Polo-like kinase 4 inhibitor suppresses hepatocellular carcinoma through inducing extensive polyploidy and activating STING pathway

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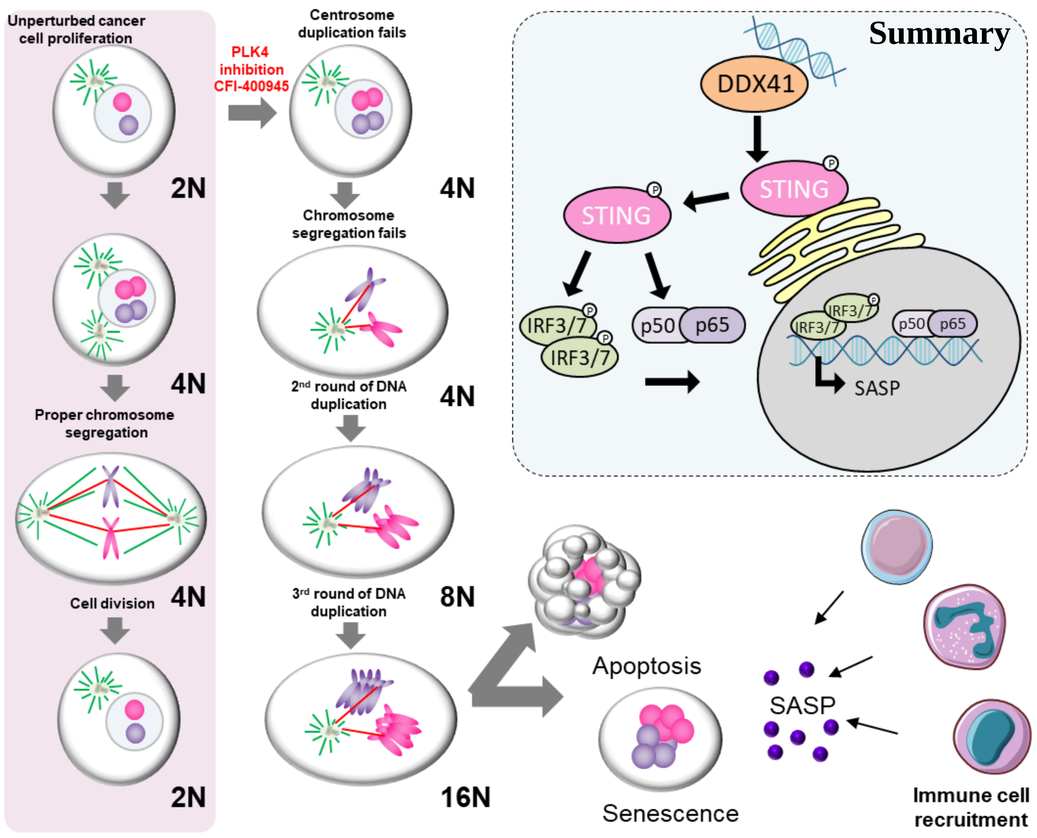
Abstract

Introduction: Aneuploidy is a typical feature in cancer cells, including hepatocellular carcinoma (HCC). A centrosome duplication regulator, polo-like kinase 4 (PLK4), is overexpressed in HCC, and is closely associated with mutations/deletions in p53 and phosphatase and tensin homolog (PTEN). Dysregulation of these two cell cycle checkpoint regulators leads to chromosome mis-segregation and aneuploidy. CFI-400945, a novel orally available PLK4 inhibitor, was shown effective in suppressing growth of other solid tumour types, including breast and pancreatic cancer. We are interested to know its effectiveness in HCC model. **Methods:** Cell cycle profiles were analysed by flow cytometry analysis using propidium iodide (PI) staining. Liver specific p53/PTEN knockout HCC models were established by hydrodynamic injection of plasmids encoding CRISPR-Cas9 and transposon systems. Infiltrated immune cell populations in tumours were analysed by flow cytometry. Transcription factors were identified by ChIP assay. Expression of senescence-associated secretory phenotypes (SASPs) was studied by transcriptome sequencing, RT-qPCR, and ELISA. **Results:** CFI-400945 selectively targeted proliferating HCC cells by blocking centrosome duplication, causing severe aneuploidy, DNA damage, and senescence. Interestingly, CFI-400945 suppressed cell division without stopping DNA replication, leading to a large amount of cytosolic DNA accumulation which elicits cytosolic DNA sensing pathway DDX41-STING-IRF3/7-NFkappaB, thereby driving the transcription of SASPs which are the secretory factors that recruit immune cells. CFI-400945 significantly impeded HCC tumour growth *in vivo* and altered the immune landscape inside tumours, increased tumour infiltration of CD4+, CD8+ T cells, macrophages and natural killer cells. The effect of CFI-400945 in tumour suppression was alleviated in STING-knockout HCC. Due to the increased number of immune infiltrates caused by CFI-400945, combination therapy of CFI-400945 with anti-PD-1 or anti-PD-L1 antibody further prolonged survival of HCC-bearing mice. **Conclusion:** CFI-400945 dramatically suppressed HCC progression through deregulating the cell cycle and activation of the STING pathway, which is originally an integral component of the anti-viral immune response. We demonstrated a novel therapeutic approach by targeting a centrosome regulator to activate the cytosolic DNA sensing mediated immune response. It will directly benefit the development of clinical trials involving CFI-400945 in HCC treatment.



