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Cell Reports

Supplemental Information

Cancer-Specific Synthetic Lethality between ATR and CHK1 Kinase Activities

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Supplemental Material and Experimental Procedures

Inhibitors. ATR inhibitor VE-821 was synthesized in house according to a previously described protocol (Charrier et al., 2011) and VX-970 (VE-822) was obtained from Vertex Pharmaceuticals (Europe) Ltd. The CHK1 inhibitors (AZD7762, LY2603618, PF-477736, SCH-900776), the DNAPK inhibitor (KU-0051777) and the CDK inhibitors (Roscovitine, and PHA-767491 (Selleck Chemical), the JNK inhibitor (SR-3306) and ATM inhibitor (KU-55933) from Calbiochem, 4-NQO (Sigma-Aldrich), Alexa 488/555 secondary antibodies, To-Pro iodide, Edu Click-it (Life Technologies), Cldu, IdU and 4-NQO (Sigma-Aldrich).

Antibodies. The following primary antibodies were used: Rabbit anti-53BP1(A300-272A, Bethyl laboratories), mouse anti-phospho Serine 139 H2AX (Upstate), rabbit anti-cleaved caspase3, rabbit anti-phospho Serine 345 CHK1, rat anti-RPA 32, rabbit anti-cleaved PARP, rabbit anti-phospho Serine 428 ATR, rabbit anti-Threonine 68 CHK2 (Cell Signaling Technologies), Mouse anti-PARP, Mouse anti-CDC25A, mouse anti-cMYC , mouse anti-GAPDH (Santa Cruz), rabbit anti-Serine 2056 DNA-PKcs, rabbit anti-53BP1, rabbit anti-phospho Serine 10 Histone H3, mouse anti CHK1, mouse anti-actin (Abcam), mouse anti-Ki67 (Dako).

Confocal and high throughput microscopy. 200,000 U2OS, VH-10, HA1EB-GFP or HA1EB-GFP-cMYC cells were seeded onto cover slips in 6 well plates and incubated overnight. The next day, the cells were treated with DMSO, VE-821, AZD7762 or the combination. At the end of incubation period, the cells were fixed in 4% PFA in PBS. For EdU experiments, 10 μ M of EdU was added in fresh media for 20 min at the end of the treatment period before fixing cells in 4% PFA for 15 min. Cells on cover slips were permeabilized with 0.3% Triton X-100 in PBS and thereafter blocked in 3% BSA in 0.1% Tween 20 in PBS (0.1% PBST). Cells were probed with primary antibodies in 0.1% PBST and incubated overnight at 4^o C, thereafter secondary antibodies in 0.1% PBST were added and cells were incubated for 1 h at room temperature. To-Pro iodide was used to stain DNA. For high content microscopy, 5000 cells per well were seeded in 96 well

plates and processed as described above. Cells were imaged using a PerkinElmer operetta high content microscope. Images from confocal microscopy were quantified manually using ImageJ software (more than 100 cells per condition). Columbus software was used to analyse images from the PerkinElmer operetta high content microscope (more than 500 cells).

Plasmid vector construction. PB-GFP plasmid (pHULK piggyBac Mammalian Expression Vector – CometGFP™, pJ503-02) and PB-RFP plasmid (pHULK piggyBac Mammalian Expression Vector – RudolphRFP™-IRES-CometGFP™, pJ549-17) were purchased from DNA2.0. PB-GFP-cMYC plasmid was generated as follows: cMYC DNA was amplified from pPB-CAG-cMYC (Wang et al., 2011) and was cloned into XbaI/BamHI sites of the PB-RFP plasmid (RFP gene was replaced with cMYC gene). The final product (PB-GFP-cMYC) was confirmed by DNA sequencing.

Generation of cell lines. HA1EB-GFP (control cells) and HA1EB-GFP-cMYC (cMYC overexpressing cells) were generated by stable transfection with PB-GFP or PB-GFP-cMYC, respectively. The respective plasmids were transfected into HA1EB cells using JetPEI® (101-10N, Polyplus-transfection). 1.5 million HA1EB cells were seeded into 75-cm² tissue culture flask. When cells were 70% confluent, they were transfected with 5 µg DNA and 10 µL JetPEI® for 4 hours. Thereafter, the medium was changed and cells were expanded for 7 days before their selection. Fluorescence Activated Cell Sorting (FACS) was used to select GFP positive cells. cMYC overexpression was confirmed by Western Blot (Figure. S3C).

Viability assay and drug interaction. 1000-3000 cells per well were seeded into 96 well plates and incubated overnight. The next day, the cells were treated with vehicle DMSO (maximum 0.05%) or various concentrations of VE-821 and/or AZD7762. After 72 h of incubation, resazurin was added to each well and further incubated for 2-6 h. Fluorescence intensity was measured at 530/590 nm (excitation/ emission) (Gad et al., 2014).

