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Chk1 inhibition after replicative stress activates a double strand break response mediated by ATM and DNA-dependent protein kinase

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Checkpoint kinase 1 (Chk1) regulates cell cycle checkpoints and DNA damage repair in response to genotoxic stress. Inhibition of Chk1 is an emerging strategy for potentiating the cytotoxicity of chemotherapeutic drugs. Here, we demonstrate that AZD7762, an ATP-competitive Chk1/2 inhibitor induces γH2AX in gemcitabine-treated cells by altering both dynamics and stability of replication forks, allowing the firing of suppressed replication origins as measured by DNA fiber combing and causing a dramatic increase in DNA breaks as measured by comet assay. Furthermore, we identify ATM and DNA-PK, rather than ATR, as the kinases mediating γH2AX induction, suggesting AZD7762 converts stalled forks into double strand breaks (DSBs). Consistent with DSB formation upon fork collapse, cells deficient in DSB repair by lack of BRCA2, XRCC3 or DNA-PK were selectively more sensitive to combined AZD7762 and gemcitabine. Checkpoint abrogation by AZD7762 also caused premature mitosis in gemcitabine-treated cells arrested in G_1/early S-phase. Prevention of premature mitotic entry via Cdk1 siRNA knockdown suppressed apoptosis. These results demonstrate that chemosensitization of gemcitabine by Chk1 inhibition results from at least three cellular events, namely, activation of origin firing, destabilization of stalled replication forks and entry of cells with damaged DNA into lethal mitosis. Additionally, the current study indicates that the combination of Chk1 inhibitor and gemcitabine may be particularly effective in targeting tumors with specific DNA repair defects.

Introduction

Genotoxic stress in proliferating cells activates a signaling network referred to as the DNA damage response (DDR) that serves to ensure genetic stability by activating cell cycle checkpoints and initiating DNA repair.[1] Central to the response are the phosphoinositide 3-kinase related kinases (PIKKs) ataxia-telangiectasia mutated (ATM), ATM and Rad 3-related kinase (ATR), and DNA-dependent protein kinase (DNA-PK)[2-5] which function as sensors and/or proximal transducers of the pathway. ATR primarily responds to replication blockage whereas ATM and DNA-PK are involved in recognition and repair of DNA double strand breaks (DSBs). However, there is significant overlap between the pathways. For example, DNA-PK is involved with repair of DNA damage resulting from replication blockage by aphidicolin[6] and ATM is activated by nucleoside analogues at concentrations insufficient for DSBs.[7] Cross-talk between the PIKKs is evidenced by ATM-dependent ATR activation in response to ionizing radiation (IR)[8] and ATR-dependent ATM phosphorylation induced by ultraviolet light (UV).[9]

Replicative block from UV, hydroxyurea, or nucleoside analogue treatment activates the ATR-Chk1 pathway. Unwinding of DNA ahead of stalled forks generates single-stranded DNA. Binding of replication protein A (RPA) to single stranded DNA recruits ATR via the ATR-interacting protein (ATRIP)[10] and the replication factor C $(RFC)_{1\text{-}5}$-Rad17 clamp-loader which loads the proliferating cell nuclear antigen (PCNA)-like Rad9-Hus1-Rad1 (9-1-1) sliding clamp onto DNA.[11,12] Rad9 binds the adaptor topoisomerase (DNA) II binding protein 1 (TopBP1)[13] which stimulates ATR, resulting in phosphorylation of histone H2A variant H2AX at Ser^{139} of its carboxy terminus to form γH2AX,[14] a marker of DNA damage. γH2AX may be important for recruitment of repair proteins and checkpoint maintenance in response to DSBs[15] and may also be integral for genomic stability following replicative stress.[16] ATR activates Chk1 by phosphorylating

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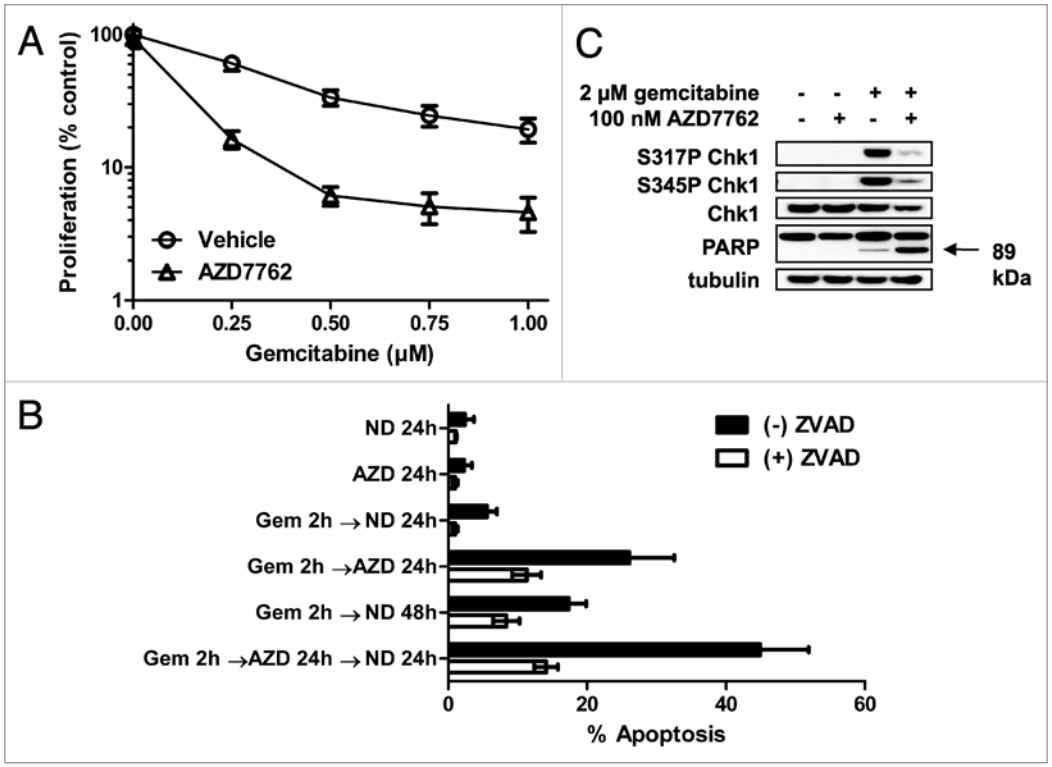