PLK4 is overexpressed in HCC

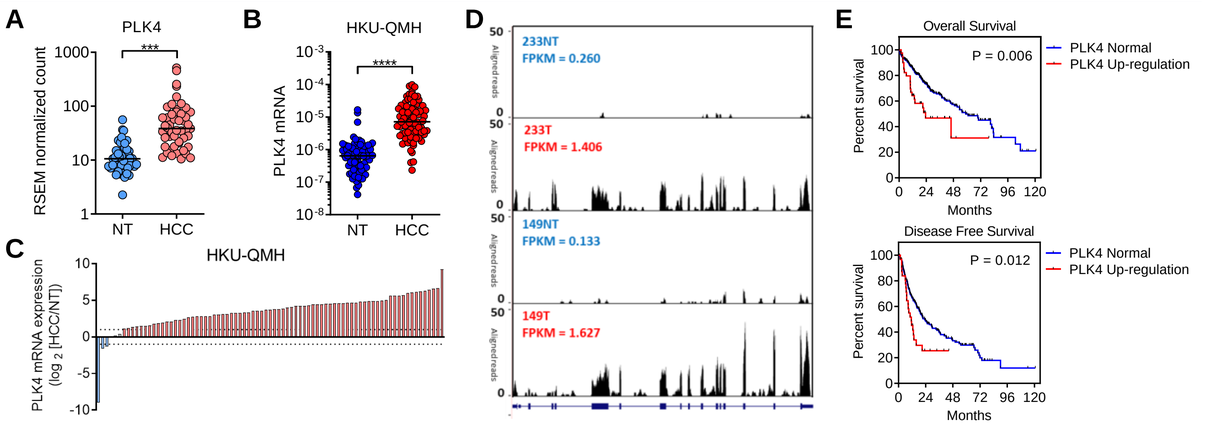


Figure 1. PLK4 is overexpressed in HCC. (A) The mRNA expression data of PLK4 in human hepatocellular carcinoma (HCC) and non-tumorous liver (NT) tissues retrieved from The Cancer Genome Atlas (TCGA) database. RSEM (RNA-Seq expression estimation by Expectation-Maximization). (B) PLK4 mRNA expression in 81 cases of paired HCC and NT tissues was evaluated by RT-qPCR. (C) Waterfall plot showed that PLK4 expression was up-regulated in 75/81 (92.6%) of HCC patients by at least 2 fold. (D) Representative pictures of raw aligned sequencing read obtained from transcriptome sequencing comparing PLK4 mRNA expression in HCC and NT tissues of our cohort of patients (HKU-QMH). FPKM (Fragments per kilobase of exon per million reads). (E) TCGA database revealed that PLK4 up-regulation was correlated with poor overall and disease free survival. A, B-Wilcoxon signed rank test, E-Kaplan Meier and Log Rank Test.