DNA fiber assay. U2OS and VH-10 cells were seeded to 70% confluence one day before the experiment. After 60 minutes of the indicated treatment, replication was labelled with CldU (25 μ M) and sequentially IdU (250 μ M) (Sigma-Aldrich, 20 minutes each) in presence of the drugs. DNA fibers were spread and stained as described previously (Groth et al., 2010). In summary, cells were lysed and DNA spread on glass slides. Acid treated DNA was stained with primary antibodies (mouse anti-BrdU pure, clone B44, BD Biosciences; rat anti-BrdU, Batch No.0412, AbD Serotec) for 1 hour at 37°C and secondary antibodies (goat anti-rat AlexaFluor555; goat anti-mouse AlexaFluor488) for 2,5 hours at room temperature(Groth et al., 2010). Pictures were taken with a Zeiss LSM-780 confocal microscope using the 63x oil objective. Fiber lengths were analysed using ImageJ software (<http://rsb.info.nih.gov/ij/>) and micrometer measurements were recalculated to kilobases with the conversion factor 1 μ M = 1.59 kb(Henry-Mowatt et al., 2003). A minimum of 450 fibers was measured per condition.

Comet assay. 200,000 U2OS cells were seeded in 6 well plates. After overnight incubation, cells were treated with VE-821, AZD7762 or the combination for 24 h. Cells were then harvested by trypsinization and washed with 1xPBS, resuspended in 1 x PBS at a concentration of approximately 1 million cells/ml. Cell suspension was mixed with 1.2 % low melting agarose and the mixture was added over 1% agarose coated fully frosted slides (Thermo-Fischer Scientific). The slides were incubated in lysis buffer containing [100 mmol/L sodium EDTA, 2.5 mol/L NaCl, 10 mmol/L Tris-HCl (pH 10)] , 1% Triton X-100 and 10% DMSO overnight at 4°C in the dark. After overnight incubation, alkaline denaturation with alkali buffer (300 mmol/L NaOH, 1 mmol/L sodium EDTA) was carried out in an electrophoresis chamber for 20 min; then electrophoresis was run at 25 V and 300 mA in the same buffer for 30 min. The slides were later neutralized with neutralizing buffer [250 mmol/L Tris-HCl (pH 7.5)] for at least 30 min. Just before imaging, the slides were stained with 20 μ M YOYO-1 dye. Images were taken with a

confocal microscope (LSM 510) using a 20X objective and analysis was performed using CometScore software (Gad et al., 2014).

Western blotting. Cells were grown and treated in 6 well plates. At the end of incubation, cells were scraped into lysis buffer (50 mM Tris, pH 7.4, 250 mM NaCl, 5 mM EDTA, 1% Triton X-100, protease inhibitor (Roche), phosphatase inhibitors (Thermo)) and further incubated on ice for 30 min followed by sonication for complete lysis. After measuring protein concentrations (BSA, Bio-Rad), proteins were separated on a 4-12% Bis-Tris acrylamide gel (Life Technologies) and transferred to nitro cellulose membrane (Bio-Rad). Membrane was blocked in 3% BSA in 0.1% PBST followed by incubation with primary and HRP conjugated or fluorescent secondary antibodies. Thereafter HRP substrate (Pierce) or secondary fluorescent antibodies (Odyssey LICOR) were used to visualize protein bands.

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Supplemental Figures and Legends

