A selective small molecule
DNA2 inhibitor for sensitization of human cancer cells to chemotherapy

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SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Inhibition mode and nonlinear regression to determine the inhibitor Ki.

For different concentrations of substrate, we increased the enzyme concentration in the same ratio to obtain the precise relative mean velocity, using 0.5nM, 1.5nM, 2.5nM, 5nM DNA2 for 5nM, 15nM, 25nM, 50nM substrates, respectively. For analysis of competitive inhibition, the Lineweaver-Burk plot was used with various concentrations of substrate and inhibitor. At a given substrate concentration, apparent inhibition constants of the inhibitor ($IC50_{observ}$) were derived from fit of the data to Eq 1,

$$\text{fraction inhibition} = \frac{[\mathrm{I}]}{\mathrm{IC50}_{obsev} + [\mathrm{I}]} \quad (1)$$

The inhibition constant K_i, which describes inhibitor binding to DNA2 in the absence of substrates, is related to observed IC_{50} values by equation (2),

$$\mathrm{IC50}_{obsev} = K_i\left(1 + \frac{[\mathrm{S}]}{K_M}\right) \quad (2)$$

in which K_M is the Michaelis constant for the substrate. At limiting substrate concentrations, the $IC50_{observ}$ values approaches K_i. Thus, the K_i value is obtained from extrapolation of the [S]-dependence of $IC50_{observ}$.

Cell culture, measurement of IC50, clonogenic assay, and cell proliferation assay

Cancerous and non-cancerous cells were cultured based on the culture instructions from the American Type Culture Collection (ATCC). Chemical compounds for the candidate DNA2 inhibitors were requested from NCI DTP (http://dtp.nci.nih.gov/). CPT was

purchased from Sigma (St. Louis, MO, purity > 99%) and MK4827 was from MedChem Express (Monmouth Junction, NJ). The cell survival rate of MES or human cancer cells under different treatments was measured by clonogenic assays following a published protocol (Franken et al, 2006). Briefly, 500 cells were seeded in a 6-well plate and incubated in culture medium containing DMSO or drugs, fresh medium with or without C5 for the cultured cells was changed every 3-4 days. For clonogenic assay, the plate was washed with PBS buffer after 14 days of culture and the colonies were fixed and stained with crystal violet solution and the number of colonies (>50 cells) was counted. The combination index, indicating the synergistic effect of the compounds, was measured following a previously published method (Chou, 2010). To measure the IC50 with the proliferation assay, 1,000–2,000 cells were seeded in a 96-well plate and incubated in culture medium containing DMSO or drugs, fresh medium with or without C5 for the cultured cells was changed every three days. After six days, the CellTiter 96 One Solution Reagent (Promega, Madison, WI) was added to the culture medium to measure the viable cells and the absorbance at 490 nm (A490), which is positively correlated to the number of cells, in each well was measured. The relative number of viable cells in the untreated control group was arbitrarily set as 1, and the relative number of viable cells in a C5-treated well was calculated by dividing its A490 by that of the control well.

Establishment of DNA2 knockout ES cells

To construct the knockout vector for the mouse DNA2 allele, two DNA fragments corresponding to the mouse DNA2 gene were inserted into the poly-linker A and B, respectively, on the gene targeting vector, PKO scrambler NTK (Invitrogen, Carlsbad, CA)

containing a neomycin or puromycin selection marker. One DNA fragment (5' arm) covered DNA sequences from intron 1 to intron 3 of the mouse DNA2 gene, and the other DNA fragment (3' arm) corresponded to DNA sequences from intron 7 to intron 12 of the mouse DNA2 gene. The knockout vector (neomycin) was electroporated into embryonic stem cells of the 129S1 genetic background. Recombination between the knockout vector and the WT DNA2 allele resulted in a mutant DNA2 allele, which deletes the exons 4-7 and disrupts the mouse DNA2 gene. DNA2+/- ES cells were selected by neomycin marker and confirmed with Southern blotting analysis. A second DNA2 knockout vector (puromycin) was electroporated into the DNA2+/- ES cells and DNA2-/- ES cells were selected by both neomycin and puromycin markers and confirmed with Southern blotting and PCR analysis.