PLK4 inhibition induced micronuclei, polyploidy and apoptosis in HCC cells

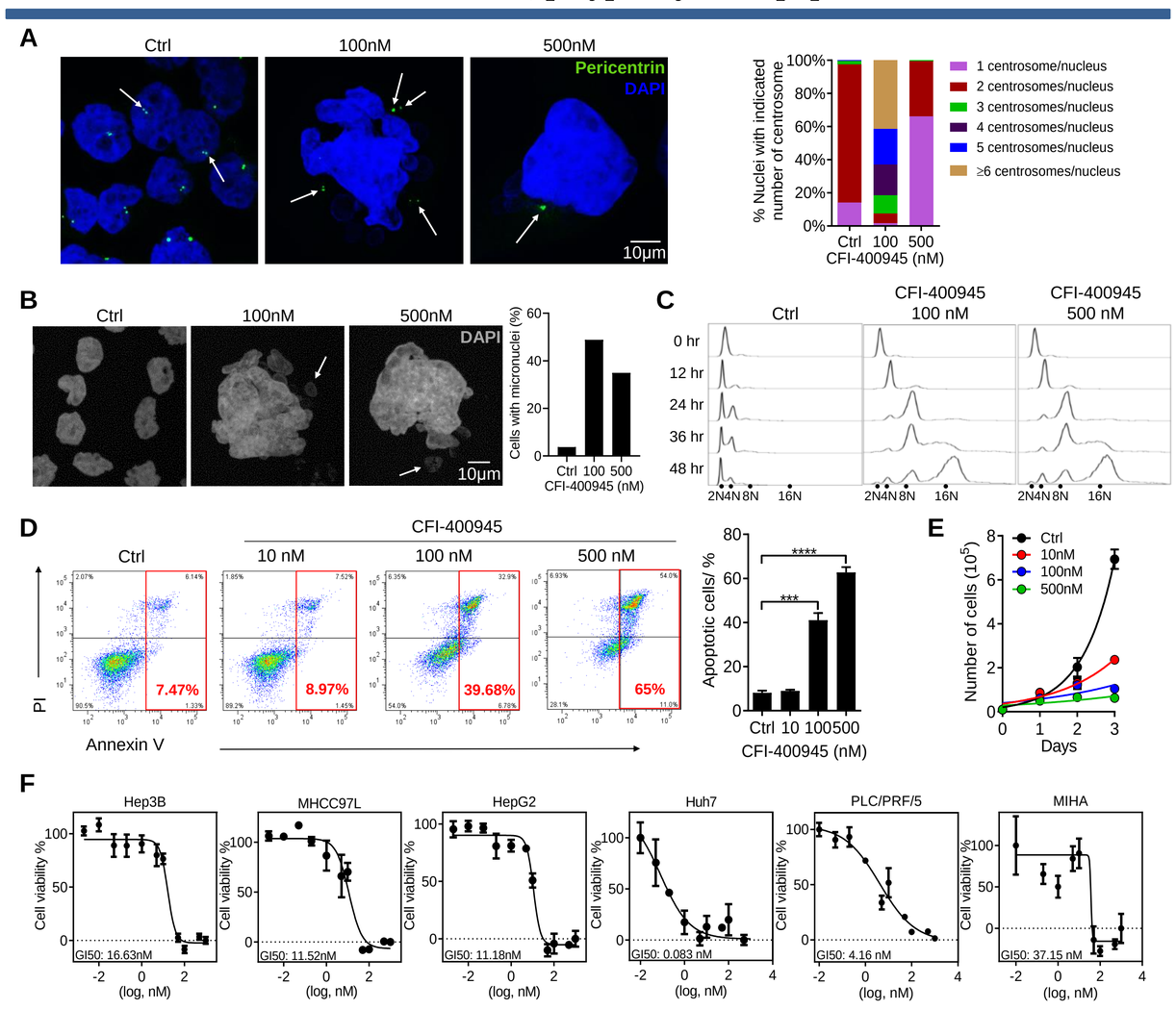


Figure 2. PLK4 inhibition induced micronuclei, polyploidy and apoptosis in HCC cells. (A) MHCC97L cells were treated with CFI-400945 for 48 hours and stained with pericentrin and DAPI for centrosome and DNA staining. Left: Representative images of centrosomes in cells. Right: Percentage of nuclei with indicated number of centrosomes. (B) MHCC97L cells were treated with CFI-400945 for 48 hours and stained with DAPI for DNA staining. Left: Representative images of micronuclei, indicated by arrows. Right: Percentage of cells with micronuclei. (C) MHCC97L cells were synchronized at G1 phase by double thymidine treatment and released for 12, 24, 36, and 48 hours in the presence of vehicle or indicated concentrations of CFI-400945. Cells were stained with PI for cell cycle analysis. Gating indicates 2N (G1), 4N (G2/M), 8N and 16N (polyploid) cells. (D) MHCC97L cells were treated with various concentrations of CFI-400945 for 48 hours and stained with apoptosis marker Annexin V and PI. Left: Cells at early and late apoptosis phases were in the right quadrants. Right: Percentage of apoptotic cells. (E) Cell proliferation of MHCC97L cells in the presence of vehicle (culture medium) and various concentrations of CFI-400945. (F) Different human HCC cell lines (Hep3B, MHCC97L, HepG2, Huh7, PLC/PRF/5) and normal hepatic cell line (MIHA) were treated with escalating doses of CFI-400945 for 5 days. XTT assay was performed to determine the GI_{50} values of different cell lines. Student's t test, mean ± SD.