Figure 1. AZD7762 potentiates gemcitabine cytotoxicity. HCT116 cells were treated with gemcitabine (Gem) for 2 h, washed, and incubated with or without 100 nM AZD7762 for 24 h. (A) Proliferation assay. (B) Quantitation of apoptosis in cultures treated with no drug (ND), 2 μM Gem, and/or AZD7762 as indicated. Cultures treated with 20 μM ZVAD were included with each condition. (A and B) are means ± standard deviation (SD) for one of three experiments. (C) Immunoblot analysis of Chk1 and PARP. Data represent at least three experiments.

it on Ser[317] and Ser[345].[17] Chk1 contributes to DDR by effecting S-phase and G_2/M phase arrest through its interaction with Cdc25 phosphatases.[18] Additionally, Chk1 coordinates Rad51-mediated homologous recombination (HR) involved with DSB break repair[19] and is important for the maintenance of viable replication structures after DNA polymerase stalling.[20]

Because of its critical role in cellular response to genotoxic stress, disruption of Chk1 function using small molecules is a strategy for improving the efficacy of DNA-targeted chemotherapeutics. Chk1 inhibition potentiates the cytotoxicity of genotoxic agents in vitro and in vivo.[21-27] Several Chk1 inhibitors are undergoing clinical evaluation in combination with topoisomerase I poison or the deoxycytidine analogue gemcitabine (2', 2'-difluoro-2'-deoxycytidine).[28] Gemcitabine is phosphorylated intracellularly and its diphosphate form (dFdCDP) depletes cellular pools of dNTPs via inhibition of ribonucleotide reductase,[29] while incorporation of gemcitabine triphosphate (dFdCTP) into DNA causes polymerase stalling one base beyond the site of addition.[30] These perturbations of DNA metabolism prevent complete replication and activate the DNA damage response pathway.[31] In cells treated with gemcitabine, Chk1 inhibition abolishes S-phase checkpoint[27,32] and causes premature mitosis.[26,27,33,34] However, a previous study indicated a lack of correlation between premature mitotic entry and cytotoxicity[35] bringing into question the mechanism for chemosensitization by Chk1 inhibitors.

In addition to its effect on checkpoints, Chk1 inhibition with 7-hydroxystaurosporine (UCN-01) or XL844 following gemcitabine treatment induces γH2AX.[32,33] The kinase mediating the increase in γH2AX and how it relates to the effect of Chk1 inhibition on stalled replication forks have not been elucidated.

The current study demonstrates that AZD7762, an ATP-competitive Chk1/2 inhibitor undergoing phase I trials, markedly induces γH2AX, abrogates cell cycle arrest, and initiates apoptosis in gemcitabine-treated cells. AZD7762 alters replication fork dynamics by allowing initiation of replicons suppressed by gemcitabine. Unexpectedly, we found γH2AX formation was mediated by ATM and DNA-PK, rather than ATR, suggesting AZD7762 converted forks stalled by gemcitabine into DSBs. Consistent with DSB formation, cells deficient in DSB repair by lack of BRCA2, Rad51 paralog XRCC3, or DNA-PK were more sensitive to combined AZD7762 and gemcitabine. These novel findings suggest the combination of AZD7762 and gemcitabine may be particularly effective in targeting tumors with specific DDR defects.

Results

The impact of Chk1 inhibition on gemcitabine sensitivity was examined via cell proliferation assay. AZD7762 alone did not affect cell proliferation (**Fig. 1A**) but potentiated the anti-proliferative effect of gemcitabine. The percentage of apoptotic