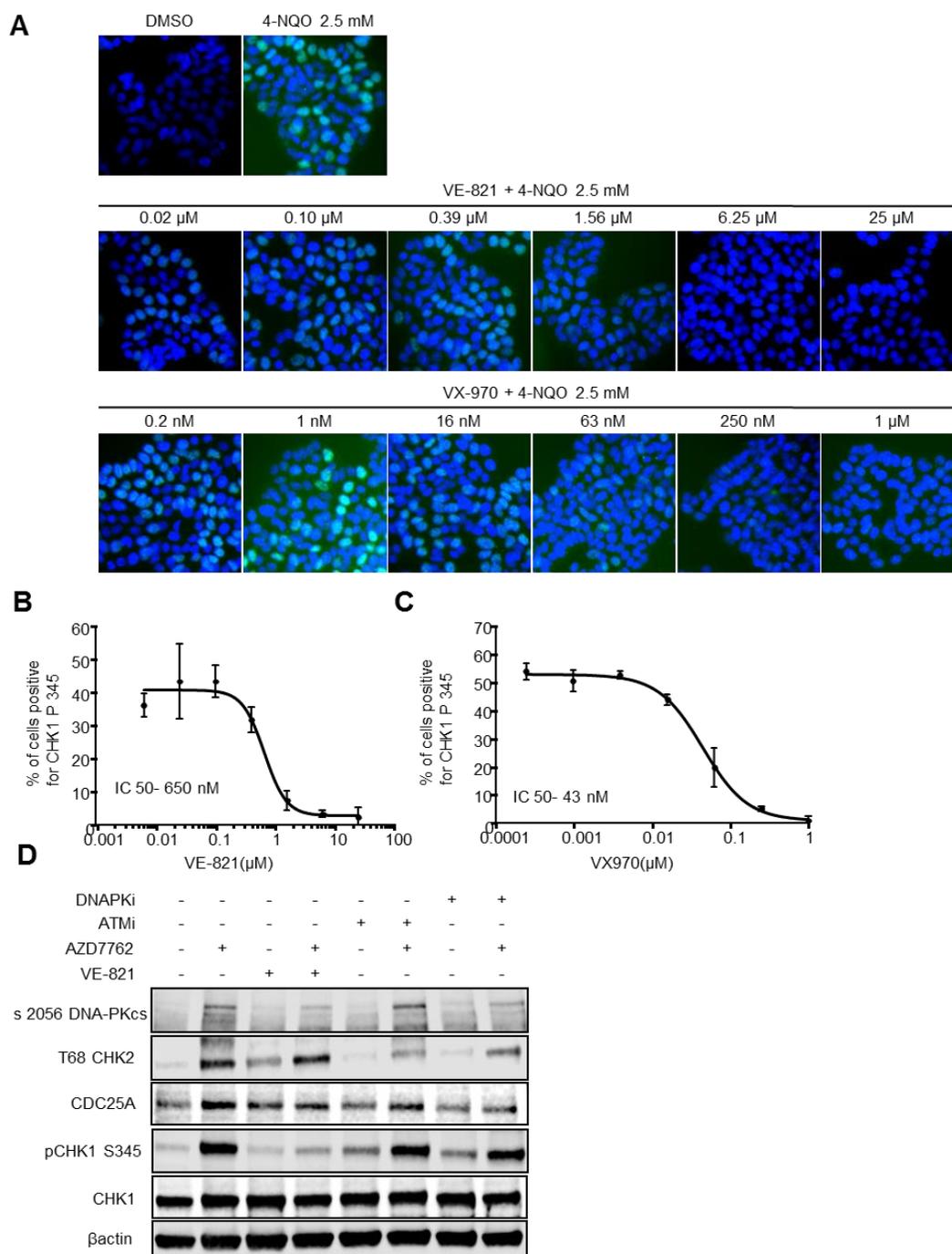


Figure S1. Inhibitory activity of ATR inhibitor, Related to Figure 1. HT29 cells were pretreated for 1 h with ATR inhibitor VE-821 or VX-970 prior to addition of 4-NQO (2.5mM). After 1 h of incubation, cells were fixed and pCHK1 Ser345 measured by IF. (A) Representative images. (B) and (C) pCHK1 Ser345 inhibition plot of VE-821 and VX-970. (D) Western blot showing inhibitory activity of ATR inhibitor VE-821, ATM inhibitor KU55933 or DNAPK inhibitor KU-0051777 in combination with CHK1 inhibitor AZD7762. Cells were treated with DMSO, AZD7762 (300 nM), VE-821 (20 μM), ATM inhibitor (20 μM), DNA-PK inhibitor (10 μM), either alone or in combination for 3 h. At the end of incubation, cells were lysed and western blotting was performed using anti-phospho (Serine 345) CHK1, anti-CHK1, anti-phospho (Serine 2056) DNA-PKcs, anti-phospho (Threonine 68) CHK2, anti-CDC25A, and anti β-actin antibodies.

Figure S2. Pre-apoptotic pan-nuclear γ -H2AX and DNA damage induction by AZD7762 and VE-821 either alone or in combination in cancer cells, Related to Figure 1. (A) U2OS cells were treated with DMSO, 60 nM of AZD7762 and 10 μ M of VE-821 or its combination for 24 h. Cells were probed with anti-phospho (Serine 139) H2AX, and anti-53BP1 antibodies as primary and Alexa 555 and Alexa 488 as secondary antibodies, respectively. Images were taken with a confocal microscope and were manually analysed using ImageJ software. Nine or more foci of γ H2AX and 53BP1 per cell were considered as positive. (B) Quantitative data presented as mean \pm S.E.M. from 3 independent experiments. (C) Comet assay showing the DNA damage induced by VE-821 and AZD7762 either alone or in combination. U2OS cells were treated with DMSO, 60 nM of AZD7762 and 10 μ M of VE-821 or its combination for 24 h, at the end of incubation cells were harvested and alkaline comet assay was performed. (D) Quantitative data of tail moment presented as mean \pm S.E.M. from 3 independent experiments. In each experiment \geq 100 comets were measured. (E) Lack of pan-nuclear γ H2AX induction in normal fibroblast VH-10 cells VH-10 cells were treated with indicated concentrations for 24 h. Cells were probed with anti-phospho (Serine 139) H2AX and anti-53BP1 antibodies as primary and Alexa 488, Alexa 555 as secondary antibody respectively. Images were taken using a confocal microscope and were manually analysed using ImageJ software. Cells with nine or more foci of γ H2AX per cell were considered as γ H2AX positive. (F) Quantitative data presented as mean \pm S.E.M. from 3 independent experiments. (G) Western blot showing minor increase in phosphorylation of H2AX. VH-10 cells were treated with indicated concentrations for 24 h. At the end of the incubation, cells were lysed, protein extracted and western blotting was performed using anti-phospho (Serine 139) H2AX, anti-cleaved PARP, anti-phospho (Serine 10) Histone H3 and anti- β -actin antibodies. (Scale bar represents 20 μ m). Statistical significance was determined using One way ANOVA. * p <0.05; ** p <0.01, *** p <0.001, **** p <0.0001.