PLK4 inhibition suppressed HCC and caused splenomegaly *in vivo*

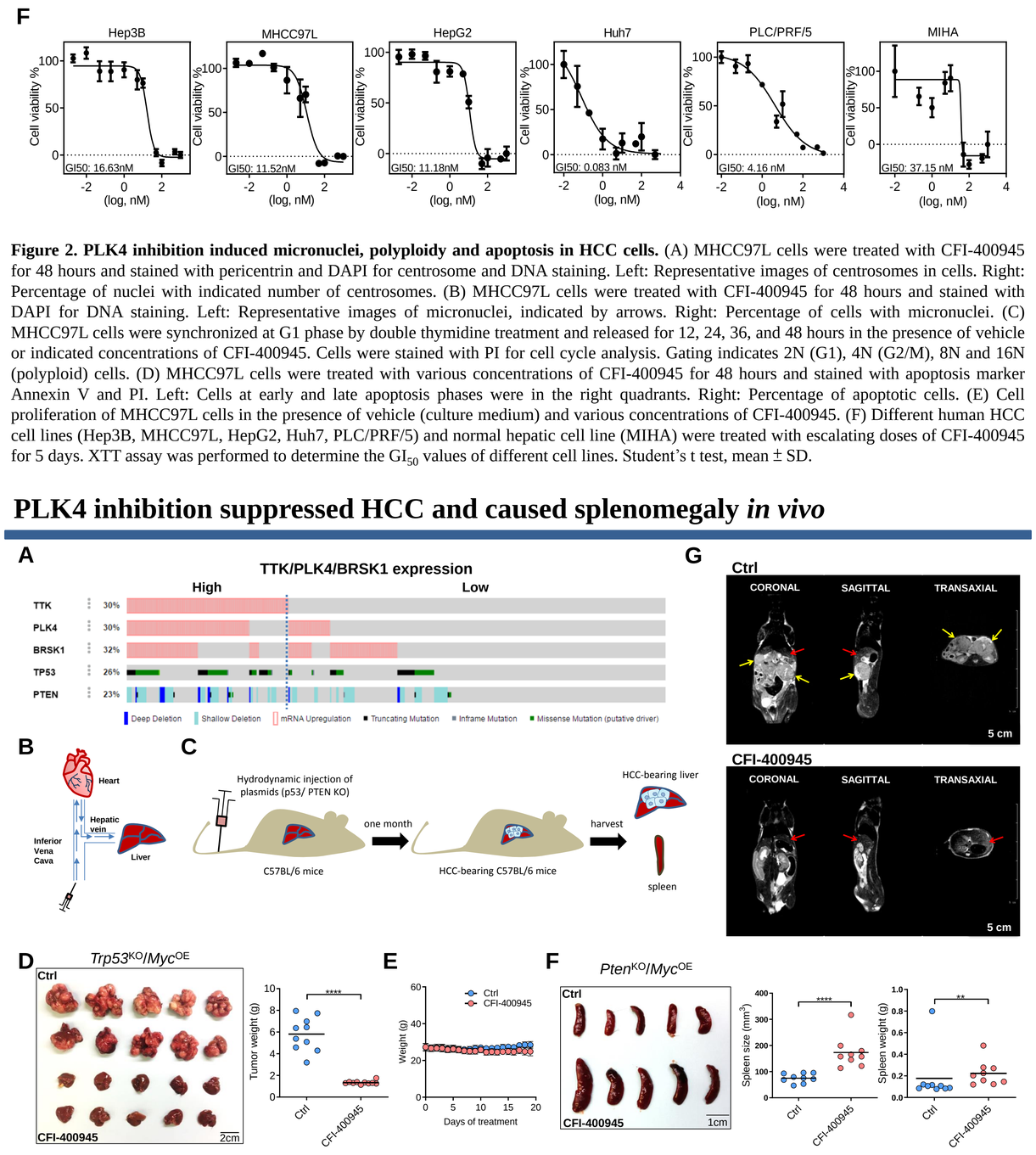


Figure 3. PLK4 inhibition suppressed HCC and caused splenomegaly *in vivo*. (A) Concurrence of cell cycle genes (TTK, PLK4, BRSK1) and tumor suppressors (TP53 and PTEN) mutations. Data retrieved from TCGA database. (B) Hydrodynamic injection scheme. (C) Schematic diagram of hydrodynamic injection to induced tumor in mouse liver. (D) Tumors formed after hydrodynamically knocked out *Trp53* and induced *Myc* expression in the livers of C57BL/6 mice. Tumors were weighed. (E) Body weighs of mice in (D) during the period of CFI-400945 oral gavage treatment. (F) Spleens in a separate experiment which tumors in livers were induced by hydrodynamically knocked out *Pten* and spleen sizes were measured. (G) MRI images of mice bearing tumors induced by the same method in (D). Red: Liver, Yellow: Tumor Student's t test, mean ± SD.