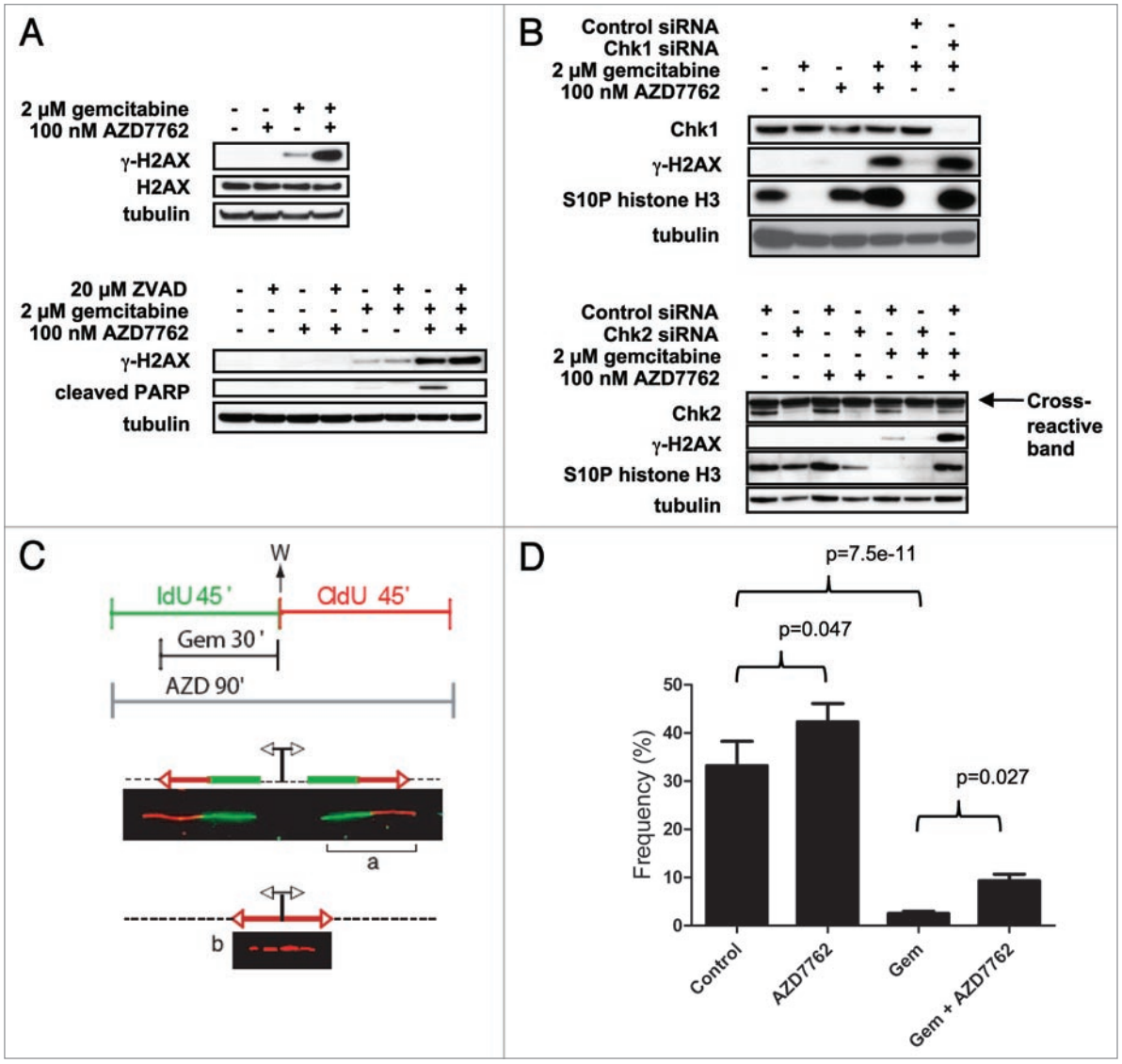

Figure 2. AZD7762 causes marked induction of γH2AX and abrogates suppression of replication origin firing in gemcitabine-treated cells. (A) HCT116 cells were treated with or without Gem for 2 h, washed, and incubated with or without AZD7762 for 24 in the absence or presence of 20 μM ZVAD. (B) Cells were treated as described in (A). Additionally, cells transfected with control, Chk1 or Chk2 siRNA were included. Data represent at least three experiments. (C and D) DNA fiber combing analysis. (C) Experimental protocol for measuring frequency of new origins. Cells were pulsed with IdU for 45 min and 2 μM Gem was added for the last 30 min. Cells were washed (W) and pulsed for 45 min with CldU. When present, AZD7762 was maintained at 100 nM for the entire experiment. (a) Image of a bidirectional replication fork firing during initial IdU pulse or (b) second CldU pulse. (D) Frequency of new origins. Bars represent SEM. Several slides from one biological replicate were analyzed.

cells after treatment was assessed with quantitative fluorescence microscopy of DAPI-stained cells (**Fig. 1B**). Treatment with AZD7762 after gemcitabine increased the percentage of apoptotic cells from 6% to 26%. When gemcitabine-treated cultures were treated with or without AZD7762 and then cultured an additional 24 h in drug-free media, AZD7762 increased the percentage of apoptotic cells from 17% to 45%. Pan-caspase inhibitor Z-VAD-FMK reduced the number of apoptotic nuclei, consistent with their development being a caspase-dependent process. In gemcitabine-treated cells, AZD7762 increased levels of 89 kDa PARP cleavage product, a marker of apoptosis (**Fig. 1C**). We confirmed gemcitabine induced Ser^{317} and Ser^{345} Chk1 phosphorylation (**Fig. 1C**). Decreased phospho-Chk1 in cells treated with gemcitabine and AZD7762 was likely due to reduced total Chk1 protein.

We then examined the impact of AZD7762 on DDR. In cells treated only with gemcitabine we observed minimal induction of γH2AX (**Fig. 2A**). Incubation with AZD7762 after gemcitabine caused a marked increase in γH2AX. Total levels of H2AX were unchanged. γH2AX formation was not diminished by Z-VAD-FMK, indicating the increase in γH2AX was not related to apoptotic DNA fragmentation (**Fig. 2A**, *lower*). To confirm increased γH2AX was due to Chk1 inhibition, Chk1 siRNA-transfected cells were treated with gemcitabine. Chk1 siRNA recapitulated AZD7762 effects (**Fig. 2B**), resulting in γH2AX induction in cells treated with gemcitabine alone comparable to that in cells

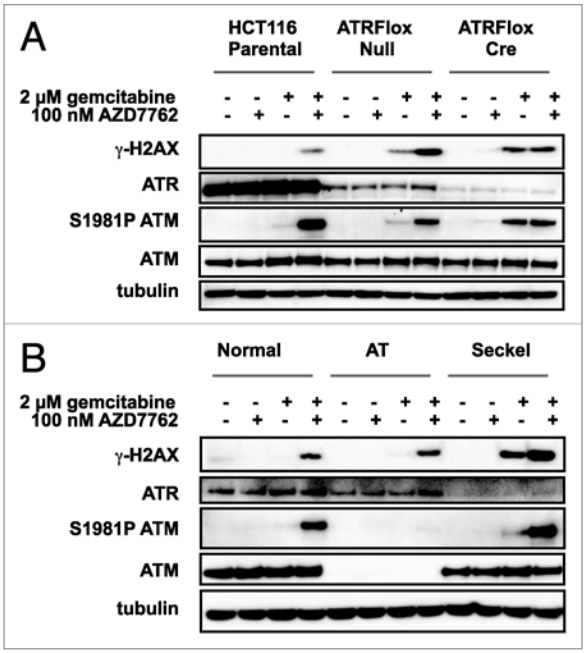

Figure 3. γH2AX induction is ATR-independent and not diminished by ATM deficiency. (A) HCT116 or ATRFlox cells. ATRFlox cells were transfected with null or Cre-recombinase adenoviral vector prior to treatment. Cells were treated with Gem for 2 h, washed, and incubated with AZD7762 for 24 h. (B) Normal, AT or Seckel syndrome lymphoblasts were treated as in (A). Data represent at least three experiments.

expressing Chk1 and treated with gemcitabine and AZD7762. Although AZD7762 also inhibits Chk2,[26] Chk2 siRNA did not phenocopy AZD7762 (**Fig. 2B**, *lower*), indicating the effects of AZD7762 were primarily due to Chk1 inhibition, consistent with another study demonstrating that Chk2 deficiency does not affect gemcitabine cytotoxicity.[36]

To examine possible causes of increased DDR related to Chk1 inhibition, we used DNA fiber combing analysis to determine the impact of AZD7762 on suppression of replication origin firing after gemcitabine. Cultures were sequentially pulse-labeled with IdU and CldU during drug treatment (**Fig. 2C**). DNA fibers incorporating IdU and CldU were visualized as green and red tracks, respectively, under immunofluorescence after labeling with halogenated nucleoside-specific antibodies. Newly synthesized DNA emanating from replication origins fired prior to the initial IdU pulse was labeled green (**Fig. 2C, a**). Contiguous with green tracts were red tracts of DNA synthesized as replication continued during the CldU pulse. Conversely, nascent DNA emanating from replication origins fired during the CldU pulse after gemcitabine was labeled only red (**Fig. 2C, b**). The percentage of new origins firing after the first pulse during various drug treatments was calculated by dividing the number of red-only tracts by the total red/green and red-only tracts. Treatment with AZD7762 increased the percentage of new origins from 33% to 42% (**Fig. 2D**), consistent with a study indicating Chk1 regulates replication initiation in unperturbed S-phase.[37] Gemcitabine suppressed the percentage of new origins to 3%. AZD7762 partially restored origin firing suppressed by gemcitabine from 3% to 9%. Replication fork velocity was also determined. Gemcitabine caused a 65% reduction in fork velocity (**Suppl. Fig. 1C**). AZD7762 demonstrated only a very modest, albeit statistically significant, restoration of the median fork velocity in gemcitabine-treated cultures. Additionally, AZD7762 did not cause an increase in DNA replication in gemcitabine-treated cells in assays utilizing tritiated thymidine incorporation to measure DNA synthesis (data not shown). Taken together, these data suggest that forks emanating from disinhibited origins stalled after limited replication.