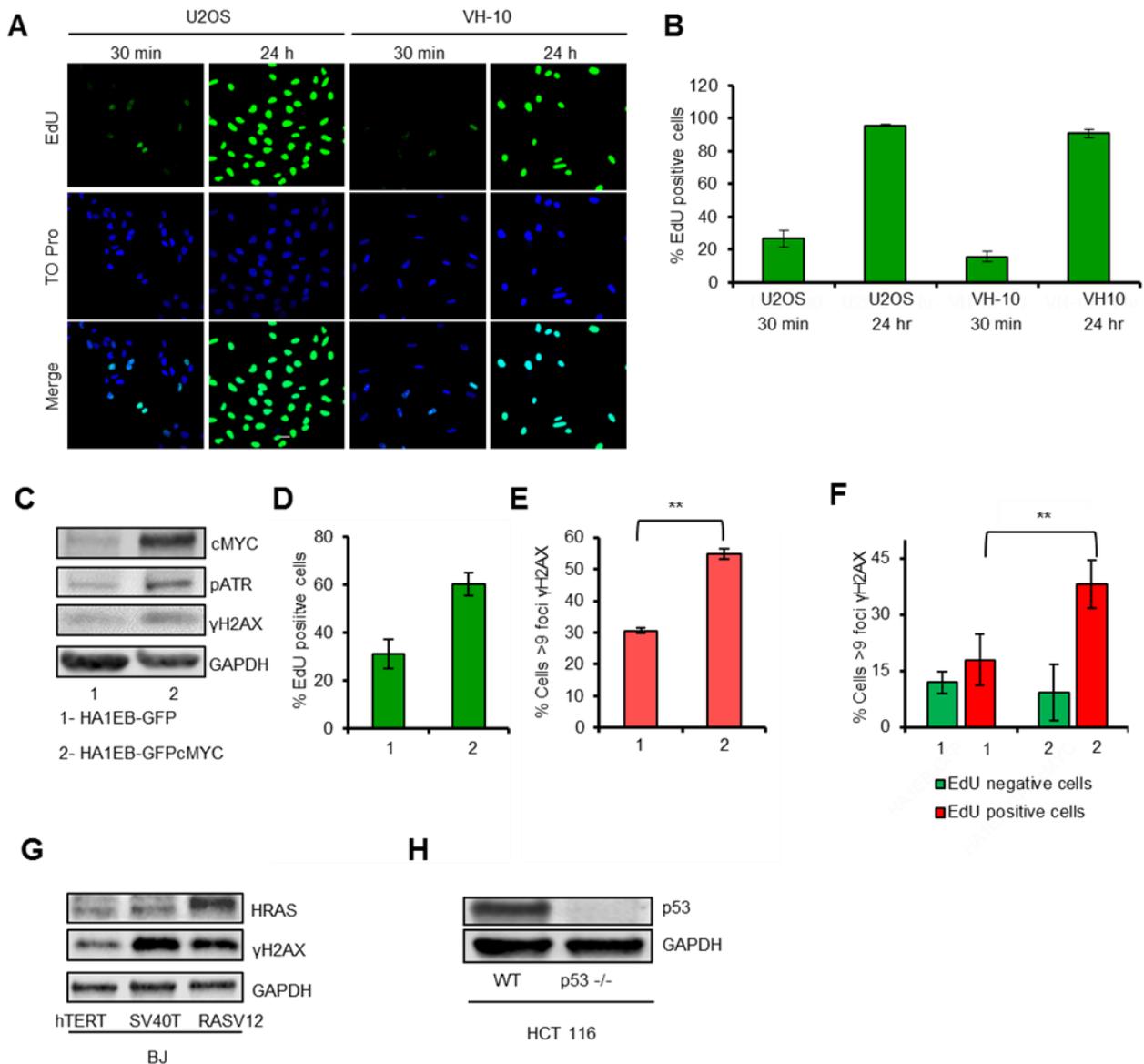


Figure S3. U2OS cancer cells and primary fibroblast VH-10 cells complete one cell cycle within 24 h, Related to Figure 1 and 2. (A) Representative images showing EdU incorporation at 30 min and 24 h post addition of EdU to U2OS and VH-10 cells. Images were taken using a confocal microscope and were analysed using ImageJ software. Mean Intensity ≥ 80 AU per cell was considered as positive. (Scale bar-20 μ m). (B) Quantitative data, mean \pm S.E.M. from 3 independent experiments. (C) cMYC overexpressed cells induce replication stress and DNA damage. Analysis of cMYC, pATR, γ -H2AX and GAPDH protein levels in HA1EB-GFP (Control) and HA1EB-GFP-cMyc (cMyc-overexpressing) cells by Western Blot. (D) Quantitative data of EdU positive cells. (E) Quantitative data of γ H2AX foci positive cells. (F) Quantitative data of γ H2AX foci in EdU negative and EdU positive cells. Data presented as mean \pm S.E.M. from 2 independent experiments. Statistical significance was determined using Student's t-test. * $p < 0.05$; ** $p < 0.01$ (G) Western blot showing transformed BJ SV40T and BJ RASV12 cancer like cells have more endogenous DNA damage compare to normal hTERT BJ cells. (H) p53 protein expression in HCT116^{WT} and HCT116^{p53^{-/-}}.