DNA fiber assay

DNA fibers performed and the data were analyzed as described previously (Schwab & Niedzwiedz, 2011; Techer et al, 2013; Thangavel et al, 2015). Briefly, A549 cells with or without DNA2 inhibition by C5 or siDNA2 were pre-incubated with 50 μM IdU for 40 min, left untreated or treated with indicated drugs in IdU medium, and then incubated with 250 μM CldU for 40 min. The cells were then collected and suspended in ice-cold PBS at 7.5×10^5 cells/ml. The labeled cells (2 μl) were dropped on a glass slide and waited for 5 min to partially dry, and then lysed with 7 μl of lysis buffer (0.5% SDS in 200 mM Tris-HCl, pH 7.4, and 50 mM EDTA) for 2 min. The slides were tilted ~15° to allow DNA fiber spreads. After drying, the slides were fixed in 3:1 methanol/acetic acid for 10 min. The DNA was then denatured in 2.5 M HCl for 80 min, followed by PBS washes for 15 min (triple), and the slides were blocked with 5% BSA for 30 min. The slides were then incubated with 1:50 rat

anti-BrdU (Abcam, clone BU1/75 (ICR1), for detection of CldU) followed by 1:50 mouse anti-BrdU (BD Biosciences, clone B44, for detection of IdU) for 1 hour. After incubation with the primary antibodies and extensive washes, slides were incubated with 1:200 Alexa Fluor 488-conjugated anti-rat (Life Technologies) and Alexa Fluor 555 anti-mouse (Life Technologies) for 1h. Next, the slides were mounted with ProLong Gold Antifade reagent (Life Technologies). Images were taken with a Zeiss AxioCam 506 Mono microscope.

References

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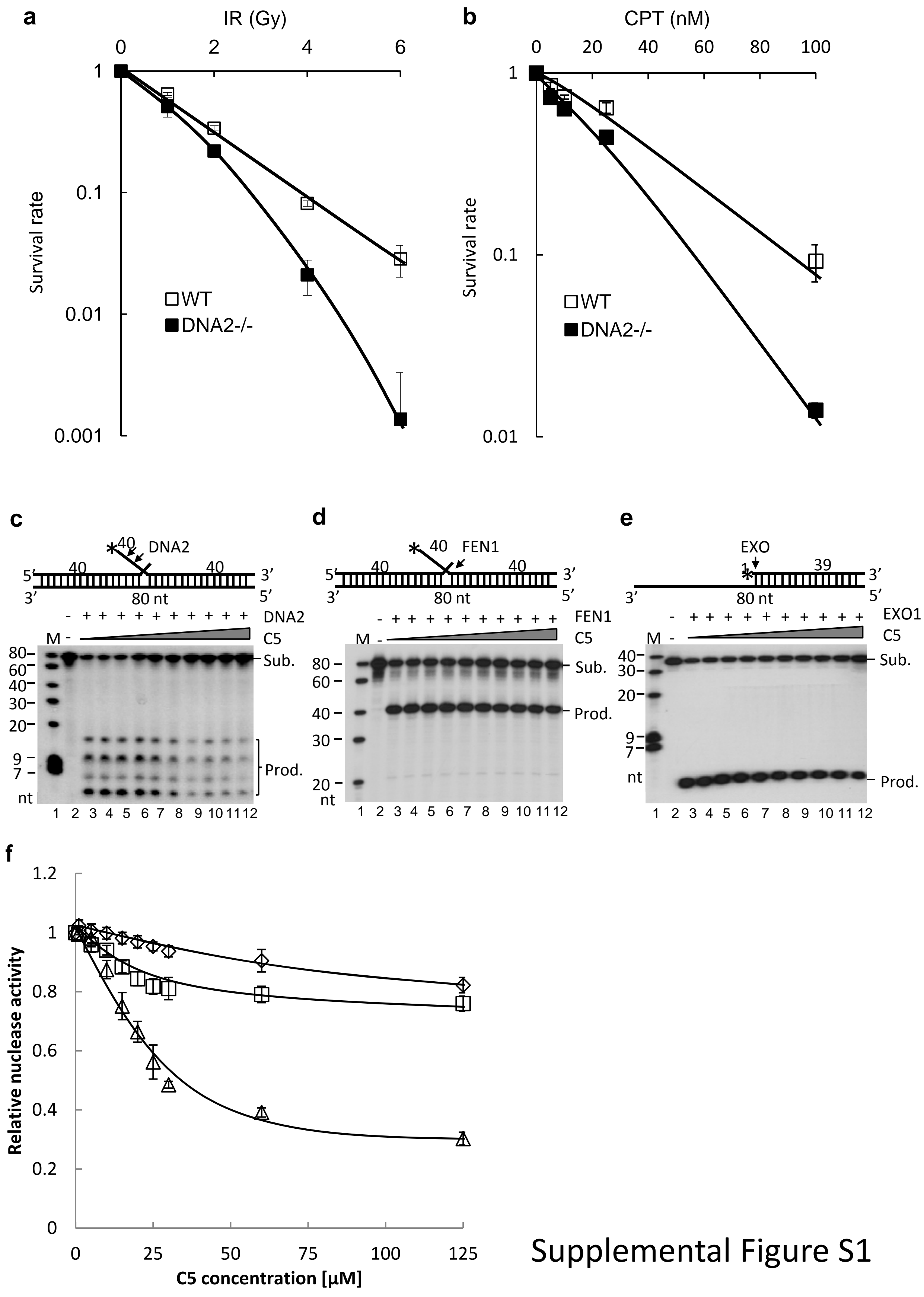
Supplemental Table 1:
Identification of the top 40 hits by virtual screening based on the DNA2 3-D model