ATR is the PIKK primarily involved with DDR to replicative stress and phosphorylates H2AX at Ser^{139}.[14] The contribution of ATR to increased γH2AX was assessed in ATRFlox cells which have one functional ATR allele flanked by LoxP sites that is susceptible to Cre recombinase-mediated deletion.[38] ATR expression in ATRFlox cells is lower than in the HCT116 cells from which they were derived, likely due to LoxP sites hindering gene expression (**Fig. 3A**). Treatment with Cre recombinase further suppressed ATR. However, ATR suppression did not diminish induction of γH2AX in cells treated with gemcitabine and AZD7762. Rather, ATR loss resulted in increased γH2AX in cells treated with gemcitabine alone. Similar results were observed in ATR hypomorphic lymphoblasts isolated from a Seckel syndrome patient when compared to normal lymphoblasts (**Fig. 3B**). These data suggest ATR functions through Chk1 to prevent increased DNA damage leading to γH2AX induction following replicative stress but is not the PIKK responsible for γH2AX induction in cells treated with gemcitabine and AZD7762.

We considered whether Chk1 inhibition after gemcitabine converted stalled forks into DSBs that might impinge upon ATM, the PIKK mediating the response to DSBs in S-phase cells. ATM is recruited and activated by the Mre11-Rad50-NBS1 (MRN) complex[39] which promotes autophosphorylation of Ser^{1981} of ATM, allowing dissolution of ATM dimers into active monomers.[40] This results in phosphorylation of targets involved in DDR, including H2AX. In HCT116 cells, AZD7762 combined with gemcitabine induced Ser^{1981} phosphorylation of ATM (**Fig. 3A**). To test whether activated ATM mediated induction of γH2AX we compared lymphoblasts from a normal individual to those from an individual with ataxia-telangiectasia (AT) deficient for ATM. Induction of γH2AX appeared intact in AT cells (**Fig. 3B**), indicating ATM is dispensable for γH2AX in these cells and strongly implicating DNA-PK involvement. DNA-PK is composed of the Ku70/Ku80 heterodimer and the catalytic subunit, DNA-PKcs.[5,41] DNA-PK mediates non-homologous end joining (NHEJ) of DSBs and its activation involves autophosphorylation at Ser^{2056} and Thr^{2609}.[42,43] In HCT116 cells, gemcitabine followed by AZD7762 induced Ser^{2056} phosphorylation of DNA-PKcs (**Fig. 4A**) and immunofluorescence analysis indicated γH2AX colocalized with phospho-Ser^{2056} DNA-PKcs (**Suppl. Fig. 2**). DNA-PK inhibitor NU7026 diminished γH2AX induction in AT cells treated with both gemcitabine and AZD7762 (**Fig. 4B**). We examined the effect of siRNA-mediated suppression of DNA-PKcs in HCT116 cells. DNA-PKcs siRNA diminished γH2AX induction (**Fig. 4C**), confirming DNA-PK was the primary mediator of increased DDR to Chk1 inhibition following

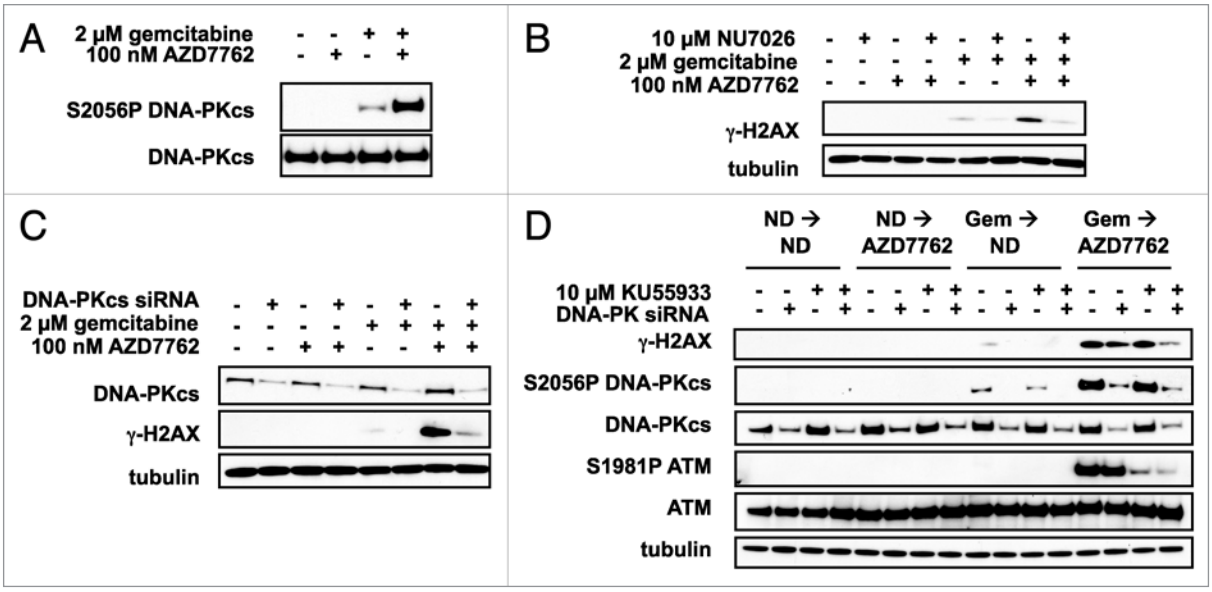

Figure 4. γH2AX induction is mediated by DNA-PK in AT and HCT116 cells, and both DNA-PK and ATM in MKN-74 cells. (A–D) Cells were treated with Gem for 2 h, washed, and treated with AZD7762 for 24 h. (A) HCT116 cells. (B) AT lymphoblasts were incubated with DMSO or NU7026 for 1 h prior to treatment. Vehicle or inhibitor was maintained throughout treatment. (C) HCT116 cells were transfected with either control or DNA-PKcs siRNA prior to treatment. (D) MKN-74 cells were transfected with control or DNA-PKcs siRNA prior to treatment. Additionally, KU55933 or DMSO was added 1 h prior to treatment and maintained throughout the experiment. Data represent at least three experiments.

replicative stress in HCT116 cells. However, in MKN-74 cells, both siRNA-mediated knockdown of DNA-PKcs and inhibition of ATM with KU55933 were required for diminution of γH2AX, indicating both PIKKs contribute to γH2AX induction in these cells (**Fig. 4D**).

