days. (C) Cell-cycle profile based on PI staining in MCF7 D1/K4 cells following 48 h exposure with PF06873600 (200 nM). (D) Western blot analysis in MCF7 cells following CDK2 knockdown. (E) Immunoblot analysis on MCF7 WT and MCF7-RB-del cells following 48 H exposure with PF06873600 (F) BrdU incorporation in 1222, 226 and the RB deficient 7310 cells treated with different concentrations of PF06873600 up to 72 H. Bars represent mean and SD from triplicates (2 independent experiments (n=3)). (G) Western blot analysis on 7310 cells treated with PF06873600 (200 nM) up to 48 h. (H) Cell-cycle profile based on PI staining in 1222 and 226 cells following 48 H exposure with PF06873600 (200 nM). Error bars represent mean and SEM from 2 independent experiments

Fig S8



Supplementary Figure 8: (A) H&E staining was performed on tumor tissues excised from the vehicle and PF06873600 treated mice. Representative images are shown (scale bar = 100 µm). (B) Relative change in body weight of mice that were treated with Palbociclib and PF06873600. Data represent the mean and SEM, p-value was calculated using 2-way ANOVA. (C) H&E staining on the indicated organs from NSG mice treated with vehicle, palbociclib and PF06873600 upto 6-days. (D) Cell-cycle profile based on PI staining in MCF7-WT, MCF7-RB-del and 226 cell lines following *CCNE1* and *CCNA2* knockdowns.