No.	NSC No.*	Formula	MW (Dalton)	Docking Scores**	Nuclease inhibition	DNA-dependent ATPase
C1	6284	$C_3H_3N_3O_3$	129.07	-8.25	-	-
C2	7861	$C_6H_6N_4O_2$	166.14	-8.63	-	-
C3	9432	$C_7H_8N_4O$	164.16	-9.25	-	-
C4	12754	$C_5H_8N_2OS$	144.19	-7.59	-	-
C5	15765	$C_{10}H_6N_2O_5$	234.17	-8.3	++	++
C6	20260	$C_9H_{15}N_2O_{15}P_3$	484.14	-10.86	+	+
C7	33120	$C_{18}H_{18}Cl_2O_6$	401.24	-7.34	-	-
C8	39858	$C_{32}H_{36}N_6O_8S_4$	760.92	-7.41	++	++
C9	42753	$C_8H_9F_3O_2$	194.15	-6.88	-	-
C10	55521	$C_9H_6F_6O$	244.13	-7.05	-	-
C11	57727	$C_5H_9N_3O_2$	143.13	-6.91	-	-
C12	64878	$C_7H_7N_3S$	165.22	-7.04	-	-
C13	65634	$C_{11}H_{14}N_2O$	190.24	-7.71	-	-
C14	79004	$C_4H_5N_5S$	155.18	-6.58	-	-
C15	79197	$C_4H_9N_3O_3$	147.13	-9.24	-	-
C16	84922	$C_{16}H_{11}NO_3$	265.26	-7.6	-	-
C17	85277	$C_{16}H_{14}N_2O_3$	282.29	-10.06	-	-
C18	99439	$C_{11}H_{13}N_3O_5$	267.24	-11.61	-	-
C19	102552	$C_6H_9N_3O_2S$	187.22	-9.28	-	-
C20	103797	$C_{13}H_{17}N_3O_6$	311.29	-11.05	-	-
C21	110391	$C_9H_6N_4O$	186.17	-7.14	-	-
C22	115812	$C_{16}H_{12}FNO_3$	285.27	-9.03	-	-
C23	119749	$C_3H_5N_3O_2$	115.09	-8.03	-	-
C24	129784	$C_{10}H_8N_2O_4$	220.18	-6.75	-	-
C25	140380	$C_{19}H_{29}ClN_6O_6S$	504.99	-7.44	-	-
C26	157740	$C_{12}H_{13}N_5O_4$	291.26	-11.14	-	-
C27	166583	$C_6H_4Cl_2N_4O$	219.03	-7.77	-	-
C28	170103	$C_{10}H_3F_7N_2$	284.13	-6.78	-	-
C29	211332	$C_5H_{11}N_5$	141.17	-7.86	-	-
C30	266142	$C_6H_6N_2O_3$	154.12	-7.27	-	-
C31	291643	$C_9H_{10}N_2O_6$	242.19	-10.87	-	-
C32	305488	$C_{14}H_{14}N_2O_4$	274.27	-9.26	-	-
C33	313976	$C_{51}H_{70}N_{10}O_{24}P_2S_2$	1340.00	-12.74	-	-
C34	321117	$C_{10}H_8N_2O_2$	188.18	-7.81	-	-
C35	329951	$C_{11}H_8N_2O$	184.19	-8.56	-	-
C36	360177	$C_6H_6N_4O_2$	166.14	-8.24	++	++
C37	367734	$C_9H_{12}N_2O_5S$	260.27	-10.05	-	-
C38	375395	$C_8H_4N_2O_6$	224.13	-8.35	++	++
C40	382898	$C_{30}H_{18}Cl_3N_{15}O_7$	806.92	-6.54	-	-