Our data supported the idea that Chk1 inhibition following gemcitabine converted stalled forks into DSBs. To assess the impact of AZD7762 on the extent of DNA damage in gemcitabine-treated cells, we used the comet assay. Cells were treated with vehicle or gemcitabine for 45 min, washed, and incubated an additional 16 h. In cultures also treated with AZD7762, the drug was added 45 min prior to gemcitabine treatment and maintained throughout the experiment. Cells exposed to IR and SN-38, the active metabolite of irinotecan, were included as positive controls for DSBs. Z-VAD-FMK was included during treatment to prevent apoptotic DNA fragmentation from contributing to the amount of DNA damage determined by the assay. AZD7762 increased the amount of DNA damage in gemcitabine-treated cells, doubling the median TM from 17 to 33 (**Fig. 5A**), a level comparable to that caused by IR and SN-38.

We hypothesized that cells defective in DSB repair by virtue of deficiency in either HR or NHEJ would be more sensitive to the combination of gemcitabine and Chk1 inhibitor. In a colony formation assay, the BRCA2-deficient VC8 cell line was more susceptible to treatment with gemcitabine and AZD7762 than its isogenic counterpart complemented with a bacterial artificial chromosome expressing BRCA2 (**Fig. 5B**). Similarly, lack of Rad51 paralog XRCC3 in irs1SF cells rendered them more sensitive than parental AA8 cells to the combination of drugs (**Fig. 5C**). Mefs from severe combined immune deficiency (SCID) mice defective for DNA-PKcs were slightly but significantly ($p < 0.05$) more sensitive to the combination than their wildtype counterpart at gemcitabine concentrations above 0.01 μM (**Fig. 5D**). These data are consistent with Chk1 inhibition following replicative stress converting stalled forks to DSBs that require both HR and NHEJ repair pathways for survival.

To examine the interplay between checkpoint abrogation and enhanced DDR due to Chk1 inhibition, the effects of AZD7762 on cell cycle progression were studied in gemcitabine-treated cells. Treatment with AZD7762 had no appreciable effect on cell cycle distribution (**Fig. 6A**) as determined by flow cytometry for DNA content and staining for mitotic marker MPM2. To monitor progression of S-phase cells through the cell cycle, asynchronous cultures were pulse-labeled with bromodeoxyuridine (BrdU) and the fate of BrdU-positive cells was observed after drug treatment. Untreated or AZD7762-treated cells traversed S-phase and cycled normally, eventually accumulating in G_1 (**Suppl. Fig. 3**). Treatment with gemcitabine caused accumulation of cells with G_1/early S-phase DNA content and concomitant reduction in the percentage of mitotic cells from 3.2% to 0.06% (**Fig. 6A**). Gemcitabine also caused the loss of mitotic marker phospho-Ser^{10}-histone H3, as determined by western blot (**Fig. 2B**, upper). Gemcitabine prevented cells incorporating BrdU from completing S-phase (**Suppl. Fig. 3**). As expected, inhibition of Chk1 by AZD7762 after gemcitabine abrogated G_1/early S-phase arrest, restoring phospho-Ser^{10}-histone H3 (**Fig. 2B**, upper). Chk1 siRNA also restored phospho-Ser^{10}-histone H3 in gemcitabine-treated cells, similar to AZD7762 (**Fig. 2B**, upper), while Chk2 siRNA failed to do so (**Fig. 2B**, lower). In gemcitabine-treated cultures, AZD7762 increased the percentage of MPM2 positive cells from 0.12% to 12% (**Fig. 6A**). Notably, the majority of these mitotic cells had G_1/early S-phase DNA

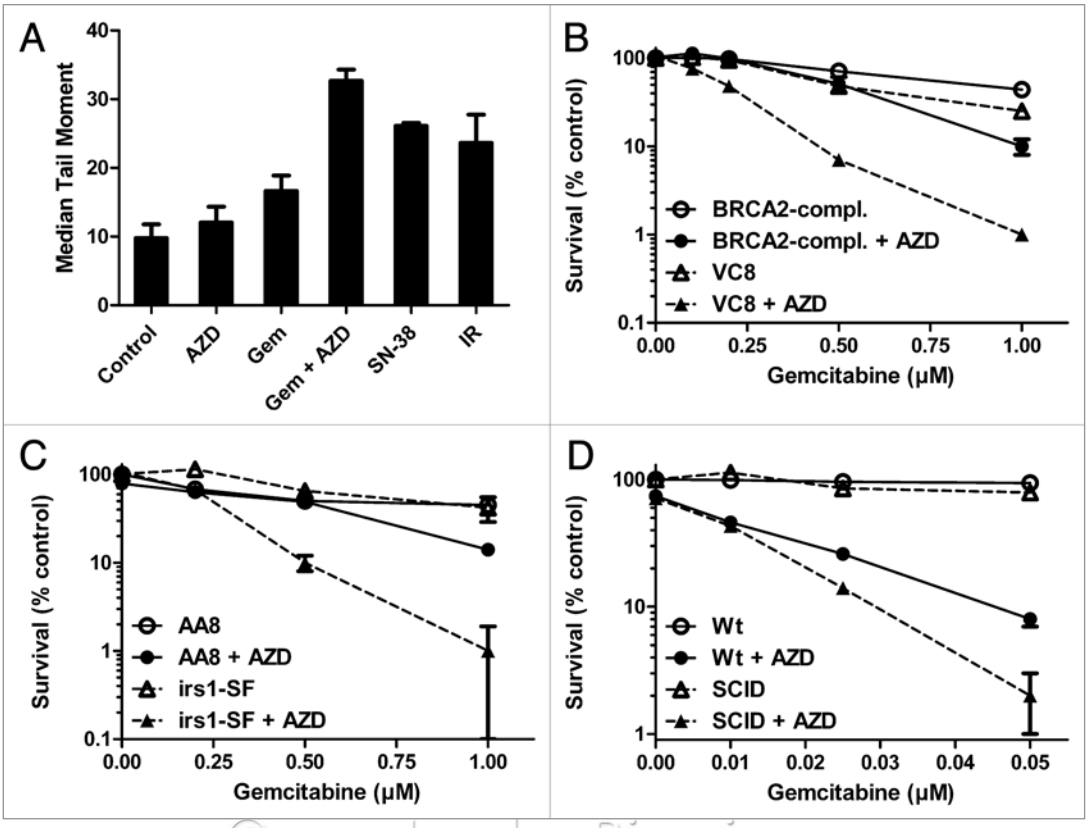

Figure 5. AZD7762 increases strand breaks and deficiency in HR or NHEJ sensitizes cells to gemcitabine and AZD7762. (A) Alkaline comet assay of HCT116 cells. Cultures were treated for 45 min with no drug or Gem, washed and incubated an additional 16 h. When present, AZD7762 was added 45 min prior to Gem treatment and maintained throughout the experiment. (B–D) Colony formation assays. Cells were treated with Gem for 2 h, washed, and incubated 24 h with no drug or 100 nM AZD7762. (B) VC8 or BRCA2-complemented VC8 cells. (C) AA8 or XRCC3-deficient irs1SF cells. (D) Wildtype (WT) or SCID Mefs. Data are means ± SD of one from 2–5 experiments with similar results.