DNA damage, cytosolic DNA and senescence are induced by PLK4 inhibition

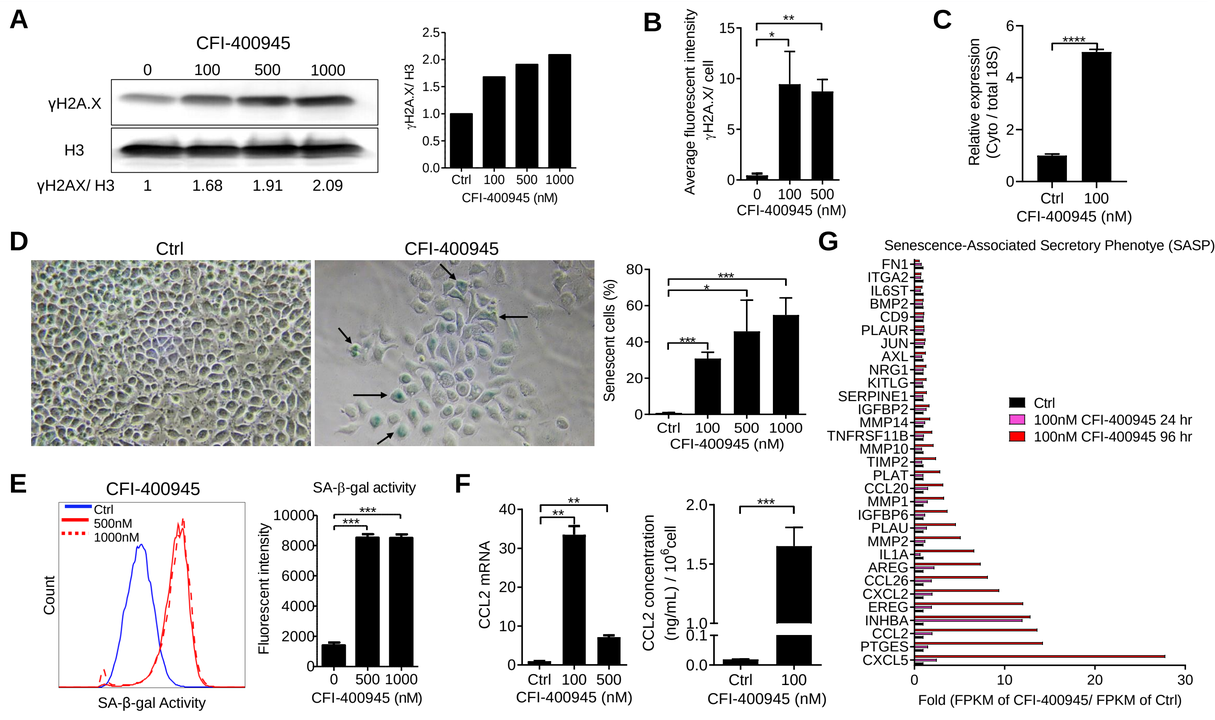


Figure 4. DNA damage, cytosolic DNA and senescence are induced by PLK4 inhibition. (A-B) MHCC97L cells treated with CFI-400945 for 48hr were probed for DNA damage marker γH2A.X by (A) Western blotting and (B) immunofluorescent staining. (C) MHCC97L cells treated with CFI-400945 for 48 hours and tested for cytosolic DNA. (D) Left: Representative images of MHCC97L after 96hr CFI-400945 treatment with senescence-associated beta-galactosidase (SA-β-gal) staining. Right: Percentage of MHCC97L cells with positive SA-β-gal staining were calculated. (E) MHCC97L cells were treated with various concentrations of CFI-400945 for 96hr and SA-β-gal activity was analyzed by flow cytometry. (F) MHCC97L cells were treated with CFI-400945 for 96hr and CCL2 expression level was determined by qPCR and ELISA. (G) MHCC97L cells were treated with