*NSC No.: the NCI's internal ID number
**Docking Score: Based on Schordinger Glide docking software at XP precision



Supplemental Figure S1

Supplemental Figure 1 (Also see the Figure 1). a-b. Elimination of *DNA2* gene sensitizes mouse ES (MES) cells to ionized radiation and camptothecin (CPT)
a. The survival curves of WT and *dna2*$^{-/-}$ MES cells after ionizing radiation (IR) treatment. WT and *dna2*$^{-/-}$ MES cells were exposed to 1, 2, 4, 6 gray (Gy) γ-irradiation. The surviving MES cell colonies were counted and normalized to the corresponding untreated control, whose survival rate was arbitrarily set as 1.
b. The survival curve of the WT and *dna2*$^{-/-}$ MES cells after CPT treatment. The WT and *dna2*$^{-/-}$ MES cells were cultured in the medium containing 6.25, 12.5, 25, and 100 nM CPT. The surviving ES cell colonies were counted and normalized to corresponding untreated control, whose survival rate was arbitrarily set as 1. In both panels, each point represents the mean ± s.d of three independent assays.
c-f. DNA2, FEN1 and EXO1 nuclease assays used to test the inhibitor specificity. c, d, e. DNA2, FEN1, and EXO1 nuclease assays with the inhibitor titration, respectively. **F.** Quantification of DNA2 FEN1 and EXO1 activities. The enzyme concentrations used for DNA2, FEN1 and EXO1 are all 10 nM. The DNA substrate concentration used was 50 nM. A range of the inhibitor C5 concentrations used was from 0 to 125 µM. The relative activities normalize to DMSO, which activity set as 1. Each experiment was repeated at least three times. The error bar represents the standard deviations. Symbols used: ◇ FEN1, □ EXO1, △ DNA2.

Supplemental Figure S1 (Continued)

a

Relative products

1
0.8
0.6
0.4
0.2
0

0 5 10 15 20 25

Time (min)