content, indicating that AZD7762 induced premature mitosis. Consistent with premature mitosis, cells labeled with BrdU prior to gemcitabine treatment failed to complete DNA replication when treated with AZD7762 (**Suppl. Fig. 3**). Interestingly, very few mid/late S-phase cells prematurely entered into mitosis when treated with AZD7762 subsequent to gemcitabine. To examine the fate of cells entering aberrant mitosis, HCT116 cells expressing GFP-linked histone H2B were used with time-lapse microscopy. In cultures treated with AZD7762 after gemcitabine, we began observing the appearance of cells with condensed DNA entering mitosis after about 18 h (Suppl. data). These cells futilely attempted cytokinesis while the DNA became highly fragmented, suggesting that premature mitosis resulted in cell death. This aberrant mitosis was not observed with the other treatments (data not shown).

To test whether increased apoptosis resulting from Chk1 inhibition was due to premature mitosis, we used Cdk1 siRNA to prevent mitotic entry in cells treated with gemcitabine and AZD7762. Cells were transfected with control or Cdk1 siRNA for 12 h and treated with gemcitabine for 2 h followed by 24 h treatment with AZD7762. Cdk1 expression was diminished at 12 h and maximally suppressed by 24 h (**Fig. 6B**). Cdk1 siRNA prevented AZD7762 from restoring phospho-Ser^{10}-histone H3 in gemcitabine-treated cells (**Fig. 6C**), indicating prevention of mitotic entry. We also confirmed these results with flow cytometry analysis of MPM2 and DNA content (data not shown). PARP cleavage was not observed in Cdk1-depleted cells, indicating premature mitosis was important for cell death. Flow analysis demonstrated gemcitabine induced accumulation of G_1/early S-phase cells equally well in cells transfected with control or Cdk1 siRNA, ruling out the possibility that Cdk1 knockdown rescued cells from apoptosis by negating the S-phase targeting effect of gemcitabine.

Discussion

Dysregulation of cell cycle checkpoints is integral for neoplastic transformation of cancer cells and results in a reduced capacity to regulate cell cycle progression after exposure to genotoxic agents. One strategy for potentiation of conventional DNA-targeted therapies exploits the loss of checkpoints in cancer cells through the pharmacologic inhibition of remaining cell cycle controls. Small molecule inhibitors of Chk1 represent a promising class of drugs shown to improve the efficacy of genotoxic agents both in vitro and in vivo. The current study demonstrates that Chk1/2 inhibitor AZD7762 increases the sensitivity of cells to gemcitabine by abrogating suppression of replication origin firing, destabilizing stalled replication forks, and inducing mitotic death.