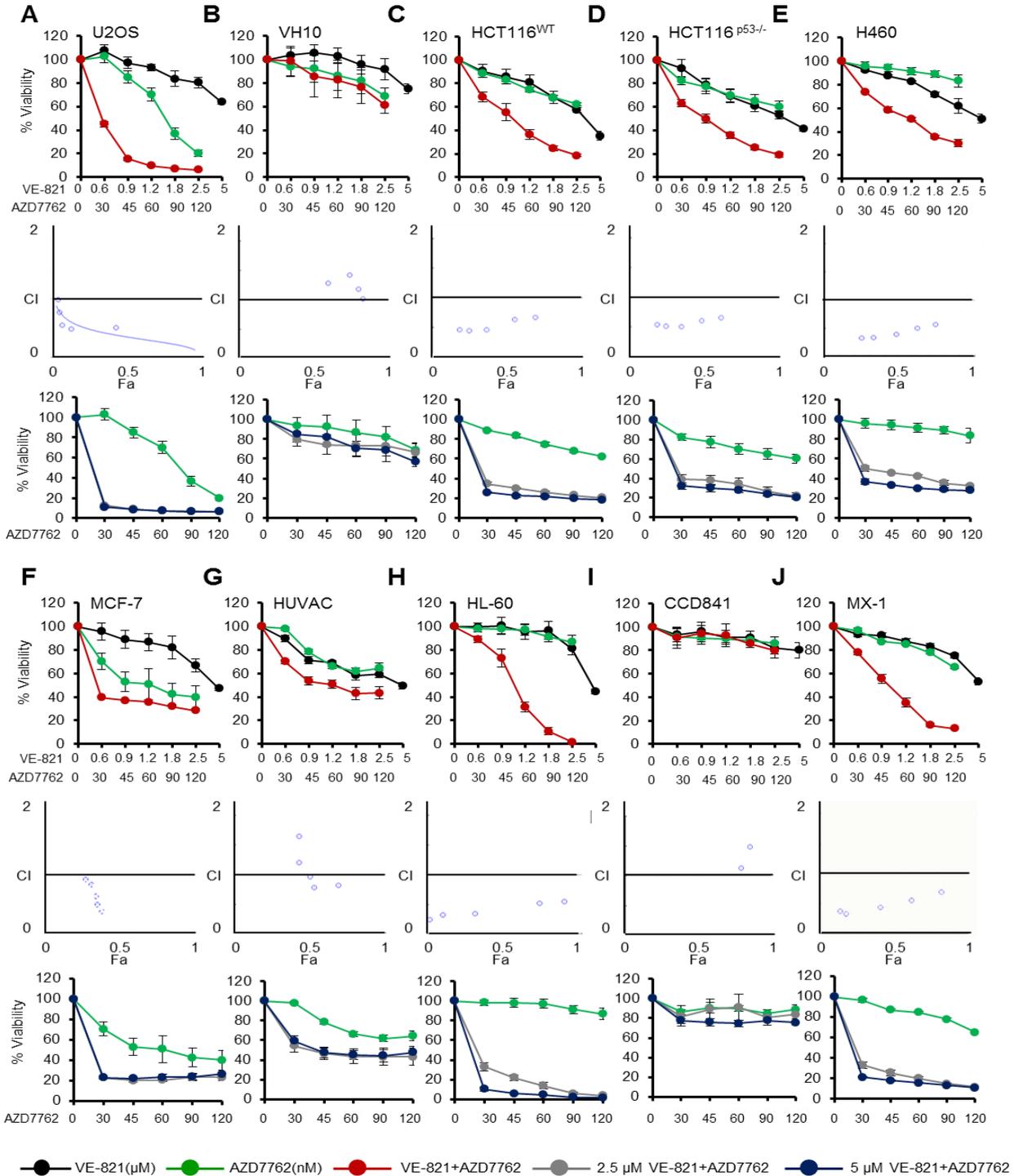


Figure S4. ATR inhibitor VE-821 and CHK1 inhibitor AZD7762 in combination synergistically kill cancer cells but not normal cells, Related to Figure 2. Different cancer cell lines as well as normal cells (U2OS, HCT-116^{WT}, HCT116^{p53-/-}, H460, MCF-7, HL 60, MX-1; normal fibroblast VH-10, normal colon epithelial cells CCD841 and normal HUVEC endothelial cells) were seeded in 96 well plates and treated with indicated doses for 72 h. Resazurin based assay was used to measure viability of cells treated with ATR and CHK1 inhibitors alone or in combination. Data represent, mean ± S.E.M from 3 independent experiment. Drug interaction was analysed using Compusyn software. CI index below 1 is considered as synergistic interaction. Quantitative data presented as mean ± S.E.M. from 3 independent experiments. (A) U2OS, (B)

VH-10, (C) HCT116^{WT}, (D) HCT116^{p53-/-}, (E) H460, (F) MCF-7, (G) HUVEC, (H) HL-60, (I) CCD841 and (J) MX-1 showing viability of particular cell lines against inhibitor alone or in combination. Drug combination index (CI) plot and potentiation of AZD7762 cytotoxicity by VE-821 are depicted below the viability figure of respective cell lines.