	[S](nM)	[E](nM)
◇	5	0.5
□	15	1.5
△	25	2.5
○	50	5

b

Dna2 – –

C5 – – – – – + + +

Δ

M13

1 2 3 4 5 6 7 8

% HELICASE REMAINING after C5

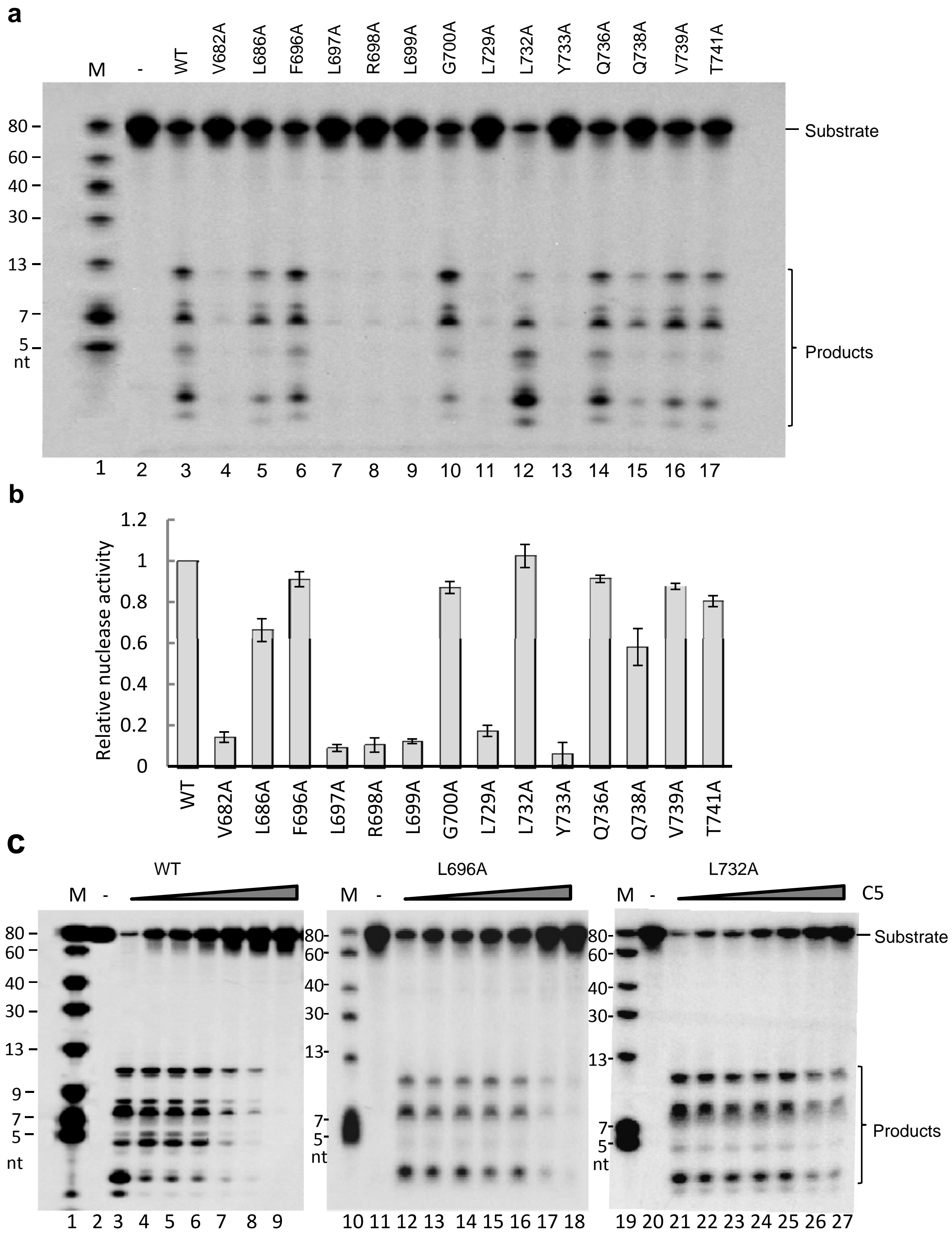
3 53 64

Supplemental Figure S2

Supplemental Figure S2 (see also Figure 2): a. Time course of flap substrate cleavage catalyzed by DNA2 enzyme. Various DNA2 concentrations and Various substrate concentrations (see the insertion) were used to determine the proper time for assays. 12 time points were tested. The efficiency is the best during 1-10 minutes.