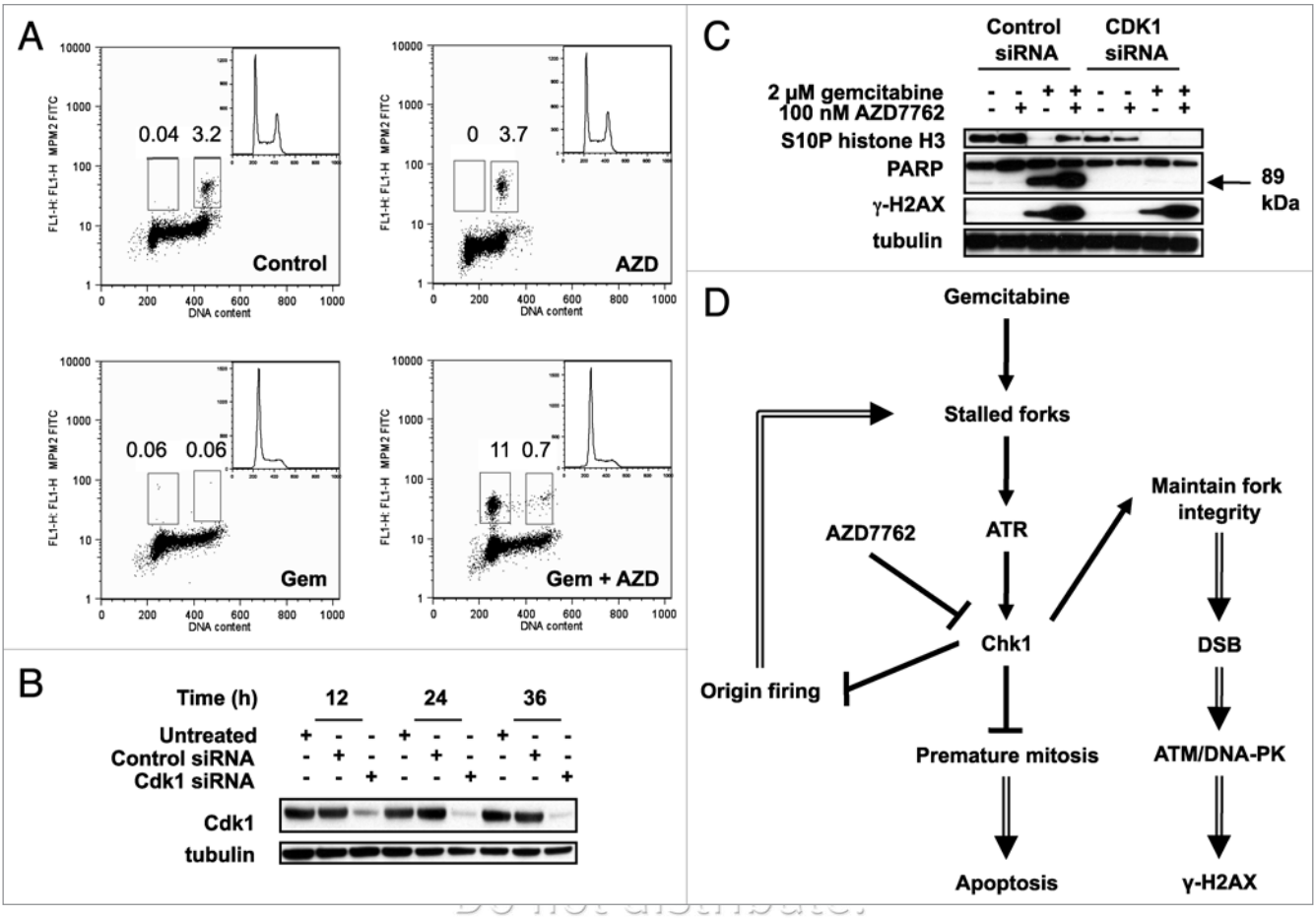

Figure 6. AZD7762 abrogates G_1/early S-phase arrest and induces lethal premature mitosis. (A) Flow cytometry analysis of HCT116 cells untreated or treated with 2 μM Gem for 2 h, washed, and treated with no drug (ND) or 100 nM AZD7762 for 24 h. Mitotic cells were detected via labeling with MPM2 antibody and DNA content was determined with propidium iodide staining. (B) HCT116 cells were untreated or transfected with control or Cdk1 siRNA and collected at the indicated times post-transfection. (C) HCT116 cells were transfected with control or Cdk1 siRNA for 12 h, treated with Gem for 2 h, washed, and incubated with AZD7762 for 24 h. Data represent at least two experiments with similar results. (D) Model for increased DNA damage and cell death resulting from Chk1 inhibition following replicative stress. Double lines represent consequences of Chk1 inhibition by AZD7762.

Chk1 blocks the activation of late replicons in cells exposed to replicative stress.[44] Using DNA fiber combing, we observed that AZD7762 partially restores firing of previously suppressed replication origins in gemcitabine-treated cells. However, in gemcitabine-treated cells, AZD7762 did not increase DNA synthesis or fork velocity. These data suggest that newly fired replicons likely stalled after limited replication. The incorporation of gemcitabine into DNA causes polymerase stalling one base beyond the site of addition[30] resulting in masked chain termination that makes its excision difficult for DNA repair systems.[45] Consequently, the inability to remove gemcitabine from DNA may present an impasse for the completion of DNA synthesis, even when Chk1-induced cell cycle controls have been inhibited. In contrast to such passive replication inhibition caused by a physical lesion, active replication inhibition by the checkpoint may be abrogated with Chk1 inhibition as illustrated by the restoration of DNA synthesis by either UCN-01 or CHIR-124 after exposure to the topoisomerase I poison camptothecin.[46] Additionally, ribonucleotide reductase inhibition by gemcitabine is irreversible. Consequently, replenishment of dNTP pools would require synthesis of new enzyme and it is unclear if the experiments in the current study would allow sufficient time for this to occur.

Chk1 inhibition following gemcitabine resulted in markedly increased γH2AX. γH2AX induction by UV- or 5-fluorouracil-induced replicative stress is mediated by ATR.[9,14] However, induction of γH2AX by gemcitabine and AZD7762 was not diminished in cells with reduced expression of ATR, indicating ATR was not the major H2AX kinase and that ATR-mediated Chk1 activation protected against development of lesions impinging upon other PIKKs. Indeed, AZD7762 caused activating phosphorylation of ATM and DNA-PKcs. ATM phosphorylation was ATR-independent (**Fig. 3**), in contrast to that induced by UV.[9] In response to IR, ATM and DNA-PK function redundantly in phosphorylating H2AX.[47] Intact γH2AX formation in ATM-deficient lymphoblasts in response to gemcitabine followed by AZD7762 suggested γH2AX formation was mediated by DNA-PK. In HCT116 cells, siRNA knockdown of DNA-PKcs diminished γH2AX formation, while neither ATM knockdown nor chemical inhibition had an impact, suggesting DNA-PK was the mediator of γH2AX formation in these cells. However,

inhibition of γH2AX induction in MKN-74 cells required inhibition of ATM in addition to DNA-PKcs knockdown, indicating redundancy within these kinases. Thus, the relative contribution of ATM and DNA-PK to γH2AX formation appears to be cell line dependent. A possible explanation is that Mre11 is mutated in HCT116 cells, resulting in lowered expression of the MRN complex.[48] While MRN deficiency does not prevent ATM activation, it does result in defective Chk2 phosphorylation[48] and likely diminishes ATM's contribution to γH2AX formation. These findings are consistent with a study demonstrating ATR-deficiency in Mefs causes increased γH2AX following exposure to aphidicolin mediated by DNA-PK and ATM.[16]

Activation of ATM and DNA-PK suggested AZD7762 converted stalled forks into DSBs, consistent with studies in yeast showing checkpoint deficiency leads to abnormal DNA structures after replicative stress likely due to collapse of stalled forks.[49] We examined whether deficiencies in pathways responsive to DSBs impacted survival of cells exposed to gemcitabine and AZD7762. AZD7762 potentiated the effects of gemcitabine in VC8 cells deficient for BRCA2 and irs1-SF cells deficient for Rad51 paralog XRCC3 suggesting HR deficiencies prevented repair of breaks induced by the combination of drugs. Although Chk1 is required for HR, we speculate the lack of epistasis between Chk1 inhibition and HR deficiency is due to recovery of Chk1-mediated functions after AZD7762 wash in the clonogenicity assay. Because Chk1 inhibition likely diminishes repair through HR, activation of DNA-PK may reflect a response of NHEJ to DSBs when HR is compromised. DNA-PKcs-deficient SCID Mefs exhibited reduced clonogenic survival when treated with AZD7762 after gemcitabine. However, the degree of sensitization by AZD7762 was smaller in SCID Mefs compared to HR-deficient cell lines, consistent with HR being the dominant DSB repair pathway in S-phase cells. These data provide evidence that DNA repair deficiencies compromise cancer cell survival after treatment with combined gemcitabine and AZD7762, providing an opportunity to target tumors with specific DDR defects.

Gemcitabine-treated cells incubated with AZD7762 progressed from G_1/early S-phase directly into mitosis without completing DNA replication. Cdk1 suppression with siRNA prevented this aberrant mitotic entry, resulting in decreased apoptosis. These data argue Cdk1 activity and/or premature mitosis in cells with incompletely replicated DNA is important for increased apoptosis through Chk1 inhibition. It is worth noting that few mid/late S-phase cells underwent premature mitosis. One explantation is that an ATR/Chk1-independent S-phase checkpoint is activated in mid/late S-phase cells that is not active in G_1/early S-phase cells. It is possible that increased DSBs resulting from fork collapse caused by AZD7762 in gemcitabine-treated mid/late S-phase cells activate the ATM-dependent intra-S phase checkpoint that would not be susceptible to Chk1 inhibition.