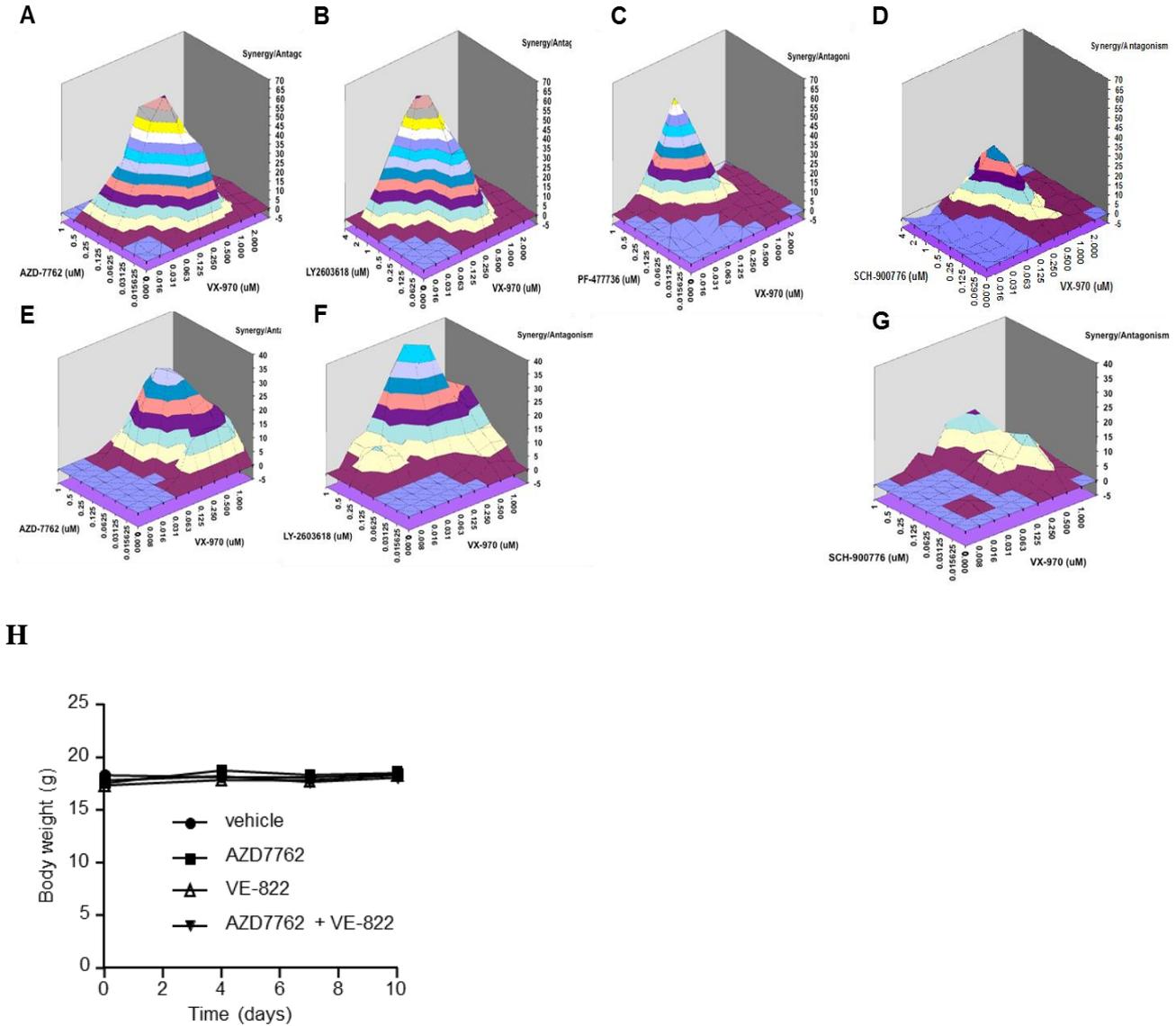


Figure S5. Various CHK1 inhibitors synergise with the ATR inhibitor VX-970 in cancer cells, Related to Figure 2. H23 (non-small cell lung carcinoma) and HT29 (colorectal carcinoma) cells were treated in triplicate with VX-970 and the indicated CHK1 inhibitor for 96 h and cell density was measured by MTS assay. Synergy was analyzed at the 95% interval using MacSynergy II software. (A-D) and (E-G) showing Synergy plot in H23 cell line and HT29 cell lines respectively. (H) ATR inhibitor VX-970 and CHK1 inhibitor AZD7762 are well tolerated either alone and in combination in H460 xenografted mice-Treatment regimen is outlined in main manuscript. Body weight data for H460 xenograft mouse.