b. Inhibition of DNA2 helicase activity by C5. hDNA2 D277A, nuclease-deficient, was purified as described previously. The helicase substrate is a 43 base oligonucleotide annealed to M13DNA over 24 bases and having an 18 nt 5' noncomplementary tail. The oligonucleotide was 5' labeled with [γ-32P]ATP by T4 polynucleotide kinase and purified with a Sepharose CL4B column. The standard reaction mixture contained 50 mM Tris-HCl, pH7.5, 2 mM DTT, 0.25mg/ml BSA, 4 mM $MgCl_2$, 4 mM ATP and 32P-labeled helicase substrate. C5 was preincubated with enzyme for 1 min. The reaction was for 30 min at 37°C. The reaction was stopped with 5x stop soluation (60 mM EDTA, 40% sucrose, 0.6% SDS, 0.25% bromphenol blue and 0.25% zylene cyanole FF). Reaction products were separated on an 8% native polyacrylamide gel containing 0.1% SDS and detected by phosphorimaging and quantified using ImageQuant. Lane 1, boiled substrate; Lane 2, no enzyme, Lanes 3-6 contained 5, 10 and 15 nM DNA2, respectively; lanes 7-10 contained 5,10, and 15 nM DNA2 plus 100 μM C5. Lanes 3, 4 and 5 represent the amount of helicase at each of the three increasing helicase concentrations in the absence of C5. Lanes 6, 7, and 8 represent the same amount of enzyme as 3, 4, 5, respectively, but in the presence of C5. The percent of helicase remaining after C5 treatment is presented as the amount of substrate unwound in lane 6 divided by the amount in lane 3, the amount of substrate unwound in lane 7 divided by the amount in lane 4, and the amount of substrate unwound in lane 8 divided by the amount in lane 5.

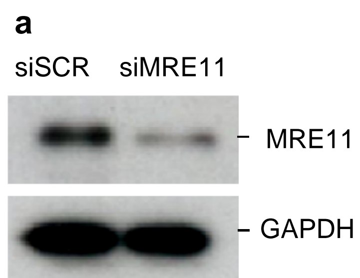
Supplemental Figure S2 (continued)

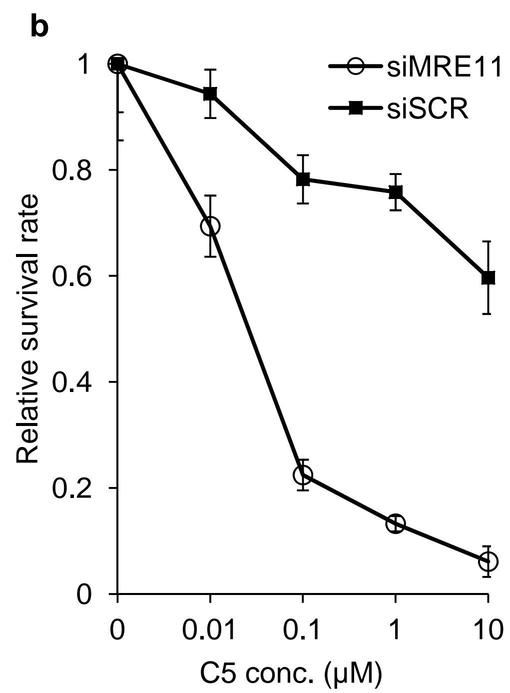


Supplemental Figure S3

Supplemental Figure S3. 14 residues in Site 1 were mutated to determine the DNA2 binding capacity to the inhibitor C5 (See also Figure 3). **a**. Nuclease assay to determine the activities of the WT and mutants. The enzyme concentrations used for DNA2 and its mutants are all 10 nM. The DNA substrate concentration used was 50 nM. **b**. Quantification of the WT and mutants nuclease activities. The WT activity was set as 1. **c**. Nuclease activity assays to measure the sensitivity of the WT (lane 1-9) and mutants F696A (lanes 10-18) and L732A (lanes 19-27) to the inhibitor C5. The enzyme concentrations used for DNA2 and its mutants are all 10 nM. The DNA substrate concentration used was 50 nM. The inhibitor concentrations used were in a range from 0 to 1000 μM.