indicated concentrations of CFI-400945 for 24hr, 96hr and RNA was sequenced. Expression levels of senescence-associated secretory phenotype (SASP) genes were shown. Student's t test, mean ± SD.

Immune surveillance cells are attracted to tumor after PLK4 inhibition

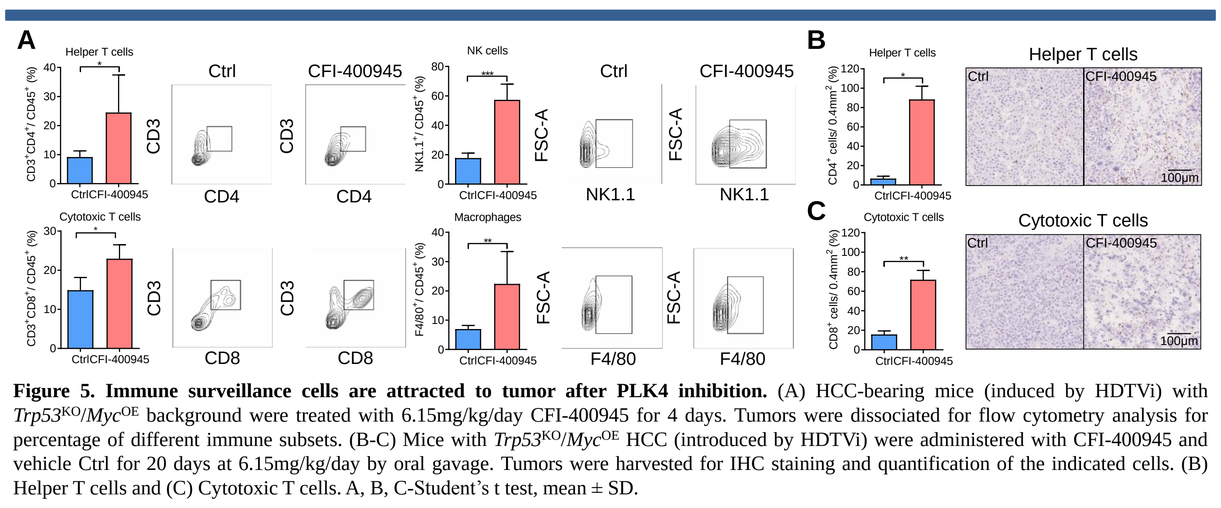


Figure 5. Immune surveillance cells are attracted to tumor after PLK4 inhibition. (A) HCC-bearing mice (induced by HDTVi) with *Trp53*^{KO}/*Myc*^{OE} background were treated with 6.15mg/kg/day CFI-400945 for 4 days. Tumors were dissociated for flow cytometry analysis for percentage of different immune subsets. (B-C) Mice with *Trp53*^{KO}/*Myc*^{OE} HCC (introduced by HDTVi) were administered with CFI-400945 and vehicle Ctrl for 20 days at 6.15mg/kg/day by oral gavage. Tumors were harvested for IHC staining and quantification of the indicated cells. (B) Helper T cells and (C) Cytotoxic T cells. A, B, C-Student's t test, mean ± SD.