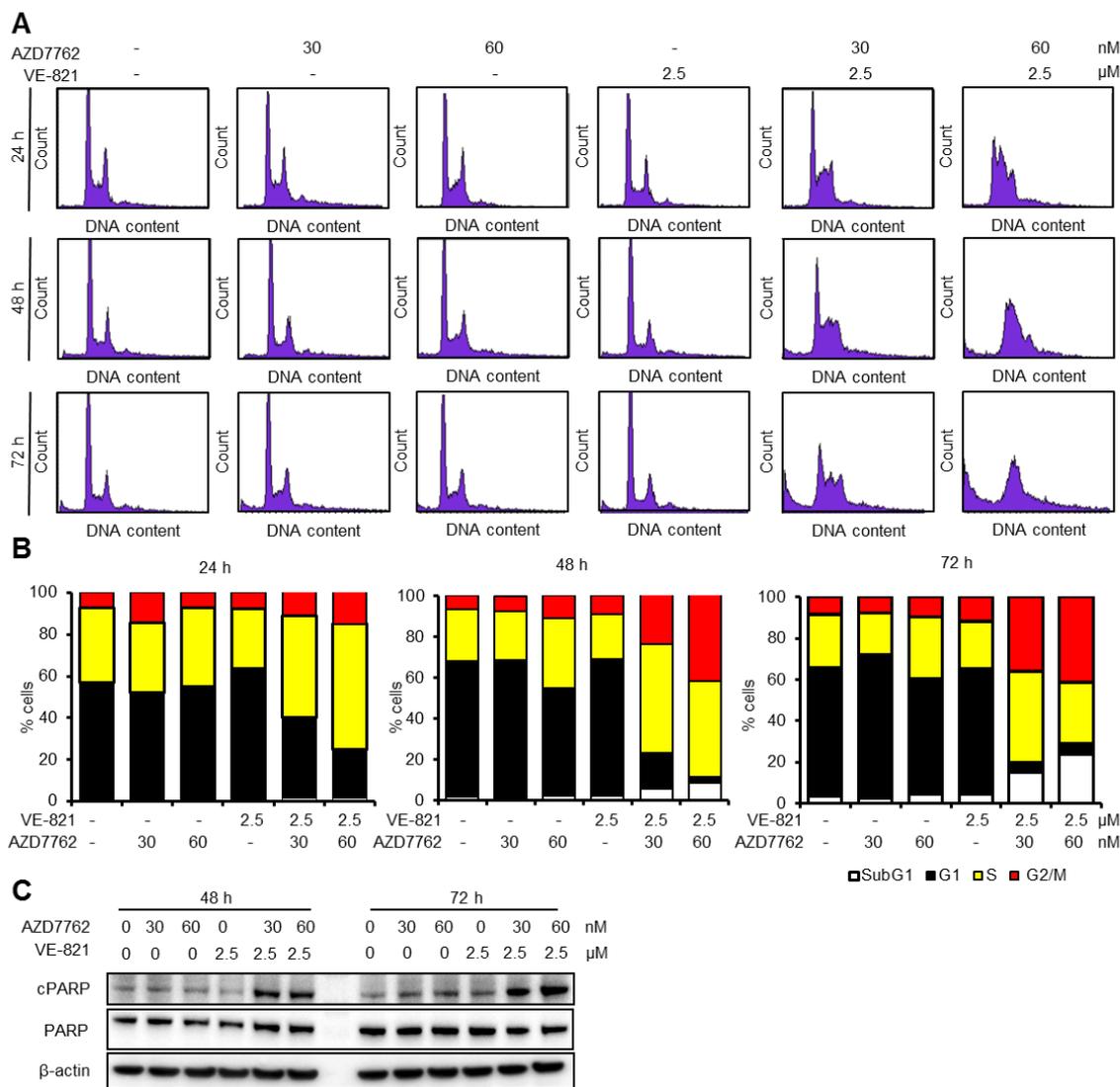


Figure S6. Combination treatment of VE-821 and AZD7762 induces apoptosis in U2OS cells after S phase arrest, Related to Figure 4. U2OS cells were treated with indicated concentrations of inhibitors, and harvested at indicated time points. Propidium iodide (PI) staining was carried out to measure cell cycle profile using flow cytometry. (A) Figures represent cell cycle profile at different time points. (B) Quantitative data was obtained using Modfit software. (C) VE-821 and AZD7762 in combination induces apoptosis in U2OS cells. Protein was harvested from U2OS cells at 48 and 72 h with the treated with the mentioned concentration. Western blot was carried out using apoptosis marker cleaved PARP, total PARP and β -actin.