Supplemental Figure S3 (continued)



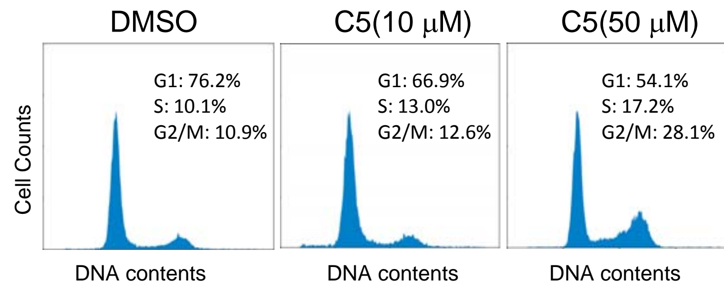


Supplemental Figure S4

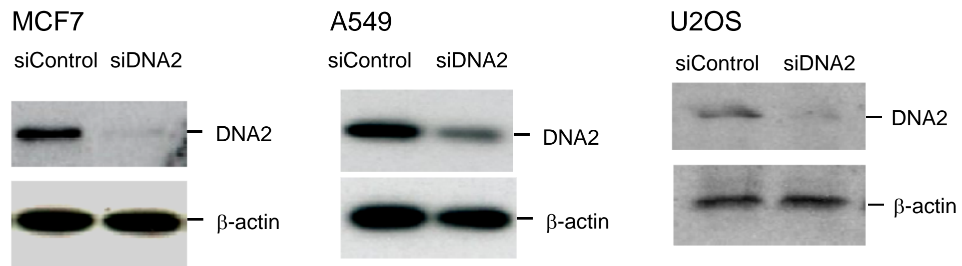
Supplemental Figure S4 (See also Figure 4): a. Knockdown of Mre11 in HCT116 cells. Cells at 50-60% confluence were transfected with scramble siRNA oligos (siSCR) or siRNA oligos against human Mre11(Sigma). After 48 hours incubation, cells were harvested and lysed. The level of Mre11 were detected by western blot using an antibody against human Mre11. The level of GADPH was used as a loading control. Antibodies were from Abcam. **b**. Synergistic effects of Mre11 knockdown and DNA2 inhibition by C5. HCT116 cells were treated with scramble siRNA oligos (siSCR) or siRNA for Mre11 knockdown (siMRE11) for 72 hours. The cells were cultured in the presence of 0, 0.01, 0.1, 1.0, 10 μM C5 for three days. The survival cells were counted. The relative survival rates were calculated with arbitrarily setting those in the absence of C5 as 1. Values are means ± s.d. of three independent assays.

Supplemental Figure S4 (continued)