SASP upregulation after PLK4 inhibition is regulated by DDX41-STING-IRF3/7-NFκ-β axis

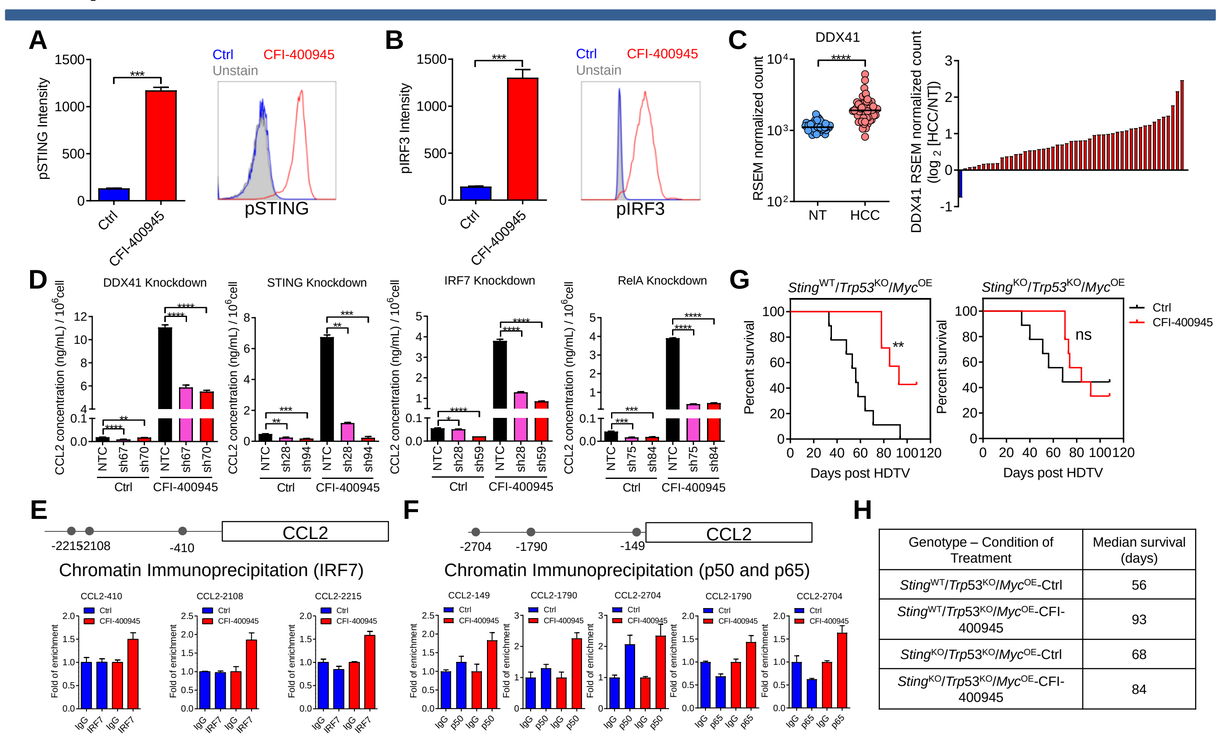


Figure 6. SASP upregulation after PLK4 inhibition is regulated by DDX41-STING-IRF3/7-NFκ-β axis. (A-B) Phosphorylation status of (A) STING and (B) IRF3 after 100nM CFI-400945 treatment in MHCC97L cells for 4 days. (C) Left: Expression of DDX41 in human HCC and NT tissues from TCGA database. Right: Waterfall plot showed that DDX41 expression was up-regulated in 48/49 (98.0%) of HCC patients. (D) CCL2 protein expression in DDX41/ STING/ IRF7/ RelA stable knockdown MHCC97L cells treated with 100nM CFI-400945 for 4 days by ELISA assay. (E-F) ChIP assay and locations of putative binding sites of (E) IRF7 and and (F) NFκ-β (p50 and p65 subunits) at CCL2 promoter. Locations refer to transcription start site (TSS) as 0. (G) Percentage survival of *Sting*$^{WT/KO}$ HCC-bearing mice (induced by HDTVi) with 6.15mg/kg/day CFI-400945 treatment. Left: *Sting*^{WT}/*Trp53*^{KO}/*Myc*^{OE}, Right: *Sting*^{KO}/*Trp53*^{KO}/*Myc*^{OE}. Treatment started on day 25 post HDTVi. (H) Median days of survival for each group in (G). Student's t test, mean ± SD.