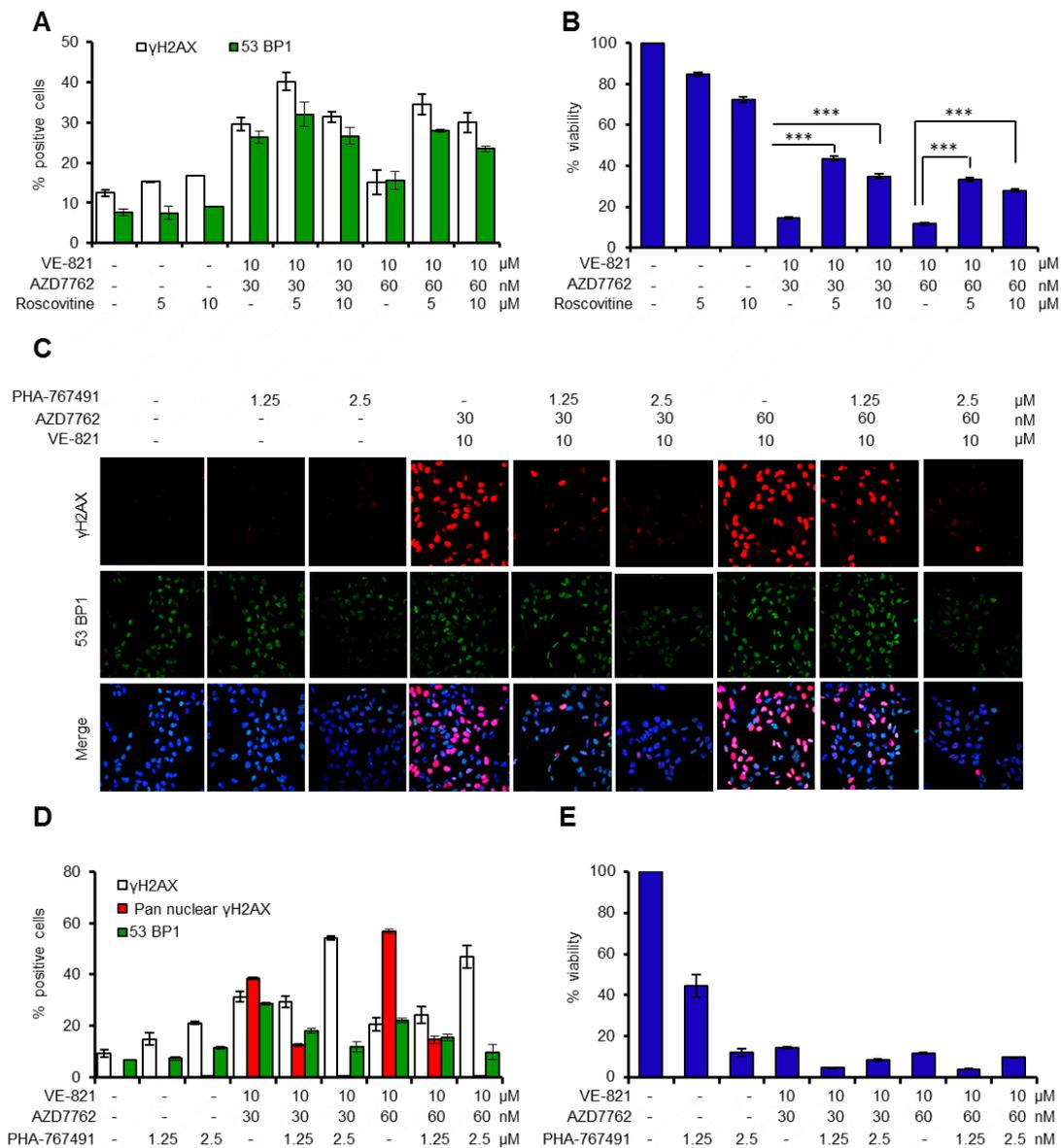


Figure S7. Cytotoxic effect in U2OS cancer cells by combination treatment of VE-821 and AZD7762 is mainly due to CDK mediated excess origin firing, Related to Figure 5. (A) U2OS cells were pre-treated with indicated concentration of the CDK inhibitor Roscovitin for 1 h followed by addition of AZD7762 and VE-821 for 24 h. Cells were probed with anti-phospho (Serine 139) H2AX antibody and anti-53BP1 and DNA was counterstained with ToPro. Quantitative data showing percentage of γ H2AX positive cells (nine or more foci per cells) or 53BP1 positive cells (nine or more foci per cells), mean \pm S.E.M. from 2 independent experiments. **(B)** Roscovitin significantly increases the U2OS cell survival against combination of AZD7762/VE-821. U2OS cells were treated with the indicated drug concentrations for 72 h and viability was measure using resazurin. Quantitative data, mean \pm S.E.M. from 4 independent experiments. Statistical significance was determined using Student's t-test. * $p < 0.05$; ** $p < 0.01$, *** $p < 0.001$. **(C)** CDK /CDC7inhibitor PHA-767491 reduce the pan-nuclear γ H2AX and 53BP1 foci in U2OS cells cotreated with VE-821 and AZD7762. U2OS cells were pretreated with PHA-

767491 for 1 h prior to combination treatment with VE-821 and AZD7762 for 24 h. Cells were probed with anti-phospho (Serine 139) H2AX antibody and anti-53BP1 and DNA was counterstained with ToPro. **(D)** Percentage of cells with γ H2AX foci or pan-nuclear γ H2AX signal after indicated treatments. Quantitative data, mean \pm S.E.M. from 2 independent experiments. **(E)** No significant increase in U2OS cell survival in cells pretreated with PHA-767491 before dual inhibition of ATR and CHK1. U2OS cells were treated with the indicated drug concentrations for 72 h and viability was measure using resazurin. Quantitative data, mean \pm S.E.M. from 4 independent experiments.