a



b

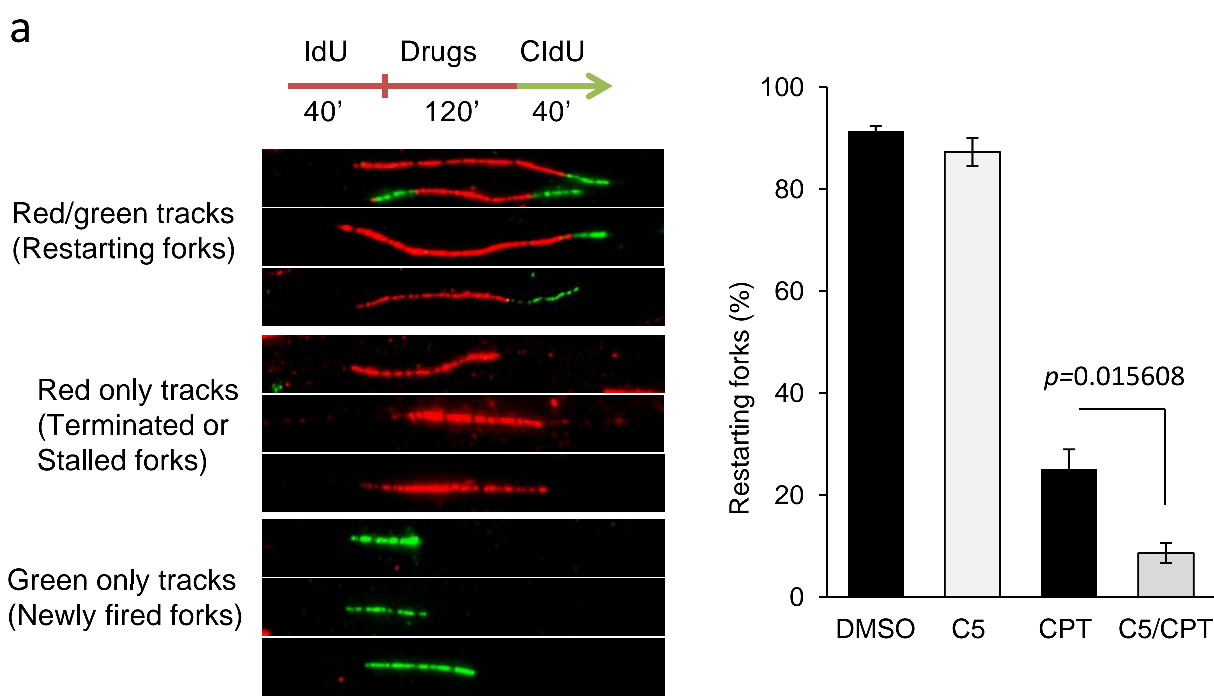


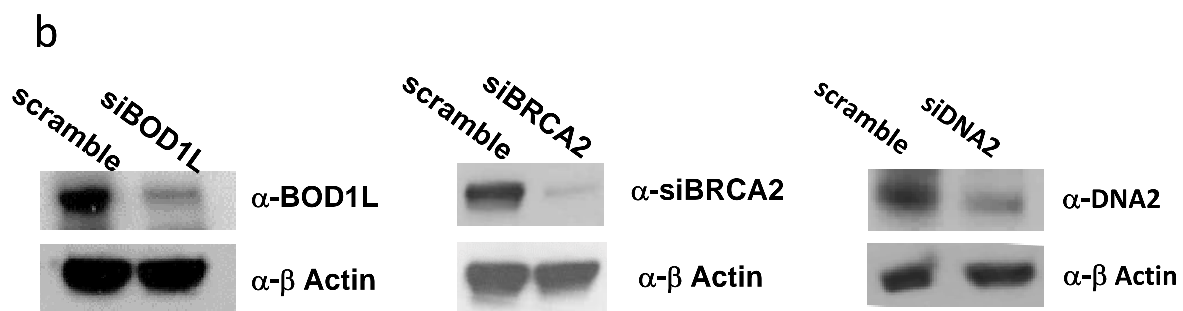
Supplemental Figure S5

Supplemental Figure S5 (See also Figure 5): a. Cell cycle analysis by flow cytometry. HCT116 cells were treated with 0, 10, 50 μM C5 for 48 h. The cells were stained by PI and the cell cycle profile was determined by flow cytometry and calculated by the Flow Jo software based on DNA contents.

b. Knockdown of DNA2 in MCF7 , A549, and U2OS cells. Cells at 50-60% confluence were transfected with scramble siRNA oligos (siControl) or siRNA oligos against human DNA2 (Sigma). After 48 hours incubation, cells were harvested and lysed. The level of DNA2 were detected by western blot using an antibody against human DNA2. The level of β-actin was used as a loading control. Antibodies were from Abgent.

Supplemental Figure S5 (continued)





Supplemental Figure S6

Supplemental Figure S6 (Also see Figure 6). a. DNA fiber assay confirms that C5 inhibits DNA replication fork restart in the presence of high concentration of CPT. A549 cells were first labeled with IdU (red) and co-cultured with the indicated drugs (10 μM C5 or 2 μM CPT) or drug combinations (10 μM C5 and 2 μM CPT), and then washed and labeled with CIdU (green). F. The different types of typical tracks. Left panels: representative images of different tracks; right panel: Quantification of the different tracks found in the cells with different treatments. Approximate 150 tracks were scored in each assay. Values are means ± s.d. from three independent experiments. The p value was calculated with the student's t-test.

b. Knockdown of DNA2, BRCA2, and BOD1L in U2OS cells. The cells at 50-60% confluence were transfected with scramble siRNA oligos or siRNA oligos (Sigma) against human BOD1L, BRCA2 and DNA2 individually or in combination. The levels of BOD1L, BRCA2, or DNA2 were detected by western blot using an antibody against human BOD1L, BRCA2, or DNA2. The level of β-actin was used as a loading control. Antibodies were from Abgent

Supplemental Figure S6 (continued)