We propose the following model for Chk1 inhibitor chemosensitization after replicative stress (**Fig. 6D**). Chk1 inhibition allows firing of suppressed replication origins after replicative stress and destabilizes stalled forks, thereby preventing completion of replication and generating DSBs. Finally, abrogation of cell cycle arrest by Chk1 inhibition allows S-phase cells to progress directly into mitosis with incompletely replicated DNA, resulting in apoptosis. The current study contributes to the growing evidence that Chk1 inhibition is a viable strategy for potentiation of DNA-targeted therapies, meriting further inquiry.

Materials and Methods

Chemicals. Gemcitabine was purchased from Eli Lilly (Indianapolis, IN). AZD7762 and KU55933 were provided by AstraZeneca (Waltham, MA and Cambridge, UK respectively). NU7026, iododeoxyuridine (IdU), and chlorodeoxyuridine (CldU) were purchased from Sigma-Aldrich (St. Louis, MO). Z-VAD-FMK was purchased from MBL International Corporation (Woburn, MA).

Cell culture. HCT116 colorectal cancer cells were provided by Dr. Bert Vogelstein (John Hopkins University, Baltimore, MD) and maintained in RPMI. ATRFlox cells were purchased from the American Type Culture Collection (Manassas, VA) and maintained in McCoy's medium. MKN-74 gastric cancer cells were provided by Dr. E. Tahara (Hiroshima University, Hiroshima, Japan) and grown in MEM. AA8 and irs1SF Chinese hamster cells were provided by Dr. Andrew Pierce (Memorial Sloan Kettering Cancer Center (MSKCC), New York, NY). VC8 and VC8-BRCA2-complemented Chinese hamster cells were provided by Dr. Maria Jasin (MSKCC). Hamster cells were maintained in DMEM. Normal (GM01953), ataxia-telangiectasia (GM01526), and Seckel syndrome (GM18367) lymphoblasts were purchased from Coriell (Camden, NJ) and maintained in RPMI. SCID and wildtype murine embryonic fibroblasts (Mefs) were provided by Dr. John Petrini (MSKCC) and cultured in DMEM. Cells were grown at 37°C, 90% humidity, and 5% CO_2; all media contained 1% penicillin/streptomycin and 10% FBS (Hyclone, Logan UT), except for lymphoblasts which received 15% FBS and Mefs cultured with 10% Cosmic Calf Serum (Hyclone).

Gene silencing by adenovirus or siRNA. ATRFlox cells were infected with Ad-CMV-null or Ad-CMV-Cre adenovirus (Vector Biolab, Philadelphia, PA) for 24 h and cultured another 48 h in fresh media before treatment.

Control and Chk1 siRNAs were purchased from Dharmacon (Lafayette, CO) and described previously.[50] CDC2/CDK1 siRNA (M-003224-03) was also purchased from Dharmacon. Chk2 (SC-29271), DNA-PKcs (SC-35200) and control (SC-37007) siRNAs were purchased from Santa Cruz Biotechnology, Inc., (Santa Cruz, CA). Transfections were performed as described.[50]

Clonogenicity and proliferation assays. Colony assays were performed as described.[23] For proliferation assays, cells were seeded in 96-well plates and allowed 24 h to attach. After treatment, cells were cultured 48 h in drug-free media and assayed with WST-8 (Dojindo Molecular Technologies, Inc., Gaithersburg, MD) as per manufacturer's directions. Absorbance at 450 nm was measured on a Spectramax 340pc (Molecular Devices, Sunnyvale, CA).

Immunoblotting. Immunoblotting was performed as described.[23] Mouse monoclonal antibodies were: ATM (2C1),

Chk1 (G-4), DNA-PKcs (Santa Cruz, Santa Cruz, CA); poly (ADP-ribose) polymerase (PARP) (19F4) (Cell Signaling, Danvers, MA); γH2AX (JBW301), and phospho-Ser^{1981} ATM (10H11.E12) (Millipore, Billerica, MA). Rabbit polyclonal antibodies were: Chk2 (H-300) (Santa Cruz); Cdk1, phospho-Ser^{317} Chk1, phospho-Ser^{345} Chk1 (133D3), α-tubulin (11H10), phospho-Ser^{10} histone H3, H2AX (Cell Signaling); ATR (Serotec, Raleigh, NC); and phospho-Ser^{2056} DNA-PKcs (Abcam, Inc., Cambridge, MA).

Flow cytometry. Cell cycle distribution analysis using flow cytometry for DNA content and mitotic marker MPM-2 was described previously.[51]

Measurement of apoptosis. Cells were harvested, fixed in 3% paraformaldehyde, stained with DAPI, and examined with fluorescence microscopy. Cells with condensed fragmented chromatin were considered apoptotic. At least 400 cells were counted per sample.

DNA fiber combing analysis. Cells were labeled with 100 μM IdU for 45 min, washed, and pulsed with 100 μM CldU. Treatment with gemcitabine and AZD7762 was performed according to **Figure 2C** or **Supplemental Figure 1A**. Cells were harvested and embedded in 1% pulse field gel electrophoresis agarose plugs at 5 x 10^4 cells per plug. Plugs were prepared as described.[46] Once DNA was combed on a sylanized surface, IdU and CldU were stained. Antibodies were diluted in ELISA blocking reagent (Roche, Indianapolis, IN). Slides were incubated with rat anti-BrdU (Accurate Chemical and Scientific) and mouse anti-BrdU (Becton Dickinson, Franklin Lakes, NJ) for 1 h at room temperature in a humid chamber. Slides were washed in PBS containing 0.05% Tween-20 and incubated with secondary antibodies: donkey anti-rat Alexa Fluor® 594, donkey anti-mouse Alexa Fluor® 488 (Molecular Probes). Slides were washed and mounted in Vectashield (Vector Laboratories, Inc., Burlingame, CA).

Single-cell gel electrophoresis (comet assay). Comet assay was performed as described previously.[52] Cells incubated with 100 nM SN-38 for 20 h, and cells irradiated with 20 Gy (Shepherd Mark-1 model 68 irradiator with a cesium-137 source delivering 200 cGy/min) and harvested on ice immediately subsequent to exposure were included as positive controls. Z-VAD-FMK (20 μM) was included with all treatments. Fifty cells from each of three replicates were randomly selected and quantified using Komet 5.5 software (Kinetic Imaging, UK). The extent of damage was quantified by the tail moment (TM), defined as the product of the percent DNA in the comet tail and the distance between the means of tail and head fluorescence distributions.[53]

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Note

Supplementary materials can be found at: www.landesbioscience.com/supplement/McNeelyCC9-5-Sup.pdf

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