CFI-400945 primed HCC cells and T cells for combination therapy with anti-PD-1/PD-L1 monoclonal antibody

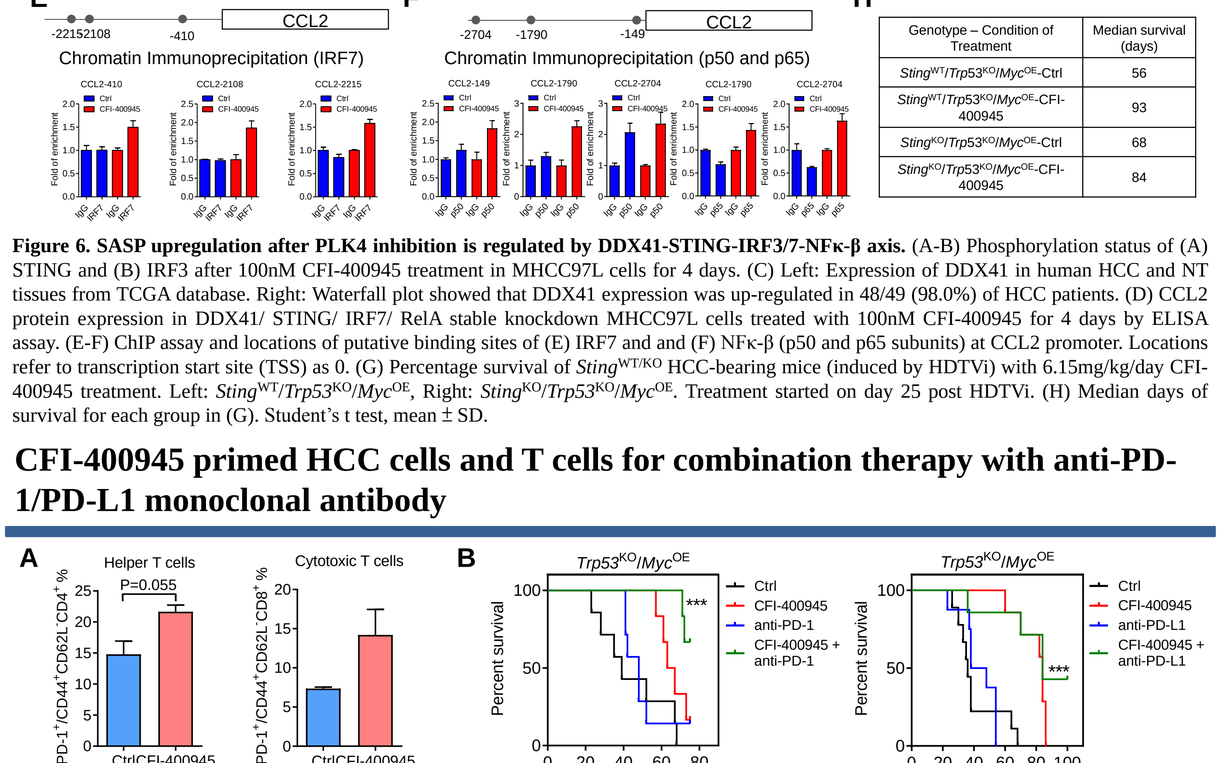


Figure 7. CFI-400945 primed HCC cells and T cells for combination therapy with anti-PD-1/PD-L1 monoclonal antibody . (A) Population of PD-1^{+} Helper T cells (left) and Cytotoxic T cells (right) at tumor sites in mice with hydrodynamically induced HCC. (B) Survival curve of combination treatment of CFI-400945 with anti-PD-1/PD-L1 monoclonal antibody in mice with hydrodynamically induced HCC. Student's t test, mean ± SD.